RICH-1, a Multi-functional RhoGAP Domain-containing Protein, Involved in Regulation of the Actin Filament System and Membrane-trafficking

BY

NINNA RICHNAU
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ABSTRACT


The Rho GTPases, which are related to the Ras family of proto-oncogenes, have been found to have important roles in regulating the morphogenic and migratory properties of eukaryotic cells. In addition, these proteins have been shown to regulate aspects of cell signaling, cell growth, cell division and cell survival. The Rho GTPases cycle between inactive GDP-bound and active GTP-bound states. In resting cells, Rho GTPases are sequestered in the cytoplasm by forming an inactive complex with guanine dissociation inhibitors (GDIs), and are, thus, unable to exchange guanine nucleotides. Rho GTPases exchange guanine nucleotides at slow rates \textit{in vivo}, and these reactions can be catalyzed by two different classes of proteins. Upon cell activation, guanine exchange factors (GEFs) stimulate the exchange of GTP for GDP and thereby activate the Rho GTPases, whereas the GTPase activating proteins (GAPs) turn off the Rho GTPase by stimulating their inherent GTP-hydrolysis activity. The active Rho GTPase associates with so-called effector proteins, which in turn mediate a plethora of responses.

In recent years a great number of Rho GTPase effectors have been identified. The Cdc42-interacting protein 4 (CIP4) is one such protein, and this thesis has focused on elucidating the role of this protein in Rho GTPase regulated activities resulting in changes in the organization of the actin filament system. Changes in actin dynamics are required for many cellular activities, such as cell migration, cytokinesis and membrane-trafficking. CIP4 is a member of the Pombe Cdc15 homology (PCH) family of proteins. Many PCH proteins have been proposed to cooperate with so-called formin homology proteins to induce changes in actin dynamics resulting in cytokinesis. We show that CIP4 interacts with the \textit{diaphanous}-related formin DAAM1 (Disheveled associated activator of morphogenesis 1). DAAM1 appeared to influence both changes in actin dynamics and microtubule dynamics, possibly by integrating signals from CIP4, Src and the Rho GTPases Rac, Cdc42.

The RhoGAP domain-containing protein RICH-1 (RhoGAP interacting with CIP4 homologues-1) was isolated in a yeast two-hybrid screen for proteins binding to CIP4. RICH-1 was shown to down-regulate the Rho GTPases Cdc42 and Rac1. In addition to the RhoGAP domain, RICH-1 possesses proline-rich motifs which confer binding to a variety of Src homology 3 (SH3) domain-containing proteins including CIP4, FBP17, Src, Abl and CIN85. Furthermore, RICH-1 exhibits a BIN/amphiphysin/Rvs (BAR) domain which associates with membrane lipids, and in addition this domain was shown to deform liposomes in an \textit{in vitro} assay, which is thought to mimic the deformation of cellular lipid bilayers, for example the invagination of the plasma membrane during endocytosis. Our results suggest a role for RICH-1 in intracellular membrane-trafficking events. RICH-1 was in addition shown to interact with the SH3 domains of two BAR domain-containing proteins, endophilin A1 and amphiphysin, which induce deformation of the plasma membrane during the specialized clathrin-mediated endocytosis. In conclusion, our data supports the notion that RhoGAPs are multi-functional proteins, fulfilling not only the role as down-regulators of Rho GTPase activity, but also as signal transducers of numerous vital cellular processes.

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To my
family
This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

The diaphanous-related formin DAAM1 is a CIP4-binding protein with a role in regulating assembly of actin filaments and microtubules. Manuscript.


The RhoGAP RICH-1 has a BAR (BIN/Amphiphysin/Rvsp) domain responsible for binding and tubulation of liposomes. Manuscript.

The RhoGAP RICH-1 interacts with Abelson tyrosine kinase, and the scaffolding proteins NHERF-2 and CIN85. Manuscript.

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Present investigations

Aim
Introduction
The diaphanous-related formin DAAM1 is a CIP4-binding protein with a role in regulating assembly of actin filaments and microtubules (Paper I)
RICH, a Rho GTPase-activating protein domain-containing protein involved in signaling by Cdc42 and Rac1 (Paper II)
The RhoGAP RICH-1 has a BAR (BIN/Amphiphysin/Rvsp) domain responsible for binding and tubulation of liposomes (Paper III)
The RhoGAP RICH-1 interacts with Abelson tyrosine kinase, and the scaffolding proteins NHERF-2 and CIN85 (Paper IV)

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<td>Abl</td>
<td>Abelson tyrosine kinase</td>
</tr>
<tr>
<td>Abp1</td>
<td>Actin-binding protein 1</td>
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<tr>
<td>Acinus</td>
<td>Apoptotic chromatin condensation inducer in the nucleus</td>
</tr>
<tr>
<td>ACK</td>
<td>Activated Cdc42-associated kinase</td>
</tr>
<tr>
<td>AP</td>
<td>Adaptor (or assembly) protein</td>
</tr>
<tr>
<td>BAR</td>
<td>BIN/Amphiphysin/Rvs1</td>
</tr>
<tr>
<td>Bni1</td>
<td>Bud neck involved 1</td>
</tr>
<tr>
<td>Bnr1</td>
<td>Bni1-related</td>
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<tr>
<td>Cbl</td>
<td>Casitas B-lineage lymphoma</td>
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<tr>
<td>CCV</td>
<td>Clathrin-coated vesicle</td>
</tr>
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<td>CIN85</td>
<td>Cdc42-interacting protein of 85 kDa</td>
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<td>CIP4</td>
<td>Cdc42-interacting protein 4</td>
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<tr>
<td>CME</td>
<td>Clathrin-mediated endocytosis</td>
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<tr>
<td>CMS/CD2AP</td>
<td>p130Cas ligand with multiple SH3 domains/CD2 associated protein</td>
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<tr>
<td>CRIB</td>
<td>Cdc42/Rac interactive binding</td>
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<tr>
<td>DAA1</td>
<td>Disheveled associated activator of morphogenesis 1</td>
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<tr>
<td>DAD</td>
<td>Dia-auto-regulatory domain</td>
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<td>Dbll</td>
<td>Diffuse B-cell lymphoma</td>
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<tr>
<td>DII</td>
<td>DII homology</td>
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<tr>
<td>Dia</td>
<td>Diaphanous</td>
</tr>
<tr>
<td>DOCK180</td>
<td>Downstream of CRK, 180kDa</td>
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<td>DRF</td>
<td>Diaphanous related formin</td>
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<tr>
<td>EB1P90</td>
<td>ERM-binding phosphoprotein 50</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>EII</td>
<td>Eps15 homology</td>
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<td>E3KARP</td>
<td>NHE3 kinase A regulatory protein</td>
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<td>Eno/VASP</td>
<td>Drosophila Enabled/Vasodilator-Stimulated Phosphoprotein</td>
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<td>ENTH</td>
<td>Epis N-terminal homology</td>
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<tr>
<td>Eps</td>
<td>Epidermal growth factor receptor pathway substrate</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>ERM</td>
<td>Ezrin-rixin-moesin</td>
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<tr>
<td>EVH</td>
<td>Ena-VASP-homology</td>
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<td>FA</td>
<td>Focal adhesion</td>
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<td>FAK</td>
<td>Focal adhesion kinase</td>
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<td>Fab1p/YOTB/Vac1p/EEA1</td>
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<td>GTGase activating protein</td>
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<td>GDP</td>
<td>Guanine diphosphate</td>
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<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<td>GPCR</td>
<td>G-protein coupled receptors</td>
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<td>GTP</td>
<td>Guanosine 5'-triphosphate</td>
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<td>GTPase</td>
<td>Guanosine triphosphatase</td>
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<td>HD</td>
<td>Huntington’s disease</td>
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<td>HIP1</td>
<td>Huntingtin interacting protein 1</td>
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<td>IRSp53</td>
<td>Insulin receptor substrate protein 53</td>
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<td>JNK</td>
<td>Jun kinase; c-jun N-terminal kinase</td>
</tr>
<tr>
<td>LARG</td>
<td>Leukemia-associated RhoGEF</td>
</tr>
<tr>
<td>LPA</td>
<td>Lysoosphosphatidic acid</td>
</tr>
<tr>
<td>LPAAT</td>
<td>Lysoosphosphatidic acid acyl transferase</td>
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<tr>
<td>MAP</td>
<td>Mitogen-activated protein</td>
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<tr>
<td>MLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>MRCK</td>
<td>Myotonic dystrophy kinase-related</td>
</tr>
<tr>
<td>N-15</td>
<td>N-WASP neuronal-WASP</td>
</tr>
<tr>
<td>PACSIN</td>
<td>PKC and CK2 substrate in neurons</td>
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<tr>
<td>PAE/PDGFRβ</td>
<td>Porcine aortic endothelial cells stably expressing the PDGF-β receptor</td>
</tr>
<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
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<tr>
<td>PCH</td>
<td>Pombe Cdc15 homology</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PIP</td>
<td>PSD-95/DlgA/ZO-1-like</td>
</tr>
<tr>
<td>PEST</td>
<td>Sequences rich in proline-, glutamic acid-, serine-, and threonine residues</td>
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<td>PI</td>
<td>Pleckstrin homology</td>
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<tr>
<td>Plk</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIP5</td>
<td>Phosphatidylinositol 4-phosphate 5-kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol 3,4,5-trisphosphate</td>
</tr>
<tr>
<td>PIP4</td>
<td>Phosphoinositides (phosphorylated derivatives of PI)</td>
</tr>
<tr>
<td>PIX</td>
<td>PAK-interacting exchange factor</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PX</td>
<td>Phox homology</td>
</tr>
<tr>
<td>PYK2</td>
<td>Proline-rich tyrosine kinase</td>
</tr>
<tr>
<td>RBD</td>
<td>Rho GTPase-binding domain</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated coiled-coil-containing protein kinase</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SCAR</td>
<td>Suppressor of CAMP receptor</td>
</tr>
<tr>
<td>SH</td>
<td>Src homology</td>
</tr>
<tr>
<td>Sos</td>
<td>Son of sevenless</td>
</tr>
<tr>
<td>Src</td>
<td>Rous sarcoma virus oncogene</td>
</tr>
<tr>
<td>SRF</td>
<td>Serum response factor</td>
</tr>
<tr>
<td>Syndapin</td>
<td>Synaptic, dynamin-associated protein</td>
</tr>
<tr>
<td>TIAM</td>
<td>T-lymphoma invasion and metastasis</td>
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<tr>
<td>WASP</td>
<td>Wiskott-Aldrich syndrome protein</td>
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<td>WAVE</td>
<td>WASP-like verprolin-homologous</td>
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<td>WIP</td>
<td>WASP-interacting protein</td>
</tr>
<tr>
<td>WW domain</td>
<td>Contains two signature tryptophan (W) residues spaced 20-23 residues apart</td>
</tr>
<tr>
<td>XMR</td>
<td>X-chromosome linked forms of mental retardation</td>
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INTRODUCTION

The different cell types that constitute the human body are in constant communication with the extracellular environment, such as the extracellular matrix (ECM), and with neighboring cells, via a vast variety of cell surface receptors. These receptors transduce extracellular signals to the cell interior via different cascades of signaling proteins, ultimately leading to distinct cellular responses, such as cell growth, division, proliferation, migration and death. Disturbance of these signaling cascades can result in pathological conditions such as cancer.

In a process referred to as endocytosis, cells can internalize material from the surrounding environment by invagination of the plasma membrane to form vesicles enclosing this material. In this way cells can take up fluid, nutrients, remove dead cells, recycle synaptic vesicles and regulate the number and activity of cell surface receptors. In the reverse process, exocytosis, a vesicle fuses with the plasma membrane whereby the cell can release substances, such as hormones or neurotransmitters to the extracellular milieu.

The Rho guanosine triphosphatases (GTPases) are key transducers of signals emerging from a wide variety of plasma membrane receptors leading to the reorganization of the actin filament system, which is the main engine for cell migration as well as other cellular processes including endocytosis and exocytosis.

The activity of Rho GTPases can be turned off by a multi-functional group of proteins called Rho GTPase activating proteins (RhoGAPs). The dysregulation of Rho GTPase activity have been shown to cause cancer and mental disorders. The scope of this thesis was to investigate the role of the RhoGAP RICH-1 (RhoGAP interacting with CIP4 homologues) in the regulation of actin dynamics and membrane-trafficking processes.

1. Cell motility

In multicellular organisms, cell migration is central to many biological processes (reviewed in Lauffenburger & Horwitz 1996; Mitchison & Cramer 1996; Stossel 1993). During embryogenesis, cells migrate to form the different parts of the emerging organism, for example, neurons migrate to form the nervous system. In the adult organism, cell migration remains prominent in both normal physiology and during pathological conditions. Under normal conditions cells within the tissue do not move much, however, cell migration is stimulated during wound healing or oncogenic transformation. Fibroblasts and epithelial cells migrate to assist in wound healing, the locomotion of leukocytes and macrophages is required for a proper immune response, and cancer cells migrate from the initial tumor mass into the circulatory system in order to metastasize and invade new tissues. An increased understanding of the molecular mechanisms behind the process of cell migration is likely to lead to new therapeutic strategies for a variety of pathological conditions, including cancer, atherosclerosis, and chronic inflammatory diseases such as rheumatoid arthritis (reviewed in Ridley 2001b; Webb et al 2002).
Although the molecular mechanisms for cell migration are complex, the basis for the process is simple; extracellular cues (diffusible factors, signals from neighboring cells, and/or signals from the ECM) stimulate transmembrane receptors to initiate intracellular signaling (Ridley 2001b). In response to these signals the cell sends out several protrusions, or filopods (Mitchison & Cramer 1996; Ridley 2001b; Stossel 1993). Then, if the conditions are favorable, some of these protrusions make stable attachments or adhesions to the underlying surface and form a leading edge or leading lamella. The stable attachment is used to drive the cell forward, and as the cell proceeds to move, adhesions are released and the cell rear retracts. Hence, cell locomotion requires the coordinated activity of the systems controlling the cytoskeleton, membranes, and adhesions (Mitchison & Cramer 1996). Moreover, a polarized morphology, or a clear distinction between the cell front and the rear parts of the cell, is required for cell migration to occur (Lauffenburger & Horwitz 1996).

2. The cytoskeleton

The cytoskeleton is formed by three distinct, yet interconnected types of filaments; microfilaments formed by actin, the microtubules formed by α/β-tubulin heterodimers, and the intermediate filament system made from fibrous proteins encoded by a multigene family consisting of more than 50 members (reviewed in Coulombe et al 2000; Insall & Machesky 2001). The actin filament system is involved in regulating a broad variety of cellular responses, such as cell motility and changes in cell shape, cell polarity, cell adhesion, membrane-trafficking and cell division (cytokinesis) (Schmidt & Hall 1998; Stossel 1993). The microtubule system is essential for cell division, cell migration, intracellular vesicle transport, and cell polarity (Gundersen 2002). In addition, the microtubule system and actin filament system cooperate in a variety of processes, including vesicle and organelle transport, directed cell migration, spindle rotation, cleavage furrow placement during cytokinesis, and nuclear migration (Goode et al 2000). Several proteins serve to bridge the two networks, including diaphanous-related formins (DRFs) and members of the ERM (ezrin-radixin-moesin) family of proteins. The major function of intermediate filaments is to stabilize the cellular architecture against physical stress (Coulombe et al 2000).

2.A. The actin filament system

Reorganization of the cortical actin cytoskeleton in response to extracellular stimuli is a key event in cell migration. The three major structures formed by the cortical actin cytoskeleton consist of spike-like surface protrusions, so-called filopodia, consisting of thin bundles of actin, a mesh-work of branched actin filaments at the cell periphery referred to as lamellipodia, and bundles of contractile actin-myosin filaments called stress-fibers, which are linked to the ECM through focal adhesions (reviewed in Hall 1998; Van Aelst & D'Souza-Schorey 1997). Veil-like membrane ruffles form when lamellipodia at the leading edge release from the substrate and fold backward. The contractile stress-fibers possess the ability to contract and exert tension, for example in the migrating cell or during cytokinesis, by virtue of a group of proteins called myosins (Mitchison & Cramer 1996; Schmidt & Hall 1998).
Regulation of the actin filament system

Actin filaments are helical protein polymers formed by the polymerization of monomeric subunits (G-actin) into filaments (F-actin) (reviewed in Pollard et al 2000; Schmidt & Hall 1998; Welch & Mullins 2002). Actin filaments are formed in an asymmetric fashion resulting in a fast-growing or barbed end (+) and a slow-growing or pointed end (-). The dynamic functions of the actin cytoskeleton make it necessary that the actin polymerization is both spatially and temporally regulated. Actin assembly can be initiated by three general mechanisms; by de novo nucleation of actin monomers, by extension of pre-existing filaments by uncapping of the barbed ends, or by creation of new ends by filament severing (Pollard et al 2000; Welch & Mullins 2002).

In resting cells, the barbed ends of actin filaments are blocked in order to prevent spontaneous elongation of the filament. When the cell receives a signal to migrate, the barbed ends will become exposed to trigger a rapid actin assembly. A tight control of actin assembly is achieved by a multitude of actin-regulatory proteins. These proteins either bind actin monomers, or regulate the length of the filaments by capping and severing, or bundle actin filaments into various supramolecular structures.

De novo nucleation

Two main mechanisms for de novo nucleation have so far been identified; one employing the Arp2/3 (actin-related proteins 2 and 3) complex, resulting in branched filaments, and another employing DRFs forming unbranched filaments (reviewed in Evangelista et al 2003; Pollard et al 2000; Welch & Mullins 2002). The latter has only been shown for Bni1 (Bud neck involved 1) and Bar1 (Bni1-related) in yeast, and remains to be demonstrated in vertebrates. Processes that require branched actin filaments include the extension of membrane protrusions during cell migration, and the intracellular motility of pathogens such as Listeria monocytogenes (Chang & Peter 2002). Linear actin filaments are found in microvilli, stress-fibers, contractile rings and the linear structures (actin cables) that enable directional transport of vesicles in yeast.

The Arp2/3 complex consists of seven subunits, including the actin related proteins Arp2 and Arp3, and was initially identified on the basis of its affinity for profilin (Machesky et al 1994). The Arp2/3 complex binds to the side of pre-existing actin filaments and nucleates a new filament as a Y-shaped branch on the side of the older filament (Pollard et al 2000). The Arp2/3 complex by itself is inactive and requires the interaction with nuclear promoting factors (reviewed in Weaver et al 2003; Welch & Mullins 2002). The major activators are the Wiskott-Aldrich syndrome protein/suppressor of cAMP receptor (WASP/SCAR) family of proteins and the cortactin family of proteins. WASPs bring actin monomers to the Arp2/3 complex, thereby promoting nucleation of a new filament, whereas cortactin binds filamentous actin and is hence proposed to stabilize branched filaments.

Recently, a report by Hudson et al. revealed that, in Drosophila, the nucleation of unbranched filaments into parallel actin bundles did not require the Arp2/3 complex (Hudson & Cooley 2002). In support of this notion, reports from other groups showed that the yeast Bni1 and Bar1 nucleated the elongation of unbranched actin cables in a manner dependent on stimulation by profilin, but independently of the Arp2/3 complex.
complex (Evangelista et al. 2002; Pruyne et al. 2002; Sagot et al. 2002a; Sagot et al. 2002b). Thus, distinct actin nucleation mechanisms appear to be used for the assembly of different actin networks. The formation of stress-fibers via Rho also involves the activity of DRFs, but occurs primarily through bundling of pre-existing filaments rather than de novo polymerization (Machesky & Hall 1997; reviewed in Chang & Peter 2002).

**The dendritic nucleation model**

Using fluorescent actin, Glacy et al. showed that the lamellipodia are the primary site of actin incorporation (reviewed in Small et al. 2002). Along with providing the cell with protrusive activity, lamellipodia are involved in macropinocytosis and phagocytosis and in the formation of adhesions to the substrate. The dendritic nucleation hypothesis, based on nucleation by the Arp2/3 complex, has been a widely used model for explaining actin assembly at the leading edge of motile cells (Pollard et al. 2000). In resting cells, the monomer-binding proteins profilin and thymosin-β4 when present (thymosin-β4 is not present in protozoa, slime molds or fungi, and hence not in *S. cerevisiae*) maintain a pool of monomeric actin ready to participate in elongation of the barbed ends of the filaments. Extracellular ligands bind to plasma membrane receptors and initiate signaling cascades in the cell, activating a variety of intracellular molecules, including the Rho GTPases. The Rho GTPases bind to and activate the WASPs, thereby causing activation of the Arp2/3 complex, resulting in de novo nucleation of branched actin filaments. The barbed ends of the growing filaments are directed toward the leading edge of the cell, pushing the membrane forward as they grow. Profilin:actin complexes are rapidly incorporated into the forming branch. At some point, elongation is terminated by capping proteins, such as CapZ and the heterodimeric capping protein, which block the free barbed ends. Actin cross-linking proteins, such as α-actinin, filamin, fimbrin, spectrin, coronin, myosin II and villin aid in the assembly of higher order actin networks (Schmidt & Hall 1998; Small et al. 2002; Stossel 1993). Disassembly of actin filaments is thought to be achieved by the ADF/cofilin family of proteins, together with severing proteins, such as gelsolin and fascin, which act by severing existing fragments where after they also cap the barbed ends. ADF/cofilin extracts actin monomers from the interior of the filaments and thereafter sequesters the released monomers. The phosphorylated membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2 or PIP2) binds to several actin binding proteins and thereby regulate their function, resulting in actin polymerization (reviewed in Takenawa & Itoh 2001). For example, PIP2 interacts with monomer-binding proteins, such as profilin, causing the release of free monomers ready for actin nucleation and subsequent actin polymerization (Takenawa & Itoh 2001). In addition, PIP2 binds to capping proteins such as CapZ and gelsolin resulting in uncapping of barbed ends, and thereby initiates actin polymerization. Furthermore, PIP2 promotes cross-linking of filaments by binding to actin cross-linking proteins.
2.B. Cell adhesion

The cell can adhere to the substrate by different types of contact sites such as focal adhesions, focal complexes and podosomes (Webb et al 2002). Cell adhesions at the leading edge of motile cells are initiated upon ligand binding and clustering of integrin family of cell adhesion molecules (Brakebusch & Fassler 2003). Focal complexes are small transient adhesions at the base of the leading edge that drive a rapid cell migration; these structures are formed in response to Rac activation, but are also found in association with filopodia formed via Cdc42 (Nobes & Hall 1995; Rottner et al 1999). These focal complexes can be either disassembled as the cell lamellae move over them or, in slowly migrating cells such as fibroblasts, mature into larger and more stable Rho-dependent adhesions, referred to as focal adhesions (FAs) at the termini of stress-fibers.

A high-level of integrin-mediated adhesion inhibits cell migration, thus, a dynamic turnover of focal complexes/adhesions is necessary (reviewed in Ridley 2001b; Webb et al 2002). Many of the FA proteins appear to be tyrosine phosphorylated, and inhibition of tyrosine phosphorylation diminish cell migration and spreading (reviewed in Burrigde et al 1992; Lauffenburger & Horwitz 1996). The FAK (focal adhesion kinase) is only present in FAs, and is activated either by tyrosine phosphorylation via integrin clustering, or by Src (Rous sarcoma virus oncogene) tyrosine kinase (Brakebusch & Fassler 2003). Activated FAK, in turn, recruits a range of adaptors and signaling molecules, such as the RhoGAP GRAF (GTPase regulator associated with FAK), the cytoskeletal proteins paxillin and talin and p130Cas. Moreover, integrins stimulate the activity of Rho GTPases, mainly via FAK and Src-like kinases (Brakebusch & Fassler 2003). Studies on cells from knock-out mice have implicated FAK and Src, as well as the tyrosine phosphatases calcineurin, SHP-2 (Src homology containing phosphatase-2) and protein tyrosine phosphatase (PTP)-PEST, as regulators of adhesion turnover (Webb et al 2002).

Podosomes are tubular invaginations of the ventral plasma membrane. These invaginations are composed of columnar arrays of F-actin surrounded by ring structures, containing proteins such as vinculin and talin (reviewed in Linder & Aepfelbacher 2003). In fact, most of the proteins found in the podosome rings are also found in FAs and other cell-substrate contacts. Podosomes were first observed in fibroblasts transformed with oncogenic protein tyrosine kinases such as v-Src (Linder & Aepfelbacher 2003; Tarone et al 1985). In similarity to FAs, podosomes are enriched in tyrosine-phosphorylated proteins, and the regulators of podosome formation include Src and its substrates, Rho GTPases, WASP/SCAR family members, lipids and microtubules (Linder & Aepfelbacher 2003). Podosomes are typically found in cells that have to cross boundaries, such as monocyte-derived cells (macrophages, osteoclasts and dendritic cells), certain transformed fibroblasts and carcinoma cells (Kanehisa et al 1990; Linder & Aepfelbacher 2003; Linder et al 1999). Podosomes are thought to contribute to tissue invasion and matrix remodeling.

In addition, cells form contact sites linking one cell to another via contact sites termed adherens junctions, which are composed of clusters of cadherins and associated protein complexes consisting of vinculin, α-actinin, catenin, ERM proteins and filamin (Schmidt & Hall 1998). Focal adhesions and adherens junctions serve in
addition to being contact sites, also to restrict generation of actin filaments to discrete nucleation sites or ‘hot spots’ for nucleation.

3. The Ras superfamily of proteins

The Ras superfamily of small monomeric 20-40 kDa GTP-binding proteins can be divided into five major groups based on sequence and functional differences, Ras, Rho, Rab, Sar1/Arf, and Ran (reviewed in Takai et al 2001). The three Ras genes, H-Ras, K-Ras and N-Ras were first discovered as oncogenes in rat sarcoma viruses and in neuroblastoma (reviewed in Downward 1990). The Ras proteins are activated upon ligand engagement of membrane receptors and mainly regulate gene expression influencing cell growth, morphology, differentiation and apoptosis (reviewed in Shields et al 2000; Takai et al 2001). The first signaling pathway found to involve Ras was the Raf/MEK/Erk cascade leading to transcriptional activation (reviewed in Vojtek & Der 1998). Other effectors for Ras include p120GAP, Ral guanine nucleotide dissociation stimulator (RalGDS), phosphatidylinositol 3-kinase (PI 3-kinase) and Ras interaction/interference (RIN). Rho proteins are key regulators in reorganization of the actin cytoskeleton, thereby influencing cellular functions such as cell shape, cell growth, motility and adhesion (reviewed in Takai et al 2001). The Rab and the Sar1/Arf proteins regulate intracellular vesicle trafficking, and the members of the Ran subfamily of proteins regulate nucleocytoplasmatic transport of RNA and, furthermore, regulate microtubule organization (Takai et al 2001). The main focus of this thesis is on the Rho subfamily, thus, the Ras, Rab, Arf and Ran subfamilies will not be discussed further.

3.A. The Ras family of proteins as molecular switches

Most Ras proteins cycle between an inactive, guanosine diphosphate (GDP)-bound form and an active, guanosine 5'-triphosphate (GTP)-bound form (Takai et al 2001). When bound to GTP the Rho GTPase interacts with a unique group of proteins (effector proteins) to elicit specific cellular responses. Figure 1 depicts the tertiary structure of Ras, comprising a hydrophobic core of six-strands of β-sheet connected by five hydrophilic loops and five α-helices (Bourne et al 1991). The five loops (designated G-1 to G-5), which form the guanine nucleotide-binding site, are the most highly conserved elements in this domain. Amino acid residues critical for Mg\(^{2+}\)-binding, GTP-binding and hydrolysis are conserved in all members of the Ras superfamily (Takai et al 2001). The overall domain structure of Ras proteins comprises an N-terminal conserved region known as the diphasosphate-binding loop (P-loop or G-1 region; residues 10-17 in Ras), which encloses the α- and β-phosphate groups of the nucleotide and provides a serine/threonine residue to the magnesium ion (Figure 1 and Figure 2) (Bourne et al 1991; Gamblin & Smerdon 1998; Sprang 1997a; Sprang 1997b; Valencia et al 1991). The switch I region (effector loop; residues 30-40 in Ras; G-2 region), contributes to the Mg\(^{2+}\) binding site and confers binding to downstream effector molecules. The switch II region (residues 60-67 in Ras; G-3 region), binds the γ-phosphate of the nucleotide and contains a catalytic glutamine (Gln61 in Ras); the guanine ring is recognized in part by the G-4 region and the G-5 region buttresses the guanine base recognition site. Further, the C-terminal CAAX (C=cysteine residue, A=aliphatic residue, X=any amino acid
residue)-box confers membrane targeting, and Rho GTPases, but not Ras, contain an ‘insert region’, which is required for interaction with some targets. The switch I and II regions adopt different conformations in the GDP- and the GTP-bound forms of the Rho GTPase. Mutations in residues Gly12 or Gln61 in Ras result in an overactive protein, unable to hydrolyze GTP, with transformation as a consequence (Valencia et al 1991). Based on their analogy with the original mutants in the Ras protein, two types of point-mutants have been widely used to study Rho GTPase function: an active mutant constitutively bound to GTP (for example, G12V and Q61L in Rac and Ras) due to inhibition of GTPase activity, and a dominant-negative Rho GTPase mutant (for example, T17N in Rac and Ras), which cannot become activated because it has reduced affinity for nucleotides and competes with the endogenous Rho GTPase for binding to guanine nucleotide exchange factors (GEFs) (Figure 2). The latter results in a complex unable to generate downstream responses (Bishop & Hall 2000; Feig 1999; Ridley 2001c). The TTF family of Rho proteins do not contain a Gly and Gln at positions 12 and 61, respectively, and hence appear to be constitutively bound to GTP (Bishop & Hall 2000).

Figure 1. The three-dimensional structure of Ras in complex with a non-hydrolyzable GTP-analogue (top), and a GDP-molecule (bottom), respectively. Adapted from Sprang, 1997.
In addition, members of the Rho family of proteins are targets of various bacterial toxins, which act to covalently modify them, resulting either in activation (deamidation and transglutamination) or inactivation [adenosine diphosphate (ADP)-ribosylation or glycosylation] of the GTPases (reviewed in Lerm et al 2000). For example, *E.coli* CNF1 (cytotoxic necrotising factor 1) and 2 deamidate Rho GTPases at Gln63/Gln61 and thereby inhibit the intrinsic and GAP stimulated GTP-hydrolysis of Rho GTPases. Moreover, the inactivation of Rho by C3 ADP-ribosyltransferase at Asn41, inhibits the activation by GEFs, and increases the affinity of Rho for GDI. Recently, several injected toxins, which are delivered into target cells by the type III-secretion system, have been shown to manipulate the GTPase cycle by acting as GAPs (ExoS and YopE) or GEFs (SopE). Toxins are widely used in studies to unravel the signaling pathways involving Rho family proteins.

### 3.B. The Rho GTPase subfamily

The first Rho gene was identified in 1985 and showed homology to Ras (*Ras homologous*) (Madaule & Axel 1985). Since then the Rho GTPase subfamily in human has expanded to include 22 members, divided into eight subgroups as shown in Table 1 (Fransson et al 2003; Ridley 2001b; Wherlock & Mellor 2002).
**Table 1.** The Rho GTPase subfamily.

<table>
<thead>
<tr>
<th>Rho GTPase subgroup</th>
<th>Members</th>
</tr>
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<tbody>
<tr>
<td>Cdc42</td>
<td>Cdc42, TC10, TCL, Chp and Wrch-1</td>
</tr>
<tr>
<td>Rac</td>
<td>Rac1, Rac2, Rac3 and RhoG</td>
</tr>
<tr>
<td>Rho</td>
<td>RhoA, RhoB and RhoC</td>
</tr>
<tr>
<td>Rnd</td>
<td>Rnd1, Rnd2 and Rnd3</td>
</tr>
<tr>
<td>RhoD</td>
<td>RhoD and Rif</td>
</tr>
<tr>
<td>RhoH/TTF</td>
<td>RhoH/TTF</td>
</tr>
<tr>
<td>Miro (mitochondrial Rho)</td>
<td>Miro1 and Miro2</td>
</tr>
<tr>
<td>RhoBTB (BTB=Broad Complex, Tram track, Bric à Brac)</td>
<td>RhoBTB1 and RhoBTB2</td>
</tr>
</tbody>
</table>

The most extensively characterized of all Rho GTPases are Cdc42 (cell division cycle 42), Rac1 (Ras-related C3 botulinum toxin substrate 1) and RhoA (Ras homologous member A). Initially, Cdc42 was shown to be an important regulator of cell polarity in budding yeast, Rac1 was implicated in regulating superoxide formation via NADPH-oxidase in phagocytic cells and RhoA was implicated in the formation of stress fibers (Abo et al 1991; Adams et al 1990; Ridley & Hall 1992). The anchoring of Ras proteins to various cellular membranes is critical for their proper function and is regulated by prenyl transferases (reviewed in Seabra 1998). In general, Rho GTPase membrane targeting is achieved by a post-translational lipid-modification in the carboxy terminal CAAX-box, and involves the prenylation of a conserved cysteine residue, followed by removal of AAX and methylation of the cysteine residue (Seabra 1998). Of note, Rac1 and RhoA are hereafter also referred to as Rac and Rho in this thesis work. When other members of the Rac or Rho subgroups are discussed I use their full names.

**3.C. Cellular responses mediated by Rho GTPases**

Members of the Rho GTPase family are the key regulatory proteins that link plasma membrane receptors to the assembly of distinct filamentous actin structures (reviewed in Hall 1998). In Swiss 3T3 fibroblasts, Rho was shown to be activated through G-protein coupled receptors (GPCRs) leading to the formation of stress fibers and focal adhesions (Ridley & Hall 1992), whereas Rac was activated through tyrosine-kinase receptors (RTKs) resulting in the assembly of lamellipodia and membrane ruffles (Ridley et al 1992). Activation of Cdc42 by GPCRs gave rise to filopodia (Kozma et al 1995; Nobes & Hall 1995). Consistent with these observations microinjection of dominant-negative mutants of Rac, Cdc42 or Rho prior to stimulation inhibited the formation of these structures. All of the structures formed were also associated with integrin-based focal adhesion complexes, although the ones formed by Rac and Cdc42 were distinct from the classical Rho-induced focal adhesions (Nobes & Hall 1995). Rho GTPase-mediated actin reorganization has been confirmed in other mammalian cell types, as well as in yeast, flies and worms (reviewed in Etienne-Manneville & Hall 2002; Hall 1998).

Initial studies in Swiss 3T3 fibroblasts showed that Cdc42, Rac and Rho are organized in a hierarchical cascade; activation of Cdc42 leads to a localized activation of Rac, hence, filopodia are often associated with lamellipodia, and in turn,
activation of Rac leads to a weak and delayed activation of Rho to produce stress fibers (Nobes & Hall 1995; Ridley et al 1992; reviewed in Hall 1998). Moreover, a Cdc42-independent cascade also exists where Ras can activate Rac and subsequently Rho. However, the hierarchical Cdc42/Rac/Rho cascade seen in Swiss 3T3 fibroblasts has been challenged by observations in other cell lines, for example, Cdc42 and Rac inhibit stress-fiber formation in a variety of cell types (Hirose et al 1998; Leeuwen et al 1997). Further, Rho was shown to inhibit the activity of Rac and Cdc42 in N1E-115 neuroblastoma cells (Sander et al 1999).

Rho, Rac and Cdc42 are involved in several processes that require the reorganization of actin, for example cell migration, cytokinesis, cell polarity, cell morphology, cell adhesion and membrane-trafficking processes (Bishop & Hall 2000; Etienne-Manneville & Hall 2002; Van Aelst & D'Souza-Schorey 1997). In addition, these Rho GTPases are involved in transcriptional control and cell cycle progression (Van Aelst & D'Souza-Schorey 1997). During cell migration, for example, Rac promotes actin polymerization at the leading edge of the lamellipodia, providing the driving force for forward movement, whereas Cdc42 defines the polarity of the cell and reorients the Golgi in the direction of movement (Allen et al 1998; Hall & Nobes 2000). Stress fibers are not needed for the migration, but Rho is required for formation of focal adhesions, whereas Ras appears to be required for focal adhesion turnover (Hall & Nobes 2000).

Actin reorganization mediated by Rac, Cdc42 and Rho has been implicated in neuronal morphogenesis involving neuronal migration and polarization, axon guidance and dendrite formation, synaptic organization and plasticity (reviewed in Luo 2000; Luo 2002). Developing neurons extend two types of processes (axons and dendrites, which are developed from neurites) which are led by a specialized end known as a growth-cone, but otherwise morphologically and functionally distinct (Luo 2002). Once a neuron has reached its target, the growth-cones in the axon ends are converted to presynaptic terminals, whereas the growth-cones in the dendritic ends form postsynaptic specializations upon proper contact with axons. Developing neurons often make more connections than required, which can be removed in a process called axon-or dendrite retraction (pruning), an event that occurs also in mature neurons. Studies in N1E-115 cells have shown that Rac and Cdc42 promote neurite outgrowth, by regulating the formation of filopodia and lamellipodia in growth-cones (Kozma et al 1997). By contrast, Rho was shown to promote neurite retraction in N1E-115 cells, presumably by generating actin-myosin-based contractility (neuronal cells cannot form stress-fibers) (Hirose et al 1998; Ridley 1999).

Many RhoGAPs have been implicated in neuronal morphogenesis, as regulators and/or as downstream effectors of Rho GTPases (see Table 3). For example, a splice variant of nadrin (the rat orthologue of the RhoGAP RICH-1) has been shown to inhibit nerve-growth factor (NGF)-dependent neurite-outgrowth in PC12 rat adrenal pheochromocytoma cells by virtue of its GAP domain (Harada et al 2000). Other RhoGAPs implicated in processes involved in neuronal morphogenesis include; p200RhoGAP, GRAF, oligophrenin, p190-A, α2-chimaerin and MEGAP (Mental disorder associated GAP protein) (Table 3). The involvement of Rho GTPases in membrane-trafficking events will be elaborated on in Chapter 6. The microtubule system is another postulated target for Rho GTPases, with Rho having a dual role in
stabilizing/destabilizing microtubules, whereas Rac and Cdc42 increase microtubule growth (Wittmann & Waterman-Storer 2001).

3.D. Rho GTPase effector proteins

The ability of the Rho GTPases to induce such a variety of cellular responses relies on their interactions with a plethora of downstream targets or effector proteins. Effector proteins have commonly been identified by the yeast two-hybrid system and affinity chromatography, based on their specific interactions with the GTP-bound form of the Rho GTPase (reviewed in Bishop & Hall 2000). Many of the targets for Rac and Cdc42 contain a conserved CRIB (Cdc42/Rac interactive binding) motif which confers binding to the effector loop in the GTPase, and is present in, for example, WASP, N-WASP (neuronal-WASP), MRCK (myotonic dystrophy kinase-related Cdc42-binding protein) and ACK (activated Cdc42-associated kinase) (Bishop & Hall 2000; Burbelo et al 1995a). However, several targets lack CRIB motifs and interact with Rho GTPases through other motifs, for example the Cdc42-interacting protein 4 (CIP4) (Aspenström 1997).

A common mechanism for effector activation by Rho GTPases is the disruption of an intramolecular autoinhibitory interaction within the effector protein (Bishop & Hall 2000). For example, WASP and N-WASP, and DRFs display an inhibitory intramolecular interaction which is disrupted by the binding of a GTPase. Most progress has been made in identifying effector proteins involved in actin reorganization, only a few of these will be described below, whereas Figure 3 gives a more complete picture of the actual diversity of target proteins and the biological responses they mediate (for extensive reviews on effector proteins see Aspenström 1999; Bishop & Hall 2000; Schmitz et al 2000; Van Aelst & D'Souza-Schorey 1997).
The Wiskott-Aldrich syndrome (WAS) is an X-linked immunodeficiency disease caused by a defect in the gene encoding the WAS protein (WASP), resulting in severe defects in platelets and cells of the immune system in the patients (Derry et al 1994; reviewed in Kirchhausen & Rosen 1996). The WASP/SCAR family of proteins includes the hematopoietic WASP, the ubiquitous but neuronally enriched N-WASP and SCAR1-3 [also known as WASP-like verprolin-homologous protein 1-3 (WAVE1-3)] (Aspenström et al 1996; reviewed in Caron 2002; Kolluri et al 1996; Symons et al 1996). Initially, filopodium formation via Cdc42 was thought to require the ubiquitous N-WASP (Machesky & Insall 1998; Miki et al 1996; Miki et al 1998; Rohatgi et al 1999). In this process, Cdc42 and PI(4,5)P2 bind N-WASP and co-operatively release the inhibitory intramolecular interaction in N-WASP, allowing for its interaction with the Arp2/3 complex. Moreover, N-WASP binds actin monomers as well as the actin monomer-binding protein profilin (Miki & Takenawa 1998).

However, recent reports proposed that N-WASP is dispensable for de novo formation of filopodia (Lommel et al 2001; Snapper et al 2001). Moreover, the Arp2/3 complex appears to be excluded from filopodia and microspikes (Small et al 2002). Instead, de novo formation of linear actin structures (actin cables) in yeast was proposed to involve the co-operativity of the DRF Bni1 and profilin, suggesting a potential role for formins in the formation of filopodia (Sagot et al 2002b). Moreover, Cdc42-mediated filopodium formation from pre-existing filaments could involve the binding of the insulin receptor substrate protein 53 (IRSp53) to the Drosophila Enabled/Vasodilator-Stimulated Phosphoprotein (Ena/VASP) family protein mammalian Enabled (Mena) (Krugmann et al 2001). The Mena/VASP proteins can reduce branching by competing with capping proteins and Arp2/3 complex for binding to barbed ends, resulting in the formation of the long unbranched filament bundles characteristic for
filopodia (Machesky 2002). Nonetheless, in hematopoetic cells, Cdc42-mediated filopodium formation is thought to occur via WASP, binding to actin monomers, the Arp2/3 complex and an indirect interaction with profilin via the WIP (WASP interacting protein) (Miki et al 1998; Ramesh et al 1997). Interestingly, WIP has also been shown to enhance actin polymerization by the Arp2/3 nuclear promoting factor cortactin (Kinley et al 2003). The Rho GTPase TC10 has also been shown to mediate the formation of filopodia (Neudauer et al 1998).

In addition, Rac interacts with IRSp53, which binds WAVE and induces Arp2/3-induced actin polymerization leading to lamellipodia formation (Miki et al 2000). Thus, IRSp53 provides a link between Rac and Cdc42 and an explanation to how Cdc42 can induce Rac-mediated ruffling (Govind et al 2001; Nobes & Hall 1995; Ridley et al 1992). IRSp53 can also bind the Rho target mouse Diaphanous 1 (mDia1, also known as p140mDia) but the physiological role of this interaction is not known (Fujiwara et al 2000). Evidently, lamellipodia formation can occur independently of Rac under some circumstances; Rab5 induced lamellipodia formation independently of Ras, Cdc42, Rac or Rho in Swiss 3T3 cells (Spaargaren & Bos 1999), membrane-ruffling in immature dendritic cells occurs independently of Rac (West et al 2000), and lamellipodia extension in colon carcinoma cells is Rho-dependent (O'Connor et al 2000).

Phosphoinositides (PIPs) are produced by phosphorylation of single or multiple sites of the inositol head group in the membrane phospholipid phosphatidylinositol (PI) (reviewed in Takenawa & Itoh 2001). The role of PIPs in signaling is well-established, for example, the hydrolysis of PIP2 generates the second messengers IP3 (inositol 1,4,5-triphosphate) and DAG (diacylglycerol) resulting in a variety of cellular responses (Toker & Cantley 1997). In addition, PIPs, and PIP2 in particular, have emerged as prominent regulators of membrane-trafficking as well as of actin organization (Takenawa & Itoh 2001). Accordingly, inositol lipid kinases and phosphatases, regulating the turnover of PIPs, play pivotal roles in these events, and act to induce temporal and spatial changes in the availability of PIPs.

There are several lines of evidence for a co-operation between Rho GTPases and PIPs in mediating actin reorganization. Phosphatidylinositol 4-phosphate 5-kinases (PIP5-kinases; there are three subtypes: α, β and γ) induce the formation of PIP2, which then binds to capping proteins and dissociates them from the barbed ends of actin filaments resulting in the polymerization of actin (reviewed in Kanaho & Suzuki 2002; Takenawa & Itoh 2001). Activated Rac and Rho have been reported to induce the formation of PIP2 via stimulation of PIP5-kinase activity (Chong et al 1994; Hartwig et al 1995; Ren & Schwartz 1998; Tolias et al 1995; Tolias et al 2000). It is not clear, however, whether the interaction between Rho and PIP 5-kinase is direct or indirect. Recent studies show that the Rho-binding serine/threonine protein kinase ROCK (Rho-associated coiled-coil-containing protein kinase) stimulates the activity of PIP 5-kinase, which may provide an explanation for how Rho regulates PIP2 levels (Oude Weernink et al 2000). Moreover, microinjections of antibodies against PIP2 inhibited lysophosphatidic acid (LPA)/Rho-induced formation of stress-fibers and focal adhesions, thus, demonstrating a role for PIP2 also in the regulation of focal adhesions (Gilmore & Burridge 1996; Takenawa & Itoh 2001). Rac is also implicated in the hydrolysis of PIP2.
by directly interacting with the endocytic PIP₂-hydrolyzing enzyme synaptojanin 2 (a polyphosphoinositide phosphatase) (Malecz et al 2000).

The Rho targets ROCK and the DRF mDia1 were shown to act cooperatively to mediate Rho-induced stress fibers and focal contacts in mammalian cells (Nakano et al 1999; reviewed in Ridley 1999; Watanabe et al 1999). Whereas ROCK appears to induce the formation of thick actin fibers, mDia1 stimulates the formation of thin actin fibers, and together they cause the formation of stress-fibers like those induced by activated Rho (Ishizaki et al 1997; Nakano et al 1999; Tominaga et al 2000; Watanabe et al 1999). ROCK can enhance the formation of contractile-actomyosin filaments in at least two ways: first, ROCK phosphorylates the myosin light chain (MLC), and, second, prevents its dephosphorylation by phosphorylating (and inactivating) the MLC phosphatase (Amano et al 1996; Kimura et al 1996). Furthermore, ROCK phosphorylates LIM-kinase, which inhibits the actin depolymerizing protein cofilin by phosphorylation, with a resulting stabilization of actin filaments (reviewed in Bishop & Hall 2000).

Other targets for ROCK include the ERM family of actin cytoskeleton membrane linking proteins, the Na’/H’ exchange (NHE) protein 1, and the actin-binding protein adducin (Bishop & Hall 2000). In addition, ROCK and mDia1 are involved in Rho-induced formation of adherens junctions, where ROCK acts inhibitory by generating contractile force and mDia1 acts as a stabilisator by acting on both the actin filament system and the microtubule system (Sahai & Marshall 2002b). Moreover, RhoD has been shown to activate the DRF hDia2C and Src to induce changes in the actin cytoskeleton and thereby regulate the motility of early endosomes (Gasman et al 2003).

The formin homology family of proteins

The founding member of the formin homology (FH) family of proteins is the mouse protein formin-1, which was identified as a gene, which if mutated results in limb deformities (reviewed in Evangelista et al 2003; Tanaka 2000). The common feature of FH proteins are the two FH domains (FH1 and FH2) (Castrillon & Wasserman 1994; reviewed in Evangelista et al 2003; Wasserman 1998). The proline-rich FH1 domain binds to profilin and, in addition, confers binding to proteins containing Src homology 3 (SH3)-and WW [contains two signature tryptophan (W) residues, spaced 20-23 amino acid residues apart] domains, perhaps linking the FH proteins to the cytoskeleton and/or regulating the interaction of the FH1 domain with profilin (Wasserman 1998). For example, mDia1 and mDia2 interact with the SH3 domain of Src, and DAAM1 (disheveled associated activator of morphogenesis 1) binds to the SH3 domains of several proteins including Src, Abl and CIP4 (Tominaga et al 2000; Paper I). Some FH proteins contain a conserved FH3 motif which might determine subcellular localization (Evangelista et al 2003). The diaphanous-related formins (DRFs) constitute a distinct subfamily of the FH proteins, by possessing a conserved N-terminal Rho GTPase-binding (RBD) and C-terminal Dia-auto-regulatory (DAD) domains (Table 2; Figure 4; reviewed in Alberts 2002; Evangelista et al 2003). DRFs have been implicated in a variety of cellular processes, including cytokinesis, cell polarization, intracellular mRNA trafficking, serum response factor (SRF)-regulated gene expression, sperm acrosome formation and hair cell stereocilia formation (Alberts 2002; Evangelista et al 2003).
Table 2. Diaphanous related formins (DRFs).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Organism</th>
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<tbody>
<tr>
<td>DFNA1</td>
<td>Human</td>
</tr>
<tr>
<td>HDia</td>
<td>“</td>
</tr>
<tr>
<td>DAAM1</td>
<td>“</td>
</tr>
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<td>“</td>
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<tr>
<td>mDia2</td>
<td>“</td>
</tr>
<tr>
<td>Diaphanous</td>
<td>Drosophila</td>
</tr>
<tr>
<td>SepA</td>
<td>A. Nidulans</td>
</tr>
<tr>
<td>Bni1p</td>
<td>S. cerevisiae</td>
</tr>
<tr>
<td>Bnr1p</td>
<td>“</td>
</tr>
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</table>

Figure 4. Schematic representation of DRFs. Rho-GTP binding to the RBD is thought to relieve the auto-inhibition by the DAD domain. Adapted from Evangelista et al. 2003.

DRFs are involved in the assembly of actin-based structures, such as the contractile ring, actin cables, and stress fibers (Chang & Peter 2002). Pruyne et al. and Sagot et al. showed that a fragment of the yeast DRF Bni1 containing the FH1 and FH2 domains was sufficient to nucleate actin filaments in vitro (Pruyne et al 2002; Sagot et al 2002b). The FH1 and the FH2 domains are thought to control actin assembly and nucleation (Evangelista et al 2003). Profilin stimulates DRF-dependent actin nucleation in vitro, most likely through the recruitment of actin monomers (Sagot et al 2002b). In addition, DRFs are regulators of microtubule function and organization (Ishizaki et al 2001; Kato et al 2001; Palazzo et al 2001), and it is likely that the effects DRFs have on cell polarity involves both the actin filament system and the microtubule system (Chang & Peter 2002). The human orthologue of mouse mDia1, DFNA1 was identified as a gene responsible for non-syndromic deafness, and it has been speculated that actin filaments are not properly organized in hair cells in DFNA1 patients (Lynch et al 1997).

Cdc42-interacting protein 4 (CIP4)

The initial purpose of this thesis work was to characterize the Cdc42 effector protein CIP4 and its effects on the actin cytoskeleton organization. Initial studies showed that over-expression of CIP4 in Swiss 3T3 fibroblasts had various effects on the actin cytoskeleton (Aspenström 1997). One effect was a decrease in stress-fiber content, which was proposed to rely on either an ability of CIP4 to sequester actin monomers, or
to activate actin-depolymerizing factors which could disassemble pre-existing actin filaments. Moreover, these cells appeared to have an elevated membrane-ruffling activity, and CIP4 localized to those areas that ruffled. In addition, clustering of CIP4 into foci on the dorsal side of the cells was induced upon co-expression with Cdc42 or Rac, or by stimulation with PDGF-BB or bradykinin. The foci were proposed to represent an initial stage in the formation of filopodia (Aspenström 1997).

CIP4 belongs to the Pombe Cdc15 homology (PCH) family of proteins, whose domain structure comprises an N-terminal FER-CIP4 homology (FCH) domain, a putative coiled-coil region, sequences rich in proline-, glutamic acid-, serine-, and threonine (PEST) residues, and a C-terminal SH3 domain (Aspenström 1997; reviewed in Lippincott & Li 2000). The function of PCH proteins in actin-based processes such as cytokinesis has been suggested to rely on an interaction with FH proteins (Lippincott & Li 2000). The SH3 domain of CIP4 was shown to bind to WASP, the RhoGAP RICH-1, the FH1 domain in the DRF DAAM1, and the proto-oncogene Cbl (Casitas B-lineage lymphoma) (Aspenström 1997; Dombrosky-Ferlan et al 2003; Paper I; Paper II; Tian et al 2000). In addition, the Src kinase Lyn interacts with the proline-rich regions in CIP4 (Dombrosky-Ferlan et al 2003).

Two proteins with high similarity to CIP4 are the formin binding protein 17 (FBP17; mouse and human) and rapostlin (rat) (Aspenström 1997; Fujita et al 2002). Rapostlin is an effector for Rnd2, and induces Rnd2-dependent reorganization of F-actin in HeLa cells, as well as neurite branching in PC12 cells (Fujita et al 2002). The human FBP17 was shown to interact with sorting nexin 2, a protein implicated in receptor trafficking (Fuchs et al 2001). The FCH domains of CIP4 and rapostlin confer binding to microtubules, and these proteins were proposed to act as linkers between the actin cytoskeleton and microtubules (Fujita et al 2002; Tian et al 2000). The formation of podosomes in primary human macrophages appears to require microtubules, WASP and CIP4 (Linder et al 1999). CIP4 and/or DAAM1 could act to mediate the Cdc42-induced polarization of the microtubule system in migrating cells (Paper I; Linder et al 1999; Nobes & Hall 1999; Tian et al 2000).

The CIP4 gene is thought to be spliced into four transcripts, where the nomenclature CIP4a (CIP4), CIP4b (also known as Felic; Fes-related, with homology to ezrin, Lyn interactor with Cdc42), CIP4c and CIP4h (also referred to as CIP4/2 or CIP4-long isoform) has been proposed (Dombrosky-Ferlan et al 2003; Wang et al 2002). CIP4b and CIP4c do not contain any SH3 domain. The Rho GTPase TC10 is activated by insulin in a cascade downstream of c-Cbl, and is required for the translocation of vesicles containing the GLUT4 (glucose transporter 4) protein to the plasma membrane, and the subsequent uptake of glucose (Chiang et al 2001). TC10 regulates the organization of the cortical actin cytoskeleton at the site of GLUT4 vesicle fusion and secretion (reviewed in Qualmann & Mellor 2003). The mouse orthologue of CIP4h (CIP4/2) was proposed to be an effecter for TC10 in regulating glucose uptake (Chang et al 2002). CIP4/2 translocated to the plasma membrane upon either insulin stimulation or co-expression with constitutively active TC10. Another protein proposed to act as an effecter for TC10 is the RhoGAP TCGAP (TC10/Cdc42-GTPase activating protein) (Chiang et al 2003). CIP4b interacts with activated Cdc42, as well as the Src kinase Lyn (Dombrosky-Ferlan et al 2003). CIP4b and Lyn co-localize to phagocytic cups, an early stage of phagosome formation, thereby integrating Src and Cdc42 pathways. Moreover,
over-expression of CIP4 (and CIP4b) was shown to inhibit NIH3T3 cell migration (Dombrosky-Ferlan et al 2003).

Three separate studies have implicated CIP4 in apoptosis; first, a microarray-approach identified CIP4 as a gene upregulated during apoptosis in human breast cancer cells (Yuan et al 2001). Second, our work shows that CIP4 interacts with acinus (apoptotic chromatin condensation inducer in the nucleus), a protein implicated in the condensation of chromatin during apoptosis (Paper I). Third, CIP4 accumulates in Huntington’s disease (HD) brain tissue, and induces cell death when over-expressed in striatal neurons (Holbert et al 2003). The pathological neurodegenerative HD is caused by an expansion of a polyglutamine (polyQ) tract in the protein huntingtin, leading to specific neuronal cell death, with symptoms such as motoric disturbances and psychiatric changes (Gusella & MacDonald 1998). The pathogenesis of HD may involve the accumulation of huntingtin aggregates in neuronal inclusions, as well as an altered interaction of huntingtin with other proteins resulting in an abnormal regulation of transcription and signal transduction. Interestingly, CIP4, and the related PACSIN 1 (PKC and CK2 substrate in neurons 1), both interact with huntingtin and are implicated in HD (Holbert et al 2003; Modregger et al 2002). Taken together, these observations suggest that CIP4 proteins and their homologues act as scaffolds, integrating Src kinase pathways, actin and microtubule systems. It will be of great interest to investigate further the involvement of CIP4 in HD and cell survival.

3.E. Rho GTPases in pathological conditions

Signaling by Rho GTPases is crucial for a variety of biological responses, thus, the dysregulation of their activities can result in diverse aberrant phenotypes such as cancer progression, mental disabilities and a group of diverse unrelated disorders (reviewed in Boettner & Van Aelst 2002; Sahai & Marshall 2002a). Importantly, mutations in genes encoding GEFs, GAPs or effector proteins may present the most prominent cause of dysregulation of Rho GTPase mediated activities.

The Ras genes are frequently mutated in human cancers, with the highest prevalence in pancreatic carcinomas (reviewed in Bos 1989; Downward 1990; Takai et al 2001). By contrast, only a single Rho gene (RhoH/TTF) has so far been found to be either rearranged or mutated, and have been proposed to have a role in the development of multiple myeloma, non-Hodgkin’s lymphoma, and diffuse large B-cell lymphoma (Sahai & Marshall 2002a). Several Rho genes have been found to be over-expressed in different types of cancers (Sahai & Marshall 2002a). For example, Rac1 and Cdc42 were over-expressed in breast tumors, and the Rac1 splice variant Rac1B is over-expressed in breast-and colorectal tumors (Jordan et al 1999; Sahai & Marshall 2002a). Several RhoGEFs were initially characterized as oncoproteins, harboring gain-of-function mutations, for example, diffuse B-cell lymphoma (Dbl; implicated in B-cell lymphoma), leukemia-associated RhoGEF (LARG; involved in acute myeloid leukemia) and T-cell lymphoma invasion and metastasis 1 (Tiam 1; an invasion promoting gene in T-cells) (Habets et al 1994; Kourlas et al 2000; Srivastava et al 1986). Many genes encoding RhoGAPs have been found upregulated, deleted or translocated in several types of cancer, for example oligophrenin is over-expressed in colon cancer and glioblastomas, Bcr (break point cluster region) is fused with the Abl (Abelson tyrosine kinase)
oncogene in leukemias, Abr (active Bcr-related) is deleted in some cases of medulloblastoma and DLC-1 (deleted in liver cancer 1) is frequently deleted in liver cancer (Table 3; Ljubimova et al 2001; McDonald et al 1994; Pinheiro et al 2001; Shtivelman et al 1985; Yuan et al 2003).

The dysregulation of Rho GTPase activities has recently been shown to result in diverse neurodegenerative disorders. The RhoGAP oligophrenin is deleted in the neuropathological disorder X-chromosome linked forms of mental retardation (XMR) (Billuart et al 1998). In addition, the RhoGEF αPIX (PAK-interacting exchange factor α; also known as ARHGEF or Cool-2) and the Rac1/Cdc42 effector p21-activated kinase (PAK) are mutated in XMR (Boettner & Van Aelst 2002; Kutsche et al 2000). The gene encoding the RhoGEF intersectin is situated on chromosome 21, and has been speculated to contribute to the malignant aspects of Down’s syndrome (Boettner & Van Aelst 2002). A loss-of-function mutation in the RhoGEF alsin has been proposed to be the cause of amyotrophic lateral sclerosis (ALS) (Boettner & Van Aelst 2002). The characteristics of ALS is the progressive degeneration of motor neurons successively leading to respiratory failure.

3.F. Regulation of RhoGTPase activity

**Signals leading to the activation of Rho GTPases**

Although the mechanism by which receptors activate Rho GTPases are far from clear, it is believed to rely on the activation of GEFs (reviewed in Kjøller & Hall 1999). PI 3-kinases have been implicated in the regulation of cell proliferation, survival, metabolism, cytoskeletal organization and membrane-trafficking (reviewed in Levers et al 1999). The role of PI 3-kinase as a link between RTKs [e.g. receptors for platelet-derived growth factor (PDGF), insulin and insulin-like growth factor (IGF)] and Rac-mediated membrane ruffling (and a subsequent Rho-mediated stress-fiber formation) became evident from studies using PI 3-kinase inhibitors (Wortmannin or LY294002), PI 3-kinase mutants, and receptor mutants unable to bind PI 3-kinase (Hawkins et al 1995; Kotani et al 1994; Nobes et al 1995; Reif et al 1996; reviewed in Van Aelst & D'Souza-Schorey 1997; Wennström et al 1994). Moreover, there exist a pathway where Ras acts through PI 3-kinase to induce membrane ruffling by Rac (Rodriguez-Viciana et al 1997). PI 3-kinases catalyze the conversion of PIP2 into phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3 or PIP3), which in turn binds to the PH (pleckstrin-homology) domain of the RacGEFs, such as Vav, Son of sevenless 1 (Sos1) and Tiam 1, and thereby regulates their activity (reviewed in Kjøller & Hall 1999; Scita et al 2000). Another route to Rac activation involves the co-operation of the Crk and DOCK180 (downstream of Crk, 180 kDa) and Cas adaptor proteins, possibly through recruitment of the RacGEF Sos (reviewed in Ridley 2001b). Interestingly, microtubule growth has been shown to activate Rac in fibroblasts, resulting in lamellipodia formation (Waterman-Storer et al 1999). Apart form being activated via GPCRs, Rho can be activated via cytokine receptors and RTKs, involving the activity of Cdc42 and Rac, respectively (Kjøller & Hall 1999). The mechanism whereby LPA activates Rho appears to involve a tyrosine kinase (Nobes et al 1995). Cdc42 can be activated via GPCRs, cytokine receptors and adhesion receptors, although the mechanisms are not known (Kjøller & Hall 1999).
addition, Rac and Cdc42 appear also to be upstream of PI 3-kinase, since Rac and Cdc42 bind to PI 3-kinase and activate the enzyme in PDGF-stimulated fibroblasts (Bokoch et al. 1996; Tolias et al. 1995; Zheng et al. 1994). Moreover, Rho appears to be upstream of PI 3-kinase (Kumagai et al. 1993; Zhang et al. 1993). However, studies regarding the placement of PI 3-kinase upstream or downstream of the Rho GTPases have gained different results depending upon which model system is used (Ren & Schwartz 1998).

Rho GTPases have been shown to interact with enzymes that affect the turnover of PIPs. One possibility is that Rho GTPases might target inositol lipid kinases and phosphatases to specific intracellular locations or vice versa. Moreover, Rho GTPases could be implicated in positive and/or negative feed-back loops, where they stimulate the generation of PIPs, which in turn could bind to and regulate the activity of RhoGEFs and RhoGAPs (by membrane-recruitment and/or changes in the structure). Regulation of GEF activity by binding of the PH domain to PIP₃ is well established. Moreover, many RhoGAPs possess PH domains and other phosphoinositide-binding domains, which could present for regulation in a similar manner.

**The Rho GTPase cycle**

In resting cells, Rho GTPases are cytosolic and exist in their inactive GDP-bound state, complexed with guanine dissociation inhibitors (GDIs) (see Figure 5; reviewed in Olofsson 1999; Zalcman et al. 1999). RhoGDIs mask the prenyl-group of some Rho GTPases and thereby prevent their interaction with membranes and inhibit nucleotide-exchange. Upon activation of plasma membrane receptors, the Rho GTPase/RhoGDI complex is recruited to the plasma membrane, whereby the Rho GTPase is released and, thus, can attach to the plasma membrane (Kjeller & Hall 1999). Rho GTPases can exchange nucleotides and hydrolyze GTP at slow rates *in vivo*. These reactions are accelerated by guanine nucleotide exchange factors (GEFs) (reviewed in Schmidt & Hall 2002; Zheng 2001), which activate the Rho GTPase by catalyzing the exchange of GTP for GDP, and GTPase activating proteins (GAPs) (reviewed in Lamarche & Hall 1994; Moon & Zheng 2003; Peck et al. 2002), which inactivate the Rho GTPase by stimulating the intrinsic hydrolysis (Figure 5).
Figure 5. Regulation of Rho GTPase activity. I. In the resting cell, a GDI sequesters the inactive GDP-bound Rho GTPase in the cytosol, inhibiting the interaction of the Rho GTPase with membranes. II. Upon activation of certain plasma membrane receptors, different factors cause the release of the Rho GTPase from the GDI and the subsequent translocation of the Rho GTPase to the plasma membrane, where a RhoGEF substitutes the GDP with a GTP, thus, activating the Rho GTPase. III. In the active GTP-bound state, the Rho GTPase can interact with a variety of downstream targets which mediate a plethora of biological responses. IV. A GAP then hydrolyzes the GTP, whereby the Rho GTPase is inactivated and can be sequestered by a GDI again. Importantly, GDIs can also sequester GTP-bound Rho GTPases, and inhibit the GTP-hydrolysis stimulated by a GAP (see text).

3.G. Rho GTPase activating proteins (RhoGAPs)

The first realization that Ras proteins required GTPase activating proteins for their down-regulation came from studies employing microinjection of recombinant GTP-bound Ras into living cells, resulting in an GTP-hydrolysis several times faster than that in vitro (Trahey & McCormick 1987). This led to the purification of the first GAP for Ras proteins, p120RasGAP (Trahey et al 1988).

The first RhoGAP identified was p50RhoGAP, and since then more than 30 RhoGAPs have been reported in eukaryotes, ranging from yeast to human (Barford et al 1993; Garrett et al 1989; Lancaster et al 1994; reviewed in Moon & Zheng 2003). Genome analysis revealed the presence of around 80 human genes containing RhoGAP domains (reviewed in Moon & Zheng 2003; Peck et al 2002). However, many of the genes containing RhoGAP domains were present as duplicate database entries, from partial sequences and as splice variants, thereby decreasing the number to 53, some of which are presented in Table 3. RhoGAP domain-containing proteins are present
throughout the genome and rarely cluster in specific chromosomal regions (Peck et al 2002).

**Table 3. Selected Rho GTPase activating proteins.**
Lipid-binding domains are noted if present, and the following abbreviations are used for these; BAR (BIN/Amphipysin/Rvsp), C1 (cysteine-rich phorbol ester binding), C2 (calcium-dependent lipid binding), PH (pleckstrin homology), PX (Phox homology) and sec14 (lipid-binding domain with homology to the yeast PI-transfer protein Sec14p). Chr. locus denotes the chromosomal locus in human. Adapted and changed from Moon, 2003 and Peck 2002.

<table>
<thead>
<tr>
<th>RhoGAP</th>
<th>GAP specificity</th>
<th>Tissue distribution and Chr. locus</th>
<th>Notes</th>
<th>References</th>
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<tr>
<td><strong>Abr [active Bcr-related] (human)</strong></td>
<td>Rac1= Rac2&gt; Cdc42</td>
<td>ND Brain Chr 17p13.3</td>
<td>Abr is deleted in seven of eight cases of medulloblastoma. Bcr and Abr are also GEFs for Cdc42&gt;RhoA&gt;Rac1=Rac2. Bcr and Abr are implicated in glial cell and vestibular morphogenesis. Bcr and Abr contain C2 domains.</td>
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<tr>
<td>RhoGAP</td>
<td>GAP specificity</td>
<td>Tissue distribution and Chr. locus</td>
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<td>β1-chimaerin/alternatively spliced SH2 domain-containing β2-chimaerin (human and rat)</td>
<td>Rac1</td>
<td>ND, chr 7p15.3</td>
<td>β1 involved in spermatogenesis. β2 expression downregulated in malignant gliomas compared to normal and low-grade astrocytoma. Receptor for phorbol esters and DAG via C1 domain.</td>
<td>(Caloca et al 1997; Caloca et al 1999; Leung et al 1993; Leung et al 1994; Yuan et al 1995)</td>
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<tr>
<td>P190-B (human)</td>
<td>RhoA, Rac1, Cdc42</td>
<td>ND, chr 14q12</td>
<td>Induced to cluster after integrin cross-linking. Regulates Rho which modulates CREB (Cyclic AMP response element binding) factor activity and thereby cell and organism size. P190-A and p190-B contain a GTPase domain, might regulate GAP activity.</td>
<td>(Burbelo et al 1995b; Sordella et al 2002)</td>
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<tr>
<td>RhoGAP</td>
<td>GAP specificity</td>
<td>Tissue distribution and Chr.locus</td>
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<td>GRAF2 (human) /PSGAP [PH- and SH3-domain-containing RhoGAP protein] (mouse)</td>
<td>Cdc42, RhoA /RhoA&gt; Cdc42</td>
<td>Ubiquitous, enriched in skeletal muscle /Ubiquitous Chr 4q31.22</td>
<td>GRAF2 interacts with PKN-β and is phosphorylated by this kinase. PSGAP interacts with proline-rich tyrosine kinase 2 (PYK2) and FAK. PYK2 inhibits PSGAP activity on Cdc42. Has a PH domain.</td>
<td>(Ren et al 2001; Shibata et al 2001)</td>
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<tr>
<td>3BP-1 [SH3-domain binding protein-1] (mouse)</td>
<td>Rac1, Rac2, Cdc42, RhoG</td>
<td>Rac1</td>
<td>Ubiquitous, enriched in spleen and brain Chr 22q11.1</td>
<td>Binds the SH3 domain in Abl. Has a BAR domain.</td>
</tr>
<tr>
<td>RICH-1 [RhoGAP interacting with CIP4 homologues] (human)/Nadrin [Neuron-associated developmentally regulated protein] (rat)</td>
<td>Rac1, Cdc42/ Rac1, Cdc42, RhoA</td>
<td>Ubiquitous, enriched in heart and placenta /Brain Chr 16p12.2</td>
<td>RICH-1 binds CIP4 and several other SH3-domain-containing proteins. Nadrin is involved in exocytosis and inhibits NGF dependent neurite outgrowth in PC12 cells, both effects proposed to rely on its GAP activity. Both RICH-1 and nadrin have BAR domains implicated in membrane-trafficking.</td>
<td>(Furuta et al 2002; Harada et al 2000; Richnau &amp; Aspenström 2001)</td>
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<tr>
<td>RICH-2 (KIAA0672) (human)</td>
<td>Rac1, Cdc42</td>
<td>ND</td>
<td>Ubiquitous, enriched in brain Chr 17p11.2</td>
<td>Has a BAR domain.</td>
</tr>
<tr>
<td>PARG [PTPL1-associated RhoGAP] (human)</td>
<td>RhoA&gt; Rac1, Cdc42</td>
<td>Ubiquitous, enriched in skeletal muscle and heart Chr 1p21.3</td>
<td>Binds PDZ (PSD-95/DlgA/ZO-1-like) domain of protein tyrosine phosphatase PTPL1. Has a C1 domain.</td>
<td>(Saras et al 1997)</td>
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<tr>
<td>RhoGAP</td>
<td>GAP specificity</td>
<td>Tissue distribution and Chr.locus</td>
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<tr>
<td>Myosin-IXb</td>
<td>RhoA</td>
<td>Ubiquitous, enriched in peripheral blood leukocytes Chr 1q13.1/Chr 15q22</td>
<td>Unconventional class IX myosin. Proposed to inhibit Rho at early stages of leukocyte spreading. Have C1 domains.</td>
<td>(Müller et al 1997; Post et al 1998; Reinhard et al 1995; Wirth et al 1996)</td>
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<tr>
<td>Myr5 (rat)/Myo9B (mouse) (Myosin-IXa less well characterized)</td>
<td>RhoA</td>
<td></td>
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<tr>
<td>MgcRacGAP (human)/MgcRacGAP (mouse)/CYK-4 (C.elegans)/RotundRacGAP (Drosophila)</td>
<td>RhoA</td>
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<tr>
<td>ARHGAP6 (human and mouse)</td>
<td>RhoA</td>
<td>Ubiquitous Chr Xp22.3</td>
<td>Deleted in male-lethal disorder microphthalmia with linear skin defects (MLS). N-terminus localizes to actin. Can in addition regulate actin dynamics independent of its GAP domain.</td>
<td>(Prakash et al 2000; Schaefer et al 1997)</td>
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<tr>
<td>ARHGAP8 (human and mouse)</td>
<td>RhoA</td>
<td>Ubiquitous (mouse) Chr 22q13.31</td>
<td>Particularly strong expression in kidney suggests a role in kidney development (mouse). Has a sec14 domain.</td>
<td>(Shan et al 2003)</td>
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<tr>
<td>ARHGAP9 (human)</td>
<td>RhoA</td>
<td>Restricted to hematopoietic tissues Chr 12q14</td>
<td>Involved in regulating adhesion of hematopoietic cells to the ECM. Has a PH domain.</td>
<td>(Furukawa et al 2001)</td>
</tr>
<tr>
<td>ARHGAP12 (human)</td>
<td>RhoA</td>
<td>Ubiquitous Chr 10p12</td>
<td>Strongly expressed in breast carcinomas. Has a PH domain.</td>
<td>(Zhang et al 2002)</td>
</tr>
<tr>
<td>RhoGAP (human and mouse)</td>
<td>GAP specificity</td>
<td>Tissue distribution and Chr. locus</td>
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<tr>
<td>ARHGAP15</td>
<td>Rac1</td>
<td>Spleen, lung, liver and lymphoid cells Chr 2q22.3</td>
<td>In vitro substrate for Rac1 effector kinases p42/44 and MRCKα. Has a PH domain.</td>
<td>(Seoh et al 2003)</td>
</tr>
<tr>
<td>KIAA1204 (human)</td>
<td>Rac1</td>
<td>ND/Ubiquitous, enriched in heart and lung Chr 3q13.32</td>
<td>CdgAP interacts with and is inhibited by the endocytic scaffold protein intersectin.</td>
<td>(Jenna et al 2002; Lamarche-Vane &amp; Hall 1998)</td>
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<tr>
<td>RhoGAP</td>
<td>GAP specificity</td>
<td>Tissue distribution and Chr.locus</td>
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<tr>
<td>KIAA0411 (MEGAP), Rac1&gt; (MEGAP), RhoA, Cdc42 (MEGAP), WRP (MEGAP), GAP1, GAP2, GAP3 (MEGAP)</td>
<td>ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND</td>
<td>Brain (MEGAP) /brain, lung and spleen (mouse)</td>
<td>KIAA0411 (MEGAP) has a putative role in severe mental retardation. WRP interacts with WAVE. SrGAPs interact with the Roundabout (Robo) repulsive guidance receptor and is involved in neuronal migration.</td>
<td>(Endris et al 2002; Ghose &amp; Van Vactor 2002; Soderling et al 2002; Wong et al 2001)</td>
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the two GAPs showed that they share a core structure made up of seven α-helices, and that the catalytic arginine residues are in approximately the same positions (Rittinger et al. 1998).

**The mechanism of GAP-stimulated GTP hydrolysis**

Determination of the three-dimensional structures of complexes between Rho GTPases and their GAPs has provided much insight into the mechanism of GAP-stimulated GTP hydrolysis. So far, the structures of p50RhoGAP complexed with RhoA and Cdc42, respectively, have been solved (Nassar et al. 1998; Rittinger et al. 1997a; Rittinger et al. 1997b). The RhoGAP interacts with the switch I and II regions as well as the P-loop of the Rho GTPase. Upon GTP-hydrolysis, there is a conformational change in the Rho GTPase, causing the development of negative charges in the active site, which are stabilized by the critical arginine in the RhoGAP. In addition, this arginine interacts with Gln61 in the Rho GTPase, a residue responsible for positioning the water molecule for GTP-hydrolysis. Moreover, the structures of the GAP domains of p50RhoGAP, p85α, GRAF have been determined (see Table 3; Barrett et al. 1997; Longenecker et al. 2000; Musacchio et al. 1996)

**Substrate specificity**

Some GAPs show specificity towards a single Rho GTPase whereas others display a broader specificity. However, since RhoGAP activity measurements usually involve the well-known Rac, Cdc42 and Rho as substrates, studies on the remaining Rho GTPases are needed to complete the picture. β-chimaerin is specific for Rac (Leung et al. 1993), whereas p190RhoGAP shows GAP activity towards Rho primarily, but also Rac and Cdc42 (Ridley et al. 1993; Settleman et al. 1992a).

An intriguing report by Minoshima et al. showed that phosphorylation induced a shift in the specificity of MgcRacGAP (male germ cell RacGAP) from Cdc42/Rac towards RhoA (Minoshima et al. 2003). As mentioned, RhoGAPs most often contain other domains in addition to their GAP domain, such as SH2 and SH3 domains, Ser/Thr kinase domains, proline-rich regions, DH (Dbh homology) motifs, PDZ (PSD-95/DlgA/ZO-1-like) binding motifs, RhoGEF domains, and domains which confer binding to membrane lipids such as PH domains, PX (Phox homology) domains and BAR (BIN/Amphiphysin/Rvsp) domains (reviewed in Chiang et al. 2003; Moon & Zheng 2003; Zalcman et al. 1999; Paper III). Possible functions for these motifs are to localize the GAP to its site of action, to regulate the GAP activity, by up-or down-regulating it, or even change its specificity, and/or to provide links between different signaling pathways. For example, β2-chimaerin translocates to Golgi upon binding to phorbolesters (Caloca et al. 2001). The BAR domain in RICH-1 and PX domain in TCGAP confer binding to membrane-lipids, and might act to localize these proteins to membranes (Chiang et al. 2003; Richnau & Aspenström 2001). The interaction between p120RasGAP and p190RhoGAP may serve to link the Ras and Rho signaling pathways, where the p120RasGAP might act as a Ras effector and regulate the activity of Rho (Settleman et al. 1992b). ARAPs (ArfGAP, RhoGAP, ankyrin repeat, and PH domains-containing proteins) contain in addition to a RhoGAP domain, an Arf GAP domain, a putative Ras
binding motif and PH domains. The ArfGAP activity is enhanced upon binding of a PH motif to the PI 3-kinase product PIP3 (Krugmann et al 2002; Miura et al 2002). In this way, ARAPs have been suggested to link Rho GTPase, Arf GTPase and PI 3-kinase pathways.

RalBP1 (Ral binding protein 1) is yet an example of a multifaceted RhoGAP, connecting signal transduction and endocytosis. In a cascade connecting Ras, Ral and Rho pathways, Ras binds to a RalGEF/RalGDS causing activation of Ral, which then binds to and stimulates RalBP1 resulting in down-regulation of Rho GTPases (Jullien-Flores et al 1995). In addition, RalBP1 interacts with endocytic proteins such as adaptor protein 2 (AP2) and the Eps15 homology (EH) proteins Reps1 (RalBP1 associated Eps-homology domain protein 1) and POB (partner of RalBP1), and has been implicated in endocytosis of epidermal growth factor (EGF)- and insulin receptors (Ikeda et al 1998; Jullien-Flores et al 2000; Nakashima et al 1999; Yamaguchi et al 1997). Importantly, some RhoGAPs have been suggested to act as effectors mediating signals downstream of Rho GTPases. For instance, N-chimaerin appears to cooperate with Cdc42 and Rac1 to induce formation of filopodia and lamellipodia, independently of its RhoGAP activity (Kozma et al 1996). Microinjection of the N-chimaerin RhoGAP domain alone, however, inhibits Rac-mediated lamellipodia formation.

TCGAP was proposed to act as a downstream effector of TC10 in regulating insulin-dependent glucose transport (Chiang et al 2003). The regulatory subunits of PI3-kinase, p85\alpha and p85\beta, contain the conserved catalytic arginine, but lacks some other residues of importance for GAP activity, and so far no GAP activity have been reported for these proteins (Musacchio et al 1996; Otsu et al 1991; Zheng et al 1993). However, activated Cdc42 and Rac1 interact with p85\alpha and stimulate the PI 3-kinase activity, suggesting a role for p85 as an effector instead of a down-regulator (Bokoch et al 1996; Zheng et al 1994). In addition, p85 subunits may act as adaptors to localize PI 3-kinase activity to its site of action.

Furthermore, there are GAP-like toxins, such as the P. aeruginosa toxin Exoenzyme S (ExoS), which is a bifunctional toxin with an N-terminal RhoGAP and C-terminal ADP-ribosyltransferase activities (Henriksson et al 2002). ExoS perturbs the activation of Rho GTPases, and other members of the Ras superfamily of proteins, in two ways; by ADP-ribosylation (Rac, Rho and Cdc42), and stimulation of the intrinsic GTP-hydrolysis (Rho and Cdc42).

**Regulation of GAP activity**

Many RhoGAPs show different substrate specificities *in vitro* and *in vivo*, possibly reflecting the influence of regulatory molecules *in vivo*. A tightly regulated GAP activity ensures a correct regulation of its cognate Rho GTPase, and regulation of GAP activity has been shown to include phosphorylation, lipid binding and protein-protein interaction.

The GAP activity of p190RhoGAP is enhanced upon phosphorylation by Src tyrosine kinase followed by binding to p120RasGAP (Hu & Settleman 1997; Roof et al 1998). The GAP activity of p190RhoGAP is then thought to be turned off by binding to and dephosphorylation by a low molecular weight protein-tyrosine phosphatase (LMW-PTP), a key mediator of PDGF receptor signaling and a Src substrate (Chiarugi et al 2000). Furthermore, p200GAP binds to Src and appears to be phosphorylated by this
tyrosine kinase (Moon et al. 2003). Protein-protein interactions can also regulate the GAP activity, for example the endocytic scaffolding protein intersectin binds CdGAP (Cdc42 GTPase activating protein) through its SH3 domains, thereby causing a conformational change in CdGAP resulting in an inhibition of the GAP activity toward Rac, both in vitro and in vivo (Jenna et al. 2002). Moreover, the proline-rich tyrosine kinase 2 (PYK2) inhibited the GAP activity of PSGAP (PH- and SH3 domain-containing RhoGAP protein) on Cdc42, although it is not clear whether it is due to protein-protein interaction, phosphorylation of PSGAP, or both (Ren et al. 2001). The members of the chimaerin family have a cysteine-rich motif similar to those found in protein kinase C. The GAP activity as well as the intracellular location of chimaerins can be regulated by binding of various membrane lipids to the cysteine-rich motif (reviewed in Moon & Zheng 2003).

The reason for the overabundance of the RhoGAPs, and RhoGEFs, in comparison with their Rho GTPase substrates is unclear. However, these regulators might be expressed in specific cell types, at specific developmental stages and might be under spatial, biochemical or/and temporal regulation. To date, gene knock-outs in mouse have been made for BCR, p190-A and p190-B, and together with targeted knock-ins of mutated GAP domains and RNA interference, useful information will be provided about the functional significance of individual GAPs (reviewed in Peck et al. 2002).

3.H. Rho guanine-exchange factors (GEFs)

When the GDP-bound Rho GTPase is released from the GDI at the plasma membrane a GEF facilitates the exchange of GTP for GDP, whereby a transient complex between a nucleotide-free Rho GTPase and the RhoGEF is formed. The subsequent binding of GTP to the Rho GTPase is favored by the high intracellular ratio of GTP to GDP (reviewed in Zheng 2001). The first characterized mammalian GEF, Dbl, was isolated as an oncogene product from a human diffuse B-cell lymphoma (Eva & Aaronson 1985). Subsequently, Dbl was shown to promote GDP-GTP exchange on Cdc42. This exchange is dependent on a region comprising 180 amino acids in Dbl, now referred to as the DH domain (Hart et al. 1991; Hart et al. 1994). Analysis of genome sequences has revealed that there are around 60 Dbl-homology domains in human (Venter et al. 2001). The DH domain is divided into three conserved regions CR1, CR2 and CR3, each 10-30 amino acids long (reviewed in Hoffman & Cerione 2002; Schmidt & Hall 2002).

GEFs show a varying degree of specificity, with Dbl and Vav acting on several Rho GTPases, while others show specificity for a single RhoGTPase, such as facio-genital dysplasia protein 1 (FGD1) (Cdc42), intersectin (Cdc42), and p115RhoGEF (Rho) (Hart et al. 1994; Hart et al. 1996; Hussain et al. 2001; Olson et al. 1996; Zheng et al. 1996). Almost all GEFs have a PH domain adjacent and C-terminal to their DH-domains. Apart from the DH and PH domains, GEFs contain additional domains, including SH2, SH3, Ser/Thr kinase, RasGEF, RhoGAP, RanGEF, PDZ or additional PH domains (Schmidt & Hall 2002). The specificity in vivo is often higher than in vitro, as exemplified by Tiam1, acting on Cdc42, Rac and Rho in vitro and only on Rac in vivo (Michiels et al. 1995).

Several mechanisms have been described for the regulation of the Dbl family of GEFs, including localization to membranes or the actin cytoskeleton mediated by the PH domain, intramolecular inhibition by DH-PH interaction or binding of
inhibitory sequences to the DH or PH domain, and stimulation by protein-protein interactions (reviewed in Hoffman & Cerione 2002; Schmidt & Hall 2002; Zheng 2001). Autoinhibition might be released by phosphorylation or by binding to proteins or PIPs, but in most cases the mechanism is not known. The intramolecular DH-PH interaction in Sos is released by binding of the PH domain to the PI 3-kinase product PIP3 (Nimnual et al 1998). One interesting example of activation of GEFs is the intermolecular oligomerization of onco-Dbl DH-domains (Zhu et al 2001). Oligomers of Dbl can recruit multiple Rho GTPases into a multimeric complex and thereby serve to coordinate multiple pathways. The Dbl family of GEFs appears to be the major class of positive regulators for Rho GTPases, although there are non-DH domain containing proteins that have been found to catalyze the GDP-GTP exchange on Rho GTPases, such as DOCK180, and the *S. typhimurium* GEF-like protein SopE (reviewed in Lerm et al 2000; Schmidt & Hall 2002). Moreover, studies in yeast have shown that RhoGEFs can act both as upstream regulators and as downstream effectors of Rho GTPases, most likely reflecting their multidomain structure (reviewed in Scita et al 2000).

3.1. Rho guanine dissociation inhibitors (RhoGDIs)

GDIs have only been found for the Rab and Rho subfamilies of the Ras superfamily, where Rab proteins have distinct GDIs (Ridley 2001a). In contrast to the RhoGAP and RhoGEF protein families, the RhoGDI family consists only of three members, namely RhoGDlz, RhoGDlb and RhoGDlγ (reviewed in Olofsson 1999; Sasaki & Takai 1998). RhoGDlz was initially isolated as a protein inhibiting the dissociation of GDP from and the subsequent binding of GTP on RhoB (Fukumoto et al 1990; Ohga et al 1989; Ueda et al 1990). However, RhoGDI appears to associate equally well with the GDP-bound and the GTP-bound form of Rho GTPases, thereby sequestering Rho GTPases from the activities of both GEFs and GAPs (Olofsson 1999; Scita et al 2000). In resting cells, Rho GTPases are found complexed with RhoGDIs in the cytosol. The RhoGDIs have a pocket which masks the C-terminal prenyl group of the Rho GTPase, thereby inhibiting their interaction with membranes (reviewed Zalcman et al 1999). Upon cell activation the Rho GTPase is translocated and released at the plasma membrane. Two possible mechanisms have been proposed for regulation of RhoGDI activity, both resulting in an inactivation and release of the sequestered Rho GTPases. The first mechanism involves protein kinase C (PKC)-α mediated phosphorylation of RhoGDI, and the second involves binding to members of the ERM-family of proteins (Mehta et al 2001; Takahashi et al 1997). The interaction of RhoGDIs with ERM proteins might be a way of directing Rho GTPases to the proper sites on the plasma membranes (Scita et al 2000).

4. Conserved protein domains

Binding of extra-cellular ligands to specific receptors on the plasma membrane on a target cell triggers signal transduction cascades inside the cell leading to diverse cellular responses. The propagation of these signals is controlled by protein-protein and protein-lipid interactions occurring through specific protein domains. These interactions can have various purposes such as targeting proteins to specific cellular locations, nucleating multimeric protein complexes, regulating the conformation and hence the activity and/or
specificity of an enzyme (reviewed in Pawson 1995; Pawson & Nash 2003). Whereas some protein domains mediate molecular interactions, others possess an enzymatic activity. Interaction domains were initially identified in the context of phosphotyrosine signaling, where SH2 domains of cytoplasmic proteins recognized phosphorylated tyrosine residues on activated autophosphorylated RTKs (Pawson & Nash 2003). Selected interaction domains which are relevant to this thesis work will be described more in detail below.

**SH3 domains** consist of 50-70 amino acid residues and bind with moderate affinity and selectivity to specific proline-rich regions (reviewed in Kay et al 2000; Mayer 2001). There are 253 SH3 domains encoded by the human genome, suggesting a role for SH3 domain-mediated interactions in a plethora of cellular processes (Pawson & Nash 2003). The ligand specificity of certain SH3 domains have been determined with the use of combinatorial peptides libraries. The SH3 domains of Abl, Src, and PI 3-kinase were the first to be characterized this way, and optimal binding was achieved by a core conserved motif, PXXP, where X denotes any amino acid (Kay et al 2000; Mayer 2001; Ren et al 1993). The ligand binding surface of the SH3 domain consists of three hydrophobic pockets. Two of the pockets are occupied by dipeptides (a hydrophobic amino acid residue followed by a proline) (Figure 6). Specificity of binding can be achieved by residues outside the PXXP core binding motif, for example the third binding pocket, where most SH3 domains prefer a positive residue such as arginine. The position of this positive residue governs in which of two orientations the peptide ligand binds with respect to the SH3 domain. The peptides can bind either N-to C-terminal [left-handed (+XXPXXP)] or C-to N-terminal [right-handed (XPXXPX+)] orientation with respect to the SH3 domain, and are denoted class I and class II ligands, respectively.
Figure 6. The interaction of SH3 domains with proline-ligands. The SH3 domain has a relatively flat, hydrophobic ligand-binding surface (white), consisting of three shallow pockets (shadowed). Two of these pockets accommodate the two hydrophobic amino acid-proline dipeptides (XP), whereas the third pocket, the specificity pocket, is occupied by a basic residue, typically arginine. The ligands can bind in either of two orientations depending on the position of the basic residue, and are classified as class I or class II ligands (See text for details). Adapted and changed from Mayer, 2001.

Moreover, the tertiary structure of a protein could fold in such a way that residues otherwise spaced apart come together to mimic a canonical PXXP motif, as exemplified by the interaction between such a motif in p53 tumor suppressor and the SH3 domain of p53BP2 (p53-binding protein 2) (Gorina & Pavletich 1996). Recently, a number of SH3 domains have been shown to have ligand preferences other than the classical PXXP motif, for example binding of Eps8 (epidermal growth factor receptor pathway substrate 8) to receptor-and nonreceptor tyrosine kinases (PXXDY), binding of the endocytic scaffold protein CIN85 (Cbl-interacting protein of 85 kDa) to Cbl, synaptotagmin 1, PAK2 and ZO-2 (zonula occulndens-2) (PX(P/A)XXR), binding of the RhoGEF PIX to PAK (PPPVIAPRPETKS), binding of the hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs)-binding protein to the deubiquitinating enzyme UBPY (ubiquitin isopeptidase Y) (PX(V/I)(D/N)XXK), and binding of the Gads T-cell adaptor to the docking protein SLP-76 (SH2 domain-containing leukocyte protein of 76 kDa) (RXXK) (Kato et al 2000; Kowanetz et al 2003; Kurakin et al 2003; Liu et al 2003; Manser et al 1998; Mongiovì et al 1999).

Proline-rich sequences are often found in proteins involved in situations that require a rapid recruitment and/or exchange of proteins, for example during transcriptional initiation, signaling cascades, membrane-trafficking events, cytoskeletal reorganization, cell polarization and organelle biogenesis (Kay et al 2000; Pawson & Nash 2003). Interactions between SH3 domains and proline motifs may serve to assemble large multimeric complexes, alter the subcellular localization to increase the local concentration of components of signaling pathways, and regulate enzyme activity through intramolecular interactions. Some SH3 domain-proline motif interactions occur
as stable complexes, whereas others are tightly regulated. Regulation of SH3 domain-proline motif interactions could be achieved by conformational changes in the protein by intramolecular or intermolecular interactions, and/or by phosphorylation/dephosphorylation events, that either expose or bury the proline-motif or the SH3 domain.

Although most SH3-domain-mediated interactions are intermolecular, some occur within one and the same molecule. For example, the SH3 domains of Src and Abl interact with a proline-rich linker region between the SH2 domain and the catalytic domain resulting in a catalytically inactive kinase (Barila & Superti-Furga 1998; Kay et al 2000). There has been a great effort to design cell permeable peptidomimetic antagonists that interfere with and disrupt specific SH3 domain-proline motif interactions, for the purpose of evaluating the functional consequences of these interactions, and to explore the possibility that such antagonists can be used in the treatment of human disease (reviewed in Kay et al 2000; Mayer 2001). There is an expanding list of modules binding to proline-rich motifs, such as WW domains and Ena-VASP-homology 1 (EVH1) domains (Kay et al 2000). Moreover, although ligands for EH domains and 14-3-3 domains are not proline-rich, they include a single proline residue (Kay et al 2000).

**PDZ domains** are repeats of ~90 amino acid residues containing the motif Gly-Leu-Gly-Phe (Saras & Heldin 1996). PDZ domains are involved in four different classes of interactions; binding to C terminal or internal motifs, PDZ-PDZ dimerization, and recognition of lipids (Nourry et al 2003). PDZ-domain-containing proteins often act as scaffolds close to the plasma membrane to aggregate receptors and signaling molecules in large multimeric complexes (reviewed in Saras & Heldin 1996). **FCH domains** are composed of about 100 amino acid residues present in a wide variety of proteins including the PCH family of proteins, RhoGAP proteins and tyrosine kinases (Aspenström 1997; Lippincott & Li 2000). FCH domains have been proposed to interact with microtubules (Fujita et al 2002; Tian et al 2000).

**Lipid binding domains.** There is a growing list of domains that confer binding to phosphorylated head-groups of membrane lipids (PIPs), such as the PH, FYVE (Fab1p/YOTB/Vac1p/EEA1), PX, epsin-N-terminal homology (ENTH), band 4.1 protein and ERM homology (FERM), Tubby and BAR domains (Farsad et al 2001; Pawson et al 2002; Takei et al 1999; Paper III). These domains could act to target proteins to specific membranes and/or regulate their activity. Whereas PH domains confer binding to PIP2 and PIP3 at the plasma membrane, FYVE domains bind certain phospholipids on endosomal membranes (Pawson et al 2002). PH domains are found in a variety of proteins, in RhoGEFs, RhoGAPs, cytoskeletal proteins, and in several proteins of the endocytic machinery, for instance dynamin, AP2 and clathrin. As shown in Table 4, BAR domains are present in the members of a wide variety of protein families (reviewed in Zhang & Zelhof 2002; SMART database and BLASTp)
Table 4. BAR domain-containing proteins in vertebrates.

<table>
<thead>
<tr>
<th>Protein family</th>
<th>Members with BAR domains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphiphysin/Bridging-integrator (BIN) family</td>
<td>Amphiphysin I</td>
</tr>
<tr>
<td></td>
<td>Amphiphysin II splice variants:</td>
</tr>
<tr>
<td></td>
<td>Amphiphysin Ila (brain)</td>
</tr>
<tr>
<td></td>
<td>BIN1[also called Amphiphysin IIb (muscle)]</td>
</tr>
<tr>
<td></td>
<td>BIN1[(ubiquitous) two splice variants]</td>
</tr>
<tr>
<td></td>
<td>BIN2</td>
</tr>
<tr>
<td></td>
<td>BIN3</td>
</tr>
<tr>
<td>Endophilin family</td>
<td>Endophilin A1</td>
</tr>
<tr>
<td></td>
<td>Endophilin A2</td>
</tr>
<tr>
<td></td>
<td>Endophilin A3</td>
</tr>
<tr>
<td></td>
<td>Endophilin B1</td>
</tr>
<tr>
<td></td>
<td>Endophilin B2</td>
</tr>
<tr>
<td>RhoGAP family</td>
<td>RICH-1/nadrin and splice variants</td>
</tr>
<tr>
<td></td>
<td>RICH-2 (KIAA0672)</td>
</tr>
<tr>
<td></td>
<td>3BP-1</td>
</tr>
<tr>
<td></td>
<td>GRAF-1</td>
</tr>
<tr>
<td>RhoGEF family</td>
<td>KIAA1010</td>
</tr>
<tr>
<td>ArfGAP family</td>
<td>Similar to KIAA1716 (or centaurin B5)</td>
</tr>
</tbody>
</table>

**Coiled-coils** are structures that consist of two to five amphiphatic α-helices twisted around one another to form a supercoil (reviewed in Burkhard et al 2001). The coiled-coil motifs enables homotypic and heterotypic oligomerization and are found in a variety of proteins including cytoskeletal proteins, motor proteins, signaling proteins and proteins involved in membrane-trafficking processes. For example, the Rho GTPase effectors Dia, ROCK, IQGAP and citron kinase contain coiled-coil regions, which at least in the case of IQGAP have been proposed to facilitate oligomerization (Bishop & Hall 2000). Oligomerization of effectors might add another level of complexity to target activation by Rho GTPases (Bishop & Hall 2000). Further, the coiled-coil regions present in endocytic proteins dynamin, amphiphysin and endophilin are proposed to mediate oligomerization required for invagination of the plasma membrane during clathrin-mediated endocytosis (Farsad et al 2001; Takei et al 1998; Takei et al 1999).

5. Endocytosis

RICH-1 has a BAR domain with homology to the members of the amphiphysin and endophilin families of endocytic proteins. These proteins are involved in the most well-studied type of endocytosis, clathrin-mediated endocytosis, partly by interacting with lipids in the plasma membrane via their BAR domains.

**The multiple pathways of endocytosis**

Endocytosis is the common term for cells internalizing material from the extracellular environment into a vesicular compartment, either by the uptake of fluid, referred to as
‘cell drinking’ (pinocytosis) or large particles, referred to as ‘cell eating’ (phagocytosis) (reviewed in Marsh 2001). Endocytosis has many functions in the cell, including internalization of nutrients, receptor regulation, recycling of synaptic vesicles, antigen presentation, and removal of apoptotic cells (reviewed in Marsh & McMahon 1999). In addition, several pathogens have been shown to use the endocytic machinery of the host cell to enter and infect it. Pinocytic events can be further divided into clathrin-dependent endocytosis, and the clathrin-independent processes; macropinocytosis, caveolae-mediated endocytosis, and a process independent of both clathrin and dynamin. The most well-studied examples of internalization events are the clathrin-mediated endocytosis of receptors in non-neuronal cells and the fast synaptic vesicle recycling in neurons.

5.A. Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME) is a receptor-mediated process and, since a vesicle cannot form without liquid content, also a pinocytic event (reviewed in Marsh & McMahon 1999). Endocytosis can be either constitutive, i.e. occur without apparent stimuli, or dependent on stimuli, such as ligand binding to growth factor receptors or recycling of synaptic vesicles after exocytosis (reviewed in Marsh 2001; Takei & Haucke 2001). Endocytosis via clathrin-coated vesicles (CCVs) in cultured cells takes less than one minute whereas, in the synapse for instance, it is an even faster process (Marsh & McMahon 1999). CCV formation is not restricted to the plasma membrane, but occurs also from the trans-Golgi network and endosomes (Stoorvogel et al 1996; reviewed in Schmid 1997). By contrast, so-called coat protein I (COPI) and COPII coats are involved in vesicular trafficking between the Golgi apparatus and the endoplasmic reticulum (ER) (Figure 7) (Schmid 1997).

Figure 7. Clathrin-mediated endocytosis and intracellular membrane-trafficking events, and the different protein-coats involved; clathrin, COP I and COP II. Changed and adapted from Kirchhausen, 2000.
A multitude of protein-protein and protein-lipid interactions are needed to accomplish CME, a tightly regulated process involving the several subsequent steps. After the assembly of the protein coat and the deformation of the plasma membrane to create an invaginated coated pit, the scission of the vesicle neck creates a free coated vesicle. In the next step the vesicle is uncoated to enable either fusion with early endosome or, in the case of synaptic vesicle recycling, refilling with neurotransmitter and re-entrance into the pool of synaptic vesicles (reviewed in Brodin et al 2000; Brodsky et al 2001; Takei & Haucke 2001). The early endosome is mainly a sorting organelle and determines whether the endocytosed material, for example certain types of receptors, are recycled back to the plasma membrane, or selected for transport to late endosomal compartments followed by degradation in lysosomes (Marsh 2001).

CME starts with the recruitment of the adaptor protein AP2, via its interaction with the transmembrane ‘docking protein’ synaptotagmin, PIP$_2$ or PIP$_3$, and endocytic sorting signals in the cytoplasmic domain of the membrane proteins that are to be internalized (Brodin et al 2000; Brodsky et al 2001; Takei & Haucke 2001). The neuron-specific adaptor protein 180 (AP180), or its ubiquitous homologue CALM (clathrin-assembly lymphoid myeloid leukemia), is recruited by binding to PIP$_2$, and appears to regulate the size of the vesicle (Figure 8).

Figure 8. The sequential steps of clathrin-mediated endocytosis and the key components involved. Adapted from Takei & Haucke, 2001.
Both AP2 and AP180 then support formation of the clathrin coat, and a plethora of so-called ‘accessory proteins’ are recruited by protein-protein and/or protein-lipid interactions to regulate the subsequent steps of the internalization. These proteins include amphiphysin, dynamin, endophilin, epsin, Eps15, and synaptojanin.

The membrane lipids themselves are important for protein-lipid interactions and signaling during the endocytic event. PIPs and consequently the enzymes controlling their turnover are important regulators of CME. During CME, PIPs, such as PIP$_2$, act to recruit endocytic proteins to the plasma membranes to form the coated pit and to deform the membrane. Subsequently, to allow for uncoating of the vesicle after detachment, phosphatases such as the polyphosphoinositide phosphatase synaptojanin hydrolyze PIP$_2$, whereas a phosphatidylinositol 4-kinase (PI 4-kinase; type IIa in particular) together with a PIP 5-kinase, primarily phosphatidylinositol phosphate kinase type I$_\gamma$ (PIPKI$_\gamma$), regenerates the PIP$_2$ levels for a new round of endocytosis (Cremona & De Camilli 2001; Guo et al 2003; Wenk et al 2001). In addition, the interactions between several endocytic proteins are regulated by protein dephosphorylation/phosphorylation events. For example, phosphorylation of amphiphysin inhibited its binding to AP2 and clathrin (Slepnev et al 1998).

Much work has been done to identify the proteins involved in CME, and at what stage(s) they act. Different approaches have been established to block the function of a protein in vivo in order to see at which stage CME is disrupted. These studies include over-expression of dominant-negative mutants, microinjection of interfering antibodies, peptide or protein domains, as well as generation of knock-out mice (reviewed in Higgins & McMahon 2002).

**Dynamin**

The dynamin family of large GTPases consists of three conventional tissue-specific isoforms (dynamin-1, -2 and -3) and their splice variants, as well as the dynamin-related Drp1 (dynamin-related protein 1), OPA1 (Optic atrophy 1), hGBP1 (human guanylate-binding protein 1) and Mx (reviewed in Danino & Hinshaw 2001; Schmid et al 1998). Dynamin was first identified as a protein interacting with microtubules, however, there is no evidence for this interaction in vivo (reviewed in Hinshaw 2000).

The first implication of dynamin in CME came from studies where the temperature sensitive mutant in the *Drosophila* dynamin gene, *Shibire*, was shown to cause a block in endocytosis at the nerve terminals of the flies, visible on thin section electron micrographs (Kosaka & Ikeda 1983). This block correlated with a lack of vesicle scission, and accumulation of deeply invaginated clathrin-coated pits with necks surrounded by an electron-dense material referred to as a ‘collar’. Accordingly, over-expression or microinjection of dynamin mutants, or functionally interfering forms of its binding partners, specifically inhibited CME in mammalian cells (Damke et al 1994; Damke et al 2001; Shupliakov et al 1997; Simpson et al 1999). Dynamin exists as a tetramer and can spontaneously self-assemble, via its coiled-coil motifs, into rings and spirals at low salt concentrations, or on lipid bilayers to form helical tubes that resemble the collared structures seen in nerve termini (Carr & Hinshaw 1997; Hinshaw & Schmid 1995; Stowell et al 1999; Sweitzer & Hinshaw 1998; Takei et al 1998; Takei et al 1995).
Although the exact mechanism for vesicle scission is not understood, it clearly involves GTP-hydrolysis by dynamin, as shown by experiments using dynamin mutants defective in GTP-binding and hydrolysis (reviewed in Song & Schmid 2003). During CME, dynamin is targeted to the coated-pits in its GDP-bound form, where binding of GTP triggers the assembly of dynamin oligomers in collar-like structures at the neck of the coated-pit (Schmid et al 1998). One model suggests that a subsequent GTP-hydrolysis triggers a conformational change that drives the membrane fission, either by constriction by dynamin collars, or by expansion of the helical pitch of dynamin rings (reviewed in Kelly 1999; Song & Schmid 2003; Stowell et al 1999; Sweitzer & Hinshaw 1998). Yet another model proposes that dynamin in its GTP-bound form acts as a ‘classical GTPase’ recruiting and/or interacting with effector proteins in order to form the vesicle, whereby dynamin self-assembly then triggers GTP-hydrolysis and termination of GTP-dependent interactions (Sever et al 1999; Song & Schmid 2003).

In contrast to other GTPases dynamin possesses a high intrinsic GTPase activity (reviewed in Hinshaw 2000). The GTPase activity can be enhanced by different factors; via oligomerization (via coiled-coil motifs), binding to SH3 domain-containing proteins (via the proline-rich motifs), association with microtubules, interaction with PIP$_2$ (via the PH domain), and phosphorylation by PKC. The interaction of dynamin with SH3 domain-containing proteins, such as endophilins and amphiphysins, appears to be of importance for targeting dynamin to the plasma membrane and/or regulating its GTPase activity (Hinshaw 2000; Huttner & Schmidt 2002). In addition to its role in membrane trafficking events, dynamin has shown involvement in apoptosis, MAP (mitogen-activated protein) kinase signaling and actin reorganization (reviewed in Sever 2002). Moreover, phosphorylation/dephosphorylation of dynamin by PKC/calcineurin regulates its interactions with other endocytic proteins. For instance, dephosphorylation is required for the binding of dynamin to amphiphysin (Slepnev et al 1998).

**Amphiphysin**

Amphiphysin I was first identified as a synaptic vesicle associated protein in chicken brain extracts, and subsequently as an autoantigen in the Stiff-man syndrome associated with breast cancer in human (De Camilli et al 1993; Lichte et al 1992). The amphiphysin members of the BAR family of proteins include the brain-specific Amphiphysin I (Amph I; also referred to as amphiphysin in this thesis), the ubiquitous amphiphysin II (Amph II) and its splice variants (reviewed in Zhang & Zelhof 2002).

Amphiphysin is proposed to recruit dynamin to the coated pit by the SH3 domain-mediated interaction with a proline-rich motif in dynamin and its additional binding sites for clathrin and AP2 (Takei et al 1999; reviewed in Wigge & McMahon 1998). Studies in which the interaction between amphiphysin and dynamin was disrupted confirmed its importance for endocytosis. For example, injection of the recombinant SH3 domain of amphiphysin into living cells caused a blockade of endocytosis at the stage of invaginated coated-pits, suggesting that the dynamin-amphiphysin interaction is required for scission of the vesicle (David et al 1996; Shupliakov et al 1997). Furthermore, over-expression of amphiphysin SH3 domain caused a block of receptor-mediated uptake of transferrin (Simpson et al 1999; Wigge et al 1997). Amphiphysin not only binds to dynamin, but also inhibits its oligomerization, and is thought to maintain.
dynamin in its dissociated form until it is needed for the scission reaction (reviewed in Wigge & McMahon 1998; Marsh & McMahon 1999). Amphiphysin also interacts with synaptojanin via the SH3 domain, with endophilin via the proline-rich motif, and with PIP$_3$ via the BAR domain (reviewed in Wigge & McMahon 1998; Zhang & Zelhof 2002). In addition to roles in CME, amphiphysins have been proposed to be involved in neurite outgrowth (Amph I), cell growth regulation (Amph I), actin reorganization via c-Abl interaction (Amph II), T-tubule biogenesis in muscle (Amph II) and signaling pathways via interaction with the RasGEF Sos (Amph II) (Floyd et al 2001; Kadlec & Pendergast 1997; Lee et al 2002; Leprince et al 1997; Mundigl et al 1998).

Endophilin

The endophilin family of proteins includes two subfamilies, the endophilin A1, 2 and 3 (also known as endophilin I, II and III, or SH3p4, SH3p8 and SH3p13, or SH3GL1, 2 and 3), and the recently identified endophilin B1 and B2 (also known as SH3GLB1 and 2) (reviewed in Huttner & Schmidt 2000; Modregger et al 2003; Pierrat et al 2001). The endophilins were initially identified as fusion partners to the human mixed-lineage-leukemia gene, and subsequently implicated in CME by virtue of a SH3-domain-dependent interaction with synaptojanin (de Heuvel et al 1997; Ringstad et al 1997; So et al 1997).

The neuron-specific endophilin A1 (also referred to as endophilin in this thesis) is the most extensively studied of the endophilin isoforms, and the SH3 domain of endophilin interacts with the proline-rich motifs of several endocytic proteins, including synaptojanin, dynamin and amphiphysin (Micheva et al 1997; Ringstad et al 1997; So et al 2000). Disrupting SH3-domain-dependent interactions of endophilin, has demonstrated a requirement for endophilin both during the early stages of invagination, and during later stages of scission and vesicle uncoating (Gad et al 2000; Hill et al 2001; Ringstad et al 1999; Simpson et al 1999). Moreover, endophilin was proposed to be recruited by the endocytic scaffold protein CIN85 during down-regulation of RTKs (Petrelli et al 2002; Soubeyran et al 2002). Furthermore, endophilin bound to proline-rich motifs in the cytoplasmic domains of membrane proteins including the G-protein-coupled β-adrenergic receptor and the metalloprotease disintegrins (Howard et al 1999; Tang et al 1999). Interestingly, endophilin is thought to regulate JNK (Jun kinase; c-jun N-terminal kinase) activation via interaction with the Germinal Center Kinase-like kinase (Ramjaun et al 2001). Other endophilin-binding-partners include huntingtin, N-WASP and RICH-1 (Otsuki et al 2003; So et al 2000; Paper III).

5.B. Mechanisms for membrane invagination

The complex cellular mechanism of endocytosis is difficult to analyze in living cells and required the development of a reliable in vitro system. Artificial liposomes provide an excellent substrate for mimicking the membrane shape changes under controlled conditions that take place during invagination and scission of the coated-pit (reviewed in Higgins & McMahon 2002). Incubation of artificial liposomes together with brain cytosol supplemented with the non-hydrolyzable GTP-analogue, GTPyS (5′-guanosine alpha-thiotriphosphate) and ATP (adenosine 5′-triphosphate), resulted in the formation of
long tube-like structures and clathrin-coated buds, and immunogold labeling confirmed the presence of clathrin and the accessory proteins, dynamin, amphiphysin, endophilin and synaptojanin in these structures (Farsad et al 2001; Takei et al 1998). In fact, purified dynamin alone was able to form tube-like structures when incubated with liposomes, and oligomers of dynamin were visualized as helices on the surface of the lipid tubes (Sweitzer & Hinshaw 1998; Takei et al 1998). Furthermore, addition of GTP caused the tubes to vesiculate, consistent with the role of dynamin in vesicle scission upon GTP-hydrolysis. Subsequently, a similar tubulating activity was demonstrated for amphiphysin, endophilin and epsin (Farsad et al 2001; Ford et al 2002; Takei et al 1999).

The common denominator for these tubulating proteins is a domain that inserts into the lipid bilayer by binding to acidic phospholipids, i.e. the PH domain in dynamin (binds PIP$_2$), the ENTH domain in epsin (binds PIP$_2$) and the BAR domain of amphiphysin (binds PIP$_2$) (Farsad et al 2001; Ford et al 2002; Sweitzer & Hinshaw 1998; Takei et al 1998; Takei et al 1999). For endophilin, amphiphysin and epsin, this domain contains an amphiphatic $\alpha$-helix with a hydrophobic face and a hydrophilic face, interacting with the side-chains and the head of membrane lipids, respectively (reviewed in Hurley & Wendland 2002; Huttner & Schmidt 2002).

In the case of dynamin, amphiphysin and endophilin, coiled-coil mediated oligomerization of the proteins on the surface of the membrane is believed to provide the force for invagination (Farsad et al 2001; Takei et al 1998; Takei et al 1999). In contrast, epsin was proposed to stimulate tubule formation in the absence of oligomerization (Ford et al 2002). Dynamin together with amphiphysin or endophilin, respectively, could cooperatively deform liposomes into tubes, and whereas amphiphysin promoted vesiculation upon GTP-addition, endophilin inhibited the process (Farsad et al 2001; Takei et al 1998). Endophilin was initially proposed to facilitate membrane curvature by acting as a lysophosphatidic acid acyl transferase (LPAAT), inducing a conformational change in certain membrane lipids and a subsequent shape change in the plasma membrane (Schmidt et al 1999; reviewed in Scales & Scheller 1999). Furthermore, the Golgi-trafficking protein CtBP/BARS (C-terminal-binding protein/brefeldin A-ADP-ribosylated substrate) was proposed to induce fission of Golgi membranes by virtue of a LPAAT activity (Weigert et al 1999).

However, the enzymatic activity is probably not the driving force for invagination, since endophilin has been shown to tubulate liposomes lacking the substrates for the enzymatic activity [lysophosphatidic acid (LPA) and phosphatidic acid (PA)], and when the enzymatic activity was inhibited (Farsad et al 2001; Hurley & Wendland 2002; Huttner & Schmidt 2002). However, the efficiency of endophilin mediated tubulation was higher when using liposomes from brain lipid extracts, as compared to synthetic liposomes (Farsad et al 2001). Thus, although the LPAAT-activity of endophilin appears to be indispensable for tubulation, it could act to speed up the tubulation process, or play a role at some other stage during CME (Hurley & Wendland 2002; Huttner & Schmidt 2002). In addition to the endophilin-and amphiphysin families, the BAR domain is present in the RhoGAP RICH-1 and its close homologue RICH-2 (Paper III). In Paper III we show that the BAR domain in RICH-1 has the ability to bind to and impose curvature on membranes. Thus, such domains appear to have evolved as functional modules in a variety of protein families (Huttner & Schmidt 2002).
The membrane shape changes involving lipid-binding and tubulation by endophilins, amphiphysins and dynamins are not restricted to endocytosis, but occur in addition during other events at the plasma membrane, as well as at intracellular membranes (Huttner & Schmidt 2002). Moreover, endocytic protein families such as the dynamins and endophilins are present as several isoforms and have, in addition, distantly related members, some of which localize to intracellular membranes. Members of the dynamin family of proteins and dynamin-related proteins have been implicated in internalization via caveolae, intracellular trafficking from late endosomes and Golgi, and fission during mitochondrial division (reviewed in Danino & Hinshaw 2001; Schmid et al 1998). The ubiquitous dynamin 2 and endophilin A2 both localize to podosomes and are implicated in generating membrane curvature to form these structures (Ochoa et al 2000; reviewed in Orth & McNiven 2003). The members of the endophilin B family localize to ER and Golgi, and are thought to play a role in intracellular membrane dynamics and signaling (Huttner & Schmidt 2002; Modregger et al 2003).

5.C. Actin reorganization during clathrin-mediated endocytosis

In budding yeast, endocytosis is dependent on actin reorganization, as shown by genetic studies, and many yeast proteins have dual functions in endocytosis and actin reorganization (Geli & Riezman 1998; Munn 2001). In vertebrates, however, studies employing actin-perturbing drugs and Rho GTPase mutants have resulted in conflicting results. Under some conditions actin reorganization facilitates, but is not critical for CME, although the events downstream of vesicle formation, for example uncoating, vesicle movement, recycling and sorting may require actin rearrangements (reviewed in Schafer 2002). Several functions for the actin cytoskeleton have been proposed during CME; the actin cytoskeleton could provide the proper localization of the endocytic machinery, into so called ‘hot spots’. Reorganization of actin could facilitate plasma membrane invagination, since the cortical actin cytoskeleton might have to be disassembled to allow for endocytosis. Moreover, actin polymerization at the neck of the vesicle could facilitate fission of the vesicle, and actin polymerization could aid in the propelling of the vesicle into the cell (reviewed in Qualmann et al 2000; Schafer 2002). ‘Actin comet tails’ have been observed to associate with endosomes, pinosomes, clathrin-coated and secretory vesicles, and resemble the mechanism whereby pathogens, such as Listeria monocytogenes, propel through the cytoplasm of infected cells (Frischknecht et al 1999; Merrifield et al 1999; Rozelle et al 2000; Schafer 2002).

There has been a great effort in order to identify proteins which could provide a direct link between CME and the actin cytoskeleton, and there is now a growing list of candidates (reviewed in McPherson et al 2001; Qualmann et al 2000; Schafer 2002). Recent research has focused much attention on the involvement of actin in intracellular vesicle movement, and, interestingly, dynamin 2 has been shown to regulate polymerization of actin comets in live cells (Lee & De Camilli 2002; Orth et al 2002). A number of proteins involved in actin organization have been shown to bind to the proline-rich motif of dynamin 2 in an SH3-domain-dependent fashion (see Figure 9; reviewed in Orth & McNiven 2003; Schafer 2002). Profilin was the first actin-binding protein found to bind to the proline-rich motif of dynamin. Furthermore, cortactin binds to and co-localizes with dynamin 2 in cortical membrane ruffles after PDGF stimulation,
and regulates actin reorganization co-operatively with dynamin 2 (Krueger et al 2003; McNiven et al 2000; Schafer et al 2002). In addition, cortactin has been shown to co-localize with dynamin 2 in podosomal structures, actin comets, clathrin-coated pits and the Golgi complex (Cao et al 2003; Ochoa et al 2000; Orth et al 2002). Abp1 (actin-binding protein 1) binds to, and co-localizes with, dynamin 2 in cortical membrane ruffles, clathrin-coated pits and at the Golgi complex (Fucini et al 2002; Kessels et al 2001). Moreover, over-expression of the SH3 domain of either cortactin or Abp1 inhibited transferrin uptake, further supporting a role for their interaction with dynamin in endocytic function (Cao et al 2003; Kessels et al 2001). Protein scaffolds that bring together a diverse set of proteins by virtue of their protein-protein interaction domains play important roles in endocytosis. For example, PKC and CK2 substrate in neurons/synaptic, dynamin-associated proteins (PACSINs/syndapins) (mouse PACSIN I, II, and III, and the rat syndapin I and II) and intersectins (intersectins I and II) are protein scaffolds that provide an indirect link between endocytosis and actin assembly by interacting with dynamin and N-WASP (Hussain et al 2001; Qualmann et al 1999). Over-expression of the PACSIN/syndapin SH3 domains was shown to inhibit receptor-mediated internalization of transferrin, and over-expression of full-length syndapins induced filopodia formation in HeLa cells (Modregger et al 2000; Qualmann & Kelly 2000; Qualmann et al 1999; Simpson et al 1999). Intersectin was shown to localize to clathrin-pits and to regulate the formation of CCVs (Hussain et al 1999; Pucharcos et al 2000; Sengar et al 1999; Simpson et al 1999; Yamabhai et al 1998).

The huntingtin-interacting protein (HIP1) and the related HIP1R localize to clathrin-coated pits (via an ENTH domain interacting with PIP2) and have in addition been shown to associate with CCVs (Engqvist-Goldstein et al 1999; Metzler et al 2001; Mishra et al 2001; Waelter et al 2001). A heterodimeric complex of HIP1-HIP1R may function to anchor newly forming coated pits to the cortical cytoskeleton, through HIP1-binding to clathrin and AP2, and HIP1R-binding to actin (McPherson 2002; Schafer 2002). HIP1 was originally characterized by virtue of its interaction with huntingtin, the gene responsible for Huntington’s disease (Gusella & MacDonald 1998).
Figure 9. A multitude of proteins are involved in linking the endocytic machinery with actin polymerization. For simplicity, all of the interactions that occur are not shown. Motifs that are shown in the figure but not discussed in the text include: NPF (arginine-proline-phenylalanine) motifs binding to EH domains and ANTH (AP180 N-terminal homology) domains interacting with membrane lipids. The BAR domain in RICH-1 might confer binding to the plasma membrane, however, this has not been established. PRD refers to proline-rich domain.

6. Rho GTPases and membrane-trafficking

The first indication of an involvement of Rho GTPases in CME came from studies by Lamaze et al., who showed that constitutively active mutants of either Rho or Rac could block transferrin-receptor mediated endocytosis (Lamaze et al 1996; Qualmann & Mellor 2003; reviewed in Ridley 2001c). Furthermore, activated Rho was shown to inhibit internalization of the muscarinic acetylcholine receptor (Vogler et al 1999).

Rho GTPases might participate in CME (and other membrane-trafficking processes) by modulating phosphoinositide metabolism, through regulation of the enzymes affecting phosphoinositide turnover (reviewed in Symons & Rusk 2003). Rac has been shown to interact with PI 3-kinases, PIP 5-kinases and synaptojanin 2. For example, activated Rac may block internalization by recruiting synaptojanin 2 to membranes, resulting in depletion of PIP 2 and a reduced formation of coated-pits (Malecz et al 2000). As mentioned above, Cdc42 and Rho have been shown to interact with PI 3-kinase, and Rho binds PIP 5-kinases. Another way for Rho GTPases to influence CME and other membrane-trafficking events, is through their effects on the actin cytoskeleton, which of course can be influenced by changes in PIP 2 levels.

Cdc42 may be coupled to CME through the endocytic scaffold intersectin-1, which is also a GEF for Cdc42, and implicated in a feed-back loop on N-WASP. N-WASP binds to and enhances the GEF activity of intersectin, resulting in
activation of Cdc42 and a subsequent N-WASP-mediated Arp2/3 actin assembly (Hussain et al 2001). Interestingly, over-expression of an intersectin-mutant unable to act on Cdc42 resulted in severe defects in T-cell receptor endocytosis, and resembles the effects caused by lack of WASP in these cells (Qualmann & Mellor 2003). The Cdc42-effector ACK is another protein that may act to link Cdc42 to CME, possibly via an interaction with clathrin (Qualmann & Mellor 2003).

Professional phagocytic cells internalize infectious particles by two types of mechanisms, both being receptor-mediated events; type I involves Cdc42 (via WASP and Arp2/3) and Rac (via PAK1 and a PIP 5-kinase), whereas type II involves Rho (via ROCK) (Caron & Hall 1998; Qualmann & Mellor 2003). Macropinocytic vesicles are formed when membrane ruffles fold back on themselves to form membrane bound vesicles (reviewed in Ridley 2001c). Ridley et al. showed in early studies that Rac stimulated macropinocytosis in Swiss 3T3 fibroblasts, and, initially, pinocytosis was thought to occur as a consequence of an increased membrane ruffling (Ridley 2001c; Ridley et al 1992). Recent reports, however, indicate that macropinocytosis can be uncoupled from membrane ruffling, and that these events are not exclusively mediated by Rac. For example, Rac is required for pinocytosis, but not for membrane-ruffling in spleen-derived dendritic cells (West et al 2000), and Ras can separately activate Rab5-dependent pinocytosis and Rac-dependent membrane ruffling in baby hamster kidney cells (Li et al 1997). Moreover, macropinocytosis in bone-marrow-derived dendritic cells requires both Rac and Cdc42 (Garrett et al 2000).

The localization of some Rho GTPases to endocytic vesicles indicated a role for these in intracellular membrane-trafficking (reviewed in Ellis & Mellor 2000). Rhob locates to late endosomes and is involved in trafficking of the EGF receptor to lysosomes for degradation, whereas RhoD localizes to early endosomes and controls the motility of these compartments (Gampel et al 1999; Gasman et al 2003; Murphy et al 1996). Under physiological conditions, the movement of endosomes is regulated by the interplay between the actin filaments and microtubules. Whereas actin filaments appear to have a role in early transport of endosomes, microtubules seem to act during endosomal sorting (Marsh 2001). Another Rho GTPase implicated in intracellular-traffic (and targeted exocytosis) is the Cdc42-related Rho GTPase TC10 (see Chapter 3.D.). Additional endocytic and exocytic events involving Rho GTPases are reviewed in (Ridley 2001c; Symons & Rusk 2003).

The future challenge will be to dissect the downstream signaling events that mediate the roles of Rho GTPases in membrane-trafficking. Future studies might also reveal additional trafficking functions for some of the less well-characterized Rho-family members. Further, it will be of outstanding interest to investigate the involvement of RhoGAPs (and RhoGEFs) as regulators and/or downstream effectors in these and other processes.
PRESENT INVESTIGATIONS

Aim

The aim of this thesis work was to identify and characterize binding partners for the Cdc42 effector protein CIP4, in order to elucidate the function of this protein in Rho GTPase-mediated activities. We isolated a previously unidentified RhoGAP (RICH-1) as a binding partner for CIP4. This thesis work focuses on the characterization of this RhoGAP, and its role in regulating actin dynamics and membrane-trafficking.

Introduction

The yeast two-hybrid system and affinity chromatography approaches have been widely used to identify the targets that mediate the plethora of responses the Rho GTPases bring about in the cell. CIP4 was previously isolated from a yeast two-hybrid screen as a binding partner for constitutively active Cdc42. In a yeast two-hybrid screen for proteins interacting specifically with the SH3 domain of CIP4, we isolated several binding partners (Paper I) including a previously unidentified RhoGAP (RICH-1). Figure 1 shows a schematic representation of RICH-1, and some of the binding partners we identified in Papers II-IV are indicated. In addition, we characterized a CIP4-binding DRF, DAAM1.

Figure 1. Schematic representation of RICH-1. The BAR domain confers binding to membrane lipids, the GAP domain is specific for Rac and Cdc42, the proline motifs confer binding to several SH3 domain-containing proteins, and the extreme C-terminal binds to the PDZ domain-containing proteins, NHERF-1 and NHERF-2.
We showed that RICH-1 is a GAP for Rac and Cdc42, but not for Rho, *in vitro* and *in vivo* (Paper II). In addition to the RhoGAP domain, RICH-1 exhibits an N-terminal BAR domain, a putative coiled-coil motif, a stretch of proline-rich motifs and a C-terminal PDZ domain-binding motif. In accordance with studies on the BAR domain-containing proteins amphiphysin I and endophilin A1, the BAR domain in RICH-1 binds lipids, and can deform lipid bilayers *in vitro*, thus, suggesting a role for RICH-1 in membrane- trafficking (Paper III). The proline motifs confer binding to a number of SH3 domain-containing proteins (Paper III and Paper IV). Furthermore, RICH-1 contains a PDZ binding motif, which binds to the first of two consecutive PDZ domains in NHERF (Na+/H+ exchanger-regulatory factor) proteins (Paper IV; Reczek & Bretscher 2001). Paper IV describes the interactions of RICH-1 with CIN85, the NHERF family of proteins, and Abl, each of which are described in more detail below.

**Cbl-interacting protein of 85 kDa (CIN85)**

The multiadaptor proteins CIN85 and its close homologue CMS/CD2AP (p130Cas ligand with multiple SH3 domains/CD2 associated protein) are composed of three SH3 domains, a proline-rich motif, a coiled-coil region and three FxDxF motifs which confer binding to AP2 (reviewed in Dikic 2002). CMS/CD2AP in addition displays four putative actin binding motifs. By virtue of their SH3 domains, CIN85 and CMS/CD2AP interact with several proteins, including the Cbl family of ubiquitin ligases, the B-cell linker protein, and the T-cell receptor CD2 (Dikic 2002; Kirsch et al 2001; Soubeyran et al 2002; Take et al 2000; Watanabe et al 2000). In addition, the proline-rich motif in CIN85 confers binding to the SH3 domains in PI 3-kinase, Grb2, p130Cas, Fyn, Src, Yes and endophilins A1-A3 (Dikic 2002). Moreover, the coiled-coil region in CIN85 mediates homo-oligomerization into tetramers (Watanabe et al 2000).

CIN85 and CMS/CD2AP have been implicated in a number of cellular functions, partly by virtue of their ability to cluster membrane receptors (Dikic 2002). These include the organization of specialized junctions in the contact area between T-cells and antigen-presenting cells (immunological synapses), formation of kidney glomeruli architecture, and down-regulation and degradation of RTKs. Growth-factor induced tyrosine phosphorylation of Cbl enhanced its association with CIN85/CMS, presumably due to a conformational change in Cbl leading to unmasking of the proline-rich motifs able to interact with CIN85/CMS (Kirsch et al 2001; Petrelli et al 2002; Soubeyran et al 2002; Take et al 2000). Recently, two groups independently of each other, presented the consensus for binding to the SH3 domains of CIN85 (PX(P/A)XXPR) (Kowanetz et al 2003; Kurakin et al 2003). CIN85 is involved in Cbl- and Cbl-b-mediated downregulation of RTKs (Petrelli et al 2002; Soubeyran et al 2002; Szymbikiewicz et al 2002). Upon ligand-induced activation of RTKs, the ubiquitin ligase Cbl binds to the phosphorylated RTKs. Cbl then mediates the addition of ubiquitin, a tag which signals the degradation of the RTKs (reviewed in Thien & Langdon 2001). CIN85/CMS are recruited to the Cbl-RTK complexes, whereby Cbl-mediated ubiquitination of CIN85 (and CMS) targets these proteins for common lysosomal degradation with Cbl and RTKs (Haglund et al 2002). In addition, Soubeyran *et al.* and Petrelli *et al.* proposed that endophilin is recruited to the RTK-Cbl-CIN85 complex in
order to promote invagination of the plasma membrane (Petrelli et al 2002; Soubeyran et al 2002).

The NHERF family of proteins

ERM proteins provide a linkage between the plasma membrane and the cortical actin cytoskeleton and participate in signal transduction (reviewed in Bretscher et al 2002). The association of ERM proteins with the cytoplasmic parts of membrane proteins can be either direct or indirect via the NHERF family of adaptor proteins [EBP50 (ERM-binding phosphoprotein 50; human)/NHERF-1(rabbit) and E3KARP (NHE3 kinase A regulatory protein; human)/NHERF-2(rabbit)]. In addition to a C-terminal ERM binding motif, NHERF proteins contain two tandem PDZ domains (PDZ-1 and PDZ-2), which confer binding to several proteins, including the cytoplasmic part of many plasma membrane receptors (reviewed in Voltz et al 2001).

Several functions for NHERFs have emerged; they regulate the activity of ion-transporters such as NHE3 upon phosphorylation by protein kinase A (PKA), they are involved in endocytosis of plasma membrane receptors to which they bind, and they appear to restrict membrane proteins to specific plasma-membrane domains by anchoring them to the cytoskeleton via ERM proteins (reviewed in Bretscher et al 2002). For example, EBP50/NHERF-1 appears to be required for recycling of the β2-adrenergic receptor upon ligand-induced endocytosis (Cao et al 1999). Moreover, the β2-adrenergic receptor appears to control Na⁺/H⁺ exchange by NHE3 through its interaction with EBP50/NHERF-1 (Hall et al 1998b). Maudsley et al. showed that EBP50/NHERF-1 regulates PDGF receptor activity by promoting PDGF receptor dimerization and autophosphorylation, consequently leading to a more efficient activation of the MAP kinase signal cascade (Maudsley et al 2000). EBP50/NHERF-1 was proposed to potentiate PDGF receptor clustering partly due to its own ability to oligomerize. A recent report showed that EBP50/NHERF-1 forms a complex with Csk-binding protein/phosphoprotein associated with glycosphingolipid-enriched microdomains (Cbp/PAG) (a Csk-associated membrane adapter protein exclusively localized to lipid-rafts) and ERM proteins. The lipid-rafts are thereby anchored to the actin cytoskeleton resulting in a restricted mobility, causing inhibition in the formation of immunological synapses (Itoh et al 2002). NHERF proteins have been implicated in human diseases such as hypertension, acute kidney failure and breast cancer (Voltz et al 2001). Several GAPs interact with PDZ domain-containing proteins; the RhoGAP PARG interacts with PTPL1, synGAP (a RasGAP) interacts with PSD-95, the RabGAP EPI64 and the RhoGAP RICH-1 interact with EBP50/NHERF-1 (Chen et al 1998; Kim et al 1998; Reczek & Bretscher 2001; Saras et al 1997).

Abelson tyrosine kinase (Abl)

c-Abl is a non-receptor tyrosine kinase, and the product of the cellular homologue of the transforming gene of Abelson murine leukemia virus (reviewed in Van Etten 1999). c-Abl has been shown to be activated in response to growth-factor stimulation, as well as integrin engagement (Lewis et al 1996; Plattner et al 1999). An F-actin binding domain (FABD) in c-Abl associates directly with F-actin in cells that have been detached from
the ECM, and F-actin acts as an inhibitor of c-Abl tyrosine kinase activity (Woodring et al. 2001). Upon integrin engagement, the kinase activity of Abl is rapidly increased upon dissociation from F-actin, and c-Abl is recruited to focal adhesions where it can phosphorylate a number of proteins involved in actin reorganization (Lewis et al. 1996; Van Etten 1999). Hence, Abl is involved in regulating cell adhesion (Kain & Klemke 2001; Lanier & Gertler 2000). The kinase activity of c-Abl is required for PDGF-induced formation of ruffles, and the formation of microspikes upon integrin clustering (Plattner et al. 1999; Woodring et al. 2002). Thus, there exist a complicated cross-talk between c-Abl and F-actin that serve to regulate cell morphology and cell motility (reviewed in Greaves 2002). In addition, the kinase activity of Abl was proposed to be regulated by proteins binding to the SH3 domain of Abl (Van Etten 1999).

The diaphanous-related formin DAAM1 is a CIP4-binding protein with a role in regulating assembly of actin filaments and microtubules (Paper I)

CIP4 was originally identified in a yeast two-hybrid screen as a protein interacting with constitutively active Cdc42 (Aspenström 1997). CIP4 was proposed to regulate the organization of the actin cytoskeleton, however, it lacked an actin binding site. Therefore, we set out to find a potential link between CIP4 and the actin cytoskeleton.

First, we performed a yeast two-hybrid screen, using the SH3 domain of CIP4 as a bait, and identified six potential binding partners. The strongest interactor was a Myb-domain containing profilin-binding protein (Mybp42POI), followed by, with decreasing strength of interaction with CIP4; RalA, acinus, the β-subunit of the Na⁺/K⁺ ATPase, the transcription factor active transcriptional factor 5 (ATF-5), and a previously unidentified RhoGAP. In spite of the weak interaction with CIP4 in the yeast two-hybrid screen, we decided to focus our attention on the RhoGAP protein (Paper II-IV). The RhoGAPs are important down-regulators of Rho GTPase activity, and can by virtue of their multidomain structure often act as effectors mediating events downstream of Rho GTPases. In addition, aberrant RhoGAP function has been implicated in diseases such as cancer and neurodegenerative disorders.

Yuan et al. identified CIP4 as a gene upregulated during apoptosis in human breast cancer cells (Yuan et al. 2001). We detected an interaction between CIP4 and acinus, a protein involved in condensation of the chromatin during apoptosis, and thus, CIP4 could affect apoptosis by its interaction with acinus. Furthermore, cytoskeletal components have been reported to translocate ion-channels to the plasma membrane through interactions with their SH3 domains and proline-rich sequences in the ion-channels, suggesting that CIP4 might localize the Na⁺/K⁺ pump to the plasma membrane (Cohen et al. 1995).

The function of members of the PCH family of proteins in actin-based processes has been suggested to rely on an interaction with FH proteins. Furthermore, FBP17 was initially identified as protein binding to the mouse formin. Therefore, in the second part of this work, we wanted to investigate whether CIP4 and FBP17 interacted with a previously uncharacterized DRF, originally identified by the HUGO cDNA cloning effort as KIAA0666. During the course of our study, Habas et al. showed that KIAA0666 was implicated in regulating cell polarity during gastrulation in Xenopus oocytes, relaying signals from the Wnt Frizzled pathway by forming a complex with
Disheveled and activated Rho (Habas et al 2001). Thus, the protein is hereafter denoted DAAM1 (Disheveled associated activator of morphogenesis 1).

We showed by employing a GST pull-down assay, that the proline-rich FH1 domain in DAAM1 conferred binding to the SH3 domains of CIP4 and FBP17. The interactions were confirmed in vivo, where CIP4, but not FBP17, was found to interact with DAAM1. In addition, DAAM1 was found to interact with the SH3 domains of Src, n-Src, and Abl, and to a lesser extent spectrin, endophilin and Btk. We furthermore showed that DAAM1 displays an inhibitory intramolecular interaction domain in similarity to other DRFs. We found that DAAM1 interacted with constitutively active Rho and Cdc42, in contrast to reports by Habas et al. describing an interaction with Rho only (Habas et al 2001). ROCK and mDia1 have been shown to cooperate in the formation of stress-fibers (Watanabe et al 1999). By contrast, our studies showed that DAAM1 over-expressing cells appeared to have a reduced content of polymerized actin, and these cells formed highly branched protrusions. This might be due to differences in primary sequences between Dia and DAAM1 causing differences in signaling, DAAM1 might sequester and thereby down-regulate intracellular Rho, and/or DAAM1 might act downstream of activated Cdc42 to induce filopodia.

We found that ectopic DAAM1 caused an increase in the amount of microtubules and that DAAM1 appeared to localize to structures surrounding the microtubule organizing center (MTOC). Thus, DAAM1 might affect MTOC localization and thereby cell polarization, however, this requires further studies. In addition, our studies revealed that DAAM1 and CIP4, but not a CIP4 mutant devoid of the SH3 domain, appeared to cooperate to inhibit PDGF-BB-induced membrane-ruffling in porcine aortic endothelial cells stably expressing the PDGF-β receptor (PAE/PDGFRβ).

DAAM1 interacted with Src in vivo, as shown by co-expression in Cos7 cells. Src mediated signaling appear to be of importance in the formation of podosomes (Tarone et al 1985). We show that over-expression of v-Src in PAE/PDGFRβ cells causes a drastic reorganization of actin with accumulation of polymerized actin at the cell margin, suggestive of podosome-like structures. A similar phenotype was seen in cells expressing ectopic v-Src and DAAM1, and the proteins co-localized to ring-like structures surrounding the podosome-like structures, indicative of an interaction in vivo. PDGF-BB stimulation of cells co-expressing these proteins, but not v-Src alone, resulted in a loss of the podosome-like structures, suggesting that the concerted action of these proteins is required for actin reorganization.

mDia1 and mDia2 have been shown to localize to endosomes in HeLa cells (Tominaga et al 2000). Further, Gasman et al. showed that RhoD activates hDia2C and Src to induce changes in the actin cytoskeleton and thereby regulate the motility of early endosomes (Gasman et al 2003). Studies employing the isolated DAD domain or an RBD truncation to disrupt the inhibitory intramolecular interaction in endogenous Dia mimics Rho-binding and has presented a potent way for its activation (Alberts 2002). Our preliminary studies show that PDGF-β-receptor endocytosis in PAE/PDGFRβ cells could be blocked by microinjection of a fragment including the DAD domain of DAAM1, thus, implicating a role for DAAM1 in receptor-endocytosis (data not shown).

Furthermore, cells microinjected with the DAAM1 DAD domain displayed a more intense vinculin staining than non-injected cells (data not shown). Copeland and Treisman showed that changes in actin dynamics can lead to the activation
of SRF and an ensuing upregulation of immediately early gene products, like vinculin (Copeland & Treisman 2002; Evangelista et al 2003). Src and DRFs could also activate the SRF in a pathway independent of actin dynamics, as shown in a report by Tominaga et al. (Tominaga et al 2000). Therefore, DAAM1 might activate the SRF to induce vinculin expression either by causing reorganization of actin, or/and by co-operating with Src.

**RICH, a Rho GTPase-activating protein domain-containing protein involved in signaling by Cdc42 and Rac1 (Paper II)**

The aim of this work was to clone and characterize the RhoGAP RICH-1, and to identify binding partners for this protein. This paper also presents initial characterization studies on RICH-2, a close homologue of RICH-1. We originally isolated RICH-1 as a partial clone, derived from a Epstein-Barr virus-transformed human B-cell library, in a yeast two-hybrid screen for proteins interacting with the SH3 domain of CIP4 (Paper I). RICH appeared to be present in at least two splice variants, a longer form of 803 amino acids (RICH-1) and a shorter form of 226 amino acids, named RICH-1B. However, recent database searches have revealed the presence of an additional twelve mRNA transcripts encoding altogether twelve protein isoforms. This is supported by the finding of several splice variants of the rat orthologue of RICH-1, nadrin (Furuta et al 2002). RICH-1 and nadrin show 83% identity, but in contrast to RICH-1, nadrin exhibits a stretch of 29 glutamines in its C-terminal. Nadrin is a GAP for Rac, Cdc42, and Rho in vitro. Moreover, nadrin enhanced Ca²⁺-dependent exocytosis and a splice variant of nadrin inhibits NGF-dependent neurite outgrowth in PC12 cells (Furuta et al 2002; Harada et al 2000). Both activities were proposed to rely on the GAP activity of nadrin; the inhibitory effect on neurite extension is probably due to down-regulation of Rac and Cdc42.

RICH-1 mRNA showed a ubiquitous expression although it was enriched in heart, placenta and skeletal muscle. Caution must be taken, however, as the presence of additional splice variants could compromise these results. Moreover, RICH-2 mRNA was expressed at low levels in all tissues tested, although at particularly high levels in brain, suggesting that RICH-2 is the neuronal form. Moreover, ectopic RICH-1 showed a uniform cytoplasmic distribution in NIH3T3 and PAE/PDGFRβ cells, whereas RICH-1B appeared to localize to vesicular structures.

RICH-1 and RICH-2 showed in vitro GAP activity towards Cdc42 and Rac but not Rho, and RICH-2 appeared to be a more effective GAP than RICH-1. Next, we wanted to investigate whether RICH-1 displayed the same substrate specificity in vivo. Over-expression of RICH-1 or the isolated RhoGAP domain blocked PDGF-BB-induced ruffling via Rac in PAE/PDGFRβ cells, but not serum-induced stress-fiber formation via Rho in NIH3T3 cells. In compliance with these observations, mutants of RICH-1 or the isolated RhoGAP domain mutated in the catalytic arginine retrieved reverse results. To verify that RICH-1 stimulated the GTP-hydrolysis on Rac and Cdc42 in vivo, we expressed the RICH-1 GAP domain, together with either wild-type Rac or Cdc42 in PAE/PDGFRβ cells. GST pull-down with either the PAK or the WASP CRIB-domain to bring down activated Rac and Cdc42, respectively, showed that the RICH-1 GAP domain stimulated GTP hydrolysis on both Rac and Cdc42. Thus, in accordance
with the \textit{in vitro} GAP activity, RICH-1 displays \textit{in vivo} specificity towards Rac and Cdc42.

A GST pull-down approach showed that the RICH-1 displayed binding to the SH3 domains of the CIP4 homologues PACSIN/syndapin and FBP17/rapostlin, endophilin A1, cortactin and Abl. The interaction between RICH-1 and CIP4 was confirmed \textit{in vivo}. The proline motifs in RICH-1 were divided into proline-rich motif 1 to 4 (P1-P4), and CIP4 was shown to interact with P2. Previously, ectopic CIP4 had been shown to relocalize into clusters at the cell periphery and at the dorsal side of Swiss 3T3 fibroblasts upon co-expression with constitutively activated Cdc42 (L61Cdc42) (Aspenström 1997). We wanted to investigate whether RICH-1 co-localized with CIP4 \textit{in vivo}, therefore we expressed RICH-1, CIP4 and L61Cdc42 in NIH3T3 fibroblasts. RICH-1 showed co-localization into the CIP4-containing clusters, which further confirmed their observed interaction.

The RhoGAP RICH-1 has a BAR (BIN/Amphiphysin/Rvsp) domain responsible for binding and tubulation of liposomes (Paper III)

RICH-1 has an N-terminal BAR domain, which comprises around 200 amino acid residues, and is found in several proteins including members of the amphiphysin and endophilin families (Table 4). The proposed function of the BAR domains in amphiphysin I and endophilin A1 is to interact with membrane lipids in order to impose curvature on the plasma membrane during CME (Farsad et al 2001; Takei et al 1999). In Paper II we show that the proline-rich motif in RICH-1 interacts with the SH3 domains in endophilin A1, as well as cortactin and PACSIN/syndapin, proteins implicated in linking endocytosis and actin reorganization. The aim of this work was to characterize the function of the BAR domain in RICH-1 and to confirm the interaction of RICH-1 with endophilin A1.

A GST fusion-protein comprising the first 125 amino acid residues of RICH-1, corresponding to the region in endophilin A1 required for lipid interaction, was shown to co-sediment with liposomes upon centrifugation, demonstrating that RICH-1 bound to lipids. Next, the ability of RICH-1 to tubulate liposomes, in an \textit{in vitro} process aiming to mimic invagination of cellular lipid bilayers, was tested. Negative stain electron microscopy showed that a GST fusion-protein of RICH-1B was able to tubulate liposomes. The tubes were encapsulated by a helical pattern, suggesting that RICH-1B oligomerized by virtue of its coiled-coil region, on the surface of the tube in a manner similar to what has been reported for amphiphysin I, endophilin A1 and dynamin 1.

We have shown that the BAR domain in RICH-1 binds to membrane lipids and can deform these into tubes, thus, indicating a role for RICH-1 in membrane-trafficking events. However, our preliminary results indicate that ectopically expressed RICH-1 and RICH-1 deletion mutants do not interfere with the endocytosis of the PDGF-and the EGF receptors (data not shown). In addition, we found that endogenous RICH-1 co-localized with a marker specific for the ER, whereas ectopic RICH-1B appeared to localize to mitochondria. It is important to stress that membrane tubulation is an event occurring not only at the plasma membrane during events such as endocytosis, but is also required for the budding of vesicles from intracellular membranes, such as the ER and Golgi. This is exemplified by finding that endophilin B1 partially localizes to the
Golgi, can deform liposomes into tubes, but is not involved in the CME of transferrin (Farsad et al 2001; Modregger et al 2003). Instead, the members of the endophilin B family are thought to be involved in intracellular membrane-trafficking events. Furthermore, dynamin-like proteins have been found to localize to Golgi and mitochondria, and are implicated in Golgi transport and regulation of mitochondrial morphology. Taken together, our data suggests that RICH-1/RICH-1B is involved in membrane-trafficking processes, possibly in the budding of vesicles originating from the ER, and/or as a regulator of mitochondrial morphology. In addition, the possibility that RICH-1 might be involved in endocytic events other than CME, as well as exocytic events, should not be excluded.

The interaction between RICH-1 and endophilin A1 was confirmed in vivo, and in addition, RICH-1 was shown to interact with amphiphysin II. Using a GST pull-down assay RICH-1 was found to interact with the SH3 domains of both endophilin A1 and amphiphysin I, via its first proline-rich motif (P1) (importantly, the SH3 domains in amphiphysin I and II are identical). In accordance with these results, we found a sequence motif in RICH-1 showing consensus for binding to both the amphiphysin I and endophilin A1 SH3 domains (Cestra et al 1999), PKPPTKR, comprising residues 590-595 within the region of P1. To ascertain the exact binding site(s), however, experiments employing mutants in the RICH-1 proline-rich regions are required. It is also possible that P1 might act in conjunction with one or more of the proline-rich motifs in binding to the SH3 domains. In conclusion, RICH-1 could affect membrane-trafficking processes by virtue of its BAR domain, and/or GAP activity, and/or its interaction with endophilins or amphiphysins, and/or proteins implicated in linking membrane-trafficking and actin organization, including cortactin and PACSIN/syndapin.

The RhoGAP RICH-1 interacts with Abelson tyrosine kinase, and the scaffolding proteins NHERF-2 and CIN85 (Paper IV)

In Paper II we showed that RICH-1 interacts with the SH3 domain in Abl. RICH-1 was also shown to display a C-terminal PDZ domain-binding motif (STAL), predicted to bind EBP50 PDZ-1, and fitting the more general consensus sequence X-(S/T)-X-(L/V/I) for a broad spectrum of PDZ domain-binding proteins (Reczek & Bretscher 2001; Saras & Heldin 1996; Songyang et al 1997). The optimal EBP50 PDZ-1 binding consensus, however, is D-(S/T)-(R/Y)-L (Hall et al 1998a; Wang et al 1998). In addition, we show that a proline-rich motif in RICH-1 binds to the SH3 domains in the endocytic scaffold protein CIN85. The aim of this work was to characterize the interactions of RICH-1 with Abl, NHERF-2 and CIN85. Of note, human EBP50 and E3KARP are roughly 80% identical to the rabbit orthologues NHERF-1 and NHERF-2, and NHERF-2 was used in our work (Weinman et al 1995; Yun et al 1997). The interaction of RICH-1 with Abl was confirmed in vivo, and Abl was shown to phosphorylate RICH-1. The GAP activity of p190RhoGAP is regulated via phosphorylation by Src, suggesting that the GAP activity of RICH-1 could be regulated in a similar manner via phosphorylation by Abl (Hu & Settleman 1997; Roof et al 1998). This is supported by the finding that RICH-1 interacts with the SH3 domain of Src (Paper II). Abl appeared to interact with the second proline rich motif in RICH-1, P2.
Moreover, the SH3 domain in Abl also interacts with a proline-rich motif in the RhoGAP 3BP-1 (Cicchetti et al 1995).

We showed that RICH-1, but not a deletion mutant of RICH-1 devoid of the PDZ-interacting motif, interacted with NHERF-2 in vivo. In addition, NHERF-2 localized RICH-1, but not the mutant lacking the PDZ-binding motif, to microvilli-like structures on the dorsal side of the cells, when co-expressed in PAE/PDGFRβ cells. The relevance of this interaction is not clear, however, one might speculate that NHERF family proteins act as scaffold proteins to recruit RICH-1 to membranes in order to perform its function as a RhoGAP and/or as a tubulator. Moreover, Reczek et al. found that RICH-1 existed in an in vivo complex which included ezrin, EBP50/NHERF-1, the RabGAP EPI64 and PLC-β3 (Reczek & Bretscher 2001). The PDZ binding motif in RICH-1 might in addition confer binding to other PDZ domain-containing proteins.

CIN85 possesses three highly similar SH3 domains and has been implicated in the endocytosis and down-regulation of certain RTKs. Kowanetz et al. found a novel atypical consensus for binding to the CIN85 SH3 domains (PXXXPR), supported by a report from Kurakin et al. (PX(P/A)XXR) (Kowanetz et al 2003; Kurakin et al 2003). Interestingly, we found that RICH-1 possessed this consensus, residing in the region of amino acid residues 721-726, (PVPKPR), and was therefore predicted to bind to CIN85. Using a GST pull-down assay, we showed that RICH-1 bound to all three SH3 domains, denoted SH3 A, B and C, independently and in combinations. In vivo studies, using HEK293T cells, show that ectopically expressed RICH-1 and CIN85 appear to interact in a phosphorylation-dependent manner upon EGF-stimulation. By contrast, a mutant of RICH-1 in the conserved arginine in the region predicted to bind CIN85 [RICH-1 (R726A)] showed no binding to CIN85 neither in vitro nor in vivo, thus, confirming that the interaction takes place through the predicted proline-rich motif in RICH-1. Immunofluorescence studies using Chinese hamster ovary (CHO) cells, showed that ectopic CIN85 relocalized RICH-1 into large aggregates characteristic for CIN85 over-expression, thus, confirming their interaction in vivo. RICH-1 might be recruited to membranes by CIN85 in a phosphorylation-dependent manner, where it could function in the regulation of Rho GTPase activity and/or facilitate membrane invagination (Petrelli et al 2002; Soubeyran et al 2002).

In conclusion, in Paper IV we showed that RICH-1 interacts with Abl, NHERF-2 and CIN85. Moreover, Abl appears to phosphorylate RICH-1, and perhaps thereby regulates its GAP activity. The scaffold proteins NHERF-2 and CIN85 could recruit RICH-1 to multimeric complexes situated at sites close to or at the plasma membrane, or intracellular membranes, in order for RICH-1 to perform its functions as a RhoGAP and/or a tubulator.
FUTURE PERSPECTIVES

Future studies on RICH-1 should aim at elucidating the role of RICH-1 in vivo. Studies employing Drosophila have shown that knock-out of the Drosophila orthologue of human RICH-1 is lethal (Billuart et al 2001). We are currently studying the cellular role of Drosophila RICH, by employing an RNA interference-based method. Preliminary results indicate an involvement of RICH-1 in the development of tracheal branches. Harada et al. showed that nadrin was involved in neuronal morphogenesis, by having an inhibitory effect on neurite outgrowth (Furuta et al 2002). Further studies employing RNA interference in mammalian cells, mouse knock-outs of full-length RICH-1 or knock-ins of the GAP domain or the BAR domain of RICH-1 will yield more information about the role of RICH-1 in vivo.

The RhoGAPs require tight regulation, otherwise the Rho GTPases they regulate will be either constitutively down-regulated or not turned off at all, resulting in aberrant Rho GTPase signaling. Therefore, it is of importance to define how RICH-1 GAP activity is regulated. We have shown that RICH-1 interacts with Abl and is in addition phosphorylated by this tyrosine kinase. In keeping with what is know about the regulation of other RhoGAPs, possible mechanisms for RICH-1 regulation might be phosphorylation, binding of lipids to the BAR domain, or interaction with SH3 domain-containing proteins via the proline-rich motifs.

As mentioned above, BAR domains are found in several protein families, and is involved in shaping membranes during endocytosis, and evidently during other membrane-trafficking events. Future studies will aim at determining at which lipid bilayers RICH-1 exerts its tubulating activity.
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