RhoGTPase Signaling in Cell Polarity and Gene Regulation

ANN-SOFI JOHANSSON
Dissertation presented at Uppsala University to be publicly examined in Room B22, BMC, Husargatan 3, Uppsala, Wednesday, April 26, 2006 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract

RhoGTPases are proteins working as molecular switches as they bind and hydrolyze GTP. They are in their active conformation when GTP is bound and are then able to interact with their effector proteins, which relay the downstream signaling. When the GTP is hydrolyzed to GDP, the RhoGTPase is inactivated. RhoGTPases have been shown to be activated by a variety of stimuli and they are implicated in regulation of diverse cellular processes, including cell migration, cell cycle progression, establishment of cell polarity and transformation.

We identified mammalian Par6 as a novel effector protein for the RhoGTPases Cdc42 and Rac1. The Caenorhabditis elegans homologue of Par6 had previously been shown to be essential for cell polarity development in the worm embryo. We found that endogenous Par6 colocalized with the tight junction protein ZO-1 in MDCKII epithelial cells. Par6 also interacted with mammalian Par3, another member of the par (for partitioning defective) gene family, first identified in C.elegans. Endogenous Par3 also localized to tight junctions in epithelial cells. This suggested that Par6 and Par3 are part of a complex regulating cell polarity also in mammalian cells. The interaction between Par6 and activated Cdc42 and Rac1 suggested a role for these RhoGTPases in the regulation of this complex.

Co-expression of Par6 together with PKCζ, induced a dramatic change in cell morphology. The cells rounded up and long cellular extensions, resembling neurites, were formed. The ability to induce these changes in cell morphology was found to be dependent on the direct interaction between Par6 and PKCζ, as well as on the kinase activity of PKCζ. We observed that cells co-expressing mPar6C and PKCζ contained bundled microtubules and microtubules that had been acetylated, indicating that the microtubules were stabilized.

To investigate the roles of RhoGTPases in PDGF-induced gene expression we performed cDNA microarray analyses on AG01518 human foreskin fibroblasts in which we over-expressed the dominant negative forms of Cdc42, Rac1 and RhoA. We found that the expression of 16 genes, out of the 45 up-regulated by PDGF-BB, were inhibited ≥50% in the presence of dominant negative Cdc42, Rac1 or RhoA. 19 other genes were down-regulated by one or two of the dominant RhoGTPases. Our data implied that the expression of many PDGF-BB induced genes can be affected by RhoGTPase signaling.

In conclusion, the work presented here has increased the knowledge of the involvement of RhoGTPase signaling in establishment of cell polarity and gene regulation.

Keywords: RhoGTPase, Par6, cell polarity, aPKC, epithelial cell, PDGF, gene regulation, microarray

Ann-Sofi Johansson, Ludwig Institute for Cancer Research, Box 595, Uppsala University, SE-75124 Uppsala, Sweden

© Ann-Sofi Johansson 2006

ISSN 1651-6206
ISBN 91-554-6505-6
urn:nbn:se:uu:diva-6698 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-6698)
To MYSELF
Utan tvivel är man inte klok
*Tage Danielsson*
List of Papers

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

I. **Johansson A-S**, Driessens M and Aspenström P
   “The mammalian homologue of the *Caenorhabditis elegans* polarity protein PAR-6 is a binding partner for the RhoGTPases Cdc42 and Rac1”
   *Journal of Cell Science* 113(18): 3267-3275, 2000

II. Bjørkøy G*, **Johansson A-S***, Øvervatn A, Aspenström P and Johansen T
   "Ectopic coexpression of mPar6C and PKCζ induce cell rounding and formation of neurite-like extensions"
   *Manuscript* *The two first authors contributed equally

   "Involvement of RhoGTPases in Platelet-derived growth factor-induced gene expression"
   *Manuscript*
### Contents

Introduction...................................................................................................11
- RhoGTPases ..................................................................................................11
  - Characteristics of RhoGTPases ...............................................................11
  - RhoGTPase Subfamilies ............................................................................14
  - Signaling Regulating RhoGTPase-activity .............................................18
  - Signaling Downstream of RhoGTPases ..................................................21
- RhoGTPases in the Regulation of Cell Polarity ...........................................24
  - Par-family Proteins ....................................................................................25
  - Establishment of Polarity in Epithelial Cells ............................................26
  - Polarization of Migrating Cells ...............................................................29
- Regulation of Gene Expression by RhoGTPase Signaling .........................33
- RhoGTPases in PDGF-signaling ................................................................40
  - PDGF-Induced Signaling ..........................................................................40
  - RhoGTPases in PDGF-induced Cellular Responses ..................................42
- Present Investigation..................................................................................46
  - Paper I ......................................................................................................46
  - Paper II ....................................................................................................47
  - Paper III ..................................................................................................48
- Future Perspective ......................................................................................51
- Acknowledgements ....................................................................................53
- References ..................................................................................................55
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACK</td>
<td>activated Cdc42 kinase</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
</tr>
<tr>
<td>aPKC</td>
<td>atypical PKC</td>
</tr>
<tr>
<td>COX2</td>
<td>cyclooxygenase 2</td>
</tr>
<tr>
<td>CIP4</td>
<td>Cdc42 interacting protein 4</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP responsive element binding</td>
</tr>
<tr>
<td></td>
<td>protein</td>
</tr>
<tr>
<td>CRIIB</td>
<td>Cdc42/Rac interacting binding domain</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DH</td>
<td>Dbl homology</td>
</tr>
<tr>
<td>Dia</td>
<td>Diaphanous</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial mesenchymal transition</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular regulated kinase</td>
</tr>
<tr>
<td>Gab1</td>
<td>Grb2 associated binder 1</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDI</td>
<td>guanine nucleotide dissociation inhibitor</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GSK3β</td>
<td>glycogen synthase 3β</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol triphosphate</td>
</tr>
<tr>
<td>JIP</td>
<td>JNK interacting protein</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LIMK</td>
<td>LIM-kinase</td>
</tr>
<tr>
<td>LPA</td>
<td>lysophosphatidic acid</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK/ERK kinase</td>
</tr>
<tr>
<td>MKK</td>
<td>MAPK kinase</td>
</tr>
<tr>
<td>MLK</td>
<td>mixed lineage kinase</td>
</tr>
<tr>
<td>MP1</td>
<td>MEK partner 1</td>
</tr>
<tr>
<td>MTOC</td>
<td>microtubule organizing center</td>
</tr>
<tr>
<td>NFAT</td>
<td>nuclear factor activated in T-cells</td>
</tr>
<tr>
<td>OSM</td>
<td>osmoensing scaffold for MEKK3</td>
</tr>
<tr>
<td>PAK</td>
<td>p21 activated kinase</td>
</tr>
<tr>
<td>PB1</td>
<td>PhoxBemp1</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PDZ</td>
<td>PSD95/DlgA/ZO-1</td>
</tr>
<tr>
<td>PH</td>
<td>plekstrin homology</td>
</tr>
<tr>
<td>PI3 K</td>
<td>phosphatidylinositol 3’ kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PKN</td>
<td>protein kinase N</td>
</tr>
<tr>
<td>PLCγ</td>
<td>phospholipase Cγ</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>POR1</td>
<td>partner of Rac1</td>
</tr>
<tr>
<td>POSH</td>
<td>plenty of SH3s</td>
</tr>
<tr>
<td>RBD</td>
<td>Rho binding domain</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>SH3</td>
<td>Src homology 3</td>
</tr>
<tr>
<td>SRE</td>
<td>serum response element</td>
</tr>
<tr>
<td>SRF</td>
<td>serum response factor</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of</td>
</tr>
<tr>
<td></td>
<td>transcription</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiskott Aldrich Syndrome Protein</td>
</tr>
</tbody>
</table>
Introduction

In a multicellular organism tissues and cells need to communicate with each other in order to maintain the physiological correct conditions. Extracellular signaling molecules transmit messages between cells, which give rise to events at the cell surface of the receiving cell. This induces intracellular signaling cascades promoting the cell to move, divide or die.

This thesis is focused on intracellular signaling mediated via RhoGTPases, which are molecular switches relaying signals from the cell surface to other compartments in the cell. It has become clear that these molecules are important for the proper control of a multitude of cellular processes in essentially all existing cell types. However, it is obvious that there still is a lot to learn about RhoGTPases and their interplay with other signaling molecules.

RhoGTPases

The RhoGTPases belong to the Ras-superfamily of small GTPases. Ras, the prototype of small GTPases, was first identified as the oncogene in the Harvey and Kirsten sarcoma viruses. Rho, Rac and Cdc42 were identified later as homologues to Ras, and were shown to induce morphological changes when microinjected into fibroblasts, suggesting their ability to regulate the cytoskeleton (Takai et al., 2001). RhoGTPases were later shown to be involved in the regulation of a number of other cellular processes, besides actin reorganization and cell migration. This includes cell polarity, apoptosis, proliferation and differentiation. These processes are important for normal function of the organism and cause diseases when deregulated.

Characteristics of RhoGTPases

RhoGTPases are proteins with a typical molecular weight of 20-25 kD that bind and hydrolyses guanosine triphosphate (GTP). They are in an active conformation when they have GTP bound, and are inactivated when the GTP is hydrolyzed to guanosine diphosphate (GDP). When the GTPase is in the active conformation it is able to interact with effector proteins, which transmit the signal downstream of the GTPase. The rate of the intrinsic hydrolysis can be enhanced by the interaction with GTP activating proteins.
Figure 1. Schematic presentation of the RhoGTPase cycle. See text for details. (Raftopoulou and Hall 2004)

(GAPs), which promote the inactivation of the GTPase. Guanine nucleotide exchange factors (GEFs) accelerate the exchange of GDP for GTP, thereby activating the GTPase (Takai et al., 2001). Rho guanine nucleotide dissociation inhibitors (RhoGDIs) keep the RhoGTPase in the GDP-bound, inactive state. RhoGDIs have turned out not only to work as inhibitors of RhoGTPases, but also be important for directing RhoGTPases to their correct site for activation (Dransart et al., 2005). RhoGDIs, GAPs and GEFs, which will be described further below, are themselves under control of signaling pathways triggered by receptor activation at the cell surface. For a graphic illustration of the GTPase cycle see figure 1.

RhoGTPases typically consists of the GTPase-domain and additional short sequences in the C-terminus and N-terminus (Takai et al., 2001; Wennerberg and Der, 2004). The Rho insert motif is an insertion of about 13 amino acid residues in the GTPase-domain of RhoGTPases, not present in GTPase-domains of other small GTPases (Vetter and Wittinghofer, 2001). The differences in the conformation of GDP-and GTP-bound small GTPases are basically manifested in two regions within the GTPase-domain, the so called Switch I and Switch II regions, see figure 2. In the GTP-bound RhoGTPase the conformation of the switches are stabilized. When GTP is hydrolyzed to GDP, the conformation is relaxing (Hakoshima et al., 2003; Vetter and Wittinghofer, 2001). Amino acid residues in the switch regions mediate the interaction between the GTPase and its effector proteins, which
Figure 2. The structure of a typical RhoGTPase. Mutations used in studies are depicted in the figure. See text for details.

bind only to the GTP-bound conformation (Vetter and Wittinghofer, 2001). The presence of Mg$^{2+}$ ions in the catalytic cleft of the GTPase-domain is crucial for both guanine nucleotide binding and catalytic function (Hakoshima et al., 2003; Vetter and Wittinghofer, 2001). Most of the small GTPases require lipid modifications to be fully active, and this occurs at the conserved CAAX-motif in the C-terminus of the protein. The most common lipid modification of RhoGTPases is isoprenylation of the cysteine residue by addition of farnesyl or geranylgeranyl isoprenoid, but palmitoylation also occur on some RhoGTPases. The lipid modifications promote the localization of RhoGTPases to membranes, which is crucial for their function (Wennerberg and Der, 2004; Winter-Vann and Casey, 2005).

**RhoGTPase mutants as research tools**

Overexpression or microinjection of mutant RhoGTPases have been an extensively used tool to investigate their role in signaling pathways and regulation of cellular processes. These mutations were first identified and evaluated in Ras, and then, based on sequence homology the corresponding mutants of RhoGTPases were produced, see figure 2.

The substitution of threonine 17 for an asparagine in Rac and Cdc42, corresponding to mutation of threonine 19 in Rho, favours a GDP-bound conformation. This renders the GTPase inactive and unable to interact with effector proteins. In addition, N17 mutants interact with GEFs with higher affinity than the wild-type GTPases, which prevents activation of the wild-type RhoGTPases within the cell. RhoGTPases bearing this type of mutation is therefore referred to as dominant negative.
Mutation of glycine in position 12 to valine, or glutamine to leucine in position 61 in Rac and Cdc42, corresponding to position 63 in Rho, decrease the intrinsic GTPase activity. This renders the GTPase locked in the GTP-bound conformation. These so called constitutively active RhoGTPases are supposed to interact continually with their effector proteins (Feig, 1999; Lim et al., 1996). Even though an obvious risk of cross-reactivity between RhoGTPase-mutants in the cell exists, since they in many cases seem to share GEFs and effector proteins, remarkably specific phenotypes have been obtained using the different mutants.

Additional mutations have been made in the effector binding domain of RhoGTPases, aiming to dissect the use of different effector proteins to mediate different cellular responses. For instance, constitutively active Rac and Cdc42 with additional mutations in position 37 or 40 was investigated in this manner. It was found that Rac and Cdc42 containing the Y40C mutation no longer interacted with the p21 activated kinase (PAK) and were unable to activate Jun N-terminal kinase (JNK). However, they still induced actin rearrangements and cell cycle progression. The F37A mutant of Rac were unable to induce changes in the actin cytoskeleton and cell cycle progression, while the corresponding mutant of Cdc42 induced this to an extent comparable to the constitutively activated Cdc42 without the additional mutation (Lamarche et al., 1996).

RhoGTPase Subfamilies

The RhoGTPase family is defined by the presence of the Rho-like GTPase-domain, and 22 human genes have been found to encode RhoGTPases. A phylogenetic tree where the RhoGTPases are organized according to sequence homology in their GTPase-domain together with functional data are shown in figure 3. The amino acid sequences of RhoGTPases show approximately 30% similarity to Ras and within the RhoGTPase family the homology is 40-95 % (Burridge and Wennerberg, 2004; Furstoss et al., 2002; Takai et al., 2001; Wennerberg and Der, 2004). RhoA, Rac1 and Cdc42 are the most well studied RhoGTPases.

Rho-proteins

RhoA was the first RhoGTPase shown to regulate the actin cytoskeleton, since microinjection of a constitutive active mutant of RhoA in Swiss3T3 fibroblast induced the formation of bundles of filamentous actin, called stress fibers. Lysophosphatidic acid (LPA) and platelet-derived growth factor (PDGF) were suggested to activate Rho, since these agents induced stress fibers in a Rho-dependent manner (Ridley and Hall, 1992). Rho also
contributes to the formation of focal adhesions, which serve as attachment sites for the stress fibers and are important for actin-myosin based contractility. RhoA, B and C have all been shown to stimulate actin polymerization and actin-myosin contractility. They basically share GEFs and effector proteins, but have different localization within the cell, likely due to differences in their C-terminals (Wennerberg and Der, 2004).

RhoA and RhoC have been shown to have growth promoting capabilities. Inhibition of RhoA, as well as inhibition of Rac and Cdc42, inhibits the progression through G1 of the cell cycle (Olson et al., 1995; Yamamoto et al., 1993). RhoA is required for cyclin D1-expression, which is important for cell cycle progression. This is believed to involve pathways promoting sustained activation of extracellular regulated kinases (ERKs) (Welsh et al., 2001). In addition, RhoA can reduce the levels of the cdk2-inhibitors p21^{cip1} and p27^{kip1}, thereby also promoting cell cycle progression. Rho is implicated in the regulation of the transcription factor serum response factor (SRF).
This seems mainly to be dependent on the ability of Rho to induce actin polymerization (Alberts et al., 1998b; Hill et al., 1995; Miralles et al., 2003; Sotiropoulos et al., 1999).

The expression of RhoC is up-regulated in certain tumors and overexpression of RhoC in tumor progenitor cells increase their tumorigenic properties (Clark et al., 2000; Suwa et al., 1998; van Golen et al., 2000). On the contrary, RhoB exhibits growth inhibitory qualities and is upregulated by the stimulation of cells with growth factors, thereby likely to serve as a modulator of the mitogenic response (Jahner and Hunter, 1991; Zalcman et al., 1995).

**Rac-proteins**

Rac1, Rac1b, Rac2, Rac3 and RhoG make up the Rac-subgroup of the RhoGTPases. Rac-proteins stimulate the formation of membrane ruffles and lamellipodia at the cell periphery. These are structures rich in filamentous actin believed to be important for cell migration. This was first seen in fibroblasts where growth factor induced membrane ruffling was shown to be dependent on Rac (Hawkins et al., 1995; Nobes and Hall, 1995; Ridley et al., 1992).

Rac also activates pathways regulating gene expression, such as the mitogen-activated protein kinases c-Jun N-terminal kinase (JNK) and p38 kinase pathways and the transcription factor NFκB (Coso et al., 1995; Minden et al., 1995). Binding of Rac to the regulatory subunit of NADPH-oxidase, p67phox, stimulate the production of reactive oxygen species (ROS). ROS production is for example important for cells in the immune systems that have phagocytic functions (Werner, 2004). Rac-induced ROS production is also an activator of the NFκB pathway and have a role in cell spreading (Sulciner et al., 1996; Werner, 2004).

Rac1 is ubiquitously expressed, whereas Rac2 expression is restricted to hematopoietic cells and Rac3 is most highly expressed in the brain. Both Rac3 and RhoG expression is induced in fibroblasts stimulated with serum (Haataja et al., 1997; Vincent et al., 1992). Although both expressed in hematopoietic cells, Rac1 and Rac2 seem to play somewhat different roles.

**Cdc42-proteins**

Cdc42 exists in two isoforms, coded for by the same gene. The Cdc42H isoform is ubiquitously expressed, while the G25K isoform is restricted to the brain. TC10, TCL, Chp/Wrch2 and Wrch1 are also members of the Cdc42-subfamily of RhoGTPases. Cdc42 was identified as a protein regulating the formation of thin cellular protrusions called filopodia and microspikes, by reorganizing the actin cytoskeleton. Bradykinin, ligand for G-protein coupled receptors, was the first ligand shown to induce these cellular protrusions in a Cdc42-dependent manner, but growth factors such as PDGF
and epidermal growth factors (EGF) also activate Cdc42 (Jimenez et al., 2000; Kozma et al., 1995; Nobes and Hall, 1995). Like Rac, Cdc42 activates JNK and p38 MAPK-pathways as well as NFκB (Coso et al., 1995; Fanger et al., 1997; Minden et al., 1995; Montaner et al., 1998; Perona et al., 1997). Cdc42 is crucial for cytokinesis and polarization of cells, which is important for cell migration and establishment of apical-basolateral polarity in epithelial cells. This mainly seems to depend on the ability of Cdc42 to regulate the microtubule system in the cells, see below.

The other members of the Cdc42 subfamily of also induce filopodia-like structures in e.g. fibroblasts, when over-expressed in their activated forms (Sorokina and Chernoff, 2005; Wennerberg and Der, 2004). TC10 and TCL are implicated in adipocyte differentiation (Chang et al., 2002; Chiang et al., 2002; Kanzaki et al., 2002; Nishizuka et al., 2003) TC10 also play a role in neurite outgrowth (Abe et al., 2003; Tanabe et al., 2000). Chp and Wrch1 have longer N-terminal sequences upstream of the GTPase-domain, compared to other RhoGTPases. These additional sequences contain proline-rich motifs able to interact with SH3-domain containing proteins. The adaptor protein Nckβ interacts with Wrch1 in this manner (Saras et al., 2004). The expression of Wrch1 is up-regulated in several type of tumors and by stimulation of the Wnt-1 signaling pathway (Tao W Genes Dev 2001 (Sorokina and Chernoff, 2005)).

*Rnd-proteins*

The Rnd subfamily consists of Rnd1, Rnd2 and Rnd3, also known as RhoE. Rnd-proteins are believed to be GTPase-deficient, and therefore always be in an active, GTP-bound confirmation in the cells. This suggests that Rnd-proteins are regulated by other means than their binding of guanine nucleotides (Nobes et al., 1998; Wennerberg and Der, 2004). The protein level of Rnd3 has for example been shown to increase after stimulation of fibroblasts with PDGF (Riento et al., 2003). Although Rnd-proteins show a quite high sequence similarity to Rho-proteins, their effects on the actin cytoskeleton are the opposite of Rho. Rnd1 and Rnd3 inhibit stress fiber formation and cause cell rounding, when over-expressed in cells (Aspenstrom et al., 2004; Nobes et al., 1998). Rnd3 has been shown to bind and activate RhoGAPs, leading to inactivation of Rho. Rnd3 also binds and inactivates the Rho-effector ROCK (Riento et al., 2003; Wennerberg et al., 2003).

*Other RhoGTPases*

RhoD and Rif show a sequence similarity of about 50% and both possess additional N-terminal sequences. Despite this they seem to play different roles in cells (Wennerberg and Der, 2004). The presence of constitutively activated RhoD in fibroblasts promotes stress fiber disassembly, while
introduction of constitutively activated Rif in fibroblasts induce formation of filopodia (Ellis and Mellor, 2000; Tsubakimoto et al., 1999). The filopodia formation is independent of Cdc42, but instead dependent on the effector protein mDia2, which originally was identified as an effector for RhoA (Alberts et al., 1998a; Pellegrin and Mellor, 2005).

RhoH-expression is restricted to hematopoietic cells and its gene has been found rearranged or fused to other genes in non-Hodgkin’s lymphoma and multiple myeloma (Dallery et al., 1995; Preudhomme et al., 2000). Like Rnd-proteins, RhoH is GTPase-deficient and proposed to be regulated at the transcriptional level. RhoH is suggested to be a negative regulator of cell growth and actin cytoskeleton rearrangement, perhaps via antagonistic effects on Rac-signaling pathways (Gu et al., 2005a; Gu et al., 2005b; Li et al., 2002).

The RhoBTB-subfamily consists of three members RhoBTB1-3. These proteins are larger than the typical RhoGTPases, since their N-terminal RhoGTPase-domain is followed by two BTB-domains (BTB for Broad-complex, Tramtrack and Brick-a brac). The structure of the GTPase-domain suggests that also this subgroup of RhoGTPases is in a constitutively active state in the cell (Ramos et al., 2002; Rivero et al., 2001). The gene coding for RhoBTB2 has been found down-regulated in certain tumors, and ectopically expression of RhoBTB2 inhibits growth, suggesting that RhoBTB2 is a tumor suppressor (Hamaguchi et al., 2002; Siripurapu et al., 2005).

Miro1 and 2 (Miro for mitochondrial Rho) contain one N-terminal and one C-terminal GTPase domain, of which the N-terminal domain show the highest similarity to RhoGTPase-domains. However, both GTPase-domains lack the Rho-insert domain, questioning the classification of Miro-proteins as RhoGTPases. Miro localize to mitochondria, and overexpression of Miro induce aggregation of mitochondria and the onset of apoptosis (Fransson et al., 2003).

**Signaling Regulating RhoGTPase-activity**

The activity of RhoGTPases is tightly regulated in the cell. As mentioned above, the regulation is mainly mediated via interacting proteins, namely RhoGEFs, RhoGAPs and RhoGDIs. Various cell surface receptors have been shown to control the activity of these factors, and thereby in turn the activity of RhoGTPases. Stimulation of cells with ligands for receptor tyrosine kinases (RTKs), such as EGF and PDGF, or G-protein coupled receptors (GPCRs), such as bombesin and LPA induce RhoGTPase activation. Stimulation of cytokine receptors and adhesion receptors such as integrins also induce downstream signaling regulating RhoGTPase activity.
RhoGEFs

The oncogene Dbl (isolated from Diffuse B-cell Lymphoma) was the first mammalian protein identified to have GEF-activities towards RhoGTPases, and hence the domain containing this activity is called Dbl-homology (DH)-domain (Eva and Aaronson, 1985; Schmidt and Hall, 2002). GEFs bind the GDP-bound form of the RhoGTPase and destabilize the GDP-GTPase complex, at the same time stabilizing a nucleotide-free intermediate. Since the intracellular concentration of GTP in the cell is high, GTP binds to the GTPase, which then becomes activated (Vetter and Wittinghofer, 2001).

The DH-domain consists of about 200 amino acid residues and most DH-GEFs also contain a pleckstrin homology (PH) domain of about 100 amino acid residues in length. PH-domains are known to interact with phospholipids, and suggested to direct the GEFs to the plasma membrane, where they can exert their action. Recent findings imply that the PH-domain participate in the interaction with the RhoGTPase. Binding of phospholipids to the PH-domain may also result in conformational changes of the GEF, thereby modulating GEF-activity (Rossman et al., 2005). Phosphorylation of GEFs can also regulate their exchange activity, e.g. phosphorylation of amino acid residue Y174 in Vav1 induce conformational changes enabling Vav1 to interact with RhoGTPases (Bustelo, 2001). GEFs of the Dbl-family contain additional domains of different functions, which supposedly give rise to GEF specificity and signaling diversity (Rossman et al., 2005; Schmidt and Hall, 2002).

In humans, 69 member of the Dbl-family of GEFs have been identified (Rossman et al., 2005). Some of these GEFs are specific for one RhoGTPase, such as Intersectin towards Cdc42 and p115RhoGEF towards Rho (Hakoshima et al., 2003; Schmidt and Hall, 2002). Other GEFs appear to activate several RhoGTPases, such as Vav1 which show exchange activity towards Cdc42, Rac and Rho (Abe et al., 2000). Measuring exchange activities in vitro and in vivo can give rise to different results. For instance, Tiam1 shows GEF-activity towards Cdc42, Rac and Rho in vitro, but in vivo it appears only to affect Rho (Michiels et al., 1995). This might be due to that additional factors are required, which presumably results in signaling specificity. So far, little is known about GEF-activities towards the more recently discovered RhoGTPases.

As mentioned earlier, Dbl was identified as an oncogene, and several other RhoGEFs have also been shown to have oncogenic potential. This is true for Vav as well as Tiam1. It has been proposed being due to their ability to, when deregulated, induce increased GDP/GTP-cycling of the RhoGTPases, thereby promoting enhanced downstream signaling (Schmidt and Hall, 2002).

Another RhoGEF family, lacking the Dbl-domain, was discovered more recently. The domain responsible for the exchange activity was first
identified in the protein DOCK180, hence the domain was called Docker (Braga, 2002; Brugnera et al., 2002). DOCK180 exhibit GEF-activity towards Rac. DOCK180 cannot exert its GEF-activity without interacting with the protein ELMO, which enhances the DOCK180 binding to Rac (Brugnera et al., 2002). DOCK180 was originally identified as a protein interacting with the adaptor protein Crk, suggested to be involved in the regulation of GEF-activity (Akakura et al., 2005). The DOCK180-Crk complexes are found at focal adhesions and have been implicated in the regulation of cell migration and phagocytosis (Braga, 2002; Meller et al., 2005). Zizimin1, which binds and activates Cdc42, is another member of the Docker-family of RhoGEFs (Meller et al., 2002).

**RhoGAPs**

The GAP-domain, approximately 140 amino acid residues in size, increases the intrinsic GTP-hydrolysis rate of RhoGTPases by interacting with the GTPase such that its conformation in the transition state during GTP-hydrolysis is stabilized (Hakoshima et al., 2003; Moon and Zheng, 2003). About 80 genes coding for RhoGAPs have been identified in the genome (Moon and Zheng, 2003). Hence, like RhoGEFs, RhoGAPs are considerably more numerous than the RhoGTPases themselves, implying that these proteins controlling RhoGTPase-activity are crucial for giving rise to signal diversity. The different RhoGAPs contain protein domains of different kinds, such as PH-domains, Src homology 2 (SH2)-domains, known to interact with phosphorylated tyrosine residues, and proline-rich sequences, known to interact with Src-homology 3 (SH3)-domains. These domains probably contribute to regulation of GAP-activity as well as enabling RhoGAPs to exert multiple roles in signaling (Moon and Zheng, 2003). As for GEFs, GAPs have been shown to have different specificities *in vitro* and *in vivo*. For instance, p50 RhoGAP show activity towards Cdc42, Rac and Rho in a cell free system, but only towards Rho in cells (Ridley et al., 1993).

Regulation of the GAP-activity can be achieved by phosphorylation or direct protein-protein interactions. Phosphorylation of p190RhoGAP by Src induce interaction between p190RhoGAP and p120RasGAP, which activate the GAP-activity of p190RhoGAP (Hu and Settleman, 1997; Roof et al., 1998). Interaction of CdcGAP, which show GAP-activity towards Cdc42 and Rho, with intersectin, implicated in endocytic trafficking, inhibit the GAP-activity. Since intersectin also possess GEF-activity towards Cdc42 and interact with the Cdc42-effector WASP, this complex can certainly serve as an example of refined regulation of RhoGTPase activity in the cell (Jenna et al., 2002).

**RhoGDIs**

In contrast to the numerous RhoGEFs and RhoGAPs identified, only three members of the RhoGDI family are known. RhoGDIs have been suggested
to have several roles in the regulation of RhoGTPase activity. RhoGDIs interaction with the RhoGTPase can inhibit the exchange of GDP, thereby blocking activation of the GTPase. The GTPase-activity of the RhoGTPase can also be inhibited by interaction with the RhoGDIs. In addition, RhoGDIs interaction with the C-terminal lipid modified tail of RhoGTPases prevent their interaction with lipid membranes, keeping them in a cytosolic fraction where they are unable to interact with effector proteins. RhoGDIs not only serve to inhibit RhoGTPase activity, but seem to be important for the appropriate activation of RhoGTPases (DerMardirossian and Bokoch, 2005; Dovas and Couchman, 2005). The interaction between RhoGDIs and so called displacement factors are thought to be one way to induce dissociation of RhoGTPases from the GDIs. The ezrin, radixin, moesin (ERM) proteins, which are associated with the plasma membrane as well as with the actin cytoskeleton, was the first proteins suggested to work in this manner (Dransart et al., 2005). Phosphorylations of RhoGDIs decrease their association with RhoGTPases. The phosphorylation of RhoGDI by the Rac-effector PAK upon PDGF or EGF-treatment of cells stimulate the release of Rac1, but not RhoA, from RhoGDI-complexes. This indicates a positive feedback loop resulting in increased Rac1-activation (DerMardirossian et al., 2004). Phosphorylations of RhoGTPases, on the other hand, seem to increase their interaction with RhoGDIs. For instance, the EGF-induced phosphorylation of Cdc42 performed by Src, increase the interaction between Cdc42 and RhoGDI, which may help direct Cdc42 to its site for activation (Tu et al., 2003).

Signaling Downstream of RhoGTPases

The interaction of RhoGTPases with their effector proteins initiate the downstream signaling events resulting in, for instance actin polymerization, gene expression, vesicular transport and apoptosis. Some effectors are shared between different RhoGTPases, while others are more specific. A summary of the effector proteins mentioned below and their RhoGTPase binding abilities are shown in table I. Intracellular signaling is however extremely interconnected, and other signaling events such as phosphorylations can also affect the RhoGTPase effector proteins and thereby the outcome of the downstream signaling.

Rac/Cdc42-effector Proteins

Cdc42 and Rac-related GTPases share some effectors, including PAK and mixed lineage kinases (MLKs). Others interact exclusively with Cdc42, such as Wiskott Aldrich Syndrome Protein (WASP) or exclusively with Rac, such as partner of Rac1 (POR1) (Aspenstrom, 1999). Most effector proteins identified to interact with Cdc42 and Rac-related GTPases contain a conserved stretch of amino acids important for the interaction, the so called
The mammalian PAK family contains six members. PAKs are serine threonine kinases which, via their CRIB-motif, interact with Cdc42 and Rac GTPases. This interaction has been shown to increase their kinase activity (Bagrodia et al., 1995b; Manser et al., 1994). PAK is able to phosphorylate a number of proteins, including myosin light chain kinase (MLCK), LIM-kinase (LIMK) and filamin, all involved in regulation of the actin cytoskeleton. Consequently, PAK has been shown to induce changes in the actin cytoskeleton such as membrane ruffling and stress fiber disassembly, when overexpressed. PAK has also been implicated in regulation of the mitogen-activated protein kinase pathways and the microtubule system, which will be described below (Hofmann et al., 2004).

MLKs are, as PAKs able to interact with both Cdc42 and Rac (Teramoto et al., 1996). They are implicated in the regulation of the JNK signaling pathways regulating gene expression, described below.

IQGAPs do not contain any CRIB-motif but interact with Cdc42 and Rac via their GAP-related domain, which should be pointed out, do not exhibit GAP-activity. IQGAPs were first shown to be involved in actin polymerization events. It has turned out that these proteins also are important for cadherin-mediated cell-cell adhesion and that they can act to stabilize microtubules (Briggs and Sacks, 2003; Noritake et al., 2005).

POR1 was identified to interact with activated Rac1 and was suggested to be involved in membrane ruffling (Van Aelst et al., 1996). POSH (for plenty of SH3s) was found to interact with Rac and induce apoptosis when overexpressed (Tapon et al., 1998). It has turned out that POSH is a scaffolding protein involved in the regulation of the JNK pathway, see below. As mentioned above, the p67phox subunit of NADPH-oxidase can also interact with Rac, which induce the production of ROS. See also below.

The Wiskott Aldrich Syndrome is an X-linked immunodeficiency disorder caused by defects in the gene coding for a protein, which therefore has been designated the Wiskott Aldrich Syndrome protein (Derry et al., 1994). WASP is expressed only in hematopoietic cells, while its homologue N-WASP is ubiquitously expressed. N-WASP and WASP were demonstrated to interact with Cdc42 in a GTP-dependant manner and be involved in the actin polymerization leading to filopodia formation (Aspenstrom et al., 1996; Symons et al., 1996). WASPs were shown to interact directly with the Arp2/3 complex and stimulate it to induce actin polymerization and branching of actin filaments. In addition, WASPs have been implicated in vesicular transport (Millard et al., 2004). Wave/Scar-proteins are homologues to WASP which also have been implicated in actin polymerization. These proteins lack a CRIB-motif and have not been shown to interact directly with RhoGTPases. Scar1 has been found in Rac-induced
lamellipodia and Rac has been suggested to activate Wave/Scar proteins but how this is achieved is not clear (Millard et al., 2004).

Cdc42 interacting protein 4 (CIP4) was identified as a protein interacting with Cdc42, but lacking the CRIB-motif. Initial studies employing Swiss 3T3 fibroblasts showed that overexpression of CIP4 induced changes in the actin cytoskeleton (Aspenstrom, 1997). CIP4 has been shown to mediate interaction between microtubules and WASP (Tian et al., 2000). ACK1 and 2 as well as MRCK α and β are other serine/threonine kinases that bind Cdc42 and thereby become activated (Aspenstrom, 1999).

**Rho-effector Proteins**

Rho kinases, ROCKs, are serine/threonine kinases that interact with Rho via their Rho-binding domain (RBD). Binding of GTP-bound Rho to ROCK is believed to induce conformational changes in ROCK resulting in relieve of autoinhibitory blockage of kinase activity. ROCKs can, as PAKs, phosphorylate LIMK. In addition ROCKs directly phosphorylate myosin light chain as well as myosin light chain phosphatase (MLCP). This synergistically leads to increased levels of phosphorylated MLC, which is important for actin-myosin fiber contractility, which in turn is supposed to be important for actin stress fiber formation (Riento and Ridley, 2003). It has, however, been shown that ROCK is not able to induce stress fibers downstream of Rho on its own, but needs to cooperate with another Rho-effector, Diaphanous (Dia), to achieve this (Watanabe et al., 1999). ROCKs are implicated in cell migration as well as regulation of gene expression. The latter for example via regulating NFκB-activity, see further below.

Dia proteins belong to the family of formin homology (FH) domain containing proteins. Formins in diverse species are implicated in the regulation of actin dynamics. Dia1 was identified as a protein interacting with Rho and profilin, an actin binding protein (Watanabe et al., 1997). Like for ROCK, binding of Rho to Dia relieve autoinhibitory interactions leading to activation of Dia. Overexpression of deletion mutants of Dia, lacking the C-terminal Dia autoinhibitory domain (DAD) cause actin bundles in cells, without coexpression of Rho (Tominaga et al., 2000; Watanabe et al., 1999). Dia has also been suggested to interact with the tyrosine kinases Src, and activate gene transcription via serum response factor (Tominaga et al., 2000). In addition, Dia as well as ROCK have been implicated in the regulation of the microtubule system. For instance, constitutively active Dia induce stable microtubules when overexpressed in cells (Ishizaki et al., 2001; Palazzo et al., 2001).

Protein kinase N (PKN) and Citron kinase are other serine/threonine kinases activated by their interaction with Rho. PKN is suggested to be involved in regulation of actin dynamics, vesicular transport and glucose transport (Mukai, 2003). Citron kinase has been implicated in regulation of contractility during cytokinesis (Madaule et al., 2000). Rhotekin and
Table 1. A summary of the effector proteins mentioned in the text. Their ability to interact with different RhoGTPases is also indicated in the table. *In most cases this means that the effectors interact with all members of the RhoGTPase subfamily. See text for details.

<table>
<thead>
<tr>
<th>Effector</th>
<th>Interacting RhoGTPase/s</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cdc42*</td>
</tr>
<tr>
<td>ACK1,2</td>
<td>+</td>
</tr>
<tr>
<td>CIP4</td>
<td>+</td>
</tr>
<tr>
<td>Citron kinase</td>
<td>-</td>
</tr>
<tr>
<td>Dia</td>
<td>-</td>
</tr>
<tr>
<td>Dia2</td>
<td>+</td>
</tr>
<tr>
<td>IQGAP</td>
<td>+</td>
</tr>
<tr>
<td>MLK</td>
<td>+</td>
</tr>
<tr>
<td>MRCΚα,β</td>
<td>+</td>
</tr>
<tr>
<td>p67 phox</td>
<td>-</td>
</tr>
<tr>
<td>PAK</td>
<td>+</td>
</tr>
<tr>
<td>Par6</td>
<td>+</td>
</tr>
<tr>
<td>PKN</td>
<td>-</td>
</tr>
<tr>
<td>POR1</td>
<td>-</td>
</tr>
<tr>
<td>POSH</td>
<td>-</td>
</tr>
<tr>
<td>Rhophilin</td>
<td>-</td>
</tr>
<tr>
<td>Rhotekin</td>
<td>-</td>
</tr>
<tr>
<td>ROCK</td>
<td>-</td>
</tr>
<tr>
<td>WASP</td>
<td>+</td>
</tr>
</tbody>
</table>

Rhophilin are two other proteins shown to interact with Rho in a GTP-dependent manner (Aspenstrom, 1999).

RhoGTPase signaling is involved in the regulation of many cellular processes, as exemplified above. In the following sections processes and signaling events related to the topics of this thesis will be described in more detail.

RhoGTPases in the Regulation of Cell Polarity

Polarization of cells is crucial for many processes such as cell migration during embryogenesis, establishment of apical-basolateral polarity in epithelial cells, directional migration of cells during wound healing as well as for metastatic tumor cells invading new tissues. RhoGTPases have been shown to be important players in the regulation of cell polarity in different cell types. Particularly the involvement of the Cdc42/Rac-effector Par6 in the establishment of cell polarity has gained a lot of attention in recent years.
Par-family Proteins

Par (Partitioning defective)-proteins were identified in screens for genetic mutations that were lethal, due to disruption of asymmetric cell division in the early embryo of Caenorhabditis elegans. Hence, Par-proteins constitute a protein family not based on amino acid similarities, but on their functional relationship (Guo and Kemphues, 1996). The asymmetric division of C. elegans embryos is dependent on asymmetric distribution of cellular components within the cell as well as on correct spindle orientation. The localization of Par-proteins within cells in the worm embryo is dependent on other Par-proteins. For instance, correct localization of Par-3 requires the presence of a functional Par6 gene, and Par-3 and Par6 co-localize in the embryo (Hung and Kemphues, 1999; Watts et al., 1996). The atypical PKC in C. elegans, PKC3, was shown to interact and co-localize with Par3, and the phenotype for PKC3-depleted embryos were similar to embryos with mutated Par3 or Par6 (Tabuse Y development 1998). Homologues to all Par-proteins, except Par2, have been identified also in Drosophila melanogaster and mammalian cells. The roles of Par-proteins in the establishment of cell polarity have also been conserved through evolution (Macara, 2004). Par1 and Par4 are serine/threonine kinases, Par3 and Par6 contain PSD95/DlgA/ZO-1 (PDZ) domains and Par5 is a 14-3-3-protein able to bind phosphoserines. The mammalian homologue of Par4, LKB1, is not only important for development of cell polarity. It has also been implicated in a number of processes such as apoptosis, cell cycle arrest and energy metabolism (Baas AF TCB 2004).

Par6

Mammalian Par6 was identified in searches for proteins interacting with RhoGTPases. Par6 was found to interact with Cdc42, TC10 and Rac1 (Joberty et al., 2000; Johansson et al., 2000; Lin et al., 2000; Qiu et al., 2000). In addition, Par6 has been found to interact also with TCL, Chp and Wrch-1 (Aspenstrom et al., 2004). Like mammalian Par3 that was identified earlier than mammalian Par6, Par6 was shown to localize mainly in tight junctions in polarized epithelial cells (Izumi et al., 1998; Joberty et al., 2000; Johansson et al., 2000; Lin et al., 2000). In one report, however, endogenous Par6 was also found in the cell nucleus, in addition to its localization in tight junctions (Johansson et al., 2000).

Par6 is a 345 amino acid residues protein, which besides its centrally situated PDZ-domain also contain a Phox/Bemp1 (PB1)-domain in the N-terminus and a partial CRIB-motif just N-terminal of the PDZ-domain, see figure 4 (Joberty et al., 2000; Lamark et al., 2003; Lin et al., 2000). The interaction between active RhoGTPases and Par6 is mediated via the partial CRIB-motif in Par6 in combination with the PDZ-domain, which helps stabilizing the interaction. The term partial CRIB-motif refers that the Par6
CRIB-motif lacks some of the normally conserved amino acid residues in a bona fide CRIB-motif (Garrard et al., 2003). The PDZ-domain in Par6 interacts with the most N-terminal of the three PDZ-domains in Par3. Par6 interacts with both isoforms of atypical PKCs (aPKC) \( \lambda \) and \( \zeta \) in mammalian cells. The designation atypical refers to that these PKC-isoforms are not regulated by binding of \( \text{Ca}^{2+} \), diacylglycerol (DAG) or phorbol esters, which are known to regulate other PKC-isoforms. Instead aPKCs is believed to be regulated by protein interactions (Moscat and Diaz-Meco, 2000). The interaction is mediated by the PB1-domain in Par6 and the PB1-domain found in aPKCs. Par3 and aPKCs are also able to interact directly with each other, see also figure 4 (Gao et al., 2002; Joberty et al., 2000; Johansson et al., 2000; Lin et al., 2000). The binding of GTP-bound Cdc42 or Rac to Par6 is believed to induce conformational changes in the Par6/aPKC/Par3-complex, which enhances the kinase activity of aPKC (Yamanaka et al., 2001). Par3, Lgl and glycogen synthase kinase 3 \( \beta \) (GSK3\( \beta \)) are proteins that can be phosphorylated by aPKC when it is activated upon Cdc42-binding to Par6 in the complex.

Four isoforms of Par6, Par6A-D, have been identified in humans. In most experiments reported Par6B or Par6C have been used. Indications of different functions of Par6-isoforms during establishment of epithelial cell polarity have been reported (Gao and Macara, 2004).

Establishment of Polarity in Epithelial Cells

The apical-basolateral polarity of epithelial cell sheets is crucial for their function as selective barriers in the organism. This type of polarity results in the separation of membrane and cytoplasmic components to different compartments of the cell. The cell-cell adhesion sites responsible for apical-basolateral polarity and selective permeability are the tight junctions. Adherens junctions and desmosomes are other types of cell-cell junctions present in epithelial cells. These junctions collaborate to hold epithelial cells together. Adherens junctions are, like tight junctions, associated with the actin microfilament system, while desmosomes are connected with intermediate filaments. The fourth type of cell-cell junctions in epithelial cells are the gap junctions, which allow exchange of small molecules between adjacent cells.

The Par6/aPKC/Par3-complex in Polarization of Epithelial Cell

Three evolutionary conserved protein complexes have been implicated in the formation of tight junctions and establishment of apical-basolateral polarity of epithelial cells. These complexes are the Par6/PKC\( \lambda \)/Par3-complex, the Crumbs/PALS1/PATJ-complex and the Scrib/Dlg/Lgl-complex. Some of the molecules of these complexes are also able to interact with each other. The
formation of tight junctions between cells is preceded by the establishment of adherens junctions between the cells (Schneeberger and Lynch, 2004).

It has been suggested that during the initial stage of polarity formation in epithelial cells Par6/aPKC is not associated with Par3, but with Lgl, which is able to interact with both Par6 and aPKC. Cdc42-induced aPKC-phosphorylation of Lgl releases Lgl from Par6/aPKC (Yamanaka et al., 2003). The Par6/aPKC-complex is then able to interact with Par3. Phosphorylation of Par3 by aPKCs has been shown to be important for tight junction formation (Hirose et al., 2002; Izumi et al., 1998; Nagai-Tamai et al., 2002; Suzuki et al., 2002). The PDZ-domain of Par6 also interacts with PALS1 in a Cdc42-GTP-dependent manner, suggesting that this interaction is initiated simultaneously as the Lgl-dissociation (Hurd et al., 2003; Miyoshi and Takai, 2005; Wang et al., 2004). The Par6/aPKC/Par3-complex and the Crumbs/PALS1/PATJ-complex seem to be dependent on each other for correct localization and promotion of tight junctions. This since overexpression of PATJ in MDCK cells results in mislocalization of PALS1, the Par6/aPKC/Par3-complex and the tight junction marker ZO-1 and overexpression of Par6 prevents localization of PALS1 to tight junctions (Hurd et al., 2003).

Nectins are Ca²⁺-dependent, transmembrane, cell-cell adhesion molecules which interact with each other on adjacent cells. Nectin interactions are involved in the formation of cadherin-based adherens junctions. Nectin-based cell adhesions induce activation of Cdc42 and Rac. This might be upstream events activating Cdc42 which then can interact with the Par6 and initiate the following exchange of Lgl for Par3 (Miyoshi and Takai, 2005; Takai et al., 2003).

JAMs are transmembrane glycoproteins with a C-terminal tail that can interact with PDZ-domains, and it interacts with the first PDZ-domain of Par3. This recruits the Par6/aPKC/Par3-complex to newly formed cell-cell adhesion sites and promotes the maturation of tight junctions (Ebnet et al., 2001; Itoh et al., 2001; Schneeberger and Lynch, 2004). Par3 can also interact with nectins, and it has been suggested that Par3 associates with nectins prior to binding to the Par6/aPKC complex (Miyoshi and Takai, 2005; Takekuni et al., 2003).

Under steady state condition of epithelial cells when tight junctions are stable, the Par6/PKCζ/Par3-complex is associated with tight junctions. Correct regulation of the Par6/aPKC/Par3-complex during the development of apical-basolateral polarity seems to be crucial, while e.g. overexpression of mutants of these proteins not affect already formed cell-cell junctions. In studies on the Par6/aPKC-complex in epithelial cell polarity PKCλ and ζ have been used. In development of polarity in migrating cells there is specificity for PKCζ, (see below). No differences in the regulation of epithelial cell polarity have been reported for PKCλ and ζ. However, a newly identified aPKC isoform, PKCζII, lacking kinase activity, have been
shown to inhibit tight junction formation. It is thought to do so by competing with other aPKCs for binding proteins, and thereby have a regulatory role in formation of tight junctions (Parkinson et al., 2004).

A schematic presentation of Par6, aPKC, Par3 and their interaction partners in epithelial cells are shown in figure 4.

![Figure 4](image-url)  

Figure 4. Par6, aPKC and Par3 and their interaction partners in epithelial cells. PB1=PhoxBemp1-domain, CRIB=Cdc42/Rac interacting binding-motif, PDZ=PSD95/DlgA/ZO-1 homology domain, PS=pseudo substrate region, C1=conserved region 1. See text for further details.

**Additional RhoGTPase Signaling in Epithelial Cell Polarity**

RhoGTPases are important for development of cell polarity in epithelial cells, probably not only via regulation of the Par6/aPKC/Par3-complex. Rac, Rho and Cdc42 are involved in the establishment of both adherens and tight junctions. In addition, signaling events initiated at e.g. adherens junctions can regulate RhoGTPase activity and influence their regulation of other cellular processes such as gene expression. Feedback loop signaling in the development of cell-cell junctions also likely occurs during the formation of junctions.

Inhibition of Rho disturbs the establishment of adherens junctions and causes displacement of cadherins from already fromed adherens junctions (Braga et al., 1999; Braga et al., 1997; Takaishi et al., 1997). Rac and Cdc42 activities are required for the establishment of adherens junctions and they are also activated at adherens junctions (Braga et al., 1999; Braga et al., 1997; Kim et al., 2000; Nakagawa et al., 2001). Overexpression of
constitutively activated as well as dominant negative mutants of Rho, Rac and Cdc42 destroys the gateway function of tight junctions and increase the paracellular permeability between epithelial cells (Jou and Nelson, 1998; Nusrat et al., 1995; Rojas et al., 2001). In addition, functional Cdc42 has been shown to be crucial for the transport of vesicles for developing and maintaining apical-basolateral polarity. The disturbance of vesicular transport may be one way in which the Cdc42-mutants influence tight junction-mediated barrier functions in epithelial cells (Kroschewski et al., 1999; Musch et al., 2001; Rojas et al., 2001). Since overexpression of mutants of members of the Par6/aPKC/Par3-complex do not affect already formed tight junctions this complex is likely not involved in the effects seen on paracellular permeability when over-expressing mutants of Cdc42 (Ebnet et al., 2001; Joberty et al., 2000; Suzuki et al., 2001; Yamanaka et al., 2001).

Epithelial-mesenchymal transition (EMT) is the process when epithelial cells sheets break up and adopt a mesenchymal, migratory phenotype. This occurs during embryogenesis, but is also an important process during cancer metastasis. EMT can be induced by extracellular signals such as scatter factor/hepatocyte growth factor (SF/HGF) and members of the TGF-β family (Van Aelst and Symons, 2002). HGF-induced EMT in MDCK-cells activates Rho, Rac as well as Cdc42 (Ridley et al., 1995). This result in reorganization of the actin cytoskeleton which is followed the disruption of cell-cell adhesions, leading to cell migration. Introduction of dominant negative versions of RhoGTPases have in some cases been shown to block HGF-induced cell scattering (Royal et al., 2000; Takaishi et al., 1994; Zondag et al., 2000). In one study, however, inhibition of Rho promoted cell scattering and in other studies both constitutively as well as dominant negative Rac blocked HGF-induced EMT (Hordijk et al., 1997; Potempa and Ridley, 1998; Ridley et al., 1995). The use of different cell types for the studies may contribute to the discrepancies. Inhibition of Rho or ROCK, but not inhibition of Rac, has been shown to inhibit TGF-β-induced EMT in mammary epithelial cells (Bhowmick et al., 2001). The regulation of gene expression by RhoGTPases has been shown to be important for EMT (see below).

Polarization of Migrating Cells

The migration of cells is induced by extracellular stimuli that elicit signal transduction events resulting in cell movement. The migratory process begins with the initial protrusion of a leading edge lamellipodium and the formation of new adhesions sites at the front of the cell. From the adhesion sites actin fibers emanate and provide the contractile forces that move the cell forward. At the rear end of the cell adhesion sites detaches from the substratum. The development of polarity in the cell is crucial for its directed migration. The involvement of RhoGTPases in the regulation of the actin
microfilament system during cell migration was early established. In recent years, however, their roles in regulating the microtubule system have been shown to probably be equally important.

**Signaling via Cdc42, Rac and Par6**

The importance of Par6 for directional movement in migrating cells was first shown in wound healing assays in primary rat astrocyte cultures. Ectopical expression of dominant negative Cdc42, kinase defective PKCζ, but not PKCδ, and full length Par6 inhibited the polarization of the astrocytes towards the wound, as assessed by the reorientation of the microtubule organizing center (MTOC) in the cells towards the wound (Etienne-Manneville and Hall, 2001). MTOC-relocalization is an important step in the polarization of a migrating cell and has been shown earlier to be dependent on Cdc42-activity (Nobes and Hall, 1999). A signaling pathway emanating from integrins activated at the wounded cell edges, which in turn activate the tyrosine kinase Src followed by activation of Cdc42, likely via phosphorylation dependent GEF-activation, was delineated. The binding of active Cdc42 to Par6 then activates PKCζ. Rac was shown not to be important for MTOC-relocalization, but for the formation of cell protrusions at the leading edge of cells. Cdc42 was also important for formation of protrusions, especially for the initiation of the protrusive activity. Par3 did not seem to be important for polarization of migrating astrocytes and were not localized to the leading edge of the cells (Etienne-Manneville and Hall, 2001).

Further studies revealed that PKCζ, activated by the interaction between Cdc42 and Par6 at the leading edge of migrating astrocytes, phosphorylated glycogen synthase kinase-3β (GSK-3β) (Etienne-Manneville and Hall, 2003). Phosphorylation of GSK3β mediates inactivation of its kinase activity. This in turn enables the adenomatous polyposis coli (APC) protein to interact with the growing plus-end of microtubules, since the GSKB-mediated phosphorylation of APC in resting cells disturbs this interaction (Zumbrunn et al., 2001). APC are involved in the stabilization of microtubules and have been found localized to elongating end of microtubules in the leading edge (Mimori-Kiyosue et al., 2000; Mogensen et al., 2002). Stabilization of microtubules at the leading edge is important for establishing polarity within the cell (Watanabe et al., 2005).

DlgA, which is the human orthologue of the *Drosophila melanogaster* disc large protein, known to be involved the establishment of epithelial cell polarity, colocalizes and interacts with APC in the leading edge of migrating astrocytes. The interaction between APC and DlgA is dependent on Cdc42/PKCζ-induced inhibition of GSK3β. The relocalization of DlgA to the leading edge of cells is however independent on GSK3β, but still dependent on Cdc42/PKCζ-signaling. DlgA was shown to be essential for
centrosome reorientation during astrocyte migration (Etienne-Manneville et al., 2005).

CLIP115- and CLIP170-associating proteins (CLASPs), dynein and dynactin are yet other microtubule binding proteins implicated in regulation of the microtubule system during migration (Etienne-Manneville and Hall, 2001; Watanabe et al., 2005). CLASPs-phosphorylation by GSK3β have been shown to be reduced by activated Rac in migrating epithelial cells, leading to increased CLASPs-binding to the microtubule lattice in the lamellipodia. The correlation to Rac-induced inhibition versus Cdc42/Par6/PKCζ is not known (Wittmann and Waterman-Storer, 2005).

Lgl, also involved in epithelial cell polarity, interacts with Par6 and is phosphorylated by PKCζ in the Par6/PKCζ-complex in migrating cells. The phosphorylation of several serine amino acids seems to be important for polarization of embryonic fibroblasts in wound healing assays. A role for Lgl in protein trafficking has also been suggested (Plant et al., 2003).

In figure 5 a summary of Par6 signaling in migrating cells are shown.

Additional RhoGTPase Signaling in Migrating Cells

Cdc42 and Rac1 are also able to affect microtubule organization and stabilization in several other ways, besides the Par6/PKCζ-pathway. PAK, which can interact with and be activated by Rac1 as well as by Cdc42, phosphorylates the microtubule destabilizing protein stathmin.

---

**Figure 5.** Signaling established to involve Par6 in migrating cells. See text for details.
Phosphorylation of stathmin inactivates it, hence promoting microtubule stabilization (Daub et al., 2001; Wittmann et al., 2004). Another Cdc42 and Rac1-effector, IQGAP1, which also can bind actin, interacts with both APC and CLIP170. IQGAP1 colocalizes with actin filaments at the leading edge and can there interact with both APC and CLIP170. CLIP170 also interacts with microtubules and it is suggested that the interaction between activated Cdc42 and Rac1 with IQGAP1 give rise to docking sites for microtubules via CLIP170 (Fukata et al., 2002; Watanabe et al., 2004).

Activation of Rho by LPA can also induce stabilization of microtubules in fibroblasts (Cook et al., 1998). The Rho-effector mDia, which interact with APC and end-binding protein 1 (EB1), another microtubule interacting protein, can mediate microtubule stabilization (Infante et al., 2000; Palazzo et al., 2001; Wen et al., 2004). In addition, mDia can interact directly with microtubules and is involved in the detyrosination that occurs on stabilized microtubules. The Rho-effector ROCK interact with and phosphorylate MAP2 and Tau, two other microtubule interacting proteins (Amano et al., 2003).

Downregulation of Rho-activity, as well as inhibition of ROCK, has however been shown to induce microtubule bundling in different cell types. In neuroblastoma N1E-115 cells, this lead to outgrowth of neurites and in NIH3T3 fibroblasts cytoplasmic extensions resembling neurites were formed (Hirose et al., 1998; Scaife et al., 2003). Degradation of RhoA mediated via the activity of the E3-ubiquitin ligase Smurf1 induce neurite outgrowth (Bryan et al., 2005) Intriguingly, it has been suggested that PKCζ-phosphorylation of Smurf1 induce degradation of RhoA (Wang et al., 2003).

As indicated above, the importance of RhoGTPases during cell migration was first identified through their regulation of the actin filament system. Activated Rac is localized in the front of the cell where it induces the polymerization of actin filaments necessary for lamellipodia formation (Kraynov et al., 2000; Nobes and Hall, 1999). Rac can induce actin polymerization via its effectors, e.g. PAK, IQGAP and IRSp53/WAVE. Rho is important for regulating cell attachment and the contractility of the actin-myosin fibers, also known as stress fibers, during cell movement.

Rho induces stress fiber formation via collaboration of its effector proteins ROCK and mDia. ROCK phosphorylates the myosin light chain phosphatase which inhibits its activity, thereby increasing phosphorylation of myosin light chain leading to increased contraction of the actomyosin fibers. In addition, ROCK phosphorylates and activates LIM kinase (LIMK) which then phosphorylate the actin binding protein cofilin, thereby inhibiting cofilin-mediated actin depolymerization. Activation of mDia also contributes to actin stress fiber formation, at least in part mediated via mDias interaction with the actin binding protein profilin, which promote actin polymerization and formation of stress fibers (Bishop and Hall, 2000; Ridley, 2001; Schmitz et al., 2000).
As seen in the sections above, the same RhoGTPase effector proteins can mediate downstream signaling regulating both the microtubule and the actin microfilament system. In fact it also seem like microtubule rearrangements can regulate the activity of RhoGTPases and not only the other way around (Etienne-Manneville, 2004; Raftopoulou and Hall, 2004). This also point towards how closely regulated these systems are and that an extensive crosstalk occur. The final outcome also depends on in what area of the cell the signaling activity occurs, as well as in which cell type. Basically all studies on RhoGTPase signaling controlling cell migration has focused on Cdc42, Rac1 and RhoA. It is likely that some of the less studied RhoGTPases also are involved in the regulation of this process, especially since many of them share effectors with Cdc42, Rac1 and RhoA, respectively.

Further information available about Par6
Two reports have shown that Par6 can induce transformation of NIH3T3 cells. Overexpression of Par6 together with PKCζ induces focus formation in NIH3T3 cells. Coexpression of constitutively active Rac1, and to a lesser extent constitutively activated Cdc42, enhanced the transforming ability of Par6/PKCζ. The kinase activity of PKCζ was crucial for transformation (Qiu et al., 2000). The novel small GTPases Rin and Rit binds to the PDZ-domain and a ternary complex between Rin/Rit, Par6 and Cdc42/Rac can be formed. Overexpression of Par6 together with constitutively active Rin/Rit and Cdc42/Rac results in transformation of NIH3T3 cells (Hoshino et al., 2005). What further downstream signaling events that mediate the transforming ability of Par6 are currently not known, but may involve its role in cell polarity and migration.

Furthermore, phosphorylation of Par6 by activated TGFβII receptors is necessary for TGFβ-dependent EMT in mammary gland epithelial cells. Smurf1 interacts with this phosphorylated serine in the amino terminus of Par6, and it is suggested that this triggers the degradation of RhoA, enabling cell-cell junction disassembly, see also above (Özdamar et al., 2005).

Regulation of Gene Expression by RhoGTPase Signaling
Gene transcription is under control of multiple pathways cooperating to achieve the desired set up of expressed genes that the cell requires at the moment. Several activated transcription factors usually interact with gene promoter sequences and with each other to initiate gene transcription. RhoGTPase signaling is implicated in the regulation of activity of several transcription factors, some of the most studied cases are described below. In
Figure 6. RhoGTPase signaling regulating gene expression. It should be noted that all these signaling pathways have not been shown to be activated simultaneously or in the same cell type. It should, however, also be noted that this figure just exemplifies what pathways that are implicated in gene regulation by RhoGTPases—see text for details.

MAP-kinase Pathways

The mitogen-activated protein kinases (MAPKs) constitute a family of kinases consisting of three major subfamilies; the extracellular regulated kinases (ERKs), the c-Jun N-terminal kinases and the p38 kinases. MAPKs signaling pathways are activated by mitogens such as cytokines and growth factors and terminate in the nucleus affecting transcription factor activity. The ERK pathway is crucial for cell cycle progression and proliferation, but also has a role in cell differentiation. JNK and p38 pathways were identified as kinases activated upon cellular stress, and were thereby linked to stress responses such as apoptosis. Later it has been shown that they also are involved in regulating the mitogenic response. Deregulation of MAPK pathways are believed to be important for tumorgenesis. This is thought to largely be due to their role in regulating proliferation and apoptosis (Davis, 2000; Roux and Blenis, 2004).

ERKs are activated when phosphorylated by the MAPK/ERK kinases (MEKs) 1 and 2, which in turn are activated by phosphorylation by Raf. Raf is itself activated by interaction with the small GTPase Ras, when it is in its activated GTP-bound form. Activated ERKs translocate to the nucleus where they phosphorylate, and hence regulate the activity of transcription factors such as Elk-1, MEF2, Fos, Myc and Stats. The MAPK kinases (MKKs) 4
and 7 are responsible for activation of JNKs, while p38 is activated upon phosphorylation by MKK3, 6 as well as 7. Activation of these MKKs can be mediated by, for example; MLKs, apoptosis-inducing kinase1 (ASK1) and transforming growth factor β activated kinase 1 (TAK1) (Davis, 2000; Gallo and Johnson, 2002). JNK and p38 pathways can, like the ERK pathway, activate the transcription factor Elk-1 and they also activate ATF2. The most well known target of JNK-activation is of course the transcription factor Jun (Aznar and Lacal, 2001; Roux and Blenis, 2004).

In 1995, it was shown that overexpression of constitutively activated Rac and Cdc42 induce activation of JNK and p38, but not ERK in both fibroblasts and epithelial cells. A dominant negative mutant of Rac inhibited JNK activation induced by EGF or constitutively activated Src and Ras (Cosso et al., 1995; Minden et al., 1995). Rac and Cdc42 can interact directly with MEKK1 and 4, and their kinase-inactive mutants block Rac and Cdc42-induced JNK activation (Fanger et al., 1997). Later it has been shown that also RhoA can interact with MEKK1 and activate its kinase activity (Gallagher et al., 2004).

Cdc42 and Rac are involved in the regulation of the JNK pathway leading to apoptosis in sympathetic neurons upon removal of nerve growth factor (NGF) (Bazenet et al., 1998). Activated Cdc42 also induce apoptosis mediated via JNK in Jurkat T-cells (Chuang et al., 1997). Rac and Cdc42 regulation of JNK and p38 pathways not only induce apoptosis. For instance, expression of certain muscle specific genes during skeletal myogenesis, was shown to be affected by the abilities of Rac and Cdc42 to regulate these pathways (Meriane et al., 2000).

MLKs interact with activated Rac and Cdc42, and this augment the ability of MLKs to induce JNK activation (Teramoto et al., 1996). Activated Cdc42 promote oligomerization of MLKs, which is required to induce JNK activation (Leung and Lassam, 1998; Vacratsis and Gallo, 2000). The overexpression of constitutively activated Cdc42 changes the phosphorylation pattern of MLK3, which may be another way of Cdc42 to influence the ability of MLK3 to activate JNK (Bock et al., 2000).

PAK was shown to induce the activation of JNK and p38 downstream of Rac and Cdc42 (Bagrodia et al., 1995a; Brown et al., 1996). In addition, PAK is able to phosphorylate Raf-1, hence cooperating with Ras to make Raf-1 fully activated and able to initiate downstream signaling of the ERK pathway (King et al., 1998; Tang et al., 1999). PAK can also phosphorylate MEK1, thereby synergizing with Raf-1 to achieve full activation (Frost et al., 1997). Kinase inactive PAK block cell transformation induced by combinations of the transforming mutants of Rac and Raf-1, and Ras and Raf-1. This suggests that PAK is necessary for cooperative transformation induced by these proteins (Tang et al., 1999).

Although RhoGTPases were first shown to activate the JNK and p38 pathways, it was later shown that they do induce ERK activation. The
sustained ERK activation necessary for cyclin D1 expression in the mid G1-phase of the cell cycle has been shown to require Rho activity, but not Rac1 and Cdc42 activity (Welsh et al., 2001). Another report, however, implicates Rac in signaling downstream of integrins required for cyclin D1 expression and cell cycle progression (Mettouchi et al., 2001). This study was performed using endothelial cells, while the other used NIH3T3 fibroblasts.

The specificity in the MAPK modules is believed to be achieved by scaffolding proteins interacting with several proteins of the same pathway. MEK partner 1 (MP1) interact simultaneously with MEK1 and ERK1, facilitating their interaction with Raf (Schaeffer et al., 1998). JNK interacting proteins (JIPs) interact with MLKs, MKK7 and JNK, and overexpression of JIP1 was shown to enhance the activation of JNK by MKK7 and MLK3 (Whitmarsh et al., 1998). The Rac-interacting protein POSH also has scaffolding ability and interacts with MLKs, MKK 4 and 7 and JNK, and increase JNK activity when over-expressed. Antisense POSH as well as small interfering RNA (siRNA) was shown to suppress e-Jun phosphorylation and neuronal apoptosis induced by withdrawal of NGF, further linking Rac to JNK-induced apoptosis (Tapun et al., 1998; Xu et al., 2003). Recently, a scaffolding protein for the p38 pathway during hypersomotic shock was identified and named osmosensing scaffold for MEKK3 (OSM). OSM interacts with actin and activated Rac in membrane ruffles. Rac then activates MEKK3 and MKK3 which also are in the complex (Uhlik et al., 2003).

**Serum Response Factor**

Serum response factor (SRF) is a transcription factor that can be activated by a variety of agents, including serum, growth factors and cytokines. The activity of SRF is regulated by phosphorylation, nuclear localization and by interaction with other transcription factors such as ternary complex factors (TCFs). Activated SRF binds to sequences in the gene promoters known as serum response elements (SREs), hereby affecting transcription (Chai and Tarnawski, 2002). The immediately early gene Fos is a transcription factor which gene expression is regulated by SRF. Activated SRF cooperate with activated TCFs, such as Elk-1, which is activated by phosphorylation by ERKs, to induce transcription of for example the Fos-gene (Eferl and Wagner, 2003).

Hill et al. showed that overexpression of activated RhoA, Rac1 and Cdc42 induced SRF-activation. Inhibition of Rho with C3 transferase blocked signaling by SRF by several stimuli, including LPA, PDGF and tetradecanoyl phorbol acetate (TPA). Induction of SRF by various extracellular stimuli, was however independent of Rac and Cdc42 (Hill et al., 1995). Activated Cdc42, but not activated Rho, induce chromosomal hyperacetylation, which is thought to be required for the transcription machinery to get access to the gene that is going to be transcribed. Both
JNK-dependent and independent mechanisms were implicated downstream of Cdc42 to induce histone acetylation (Alberts et al., 1998b). TC10 is also suggested to induce SRF-activation (Murphy et al., 2001; Murphy et al., 1999).

Rho-activation of SRF was shown to be dependent on the ability of Rho to promote polymerization of actin filaments, as well as on the threadmilling capacity of actin filaments (Sotiropoulos et al., 1999). It was later shown that the SRF-coactivator MAL binds to monomeric actin and is localized in the cytoplasm of resting fibroblasts. MAL is released from the actin monomers upon stimulation of cells with serum, which induces actin polymerization, and is translocated to the nucleus where it interacts with and activities SRF (Miralles et al., 2003; Settleman, 2003). SRF-activation by RhoA is dependent on ROCK, LIMK and mDia, which all are known to induce actin polymerization downstream of Rho (Copeland and Treisman, 2002; Geneste et al., 2002; Sotiropoulos et al., 1999).

It has been suggested that RhoA is important for activation of certain SRF-target genes but not others. Transcription of the SRF-target genes SRF and vinculin induced by stimulation with serum, PDGF or phorbol 12-myristate 13-acetate (PMA) is dependent on Rho and actin dynamics, while largely independent of MEK activation. The same stimuli also induce the expression of Fos and Egr-1 genes, but this was shown to be independent of Rho and actin dynamics and instead almost completely dependent on MEK-activation (Gineitis and Treisman, 2001).

The Rho/SRF pathway is implicated in tumor development. For instance, RhoA-dependent SRF activation is important for G12-induced cellular transformation (Fromm et al., 1997). Furthermore, Rho signaling to SRF is necessary for EMT of mouse carcinoma cell lines (Psichari et al., 2002). The PDGF-induced EMT of smooth muscle cells is also dependent on Rho signaling to SRF (Lu et al., 2001).

NFkB
The NFkB transcription factor exists as homo-and heterodimers located in the cytoplasm of resting cells. The cytoplasmic localization is achieved through the interaction with IxB-proteins, which are thought to mask the nuclear localization signal (NLS) of NFkB (Foo and Nolan, 1999). The NFkB-pathway is activated by stimulation of cells with cytokines and growth factors and can be involved in apoptotic as well as anti-apoptotic signaling depending on the context (Foo and Nolan, 1999; Graham and Gibson, 2005).

Overexpression of Cdc42, Rac and Rho induce transcriptional activity of NFkB by inducing phosphorylation of IxB, thereby releasing NFkB-dimers which enable their nuclear translocation. Activation of NFkB by stimulation of cells with tumor necrosis factor α (TNFα) was inhibited by overexpression of dominant negative Cdc42 and RhoA, but not Rac1. NFkB
activation induced by exposure to UV-light was not dependent on RhoGTPases, suggesting specificity in the involvement of RhoGTPases in the regulation of NF\(\kappa\)B (Perona et al., 1997). Rac1 was shown to activate NF\(\kappa\)B by inducing the production of reactive oxygen species upon cytokine stimulation of HeLa cells (Sulciner et al., 1996). JNK dependent activation of NF\(\kappa\)B downstream of Rac1 and Cdc42 has also been suggested (Montaner et al., 1998). PAK1 has been reported to mediate activation of NF\(\kappa\)B downstream of Rac1, but it was not investigated if that was mediated via the JNK pathway (Frost et al., 2000).

Activation of NF\(\kappa\)B has been coupled to cell migration and anchorage-dependent growth of tumor cells (Dempke et al., 2001). Cyclooxygenase 2 (COX-2) is an enzyme implicated in tumorgenesis, which expression can be induced by NF\(\kappa\)B. RhoA, Rac1 and Cdc42 can induce expression of COX-2, and in epithelial cells this occurs via an NF\(\kappa\)B-dependent mechanism (Benitah et al., 2003a; Hahn et al., 2002; Slice et al., 2000). A ROCK inhibitor blocked tumor growth in mice by preventing NF\(\kappa\)B activation and the following COX-2 production, implicating ROCK in the NF\(\kappa\)B pathway downstream of Rho (Benitah et al., 2003a). It should be mentioned that several pathways, including ERK, JNK and p38 pathways, also are implicated in the regulation of COX-2 expression (Chen et al., 2005; Slice et al., 2005; Zaric and Ruegg, 2005).

Activation of Rac1 in NIH3T3 fibroblasts induces expression from the cyclin D1 promotor in an NF\(\kappa\)B-dependent manner (Joyce et al., 1999). Cyclin D1 abundance is important for cell cycle progression and oncogenic mutants of Ras also induce cyclin D1 expression, coupling this pathway to tumorgenesis (Albanese et al., 1995; Finco et al., 1997).

**Stats**

The signal transducer and activator of transcription (Stat) factors dimerize via SH2-domains when they become phosphorylated and are then translocated to the nucleus where they can exert their action on gene transcription. Stats can be phosphorylated by the Janus kinases (JAKs), by non-receptor tyrosine kinase such as Src and in some cases, by the tyrosine receptor kinase itself (Levy and Darnell, 2002). Serine phosphorylation of Stats can modulate their activity in a positive or negative manner (Aznar et al., 2001).

Several studies have implicated RhoGTPases in the regulation of Stats. RhoGTPases can influence the phosphorylation status of Stat3 and Stat5a, but not of Stat1 and Stat5b (Aznar et al., 2001; Benitah et al., 2003b). RhoA induce tyrosine phosphorylation of Stat3 via the JAK2 kinase, while the JNK pathway mediates serine phosphorylation. Constitutively active Rho co-expressed together with wild-type Stat3 synergistically reduced contact inhibition of cells. Coexpression together with Stat3 dominant negative mutants instead blocked development of a tumorgenic phenotype, suggesting
a role for Stat3 in RhoA induced tumorgenesis (Aznar et al., 2001). Stat3 regulate the expression of many cell-cycle related genes and this is line with the fact that Stat3 seems to play a role in tumorgenesis.

Both RhoA and Rac1 stimulate Stat3 activity downstream of different G-protein coupled receptors (Faruqi et al., 2001; Lo et al., 2003; Pelletier et al., 2003). In the case of Rac1, this was shown to be dependent on its ability to interact with the NADPH-subunit p67phox which lead to the production of reactive oxygen species. This was necessary for the activation of JAK2 phosphorylation of Stats (Pelletier et al., 2003). Rac1 has been shown to activate Stat3 by different mechanisms. In one study, Rac1-induced serine phosphorylation of Stat3 was dependent on p38 and JNK activities, while in another study it was shown not to be dependent on JNK (Schuringa et al., 2001; Turkson et al., 1999). Rac1 can also interact directly with Stat3 in a GTP-dependent manner. The interaction between Rac1 and Stat3 was enhanced upon stimulation of cells with EGF or PDGF. Furthermore, dominant negative Rac1 was shown to inhibit Stat3-driven transcription in response to EGF and PDGF. It was suggested that Rac1, via its direct interaction could direct Stat3 to the proper kinase complex, as well as activating kinases in the complex (Simon et al., 2000).

Cdc42, Rac1, RhoA and RhoC can all activate Stat5a in epithelial cells. RhoA had the most pronounced effect and it was shown that RhoA triggered tyrosine phosphorylation of Stat5a via a JAK2-dependent mechanism, which enhanced the DNA-binding activity of Stat5a. RhoA also mediated the reduction of serine phosphorylation, which further increased the activity of Stat5a. Furthermore, it was shown that Stat5a was important for RhoA-induced EMT (Benitah et al., 2003b).

Other transcription factors
Nuclear factor of activated T-cells (NFAT) is important for proliferation and cytokine production in lymphocytes. RhoGTPases have been implicated in signaling regulating NFAT-activation downstream of both the T-cell receptor and the B-cell receptor (Hao et al., 2003; Yablonski et al., 1998). Constitutively activated mutants of Rac and Cdc42 induce transcriptional activation of E2F, a transcription factor crucial for cell proliferation (Gjoerup et al., 1998). Overexpression of RhoE/Rnd3 on the other hand has been shown to prevent the activation of E2F in response to serum, in line with the suggested inhibitory function of RhoE/Rnd3 (Riento et al., 2003; Wennerberg et al., 2003; Villalonga et al., 2004). cAMP-responsive element-binding protein (CREB) is yet another transcription factor suggested to be regulated by RhoGTPase signaling. In hippocampal progenitor cells LIMK1 was shown to phosphorylate and activate CREB during cell differentiation. Rac and Cdc42 signaling to PAK was shown to mediate the upstream signaling activating LIMK1 (Yang et al., 2004).
RhoGTPases in PDGF-signaling

PDGF is, as mentioned above, one of the growth factors known to regulate RhoGTPase activity. Here, a general introduction to PDGF-induced signaling is followed by a description of what is known about the involvement of RhoGTPases in PDGF-induced cellular responses.

PDGF-Induced Signaling

PDGFs constitute a family of polypeptide chains denoted, PDGF A-D. They are secreted as homodimeric ligands, and in addition A and B can also form PDGF-AB heterodimers. Two PDGF-receptor (PDGFR) subtypes, α and β, exist, which upon ligand binding become dimerized. Depending on which dimeric PDGF ligand is present, receptor hetero- or homodimers can be formed. Dimerization induces conformational changes which activate the kinase domains of the receptors and autophosphorylation of tyrosine residues in the receptors occurs (Fredriksson et al., 2004; Heldin et al., 1998). Phosphorylations on amino acid residues within the kinase domains enhance kinase activity, while phosphorylation of other residues provide binding sites for downstream signaling molecules (Kazlauskas et al., 1991; Kelly et al., 1991).

Src family tyrosine kinases, the regulatory subunit of phosphatidylinositol 3’ kinase (PI3’K), phospholipase C γ1, the protein tyrosine phosphatase SHP2 as well as adaptor molecules such as Grb2, Shc and Nck can interact directly with the phosphorylated PDGF-receptor. These molecules mediate the downstream signaling cascade controlling cellular responses to PDGF such as cell proliferation and chemotaxis (Heldin et al., 1998).

Src has been suggested to play a crucial role in PDGF-induced cell proliferation. Microinjection of Src inhibitory antibodies blocked DNA synthesis and led to inhibition of PDGF-mediated cell growth (Twamley-Stein et al., 1993). The employment of Src mutants in another study gave a similar result (Broome and Hunter, 1996). Src is implicated in PDGF-induced expression of Myc, which is a prerequisite for the mitogenic response (Barone and Courtneidge, 1995). Recent findings concerning this, involving RhoGTPases signaling will be discussed below. Conflicting data concerning the involvement of Src in PDGF-induced mitogenesis however exists. Receptor mutants not able to bind Src, have been shown to exhibit a normal mitogenic response to PDGF (DeMali and Kazlauskas, 1998; Hooshmand-Rad et al., 1998). In some reports it has been suggested that the phosphorylations of PLCγ1, SHP2, RasGAP and Shc are not only mediated via direct phosphorylation by the PDGF-receptor, but also require Src (DeMali et al., 1999). Src has also been shown to phosphorylate the PDGFβR itself. Mutation of this phosphorylation site in the receptor caused an increased chemotaxis towards PDGF, but decreased the mitogenic
response (Hansen et al., 1996). It is not clear why different approaches to block Src signaling in cells have given different results concerning the importance of Src in PDGF-induced responses. The use of different cell lines also suggests that the dependence on Src signaling may be cell type specific. But overall, it seems like Src is involved in PDGF-induced gene expression. In a recently published gene array study using a Src inhibitor it was shown that Src was important for expression of a subset of immediately early genes, including Myc, induced by PDGF-BB. Furthermore, it was suggested that the increased expression of this particular genes were not due to enhanced transcription, but to increased stabilization of their mRNA, mediated via a Src-signaling pathway (Bromann et al., 2005).

PI3’Ks is a family of lipid kinases, phosphorylating membrane phosphoinositides which then bind and activate downstream molecules such as Akt/protein kinase B (PKB), GEFs such as Vav and Sos, and atypical protein kinase Cs. PI3’Ks consists of one regulatory and one catalytic subunit. The binding of the regulatory subunit to, for example, the activated PDGF-receptors induce catalytic activity (Heldin et al., 1998). PI3’K activation is important for PDGF-stimulated actin reorganization, cell migration and chemotaxis, at least in part mediated via its effects on RhoGTPase activity (Hawkins et al., 1995; Wennstrom et al., 1994). Activation of Akt/PKB is important for PDGF signaling to cell survival and anti-apoptotic pathways. In addition, activated Ras can interact with the regulatory subunit of PI3’K, which causes a reciprocal activation of these two enzymes (Hu et al., 1995; Rodriguez-Viciana et al., 1994). PI3’K mediated signal transduction is also required for cell proliferation. Many signaling transduction pathways are activated in a biphasic manner during prolonged growth factor stimulation, which is required for initiation of cell cycle progression (Jones and Kazlauskas, 2001). It has been shown that only the late phase of increased PI3’K activation, occuring 3-7 hours after addition of PDGF, is important for cell cycle progression (Jones et al., 1999).

PLCγ1 catalyzes the hydrolysis of phosphatidylinositol(4,5)bisphosphate (PIP2) to inositol(1,4,5)trisphosphate (IP3) and diacylglycerol (DAG). IP3 increase the intracellular Ca\(^{2+}\) concentration, activating Ca\(^{2+}\)-dependent proteins, while DAG mediates activation of PKCs. PLCγ1 can be activated directly by the PDGF-receptor or by phosphorylation by Src (Kashishian and Cooper, 1993; Kim et al., 1991; Ronnstrand et al., 1992). PI3’K can also mediate activation of PLCγ1 (Rameh et al., 1995). PDGF α-and β-receptors have been shown to activate PLCγ1 differently (Eriksson A JBC 1995). PLCγ1 is suggested to play a role in the mitogenic as well as chemotatic response to PDGF-stimulation (Kundra et al., 1994; Roche et al., 1996).

As described in previous section, Ras is a crucial activator of MAPK-pathways. Ras can be activated in several ways downstream of the activated PDGF-receptors. Grb2 interacting directly with the receptor, or via indirect
interaction mediated via the adaptor protein Shc, induce Grb2-binding to Sos (Arvidsson et al., 1994; Rozakis-Adcock et al., 1992; Yokote et al., 1994). Furthermore, SHP2 can also serve as a link between Grb2 and the receptor (Li et al., 1994). The Ras-GEF activity of Sos is then activated, which promote formation of active Ras-GTP (Schlessinger, 1993).

The role of Ras as inducer of cell proliferation and growth is well established. SHP2 has also been suggested to mediate increased Ras-activation by dephosphorylating the tyrosine residue in the PDGF-receptor interacting with RasGAP, thereby preventing RasGAP-activation, leading to increased Ras-signaling (Ekman et al., 2002). In addition to its role in mitogenic signaling, SHP2 is suggested to be important for chemotaxis induced by PDGF (Ronnstrand et al., 1999).

Internalization and degradation of activated PDGF-receptor complexes as well as deactivation of signaling molecules by various negative feedback mechanisms determines the duration and strength of the PDGF-induced signaling.

**RhoGTPases in PDGF-induced Cellular Responses**

The involvement of RhoGTPases in PDGF-induced cellular responses, including actin rearrangements and gene regulation has been mentioned in previous sections. Here follows a more detailed summary on the subject.

*Reorganization of the actin cytoskeleton and chemotaxis*

PDGF-BB-induced actin rearrangements resulting in membrane ruffling was shown to be dependent on the PI3’K-activation of Rac in several reports (Hawkins et al., 1995; Nobes et al., 1995; Ridley et al., 1992; Wennstrom et al., 1994). Phospholipids produced by PI3’K bind to the PH-domains of RhoGEFs and are believed to activate their GTP-exchange activity, as described above. Moreover, the PI3’K-Rac pathway was shown be important for PDGF-induced chemotaxis (Hooshmand-Rad et al., 1997).

The exchange factor Tiam1 can mediate Rac activation upon PDGF-stimulation. Tiam1 was shown to interact with GTP-bound Ras which lead to synergistic activation of Rac by Ras and PI3’K (Lambert et al., 2002). Induction of Sos Rac GEF activity seems to be another way in which PDGF can regulate Rac. Sos is a well known activator of Ras, but it also contain a RhoGEF domain. It has been shown that the interaction of Sos with Grb2 mediates activation of Ras, while another pool of Sos, interacting with a protein complex containing eps8, Abi and p85 display RacGEF activity (Innocenti et al., 2003; Innocenti et al., 2002; Scita et al., 2001). Phosphorylation of Sos1 by Abl has also been shown to induce its Rac GEF activity (Sini et al., 2004).

PDGF was first shown to not be able to induce Cdc42-mediated filopodia formation in Swiss 3T3 fibroblasts (Kozma et al., 1995). It was however...
later demonstrated that PDGF activated Cdc42 in NIH3T3 fibroblasts and that this overexpression of dominant negative Cdc42 blocked PDGF-induced stress fiber disassembly. It was suggested that the activation of Cdc42 was mediated via the interaction between Cdc42 and the regulatory p85 subunit of PI3’K and not by PI3’K catalytic activity. N-WASP was suggested to mediate the cytoskeletal effects downstream of Cdc42 and disturbing this pathway resulted in decreased PDGF-induced cell migration (Jimenez et al., 2000).

From first studies made in Swiss 3T3 fibroblasts it was suggested that Rho activation was responsible for PDGF-induced formation of actin stress fibers (Ridley and Hall, 1992). As indicated here, other studies have shown that PDGF induce stress fiber disassembly. Activation of Rac and Cdc42 has been proposed to downregulate Rho activity upon stimulation by PDGF. Tiam1 was also here shown to specifically activate Rac, but not Cdc42 (Sander et al., 1999). Rac-induced production of ROS has been suggested to mediate downregulation of Rho activity in cells. ROS induce inactivation of the low molecular weight protein tyrosine phosphatase (LMW-PTP). This results in enhanced phosphorylation of p190RhoGAP, which otherwise is dephosphorylated by LMW-PTP. p190RhoGAP then downregulates Rho-activity (Nimnual et al., 2003).
The involvement of RhoGTPases in PDGF-induced cell movement through three-dimensional collagen matrices has also been investigated. This type of assay is supposed to illustrate cell invasion. It was shown that expression of dominant negative Cdc42, Rac and Rho, all inhibited PDGF-BB-stimulated Rat1 fibroblast movement through the 3D matrices. Expression of constitutively activated Cdc42 and Rho, and to a smaller extent constitutively activated Rac also inhibited cell invasion (Banyard et al., 2000). The finding that both dominant negative and constitutively active mutants of RhoGTPases blocked the same cellular response is not so surprising. The activity of RhoGTPases can be different in different areas of a cell and overexpression of a protein in large amount might lead to that the localized activation is lost.

In a similar type of invasion assay, using mammary epithelial cells, integrin-mediated cell invasion was shown to be dependent on Cdc42 and Rac1, but not RhoA. Interestingly, in terms of integrin-mediated invasiveness PI3’K was shown to be downstream of Cdc42 and Rac1 (Keely et al., 1997). PI3’K has been reported to also interact directly with Cdc42 and Rac1 (Zheng et al., 1994).

It should be mentioned that also MAPK-pathways have been implicated in the migratory response to growth factors. The importance of ERK signaling in PDGF-induced chemotaxis seems to be cell type specific (Anand-Apte et al., 1997; Choudhury et al., 1997; Hinton et al., 1998). In endothelial cells, p38 was shown to be involved in a Ras-dependent pathway important for PDGF-mediated cell migration (Matsumoto et al., 1999).

In a recent report, overexpression of the SHP2 and Grb2 interacting protein Gab1 was shown to enhance membrane ruffling induced by PDGF. The ruffling could be inhibited by coexpression of dominant negative Rac, but not by inhibition of p38 or ERK, although Gab1 expression increased their activation in response to PDGF (Kallin et al., 2004).

Gene expression and proliferation
The PDGF-induced Myc expression mediated by Src has been reported to be dependent on Rac and Cdc42 in NIH3T3 fibroblasts. It was shown that PDGF-induced Src activation of a Myc promotor was inhibited by dominant negative Rac, to a lesser extent by dominant negative Cdc42, but not by dominant negative Rho. It was suggested that Vav2 mediated the activation of Rac and Cdc42 downstream of Src. In the same report it was shown that the Src-induced expression of Myc was independent of the Ras-ERK pathway (Chiariello et al., 2001). In a following study it was demonstrated that the transcriptions factors c-Jun and JunD constituted the activator protein-1 (AP-1) transcription factor complex mediating the JNK-activation of the Myc promotor (Iavarone et al., 2003).

The tyrosine kinase Abl has previously been reported to be a downstream target for Src in PDGF-induced Myc expression and DNA synthesis
(Furstoss et al., 2002; Plattner et al., 1999). It was recently shown that Rac was activated downstream of Abl. The activation of Rac induced activation of both JNK and NADPH-oxidase. This mediated an induction of Myc expression (Boureux et al., 2005). This suggests that also the production of ROS is important for the expression of Myc.

In another report, when a different cell line was used, PDGF-induced JNK activation was shown to be dependent on PI3′K. JNK activation induced by the overexpression of the p110α catalytic subunit of PI3′K was inhibited by dominant negative Rac and dominant negative Ras (Lopez-Illasaca et al., 1997).

A dominant negative Rac1 have been shown to inhibit PDGF-induced expression from a cyclin D1 promoter in airway smooth muscle cells. ROS production downstream of Rac1 seemed to be involved in the induction of cyclin D1. ERK signaling also mediated induction of cyclin D1, independent of Rac1. This suggests that Rac1 and ERK belong to parallel pathways inducing cyclin D1 expression (Page et al., 1999).

PDGF has, as described above been shown to induce inactivation of Rho via a Rac-dependent pathway. However, there are reports implicating the involvement of Rho in cellular processes induced by PDGF. Cell type specific features can of course be one explanation, but no thorough clarification has been made. PDGF-mediated degradation of the cyclin inhibitor p27kip1 has been reported to be dependent on RhoA in Chinese hamster embryonic fibroblasts (Weber et al., 1997).

RhoGTPases have also been implicated in the PDGF-induced activation of other transcription factors, including SRF and Stats as described in the previous section. It should be mentioned that Stat3 also has been shown to be involved in Src mediated Myc expression upon PDGF stimulation (Bowman et al., 2001).
Present Investigation

Paper I

*The Mammalian Homologue of the Caenorhabditis elegans Polarity Protein PAR6 is a Binding Partner for the RhoGTPases Cdc42 and Rac1*

Par6 was identified in a yeast-two hybrid screen for proteins interacting with Cdc42. The identification of WASP and CIP4 as Cdc42-interacting proteins done in the same screen, had been reported previously (Aspenstrom, 1997; Aspenstrom et al., 1996). In the present study, our aim was to characterize the interactions between Par6 and RhoGTPases as well as investigate the role of Par6 in mammalian cells.

The *C. elegans* homologue of Par6 had been shown to be essential for establishment of cell polarity during asymmetric cleavage of the worm embryo. Another Par-protein, Par3 had been found to colocalize with Par6 in the embryo. In addition, Par3 and Par6 were dependent on each other for localizing correctly within the embryo (Hung and Kemphues, 1999; Watts et al., 1996).

We showed that Par6 interact with Cdc42 and Rac1 in a GTP-dependent manner, not only in yeast two hybrid system but also in co-immunoprecipitation experiments in transfected COS1 cells and in *in vitro* binding experiments.

In order to study endogenous Par6, an antiserum raised against amino acid residues 2-16 of mouse Par6 was produced. This was used to perform immunofluorescence staining of epithelial MDCKII cells. Par6 was found localized to the nucleus and to cell-cell contact areas. We stained the cells for proteins known to localize to cell-cell contacts in epithelial cells and found that the localization pattern of Par6 corresponded well with the one for the tight junction protein ZO-1.

Treatment of MDCKII cells with HGF/SF had previously been shown to induced breakdown of cell-cell contacts and promote cell spreading. Moreover, this had been shown to be dependent on RhoGTPase activity (Van Aelst and Symons, 2002). This made us investigate the localization of endogenous Par6 during stimulation of MDCKII cells with HGF/SF. We found that loss of Par6 from tight junctions was concomitant with their progressive breakdown and seemed to follow the same dislocation pattern as
ZO-1. Par6 localization to the nucleus appeared to be unaffected by HGF/SF-treatment of the cells.

It had previously been shown that the mammalian homologue of Par3 localized to tight junctions in epithelial cells (Izumi et al., 1998). We were not able to perform double staining experiments for Par6 and Par3 in the same cell, since the antibodies we had access to were raised in the same species. However, Par3 colocalized with ZO-1 in the same manner as Par6 did. Furthermore, co-immunoprecipitation experiments in transfected COS1 cells showed that Par6 could interact with the 100, 150 and 180 kD splice variants of Par3.

In conclusion, we identified a mammalian homologue of the C.elegans Par6 as a protein interacting with active Cdc42 ad Rac1. We also showed that endogenous Par6 localized to the nucleus as well as to areas of cell-cell contacts, where it colocalized with the tight junction protein ZO-1, in epithelial cells. In addition, Par6 was shown to interact with Par3, a protein previously shown to localize to tight junctions of epithelial cells. This implied Par6 and Par3 as parts of a complex regulating cell polarity also in mammalian cells. Furthermore, the interaction between Par6 and activated Cdc42 and Rac1 suggested a role for these RhoGTPases in the regulation of the activity of this complex.

PaperII

Ectopic Coexpression of mPar6C and PKCζ Cause Cell Rounding and Formation of Neurite-like Extensions

Prior to the initiation of this study Par6 had been shown to, in addition to interacting with Cdc42, Rac1 and Par3, also interact with atypical PKCs (Joberty et al., 2000; Lin et al., 2000). In an attempt to gain further insight into the role of the Par6 interaction with atypical PKCs we cotransfected these proteins into NIH3T3 fibroblasts. A dramatic change in cell morphology in the NIH3T3 cells coexpressing Par6 and PKCζ occurred. The cells rounded up and usually two long neurite-like extensions were formed. The induction of cell morphology alterations was specific for the ζ-isof orm of aPKCs, since overexpression of PKCλ together with mPar6C did not induce these alterations. Similar changes in morphology was seen when mPar6C was coexpressed with PKCζ in MDCKII cells.

Overexpression of a PKCζ-mutant, not able to interact with mPar6C, together with mPar6C did not induce cell rounding and formation of cytoplasmic extensions. This implies that a direct interaction between these proteins is required to induce changes in cell morphology. Furthermore, kinase activity of PKCζ was required, since a kinase defective mutant of
PKCζ failed to induce morphological alterations when co-expressed with mPar6C.

Cells co-expressing N17Cdc42, mPar6C and PKCζ did not exhibit cell rounding and long extensions, implying that functional Cdc42 is required for these changes in morphology. Coexpression of N17Rac1 or N19RhoA did not block the mPar6C/PKCζ-induced morphology changes.

It has previously been shown that induction of neurite-like extensions in cells is linked to alterations in the microtubule system (Hirose et al., 1998; Scaife et al., 2003). Immunofluorescence labelings using an antibody recognizing α-tubulin showed that the microtubules in the cells co-expressing mPar6C and PKCζ were arranged in bundles, resembling stabilized microtubules. Acetylation is a post-translational modification found on stabilized microtubules (Palazzo et al., 2003). By the use of an antibody against acetylated tubulin we could show that microtubules in the cytoplasmic extensions were acetylated.

We also performed an initial study on mPar6C phosphorylation. Coexpression of mPar6C together with PKCζ or PKCα increased the general phosphorylation of mPar6C in in vitro kinase experiments. PKCζ and PKCα were also able to phosphorylate GST-mPar6C in an in vitro kinase assay with purified proteins. Several phosphorylated amino acid residues in mPar6C were identified by two-dimensional tryptic phosphopeptide mapping. The importance of these phosphorylations for mPar6C-function, however, requires further investigations.

In conclusion, coexpression of mPar6C and PKCζ, but not PKCα, induced dramatic changes of cell morphology in NIH3T3 as well as MDCKII cells. These changes in morphology probably involve the microtubule system of the cells.

**Paper III**

*Involvement of RhoGTPases in Platelet-Derived Growth Factor-Induced Gene Expression*

RhoGTPases have been implicated in the regulation of signaling pathways regulating gene expression. However, little is still known about how transcription of specific genes is influenced by RhoGTPase signaling. Activation of the PDGF-receptor and RhoGTPases have been shown to regulate the same signaling pathways such as MAP-kinase and SRF activation. It has been shown in some cases that RhoGTPases are involved in regulating gene expression downstream of the PDGF-receptor (Chiariello et al., 2001; Gineitis and Treisman, 2001; Page et al., 1999). This made us interested to see what effects RhoGTPases have on the global gene expression pattern elicited by PDGF stimulation.
We decided to investigate the roles of RhoGTPases in PDGF-induced gene expression by cDNA microarray analyses. We chose AG01518 human primary foreskin fibroblasts for our experiment. This model system had been used previously by us to study global gene expression pattern induced by PDGF (Demoulin et al., 2004).

It has previously been shown that PDGF-BB stimulation of cells induces the activation of Cdc42 and Rac1 (Hawkins et al., 1995; Jimenez et al., 2000; Wennstrom et al., 1994). To confirm that this was the case also in AG01518 fibroblasts we performed GST-pulldown experiments detecting the amount of activated Cdc42, Rac1 and RhoA upon PDGF-BB treatment of cells. We found that Cdc42 was activated upon PDGF-BB stimulation. To our surprise, we could not detect increased Rac-activation. On the contrary, the amount of activated Rac1 was high in non-stimulated fibroblasts, and decreased upon stimulation with PDGF-BB. No activation of RhoA upon PDGF-BB stimulation of cells was observed, which was in line with previous reports.

Since we intended to block RhoGTPase-signaling downstream of the PDGF-receptor by using dominant negative mutants of Cdc42, Rac1 and RhoA in the gene expression study, we wanted to confirm that these dominant negative proteins worked as expected in this system. Overexpression of dominant negative Cdc42 and Rac1 blocked PDGF-BB induced rearrangements of the actin microfilament system. As could be expected, dominant negative RhoA did not seem to have any effect.

Even though we could not detect any increased activation of Rac1 upon PDGF-BB treatment the ability of dominant negative Rac1 to prevent PDGF-BB induced membrane ruffling yet indicated that functional Rac is important for the cellular response to PDGF-BB in AG01518 fibroblasts.

We then went on to perform the gene expression experiment. Adenovirus infection was used to over-express the dominant negative versions of Cdc42, Rac1 and RhoA as well as GFP, as a control, in AG01518 fibroblasts. The gene expression induced by PDGF-BB was monitored by cDNA microarray analysis and the differences in expression of genes in the cells expressing different dominant negative RhoGTPases were determined.

The expression of 16 genes, out of the 45 genes upregulated by PDGF-BB stimulation for one hour, was inhibited ≥50% in the presence of dominant negative Cdc42, Rac1 or RhoA. 19 other genes were down-regulated by one or two of the dominant negative RhoGTPases used. In addition, 5 genes were further upregulated by PDGF-BB in the presence of dominant negative Cdc42 and 4 other genes in the presence of dominant negative Rac1, compared to control.

The expression of the genes coding for cyclooxygenase 2 and RhoB were found to be affected by the presence of dominant negative RhoGTPases. These genes have previously been shown to be regulated by RhoGTPases, hence serving as an indicator of the relevance of our data (Benitah et al.,
Teramoto et al. have reported a study on global gene expression when constitutively activated Cdc42, Rac1, RhoA, as well as Ras, were stably expressed in NIH3T3 fibroblasts (Teramoto et al., 2003). The genes regulated in their study showed few similarities to the genes we found affected in our study. This is probably due to the different approaches employed. We used a method based on blockage of induced RhoGTPase activity, while Teramoto et al. used cell lines overexpressing constitutively activated RhoGTPases. In addition, different cell lines and microarray DNA-chips were used.

Our data suggests that RhoGTPase signaling do play a role in PDGF-BB induced gene expression. We, however, still need to validate the expression levels we found in the microarray experiment with real-time PCR (RT-PCR). Studies of changes of protein levels will also be important. We have so far not investigated the activity of MAPK pathways in the AG01518 fibroblasts that express dominant negative RhoGTPases. This, as well as looking into the activity of transcription factors, such as Stats, would be of interest to clarify what signaling pathways that are involved.

In some of the previous published microarray studies on PDGF-induced gene expression, Src, MEK/ERK and PI3´K pathways have been blocked by different approaches to investigate these pathways contribution to gene transcription (Bromann et al., 2005; Fambrough et al., 1999; Tullai et al., 2004). Comparisons of our data with these previous gene array studies will also give clues to how the signaling pathways cooperate to regulate gene expression. Such comparisons can hopefully serve as guidance for future studies.
Future Perspective

The involvement of RhoGTPase signaling in the regulation of numerous cellular processes as well as their potential role in disease development, such as tumorgenesis, make continued signal transduction research in this area essential.

Par6 was quite recently identified as an effector protein for Cdc42 and their collaboration in the establishment of cell polarity in different cell types began to be unraveled. However, Par6 has also been shown to interact with other RhoGTPases, including Rac1, TC10, TCL, Chp and Wrch-1 (Aspenstrom et al., 2004; Joberty et al., 2000; Johansson et al., 2000). The knowledge of signaling mediated via these RhoGTPases and Par6 is minimal. It will be interesting to see what roles Par6 play together with these GTPases. On the whole, information about the more recently discovered RhoGTPases, such as Chp and Wrch1, is limited. Studies will be required to establish what roles they play in intracellular signaling and how and if they collaborate with other RhoGTPases. As exemplified here by Par6, many RhoGTPases share effector proteins. How the interactions between the same effector protein and different RhoGTPases is regulated in the cell and how this affect the outcome cellular response is another area for further studies.

The ability of Par6 to induce transformation when over-expressed together with PKCζ, has so far gained quite little attention (Qiu et al., 2000). The Par6/PKCζ-complex can regulate RhoA-degradation via Smurf1. Blockage of Smurf1-mediated degradation of RhoA lead to decreased cell motility and reversion of the transformed phenotype of HEK293T cells (Wang et al., 2003). It is possible that our finding that coexpression of Par6 and PKCζ induce cell rounding and the formation of long cytoplasmic extensions are coupled to these previous findings. The cell rounding may indicate cell detachment, which might be coupled to cell transformation. Furthermore, Par6 has been implicated in TGFβ-induced EMT, a process closely linked to tumor progression (Ozdamar et al., 2005). The connection between cell transformation and increased cell migration is an interesting area, and in this respect it seems like Par6 might be an important player.

Our initial study of the phosphorylation of mPar6C suggested that it could be phosphorylated on several serine and threonine residues in cells. Combination of our in vitro and in vivo data suggests that aPKCs are candidate kinases for phosphorylating mPar6C. Further studies will be needed to determine if this is the case and what role phosphorylations of
mPar6C mean for its function. Phosphorylation may for example change the ability of Par6 to interact with other proteins. Indeed, it was recently shown that phosphorylation of a serine in the C-terminus of Par6 by the TGFβ type II receptor was required for Par6 to interact with Smurf1 (Ozdamar et al., 2005). Potentially, phosphorylation can be performed by kinases activated downstream of the Par6/aPKC mediating positive or negative feedback signaling during cell migration. This would also be interesting to investigate.

In our gene array study on the involvement of RhoGTPases in PDGF-BB induced gene expression we found that the expression of many genes were affected by the presence of dominant negative RhoGTPases in the cells. More studies are required to establish which signaling pathways are affected by RhoGTPase signaling in this system. Furthermore, performing another set of gene array experiments in the presence of dominant negative RhoGTPases but with a longer time of PDGF-BB stimulation would be interesting. Demoulin et al showed that PDGF-BB stimulation of AG01518 fibroblasts for 24 hours induced a striking up-regulation of genes related to lipid metabolism (Demoulin et al., 2004). It would be interesting to see how the effect of blockage of RhoGTPase signaling during prolonged PDGF-BB stimulation would affect the expression of this subset of genes.

As mentioned above, comparisons with other gene array studies performed on cells stimulated with PDGF should be helpful. With the advances of bioinformatic technologies this can hopefully be done in a systematic manner. This type of technology will also be a useful aid in integrating data obtained from gene array experiments, proteomic investigations and traditional signal transduction research.

RhoGTPases seem to be important for both cell migration and gene expression induced by PDGF. In some cases the signaling pathways responsible for these responses have been delineated as described in previous sections.

However, how coordination of migratory and mitogenic responses occurs in the cell upon growth factor stimulation is still not comprehensively understood. It is certainly an intriguing topic, not the least in light of tumor cell growth and metastasis.
Acknowledgements

The work presented in this thesis was performed at the Ludwig Institute for Cancer Research in Uppsala, Sweden.

I would like to thank:

Carl-Henrik Heldin, director of the Ludwig Institute in Uppsala, for giving me the opportunity to start and finish my PhD studies at the institute. Your beliefs in science as well as in people are astonishing.

Jean-Baptiste Demoulin for supervision during my last project. I enjoyed the, unfortunate, short time we worked together at Ludwig. Thank you for the help with the project and the manuscript that you have provided me with from Brussels.

Johan Lennartsson for including me in your group and for all the help you have provided me with during the end of my thesis work. It was great that you came back from the U.S. just in the right moment! Thank you for also being a good friend.

Pontus Aspenström for financing and for supporting my attendance in conferences.

The group of Terje Johansen, Tromsø University, Norway, for the collaboration on the Par6-project. Especially I would like to thank Terje Johansen and Geir Bjørkøy for all their help with the manuscript. I also would like to thank the entire group for making my short visit in Tromsø informative as well as pleasant.

The Department of Natural Sciences Högskolan i Kalmar, for providing me with an excellent master education-which I really enjoyed!

All funny people participating in “spex” at Ludwig. I always enjoyed planning, performing or being a spectator of these events!

Maria Simonsson, Åsa Fransson, Susann Karlsson, Maria Ekman and Katerina Pardali for nice company at the institute during the last years.
Maréne Landström for all that female bonding!

Valeria Giandomenico for including me in your social community and thereby also inviting me to many excellent Italian dinners.

Valeria Giandomenico, Magnus Essand, Marcin Kowanetz, Kaska Kowanetz, Jean-Baptiste Demoulin, Philippe Soubeyran, Enrico Bracco and Anders Kallin for all social events such as dinners, movie-visits and trips.

Simon Ekman, for being a good, warmhearted friend during your time at Ludwig and for still being so.

The friends I got to know during my studies in Kalmar: Karin Odenô, Karin Hygge Blakeman, Elin Gustafsson, Susanna Eketjäll, Lotten Ragnarsson and Maria Lokrantz. You are all very sensible people with a good sense of humor, which make your company very joyful. I am happy that you are my friends!

Lucie Hájková, my dear sister. Your caring and love mean more to me than I can express in words. Thank you for recognizing and reminding me about my good sides. Our common humor also makes our friendship special- I do not giggle with anyone else like I do together with you!

Anders Kallin, the best friend imaginable! Thank you for your sensibility, vacation trips (including advices for fridge-magnet purchases!), movie-visits, dinners, phonecalls, crazy jokes, crazy conversations, Näsapan and all your support! I appreciate very much that I can talk to you about everything and that I can say anything to you. Thank you for being there!
References


PDGF stimulates an increase in GTP-Rac via activation of phosphoinositide 3-kinase. *Curr Biol* 5, 393-403.


63


Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 128

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)

Distribution: publications.uu.se

urn:nbn:se:uu:diva-6698

ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2006