Signalling Pathways Involved in Controlling Polarity, Cell Migration and Cytoskeletal Organization in Walker 256 Carcinosarcoma Cells.

Inauguraldissertation

der Philosophisch-naturwissenschaftlichen Fakultät

Der Universität Bern

vorgelegt von

Marc Gutjahr

von Rohrbach/BE

Leiterin der Arbeit:

Prof. Dr. Verena Niggli

Institut für Pathologie

Universität Bern

1. Abbreviations

BSA	bovine serum albumin
DMSO	dimethylsulphoxide
EDTA	ethylenediaminetetraacetic acid
ERM	ezrin/radixin/moesin
F-actin	filamentous actin
G-actin	globular actin
GAP	GTPase-activating protein
GEF	guanine nucleotide exchange factor
GDI	guanine nucleotide dissociation inhibitor
HA	haemagglutinine
MLC	myosin light chain
MLCP	myosin light chain phosphatase
MLCK	myosin light chain kinase
OA	okadaic acid
PBS	phosphate buffered saline
PI3-kinase	phosphatidylinositol 3-kinase
PIP4,5K	phosphatidylinositol 4-phosphate 5-kinase
PI	phosphatidylinositol
PIP	phosphatidylinositol-mono-phosphate
PIP3,4P_2	phosphatidylinositol 3,4-bisphosphate
PIP4,5P_2	phosphatidylinositol 4,5-bisphosphate
PIP3,4,5P_3	phosphatidylinositol 3,4,5-trisphosphate
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PS	phosphatidylserine
ROCK	Rho-associated coiled-coil forming protein kinase
SDS	sodium dodecyl sulfate
TBS	tris buffered saline

2. Introduction

Among the diseases in the world, cancer is maybe the most devastating every year causing a high number of deaths.

What is it that makes cancer such a dangerous disease? The high mortality is mainly caused by metastasis formation (around 90% of all cancer deaths). Tumour cells spread from their origin all over the body to form secondary tumors (Condeelis and Segall, 2003).

Directed cell migration is very important for many physiological processes in metazoan organisms. Especially in embryogenesis cells have to migrate to form different tissues so that a whole organism can be formed (Kulesa et al., 2004). In host defense cell migration is required to fight pathogens, and in wound-healing fibroblasts have to migrate to the site of tissue damage (Geng, 2001). But also in pathological events cell migration is highly important (Entschladen et al., 2004).

Active tumor cell locomotion is known to play a crucial role in invasion and metastasis. There are many examples that show that enhanced motility correlates with high metastatic potential (Itoh et al., 1999). Therefore, it is of interest to know how shape changes and locomotion of tumor cells are regulated and how locomotion can be stopped. A better understanding of these mechanisms could lead to the development of novel therapeutic strategies.

2.1. Tumor invasion and metastasis

Tumor cell migration and invasion of basement membranes is a crucial step in the multistage process which leads to the formation of metastasis. Transformation of normal cells into cancer cells (tumorigenesis) occurs by successive mutations. Cancer cells proliferate independently and no longer respond to their environment (Hanahan and Weinberg, 2000).

Metastasis is a process that usually takes years but can be accelerated by many environmental factors, especially smoking. The mechanisms that causes metastatic cells is not well understood up to date but it is comprised of distinct events.

Cancer cells penetrate the boundaries of the tumor tissue and infiltrate the walls of blood vessels or lymph vessels, gaining a means of transport to other parts of the body far from the original tumor site (Woodhouse et al., 1997).

Cellular invasion includes the attachment of cells to the basement membrane, secretion of enzymes which degrade the basement membrane, the migration of cells into the target tissue in response to specific chemotactic stimuli, and tumor cell colony formation.

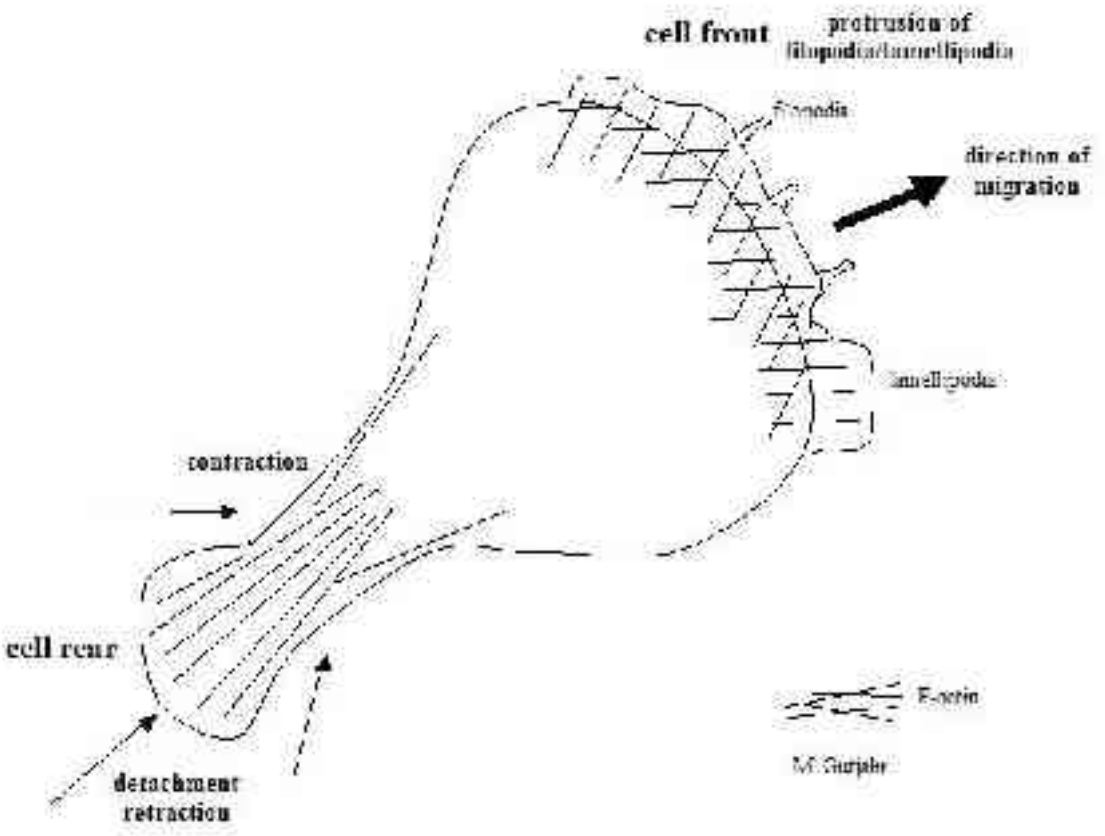


Fig. 1. Polarized and migrating cell. At the leading front F-actin polymerisation pushes the cell forward and formation of new focal contacts stabilizes new extensions. At the rear old focal contacts detach and the cell slides forward by contraction of actin filaments.

A necessary feature of invasion is tumor cell locomotion (Lin et al., 2004). This active movement is required to get through the basement membrane and stroma. To migrate a cell needs to build up a polarized shape (Fig. 1). At the leading front the formation of pseudopodia protrusions is induced which is regulated by surface ligand binding. Cytoskeletal components are being mobilized and interact with the inner membrane surface (Ridley, 2004). The motility of tumor cells is thought to be regulated by autocrinely secreted cytokines (Nabi et al., 1991) that are acting through receptor-activated G-proteins (Morikawa et al., 1999). Moreover, host organ-derived chemokines are influencing the direction of locomoting tumor cells and might play a crucial role in the organ-selective homing of metastasis. These signals from outside are transduced into the cell where they are processed into very precisely tuned changes within the cell. This is roughly how a cell responds to diverse signals from the environment (Balkwill, 2004).

One of the aims of cancer research is to understand which signal transduction pathways are altered in a cancer cell that spontaneously migrates and which are the targets of these pathways involved in migration.

2.2. Walker 256 carcinosarcoma cells

Walker 256 carcinosarcoma cells were derived from Spraque-Dawley rats. This Walker 256 tumor arose spontaneously in the region of the mammary gland and causes osteoclastic bone metastasis and hypercalcemia. It grew as a carcinosarcoma with 2 to 3 morphologically different cell types. It is suggested that the origin of the tumor was a multipotential stem cell of hematopoietic origin (Stewart et al., 1959). Walker 256 carcinosarcoma cells are most likely of monocytoid origin (Simpkins et al., 1991).

A special feature of these cells is that they are able to polarize and migrate spontaneously in the absence of added stimuli at high speed (5-10 μm/min). Polarized locomoting cells show a clear front-tail polarity with a contracted tail at the trailing end and ruffles and spikes at the leading front (Keller, 1986).

It has been shown that migration can be enhanced by microtubule-disassembling drugs such as colchicine, vinblastine and nocodazole or decreased by the microtubule-stabilizing drug taxol or by the actin filament-stabilizing agent cytochalasin B. These data suggest that both microtubules as well as actin filaments are involved in the development of polarization and migration (Keller, 1986).

Recent findings suggest that spontaneous locomotion of Walker carcinosarcoma occurs due to constitutively activated intracellular signalling proteins. Their activation may be induced either by mutation or overexpression. PKC, PI 3-kinase, Rho and ROCK are implicated in migration of these cells (Wicki and Niggli, 1999; Wicki and Niggli 2001).

2.3. Small GTP-binding proteins: the Rho-family

The Rho proteins belong to the widespread Ras superfamily of small G proteins. Therefore they are called Ras homologues (Macara et al., 1996). Rho GTPases are key regulators of the actin cytoskeleton. By their action on the actin cytoskeleton, they play a major role in fundamental processes, such as cell contraction, cell motility, cell adhesion, cell shape, and also transcriptional

activation and vesicle trafficking. Therefore, it is not surprising that knockout mice lacking Rho GTPases often are not viable (Sugihara et al., 1998).

The nomenclature has become more and more confusing with the increasing number of identified members and isoforms. The Rho protein family can be devided into 6 different classes consisting of the following members: Rho (RhoA, RhoB, and RhoC), Rac (Rac1, Rac2, and Rac3, which is also known as Rac1B [RhoG]), Cdc42 (Cdc42Hs, Chp, G25K, and TC10), Rnd (RhoE/Rnd3, Rnd1/Rho6, and Rnd2/Rho7), RhoD, and TTF (Bishop and Hall, 2000).

Rho, Rac, and Cdc42 are the best studied isoforms. Each of them has its own specific effects on the actin cytoskeleton. Like other Ras superfamily members, Rho family GTPases cycle between a GDP-bound (inactive) state and a GTP-bound (active) state (Fig. 2). Therefore, Rho proteins are also called molecular switches (Symons and Settleman, 2000).

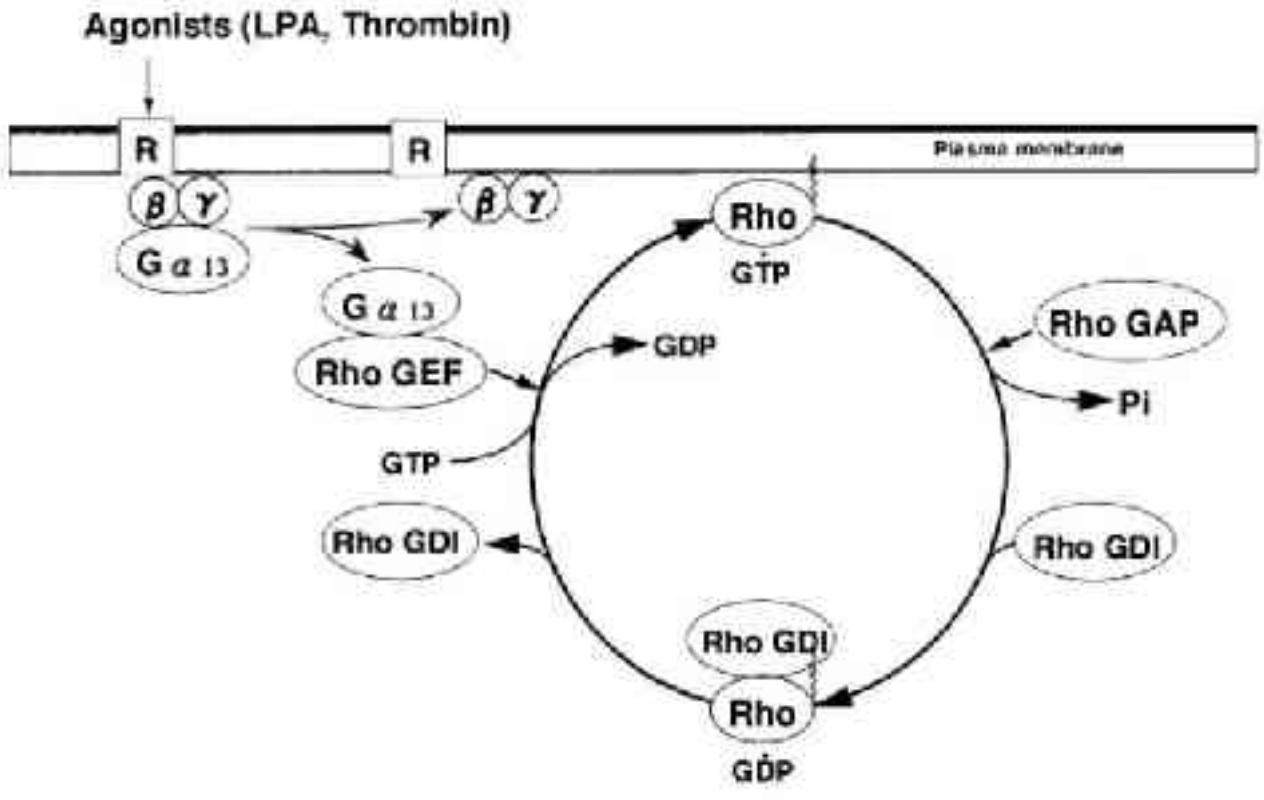


Fig. 2. Mode of Rho activation. R, Receptor for agonists; GEF, Guanine nucleotide exchange factor; GDI, guanine dissociation inhibitor; GAP, GTPase activating protein. (Kawano et al., 2002).

Biochemical and genetic studies have led to the identification of three classes of regulatory proteins that control the nucleotide state of Rho family proteins. These are the guanine nucleotide exchange factors (GDP-GTP exchange factors or GEFs), the GTPase-activating proteins (GAPs) and the

guanine nucleotide dissociation inhibitors (GDIs). GEFs are the proteins that catalyse the exchange of GDP for GTP in response to extracellular signals. This depends on the ability of GEFs to facilitate the release of GDP and to transiently stabilize the nucleotide-free state of the protein. Then, binding of GTP stimulates the release of the protein from GEF and GAPs promote the intrinsic GTP-hydrolysing activity of Rho proteins, thereby leading to their rapid conversion to the inactive GDP-bound form. Finally the GDIs appear to function by preferentially binding to GDP-bound GTPases and preventing spontaneous and GEF-catalysed release of nucleotide. Thus, they maintain the GTPase in its inactive state (Wheeler and Ridley, 2004).

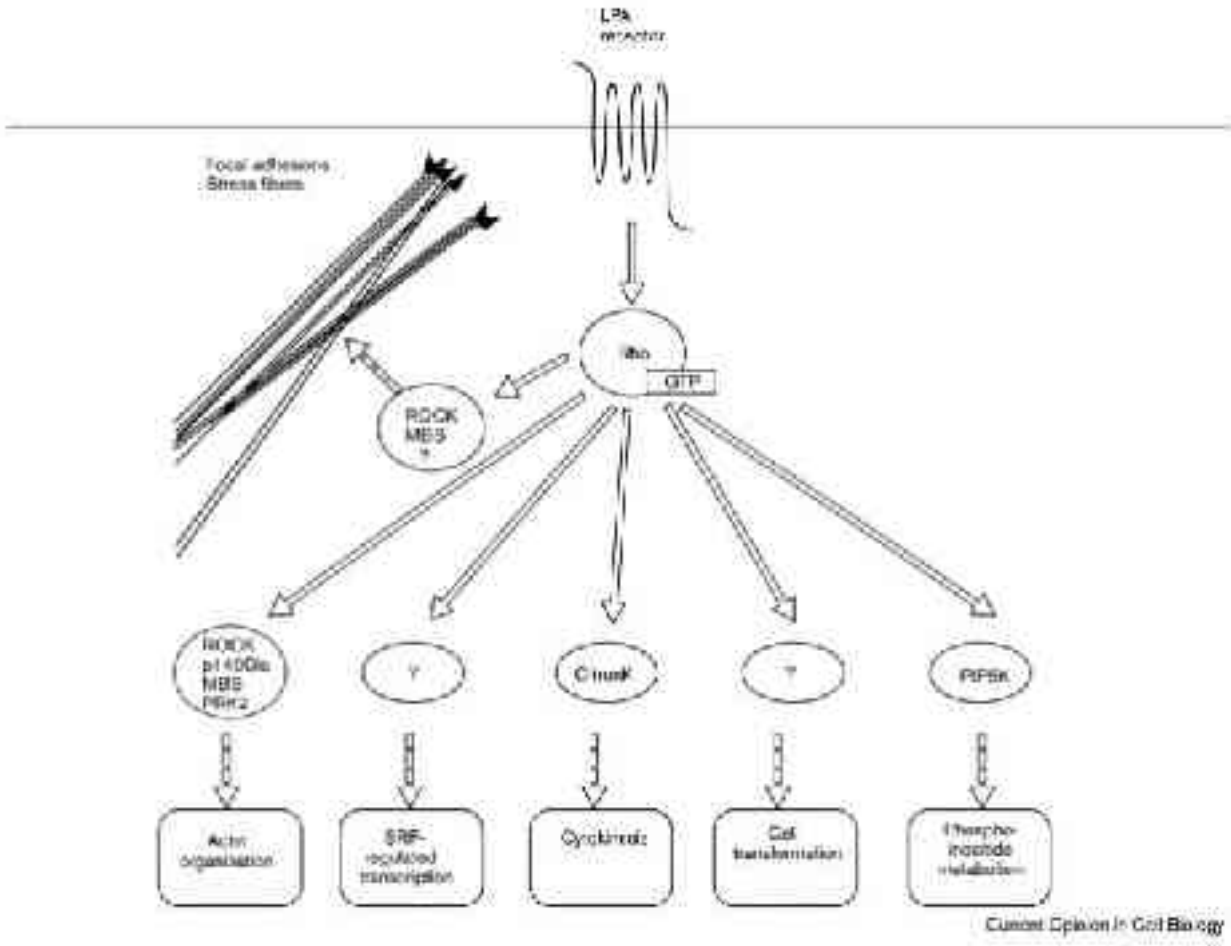


Fig. 3. Rho-regulated signalling pathways. (Aspenstrom, 1999)

A number of studies have indicated that an intracellular translocation of Rho family GTPases to the membrane occurs following activation (Bokoch et al., 1994; Abo et al., 1994; Kranenburg et al., 1997). Whether this membrane translocation is necessary for the activation of Rho proteins or whether it is involved in the recruitment of downstream signalling components remains not clear up to now.

Upon activation of Rho GTPases a subset of many downstream targets become active that initiate further signalling pathways (Fig. 3). Among these downstream targets there are protein kinases,

phosphoinositide kinases and adaptor proteins. Rho proteins play a crucial role in the development of cell polarity and migration. It has been shown that in resting fibroblasts, Rho activation leads to assembly of stress fibers and focal adhesions (Ridley and Hall, 1992). Furthermore, activation of Rac results in the formation of lamellipodia and membrane ruffles whereas activated Cdc42 is involved in initiating filopodial protrusions (Ridley et al., 1992).

A first indication that Rho affects the actin cytoskeleton came from the use of C3 exoenzyme, a *Clostridium botulinum*-derivate ADP-ribosyl transferase that covalently modifies the Asn residue in position 41, located in the effector domain of Rho (Aktories et al., 1988; Sekine et al., 1989). The effect of this very potent Rho inhibitor, which inhibits RhoA, RhoB, and RhoC, is the induction of cell rounding and loss of stress fibers in many cell types.

2.4. Rho-associated coiled-coil forming protein kinase (ROCK)

A number of Rho effectors have been identified which associate specifically with the GTP-bound forms of Rho GTPases, including Rho-kinase, protein kinase N (PKN), rhophilin, rhotekin, citron, citron kinase, mDia, and kinectin (Shimizu et al, 2003).

One of them, Rho-kinase, has received much attention because it is implicated in many processes downstream of Rho including smooth muscle contraction, stress fiber and focal adhesion formation, intermediate filament disassembly, neurite retraction, and cell migration.

Rho-kinase was identified as a GTP-Rho-binding protein by affinity column chromatography of bovine brain membrane extract fraction (Matsui et al., 1996). It is a 160 kDa multidomain serine/threonine protein kinase composed of four domains: the N-terminal kinase domain, the long coiled coil domain encompassing about 600 amino acid residues, the Rho-binding domain (RhoBD), and the C-terminal pleckstrin homology (PH) domain with an inserted Cys-rich Zn finger motif. In absence of the C-terminal PH domain or the coiled-coil and PH domains, the kinase domain is constitutively active.

It exists in two isoforms, ROCK-I (also called p160ROCK and ROKβ) and ROCK-II (also called ROKα) that share an overall amino acid sequence homology of 60% whereas their kinase domains are 86% identical. Furthermore the kinase domain sequence is 72% identical to that of myotonic dystrophy kinase (Bush et al, 2000).

Substrates of Rho-kinase have been shown to be myosin light chain (MLC) and myosin binding subunit (MBS) of myosin phosphatase. These targets regulate stress fiber and focal adhesion formation, neurite retraction, and tumor cell invasion. Other targets of ROCK are ERM (ezrin,

radixin, moesin) family proteins, which regulate microvilli formation. Glial fibrillary acidic protein is involved in regulate cytokinesis. Collapsin response mediator protein (CRMP) is involved in neuronal growth cone collapse induced by lysophosphatidic acid. The ROCK target LIM kinase acts on cofilin and thus regulates actin cytoskeleton rearrangement. Adducin contributes to actin assembly in the spectrin-F-actin meshwork beneath the plasma membrane (Amano et al., 2000) (Fig. 4).

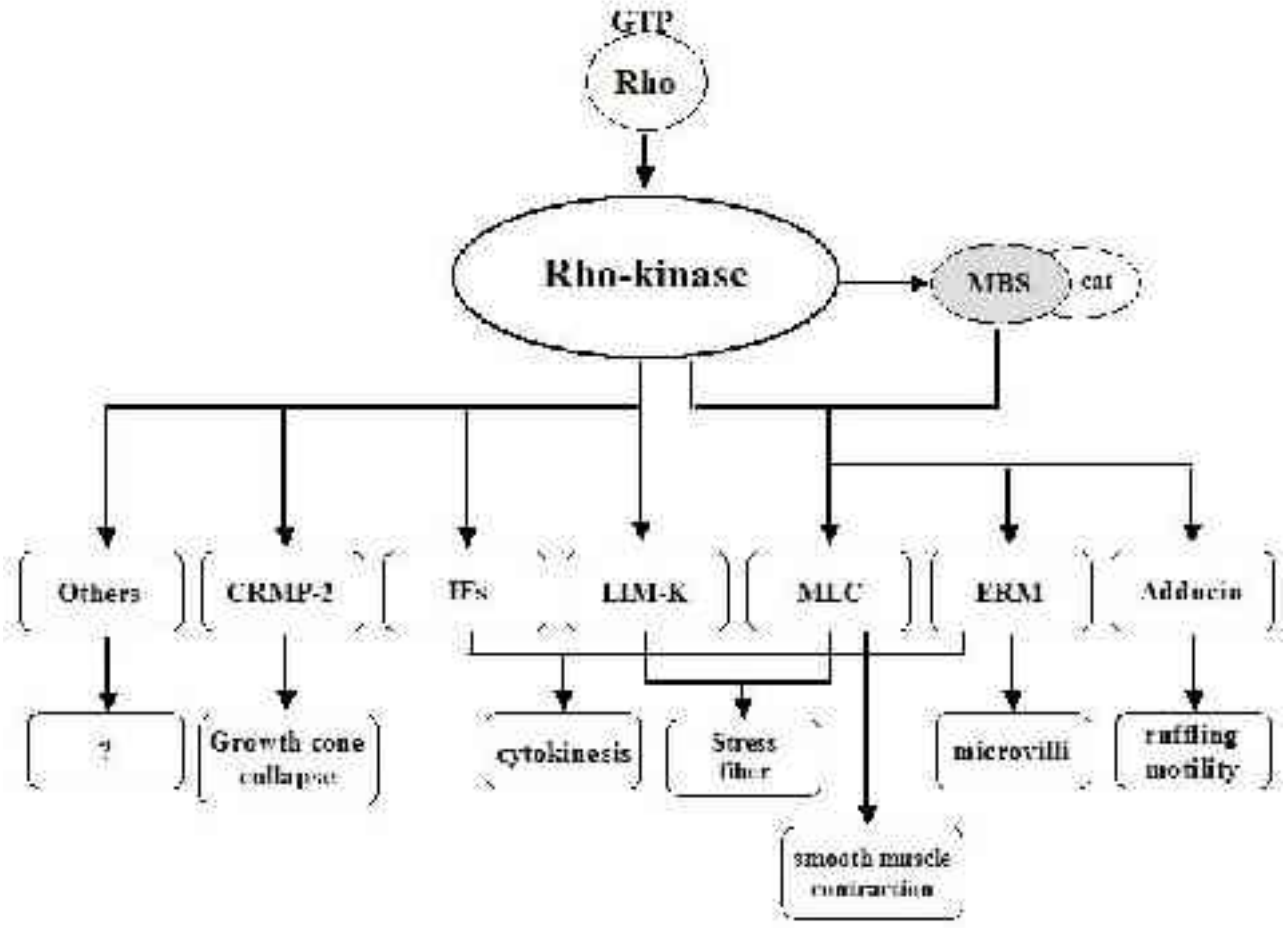


Fig. 4. Substrates for Rho-kinase. Cat, catalytic subunit of myosin phosphatase; Ifs, intermediate filaments; LIM-K, LIM-kinase. (Amano et al., 2000)

Y-27632, a pyridine-derived smooth muscle relaxant was shown to act as a potent Rho-kinase inhibitor with a high selectivity for ROCK (K_i of 0.14-1.0 μM *in vitro*) without inhibiting other kinases such as PRK1/PKN, citron kinase, protein kinase C, and protein kinase A at these concentrations (Davies et al., 2000). Much of what is known about the function of Rho-kinase is based on studies using this compound.

2.5. Phosphatidylinositol 3-kinase (PI 3-kinase)

Research on phosphatidylinositol 3-kinases has demonstrated that this family of enzymes importantly contributes to cellular signalling. On the basis of their primary structure, regulation and lipid substrate specificity, three classes of phosphatidylinositol 3-kinases have been defined.

PI 3-kinases are activated by G-protein-coupled receptors, or by receptors with an intrinsic or associated protein tyrosine kinase activity and/or proteins that are tyrosine phosphorylated in response to extracellular stimuli (Kapeller et al., 1994). Another way in which phosphatidylinositol 3-kinases are activated is by a direct interaction with the small GTPase Ras (Rodriguez-Viciana et al., 1994 and 1996).

Upon activation, inositol lipids are phosphorylated at the D-3 position of the inositol ring to generate the 3-phosphoinositides, phosphatidylinositol 3-phosphate [PtdIns(3)P], phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)P_2] and phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3].

Class I phosphatidylinositol 3-kinases exist as a heterodimer composed of an 85 kDa regulatory and a 110 kDa catalytic subunit (Vanhaesebroeck et al., 1999). There are four mammalian p110 catalytic isoforms (p110α, β, γ, and δ) which associate with the p85-family of regulatory subunits, except for p110γ which binds to a p101 adaptor (Stephens et al., 1997; Stoyanov et al., 1995; Toker et al., 1997). They are regulated by stimulation of cell surface receptors. Type IA PI3Ks (PI3Ks α, β, and γ) are activated by receptor-tyrosine kinases and intracellular protein-tyrosine kinases, whereas type IB PI3K (PI3Kγ) is activated by G protein-coupled receptors via the βγ subunits of heterotrimeric G proteins. Both type IA and type IB PI3Ks are also activated by Ras (Marte et al., 1997) and generate PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3.

Class II phosphatidylinositol 3-kinases generate PtdIns(3)P and PtdIns(3,4)P_2 and are large (170-210 kDa) proteins which all contain a characteristic C-terminal region with homology to C2 domains (Fruman et al., 1998).

The Class III enzymes only produce PtdIns(3)P and only contain a catalytic and a phosphoinositide kinase domain (Fruman et al., 1998; Vanhaesebroeck et al., 1997).

The generated phosphoinositides are functional as second messengers and serve as binding targets for proteins containing pleckstrin-homology (PH) domains or FYFE-fingers (Leevers et al., 1999; Wiedemann et al., 1998). PH domain-containing proteins such as PKB/Akt, 3-phosphoinositide-dependent kinase-1, guanine nucleotide exchange factors, and PLCγ preferentially bind to PtdIns (3,4)P_2 and PtdIns(3,4,5)P_3 whereas FYFE-finger-containing proteins like early endosome antigen 1 and Vsp27p, implicated in vesicular trafficking, bind to PtdIns(3)P but not to PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 (Gaullier et al., 1998; Patki et al., 1998).

Moreover, phosphatidylinositol 3-kinases have protein kinase activity (Carpenter et al., 1993; Dhand et al., 1994; Wymann et al., 2000) and are also able to exert their regulatory function by binding to other proteins (Kapeller et al., 1995; Shibasaki et al., 1994).

Phosphatidylinositol 3-kinases are involved in a number of physiological processes such as the regulation of cell proliferation (Roche et al., 1994), differentiation (Magun et al., 1996; Qiu et al., 1998), apoptosis (Yao et al., 1995), sugar metabolism (Cross et al., 1995; Deprez et al., 1997) and vesicle trafficking (Herman et al., 1990).

Neutrophils from PI 3-kinase γ knock-out mice have been shown to be unable to produce PtdIns $(3,4,5)P_3$ upon stimulation, and migration towards chemokines *in vitro* and *in vivo* was reduced (Wymann et al., 2000).

2.6. Myosin light chain (MLC)

Non-muscle myosin II is one of the major very well characterized motor proteins of animal cells. It is involved in a wide range of processes including cell locomotion, cell division and receptor capping (Kamm and Stull, 2001; Amano et al., 1998). In our study we focussed on the role of myosin light chain (MLC) in the generation of the contractile force for cell migration.

Myosin II activity is mainly controlled through the phosphorylation of MLC, which is basically regulated by two classes of enzymes: MLC kinases and myosin phosphatases (Hartshorne et al., 1998; Kamm and Stull, 2001; Somlyo and Somlyo, 2003). Myosin light chain kinase (MLCK) and Rho-kinase are to be the two major kinases that phosphorylate MLC in vitro as well as in vivo (Totsukawa et al., 2004). Phosphorylation induces a conformational change in MLC that allows actin-myosin interaction and activates the Mg^{2+}-ATPase activity of myosin (Sellers et al., 1981) (Fig. 5).

An increase in intracellular Ca^{2+} concentration leads to activation of Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK), which then phosphorylates Thr^{18} and Ser^{19} of MLC (Goeckeler et al., 1995).

Monophosphorylation at Ser^{19} increases both the actin-activated Mg^{2+}-ATPase activity and the stability of myosin II filaments. Phosphorylation at both sites (Thr^{18} and Ser^{19}) results in higher myosin II activity and stability of myosin II filaments compared to only monophosphorylation (Ikebe et al., 1986 and 1989).

Rho-kinase can increase MLC phosphorylation in two ways: on the one hand mainly by phosphorylating the myosin binding subunit (MBS) of myosin phosphatase and thereby inhibiting

myosin phosphatase activity (Kimura et al., 1996) and on the other hand by direct phosphorylation of MLC (Amano et al., 1996 and Kureishi et al., 1997).

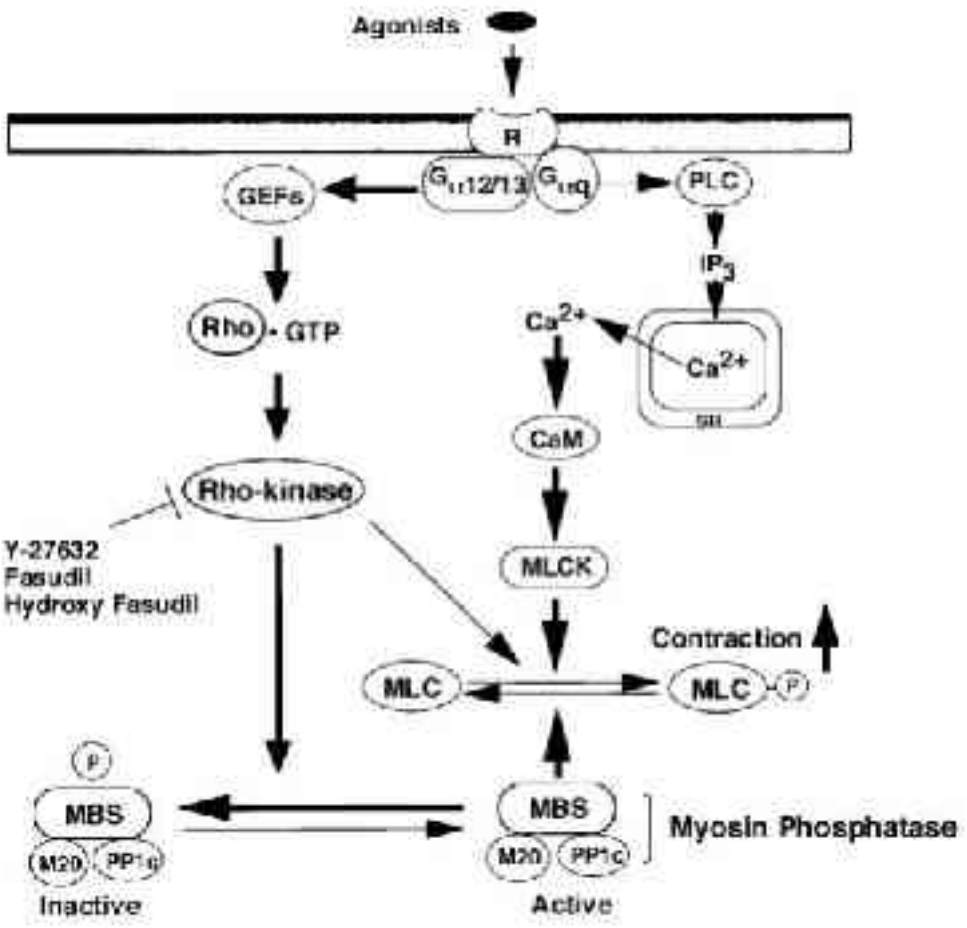


Fig. 5. Regulation of MLC phosphorylation by Rho-kinase, MLCP, and MLCK. (Kawano et al., 2002)

There are 5 phosphorylatable sites on MLC: Thr^{18} and Ser^{19} (MLCK) (Ikebe et al., 1985), Ser^{1}, Ser^{2} and Thr^{9} (PKC, cdc2 kinase) (Ikebe et al., 1987; Satterwhite et al., 1992).

Other kinases including DAPK (Jin et al., 2001), PAK (Chew et al., 1998), ZIP-kinase (Murata-Hori et al., 1999), and citron kinase (Yamashiro et al., 2003) have also been reported to phosphorylate MLC at both Thr^{18} and Ser^{19}, but the physiological significance of these processes has not yet been demonstrated.

Myosin phosphatase, the other major regulator of MLC phosphorylation, consists of a catalytic subunit (PP1c-delta), a large subunit called the myosin phosphatase targeting subunit (MYPT, also called MBS or M130) and a 20 kDa small subunit (Alessi et al., 1992).

The involvement of a phosphatase in regulating MLC phosphorylation was shown by the use of pharmacological inhibition: inhibitors directed against protein phosphatase 1 (PP1) increased both MLC phosphorylation and cell contraction (Verin et al., 1995). Microinjection of active PP1

decreased MLC phosphorylation and disturbed the interaction between actin and myosin (Fernandez et al., 1990).

Phosphorylated myosin II is known to be localized at the rear of migrating cells (Post et al., 1995; Matsumura et al., 1998; Xu et al., 2003). There it plays a role in translocating the cell body forward by contracting the posterior region, the last step in the process of cell migration (Lauffenberger and Horwitz, 1996; Mitchison and Cramer, 1996). Myosin II may play an additional role in cell migration: it was observed that phosphorylated myosin II is also localized in the anterior regions of motile fibroblasts (Matsumura et al., 1998).

2.7. Rac

Rac proteins comprise a subfamily of the Rho family of GTPases that belongs to the Ras superfamily of proteins. The Rac subfamily consists of 3 isoforms (Rac1, Rac2, and Rac3, which is also known as Rac1B) that share a sequence homology of 92% (Haataja et al., 1997).

As well as Rho GTPases they are key regulators in cell morphology, adhesion and migration. Rac isoforms mediate the formation of lamellipodia, membrane ruffles, and focal complexes in the leading front that stabilize lamellipodia (Nobes and Hall, 1995).

Like other Ras superfamily members, Rac GTPases cycle between a GDP-bound (inactive) state and a GTP-bound (active) state (see above). Rac can be activated by tyrosine kinases downstream of G-protein-coupled receptors. PI 3-kinase is also involved in Rac activation, as shown using inhibitors of this protein (Sander et al., 1998).

Activation of Rac results in the formation of lamellipodia and membrane ruffles in fibroblasts (Kozma, 1997). Rac is located preferentially at the leading front of migrating cells (Kraynov et al., 2000). Integrins regulate Rac by targeting them to specific plasma membrane domains and coupling them to their downstream effector molecules such as PAK (p21-activated kinase) (del Pozo et al., 2004). Many studies revealed that Rac is required for cell migration. However there are exceptions: dominant-negative Rac for example did not inhibit membrane ruffling, cell spreading, and migration in colon carcinoma cells (O`Connor et al., 2000).

Rac coordinates lamellipodium extension by different ways. First, Rac stimulates actin polymerisation. It interacts with IRSp53, which in turn, through WAVE, activates the Arp2/3 complex, which nucleates new actin filaments on the sides of existing filaments (Pollard et al., 2000). Furthermore, Rac stimulates actin polymerization via phosphatidylinositide 4-phosphate 5-kinase (PIP 5-kinase) that produces PtdIns(4,5)P_2. PtdIns(4,5)P_2 then promotes the uncapping of actin filaments by removing capping proteins from their barbed ends (Carpenter et al., 1995).

Rac1-deficient neutrophils showed marked defects in inflammatory recruitment in vivo, migration to chemotactic stimuli, and chemoattractant-mediated actin assembly (Glogauer et al., 2003). Rac1 also plays a crucial role in morphogenesis as Rac1 knock-out mice die early in development during gastrulation (Sugihara et al., 1998). In contrast Rac1-deficient macrophages exhibited normal migration and chemotaxis, although they assumed an elongated morphology. This suggests that in macrophages Rac1 is primarily responsible for regulating cell morphology, but is not required for migration (Wells et al., 2003). Rac2 is about 4-fold less abundant in murine macrophages. Macrophages lacking Rac2 show reduced, but not abolished accumulation at inflamed sites in vivo (Yamauchi et al., 2004).

In neutrophils and macrophages Rac activity is required for activation of a multiprotein complex that produces superoxide in phagocytic cells, the NADPH oxidase (Abo, 1991). Rac is also involved in signal transduction from the membrane to the nucleus via mitogen-activated protein kinase cascades (Zhang, 1995).

2.8. ERM (Ezrin, Radixin, Moesin)-family of proteins

The ezrin-radixin-moesin (ERM) family of proteins has emerged as key regulators in linking F-actin to specific membrane proteins (Martin et al., 2003). They are involved in the formation of microvilli, cell adhesion sites, membrane ruffling, and cleavage furrows (Jeon et al., 2002).

The ERM family belongs to a superfamily of proteins. The prototypes of this superfamily are talin and band 4.1, two proteins whose membrane-cytoskeleton interactions are well documented (Crepaldi et al., 1997). The cortical cytoskeleton provides structural support for the plasma membrane. In this context ERM proteins play both a structural and a regulatory role in the assembly and stabilization of plasma membrane domains (Bretscher et al., 1997).

The prototype of the ERM protein family is ezrin. Other members of this highly conserved group are radixin, moesin, and merlin. They share homologies of 70% and are composed of three main domains: 1) the highly conserved globular N-terminus (85% identity) referred to as the FERM (4.1 and ERM) domain that is involved in membrane binding, 2) followed by an extended α-helical domain and 3) a positively charged C-terminus which includes a conserved actin-binding domain (Granes et al., 2000; Tsukita et al., 1997).

The amino-terminus of Ezrin is able to interact and associate with the carboxy-terminus of any ERM member. The amino-terminal residues bind directly or indirectly to the plasma membrane, the carboxy-terminal residues bind laterally to actin (Bretscher et al., 1997).

Ezrin exists in an activated and in an inactivated state. In the dormant conformation, association sites are masked: The carboxy-terminal domain (called C-ERM Association Domain (C-ERMAD)) is able to bind to the amino-terminal domain (called N-ERM Association Domain (N-ERMAD)) in an intramolecular and an intermolecular association that inactivates the protein and prevents binding to other molecules.

ERM proteins are maintained in the inactive conformation through the strong intramolecular N-/C-ERMAD interactions (Bretscher et al., 2000).

When phosphorylated at the C-terminal threonine (Thr567 in ezrin, Thr564 in radixin, Thr558 in moesin), intramolecular association between N-terminus and C-terminus is disrupted (Matsui et al., 1998;). Another factor that is required for ERM activation is phosphatidylinositol-4,5-bisphosphate (PIP_2), that binds to the FERM domain of ERM proteins and might alter their conformation. This interaction of PIP_2 with ERM involves three clusters of lysines that are part of a groove in the FERM domain. Mutations in these residues abrogates membrane localization of ezrin, indicating that PIP_2 is a crucial determinant of ERM membrane localization (Barret et al., 2000). Interaction with PIP_2 has been shown to be a prerequisite for ERM phosphorylation in the LLC-PK1 epithelial cell line (Fievet et al., 2004). Subsequently the N-terminal FERM domain is exposed, which in turn links the C-terminal tail to the actin cytoskeleton (Matsui et al., 1998).

2.9. Protein kinase C

Protein kinase C (PKC) molecules are members of the AGC serine-threonine protein kinase family. The PKC subfamily comprises three groups: the classical or conventional PKC isoforms (α, βI, βII, γ) which are regulated by calcium, diacylglycerol (DAG) and phospholipids; the novel PKC isoforms (δ, ε, η, θ), regulated by DAG and phospholipids; and the atypical PKC isoforms (ζ, and λ), that are insensitive to calcium and DAG (Spitaler et al., 2004).

The structural characteristics and the classification of PKC isoforms is based on the differences in the regulatory domain called C1-C4. Whereas the catalytic domain is conserved, the regulatory domains of the three subgroups of PKC differ. Classical PKCs all have an autoinhibitory pseudosubstrate motif, two DAG-binding C1 domains and a calcium-binding C2 domain. Novel PKCs lack a calcium-binding domain but have an extended N-terminal domain that can receive regulatory signals. Atypical PKCs neither posses a calcium- nor a DAG-binding motif and seem to be mainly regulated by intracellular localization (Mellor et al., 1998).

PKCs activation starts with stimulation of a cell surface receptor, which in turn activates a heterotrimeric GTP-binding protein. Then, phospholipase C is activated generating IP_3 and DAG by cleaving PIP_2. DAG, calcium and phospholipids together finally activate PKC, which then translocates to the membrane. The membrane-permeable phorbol ester PMA (phorbol-12-myristate-13-acetate) can act as an activator of PKC as well and has been shown to be a potent tumor promotor capable of altering many cell properties (Kefalas et al., 1995).
The role of PKC in tumor cell locomotion is still not clear. Short-term and long-term incubation of cells with the phorbol ester PMA have opposing effects: short-time treatment of Walker carcinosarcoma cells inhibited spontaneous locomotion concomitant with translocation of PKC activity to the membrane whereas long-term treatment leads to a downregulation of PKC isoforms and recovery of migration (Wicki and Niggli, 1999).

2.10. Projects and aims of the thesis

In the first part of the thesis I investigated the effects of modulating activities of the Rho/Rho-kinase pathway on the downstream effector myosin light chain. Recent findings revealed that in the spontaneously migrating Walker 256 carcinosarcoma cells the proteins Rho, and Rho-kinase appear to be constitutively activated and that inhibition of these proteins resulted in suppression of spontaneous development of polarity and migration (Wicki and Niggli, 2001). Whether this constitutive activation is due to a mutation or to overexpression remains to be investigated. The downstream targets of this pathway in Walker carcinosarcoma cells have not yet been elucidated. I used C3 exoenzyme for Rho inhibition and Y-27632 for Rho-kinase inhibition and also introduced bacterially expressed Rho and Rac mutants into Walker carcinosarcoma cells. Moreover I transfected these cells with dominant-negative Rac and Rho-kinase and with dominant-active Rho and Rho-kinase. By means of these tools we wanted to study the roles of Rho, Rac, and Rho-kinase in cell morphology, migration, F-actin localization, and phosphorylation of myosin light chain.
In a second project I investigated the regulation of another downstream effector of Rho, the ERM (ezrin/radixin/moesin) family in Walker carcinosarcoma cells. For this we used the same tools as in the first project to modulate activities of Rho, Rac, and ROCK.
In the third part of my thesis I analyzed the role of phosphatases in modulating activities and localization of Rho, Rho-kinase and PI 3-kinase, and phosphorylation of myosin light chain and ERM proteins. Previous findings of our laboratory showed that treatment of Walker carcinosarcoma cells with the phosphatase 1/2A inhibitor okadaic acid, that also inhibits myosin light chain

phosphatase (Ishihara et al., 1989), inhibits establishment of spontaneous polarity and migration (Niggli and Keller, 1997).

2.11. References

Abo A., Webb M.R., Grogan A., Segal A.W. (1994) Activation of NADPH oxidase involves the dissociation of p21rac from its inhibitory GDP/GTP exchange protein (rhoGDI) followed by its translocation to the plasma membrane. Biochem J. 298:585-591.

Aktories K., and Hall A. (1989) Botulinum ADP-ribosyltransferase C3: a new tool to study low molecular weight GTP-binding proteins. Trends Pharmacol. Sci. 10:415-418.

Alessi D., MacDougall L.K., Sola M.M., Ikebe M., and Cohen P. (1992) The control of protein phosphatase-1 by targeting subunits. The major myosin phosphatase in avian smooth muscle is a novel form of protein phosphatase-1. Eur. J. Biochem. 210:1023-1035.

Amano M., Chihara K., Nakamura N., Fukata Y., Yano T., Shibata M., Ikebe M., and Kaibuchi K. (1998) Myosin II activation promotes neurite retraction during the action of Rho and Rho-kinase. Genes Cells 3:177-188.

Amano M., Fukata Y., Kaibuchi K. (2000) Regulation and functions of Rho-associated kinase. Exp Cell Res. 261:44-51.

Amano M., Ito M., Kimura K., Fukata Y., Chihara K., Nakano T., Matsuura Y., and Kaibuchi K. (1996) Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). J. Biol. Chem. 271:20246-20249.

Amano M., Mukai H., Ono Y., Chihara K., Matsui T., Hamalima Y., Okawa K., Iwamatsu A., and Kaibuchi K. (1996) Identification of a putative target for Rho as the serine-threonine kinase protein kinase N. Science 271:648-650.

Arimura N., Inagaki N., Chihara K., Menager C., Nakamura N., Amano M., Iwamatsu A., Goshima Y., Kaibuchi K. (2000) Phosphorylation of collapsin response mediator protein-2 by Rho-kinase. Evidence for two separate signaling pathways for growth cone collapse. J Biol Chem. 275(31): 23973-80.

Aspenstrom P. (1999) Effectors for the Rho GTPases. Curr Opin Cell Biol. 11:95-102.

Bain J., H. McLauchlan, M. Elliot, P. Cohen, The specificities of protein kinase inhibitors: an update, Biochem. J. 371 (2003) 199-204.

Balkwill F. (2004) The significance of cancer cell expression of the chemokine receptor CXCR4. Semin Cancer Biol. 14:171-179.

Barret C., Roy C., Montcourrier P., Mangeat P., and Niggli V. (2000) Mutagenesis of the phosphatidylinositol 4,5-bisphosphate (PIP(2)) binding site in the NH(2)-terminal domain of ezrin correlates with its altered cellular distribution. J. Cell Biol. 151:1067-1080.

Barsky S.H., Siegal G.P., Jannotta F., and Liotta L.A. (1983) Loss of basement membrane components by invasive tumors but not by their benign counterparts. Lab. Invest. 49:140-147.

Bishop A.L., Hall A. (2000) Rho GTPases and their effector proteins. Biochem J. 348:241-255.

Boguski M.S., and McCormick F. (1993) Proteins regulating Ras and its relatives. Nature 366:643-654.

Bokoch G.M., Bohl B.P., Chuang T.H. (1994) Guanine nucleotide exchange regulates membrane translocation of Rac/Rho GTP-binding proteins. J. Biol. Chem. 269:31674-31679.

Bonev B.B., Gilbert R.J.C., Andrew P.W., Byron O., Watts A. (2001) Structural analysis of the protein/lipid complexes associated with pore formation by the bacterial toxin pneumolysin. J Biol Chem. 276:5714-5719.

Bretscher A. (2000) ERM-Merlin and EBP50 protein families in plasma membrane organization and function. Annu. Rev. Cell Dev. Biol. 16:113-143.

Bretscher A., Reczek D., and Berryman M. (1997) Ezrin: a protein requiring conformational activation to link microfilaments to the plasma membrane in the assembly of cell surface structures. J. Cell Sci. 110:3011-3018.

Bush E.W., Helmke S.M., Birnbaum R.A., and Perryman M.B. (2000) Myotonic dystrophy protein kinase domains mediate localization, oligomerization, novel catalytic activity, and autoinhibition. Biochemistry 39:8480-8490.

Candelis J.S., Wyckoff J., Segall J.E. (2000) Imaging of cancer invasion and metastasis using green fluorescent protein. Eur J Cancer 36:1671-1680.

Carpenter C.L., Auger K.R., Duckworth B.C., Hou W., Schaffhausen B., and Cantley L.C. (1993) A tightly associated serine/threonine protein kinase regulates phosphoinositide 3-kinase activity. Mol. Cell. Biol. 13:1657-1665.

Chartier L, Rankin L.L., Allen R.E., Kato Y., Fusetani N., Karaki H., Watanabe S., Hartshorne D.J. (1991) Calyculin-A increases the level of protein phosphorylation and changes the shape of 3T3 fibroblasts. Cell Motil. Cytoskel. 18:26-40.

Chen J., Wagner M.C. (2000) Altered membrane-cytoskeleton linkage and membrane blebbing in energy-depleted renal proximal tubular cells. Am J Physiol Renal Physiol. 280:619-627.

Chen X.Q., Tan I., Ng C.H., Hall C., Lim L., Leung T. (2002) Characterization of RhoA-binding kinase ROKα implication of the pleckstrin homology domain in ROK(function using region-specific antibodies. J Biol Chem. 277:12680-12688.

Chew T.L., Masaracchia R.A., Goeckeler Z.M., Wysolmerski R.B. (1998) Phosphorylation of non-muscle myosin II regulatory light chain by p21-activated kinase (gamma-PAK). J. Muscle Res. Cell Motil. 19:839-854.

Cohen P., C.F. Holmes, Tsukitani Y. (1990) Okadaic acid: a new probe for the study of cellular regulation. Trends Biochem Sci. 15:98-102.

Coleman M.L., E.A. Sahai, M. Yeo, M. Bosch, A. Dewar, M.F. Olson, Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I, Nat. Cell Biol. 3 (2001) 339-345.

Crepaldi T., Gautreau A., Comoglio P.M., Louvard D., Arpin M. (1997) Ezrin is an effector of hepatocyte growth factor-mediated migration and morphogenesis in epithelial cells. 138:423-434.

Cross D.A.E., Alessi D.R., Cohen P., Andjelkovich M., and Hemmings B.A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated protein kinase B. Nature 378:785-789.

Davies S.P., Reddy H., Caivano M., Cohen P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J. 351:95-105.

Del Pozo M.A., Alderson N.B., Kiosses W.B., Chiang H.H., Anderson R.G., Schwartz M.A. (2004) Integrins regulate Rac targeting by internalization of membrane domains. Science 303:839-842.

Deprez J., Vertommen D., Alessi D.R., Hue L., and Rider M.H. (1997) Phosphorylation and activation of heart 6-phosphofructo-2-kinase by protein kinase B and other protein kinases of the insulin signaling cascades. J. Biol. Chem. 272:17269-17275.

Dhand R., Hiles I., Panayotou G., Roche S., Fry M.J., Gout I., Totty N.F., Truong O., Vicendo P., Yonezawa K., Kasuga M., Courtneidge S.A., and Waterfield M.D. (1994) PI 3-kinase is a dual specificity enzyme: autoregulation by an intrinsic protein-serine kinase activity. EMBO J. 13:522-533.

Entschladen F., Drell T.L. 4th, Lang K., Joseph J., Zaenker K.S. (2004) Tumour-cell migration, invasion, and metastasis: navigation by neurotransmitters. Lancet Oncol. 5:254-258.

Etienne-Manneville S., A. Hall, Rho GTPases in cell biology, Nature 420 (2002) 629-635.

Eto M., Bock R., Brautigan D.L., and Linden D.L (2002) Cerebellar long-term synaptic depression requires PKC-mediated activation of CPI-17, a myosin/moesin phosphatase inhibitor. Neuron 36:1145-1158.

Fernandez A., Brautigan D.L., Mumby M., and Lamb N.J.C. (1990) J. Cell Biol. 111:103-112.

Fidler I.J., and Hart I.R. (1982) Biological diversity in metastatic neoplasms: origins and implications. Science 217:998-1001.

Fievet B.T., Gautreau A., Roy C., Del Maestro L., Mangeat P., Louvard D., Arpin M. (2004) Phosphoinositide binding and phosphorylation act sequentially in the activation mechanism of ezrin. J Cell Biol. 164:653-659.

Fruman D.A., Meyers R.E., and Cantley L.C. (1998) Phosphoinositide kinases. Ann. Rev. Biochem. 67:481-507.

Fujita A., Y. Saito, T. Ishizaki, M. Maekawa, K. Fujisawa, F. Ushikubi, Integrin-dependent translocation of p160ROCK to cytoskeletal complex in thrombin-stimulated human platelets, Biochem. J. 328 (1997) 769-775.

Fukata Y., N. Oshiro, N. Kinoshita, Y. Kawano, Y. Matsuoka, V. Bennet, Y. Matsura, K. Kaibuchi, Phosphorylation of adducin by Rho-Kinase plays a crucial role in cell motility, J. Cell Biol. 145 (1999) 347-361.

Gaullier L.M., Simonsen A., D'Arrigo A., Bremnes B., Stenmark H., andAasland R. (1998) FYVE fingers bind PtdIns(3)P. Nature 394:432-433.

Gautreau A., Louvard D., and Arpin M. (2002) ERM proteins and NF2 tumor suppressor: the Yin and Yang of cortical actin organization and cell growth signaling. Curr. Opin. Cell Biol. 14:104-109.

Geng J.G. (2001) Directional migration of leukocytes: their pathological roles in inflammation and strategies for development of anti-inflammatory therapies. Cell Res. 11:85-88.

Glogauer M., C.C. Marchal, F. Zhu, A. Worku, B.E. Clausen, I. Foerster, P. Marks, G.P. Downey, M. Dinauer, D.J. Kwiatkowski, Rac1 deletion in mouse neutrophils has selective effects on neutrophil functions, J. Immunol. 170 (2003) 5652-5657.

Goeckeler Z.M., and Wysolmerski R.B. (1995) Myosin light chain kinase-regulated endothelial cell contraction: the relationship between isometric tension, actin polymerization, and myosin phosphorylation. J. Cell Biol. 130:613-627.

Goto H., Kosako H., Tanabe K., Yanagida M., Sakurai M., amano M., Kaibuchi K., and Inagaki M. (1998) Phosphorylation of vimentin by Rho-associated kinase at a unique amino-terminal site that is specifically phosphorylated during cytokinesis. J. Biol. Chem. 273: 11728-11736.

Granes F., Urena J.M., Rocamora N., Vilaro S. (2000) Ezrin links syndecan-2 to the cytoskeleton. J Cell Sci. 113:1267-1276.

Guirguis R., Margulies I., Taraboletti G., Schiffman E., and Liotta L. (1987) Cytokine-induced pseudopodial protrusion is coupled to tumour cell migration. Nature 329 :261-263.

Haataja L., Groffen J., eisterkamp N. (1997) Characterization of Rac3, a novel member of the Rho family. J Biol Chem. 33:20384-20388.

Hall A. (1998) G proteins and small GTPases: distant relatives keep in touch. Science 280:2074-2075.

Hall A. (1998) Rho GTPases and the actin cytoskeleton. Science 279:509-514.

Hanahan D., and Weinberg R.A. (2000) The hallmarks of cancer. Cell 100:57-70.

Hartshorne D.J., Ito M., Erdodi F. (1998) Myosin light chain phosphatase: subunit composition, interactions and regulation. J. Muscle Res. Cell Motil. 19:325-341.

Herman P.K., and Emr S.D. (1990) Characterization of VPS34, a gene required for vacuolar protein sorting and vacuole segregation in Saccharomyces cerevisiae. Mol. Cell. Biol. 10:6742-6754.

Hirano K., Chartier L. Taylor R.G., Allen R.E., Fusetani N., Karaki H., Hartshorne D.J. (1992) Changes in the cytoskeleton of 3T3 fibroblasts induced by the phosphatase inhibitor, calyculin-A. Res. Cell Motil. 13:341-353.

Ikebe M. (1989) Biochemistry 28:8750-8755.

Ikebe M., and Hartshorne D.J. (1985) Phosphorylation of smooth muscle myosin at two distinct sites by myosin light chain kinase. J. Biol. Chem. 260:10027-10031.

Ikebe M., Hartshorne D.J., and Elzinga M. (1986) Identification, phosphorylation and dephosphorylation of a second site for myosin light chain kinase on the 20,000-Dalton light chain of smooth muscle myosin. J. Biol. Chem. 261:36-39.

Ikebe M., Hartshorne D.J., and Elzinga M. (1987) Phosphorylation of the 20,000-Dalton light chain of smooth muscle myosin by the calcium-activated, phospholipid-dependent protein kinase. J. Biol. Chem. 262:9569-9573.

Ishihara H., Martin B.L., Brautigan D.L., Karaki H., Ozaki H., Kato Y., Fusetani N., Watabe S., Hashimoto K., Uemura D., Hartshorne D.J. (1989) Calyculin A and okadaic acid: inhibitors of protein phosphatase activity. Biochem. Biophys. Res. Commun. 159:871-877.

Ishizaki T., M. Naito, K. Fujisawa, N. Watanabe, Y. Saito, and S. Narumiya (1997) p160ROCK, a Rho-associated coiled-coil forming protein kinase, works downstream of Rho and induces focal adhesions. FEBS Lett. 404:118-124.

Ishizaki T., Maekawa K., Fujisawa K., Okawa A., Iwamatsu A., Fujita N., Watanabe Y., Saito A., Kakizuka A., Morii N., and Narumiya S. (1996) The small GTP-binging protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. EMBO J. 15: 1885-1893.

Ishizaki T., Naito M., Fujisawa K., Watanabe N., Saito Y., and Narumiya S. (1997) p160ROCK, a Rho-associated coiled-coil forming protein kinase, works downstream of Rho and induces focal adhesions. FEBS Lett. 404:118-124.

Itoh K., Yoshioka K., Akedo H., Uehata M., Ishizaki T., Narumyia S. (1999) An essential part for Rho-associated kinase in the transcellular invasion of tumor cells. Nat Med. 5:221-225.

Jeon S., Kim S., Park J.B., Suh P.G., Kim Y.S., Bae C.D., and Park J. (2002) RhoA and Rho-kinase-dependent phosphorylation of moesin at Thr-558 in hippocampal neuronal cells by glutamate. J. Biol. Chem. 277:16576-16584.

Jin Y., Blue E.K., Dixon S., Hou L., Wysolmerski R.B., and Gallagher P.J. (2001) Identification of a new form of death-associated protein kinase that promotes cell survival. J. Biol. chem. 276:39667-39678.

Kamm K.E., and Stull J.T. (2001) Dedicated myosin light chain kinases with diverse cellular functions. J Biol. Chem. 276:4527-4530.

Kapeller R., and Cantley L.C. (1994) Phosphatidylinositol 3-kinase. Bioessays 16:565-576.

Kapeller R., Toker A., Cantley L.C., and Carpenter C.L. (1995) Phosphoinositide 3-kinase binds constitutively to alpha/beta-tubulin and binds to gamma-tubulin in response to insulin. J. Biol. Chem. 270:25985-25991.

Kawano Y., Yoshimura T., Kaibuchi K. (2002) Involvement of small GTPase Rho in cardiovascular diseases. Nippon Yakurigaku Zasshi. 120:149-158.

Kefalas P., Brown T.R., Katz D.R., Brickell P.M. (1995) Identification of a phorbol ester responsive region in the myeloid-specific promoter of the c-fgr proto-oncogene. Biochem Biophys Res Commun. 209:60-65.

Keller H.U., A. Dehghani Zadeh, P. Eggli, Localized depletion of polymerised actin at the front of Walker carcinosarcoma cells increases the speed of locomotion, Cell Motil. Cytoskel. 53 (2002) 189-202.

Keller H.U., and Zimmermann A., (1986) Shape changes and chemokinesis of Walker 256 carcinosarcoma cells in response to colchicine, vinblastine, nocodazole and taxol. Invasion Metastasis 6:33-43.

Keller H.U., Redundancy of lamellipodia in locomoting Walker carcinosarcoma cells, Cell Motil. Cytoskel., 46 (2000) 247-256.

Kim S.U., Jeon S., Shin E.Y., Kim E.G., Park J., and Bae C.D. (2004) AMPA, not NMDA, activates RhoA GTPases and subsequently phosphorylates moesin. Exp Mol Med. 36:98-102.

Kimura K., Ito M., Amano M., Chihara K., Fukata Y., Nakafuku M., Yamamori B., Feng J., Nakano T., Okawa K., Iwamatsu A., and Kaibuchi K. (1996) Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science 273:245-248.

Kjoller L., and Hall A., (1999) Signaling to rho GTPases. Exp Cell Res. 253:166-179.

Klippel A., Reinhard C., Kavanaugh W.M., Apell G., Escobedo M.A., Williams L.T. (1996) Membrane localization of phosphatidylinositol 3-kinase is sufficient to activate multiple signal-transducing kinase pathways. Mol Cell Biol. 16:4117-4127.

Kondo T., Takeuchi K., Doi Y., Yonemura S., Nagata S., Tsukita S., Tsukita S. (1997) ERM (Ezrin/Radixin/Moesin)-based molecular mechanism of microvillar breakdown at an early stage of apoptosis. J Cell Biol. 139:749-758.

Kosako H., Amano M., Yanagida M., Tanabe K., Nishi Y., Kaibuchi K., Inagaki M. (1997) Phosphorylation of glial fibrillary acidic protein at the same sites by cleavage furrow kinase and Rho-associated kinase. J Biol Chem. 272(16): 10333-10336.

Kozma R., Sarner S., Ahmed S., Lim L. (1997) Rho family GTPases and neuronal growth cone remodelling: relationship between increased complexity induced by Cdc42Hs, Rac1, and acetylcholine and collapse induced by RhoA and lysophosphatidic acid. 17:1201-1211.

Kranenburg O., Poland M, Gebbink M, Oomen L, Moolenaar WH. (1997) Dissociation of LPA-induced cytoskeletal contraction from stress fiber formation by differential localization of RhoA. J. Cell Sci. 110:2417-2427.

Kraynov V.S., Chamberlain C., Bokock G.M., Schwartz M.A., Slabaugh S., Hahn K.M. (2000) Localized Rac activation dynamics visualized in living cells. Science 290:333-337.

Kreienbuhl P., Keller H.U., Zimmermann A. (1992) Protein phosphatase inhibitors okadaic acid and calyculin A alter cell shape and F-actin distribution and inhibit stimulus-dependent increases in cytoskeletal actin of human neutrophils. Blood 80:2911-2919.

Kulesa P., Ellies D.L., Trainor D.L. (2004) Comparative analysis of neural crest cell death, migration, and function during vertebrate embryogenesis. Dev Dyn. 229:14-29.

Kureishi Y., Kobayashi S., Amano M., Kimura K., Kanaide H., Nakano T., Kaibuchi K., and Ito M. (1997) Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation. J. Biol. Chem. 272:12257-12260.

Lauffenberger D.A., and Horwitz A.F. (1996) Cell migration: a physically integrated molecular process. Cell. 84:359-369 .

Lee J.H., Katakai T., Hara T., Gonda H., Sugai M., and Shimizu A. (2004) Roles of p-ERM and Rho-ROCK signaling in lymphocyte polarity and uropod formation. J Cell Biol. 167:327-337.

Leevers S.J., Vanhaesebroeck B., and Waterfield M.D. (1999) Signalling through phosphoinositide 3-kinase: the lipids take centre stage. Curr. Opin. Cell Biol. 11:219-225.

Lenzen C., (1998) Kinetic analysis by fluorescence of the interaction between Ras and the catalytic domain of the guanine nucleotide exchange factor Cdr25Mm. Biochemistry 37:7420-7430.

Leung T., Chen X.Q., Manser E., and Lim L. (1996) The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the reorganization of the cytoskeleton. Mol. Cell Biol. 16:5313-5327.

Leung T., Manser E., Tan L., and Lim L. (1995) A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. J Biol Chem. 256:29051-29054.

Limouze J., A.F. Straight, T. Mitchison, J.R. Sellers, Specificity of blebbistatin, an inhibitor of myosin II. J. Muscle Res. Cell Motil. 25 (2004) 337-341.

Lin M., van Golen K.L. (2004) Rho-regulatory proteins in breast cancer cell motility and invasion. Breast Cancer Res Treat. 84:49-60.

Liotta L.A., Steeg P.S., and Stetler-Stevenson W.G. (1991) Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell 64:327-336.

Luna E.J., Wuestehube L.J., Ingalls H.M., and Chia C.P. (1989) Adv. Cell Biol. 3:33.

Macara I.G., Lounsbury K.M., Richards S.A., McKiernan C., and Bar-Sagi D. 1996 The Ras superfamily of GTPases. FASEB J. 10:625-630.

Maciver S.K., Myosin II function in non-muscle cells, Bioessays 18 (1996) 179-182.

Maekawa M., Ishizaki T., Boku S., Watanabe N., Fujita A., Iwamatsu A., Obinata T., Ohashi K., Mizuno K., Narumiya S. (1999) Signalling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. Science 285(5429): 895-898.

Magun R., Burgering B.M.T., Coffer P.J., Pardasani D., Lin Y., Chabot J., and Sorisky A., (1996) Expression of a constitutively activated form of protein-kinase-β (c-AKT) in 3T3-Il preadipose cells causes spontaneous differentiation. Endocrinology 137:3590-3593.

Maier G.D., Wright M.A., Lozano Y., Djordjevic A., Matthews J.P., Young M.R. (1995) Regulation of cytoskeletal organization in tumor cells by protein phosphatases-1 and -2A. Int. J. Cancer 61:54-61.

Manishen W.J., Sivanathan K., and Orr F.,W. (1986) Resorbing bone stimulates tumor cell growth. A role for the host microenvironment in bone metastasis. Am. J. Physiol. 123:39-45.

Marte B.M., Rodriguez-Viciana P., Wennstrom S., Warne P.H., and Downward J. (1997) R-Ras can activate the phosphoinositide 3-kinase but not the MAP kinase arm of the Ras effector pathways. Curr. Biol. 7:63-70.

Martin T.A., Harrison G., Mansel R.E., Jiang W.G. (2003) The role of the CD44/ezrin complex in cancer metastasis. Crit. Rev. Oncol. Hematol. 46:165-86.

Matsui T., Amano M., Yamamoto T., Chihara K., Nakafuku M., Ito M., Kanano T., Okawa K., Iwamatsu A., and Kaibuchi K. (1996) Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP-binding protein Rho. EMBO J. 15:2208-2216.

Matsui T., Maeda M., Doi Y., Yonemura S., Amano M., Kaibuchi K., and Tsukita S. (1998) Rho-kinase phosphorylates COOH-terminal threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to-tail association. J. Cell Biol. 140:647-657.

Matsui T., Yonemura S., Tsukita S., and Tsukito S. (1999) Activation of ERM proteins in vivo by Rho involves phosphatidylinositol 4-phosphate 5-kinase and not ROCK kinases. Curr Biol. 9:1259-1262.

Matsumura F., Ono S., Yamakita Y., Totsukawa G., and Yamashiro S. (1998) Specific localization of serine 19 phosphorylated myosin II during cell locomotion and mitosis of cultured cells. J. Cell. Biol. 140:119-129.

McGee K., M. Zettl, M. Way, M. Fallman, A role of N-WASP in invasin-promoted internalisation, FEBS Lett. 509 (2001) 59-65.

Mellor H., Parker P.J. (1998) The extended protein kinase C superfamily. Biochem J. 332:281-292.

Michaelson D., Silletti J., Murphy G., D'Eustachio P., Rush M., Philips M.R., Differential localization of Rho GTPases in live cells: regulation by hypervariable regions and RhoGDI binding, J. Cell Biol. 152 (2001) 111-126.

Mills J.C., Stone N.L., Erhardt J., Pittman R.N. (1998) Apoptotic membrane blebbing is regulated by myosin light chain phosphorylation. J. Cell Biol. 140:627-636.

Mitchison T.J., and Cramer L.P. (1996) Actin-based cell motility and cell locomotion. Cell. 84:371-379.

Morikawa H., Mima H., Uga H., Shoda T., and Fukuda K. (1999) Opioid potentiation of N-type Ca^{2+} channel currents via pertussis-toxin-sensitive G proteins in NG108-15 cells. Pflugers Arch. 438:423-426.

Murata-Hori M., Suizu F., Iwasaki T., Kikuchi A., and Hosoya H. (1999) ZIP kinase identified as a novel myosin regulatory light chain kinase in HeLa cells. FEBS Lett. 451:81-84.

Nabi I.R., Watanabe H., Silletti S., and Raz A. (1991) Tumor cell autocrine motility factor receptor. EXS. 59:163-177.

Nakagawa O., Fujisawa K., Ishizaki T., Saito Y., Nakao K., and Narumiya S. (1996) ROCK-I and ROCK-II, two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice. FEBS Lett. 392:189-193.

Niggli V., H. Keller (1997) Low concentrations of the phosphatase inhibitor okadaic acid stop tumor cell locomotion. Eur J Pharmacol. 324:99-108.

Niggli V., Rho-kinase in human neutrophils: a role in signalling for myosin light chain phosphorylation and cell migration, FEBS Lett. 445 (1999) 69-72.

Niggli V., Signaling to migration in neutrophils: importance of localized pathways, Int. J. Biochem. Cell Biol. 35 (2003) 1619-1638.

Niggli V., Zimmermann A., Keller H. (1996) Inhibition of protein kinase C-dependent protein phosphorylation correlates with increased polarity and locomotion in Walker 256 carcinosarcoma cells. Int J Cancer 65:473-478.

Nobes C.D., Hall A. (1995) Rho, Rac and Cdc42 GTPases: regulators of actin structures, cell adhesion and motility. Biochem Soc Trans. 23:456-459.

O'Connor K.L., Mercurio A.M. (2001) Protein kinase A regulates Rac and is required for the growth factor-stimulated migration of carcinoma cells. J Biol Chem. 276:47895-47900.

Oshiro N., Fukata Y., and Kaibuchi K. (1998) Phosphorylation of Moesin by Rho-associated Kinase (Rho-kinase) plays a crucial role in the formation of microvilli-like structures. J. Biol. Chem. 273:34663-34666.

Partin A.W., J.S. Schoeniger, J.L. Mohler, D.S. Coffey, Fourier analysis of cell motility: correlation of motility with metastatic potential, Proc. Natl. Acad. Sci. (USA) 86 (1989) 1254-1258.

Patki V., Lawe D.C., Corvera S., Virbasius J.V., and Chawla A. (1998) A functional PtdIns(3)P-binding motif. Nature 394:433-434.

Pietromonaco S.F., Simons P.C., Altman A., and Elias L. (1998) Protein kinase C-θ phosphorylation of moesin in the actin-binding sequence. J Biol Chem. 273:7594-7603.

Pollard T.D., Blanchoin L., Mullins R.D. (2000) Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. Annu Rev Biophys Biomol Struct. 29:545-576.

Post P.L., DeBiaso R.L., and Taylor D.L. (1995) A fluorescent protein biosensor of myosin II regulatory light chain phosphorylation reports a gradient of phosphorylated myosin II in migrating cells. Mol. Biol. Cell. 6:1755-1768.

Qiu Y., Robinson D., Pretlow T.G., and Kung H.J., (1998) Etk/Bmx, a tyrosine kinase with a pleckstrin-homology domain, is an effector of phosphatidylinositol 3-kinase and is involved in interleukin 6-induced neuroendocrine differentiation of prostate cancer cells. Proc. Natl Acad. Sci. USA 95:3644-3649.

Ren X.D., R. Wang, Q. Li, L.A.F. Kahek, K. Kaibuchi, R.A.F. Clark, Disruption of Rho signal transduction upon cell detachment, J. Cell Sci. 117 (2004) 3511-3518.

Ridley A.J. (2004) Rho proteins and cancer. Breast Cancer ResTreat. 84:13-19.

Ridley A.J. and Hall A. (1992) The small GTP-binding protein Rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 70:389-399.

Ridley A.J., Paterson H.F., Johnston C.L., Diekmann D., and Hall A. (1992) The small GTP-binding protein Rac regulates growth factor-induced membrane ruffling. Cell 70:401-410.

Roche S., Koegl M., and Courtneidge S.A. (1994) The phosphatidylinositol 3-kinase alpha is required for DNA synthesis induced by some, but not all, growth factors. Proc. Natl Acad. Sci. USA 91:9185-9189.

Rodriguez-Viciana P., Warne P.H., Dhand R., Vanhaesebroeck B., Gout I., Fry M.J., Waterfield M.D., and Downward J. (1994) Phosphatidylinositol 3-OH kinase as a direct target of Ras. Nature. 370:527-532.

Rodriguez-Viciana P., Warne P.H., Vanhaesebroeck B., Waterfield M.D., and Downward J. 1996 Activation of phosphoinositide 3-kinase by interaction with Ras and by point mutation. EMBO J. 15:2442-2451.

Sanchez-Madrid F. and del Pozo M.A. (1999) Leukocyte polarization in cell migration and immune interactions. EMBO J. 18:501-511.

Sander E.E., van Delft S., ten Klooster J.P., Reid T., van der Kammen R.A., Michiels F., Collard J.G. (1998) Matrix-dependent Tiam1/Rac signaling in epithelial cells promotes either cell-cell adhesion or cell migration and is regulated by phosphatidylinositol 3-kinase. J Cell Biol. 143:1385-1398.

Sanders L.C., F. Matsumara, G.M. Bokoch, P. de Lanerolle, Inhibition of myosin light chain kinase by p21-activated kinase, Science 283 (1999) 2083-2085.

Satterwhite L.L., Lohka M.J., Wilson K.L., Scherson T.Y., Cisek L.J., Corden J.L., and Pollard T.D. (1992) Phosphorylation of myosin-II regulatory light chain by cyclin-p34cdc2: a mechanism for the timing of cytokinesis. J. Cell Biol. 118:595-605.

Sebbagh M., C. Renvoize, J. Hamelin, N. Riche, J. Bertoglio, and J. Breard (2001) Caspase-3-mediated cleavage induces MLC phosphorylation and apoptotic membrane blebbing. Nature Cell Biol. 3:346-352.

Sekine A., Fujiwara M., and Narumiya S. (1989) Asparagine residue in the rho gene product is the modification site for botulinum ADP-ribosyltransferase. J. Biol. Chem. 264:8602-8605.

Sellers J.R. (1991) Regulation of cytoplasmic and smooth muscle myosin. Curr. Opin. Cell Biol. 3:98-104.

Shibasaki F., Fukami K., Fukui Y., and Takenawa T. (1994) Phosphatidylinositol 3-kinase binds to alpha-actinin through the p85 subunit. Biochem. J. 302:551-557.

Shimizu T., Ihara K., Maesaki R., Amano M., Kaibuchi K., and Hakoshima T. (2003) Parallel coiled-coil association of the RhoA-binding domain in Rho-kinase. J. Biol. Chem. 278:46046-46051.

Simpkins H., Lehmann J.M., Mazurkiewicz J.E., Davies B.H. (1991) A morphological and phenotypic analysis of Walker 256 cells. Cancer Res. 51:1334-1338.

Somlyo A.P., and Somlyo A.V. (2003) Ca^{2+} sensitivity of smooth muscle and non-muscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. Physiol. Rev. 83:1325-1358.

Spiller D.G., D.M. Tidd, Nuclear delivery of antisense oligodeoxynucleotides through reversible permeabilization of human leukemia cells with streptolysin O, Antisense Res. Dev. 5 (1995) 13-21.

Spitaler M., Cantrell D.A. (2004) Protein kinase C and beyond. Nat Immunol. 555:785-790.

Spudich J.A. (1989) In pursuit of myosin function. Cell Regul. 1:1-11.

Stephens L.R., Eguinoa A., Erdjument-Bromage H., Lui M., Cooke F., Coadwell J., Smrcka A.S., thelen M., Cadwallader K., Tempst P., and Hawkins P.T. (1997) The Gβ sensitivity of a PI3-K is dependent upon a tightly associated adaptor, p101. Cell 89:105-114.

Stewart H.L., Snell K.C., Dunham L.J. and Schlyen S.M. (1959) Fasc. 40:216-271.

Stohr M., Lohrke H., and Sandbrink H. (1975) Automated cytofluorometry of solid malignant tumors after cell dispersal by means of intraperitoneal cultivation of cell complexes in young rats. Beitr. Pathol. 155:36-43.

Stoyanov B., Volinia S., Hanck T., Rubio I., Loubtchenkov M., Malek D., Stoyanova S., Vanhaesebroeck B., Dhand R., Nürnberg B., Gierschick P., Seedorf K., Hsuan J.J., Waterfield M. D., and Wetzker R. (1995) Cloning and characterization of a G protein-activated human phosphoinositide 3-kinase. Science 269:690-693 .

Straight A.F., A. Cheung, J. Limouze, I. Chen, N.J. Westwood, J.R. Sellers, T.J. Mitchison, Dissecting temporal and spatial control of cytokinesis with a myosin II inhibitor, Science 299 (2003)1743-1747.

Sugihara K., Nakatsuji N., Nakamura K., Nakao K., Hashimoto R., Otani H., Sakagami H., Kondo H., Nozawa S., and Aiba A. (1998) Rac1 is required for the formation of three germ layers during gastrulation. Oncogene 17:3427-3433.

Symons M., and Settleman J. (2000) Rho family GTPases: more than simple switches. Trends in Cell Biol. 10:415-419.

Takahashi K., Sasaki T, Mammoto A, Takaishi K, Kameyama T, Tsukita S, Takai Y. (1997) Direct interaction of the Rho GDP dissociation inhibitor with ezrin/radixin/moesin initiates the activation of the Rho small G protein. J. Biol. Chem. 272:23371-23375.

Toker A., and Cantley L.C., (1997) Signalling through the lipid products of phosphoinositide-3-OH kinase. Nature 387:673-676.

Totsukawa G., Wu Y., Sasaki Y., Hartshorne D.J., Yamakita Y., Yamashiro S., and Matsumara F. (2004) Distinct roles of MLCK and ROCK in the regulation of membrane protrusions and focal adhesion dynamics during cell migration of fibroblasts. J Cell Biol. 164(3): 427-439.

Trauger J.W., Turner M.S., Fen-Fen-Lin, Stephens J., LoGrasso P. (2002) Characterization and purification of truncated human Rho-kinase II expressed in Sf-21 cells. Archives of Biochem. and Biophys 405:13-20.

Tsukita S., Yonemura S. (1997) ERM (ezrin/radixin/moesin) family: from cytoskeleton to signal transduction. Curr Opin Cell Biol. 9:70-75.

Uehata M., Ishizaki T., Satoh H., Ono T., Kawahara T., Morishita T., Tamakawa H., Yamagami K., Inui J., Maekawa M., and Narumiya S. (1997) Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. Nature. 389:990-994.

Vanhaesebroeck B., and Waterfield M.D. (1999) Signaling by distinct classes of phosphoinositide 3-kinases. Exp. Cell Res. 253:239-254.

Vanhaesebroeck B., Leevers S.J., Panayotou G., and Waterfield M.D. (1997) Phosphoinositide 3-kinases: a conserved family of signal transducers. Trends Biochem. Sci. 22:267-272.

Verin A.D., Patterson C.E., Day M.A., Garcia J.G. (1995) Regulation of endothelial cell gap formation and barrier function by myosin-associated phosphatase activities. Am J Physiol. 269:99-108.

Wells C.M., M. Walmsley, S. Ooi, V. Tybulewicz, A.J. Ridley, Rac1-deficient macrophages exhibit defects in cell spreading and membrane ruffling but not migration, J. Cell Sci. 117 (2004) 1259-1268.

Wheeler A.P., A.J. Ridley, Why three Rho proteins? RhoA, RhoB, RhoC, and cell motility, Exp. Cell Res. 301 (2004) 43-49.

Wicki A. and Niggli V. (1999) Role of Protein kinase C isoforms in locomotion of Walker 256 carcinosarcoma cells. Int. J. Cancer 81:255-261.

Wicki A., and Niggli V. (2001) The Rho/Rho-kinase and the phosphatidylinositol 3-kinase pathway are essential for spontaneous locomotion of Walker 256 carcinosarcoma cells. Int. J. Cancer 91:763-771.

Wiedemann C., and Cockcroft S., (1998) Sticky fingers grab a lipid. Nature 394: 426-427.

Wilson A.K., Takai A., Ruegg J.C., de Lanerolle P. (1991) Okadaic acid, a phosphatase inhibitor, decreases macrophage motility. Am J Physiol. 260:105-112.

Woodhouse E.C., Chuaqui R.F., Liotta L.A. (1997) General mechanisms of metastasis. Condeelis J., J.E. Segall, Intravital imaging of cell movement in tumours, Nat. Rev. Cancer 3 (2003) 921-930.

Wymann M.P., Sozzani S., Altruda F., Mantovani A., Hirsch E. (2000) Lipids on the move: phosphoinositide 3-kinases in leukocyte function. Immunol Today 21:260-264.

Wymann M.P., Bulgarelli-Leva G., Zvelebil M.J., Pirola L., Vanhaesebroeck B., Waterfield M.D., Panayotou G. (1996) Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. Mol Cell Biol. 16:1722-1733.

Xu J., F. Wang, A. Van Keymeulen, P. Herzmark, A. Straight, K. Kelly, Y. Takuwa, N. Sugimoto, T. Mitchison, H.R. Bourne, Divergent signals and cytoskeletal assemblies regulate self-organizing polarity in neutrophils, Cell 114 (2003) 201-214.

Yamashiro S., Totsukawa G., Yamakita Y., Sasaki Y., Madaule P., Ishizaki T., Narumiya S., and Matsumura F. (2003) Citron kinase, a Rho-dependent kinase, induces di-phosphorylation of regulatory light chain of myosin II. Mol. Biol. Cell. 14:1745-1756.

Yamauchi A., C. Kim, S. Li, C.C. Marchal, J. Towe, S.J. Atkinson, M.C. Dinauer, Rac2-deficient murine macrophages have selective defects in superoxide production and phagocytosis of opsonized particles, J. Immunol. 173 (2004) 5971-5979.

Yao R., and Cooper G.M., (1995) Requirement for phosphatitylinositol 3-kinase in the prevention of apoptosis by nerve growth factor. Science 2675:2003-2006.

Yonemura S., Matsui T., Tsukita S., Tsukita S. (2002) Rho-dependent and -independent activation mechanisms of ezrin/radixin/moesin proteins: an essential role for polyphosphoinositides in vivo. J Cell Sci. 115:2569-2580.

Zhang S., Han J., Sells M.A., Chernoff J., Knaus U.G., Ulevitch R.J., Bokoch G.M. (1995) Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. 270: 23934-23936.

Zheng B., Woo C.F., Kuo J.F. (1991) Mitotic arrest and enhanced nuclear protein phosphorylation in human leukemia K562 cells by okadaic acid, a potent protein phosphatase inhibitor and tumor promoter. J. Biol. Chem. 266:10031-10034.

3. MANUSCRIPT I

Role of Rho/Rho-kinase in phosphorylation of myosin light chain, development of polarity and spontaneous migration of Walker 256 carcinosarcoma cells

(submitted to Experimental Cell Research)

Marc C. Gutjahr and Verena Niggli

3.1. Abstract

We have previously shown that constitutive activation of the small GTPase Rho and its downstream target Rho-kinase are crucial for spontaneous migration of Walker carcinosarcoma cells.
We now show that after treatment of cells with either the Rho inhibitor C3 exoenzyme or the Rho-kinase inhibitor Y-27632, constitutive myosin light chain (MLC) phosphorylation is significantly decreased, correlating with inhibition of cell polarization and migration. Treatment with the myosin light chain kinase inhibitor ML-7 parallels these effects.
Transfection with a dominant-negative Rho-kinase mutant similarly inhibits cell polarization and MLC phosphorylation. Transfection with a dominant-active Rho-kinase mutant leads to significantly increased MLC phosphorylation and membrane blebbing. This Rho-kinase-induced membrane blebbing can be inhibited by Y-27632, ML-7 and blebbistatin
Unexpectedly, overactivaton of RhoA has similar effects as its inhibition. Introduction of a bacterially expressed constitutively activated mutant protein, L63-RhoA (but not of wild-type RhoA) into the cells or transfection of cells with constitutively active V14-RhoA, inhibits polarization and migration and significantly decreases MLC phosphorylation.
Our findings strongly suggest an important role of both Rho/Rho-kinase and MLCK in controlling myosin activity in Walker carcinosarcoma cells and show that an appropriate level of RhoA and Rho-kinase activity is required to regulate cell polarity and migration.

3.2. Introduction

Tumor cell locomotion is known to play a crucial role in invasion and metastasis (Condeelis et al., 2003) . Enhanced cell motility correlates with high metastatic potential (Partin et al., 1989. A better understanding of tumor cell migration could lead to novel therapeutic strategies.
The Rho family of small GTPases, molecular switches that cycle between a GDP-bound (inactive) state and a GTP-bound (active) state (Bokoch et al., 1994; Symons et al., 2000; Etienne-Manneville et al., 2002), have been implicated to play an important role in cell migration (Wheeler and Ridley, 2004). A number of Rho effectors have been identified which associate specifically with the GTP-bound forms of Rho GTPases. One of them, Rho-kinase (also called ROCK, Rho-associated coiled-coil forming protein kinase) (Matsui et al., 1996; Nakagawa et al., 1996), is implicated in many processes downstream of Rho including smooth muscle contraction (Amano et al., 1996), stress fiber and focal adhesion formation (Leung et al., 1996), intermediate filament disassembly (Goto et

al., 1998), neurite retraction (Amano et al., 1998), and cell migration (Fukata et al., 1999; Niggli, 1999).

Targets of Rho-kinase have been shown to be myosin light chain (MLC) (Amano et al., 1996) and myosin binding subunit (MBS) of myosin phosphatase (Kimura et al., 1996). Myosin II, a major motor protein in animal cells, is involved in a wide range of processes including muscle contraction, cell locomotion, cell division and receptor capping (Maciver, 1996). Myosin II activity is mainly controlled through the phosphorylation of MLC, which is regulated by two classes of enzymes: MLC kinases and myosin phosphatases (Hartshorne et al., 1998; Kamm and Stull, 2001; Somlyo and Somlyo, 2003). Myosin light chain kinase (MLCK) and Rho-kinase seem to be the two major kinases that phosphorylate MLC in vitro as well as in vivo (Somlyo and Somlyo, 2003). Phosphorylation induces a conformational change in MLC that allows actin-myosin interaction and activates the Mg^{2+}-ATPase activity of myosin (Somlyo and Somlyo, 2003; Totsukawa et al., 2004). An increase in intracellular Ca^{2+} concentration leads to activation of Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK), which then phosphorylates Thr-18 and Ser-19 on MLC (Somlyo and Somlyo, 2003; Totsukawa et al., 2004). Rho-kinase can increase MLC phosphorylation in two ways: on one hand mainly by phosphorylating the myosin binding subunit (MBS) of myosin phosphatase and thereby inhibiting myosin phosphatase activity (Kimura et al., 1996) and on the other hand by direct phosphorylation of MLC (Amano et al., 1996).

Rat Walker carcinosarcoma cells migrate spontaneously, apparently without requirement for extracellular signals. Recent findings in our laboratory revealed that activities of Rho and Rho-kinase are essential for polarization and migration in these cells, as inhibition of Rho proteins with C3 exoenzyme and Rho-kinase with Y-27632 abrogated cell polarity and migration. Rho-kinases are predominantly located in the membrane fraction in Walker carcinosarcoma cells, indicative of their constitutive activation (Wicki and Niggli, 2001). The downstream targets of Rho/Rho-kinase have not yet been elucidated in this cell line. In the present study we focused on the role of myosin light chain (MLC) in the generation of the contractile force for cell migration, as a target of the Rho/Rho-kinase signalling pathway in these cells. We investigated the regulation of myosin light chain (MLC) phosphorylation, as this process may contribute to the contractile force which is required for the development of cell polarity. For this we used expression of constitutively active or dominant-inactive signaling proteins in combination with pharmacological tools. Our data show that overactivation as well as inhibition of Rho/Rho-kinase activity results in inhibition of spontaneous polarization and migration. Thus an appropriate level of RhoA/Rho-kinase activity is required to control myosin light chain phosphorylation, spontaneous development of cell polarity and migration. Rac activity however appears not to be required for these processes, in contrast to

other cells such as neutrophils (Xu et al., 2003; Niggli, 2003). Unexpectedly our data also show that constitutive activation of RhoA results in reduced MLC phosphorylation.

3.3. Materials and Methods

Materials

Reagents and suppliers were: Y-27632 (Calbiochem, La Jolla, CA, USA) was prepared as a 10 mM stock solution in H_2O. ML-7 (Alexis, Lausen, Switzerland) was prepared as a stock solution of 10 mM in dimethylsulfoxide (DMSO). Blebbistatin (Tocris, Avonmouth, UK) was prepared as a stock solution of 100 mM in DMSO. Aliquots of these stock solutions were stored at -20°C. DMSO alone had no significant effect in these experiments at the final concentrations used. Enhanced chemiluminescence Western blotting detection reagents were obtained from Pierce, Rockford, IL, USA; rhodamine-phalloidin was from Molecular Probes, Eugene, OR, USA.

GA (Glutaraldehyde) and BSA were obtained from Serva, Heidelberg, Germany. Lysolecithin, PFA (Paraformaldehyde), DMSO, and SLO (Streptolysin O) were from Sigma, St. Louis, MO, USA. Human serum albumin (HSA) was from ZLB Bioplasma AG, Bern, Switzerland.

C3 exoenzyme and bacterially expressed RhoA and Rac1 proteins were obtained from Cytoskeleton, Denver, CO, USA.

Gey's solution contained 138 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.1 mM $CaCl_2$, 100 µM EGTA, 1 mM Na_2HPO_4, 5 mM $NaHCO_3$, 5.5 mM glucose and 20 mM HEPES (pH 7.4).

Plasmids

pCAG-myc-WT-ROCKI, pCAG-myc-KD-IA-ROCKI, and pCAG-myc-Δ3-ROCKI were kindly provided by S. Narumiya (Department of Pharmacology, Kyoto University, Faculty of Medicine, Kyoto, Japan). pEXV3-myc-V14-RhoA was a kind gift of A.J. Ridley (Ludwig Institute for Cancer Research, London, UK). pCB6-GFP(green fluorescent protein)-Rac1 and pCB6-GFP-N17-Rac1 were kindly provided by M. Way (Cancer Research UK, London Research Institute, London, UK). pEGFP was obtained from Clontech BD (Palo Alto, CA, USA).

Antibodies

Antibodies were obtained from the following sources: a monoclonal murine antibody directed against RhoA (Cat. Nr. ARH01) from Cytoskeleton, Denver, CO, USA. A polyclonal anti-ROCK I antibody was prepared as described (Fujita et al., 1997). A polyclonal anti-phospho MLC2 (Thr-18/Ser-19) antibody (Cat. Nr. 3674S) was from Cell Signaling, Beverly, MA, USA. An anti-cMyc antibody (Cat. Nr. sc-40) was from Santa Cruz Biotechnology, Santa Cruz, CA, USA. A monoclonal murine antibody against actin (Cat. Nr. 010056) was from Bio-Science Products. The Alexa 488 goat-anti-mouse antibody (Cat. Nr. A11001) was from Molecular Probes, Eugene, OR, USA. Secondary antibodies, that is, goat-anti-mouse horseradish peroxidase conjugated (Cat. Nr. 170-6516), and goat-anti-rabbit horseradish peroxidase conjugated (Cat. Nr. 170-6515) were obtained from BioRad, Hercules, CA, USA.

Tumor cell culture

Walker 256 carcinosarcoma cells were kindly provided by Dr. B. Sordat (ISREC, Lausanne, Switzerland) and cultured in vitro in RPMI 1640 supplemented with 10% FCS (fetal calf serum), penicillin (25 µg/ml) and streptomycin (25 µg/ml). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

Determination of cell morphology

Cells were washed twice in Gey's medium and resuspended in this medium (1 x 10^6 cells/ml). Details of the incubation procedure are given in the Results section. The incubation was terminated by fixation with 1% glutaraldehyde (final concentration) at 37°C for 30 min. At least 100 cells per condition were analyzed in each experiment using DIC (differential interference contrast) optics (Zeiss IM 35 microscope, x 100 objective). Cells were classified by shape. The following shape categories were distinguished: 1) spherical cells, 2) polarized cells with a lamellipod and a contracted tail (uropod), 3) non-polar cells with surface protrusions, 4) non-polar cells with blebs.

Cell locomotion assay

Cells (1 x 10^6 cells/ml) were washed twice and then incubated in Gey's medium plus 2% HSA without or with inhibitors (Results section). Cells were studied in narrow, paraffin-sealed, slide-coverslip preparations (depth 5-8 µm) to prevent passive cellular translocation. The preparation was placed on a heated (37°C) stage of an inverted microscope. The path of the cells was recorded on

videotape for 10 min using a x 40 objective. The initial position was outlined, and only cells that had completely left the initial position were scored as locomoting. Speed was determined by means of a morphometry unit (KS 300; Kontron, Eching, Germany).

TCA precipitation, electrophoresis and immunoblotting

Cells were precipitated by adding 1 vol. of an ice-cold solution containing 200 mg/ml trichloracetic acid (TCA), 40 mM NaF and 10 mM Na_2HPO_4. After incubation on ice for 20 min, precipitates were collected by centrifugation (10 min) in an Eppendorf centrifuge, 10'000 g at 4°C. Pellets were washed once with 1 ml of 5% TCA and once with 1 ml of 0.5% TCA and then solubilized in 100 µl of sample buffer containing 1% SDS, 50 mM dithiothreitol, 15% glycerol, 62.5 mM Tris/HCl (pH 6.8) and 0.001% bromphenol blue by incubation at 95°C for 10 min. Fractions were electrophoresed through a SDS-polyacrylamide gel followed by transblotting to nitrocellulose using a Genie blotter (Idea Scientific, Minneapolis, MN). The blots were incubated for 1h in a blocking buffer containing 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-20 and 5% defatted milk powder (TBSTM), followed by overnight incubation at 4°C with the indicated antibodies, diluted in PBS containing 3% BSA and 0.02% NaN3. After 3 washes with TBST, the blots were incubated for 1 h either with a second horseradish peroxidase-labeled goat anti-rabbit IgG antibody (Bio Rad Laboratories, Hercules, CA) for polyclonal antibodies, diluted 1:10'000 in TBSTM, or with a horseradish peroxidase-labeled goat anti-mouse IgG antibody for monoclonal antibodies, diluted 1:3000 in TBSTM. Detection was performed with an ECL Western detection system (Pierce, Rockford, IL). Bands were quantified by densitometry of the films using a GS-800 Calibrated Densitometer from Bio-Rad.

MLC Phosphorylation

Cells were washed twice in Gey's medium, resuspended in this medium (1.0×10^6 cells/0.5 ml) and then incubated at 37°C for 30 min with the indicated concentrations of Y-27632, ML-7 or blebbistatin. The reaction was stopped with TCA (see above) and the precipitated proteins were subjected to immunoblotting using a polyclonal rabbit anti-phospho MLC2 (Thr-18/Ser-19) antibody (1:500 dilution).

Streptolysin O permeabilization of Walker Carcinosarcoma cells

Cells were washed twice in RPMI 1640 medium without FCS. Then cells are resuspended to a final volume of 100 µl (10 x 10^6 cells/ml) in this medium. The protein to be introduced (30 µg/ml) was placed in the wells of a 24-well plate after addition of the cells (100 µl) and 5-20 U of SLO. The optimal amount of SLO was determined empirically in advance for each batch (Spiller et al., 1995). The cells were incubated at 37°C in a CO2 incubator for 10 minutes and were agitated three times during this period. Then 1 ml RPMI 1640 containing 10% FCS was added and the plate was placed back into the incubator for another hour. Cells were subsequently washed twice with Gey's buffer and incubated (1 x 10^6 cells/0.5 ml) at 37°C for 30 min followed by TCA-precipitation for immunoblot analysis or fixed with 1% glutaraldehyde (final concentration) at 37°C for 30 min for analysis of cell shape as described above.

Nucleofection

Cells were washed twice with Gey's buffer. 2-3 x 10^6 cells were resuspended in 100 µl Nucleofector Solution (diluted 1:3 with PBS) supplied by Amaxa (Köln, Germany) and 5 µg of plasmid-DNA was added. For cotransfection experiments 0.5 µg of pEGFP were mixed with 5 µg of the appropriate plasmid. Then the cell suspension and the plasmid DNA were transferred to a supplied cuvette and Nucleofection was carried out (Amaxa Nucleofector, program Q23). Immediately 500 µl of RPMI 1640 medium with FCS was added and the cells were transferred to a prewarmed 12-well plate containing 1.5 ml of RPMI 1640 medium with FCS, followed by incubation at 37°C in a CO_2 incubator for 12 hours. Transiently transfected cells were subjected to cell sorting. Cells were harvested and centrifuged at 300 g for 5 min. The cell pellet was resuspended in culture medium containing serum and supplements to a final concentration of 10 x 10^6 cells/ml. Then the viable GFP-positive cells were separated from the viable non-fluorescent and the dead cells using a Vantage Diva Cell Sorter from BD. The sorted GFP expressing cells were incubated in Gey's medium at 37°C for 30 min, followed by TCA precipitation and immunoblotting (see above).

Immunofluorescence staining

6 h after transfection cells were collected and washed twice with Gey's buffer. After incubation at 37°C for 30 min cells were fixed with 4% paraformaldehyde for 15 min at 37°C. Cells then were washed with PBS containing 10 mM EDTA, permeabilized with lysolecithin (5 µg/ml) for 15 min at RT and washed 2 times with PBS containing 10 mM EDTA. Cells were cytocentrifuged and blocked for 1 hour with PBS containing 4% BSA. After that cells were incubated with the primary

antibody (anti-cmyc, 1:350 dilution) at RT for 45 min, rinsed 3 times with PBS, blocked again for 15 min with PBS containing 4% BSA, incubated with the secondary antibody (Alexa 488 goat-anti-mouse, 1:4000 dilution), at RT for 45 min and then rinsed 3 times with PBS.
Subsequently F-actin staining was added performed as follows: Cells were washed once with 0.1 M Tris/NaCl buffer pH 8.0 containing 0.05% BSA and then incubated with rhodamine-phalloidin (200 U/ml) diluted in 0.1 M Tris/NaCl buffer pH 8.0 containing 0.05% BSA at RT for 10 min. The reaction was stopped by the addition of 1 ml of 0.1 M Tris/NaCl buffer pH 8.0 containing 4% BSA. Cells were washed 2 times with 0.1 M Tris/NaCl buffer pH 8.0. Images were taken with a confocal microscope (Zeiss LSM 510 META, x 63 objective).

Cell viability assay

Nonviable and/or apoptotic cells were determined by detection of phosphatidylserine externalization using annexin-V-FITC staining. Maximally used concentrations of every inhibitor were tested on inducing cell membrane permeabilization and/or apoptosis. Cells were incubated with inhibitors (Y-27632: 30 µM; ML-7: 30 µM; blebbistatin: 100 µM) for 30 min, harvested and washed twice with annexin-V-buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2). Cell pellets (0.5 x 106 cells) were resuspended in annexin-V-buffer containing annexin-V-FITC (0.5 (g/ml) for 20 min at 4°C under light protection. Cells were washed twice with annexin-V-buffer and then fixed with annexin-V-buffer containing 1% paraformaldehyde. Evaluation of annexin-V-positive cells was done with a FACScan measuring FL-1. None of the tested inhibitors significantly affected cell viability (data not shown).

Statistics

Differences between data were analysed with the Student's *t* test, with a P value < 0.05 considered significant. Data correspond to the mean ± SD.

3.4. Results

Modulation of activity of Rho but not Rac affects cell polarity, migration, F-actin localization, and MLC phosphorylation of Walker carcinosarcoma cells.

About 71% of the Walker carcinosarcoma cells in the culture used were spontaneously polarized in the absence of added stimuli (Fig. 1Aa). The remaining cells assumed a spherical morphology (Fig. 1Ab). As shown previously (Wicki and Niggli, 2001), the Rho inhibitor C3 exoenzyme, which specifically inhibits RhoA, RhoB, and RhoC, suppressed polarization by 94% (Figs. 1Ad, 1B) and migration by 90% (Table 1). Activity of Rho is thus required for motility. We now checked the impact of introducing bacterially expressed activated RhoA (L63-RhoA) into SLO-permeabilized cells on morphology. Introduction of constitutively activated L63-RhoA into cells had a similar effect as C3 exoenzyme, inhibiting polarity maximally by 72% (Figs. 1Ae, 1B) and migration by 79% (Table 1). This inhibition was dependent on the concentration of constitutively activated RhoA. Maximal effects were achieved when cells (10×10^6 cells/ml) were incubated with 30 μg/ml of the protein resulting in 79% spherical cells. In contrast when SLO-permeabilized cells were incubated with bacterially expressed wild-type RhoA or with dominant-negative N17-Rac1 polarity was not affected (Figs. 1Ac, 1B). Activity of the constitutively activated RhoA mutant L63-RhoA was checked using a pull-down assay with the GST-tagged Rhotekin Rho-binding domain. The constitutively active mutated Rho protein indeed bound to Rhotekin (data not shown).

In order to confirm these findings with another technique we transfected cells with a myc-tagged dominant-active RhoA mutant, V14-RhoA (Ridley et al., 1992) or with a GFP-tagged dominant-negative Rac1 mutant, N17-Rac1 (McGee et al., 2001). Transfection of cells with the dominant-active V14-RhoA resulted in an almost complete suppression of cell polarization. Cells instead assumed a spherical morphology (Figs. 2Ab, 2B). The myc-tagged protein showed a punctate localization in the cytosol. In order to confirm that the myc-tagged V14-RhoA is indeed expressed in the cells, we carried out immunoblotting using anti-RhoA and anti-cmyc antibodies (Fig. 3A). In contrast transfection of cells with the dominant-negative N17-Rac1 had no effect on cell polarization (data not shown).

We also assessed the role of Rho proteins in F-actin organization using rhodamine-phalloidin staining of the cells to visualize polymerised actin. As shown in Fig. 1Aa`, in spontaneously polarized control cells F-actin was accumulated mainly in the tail and along the cell membrane (see also Keller, 2000). In spherical control cells F-actin was equally distributed along the cell membrane (Fig. 1Ab`). After treatment with the Rho inhibitor C3 exoenzyme F-actin was still

localized along the cell membrane (Fig. 1Ac`) but the cortical F-actin staining was significantly weaker than in untreated control cells (Fig. 1Ab`). In contrast, neither introduction of bacterially expressed WT-RhoA (Fig. 1Ac`) nor of dominant-active RhoA (L63-RhoA) (Fig. 1Ae`) or transfection with V14-RhoA (Fig. 2Ab``) reduced cortical F-actin staining. In these cells F-actin was still linearly associated with the plasma membrane showing no difference as compared to spherical control cells (Fig. 1Ab`).

One target of the RhoA/Rho-kinase pathway is myosin light chain (MLC) (Amano et al., 1996; Kimura et al., 1997; Somlyo and Somlyo, 2003). We therefore investigated the role of RhoA in controlling MLC-phosphorylation in Walker carcinosarcoma cells. Cells were SLO permeabilized in the presence of the indicated amount of proteins. As shown in Fig. 1C, both inhibition of RhoA by C3 transferase and unexpectedly constitutively activated L63-RhoA strongly reduced MLC-phosphorylation as compared to untreated cells. Treatment of cells with C3 transferase resulted in 90% inhibition of MLC-phosphorylation and introduction of constitutively activated RhoA in 77% inhibition. In cells expressing the dominant-active RhoA mutant (V14-RhoA), MLC phosphorylation was also inhibited significantly by 30 ± 5% ($P < 0.05$) as compared to untransfected control cells (Fig. 2D) similar to effects of introducing bacterially expressed L63-RhoA. Inhibition of MLC phosphorylation thus correlates with inhibition of polarization.

In contrast in cells permeabilized in the presence of WT-RhoA no significant changes in MLC phosphorylation were observed correlating with a lack of effect on polarization. Introduction of bacterially expressed dominant-negative N17-Rac1 also had no significant effect on MLC phosphorylation (Fig. 1C) correlating with a lack of effect on cell morphology.

The role of Rho-kinase in regulating morphology, F-actin localization, and MLC phosphorylation of Walker carcinosarcoma cells.

As shown previously, inhibition of ROCK by Y-27632 inhibits development of polarity and migration in Walker cells (Wicki and Niggli, 2001). In order to confirm our data and to obtain more detailed information on the role of Rho-kinase in cellular processes such as development of polarity and MLC phosphorylation, we transiently expressed myc-tagged wild type Rho-kinase (WT-ROCKI) as well as mutants of this protein (Ishizaki et al., 1997) in Walker carcinosarcoma cells. The mutant Δ3-ROCKI acts as a dominant-active enzyme. It lacks the C-terminus with the PH domain, the Rho-binding domain and the cystein-rich domain and is thus similar to the fragment that is generated by caspase-3 during apoptosis. The KD-IA mutant corresponds to the full length protein with two point mutations in the kinase and the Rho-binding domain (KD-IA), acting as a

dominant-negative form of the enzyme (Ishizaki et al., 1997). Evidence for cellular expression of mutants obtained by immunoblotting of cell lysates is shown in Fig. 3B.

When cells were transfected with WT-ROCKI, we observed that the ectopically expressed Rho-kinase was enriched in the rear of the cells. Wild type and mutant proteins all showed a punctated localization in the cytosol, comparable to V14-RhoA (Fig. 2a`-e`). The morphology of the transfected cells was not markedly affected (Figs. 2Ac, 2Ac`, 2C). F-actin was predominantly located in the rear of the cell comparable to untransfected polarized control cells (Figs. 2Aa``, 2Ac``). In contrast transfection of cells with the dominant-negative KD-IA-ROCKI mutant completely inhibited development of polarity. Cells assumed a spherical morphology with a smooth surface. The mutant was evenly distributed in the cell (Figs. 2Ad, 2C). No significant change in F-actin localization could be detected in cells transfected with KD-IA. It was still linearly associated with the plasma membrane (Fig. 2Ad``) as in untreated spherical control cells (Fig. 1Ab`).

Similarly in cells treated with the Rho-kinase inhibitor Y-27632 F-actin was concentrated at the cell membrane comparable to spherical control cells (Figs. 4Ab`, 4Ac`). Inhibition of Rho-kinase with Y-27632 thus has the same effect as transfection with dominant-negative ROCK mutant. In cells transfected with the constitutively active mutant, Δ3-ROCKI, increased cell contraction, inhibition of cell polarization, and extensive membrane blebbing was observed (Figs. 2Ae, 2C) that finally resulted in cell death (see below). In these transfected cells F-actin was located along the cell membrane and along the blebs. Local aggregates at the membrane were observed (Fig. 2Ae``).

To confirm the role of ROCK in modulating myosin light chain phosphorylation, we cotransfected the cells with dominant-negative Rho-kinase KD-IA and pEGFP followed by cell sorting and immunoblotting. Phosphorylation of MLC could be reduced to a level of 26% ± 15 ($P < 0.01$) as compared to control cells only transfected with pEGFP or cells cotransfected with WT-ROCKI and pEGFP (Fig. 2E). Similarly, incubation of Walker carcinosarcoma cells with 10 μM to 20 μM of Y-27632 resulted in a very marked decrease (79% and 81.5% respectively) in constitutive MLC-phosphorylation (Fig. 4D), correlating with effects of Y-27632 observed previously on morphology and migration.

Transfection of cells with constitutively active Δ3-ROCKI in contrast resulted in a significant 1.9 ± 0.1-fold increase ($n=3$, $P < 0.01$) in MLC phosphorylation as compared to pEGFP-transfected control cells (Fig. 2E). This is different from the decrease in phosphorylation observed in cells transfected with constitutively active RhoA (Fig. 2D). Increased MLC phosphorylation thus does not result in increased development of polarity, but rather correlates with inhibition of polarization and increased blebbing.

The role of MLCK and myosin II in regulatin morphology, F-actin localization, and MLC phosphorylation in Walker carcinosarcoma cells.

We assessed also the role of MLCK in constitutive MLC phosphorylation, polarization and migration of Walker carcinosarcoma cells. ML-7 inhibits MLCK with some specificity (Bain et al., 2003). 20-30 μM of ML-7 maximally suppressed polarity by 95% (Figs. 4Ad, 4B). MLCK is thus as important as ROCK in regulating Walker cell polarity. Similarly migration could be inhibited by 84% by 10 μM of ML-7 (Table 2).

The effects of ML-7 on polarity correlate with inhibition of MLC phosphorylation. We observed a concentration-dependent effect with maximal inhibition (74%) observed at 20-30 μM which correlates with inhibition of polarization (Fig. 4E). Inhibition of MLCK with ML-7 resulted in cells with F-actin still associated linearly along the plasma membrane, but we also observed a characteristic accumulation of F-actin into aggregates (Fig. 4Ad`). Cells treated with blebbistatin or Y-27632 lacked these aggregates (Figs. 4Ac`, 4e`).

Both for ML-7 and Y-27632 inhibition of MLC phosphorylation was not complete (74-81%). A combination of 20 μM Y-27632 and ML-7 further reduced MLC phosphorylation to 11% of controls (Fig. 4D). Our data show that Rho-kinase and MLCK are equally important for the control of MLC phosphorylation, polarization and migration in Walker carcinosarcoma cells.

To further provide evidence that the target of MLCK, myosin, is crucial for development of cell polarity and migration we tested effects of inhibiting myosin II activity directly using blebbistatin, a compound which inhibits the actin-activated Mg^{2+}-ATPase of myosin II from muscle and non-muscle cells with some specificity (Straight et al., 2003; Limouze et al., 2004). Maximal inhibition of cell polarity was achieved with 100 μM of blebbistatin (Figs. 4Ae, 4C). Half-maximal effects were observed at 21 ± 4 μM. Cells assumed a spherical morphology and migration was blocked (Table 2).

Inhibition of ROCKI, MLCK, and myosin II suppresses membrane blebbing of Walker carcinosarcoma cells transfected with constitutively active ROCKI.

As shown above, transfection of cells with Δ3-ROCKI results in blebbing cells (Figs. 2Ae, 2C). 3 hours after transfection microscopic observations revealed a highly dynamic protrusion and retraction of blebs in cells cotransfected with Δ3-ROCKI and pEGFP. Approximately 12 hours after transfection blebbing intensity decreased, the cells assumed a spherical morphology, followed by cell death (data not shown).

The Δ3-ROCKI mutant lacks the autoinhibitory domain and thus is constitutively active and not controlled by cellular signals. It therefore mimics the caspase-3-cleaved form of truncated ROCKI created during apoptosis (Sebbagh et al., 2001). We tested whether caspase inhibition with the caspase inhibitor z-VAD-fmk prevents blebbing in order to rule out the involvement of putative electroporation-induced activation of caspases. However this was not the case (data not shown) indicating that these processes are caspase-independent and that this truncated active ROCKI fragment alone is sufficient to induce membrane blebbing and increased cell death.

In Δ3-ROCKI-transfected cells that were incubated with 30 μM of the ROCK inhibitor Y-27632 membrane blebbing was markedly inhibited by 77% and the cells assumed a spherical morphology with sometimes a few small blebs (Fig. 5). Similarly 30 μM of the MLCK inhibitor ML-7 inhibited membrane blebbing by 98%. After treatment most of the cells assumed a spherical morphology. Inhibition of myosin II with blebbistatin (100 μM) completely suppressed Δ3-ROCKI-induced blebbing and was thus most effective as compared to Y-27632 and ML-7. Our results thus suggest a crucial role of myosin II in membrane blebbing induced by constitutive active ROCKI.

3.5. Discussion

We focus on the role of Rho family proteins and Rho-kinase in spontaneous locomotion of Walker carcinosarcoma cells. We have previously provided evidence for constitutive activation of Rho and ROCK being required for spontaneous random migration of Walker carcinosarcoma cells (Wicki and Niggli, 2001). We now identify myosin II as a major target of the Rho/ROCK pathway in these cells. We furthermore provide evidence that not only inhibition but also non-localized overactivation of Rho and ROCK suppress polarization and migration, while interestingly differing in effects on MLC phosphorylation. Rac, in contrast to other cell types, appears not to be required for migration.

Rho

As previously shown, C3 exoenzyme which suppresses specifically activity of RhoA, B, and C by ADP-ribosylation, inhibits polarization of Walker carcinosarcoma cells. We now provide evidence that suppression of Rho activity also resulted in a decrease in constitutive MLC phosphorylation correlating with decreased polarization, indicating decreased activity of the downstream target of Rho, ROCK, which results in increased activation of MLC phosphatase. Furthermore, Rho

inhibition by C3 exoenzyme reduced rhodamine-phalloidin staining as compared to spherical control cells. This effect was not observed in experiments where ROCK activity was directly inhibited, indicating that a target of Rho different than ROCK is required for maintenance of cortical F-actin (Figs. 1Ab`, 1Ad`).

Not only inhibition, but also introduction of constitutively active, mutated L63-RhoA or transfection with another dominant-active RhoA, V14-RhoA, inhibited cell polarity (Figs. 1B, 2B), and migration (Table 1). These findings are similar to observations in human neutrophil-like HL-60 cells transfected with L63-RhoA (Xu et al., 2003). Introduction or expression of a constitutive activated RhoA protein had no effect on cortical F-actin (Figs. 1Ae`, 2Ab``) different from the effects of C3 exoenzyme (Fig. 1Ad`).

Inhibition of polarization induced by increased Rho activation can be explained by the observed unexpected decrease in MLC phosphorylation (Figs. 1C, 2D). This is in contrast to the stimulatory effect of expressing activated ROCK, which increased MLC phosphorylation. Our findings suggest that constitutively active RhoA does not activate ROCK further. Rather, a downstream target of Rho different from ROCK may inhibit an enzyme contributing to MLC phosphorylation via a negative feedback loop. For example PAK, a downstream effector of Rac and Cdc42, has been reported to phosphorylate and inactivate MLCK (Sanders et al.,1999).

Rho proteins are mainly located in the rear of polarized neutrophils, organizing contractile actin-myosin complexes (Xu et al., 2003). In Walker carcinosarcoma calls transfected with constitutively active V14-RhoA, this protein is evenly distributed in the cell showing a punctate distribution indicating association with internal membranes, similar to findings in MDCK cells (Michaelson et al., 2001). We suggest that an exactly defined spatial activity of Rho is required to establish polarity. Thus delocalised activation of RhoA is enough to inhibit polarity. According to Xu et al. overexpression of constitutively active RhoA inhibits Rac, whose spatially defined localized activation in the leading lamellae is required for neutrophil migration. This mechanism is very likely not operative in Walker carcinosarcoma cells as Rac is not required for migration in these cells (see below).

Rac

The Rho family protein Rac is crucial for migration in neutrophils. Rac1-deficient neutrophils showed clear defects in inflammatory recruitment in vivo, migration to chemotactic stimuli, and chemoattractant-mediated actin assembly (Glogauer et al., 2003). Rac1 also plays a crucial role in morphogenesis as Rac1 knock-out mice die early in development during gastrulation (Sugihara et al., 1998). In contrast Rac1-deficient macrophages exhibited normal migration and chemotaxis,

although they assumed an elongated morphology. This suggests that in macrophages Rac1 is primarily responsible for regulating cell morphology, but is not required for migration (Wells et al., 2004). Rac2 is about 4-fold less abundant in murine macrophages. Macrophages lacking Rac2 show reduced, but not abolished accumulation at inflamed sites in vivo (Yamauchi et al., 2004).
Unexpectedly, introduction or transfection with a dominant-negative Rac mutant did not have any effect on cell polarity (Fig. 1B), or migration (Table 1) and also did not affect MLC phosphorylation (Fig. 1C) in Walker carcinosarcoma cells. In polarized leukocytes Rac is localized in the leading front, where it is involved in protrusion by locally inducing actin polymerization. A positive feedback loop involving PI 3-kinase, whose products activate Rac which in turn activates PI 3-kinase, is also implicated in this process (Xu et al., 2003; Niggli, 2003). In Walker carcinosarcoma cells PI 3-kinase is required for polarization and migration, as polarization and migration are suppressed upon inhibition of PI 3-kinase by wortmannin or LY-294002 (Wicki and Niggli, 2001). However, the target of PI 3-kinase must be different from Rac, as the latter protein is not required for migration in Walker carcinosarcoma cells (this work).
In contrast to neutrophils relatively little F-actin is located in the leading front of polarized Walker carcinosarcoma cells (Fig. 1Aa`). Furthermore, observation of migrating Walker cells revealed that ruffling is much less extensive at the leading front as compared to neutrophils, and migration may be driven rather by rear contraction than by F-actin directly pushing the membrane forward. However, at least in the rear of the cells actin polymerization is required as latrunculin A, an F-actin depolymerising agent, inhibits migration (Keller et al., 2002). Possibly, mechanisms of actin polymerisation and lamellipodia protrusion are different in neutrophils and Walker carcinosarcoma cells, only the former requiring localized Rac activation.

Rho-kinase

Rho-kinase is a direct downstream effector of RhoA and involved in regulating MLC phosphorylation (Somlyo and Somlyo, 2003). We could clearly show that inhibition of cell polarization with the Rho-kinase inhibitor Y-27632 (Fig. 4Ac) correlates with inhibition of MLC phosphorylation (Fig. 4D). These data could be confirmed with expression of the dominant-negative Rho-kinase mutant KD-IA (Figs. 2C, 2E). In contrast to the Rho inhibitor C3 exoenzyme which reduced F-actin staining (Fig. 1Ad`), neither Y-27632 (Fig. 4Ac`) nor the dominant-negative mutant KD-IA (Fig. 2Ad``) decreased the amount of cortical F-actin. Rho, being upstream of Rho-kinase may thus play a more central role in maintaining polymerized actin in these cells.
The relative roles of MLCK and ROCK in controlling MLC phosphorylation appear to differ depending on the cell type (Ren et al., 2004). We could show that MLCK is as important as ROCK

for constitutive MLC phosphorylation (Fig. 4E), polarization (Fig. 4Ad, 4B) and migration (Table 2) of Walker carcinosarcoma cells.

Effects comparable to those seen with Y-27632, ML-7, and KD-IA-ROCKI on Walker carcinosarcoma cell morphology were obtained with blebbistatin (Fig. 4). Blebbistatin was reported to inhibit selectively nonmuscle myosin IIA and IIB but not novel isoforms such as myosin I, V, and X (Limouze et al., 2004). Assuming blebbistatin to be a specific inhibitor of myosin II, this would conclusively show that MLC phosphorylation and concomitant myosin II activation are involved directly in spontaneous polarization and migration of Walker carcinosarcoma cells.

Constitutively active RhoA and constitutively active ROCKI caused two quite different phenotypes. Expression or introduction of constitutively active RhoA inhibited cell polarity and decreased MLC phosphorylation resulting in spherical cells with a smooth surface, whereas expression of constitutively active Δ3-ROCKI induced increased cell contraction and membrane blebbing and inhibited polarization (Fig. 2Ac). We assume that formation of the contracted tail (uropod) during polarization was prevented by membrane blebbing. Rhodamine-phalloidin staining revealed local aggregates of F-actin at the membrane, possibly indicating the region where the F-actin rich uropod may have been located prior to Δ3-ROCKI expression (Fig. 2Ae``). Upon Δ3-ROCKI expression MLC phosphorylation was significantly increased, indicating that activity of myosin phosphatase is only partially suppressed by constitutive ROCK activity in untreated cells. Additionally, activated ROCK may directly phosphorylate myosin light chain. However inhibition of MLCK by ML-7 was at least as efficient as inhibition of ROCK in suppressing blebbing in cells transfected with Δ3-ROCKI, suggesting that ROCK mainly acts by inhibiting myosin phosphatase. Increased MLC phosphorylation does thus not result in increased polarization but rather is inhibitory in this process. Similar to our findings, expression of the Δ3-ROCKI mutant lacking the autoinhibitory domain in Jurkat cells (Sebbagh et al., 2001) or in NIH 3T3 cells (Coleman et al., 2001) induced membrane blebbing. Thus overactivation of ROCKI alone is sufficient for cell contraction and membrane blebbing. Cell death resulting from transfection with Δ3-ROCKI does not seem to correspond to classical apoptosis, as the cells undergo blebbing and cell death also in the presence of the caspase inhibitor z-VAD-fmk. The cells probably die because of ATP depletion as a result of extensive myosin activation which uses up cellular ATP. Indeed, myosin II plays a very important role in membrane blebbing in Δ3-ROCKI-transfected cells as it could be inhibited by inhibition of ROCK, MLCK and most efficiently by inhibition of myosin II activity by blebbistatin (Fig. 5).

In summary, our results show that both Rho/Rho-kinase and MLCK control myosin activity in Walker carcinosarcoma cells, and that myosin activation is crucially required for development of

polarity and migration. An appropriate level of Rho/Rho-kinase activity seems to be important for the regulation of MLC phosphorylation, cell polarity and migration. Rho/Rho-kinase inhibition as well as delocalised strong activation appear to prevent motile responses. Delocalized strong activation of ROCKI leads to increased MLC phosphorylation, membrane blebbing, and cell death. The decrease in MLC phosphorylation observed after introduction or expression of constitutively activated RhoA mutants indicates that high delocalised RhoA activity negatively regulates downstream signalling impacting on myosin regulation.

3.6. References

Amano M., K. Chihara, N. Nakamura, Y. Fukata, T. Yano, M. Shibata, M. Ikebe, K. Kaibuchi, Myosin II activation promotes neurite retraction during the action of Rho and Rho-kinase, Genes Cells 3 (1998) 177-188.

Amano M., M. Ito, K. Kimura, Y. Fukata, K. Chihara, T. Nakano, Y. Matsuura, K. Kaibuchi, Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase), J. Biol. Chem. 271 (1996) 20246-20249.

Bain J., H. McLauchlan, M. Elliot, P. Cohen, The specificities of protein kinase inhibitors: an update, Biochem. J. 371 (2003) 199-204.

Bokoch G.M., B.P. Bohl, T.H. Chuang, Guanine nucleotide exchange regulates membrane translocation of Rac/Rho GTP-binding proteins, J. Biol. Chem. 269 (1994) 31674-31679.

Coleman M.L., E.A. Sahai, M. Yeo, M. Bosch, A. Dewar, M.F. Olson, Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I, Nat. Cell Biol. 3 (2001) 339-345.

Condeelis J., J.E. Segall, Intravital imaging of cell movement in tumours, Nat. Rev. Cancer 3 (2003) 921-930.

Etienne-Manneville S., A. Hall, Rho GTPases in cell biology, Nature 420 (2002) 629-635.

Fujita A., Y. Saito, T. Ishizaki, M. Maekawa, K. Fujisawa, F. Ushikubi, Integrin-dependent translocation of p160ROCK to cytoskeletal complex in thrombin-stimulated human platelets, Biochem. J. 328 (1997) 769-775.

Fukata Y., N. Oshiro, N. Kinoshita, Y. Kawano, Y. Matsuoka, V. Bennet, Y. Matsura, K. Kaibuchi, Phosphorylation of adducin by Rho-Kinase plays a crucial role in cell motility, J. Cell Biol. 145 (1999) 347-361.

Glogauer M., C.C. Marchal, F. Zhu, A. Worku, B.E. Clausen, I. Foerster, P. Marks, G.P. Downey, M. Dinauer, D.J. Kwiatkowski, Rac1 deletion in mouse neutrophils has selective effects on neutrophil functions, J. Immunol. 170 (2003) 5652-5657.

Goto H., H. Kosako, K. Tanabe, M. Yanagida, M. Sakurai, M. Amano, K. Kaibuchi, M. Inagaki, Phosphorylation of vimentin by Rho-associated kinase at a unique amino-terminal site that is specifically phosphorylated during cytokinesis, J. Biol. Chem. 273 (1998) 11728-11736.

Hartshorne D.J., M. Ito, F. Erdodi, Myosin light chain phosphatase: subunit composition, interactions and regulation, J. Muscle Res. Cell Motil. 19 (1998) 325-341.

Ishizaki T., M. Naito, K. Fujisawa, N. Watanabe, Y. Saito, S. Narumiya, p160ROCK, a Rho-associated coiled-coil forming protein kinase, works downstream of Rho and induces focal adhesions, FEBS Lett. 404 (1997) 118-124.

Kamm K.E., J.T. Stull, Dedicated myosin light chain kinases with diverse cellular functions, J. Biol. Chem. 276 (2001) 4527-4530.

Keller H.U., A. Dehghani Zadeh, P. Eggli, Localized depletion of polymerised actin at the front of Walker carcinosarcoma cells increases the speed of locomotion, Cell Motil. Cytoskel. 53 (2002) 189-202.

Keller H.U., Redundancy of lamellipodia in locomoting Walker carcinosarcoma cells, Cell Motil. Cytoskel., 46 (2000) 247-256.

Kimura K., M. Ito, M. Amano, K. Chihara, Y. Fukata, M. Nakafuku, B. Yamamori, J. Feng, T. Nakano, K. Okawa, A. Iwamatsu, K. Kaibuchi, Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase), Science 273 (1996) 245-248.

Leung T., X.Q. Chen, E. Manser, L. Lim, The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the reorganization of the cytoskeleton, Mol. Cell Biol. 16 (1996) 5313-5327.

Limouze J., A.F. Straight, T. Mitchison, J.R. Sellers, Specificity of blebbistatin, an inhibitor of myosin II, J. Muscle Res. Cell Motil. 25 (2004) 337-341.

Maciver S.K., Myosin II function in non-muscle cells, Bioessays 18 (1996) 179-182.

Matsui T., M. Amano, T. Yamamoto, K. Chihara, M. Nakafuku, M. Ito, T. Kanano, K. Okawa, A. Iwamatsu, K. Kaibuchi, Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP-binding protein Rho, EMBO J. 15 (1996) 2208-2216.

McGee K., M. Zettl, M. Way, M. Fallman, A role of N-WASP in invasin-promoted internalisation, FEBS Lett. 509 (2001) 59-65.

Michaelson D., Silletti J., Murphy G., D'Eustachio P., Rush M., Philips M.R., Differential localization of Rho GTPases in live cells: regulation by hypervariable regions and RhoGDI binding, J. Cell Biol. 152 (2001) 111-126.

Nakagawa O., K. Fujisawa, T. Ishizaki, Y. Saito, K. Nakao, S. Narumiya, ROCK-I and ROCK-II, two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice, FEBS Lett. 392 (1996) 189-193.

Niggli V., Rho-kinase in human neutrophils: a role in signalling for myosin light chain phosphorylation and cell migration, FEBS Lett. 445 (1999) 69-72.

Niggli V., Signaling to migration in neutrophils: importance of localized pathways, Int. J. Biochem. Cell Biol. 35 (2003) 1619-1638.

Partin A.W., J.S. Schoeniger, J.L. Mohler, D.S. Coffey, Fourier analysis of cell motility: correlation of motility with metastatic potential, Proc. Natl. Acad. Sci. (USA) 86 (1989) 1254-1258.

Ren X.D., R. Wang, Q. Li, L.A.F. Kahek, K. Kaibuchi, R.A.F. Clark, Disruption of Rho signal transduction upon cell detachment, J. Cell Sci. 117 (2004) 3511-3518.

Ridley A.J., H.F. Paterson, C.L. Johnston, D. Diekmann, A. Hall, The small GTP-binding protein rac regulates growth factor-induced membrane ruffling, Cell 70 (1992) 401-410.

Sanders L.C., F. Matsumara, G.M. Bokoch, P. de Lanerolle, Inhibition of myosin light chain kinase by p21-activated kinase, Science 283 (1999) 2083-2085.

Sebbagh M., C. Renvoize, J. Hamelin, N. Riche, J. Bertoglio, J. Breard, Caspase-3-mediated cleavage induces MLC phosphorylation and apoptotic membrane blebbing, Nat. Cell Biol. 3 (2001) 346-352.

Somlyo A.P. and A.V. Somlyo, Ca^{2+} sensitivity of smooth muscle and non-muscle myosin II: modulated by G proteins, kinases, and myosin phosphatase, Physiol. Rev. 83 (2003) 1325-1358.

Spiller D.G., D.M. Tidd, Nuclear delivery of antisense oligodeoxynucleotides through reversible permeabilization of human leukemia cells with streptolysin O, Antisense Res. Dev. 5 (1995) 13-21.

Straight A.F., A. Cheung, J. Limouze, I. Chen, N.J. Westwood, J.R. Sellers, T.J. Mitchison, Dissecting temporal and spatial control of cytokinesis with a myosin II inhibitor, Science 299 (2003)1743-1747.

Sugihara K., N. Nakatsuji, K. Nakamura, K. Nakao, R. Hashimoto, H. Otani, H. Sakagami, H. Kondo, S. Nozawa, A. Aiba, Rac1 is required for the formation of three germ layers during gastrulation, Oncogene 17 (1998) 3427-3433.

Symons M., J. Settleman, Rho family GTPases: more than simple switches, Trends Cell Biol. 10 (2000) 415-419.

Totsukawa G., Y. Wu, Y. Sasaki, D.J. Hartshorne, Y. Yamakita, S. Yamashiro, F. Matsumara, Distinct roles of MLCK and ROCK in the regulation of membrane protrusions and focal adhesion dynamics during cell migration of fibroblasts, J. Cell Biol. 164 (2004) 427-439.

Wells C.M., M. Walmsley, S. Ooi, V. Tybulewicz, A.J. Ridley, Rac1-deficient macrophages exhibit defects in cell spreading and membrane ruffling but not migration, J. Cell Sci. 117 (2004) 1259-1268.

Wheeler A.P. and A.J. Ridley, Why three Rho proteins? RhoA, RhoB, RhoC, and cell motility, Exp. Cell Res. 301 (2004) 43-49.

Wicki A. and V. Niggli, The Rho/Rho-kinase and the phosphatidylinositol 3-kinase pathway are essential for spontaneous locomotion of Walker 256 carcinosarcoma cells, Int. J. Cancer 91 (2001) 763-771.

Xu J., F. Wang, A. Van Keymeulen, P. Herzmark, A. Straight, K. Kelly, Y. Takuwa, N. Sugimoto, T. Mitchison, H.R. Bourne, Divergent signals and cytoskeletal assemblies regulate self-organizing polarity in neutrophils, Cell 114 (2003) 201-214.

Yamauchi A., C. Kim, S. Li, C.C. Marchal, J. Towe, S.J. Atkinson, M.C. Dinauer, Rac2-deficient murine macrophages have selective defects in superoxide production and phagocytosis of opsonized particles, J. Immunol. 173 (2004) 5971-5979.

3.7. Legends to Figures

Fig. 1. Effects of modulation of activity of Rho on morphology, F-actin localization, and MLC phosphorylation in Walker carcinosarcoma cells. (A) Cells were permeabilized with SLO in the absence (control) or presence of the indicated proteins (30 μg/ml), followed by fixation, visualization of F-actin with rhodamine-phalloidin and determination of the percentage of polarized cells. Images were taken with a confocal imaging system. a-e: DIC optics; a`-e`: visualization of rhodamine-phalloidin. Scale bar, 10 μm. (B) Quantitative evaluation of cell morphology for the experiment shown in (A). Mean ± SD of 3 independent experiments. (C) Cells were permeabilized with SLO in the absence (control) or presence of the indicated proteins, precipitated using TCA and subjected to immuno-blotting using a polyclonal rabbit anti-phospho MLC2 (Thr-18/Ser-19) antibody (1:500). PP-MLC indicates MLC phosphorylated on Thr-18 and Ser-19. Mean ± SD of 3 independent experiments.

Fig. 2. Effects of transfection of Walker carcinosarcoma cells with Rho and Rho-kinase mutants on morphology, F-actin localization, and MLC phosphorylation of Walker carcinosarcoma cells. (A)

Cells were transfected with the indicated plasmids followed by immunostaining with the specific antibody against cmyc and visualization of F-actin with rhodamine-phalloidin. Images were taken with a confocal imaging system. a-e: DIC optics; a`-e`: cmyc immunofluorescence; a``-e``: visualization of rhodamine-phalloidin. Scale bar, 10 μm. (B), (C) Quantitative evaluation of cell morphology of the cells expressing cmyc for the experiment shown in (A). Mean ± SD of 3 independent experiments. (D), (E) Cells were cotransfected with the indicated plasmids and pEGFP. After 12 h the viable GFP-positive cells were sorted, precipitated using TCA and subjected to immuno-blotting using a polyclonal rabbit anti-phospho MLC2 (Thr-18/Ser-19) antibody (1:500). PP-MLC indicates MLC phosphorylated on Thr-18 and Ser-19. Mean ± SD of 3 independent experiments.

Fig. 3. Expression of ROCKI and RhoA mutants in Walker carcinosarcoma cells. Cells were transfected, lysed and subjected to immuno-blotting (0.75 x 10^6 cells per lane) with anti-ROCKI, anti-RhoA, or anti-cmyc antibodies. (A) Cells transfected with V14-RhoA, (B) cells transfected with ROCKI mutants.

Fig. 4. Effects of inhibition of ROCKI, MLCK or myosin II on morphology, F-actin localization, and MLC phosphorylation of Walker carcinosarcoma cells. (A) Cells were incubated with the indicated inhibitors (Y-27632: 10 μM; ML-7: 10 μM; blebbistatin: 50 μM) for 30 min at 37°C, followed by fixation, visualization of F-actin with rhodamine-phalloidin and determination of the percentage of polarized cells. Images were taken with a confocal imaging system. a-e: DIC optics; a`-e`: visualization of rhodamine-phalloidin. Scale bar, 10 μm. (B), (C) Quantitative evaluation of cell morphology for the experiments shown in (A). Mean ± SD of 3 independent experiments. (D), (E) After incubation with the inhibitors as described under (A) cells were precipitated using TCA and subjected to immuno-blotting using a polyclonal rabbit anti-phospho MLC2 (Thr-18/Ser-19) antibody (1:500). PP-MLC indicates MLC phosphorylated on Thr-18 and Ser-19. Mean ± SD of 3 independent experiments.

Fig. 5. Inhibition of ROCKI, MLCK or myosin II prevents blebbing induced by transfection of Walker carcinosarcoma cells with Δ3-ROCKI. Cells were co-transfected with pCAG-myc-Δ3-ROCKI and with pEGFP as described in the methods section. 1 hour after transfection cells were

incubated for 4h in medium containing the indicated inhibitors and then fixed with 1% glutaraldehyde. Morphology of GFP-expressing cells was analyzed using DIC optics. Mean ± SD of 3 independent experiments.

3.8. Tables

Table 1

Effect of modulating activity of RhoA and Rac1 on locomotion of Walker carcinosarcoma cells

Proteins [a]	migrating cells (%)	mean speed of migrating cells (μm/min)
-	29 ± 3	5 ± 2
WT-RhoA	25 ± 5	5 ± 1
L63-RhoA	6 ± 1	5 ± 0.5
C3 exoenzyme	3 ± 1	5 ± 1
N17-Rac1	28 ± 4	4 ± 1

[a] Bacterially expressed proteins (30 μg/ml) were introduced by SLO treatment of cells. Mean ± SD of 3 independent experiments.

Table 2

Effect of the MLCK inhibitor ML-7 and the myosin II inhibitor blebbistatin on locomotion of Walker carcinosarcoma cells as determined by videomicroscopy

Additions [a]	migrating cells (%)	mean speed of migrating cells (μm/min)
Medium	29 ± 9	5 ± 2
ML-7 (10 μM)	5 ± 2	4 ± 1
Blebbistatin (50 μM)	0	-
Blebbistatin (100 μM)	0	-

[a] time of incubation with inhibitors: 30 min. Mean ± SD of 3 independent experiments.

3.9. Figures

Fig. 1

A

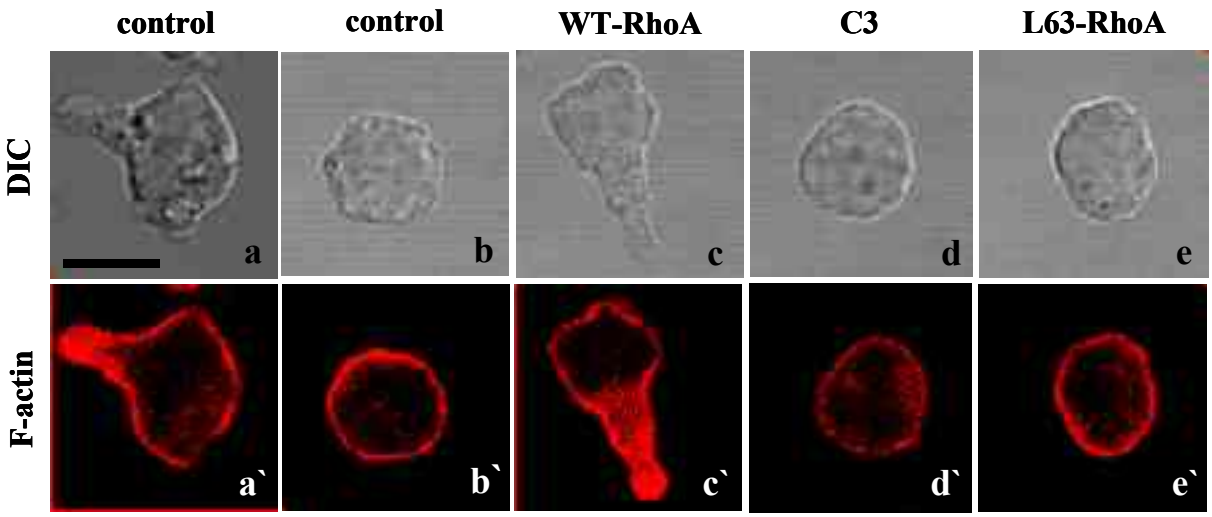
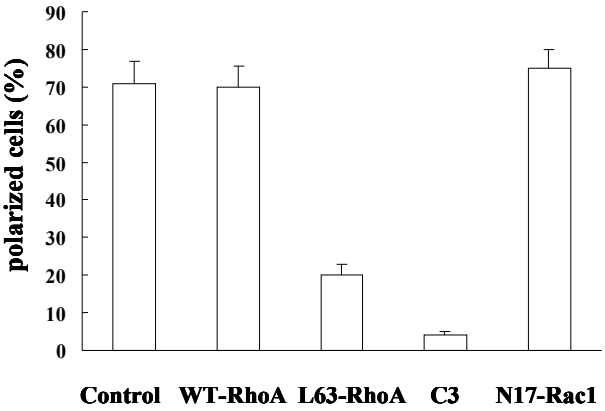


Fig. 1

B


polarized cells (%)
90
80
70
60
50
40
30
20
10
0
Control
WT-RhoA
L63-RhoA
C3
N17-Rac1

C

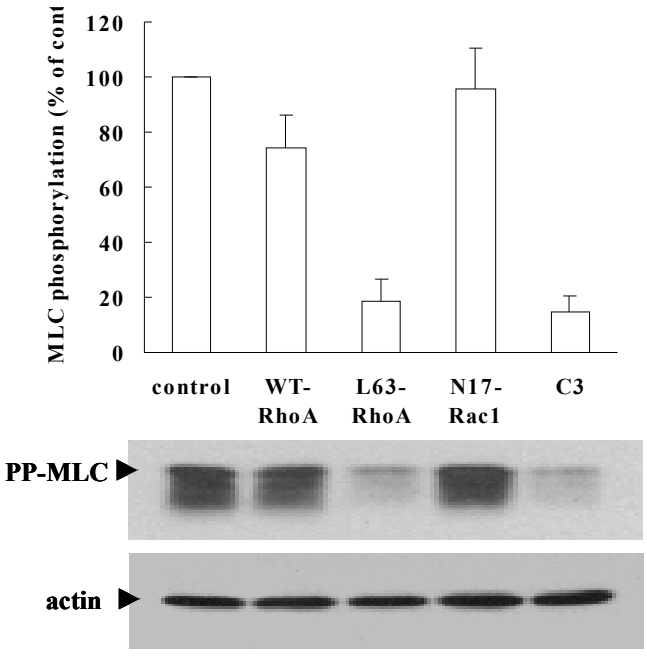

MLC phosphorylation (% of cont
120
100
80
60
40
20
0
control
WT-RhoA
L63-RhoA
N17-Rac1
C3
PP-MLC
actin

Fig. 2

A

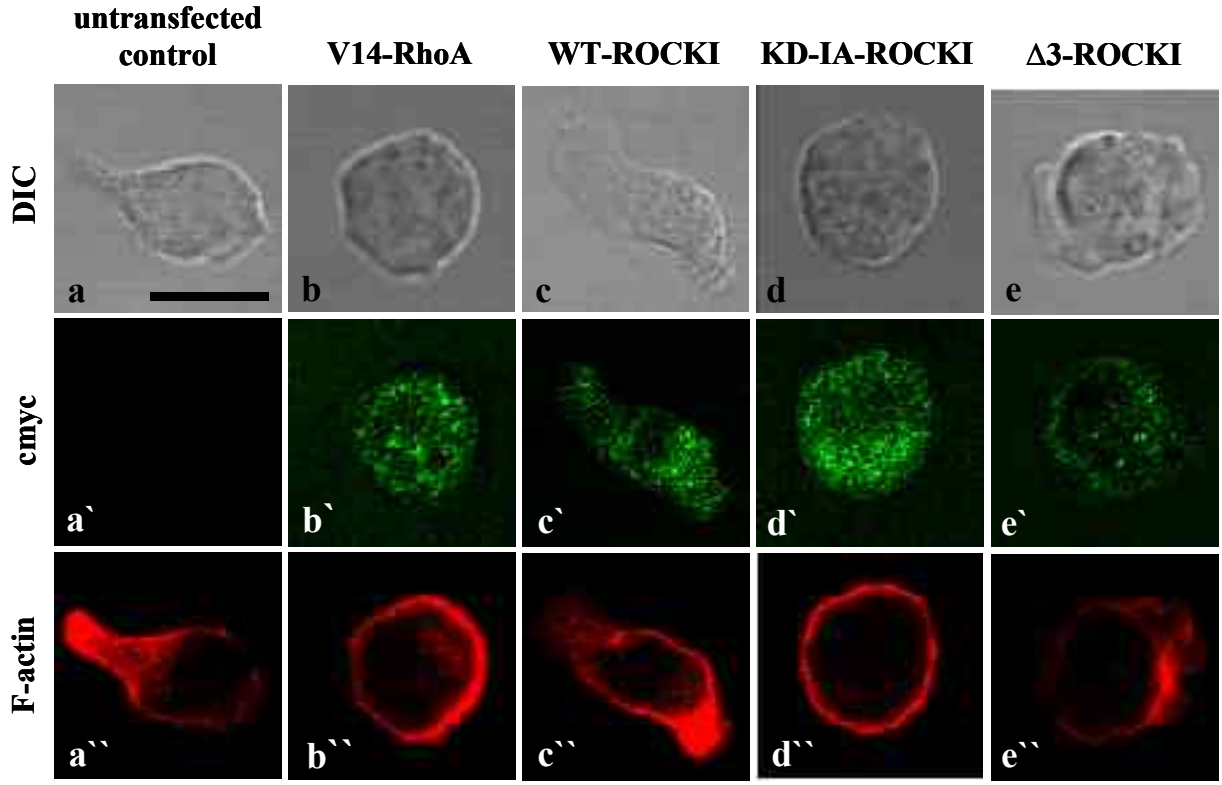

untransfected control
V14-RhoA
WT-ROCKI
KD-IA-ROCKI
Δ3-ROCKI
DIC
cmyc
F-actin
a
b
c
d
e
a`
b`
c`
d`
e`
a``
b``
c``
d``
e``

Fig. 2

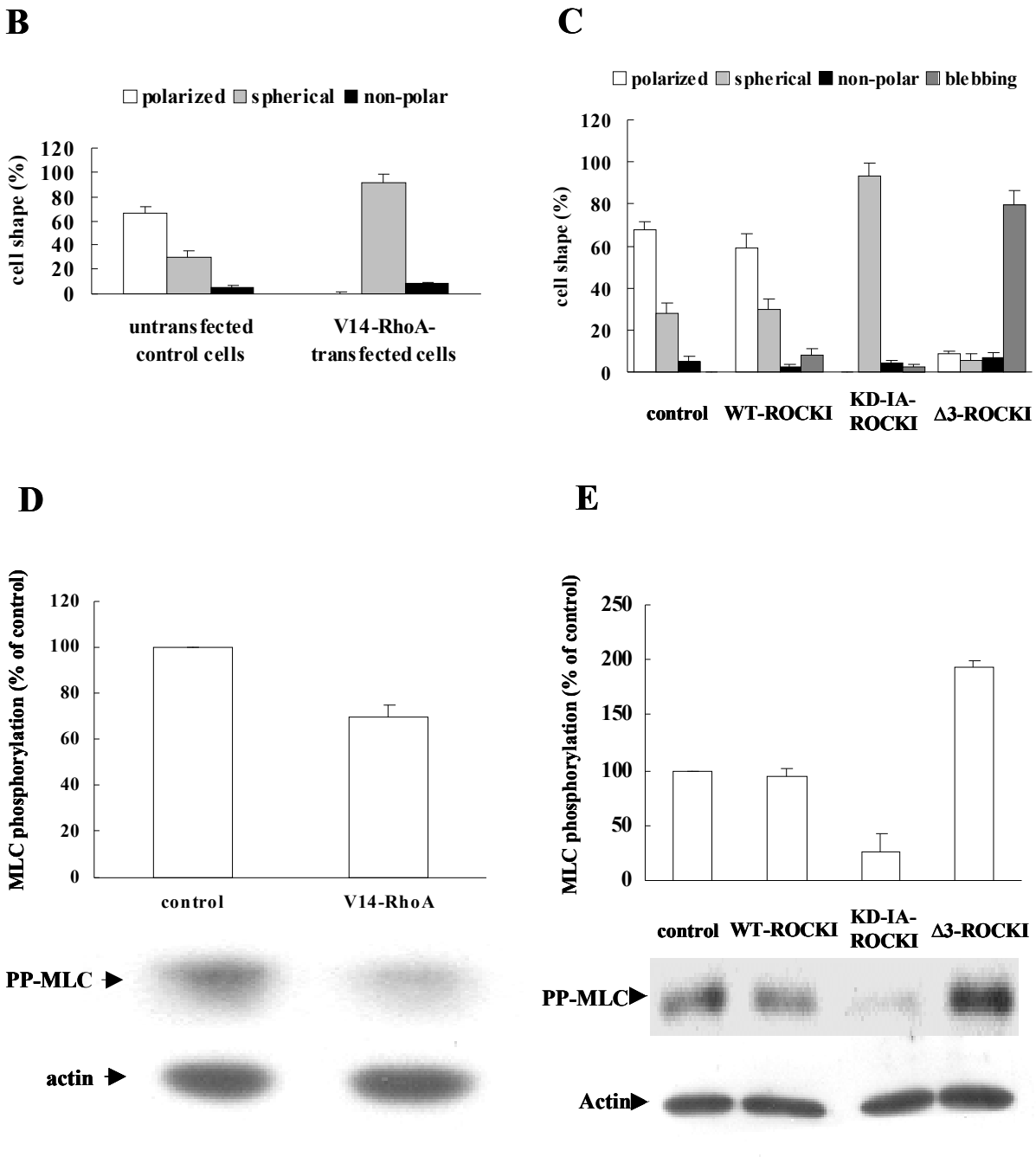

B
polarized
spherical
non-polar
cell shape (%)
120
100
80
60
40
20
0
untransfected control cells
V14-RhoA-transfected cells
C
polarized
spherical
non-polar
blebbing
cell shape (%)
120
100
80
60
40
20
0
control
WT-ROCKI
KD-IA-ROCKI
Δ3-ROCKI
D
MLC phosphorylation (% of control)
120
100
80
60
40
20
0
control
V14-RhoA
PP-MLC
actin
E
MLC phosphorylation (% of control)
250
200
150
100
50
0
control
WT-ROCKI
KD-IA-ROCKI
Δ3-ROCKI
PP-MLC
Actin

Fig. 3

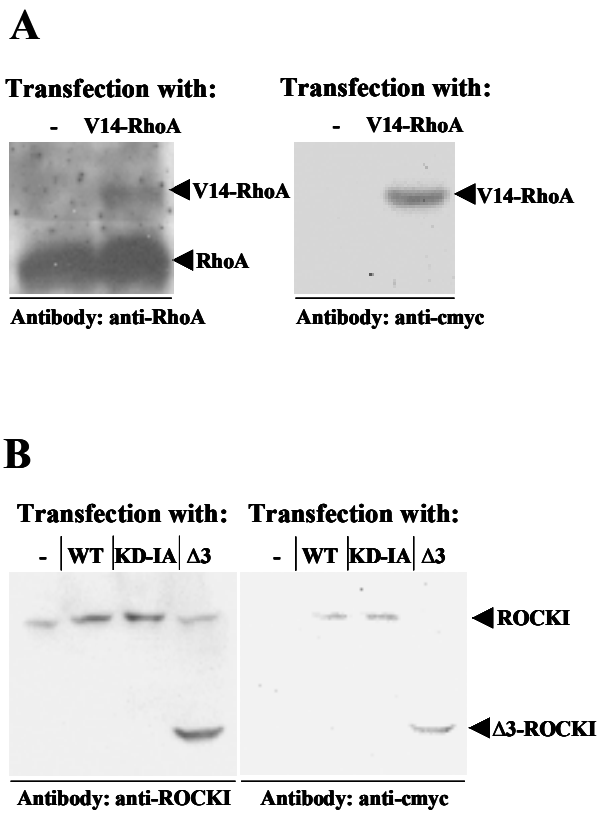

A
Transfection with:
- V14-RhoA
V14-RhoA
RhoA
Antibody: anti-RhoA
Transfection with:
- V14-RhoA
V14-RhoA
Antibody: anti-cmyc
B
Transfection with:
- WT KD-IA Δ3
Transfection with:
- WT KD-IA Δ3
ROCKI
Δ3-ROCKI
Antibody: anti-ROCKI
Antibody: anti-cmyc

Fig. 4

A

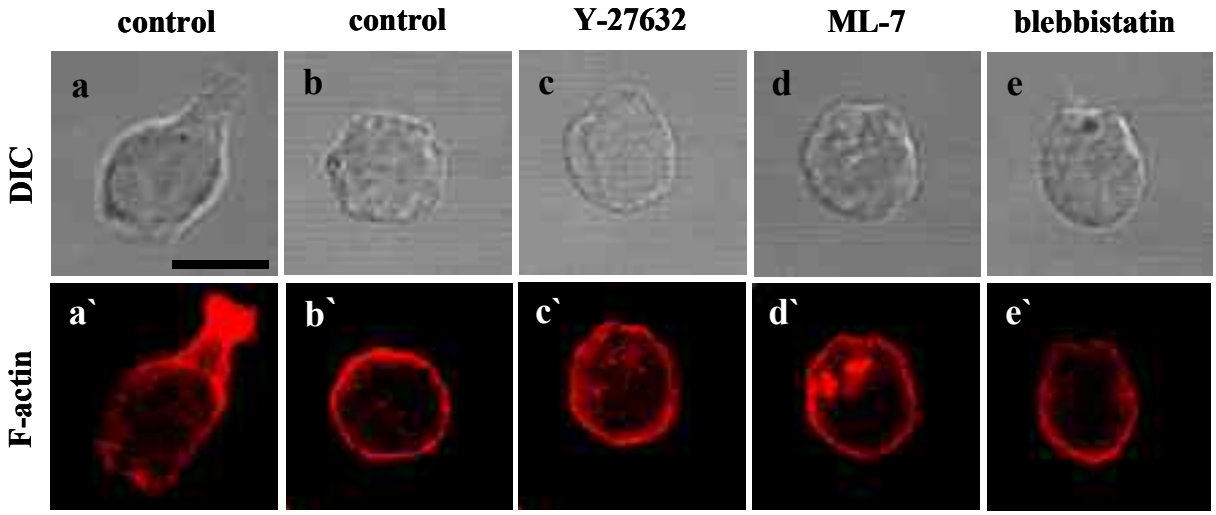

control
control
Y-27632
ML-7
blebbistatin
DIC
F-actin
a
b
c
d
e
a`
b`
c`
d`
e`

Fig. 4

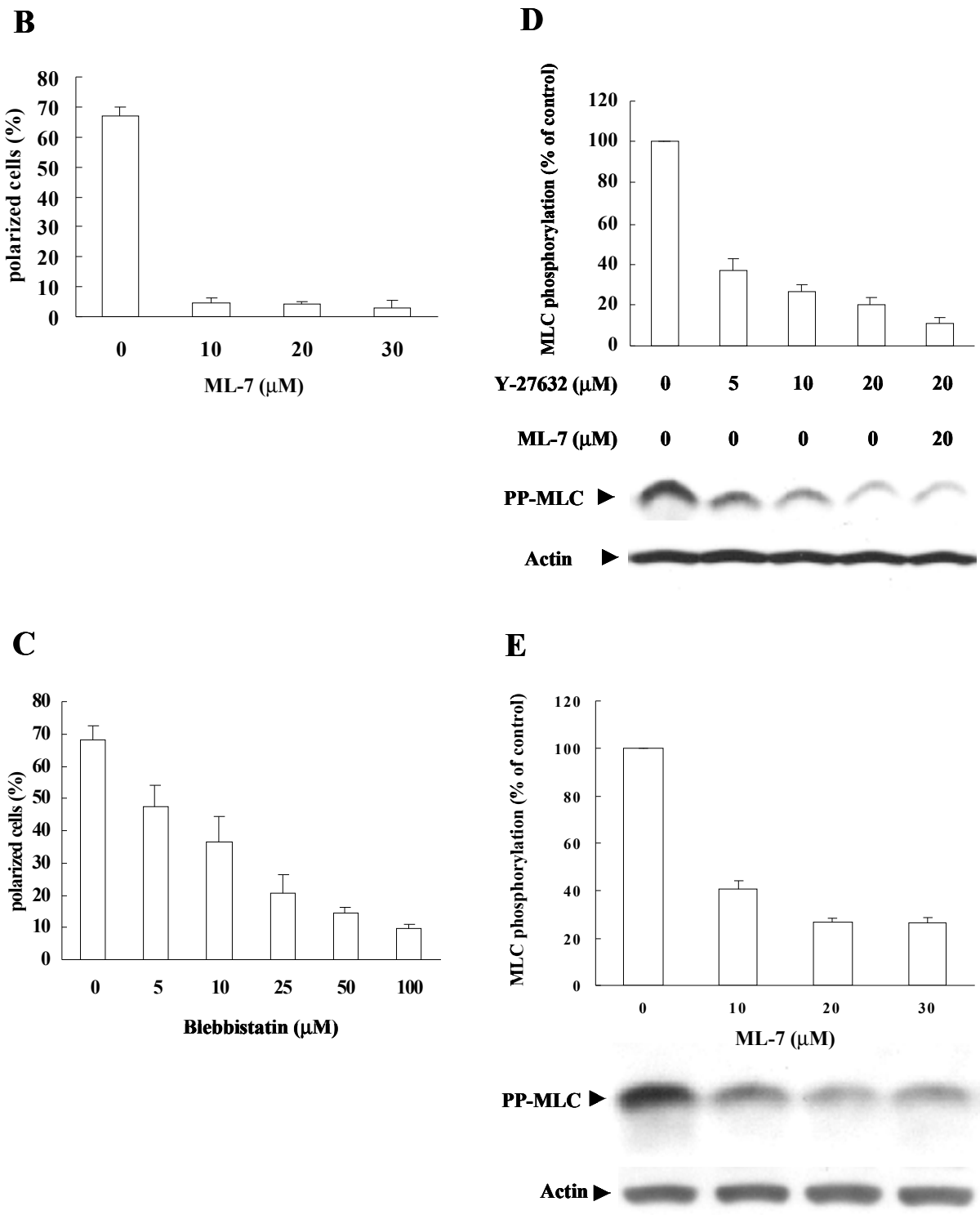
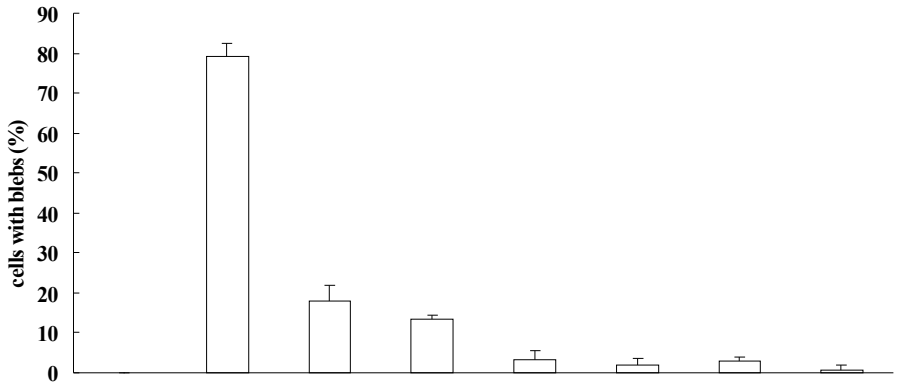

B
polarized cells (%)
80
70
60
50
40
30
20
10
0
0
10
20
30
ML-7 (μM)
D
MLC phosphorylation (% of control)
120
100
80
60
40
20
0
Y-27632 (μM) 0 5 10 20 20
ML-7 (μM) 0 0 0 0 20
PP-MLC ▶
Actin ▶
C
polarized cells (%)
80
70
60
50
40
30
20
10
0
0
5
10
25
50
100
Blebbistatin (μM)
E
MLC phosphorylation (% of control)
120
100
80
60
40
20
0
0
10
20
30
ML-7 (μM)
PP-MLC ▶
Actin ▶

Fig. 5



Δ3-ROCKI	-	+	+	+	+	+	+	+
Y-27632 (μM)	0	0	30	40	0	0	0	0
ML-7 (μM)	0	0	0	0	30	40	0	0
Blebbistatin (μM)	0	0	0	0	0	0	50	100

4. MANUSCRIPT II

Phosphorylation of ERM (Ezrin/Radixin/Moesin) proteins in Walker 256 carcinosarcoma cells: roles of Rho, Rho-kinase and protein kinase C

Marc C. Gutjahr and Verena Niggli

4.1. Abstract

ERM proteins are plasma membrane-actin filament cross-linkers that play an important role in regulating the stability of the cortical cytoskeleton which provides structural support for the plasma membrane. They are activated by phosphorylation.

We show that after permeabilization of Walker carcinosarcoma cells in the presence of the Rho inhibitor C3 exoenzyme, constitutive ERM phosphorylation is significantly inhibited as compared to cells permeabilized in absence of C3, correlating with inhibition of cell polarity and migration. Unexpectedly, permeabilization of cells in the presence of a bacterially expressed constitutively active RhoA mutant (L63-RhoA) showed the same effect as Rho inhibition. In contrast to the findings with C3 exoenzyme, treatment of cells with the Rho-kinase inhibitor Y-27632 had no effect on ERM phosphorylation, whereas it suppressed cell polarity and migration.

Transfection with a dominant-negative Rho-kinase mutant similarly inhibited cell polarization and migration but also had no effect on ERM phosphorylation correlating with data obtained with the Rho-kinase inhibitor Y-27632. Transfection with a dominant-active Rho-kinase mutant caused membrane blebbing and unexpectedly decreased ERM phosphorylation.

Treatment of cells with the PKC activator PMA (phorbol-12-myristate-13-acetate) completely inhibited ERM phosphorylation.

These data suggest that regulation of ERM phosphorylation in Walker carcinosarcoma cells is under the control of Rho and may be indirectly modulated by Rho-kinase. An appropriate level of RhoA activity is required to maintain constitutive ERM phosphorylation. A member of the PKC family might be involved in the suppression of ERM phosphorylation.

4.2. Introduction

Important and necessary characteristics of motile cells are low adhesiveness, the capacity for rapid cytoskeletal reorganization, and definite asymmetry of cellular components along the front-rear axis that is required for effective directional movement. At the leading front forward-pushing of the cell body in the direction of movement takes place. At the rear part, migrating cells have a tail-like cellular structure, called the uropod. This part of the migrating cell is also called the trailing edge. It is responsible for detachment from the substratum and retraction of the cell body enabling the cell to move forward (Sanchez-Madrid and del Pozo, 1999).

It has been shown that proteins of the ERM (Ezrin,Radixin,Moesin) family are predominantly located in the uropod of polarized cells (Lee et al., 2004).

ERM proteins have emerged as key regulators linking F-actin to specific membrane proteins (Martin et al., 2003). They are involved in the formation of microvilli, cell adhesion sites, membrane ruffling, and cleavage furrows (Jeon et al., 2002).

The cortical cytoskeleton provides structural support for the plasma membrane. In this context ERM proteins play both a structural and regulatory role in the assembly and stabilization of plasma membrane domains (Bretscher et al., 1997). Activation of ERM proteins requires phosphorylation on threonine 567 (ezrin) /564 (radixin) /558 (moesin).

Recently ERM proteins have been implicated as down-stream effectors of the Rho/Rho-kinase pathway. Two models for regulation of activity of ERM proteins have been proposed, both implicating a role of Rho, but only one also including ROCK. The first model predicts that ERM phosphorylation requires Rho and PIP4,5K (phosphatidylinositol 4-phosphate 5-kinase) but not ROCK (Matsui et al., 1999; Lee et al., 2004). The second model shows a role for Rho in regulating phosphorylation of ERM proteins requiring as downstream effectors both Rho-kinase and PIP4,5K (Jeon et al., 2002; Kim et al., 2004;).

As we are interested in down-stream targets of the Rho/ROCK pathway, we investigated how phosphorylation of ERM proteins is regulated in the spontaneously migrating Walker carcinosarcoma cells.

We tested the effect of inhibition of Rho by C3 exoenzyme on ERM phosphorylation. In fact, permeabilization of cells in the presence of C3 exoenzyme clearly reduced constitutive ERM phosphorylation as compared to permeabilized control cells. Unexpectedly, permeabilization of cells in the presence of the constitutively active RhoA mutant L63-RhoA also inhibited ERM phosphorylation as compared to cells permeabilized in the presence of wild-type RhoA.

To investigate a possible role of Rho-kinase in directly phosphorylating ERM, we inhibited Rho-kinase with the Rho-kinase inhibitor Y-27632. Incubation of Walker carcinosarcoma cells with Y-27632 did not affect ERM phosphorylation. Similarly, transfection of Walker carcinosarcoma cells with the dominant-negative Rho-kinase mutant KD-IA-ROCKI also had no effect on ERM phosphorylation compared to untransfected cells or cells transfected with wild-type-ROCKI.

In conclusion we could show that Rho is involved in regulating ERM phosphorylation, whereas we have no evidence for a direct role of Rho-kinase in controlling ERM phosphorylation in Walker carcinosarcoma cells.

4.3. Materials and Methods

Materials

Reagents and suppliers: Y-27632 (Calbiochem, La Jolla, CA, USA), was prepared as a 10 mM stock solution in H_2O. PMA (phorbol-12-myristate-13-acetate) (Sigma, St.Louis, MO, USA), was prepared as a 1 mM stock solution in DMSO. Enhanced chemiluminescence Western blotting detection reagents were obtained from Pierce, Rockford, IL, USA; rhodamine-phalloidin was from Molecular Probes, Eugene, OR, USA.

BSA was obtained from Serva, Heidelberg, Germany. Lysolecithin, PFA (Paraformaldehyde), DMSO, and SLO (Streptolysin O) were from Sigma, St. Louis, MO, USA.

C3 exoenzyme and bacterially expressed RhoA and Rac1 proteins were obtained from Cytoskeleton, Denver, CO, USA. PIP_2 (phosphatidylinositol 4,5-trisphosphate; PtdIns(4,5)P_2) was from Lipid products, South Nutfield, Surrey, England. PIP_3 (phosphatidylinositol 3,4,5-trisphosphate; PtdIns(3,4,5)P_3) (Cat. Nr. P-3916) was from Echelon, Salt Lake City, UT, USA.

Gey's solution contained 138 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.1 mM $CaCl_2$, 100 µM EGTA, 1 mM Na_2HPO_4, 5 mM $NaHCO_3$, 5.5 mM glucose and 20 mM HEPES (pH 7.4).

Complete protease inhibitor (Cat. Nr. 1 873 580) was from Roche, Penzberg, Germany.

Plasmids

Plasmids were from the following sources: pCAG-myc-WT-ROCKI, pCAG-myc-KD-IA-ROCKI, and pCAG-myc-Δ3-ROCKI were kindly provided by S. Narumiya (Department of Pharmacology, Kyoto University, Faculty of Medicine, Kyoto, Japan). pEXV3-myc-V14-RhoA was a kind gift of A.J. Ridley (Ludwig Institute for Cancer Research, London, UK). pEGFP was obtained from Clontech BD (Palo Alto, CA, USA).

Antibodies

Antibodies were obtained from the following sources: A polyclonal anti-phospho Ezrin (Thr567)/ Radixin (T564)/ Moesin (T558) antibody (Cat. Nr. 3141S) was from Cell Signaling, Beverly, MA, USA. An Mab against Actin (Cat. Nr. 010056) was from Bio-Science Products. Secondary antibodies (goat-anti-mouse horseradish peroxidase conjugated (Cat. Nr. 170-6516), and goat-anti-rabbit horseradish peroxidase conjugated (Cat. Nr. 170-6515) were obtained from BioRad, Hercules, CA, USA. A polyclonal antibody against ezrin was obtained from P. Mangeat (Département Biologie-Santé, Université Montpellier II, France) (Andreoli et al., 1994).

Tumor cell culture

Walker 256 carcinosarcoma cells were kindly provided by Dr. B. Sordat (ISREC, Lausanne, Switzerland) and cultured in vitro in RPMI 1640 supplemented with 10% FCS (fetal calf serum), penicillin (25 µg/ml) and streptomycin (25 µg/ml). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

TCA precipitation, electrophoresis and immunoblotting

Cells were precipitated by adding 1 vol. of an ice-cold solution containing 200 mg/ml TCA, 40 mM NaF and 10 mM Na_2HPO_4. After incubation on ice for 20 min, precipitates were collected by centrifugation (10 min) in an Eppendorf centrifuge, 10,000 g at 4°C. Pellets were washed once with 1 ml of 5% TCA and once with 1 ml of 0.5% TCA and then solubilized in 100 µl of sample buffer containing 1% SDS, 50 mM dithiothreitol, 15% glycerol, 62.5 mM Tris/HCl (pH 6.8) and 0.001% bromphenol blue by incubation at 95°C for 10 min. Fractions were electrophoresed through a SDS-polyacrylamide gel followed by transblotting to nitrocellulose using a Genie blotter (Idea Scientific, Minneapolis, MN). The blots were incubated for 1h in a blocking buffer containing 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.05 % Tween-20 and 5 % defatted milk powder (TBSTM), followed by overnight incubation at 4°C with the indicated antibodies, diluted in PBS containing 3 % BSA and 0.02 % NaN_3. After 3 washes with TBST, the blots were incubated for 1 hr either with a second horseradish peroxidase-labeled goat anti-rabbit IgG antibody (Bio Rad Laboratories, Hercules, CA) for polyclonal antibodies, diluted 1:10000 in TBSTM, or with a horseradish peroxidase-labeled goat anti-mouse IgG antibody for monoclonal antibodies, diluted 1:3000 in TBSTM. Detection was performed with an ECL Western detection system (Pierce, Rockford, IL). Bands were quantified by densitometry of the films using a GS-800 Calibrated Densitometer from Bio-Rad.

ERM-Phosphorylation

Cells were washed twice in Gey's medium, resuspended in this medium (1.0 x 10^6 cells/0.5 ml) and then incubated at 37°C for 30 min with the indicated concentrations of Y-27632. The reaction was stopped with TCA (see above) and the precipitated proteins were subjected to immunoblotting using an anti-phospho Ezrin (Thr567)/ Radixin (T564)/ Moesin (T558) antibody (1:10000).

Streptolysin O permeabilization of Walker Carcinosarcoma cells

Cells were washed twice in RPMI 1640 medium without FCS. Then cells were resuspended to a final volume of 100 µl (10 x 10^6 cells/ml) in this medium. The protein to be introduced was placed in the wells of a 24-well plate after addition of the cells and 5-20 U of SLO. The optimal amount of SLO was determined empirically in advance for each batch (Spiller et al., 1995). The cells were incubated at 37°C in a CO_2 incubator for 10 minutes and were agitated three times during this period. Then 1 ml RPMI 1640 containing 10% FCS was added and the plate was placed back into the incubator for another hour. Cells were subsequently washed twice with Gey's buffer and incubated (1.0 x 10^6 cells/0.5ml) at 37°C for 30 min followed by TCA-precipitation for immunoblot analysis or fixed with 1% glutaraldehyde (final concentration) at 37°C for 30 min for analysis of cell shape as described above.

Nucleofection

Cells were washed twice with Gey's buffer. 2-3 x 10^6 cells were resuspended in 100 µl Nucleofector Solution (diluted 1:3 with PBS) supplied by Amaxa (Köln, Germany) and 5 µg of plasmid-DNA was added. For co-transfection experiments 0.5 µg of pEGFP were mixed with 5 µg of the appropriate plasmid. Then the cell suspension and the plasmid-DNA were transferred to a supplied cuvette and nucleofection was carried out (Amaxa Nucleofector, program Q23). Immediately 500 µl of RPMI 1640 medium with FCS was added and the cells were transferred to a prewarmed 12-well plate containing 1.5ml of RPMI 1640 medium with FCS, followed by incubation at 37°C in a CO2 incubator for 12 hours. Transiently transfected cells were subjected to cell sorting. Cells were harvested and centrifuged at 300g for 5min. The cell pellet was resuspended in culture medium containing serum and supplements to a final concentration of 10 x 10^6 cells/ml. Then the viable GFP-positive cells were separated from the viable non-fluorescent and the dead cells using a Vantage Diva Cell Sorter from BD. The sorted GFP expressing cells were incubated in Gey's medium at 37°C for 30 min followed by TCA precipitation and immunoblotting (see above).

Cell viability assay

Nonviable and/or apoptotic cells were determined by detection of phosphatidylserine externalisation using annexin-V-FITC staining. Maximally used concentration of Y-27632 was tested on inducing cell membrane permeabilization and/or apoptosis. Cells were incubated with inhibitors as described before, harvested and washed twice with annexin-V-buffer (10 mM HEPES ph 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$). Cell pellets (0.5 x 10^6 cells) were

resuspended in annexin-V-buffer containing annexin-V-FITC (0.5 µg/ml) for 20 min at 4°C under light protection. Cells were washed twice with annexin-V-buffer and then fixed with annexin-V-buffer containing 1% paraformaldehyde. Evaluation of annexin-V-positive cells was done with a FACScan measuring FL-1. Y-27632 did not significantly affected cell viability.

ADP-Ribosylation

Cells were washed twice with Gey's buffer. Then cells were resuspended to a final volume of 100 µl (20 x 10^6 cells/ml) in this buffer. C3 exoenzyme (15 µg/ml) and/or the appropriate phospholipid (380 µg/ml) were placed in the wells of a 24-well plate after addition of the cells and 5-20 U of SLO. The optimal amount of SLO was determined empirically in advance for each batch (Spiller et al., 1995). The cells were incubated at 37°C in a CO_2 incubator for 10 minutes and was agitated three times during this period. Then 1 ml RPMI 1640 containing 10% FCS was added and the plate was placed back into the incubator for another hour. Cells were washed 5 times with Gey's buffer without Ca^{2+} and then resuspended in 0.4 ml of an ice-cold buffer containing 10 mM triethanolamine-hydrochloride, pH 7.5, 2 mM $MgCl_2$, 4 % (v/v) complete protease inhibitor. After incubation at 4°C for 10 min, 0.5 µM [^{32}P]NAD (3 µCi) was added to the cells followed by 15 passages through a fine needle (gauge 30). After centrifugation at 700 x g for 10 min (4°C), aliquots were subjected to ADP-ribosylation in the absence or presence of C3 exoenzyme as described (Aktories, 1995).

Statistics

Differences between data were analysed with the Student's *t* test, with a P value < 0.05 considered significant. Data correspond to the mean ± SD.

4.4. Results

C3 transferase and constitutively activated RhoA both reduce ERM-phosphorylation

ERM proteins have been shown to be involved in establishing cell polarity. The phosphorylated activated form of ezrin, but not the latent form colocalized with CD44, a transmembrane adhesion molecule, at the uropod (Sanchez-Madrid and del Pozo, 1999). It was shown that transfection with

phosphorylation-mimetic ezrin induced the construction of a polar cap accompanied by CD44 accumulation in a mouse T lymphoma cell line, although the formation of the uropod was not induced. Thr567-D ezrin has thus the ability to organize plasma membrane polarity also when there is no existing uropod, whereas uropod protrusion requires additional mechanisms (Lee et al., 2004). Phosphorylation and activation of proteins of the ERM family can occur downstream of Rho/Rho-kinase (Yonemura et al., 2002). However, whereas the small GTPase Rho always has been shown to be implicated, depending on the cell type there was not always evidence for a role for Rho-kinase (Matsui et al., 1999).

Since it is well established that Rho is involved in ERM-Phosphorylation, we tested the effect of C3 transferase (Aktories and Hall, 1989) and bacterially expressed constitutively activated RhoA (L63-RhoA) on ERM-phosphorylation. Therefore, we introduced these proteins into SLO permeabilized cells. As shown in Fig. 1A, both C3 transferase, and unexpectedly constitutively activated RhoA, reduced the level of constitutive ERM-phosphorylation as compared to permeabilized control cells and cells permeabilized in the presence of wild-type RhoA.

Rho inhibition by C3 transferase resulted in 74 ± 4% ($p < 0.01$) inhibition of ERM phosphorylation and constitutively activated L63-RhoA decreased ERM phosphorylation to 47 ± 6% ($p < 0.025$) of control cells, whereas there was no significant inhibition of ERM phosphorylation with wild-type RhoA. We also investigated a possible role of another member of the family of small GTPases, Rac1, on ERM phosphorylation. There was no significant effect on ERM phosphorylation when cells were permeabilized in the presence of the dominant-negative N17-Rac1 (Fig. 1A). These results correlate with effects of C3 exoenzyme and constitutively activated RhoA on cell morphology and MLC phosphorylation (see manuscript I).

Effect of transfection of Walker cells with the dominant active RhoA mutant V14-RhoA

In order to confirm our results with the bacterially expressed constitutively active L63-RhoA mutant protein with a complementary approach, we transfected Walker carcinosarcoma cells with another dominant-active RhoA mutant, V14-RhoA, which has a glycine to valine substitution at amino acid position 14 (Ridley and Hall, 1992).

We cotransfected cells with dominant-active V14-RhoA and pEGFP, followed by cell sorting and immunoblotting. After 12 h of incubation there was only a slight non-significant decrease (17 ± 11%) in ERM phosphorylation compared to pEGFP-transfected control cells (Fig. 1B).

Effect of Y-27632 on the phosphorylation of Ezrin/Radixin/Moesin (ERM)

As observed previously, the pyridine-derived smooth muscle relaxant, Y-27632, which acts as a ROCK inhibitor suppresses spontaneous polarization and locomotion of Walker carcinosarcoma cells. The cells assumed a spherical morphology mostly lacking protrusions (Wicki and Niggli, 2001).

As both C3 exoenzyme and constitutively active RhoA inhibit ERM phosphorylation (Fig. 1A), Rho GTPases seem to play a crucial role in the regulation of ERM phosphorylation. We wanted to know whether signalling to ERM phosphorylation goes via the direct RhoA-effector Rho-kinase or via another downstream effector of Rho. Thus, we investigated whether the Rho-kinase inhibitor Y-27632 has an inhibitory effect on ERM-phosphorylation as well. Despite the fact that the uropod was completely disrupted and cells assumed a spherical morphology (see manuscript I), incubation of Walker carcinosarcoma cells with 10 μM or 20 μM resp. of Y-27632 did not affect ERM-phosphorylation (Fig. 2A). This indicates that ERM phosphorylation is independent of Rho-kinase, but that ROCK activity is required for uropod formation.

Effect of transfection of Walker cells with the dominant negative Rho-kinase KD-IA

To confirm the findings obtained with the Rho-kinase inhibitor Y-27632 we transfected cells with the dominant-negative Rho-kinase mutant KD-IA (Ishizaki et al., 1997). We cotransfected cells with KD-IA-ROCKI and pEGFP, followed by cell sorting and immunoblotting. In good agreement with the effects of the Rho-kinase inhibitor Y-27632, also the dominant-negative Rho-kinase mutant had no effect on ERM phosphorylation, but inhibited the formation of the uropod and thus inhibited cell polarity and migration (Fig. 2B).

Effect of transfection of Walker cells with the dominant active Rho-kinase Δ3-ROCKI

As shown previously, cells transfected with Δ3-ROCKI, a constitutive active Rho-kinase mutant (Ishizaki et al., 1997), assumed a blebbing morphology (see manuscript I).

We cotransfected Walker carcinosarcoma cells with the dominant-active Δ3-ROCKI and pEGFP followed by cell sorting and immunoblotting. As shown in Fig. 2B, unexpectedly, phosphorylation of ERM proteins was markedly reduced to 29 ± 5% ($p < 0.025$) of the phosphorylation level of cells only transfected with pEGFP or of cells cotransfected with wild type ROCKI and pEGFP.

Effect of PMA (phorbol-12-myristate-13-acetate) on ERM phosphorylation

PKC isoforms have been shown to play a role in tumor cell locomotion (Niggli et al., 1996). Short-term incubation of Walker carcinosarcoma cells with the PKC activator PMA acted as a stop-signal for spontaneous locomotion (Wicki and Niggli, 1999). We investigated whether activation of PKC by PMA affects the phosphorylation state of ERM proteins. Incubation of cells with 1 nM of PMA resulted in 62 ± 2% ($p < 0.025$) inhibition, whereas 5 nM of PMA resulted in an almost complete inhibition (93 ± 4%) ($p < 0.01$) of ERM phosphorylation as compared to untreated control cells, thus implicating a role for PKC in negatively regulating ERM phosphorylation (Fig. 3). These data correlate with the inhibitory effect of PMA on cell polarization (Wicki and Niggli, 1999). Treatment of Walker carcinosarcoma cells with 1 nM of PMA inhibited cell polarity by 25.5 ± 3% as compared to untreated control cells. 5 nM of PMA led to a complete inhibition of cell polarity and cells assumed a spherical shape (data not shown). In contrast to ERM phosphorylation, MLC phosphorylation was not significantly affected by incubation of cells with PMA (data not shown).

Does introduction of PIP_2 (but not of PIP_3 or other phospholipids) into Walker carcinosarcoma cells overcome the inhibitory effect of C3 exoenzyme on cell morphology and ERM phosphorylation?

Permeabilization of Walker carcinosarcoma cells in the presence of C3 exoenzyme abrogates cell polarity and ERM phosphorylation (Fig. 1A). There is strong evidence that the phospholipid PIP_2 produced by phosphatidylinositol 4-phosphate 5-kinase (PI4P5-kinase) is involved in and necessary for ERM activation and that PI4P5-kinase is under the control of Rho (Matsui et al., 1999). We asked whether it is possible to compensate the inhibitory effect of C3 by introducing PIP_2 into the cells. Cells were permeabilized in the presence of C3 and PIP_2, followed by analysis of cell morphology and immunoblotting. After this treatment, cells again assumed a polarized morphology (data not shown) and showed a restored ERM phosphorylation as did the permeabilized control cells (Fig. 4A), whereas cells that were permeabilized in the presence of C3 and PIP_3 still assumed a spherical morphology, and ERM phosphorylation was inhibited as in cells permeabilized in the presence of only C3 (Fig. 4B). Other lipids such as PS (phosphatidyldserine), PI (phosphatidylinositol), and PIP (phosphatidylinositol phosphate) were also not active (Fig. 4C). It is known that streptolysin O interacts with membrane lipids by extracting them out of the membrane in the process of pore formation (Bonev et al., 2001). Our results can be explained in 2 ways: (A) ERM phosphorylation and cell polarity are restored by the action of PIP_2; (B) PIP_2 only absorbs streptolysin, thus inhibiting membrane permeabilization and C3 exoenzyme introduction into the

cells. Therefore, we examined whether C3 exoenzyme enters the cells and effectively ADP-ribosylated Rho also in cells exposed to PIP_2. To test this, in vitro ADP-ribosylation using [^{32}P] NAD was carried out in lysates of untreated cells, cells treated with C3 exoenzyme alone, cells treated with C3 exoenzyme in combination with PIP_2, and cells treated with C3 exoenzyme in combination with PIP_3. Indeed, in lysates of cells permeabilized in the absence of C3 exoenzyme, a band of 25 kDa was [^{32}P]ADP-ribosylated dependent on the addition of C3 exoenzyme to the lysates. In lysates derived from cells permeabilized in the presence of C3 exoenzyme, [^{32}P]ADP-ribosylation was inhibited indicating that Rho was modified already before adding C3 exoenzyme to lysates. However, in lysates derived from cells permeabilized in the presence of C3 exoenzyme and PIP_2, Rho was [^{32}P]ADP-ribosylated comparable to results with untreated controls whereas in lysates of cells permeabilized in the presence of C3 exoenzyme and PIP_3, Rho was not [^{32}P]ADP-ribosylated (Fig. 5). These findings suggest that PIP_2 selectively interacts with streptolysin thereby inhibiting pore formation in the cell membrane, C3 exoenzyme entry into the cell and thus inactivation of Rho.

4.5. Discussion

Rho

We have shown here that ERM phosphorylation in Walker carcinosarcoma cells is dependent on Rho but not on ROCK. Inhibition of Rho by C3 exoenzyme abrogated ERM phosphorylation (Fig. 1A). Unexpectedly, also permeabilization of cells in the presence of the bacterially expressed constitutively active L63-RhoA mutant downregulated ERM phosphorylation (Fig. 1A) correlating with suppression of cell polarity and MLC phosphorylation (see manuscript I). To confirm these data we transfected Walker carcinosarcoma cells with another dominant-active RhoA mutant, the V14RhoA. V14-RhoA inhibited cell polarity (see manuscript I), but we only observed a slight not significant decrease (17%) in ERM phosphorylation as compared to control cells (Fig. 1B). A similar difference obtained with bacterially expressed L63-RhoA and transcriptionally expressed V14-RhoA was observed concerning MLC phosphorylation (see manuscript I). This might be due to a low expression level of V14-RhoA as compared to the high intracellular concentration of L63-RhoA in SLO permeabilized cells. An unknown target of RhoA may thus act negatively on phosphorylation of MLC and ERM.

In contrast, neither inhibition of Rho-kinase by Y-27632 (Fig. 2A) nor transfection of cells with the dominant-negative Rho-kinase mutant KD-IA-ROCKI (Fig 2B) had an effect on ERM phosphorylation whereas MLC phosphorylation was inhibited by these treatments (see manuscript I).

The results using C3 exoenzyme and Y-27632 could be confirmed by immunofluorescence experiments. In untreated polarized control cells, phosphorylated ERM proteins are mainly located in the uropod and to a lesser extent also along the peripheral membrane (Niggli and Blaser, unpublished observations). Cells treated with the Rho inhibitor C3 lost their polarized shape and assumed a spherical morphology (Wicki and Niggli, 2001). In this case ERM proteins were distributed diffusely in the cytoplasm and the anti-phospho ERM antibody failed to bind to the cells. Cells treated with the Rho-kinase inhibitor Y-27632 also assumed a spherical shape (Wicki and Niggli, 2001). Despite abolishment of the uropod ERM phosphorylation remained unchanged and phosphorylated ERM was predominantly located along the membrane over one half of the spherical cells, forming a so-called polar cap that covers the posterior cytoplasm, whereas the anterior cytoplasm was free of phosphorylated ERM (Niggli and Blaser, unpublished observations) correlating with observations in mouse T lymphoma cells (Lee et al., 2004).

These findings suggest that phosphorylated ERM proteins have the ability to construct a polar cap in spherical cells preceding uropod formation. ROCK activity is not required for ERM phosphorylation, but is indispensable for cell polarity and thus for the formation of the uropod.

Rho-kinase

Both inhibition of ROCK by Y-27632 and expression of a dominant-negative mutant of ROCKI (KD-IA-ROCKI) did not affect ERM phosphorylation (Fig. 2A,B) strongly suggesting that ERM phosphorylation is not regulated by Rho-kinase in Walker carcinosarcoma cells. Two models for the regulation of ERM activation have been proposed. The first model predicts a Rho-dependent regulation of ERM proteins but without an involvement of ROCK. Rho is supposed to activate PIP4,5K which in turn produces PIP_2. PIP_2 is suggested to be involved in activating dormant ERM proteins by opening the closed form of head-to-tail associated ERM dimers. Activated ERM proteins then are phosphorylated at the conserved carboxy-terminal threonine residue by an as yet unidentified kinase (Matsui, 1999; Fievet et al., 2004). The second model suggests that Rho transmits the signal for ERM activation via Rho-kinase to PIP4,5K that produces PIP_2. Rho-kinase is also thought to phosphorylate ERM directly (Matsui et al., 1998; Oshiro et al., 1998).

Considering our data, we suggest the first model to be true for Walker carcinosarcoma cells. ERM phosphorylation is independent of Rho-kinase and the kinase that phosphorylates ERM proteins at their conserved carboxy-terminal threonine residues still has to be identified.

Δ3-ROCKI-induced membrane blebbing

Unexpectedly, transfection of Walker carcinosarcoma cells with Δ3-ROCKI significantly inhibited ERM phosphorylation by 61%. Earlier we demonstrated a blebbing phenotype for Walker carcinosarcoma cells when transfected with the dominant-negative mutant of Rho-kinase, Δ3-ROCKI, correlating with increased phosphorylation of MLC (see manuscript I). During membrane blebbing, the plasma membrane dissociates from the cytoskeleton. It has been shown that there is a close correlation between ezrin phosphorylation/dephosphorylation and association/dissociation of ezrin with the cytoskeleton (Kondo et al., 1997). We suggest an indirect role for Δ3-ROCKI in reducing ERM phosphorylation. Expression of Δ3-ROCKI induces myosin activation and strong membrane blebbing in Walker carcinosarcoma cells. Continuous myosin activation consumes large amounts of cellular ATP resulting in ATP depletion. ERM proteins may thus become dephosphorylated and are no longer able to link the membrane with the cytoskeleton. It has been shown that energy depletion in rabbit renal proximal tubular cells weakened membrane-cytoskeleton interactions, which in turn facilitated membrane blebbing (Chen et al., 2000). Whether ATP-depletion as a consequence of Δ3-ROCKI expression and extensive myosin activation is indeed the cause of ERM dephosphorylation in Walker carcinosarcoma cells still has to be proved. A possible approach would be to inhibit myosin II by blebbistatin in cells that are transfected with Δ3-ROCKI and then to check the level of ERM phosphorylation. If ERM phosphorylation can be restored, then this would confirm the above hypothesis.

The role of PIP_2 in regulating ERM phosphorylation

In order to obtain further information on the signalling pathways involved in modulating ERM phosphorylation in Walker carcinosarcoma cells, we permeabilized Walker carcinosarcoma cells in the presence of the Rho inhibitor C3 exoenzyme (which is known to suppress ERM phosphorylation completely) and different phospholipids (Fig. 4). Our hypothesis was that Rho induces synthesis of PIP_2 which activates ERM and allows phosphorylation. C3 exoenzyme thus would inhibit PIP_2 formation. By adding back PIP_2 we wanted to overcome inhibition if PIP_2 production by C3 exoenzyme.

As shown in Fig. 5, Rho was [^{32}P]ADP-ribosylated in lysates derived from cells permeabilized in the presence of C3 exoenzyme and PIP_2 indicating that Rho was not inhibited before adding C3 exoenzyme to lysates. In contrast, in lysates of cells permeabilized in the presence of C3 exoenzyme and PIP_3, Rho was not [^{32}P]ADP-ribosylated showing that Rho was already ADP-ribosylated before adding C3 to the lysate. We suggest thus that PIP_2 selectively interacts with streptolysin thereby inhibiting pore formation in the cell membrane. C3 exoenzyme cannot penetrate the cell membrane and thus Rho is not inhibited. These experiments are thus not feasible using streptolysin O. We have to find another method to introduce C3 and lipids into the cells.

PKC

We provide evidence for PKC to be involved in the regulation of ERM phosphorylation. Treatment of Walker carcinosarcoma cells with the PKC activator PMA (phorbol-12-myristate-13-acetate) completely inhibited ERM phosphorylation (Fig. 3). It was previously shown that PKC-θ phosphorylates moesin in the actin-binding domain in vitro (Pietromonaco et al., 1998) and could thus be the kinase responsible for ERM phosphorylation. We showed previously that PKC-θ is not expressed in Walker carcinosarcoma cells (Wicki and Niggli, 1999). Thus in these cells ERM phosphorylation cannot be controlled by this PKC isoform. Another isoform of the PKC family might be involved in the suppression of ERM phosphorylation, but the exact mechanism still has to be elucidated.

In conclusion, our data suggest that phosphorylation and thus activation of ERM proteins requires activity of Rho but not Rho-kinase in Walker carcinosarcoma cells. An appropriate level of Rho activity is required for maintaining cell polarity and constitutive ERM phosphorylation as inhibition and constitutive activation of Rho both decrease the level of ERM phosphorylation. It remains to be elucidated whether ATP-depletion caused by Δ3-ROCKI-induced membrane blebbing is responsible for decreasing the level of phosphorylated ERM. A possible approach would be to inhibit myosin II directly with blebbistatin (Limouze et al., 2004) in Δ3-ROCKI-transfected cells and then to examine ERM phosphorylation.

4.6. References

Aktories K., and Hall A. (1989) Botulinum ADP-ribosyltransferase C3: a new tool to study low molecular weight GTP-binding proteins. Trends Pharmacol. Sci. 10:415-418.

Andreoli C., Martin M, Le Borgne R, Reggio H, Mangeat P. (1994) Ezrin has properties to self-associate at the plasma membrane. J Cell Sci. 107:2509-2521.

Bonev B.B., Gilbert R.J.C., Andrew P.W., Byron O., Watts A. (2001) Structural analysis of the protein/lipid complexes associated with pore formation by the bacterial toxin pneumolysin. J Biol Chem. 276:5714-5719.

Bretscher A., Reczek D., and Berryman M. (1997) Ezrin: a protein requiring conformational activation to link microfilaments to the plasma membrane in the assembly of cell surface structures. J. Cell Sci. 110:3011-3018.

Chen J., Wagner M.C. (2000) Altered membrane-cytoskeleton linkage and membrane blebbing in energy-depleted renal proximal tubular cells. Am J Physiol Renal Physiol. 280:619-627.

Ishizaki T., M. Naito, K. Fujisawa, N. Watanabe, Y. Saito, and S. Narumiya (1997) p160ROCK, a Rho-associated coiled-coil forming protein kinase, works downstream of Rho and induces focal adhesions. FEBS Lett. 404:118-124.

Jeon S., Kim S., Park J.B., Suh P.G., Kim Y.S., Bae C.D., and Park J. (2002) RhoA and Rho-kinase-dependent phosphorylation of moesin at Thr-558 in hippocampal neuronal cells by glutamate. J. Biol. Chem. 277:16576-16584.

Kim S.U., Jeon S., Shin E.Y., Kim E.G., Park J., and Bae C.D. (2004) AMPA, not NMDA, activates RhoA GTPases and subsequently phosphorylates moesin. Exp Mol Med. 36:98-102.

Kondo T., Takeuchi K., Doi Y., Yonemura S., Nagata S., Tsukita S., Tsukita S. (1997) ERM (Ezrin/Radixin/Moesin)-based molecular mechanism of microvillar breakdown at an early stage of apoptosis. J Cell Biol. 139:749-758.

Lee J.H., Katakai T., Hara T., Gonda H., Sugai M., and Shimizu A. (2004) Roles of p-ERM and Rho-ROCK signaling in lymphocyte polarity and uropod formation. J Cell Biol. 167:327-337.

Martin T.A., Harrison G., Mansel R.E., Jiang W.G. (2003) The role of the CD44/ezrin complex in cancer metastasis. Crit Rev Oncol Hematol. 46:165-186.

Matsui T., Maeda M., Doi Y., Yonemura S., Amano M., Kaibuchi K., and Tsukita S. (1998) Rho-kinase phosphorylates COOH-terminal threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to-tail association. J. Cell Biol. 140:647-657.

Matsui T., Yonemura S., Tsukita S., and Tsukito S. (1999) Activation of ERM proteins in vivo by Rho involves phosphatidylinositol 4-phosphate 5-kinase and not ROCK kinases. Curr Biol. 9:1259-1262.

Niggli V., Zimmermann A., Keller H. (1996) Inhibition of protein kinase C-dependent protein phosphorylation correlates with increased polarity and locomotion in Walker 256 carcinosarcoma cells. Int J Cancer 65:473-478.

Oshiro N., Fukata Y., and Kaibuchi K. (1998) Phosphorylation of Moesin by Rho-associated Kinase (Rho-kinase) plays a crucial role in the formation of microvilli-like structures. J. Biol. Chem. 273:34663-34666.

Pietromonaco S.F., Simons P.C., Altman A., and Elias L. (1998) Protein kinase C-θ phosphorylation of moesin in the actin-binding sequence. J Biol Chem. 273:7594-7603.

Ridley A.J. and Hall A. (1992) The small GTP-binding protein Rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 70:389-399.

Sanchez-Madrid F. and del Pozo M.A. (1999) Leukocyte polarization in cell migration and immune interactions. EMBO J. 18:501-511.

Takahashi K., Sasaki T, Mammoto A, Takaishi K, Kameyama T, Tsukita S, Takai Y. (1997) Direct interaction of the Rho GDP dissociation inhibitor with ezrin/radixin/moesin initiates the activation of the Rho small G protein. J. Biol. Chem. 272:23371-23375.

Wicki A. and Niggli V. (1999) Role of Protein kinase C isoforms in locomotion of Walker 256 carcinosarcoma cells. Int. J. Cancer 81:255-261.

Wicki A., and Niggli V. (1999) Role of protein kinase C isoforms in locomotion of Walker 256 carcinosarcoma cells. Int. J. Cancer 81:255-261.

Wicki A., and Niggli V. (2001) The Rho/Rho-kinase and the phosphatidylinositol 3-kinase pathway are essential for spontaneous locomotion of Walker 256 carcinosarcoma cells. Int J Cancer 91:763-771.

Yonemura S., Matsui T., Tsukita S., Tsukita S. (2002) Rho-dependent and -independent activation mechanisms of ezrin/radixin/moesin proteins: an essential role for polyphosphoinositides in vivo. J Cell Sci. 115:2569-2580.

4.7. Legends to Figures

Fig. 1. Effects of modulation of activity of Rho on ERM phosphorylation in Walker carcinosarcoma cells. (A) Cells were permeabilized with SLO in the absence (control) or presence of the indicated proteins, or (B) transfected with the indicated plasmid and pEGFP. After 12 h the viable GFP-positive cells were sorted. Cells were precipitated using TCA and subjected to immuno-blotting using a polyclonal rabbit anti-phospho ERM (Ezrin(Thr567)/Radixin(Thr564)/Moesin(Thr558))

antibody (1:10'000). P-ERM indicates ERM phosphorylated on (Ezrin(Thr567)/Radixin(Thr564)/ Moesin(Thr558)). Two bands of approximately 75 kDa (ezrin) and 70 kDa (radixin and/or moesin) were detected. Mean ± SD of 3 independent experiments.

Fig. 2. (A) Effect of modulation of activity of Rho-kinase on phosphorylation of ERM in Walker carcinosarcoma cells. Cells (1 x 10^6/ml) were incubated for 30 min at 37°C in Gey's solution in the presence of Y-27632 as indicated. (B) Walker carcinosarcoma cells were cotransfected with the indicated plasmids and pEGFP. After 12 h the viable GFP-positive cells were sorted. Cells then were precipitated using TCA and subjected to immuno-blotting using a polyclonal rabbit anti-phospho ERM (Ezrin (Thr567) Radixin (Thr564) Moesin (Thr558)) antibody (1:10'000). P-ERM indicates ERM phosphorylated on (Ezrin(Thr567)/Radixin(Thr564)/Moesin(Thr558)). Two bands of approximately 75 kDa (ezrin) and 70 kDa (radixin and/or moesin) were detected. Mean ± SD of 3 independent experiments.

Fig. 3. Effect of the PKC activator PMA (phorbol-12-myristate-13-acetate) on ERM phosphorylation in Walker carcinosarcoma cells. Cells (1 x 10^6/ml) were incubated for 30 min at 37°C in Gey's solution in the absence or presence of PMA as indicated. Cells then were precipitated using TCA and subjected to immuno-blotting using a polyclonal rabbit anti-phospho ERM (Ezrin (Thr567)/Radixin(Thr564)/Moesin(Thr558)) antibody (1:10'000). P-ERM indicates ERM phosphorylated on (Ezrin(Thr567)/Radixin(Thr564)/Moesin(Thr558)). Two bands of approximately 75 kDa (ezrin) and 70 kDa (radixin and/or moesin) were detected. Mean ± SD of 3 independent experiments.

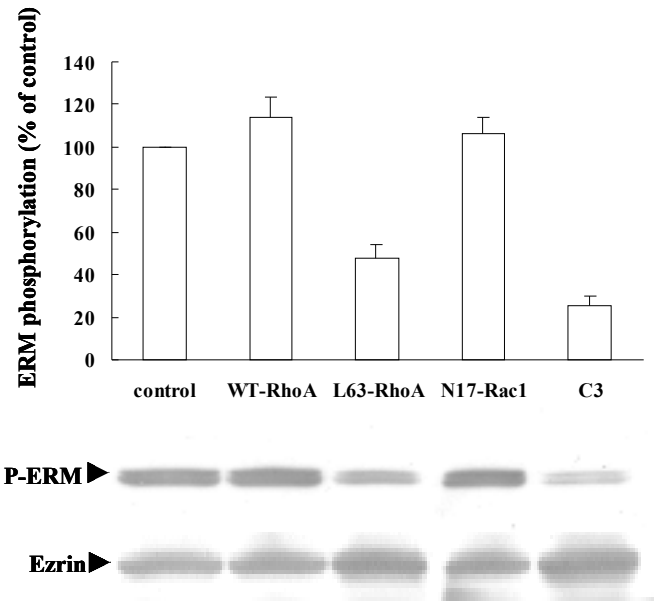
Fig. 4. ERM phosphorylation in Walker carcinosarcoma cells treated with C3 and different phospholipids. Cells were permeabilized with SLO in the presence of (A) C3 exoenzyme, or C3 exoenzyme plus PIP_2, or PIP_2 alone, (B) C3 exoenzyme, both C3 exoenzyme plus PIP_3, or PIP_3 alone, or (C) C3 exoenzyme, C3 exoenzyme plus the indicated phospholipid, or the indicated phospholipid alone. Cells then were precipitated using TCA and subjected to immuno-blotting using a polyclonal rabbit anti-phospho ERM (Ezrin(Thr567)/Radixin(Thr564)/Moesin(Thr558)) antibody (1:10'000). P-ERM indicates ERM phosphorylated on (Ezrin(Thr567)/Radixin(Thr564)/ Moesin(Thr558)). Two bands of approximately 75 kDa (ezrin) and 70 kDa (radixin and/or moesin) were detected. Mean ± SD of 3 independent experiments.

Fig. 5. ADP-ribosylation of Rho by C3 exoenzyme in Walker carcinosarcoma cells treated without or with C3 and phosphoinositides. Cells were permeabilized with SLO in the absence of C3 and phospholipids (A), in the presence of C3 exoenzyme (B), in the presence of C3 exoenzyme and PIP_2 (C), and in the presence of C3 exoenzyme and PIP_3. After 1 hour at 37°C ADP-ribosylation was performed with cell lysates using [^{32}P]NAD (see the Methods section for details) in the absence (lanes A1, B1, C1, D1) or presence (lanes A2, B2, C2, D2) of added C3 exoenzyme.

4.8. Figures

Fig. 1

A



B

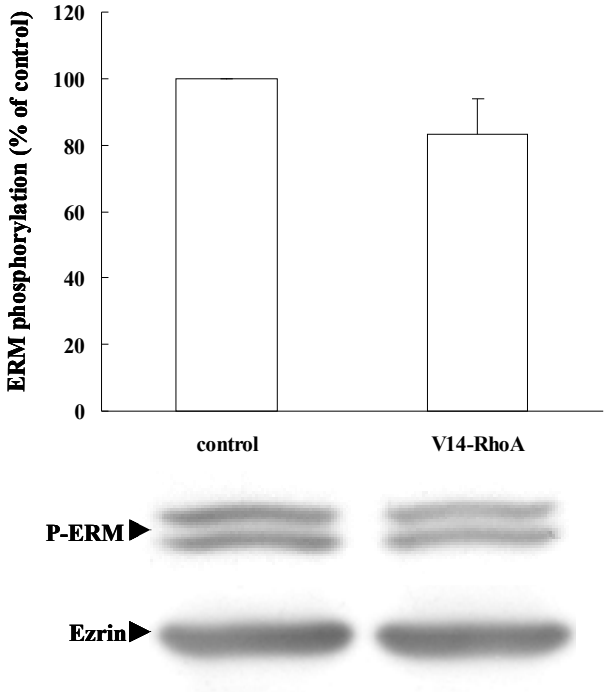


Fig.2

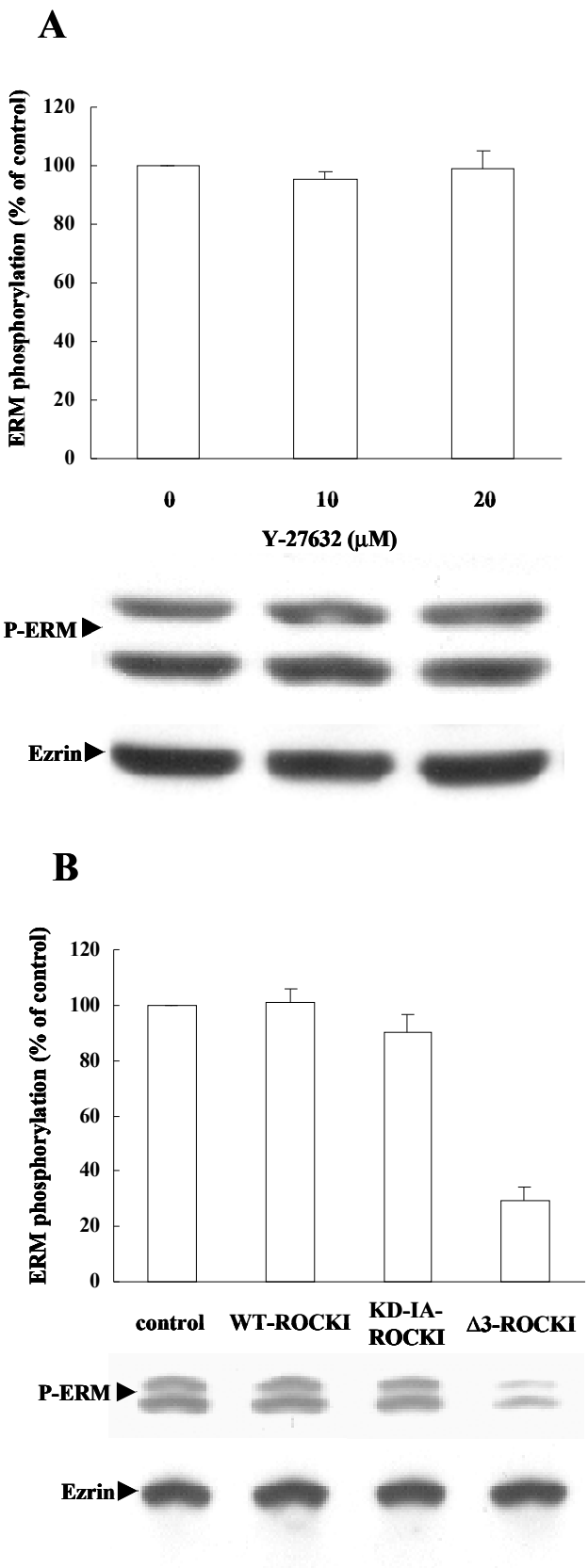

A
ERM phosphorylation (% of control)
120
100
80
60
40
20
0
0
10
20
Y-27632 (μM)
P-ERM
Ezrin
B
ERM phosphorylation (% of control)
120
100
80
60
40
20
0
control
WT-ROCKI
KD-IA-ROCKI
Δ3-ROCKI
P-ERM
Ezrin

Fig. 3

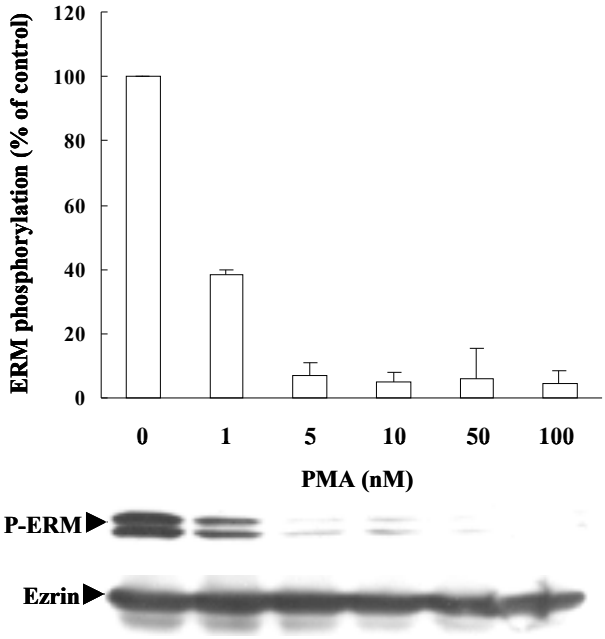

ERM phosphorylation (% of control)
120
100
80
60
40
20
0
0
1
5
10
50
100
PMA (nM)
P-ERM
Ezrin

Fig. 4

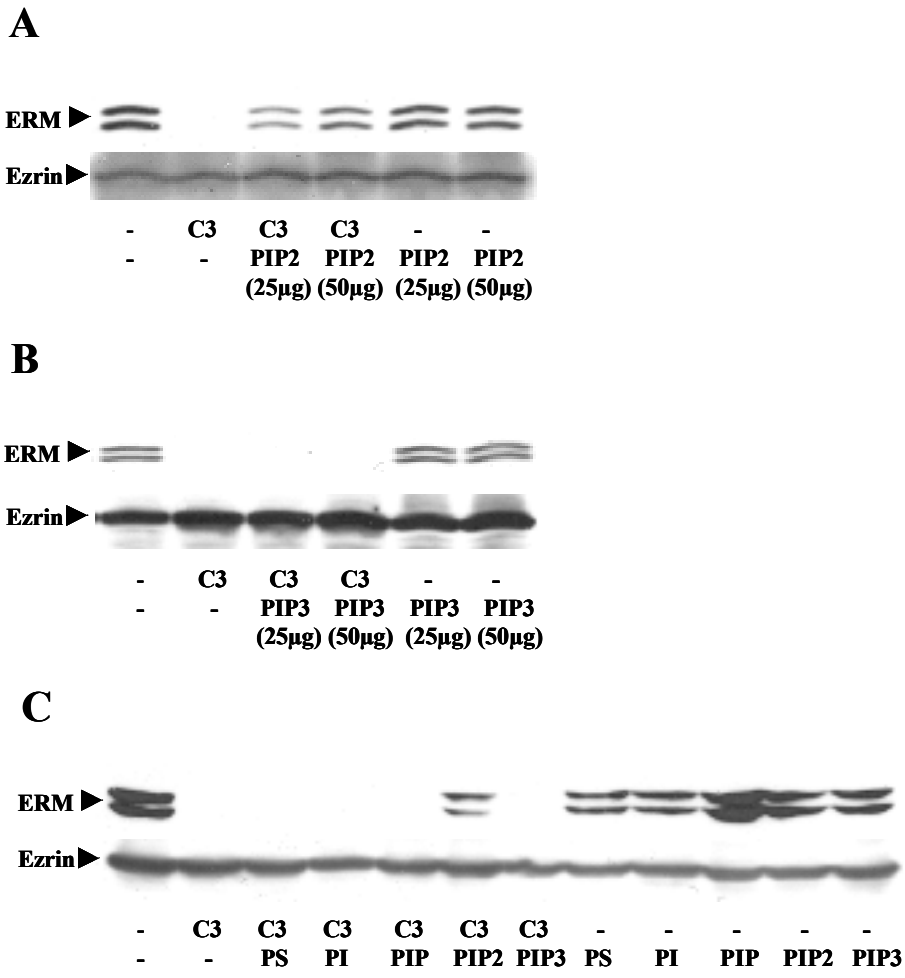
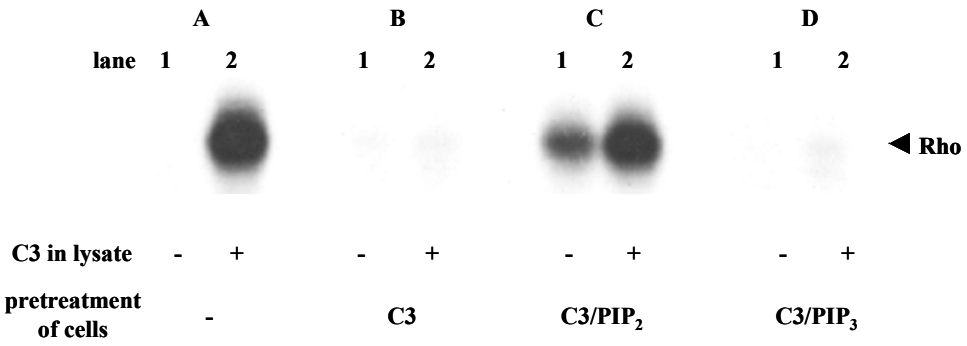

A
ERM
Ezrin
- C3 C3 C3 - -
- - PIP2 PIP2 PIP2 PIP2
(25µg) (50µg) (25µg) (50µg)
B
ERM
Ezrin
- C3 C3 C3 - -
- - PIP3 PIP3 PIP3 PIP3
(25µg) (50µg) (25µg) (50µg)
C
ERM
Ezrin
- C3 C3 C3 C3 C3 C3 - - - - -
- - PS PI PIP PIP2 PIP3 PS PI PIP PIP2 PIP3

Fig. 5


A
B
C
D
lane 1 2 1 2 1 2 1 2
Rho
C3 in lysate - + - + - + - +
pretreatment of cells
-
C3
C3/PIP2
C3/PIP3

5. MANUSCRIPT III

The role of an okadaic acid-sensitive phosphatase in regulating RhoA, ROCK, PI 3-kinase, and phosphorylation of MLC in Walker carcinosarcoma cells

Marc C. Gutjahr and Verena Niggli

5.1. Abstract

In the past the main focus in intracellular signaling rather concerned protein kinases than protein phosphatases. Nevertheless, protein phosphatases comprise a very important family of proteins involved in regulating signalling cascades within a cell.

Often a certain protein is under the control of a kinase/phosphatase couple that keeps the activity of that protein in balance.

We have investigated the role of serine/threonine protein phosphatases in motile responses of Walker carcinosarcoma cells. Our tool was the phosphatase type 1 and 2A inhibitor okadaic acid (OA) which has been shown to have a biphasic effect on the shape of these cells. Low concentrations (0.25 μM) suppressed cell polarity and locomotion and cells assumed a spherical shape. Higher concentrations (> 0.25 μM) induced a blebbing phenotype.

We now show that treatment of cells with okadaic acid affects the subcellular localization of ROCK I and II and PI 3-kinase 110γ. These proteins translocate from the membrane to the cytosol. Whether this translocation affects also the activity of these proteins remains to be investigated.

Furthermore, okadaic acid induced an increase in phosphorylation of myosin light chain and of the membrane-cytoskeleton linker proteins of the ERM (Ezrin/Radixin/Moesin) family, and reduced RhoA activity.

Okadaic acid-induced membrane blebbing could be efficiently suppressed by inhibiting myosin light chain kinase or myosin II. Only a partial suppression of membrane blebbing was observed by inhibiting Rho-kinase. Localization of PI 3-kinase and ROCK, and phosphorylation of MLC and ERM thus are controlled by an okadaic acid-sensitive phosphatase in Walker carcinosarcoma cells.

5.2. Introduction

Activities of proteins are controlled by phosphorylation and dephosphorylation of amino-acid residues. Thus, there is evidence for both kinases and phosphatases playing a crucial role in keeping the phosphorylation state of a protein in balance. Whereas kinases have been extensively studied, few studies concern the role of phosphatases in tumor cell locomotion (Niggli + Keller, 1997).

Okadaic acid, isolated from marine sponges, is a complex polyether fatty acid that enters intact cells. It is a highly selective and potent inhibitor of two widely distributed major protein phosphatases that dephosphorylate serine and threonine residues, phosphatases 1 and 2A (Cohen et al., 1990).

In Walker carcinosarcoma cells it has been shown that inhibition of phosphatases is a stop-signal for locomotion. There was a biphasic effect on cell shape. Cell polarity was suppressed at low concentrations and cells assumed a spherical shape. At higher concentrations marked bleb formation was observed, concomitant with increased phosphorylation of the intermediate filament protein vimentin (Niggli + Keller, 1997).

Recently it has been shown that in Walker carcinosarcoma cells the two Rho-kinase isoforms ROCKI+II and also a part of the p85 subunit of PI 3-kinase are predominantly located in the membrane fraction, indicating constitutive activation. Inhibition of Rho-kinases or inhibition of PI 3-kinase both inhibit spontaneous polarization and migration suggesting that activation of these proteins is required for these processes (Wicki and Niggli, 2001).

We now wanted to check the subcellular distribution of these proteins upon okadaic acid treatment of the cells, as phosphatase inhibition suppresses polarity (Keller and Niggli, 1997). Further, we investigated myosin light chain phosphorylation after treatment of cells with okadaic acid. In macrophages, high concentrations of okadaic acid suppressed cell motility and this correlated with increased MLC phosphorylation (Wilson et al., 1991). One of the main regulatory proteins of MLC, myosin light chain phosphatase, a type 1 phosphatase, has been identified to be a target of okadaic acid (Ishihara et al., 1989).

In this study we demonstrate that treatment of Walker carcinosarcoma cells with increasing concentrations of okadaic acid results in a redistribution of ROCK I and II from the membrane to the cytosol, correlating with inhibition of polarity. Also PI 3-kinase p110γ, but not PI 3-kinase p85α, significantly redistributes to the cytosol in okadaic acid treated cells. RhoA activity was also decreased by okadaic acid. Increased phosphorylation of MLC and of ERM proteins could be observed upon okadaic acid treatment of cells.

5.3. Materials and Methods

Materials

Reagents and suppliers were: Y-27632 (Calbiochem, La Jolla, CA, USA) was prepared as a 10 mM stock solution in H_2O. Okadaic acid (Alexis, Lausen, Switzerland) was prepared as a stock solution of 0.5 mM in DMSO and was stored at -20°C. DMSO (Sigma, St. Louis, MO, USA) alone had no significant effect in these experiments at the final concentrations used. GA (Glutaraldehyde) and BSA were obtained from Serva, Heidelberg, Germany. Human serum albumin (HSA) was from

ZLB Bioplasma AG, Bern, Switzerland. Complete protease inhibitor was from Roche, Penzberg, Germany.

Gey's solution contained 138 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.1 mM $CaCl_2$, 100 µM EGTA, 1 mM Na_2HPO_4, 5 mM $NaHCO_3$, 5.5 mM glucose and 20 mM HEPES (pH 7.4).

Antibodies

Antibodies were obtained from the following sources: a MAb directed against RhoA (Cat. Nr. ARH01) from Cytoskeleton, Denver, CO, USA. A polyclonal anti-ROCK I antibody was prepared as described (Fujita et al., 1997). A MAb against ROCK II was obtained from Transduction Laboratories (Lexington, KY). A polyclonal anti-phospho MLC2 (Thr18/Ser19) antibody (Cat. Nr. 3674S) was from Cell Signaling, Beverly, MA, USA. A polyclonal antibody against the p85α subunit of PI 3-kinase, kindly provided by Dr. M. Wymann, was obtained in rabbits by immunization with $(His)_6$ tag fusion protein containing amino acids 6 to 112 of bovine p85α (Wymann et al., 1996).

Tumor cell culture

Walker 256 carcinosarcoma cells were kindly provided by Dr. B. Sordat (ISREC, Lausanne, Switzerland) and cultured in vitro in RPMI 1640 supplemented with 10% FCS, penicillin (25 µg/ml) and streptomycin (25 µg/ml). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

Subcellular distribution of ROCK I, ROCK II and PI 3-kinase in Walker carcinosarcoma cells.

Cells (2.4 x 10^6/ml, 0.5 ml /tube) were exposed to inhibitors as described in the Results section. The reaction was stopped by centrifugation of cells for 5 min at 240 g at room temperature. Pellets were resuspended in 500 µl of a buffer containing 20 mM Tris/HCl (pH 7.5), 2 mM EDTA and 1 mM dithiothreitol and incubated for 10 min on ice. Cells were subsequently homogenized by 15 passages through a fine needle (gauge 30, length 13 mm) using a 1 ml plastic syringe, followed by centrifugation for 10 min, 4°C, at 1,000 g. Supernatants were centrifuged at 100,000 g for 25 min. The resulting pellets (representing the membrane fraction) were solubilized in 100 µl of sample buffer containing 1% SDS, 50 mM dithiothreitol, 15% glycerol, 62.5 mM Tris/HCl (pH 6.8) and 0.001% bromphenol blue by incubation at 95°C for 10 min. Supernatants (representing the

cytosolic fraction) were precipitated by adding 1 vol. of an ice-cold solution containing 200 mg/ml TCA, 40 mM NaF and 10 mM Na_2HPO_4. After incubation on ice for 20 min, precipitates were collected by centrifugation (10 min) in an Eppendorf centrifuge, 10,000 g at 4°C. Pellets were washed once with 1 ml of 5% TCA and once with 1 ml of 0.5% TCA and solubilized in 100 µl of sample buffer. Pellets and supernatants were electrophoresed through a 5-10 % SDS-polyacrylamide gradient gel (Laemmli, 1970).

Determination of cell morphology

Cells were washed twice in Gey's medium and resuspended in this medium (1 x 10^6 cells/ml). Details of the incubation procedure are given in the Results section. The incubation was terminated by fixation with 1% glutaraldehyde (final concentration) at 37°C for 30 min. At least 100 cells per condition were analyzed in each experiment using DIC (differential interference contrast) optics (Zeiss IM 35 microscope, x 100 objective).

TCA precipitation, electrophoresis and immunoblotting

Cells were precipitated by adding 1 vol. of an ice-cold solution containing 200 mg/ml trichloracetic acid (TCA), 40 mM NaF and 10 mM Na_2HPO_4. After incubation on ice for 20 min, precipitates were collected by centrifugation (10 min) in an Eppendorf centrifuge, 10'000 g at 4°C. Pellets were washed once with 1 ml of 5% TCA and once with 1 ml of 0.5% TCA and then solubilized in 100 µl of sample buffer containing 1% SDS, 50 mM dithiothreitol, 15% glycerol, 62.5 mM Tris/HCl (pH 6.8) and 0.001% bromphenol blue by incubation at 95°C for 10 min. Fractions were electrophoresed through a SDS-polyacrylamide gel followed by transblotting to nitrocellulose using a Genie blotter (Idea Scientific, Minneapolis, MN). The blots were incubated for 1h in a blocking buffer containing 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.05 % Tween-20 and 5 % defatted milk powder (TBSTM), followed by overnight incubation at 4°C with the indicated antibodies, diluted in PBS containing 3% BSA and 0.02 % NaN3. After 3 washes with TBST, the blots were incubated for 1 h either with a second horseradish peroxidase-labeled goat anti-rabbit IgG antibody (Bio Rad Laboratories, Hercules, CA) for polyclonal antibodies, diluted 1:10'000 in TBSTM, or with a horseradish peroxidase-labeled goat anti-mouse IgG antibody for monoclonal antibodies, diluted 1:3000 in TBSTM. Detection was performed with an ECL Western detection system (Pierce, Rockford, IL). Bands were quantified by densitometry of the films using a GS-800 Calibrated Densitometer from Bio-Rad.

MLC-Phosphorylation

Cells were washed twice in Gey's medium, resuspended in this medium (1.0 x 10^6 cells/0.5 ml) and then incubated at 37°C for 30 min with the indicated concentrations of Y-27632, and/or okadaic acid. The reaction was stopped with TCA (see above) and the precipitated proteins were subjected to immunoblotting using a polyclonal rabbit anti-phospho MLC2 (Thr18/Ser19) antibody (1:500).

ERM-Phosphorylation

Cells were washed twice in Gey's medium, resuspended in this medium (1.0 x 10^6 cells/0.5 ml) and then incubated at 37°C with okadaic acid as indicated in the Results section. The reaction was stopped with TCA (see above) and the precipitated proteins were subjected to immunoblotting using an anti-phospho Ezrin (Thr567)/ Radixin (T564)/ Moesin (T558) antibody (1:10'000).

Measurement of Rho Activity

Cells were washed twice in Gey's medium w/o magnesium and phosphate and then incubated (10 x 10^6 cells/0.5ml) at 37°C with the appropriate agents (as described in the Results section). Cells were then centrifuged in a microfuge at 4°C and 2,000 rpm (400 x g) and the pellets were resuspended and lysed in 500 µl of a buffer containing 50 mM Tris, pH 7.4, 100 mM NaCl, 10 % glycerol, 1 % NP-40 and 2 mM $MgCl_2$. Lysates were clarified by centrifugation at 14,000 x g at 4°C for 10 min. For positive and negative controls lysates were incubated with GTPγS (10 mM) or with GDP (100 mM) for 15 min at 30°C and then the reaction was terminated by placing the tubes on ice and adding $MgCl_2$ (final concentration 60 mM). Then equal amounts of lysates (450 µl) were incubated with 20 µg GST-RBD beads (Upstate Biotechnologies) at 4°C for 45 min with rotation. The beads were washed 3 times with 500 µl lysis buffer, and bound Rho was eluted by boiling each sample in Laemmli sample buffer (33 µl 3 x conc. sample buffer + 66 µl lysis buffer). Eluted samples and aliquots (66 µl) of total cell lysates were then electrophoresed on 12.5% SDS-polyacrylamide gel electrophoresis gels, transferred to nitrocellulose, blocked with 5% nonfat milk, and analyzed by Western blotting using a anti-Rho A antibody (dilution: 1:300).

Statistics

Differences between data were analysed with the Student's *t* test, with a P value < 0.05 considered significant. Data correspond to the mean ± SD.

5.4. Results

There is evidence that shape changes and locomotion are regulated by selective phosphorylation and dephosphorylation of distinct proteins and therefore not only kinases but also phosphatases might play a crucial role. Up to now only little is known about the role of phosphatases in cell migration. However, it was shown that in neutrophils and fibroblasts morphology was affected by the phosphatase inhibitors okadaic acid or calyculin A. Cell polarization was inhibited and cells rounded up (Chartier, 1991; Kreienbühl, 1992). These findings support the idea of cell shape being controlled by the concerted actions of kinases and phosphatases.

Walker carcinosarcoma cells show a substantial proportion of spontaneously polarized cells (about 70%). Recent findings in our lab showed that when cells were treated with the phosphatase inhibitor okadaic acid (OA) two major phases in the dose-response curve could be distinguished. At about 0.25 μM OA, cell polarity was almost completely suppressed and the cells were mostly spherical. At higher concentrations, cells developed first small and then (2.0 μM OA) large blebs (Niggli and Keller, 1997). This suggests that the shape of Walker carcinosarcoma cells is regulated by a kinase/phosphatase couple.

Effect of okadaic acid on the subcellular distribution of ROCK I and ROCK II

It has been shown that in Walker carcinosarcoma cells Rho-kinases I and II predominantly accociate with membranes, suggesting constitutive activation of these proteins (Wicki and Niggli, 2001). In untreated control cells 55 ± 4% of ROCK I and 66 ± 14% of ROCK II were associated with membranes (n = 3). In cells treated with okadaic acid, the fraction of ROCK I and II associated with the membrane was almost completely abolished with increasing concentrations of okadaic acid. Half-maximal effects were obtained at 0.3 ± 0.03 μM for ROCK I and 0.3 ± 0.04 μM for ROCK II (Fig. 1A,B).

Effect of okadaic acid on the subcellular distribution of PI 3-kinase subunits p85α and p110γ

Similar findings as for ROCK resulted for the PI 3-kinase subunits p85α (at high concentrations of okadaic acid) and p110γ. In untreated control cells 54 ± 7% of PI 3-kinase p110γ and 62 ± 10% of PI 3-kinase p85α were predominantly located in the membrane fraction. Upon treatment of cells

with okadaic acid both subunits relocalized into the cytosolic fraction. At a concentration of 2.0 µM OA 14 ± 8% of PI 3-kinase p110γ and 33 ± 10% of PI 3-kinase p85α remained in the membrane. Half-maximal effects were obtained at 0.5 ± 0.1 µM for PI 3-kinase p110γ and at 1.0 ± 0.2 µM for PI 3-kinase p85α (Fig. 2A,B).

Effect of okadaic acid on Rho A activity

As observed above there is a shift of Rho-kinase I and II from the membrane to the cytosolic fraction upon treatment of cells with okadaic acid, suggesting a decrease in activation. Since ROCK is a direct downstream effector of the small GTPase RhoA, we tested whether okadaic acid exerts its effect directly on ROCK or via inhibition of RhoA. The extent of RhoA activation was assessed by a pull-down assay in which a GST fusion protein of the Rho-binding domain of the Rho effector Rhotekin is used to affinity-precipitate the active (GTP-bound) RhoA. As shown in Fig. 3, a significantly smaller amount (32 ± 9%; $p < 0.025$) of active RhoA was affinity-precipitated in cells treated with 2.0 µM OA as compared to untreated control cells. Thus, okadaic acid might inhibit RhoA activity which in turn could result in reduced activity of Rho-kinases I and II.

Effect of okadaic acid on MLC-phosphorylation

Walker carcinosarcoma cells show a blebbing phenotype upon treatment with 2.0 µM of okadaic acid (Niggli + Keller, 1997). Phosphorylation of MLC has been shown to be involved in membrane blebbing in apoptotic cells (Sebbagh et al., 2001). MLC phosphorylation is not only controlled by ROCK but also by myosin light chain phosphatase (MLCP) and by myosin light chain kinase (MLCK) (Hartshorne et al., 1998; Somlyo and Somlyo, 2003). MLC-phosphatase (MLCP) is inhibited at the okadaic acid concentrations used (Ishihara et al., 1989).

Therefore we investigated the effect of okadaic acid on MLC phosphorylation. In fact, incubation of cells with increasing concentrations of okadaic acid resulted in a marked increase in phosphorylation of MLC. At a concentration of 2.0 µM okadaic acid MLC phosphorylation was 12 ± 2-fold increased ($p < 0.025$) as compared to untreated control cells (Fig. 4).

Rho-kinase contributes to MLC phosphorylation in two ways. First, indirectly by phosphorylating and inhibiting the myosin binding subunit of myosin light chain phosphatase (Kimura et al., 1996), second, by direct phosphorylation of MLC (Kureishi et al., 1997). We tested the effect of preincubating the cells with 20 µM Y-27632 prior to the incubation with okadaic acid on MLC phosphorylation. In fact, the increase in MLC-phosphorylation could be reduced (48 ± 3%

inhibition at 2.0 μM OA; $p < 0.025$) by pretreatment of cells with Y-27632 as compared to incubation of cells with okadaic acid alone (Fig. 4). Nevertheless, MLC phosphorylation still increased with increasing amounts of okadaic acid as compared to control cells, suggesting that this increase in MLC-phosphorylation is not only due to ROCK that is inhibited by Y-27632, and that there is another protein contributing to MLC phosphorylation. This protein could be MLCK. We treated the cells with 20 μM of the MLCK inhibitor ML-7 and with 20 μM of the ROCK inhibitor Y-27632 (concentrations that almost completely inhibit constitutive MLC phosphorylation in Walker carcinosarcoma cells; see manuscript I) prior to incubation with 0.25 μM or 0.5 μM of okadaic acid. The concerted inhibition of Rho-kinase and of MLCK did however not further reduce the okadaic acid-induced increase in MLC phosphorylation (data not shown).

Effect of inhibition of ROCK, MLCK, and myosin II on okadaic acid-induced membrane blebbing of Walker carcinosarcoma cells.

We previously showed (see manuscript I) that membrane blebbing induced by transfection of a constitutively active Rho-kinase, Δ3-ROCKI (Ishizaki, 1997), could be suppressed by inhibition of ROCK, MLCK, or myosin II. We wondered whether the okadaic acid-induced blebbing can be inhibited in the same way. As shown in Fig. 5 Walker carcinosarcoma cells were preincubated with 2 μM okadaic acid for 45 min followed by 30 min incubation of cells with either the ROCK inhibitor Y-27632, the MLCK inhibitor ML-7, or the myosin II inhibitor blebbistatin. Membrane blebbing could not be reversed efficiently (inhibition: 19.5 ± 8%) by inhibiting ROCK with 40 μM of Y-27632. In contrast, membrane blebbing could be suppressed by 83.7 ± 4% by inhibition of MLCK with 40 μM ML-7. Inhibition of myosin II with blebbistatin was the most effective way to suppress okadaic acid-induced blebbing (inhibition: 97.8 ± 1%) (Fig. 5).

Effect of okadaic acid on ERM-phosphorylation

We also addressed the question whether okadaic acid affects the phosphorylation of ERM proteins. We showed that Rho is involved in the regulation of ERM phosphorylation (see manuscript II) and that okadaic acid lowered the amount of active RhoA. When Walker carcinosarcoma cells were treated with 0.5 μM of okadaic acid ERM phosphorylation was significantly increased (239.6 ± 24%; $p < 0.05$) as compared to untreated control cells (Fig 6).

5.5. Discussion

In this study we showed that treatment of Walker carcinosarcoma cells with the phosphatase inhibitor okadaic acid lead to a change in the subcellular distribution of important signalling proteins. Rho-kinases I and II as well as PI 3-kinase that are predominantly located in the membrane fraction of untreated cells, shifted to the cytosolic fraction. Moreover incubation of cells with OA decreased the constitutive activity of the small GTPase RhoA.

RhoA/Rho-kinase

In Walker carcinosarcoma cells Rho-kinases I and II are predominantly localized in the membrane. It has been reported that ROCK II translocates from the cytosol to the membrane upon activation by Rho in Cos-7 cells (Leung et al., 1995). In unstimulated human neutrophils ROCK II is mainly located in the cytosol and translocates to the membrane upon chemotactic activation of the neutrophils (Wicki and Niggli, 2001). However, the Rho-kinase inhibitor Y-27632 did not affect the membrane association of both ROCK I and II in Walker carcinosarcoma cells (Wicki and Niggli, 2001). It is thus not clear whether membrane localization of ROCK correlates with activation of the kinase. How exactly Rho-kinase is targeted to the membrane is also not clear. It has been shown that in the inactive state Rho-kinase can form dimers through parallel self-association at the Rho-binding domain. Binding of the GTPase RhoA could trigger conformational changes resulting in disruption of the dimer and activation of Rho-kinase (Shimizu et al., 2003). It was suggested that trans-autophosphorylation is involved in regulating the kinase activity as dimerization of wild-type kinase with a kinase-dead ROCK kinase domain resulted in an inactive kinase (Chen et al., 2002). The C-terminal PH domain of ROCK might play a key role in specific localization of the protein. Endogenous localization of ROCK to the acto-myosin network was shown to be disrupted by overexpression of the PH domain of ROCK in HeLa cells. Moreover, an antibody directed against the PH domain had an inhibitory effect on ROCK activity, cell contraction and actomyosin assembly (Chen et al., 2002).

In this study we could show that the subcellular localization of Rho-kinases is dependent on the action of phosphatases. This is a novel finding not observed previously to our knowledge. Treatment of cells with okadaic acid led to a shift of ROCK I and II from the membrane to the cytosol (Fig. 1A,B). If we assume that in this case the translocation of ROCK from the membrane to the cytosol is paralleled by deactivation of the protein, then this would explain inhibition of cell polarity upon treatment of cells with low concentrations of okadaic acid, as ROCK activity is

necessary for polarization (Wicki and Niggli, 2001). Nevertheless, to be sure about the activation state of Rho-kinase after okadaic acid treatment, a kinase assay in cell lysates is required.

To find out why Rho-kinases translocate form the membrane to the cytosol upon okadaic acid treatment we investigated the activity of the small GTPase RhoA, which activates ROCK directly (Ishizaki et al., 1996; Matsui et al., 1996). In fact, high concentrations of okadaic acid reduced RhoA activity (Fig. 3). This supports the notion that membrane association of Rho-kinase might be decreased because of reduced RhoA activity.

PI 3-kinase

Phosphatidylinositol 3-kinase was shown to be predominantly located in the membrane in Walker carcinosarcoma cells and to be essential for spontaneous locomotion of these cells. In resting human neutrophils, in contrast PI 3-kinase was mainly located in the cytosol. Inhibition of PI 3-kinase with two different inhibitors (wortmannin and LY-294002) suppressed spontaneous development of Walker cell polarity and migration. Thus PI 3-kinase was considered to be constitutively activated (Wicki and Niggli, 2001). It has been shown that membrane localization of PI 3-kinase is sufficient for activation of multiple signalling pathways including pp70 S6 kinase, Akt/Rac, and Jun N-terminal kinase (JNK) in Cos-7 cells (Klippel et al., 1996).

Upon okadaic acid treatment of Walker carcinosarcoma cells the catalytic subunit of PI 3-kinase, p110γ, translocates from the membrane to the cytosol already at low OA concentrations correlating with suppression of polarity (Fig. 2B). In contrast, the regulatory subunit p85α only significantly shifts to the cytosol at higher concentrations of OA and to a lesser extent (Fig. 2A). Our findings indicate a possible role of okadaic acid-sensitive phosphatases in regulating especially activity of PI 3-kinase p110γ. Further investigations of PI 3-kinase activity in okadaic acid-treated cells are necessary.

Myosin light chain

Treatment of Walker carcinosarcoma cells with high concentrations of okadaic acid induced membrane blebbing (Niggli and Keller, 1997). Cell blebbing has previously been shown to require increased myosin activity (Mills et al., 1998). Indeed, also in blebbing Walker carcinosarcoma cells treated with okadaic acid MLC phosphorylation was strongly increased (Fig. 4). MLC phosphorylation is under the control of Rho-kinase, myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP). Rho-kinase contributes to MLC phosphorylation first by direct phosphorylation or second indirectly by inhibiting MLCP (Hartshorne et al., 1998; Somlyo and

Somlyo, 2003). Our results could be explained by inhibition of MLCP by okadaic acid (Ishihara et al., 1989).

We asked whether okadaic acid treatment of cells inhibits Rho-kinase or whether Rho-kinases just dissociate from the membrane without being inhibited and stay activated in the cytosol. The increase in MLC-phosphorylation could be reduced by pretreatment of cells with Y-27632 prior to okadaic acid treatment, indicating that ROCK was still indeed partially active (Fig. 4). However, MLC phosphorylation still was about 6-fold increased in the presence of Y-27632 as compared to untreated control cells. Also membrane blebbing could not efficiently be inhibited by Y-27632. In contrast, ML-7, a MLCK inhibitor efficiently inhibited okadaic acid-induced membrane blebbing at 30 μM (Fig. 5). Future experiments should show whether okadaic-acid-induced MLC phosphorylation can be inhibited by MLCK inhibition with higher concentrations of ML-7 (30 μM). Our data suggest that MLCK or another kinase different from ROCK might play a key role in phosphorylating MLC in okadaic acid treated cells. Also direct inhibition of myosin II activity with the myosin II inhibitor blebbistatin suppressed okadaic acid-induced membrane blebbing almost completely. This, together with the observation that MLC phosphorylation is increased in blebbing cells, supports the notion that myosin activity is required for membrane blebbing induced by okadaic acid.

In contrast to Δ3-ROCKI-induced membrane blebbing (see manuscript I) okadaic acid-induced membrane blebbing could not be suppressed efficiently by subsequent inhibition of Rho-kinase. These two forms of blebbing may thus not involve the same mechanisms. In the former case, blebbing is induced by myosin activation via ROCK-dependent inhibition of MLCP and possibly direct MLC phosphorylation. In the latter case, MLC phosphorylation is increased via OA-dependent inhibition of MLCP. In this case MLCK, and to a smaller extent ROCK, may directly phosphorylate MLC.

ERM

Already low concentrations of okadaic acid (up to 0.5 μM) induced a significant (239.6 ± 24%; $p < 0.05$) increase in ERM phosphorylation in Walker carcinosarcoma cells (Fig. 6). At these concentrations of okadaic acid, cell polarity and migration are inhibited and cells assume a spherical shape but no membrane blebbing occurs (Niggli and Keller, 1997). Our data show that an okadaic acid-sensitive phosphatase controls ERM phosphorylation, comparable to MLC phosphorylation. In fact it has been shown by coimmunoprecipitation that the myosin-binding

subunit of myosin light chain phosphatase associates with moesin in MDCK cells (Fukata et al., 1998).

In summary, we conclude that constitutive phosphatase activity seems to be required for membrane localization of Rho-kinase I and II and of PI 3-kinase 110γ, and for maintaining constitutive levels of MLC and ERM phosphorylation and constitutive RhoA activity optimal for migration. Whether activities of Rho-kinase and PI 3-kinase are indeed decreased upon okadaic acid treatment, remains to be elucidated.

5.6. References

Chartier L, Rankin L.L., Allen R.E., Kato Y., Fusetani N., Karaki H., Watanabe S., Hartshorne D.J. (1991) Calyculin-A increases the level of protein phosphorylation and changes the shape of 3T3 fibroblasts. Cell Motil. Cytoskel. 18:26-40.

Chen X.Q., Tan I., Ng C.H., Hall C., Lim L., Leung T. (2002) Characterization of RhoA-binding kinase ROKα implication of the pleckstrin homology domain in ROKα function using region-specific antibodies. J Biol Chem. 277:12680-12688.

Cohen P., C.F. Holmes, Tsukitani Y. (1990) Okadaic acid: a new probe for the study of cellular regulation. Trends Biochem Sci. 15:98-102.

Fujita A., Y. Saito, T. Ishizaki, M. Maekawa, K. Fujisawa, F. Ushikubi, Integrin-dependent translocation of p160ROCK to cytoskeletal complex in thrombin-stimulated human platelets, Biochem. J. 328 (1997) 769-775.

Fukata Y., Kimura K., Oshiro N., Saya H., Matsuura Y., Kaibuchi K. (1998) Association of the myosin-binding subunit of myosin phosphatase and moesin: dual regulation of moesin phosphorylation by Rho-kinase and myosin phosphatase. J Cell Biol. 141:409-418.

Hartshorne D.J., Ito M., Erdodi F. (1998) Myosin light chain phosphatase: subunit composition, interactions and regulation. J. Muscle Res. Cell Motil. 19:325-341.

Hirano K., Chartier L. Taylor R.G., Allen R.E., Fusetani N., Karaki H., Hartshorne D.J. (1992) Changes in the cytoskeleton of 3T3 fibroblasts induced by the phosphatase inhibitor, calyculin-A. Res. Cell Motil. 13:341-353.

Ishihara H., Martin B.L., Brautigan D.L., Karaki H., Ozaki H., Kato Y., Fusetani N., Watabe S., Hashimoto K., Uemura D., Hartshorne D.J. (1989) Calyculin A and okadaic acid: inhibitors of protein phosphatase activity. Biochem. Biophys. Res. Commun. 159:871-877.

Ishizaki T., Maekawa K., Fujisawa K., Okawa A., Iwamatsu A., Fujita N., Watanabe Y., Saito A., Kakizuka A., Morii N., and Narumiya S. (1996) The small GTP-binging protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. EMBO J. 15: 1885-1893.

Kimura K., Ito M., Amano M., Chihara K., Fukata Y., Nakafuku M., Yamamori B., Feng J., Nakano T., Okawa K., Iwamatsu A., and Kaibuchi K. (1996) Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science 273:245-248.

Klippel A., Reinhard C., Kavanaugh W.M., Apell G., Escobedo M.A., Williams L.T. (1996) Membrane localization of phosphatidylinositol 3-kinase is sufficient to activate multiple signal-transducing kinase pathways. Mol Cell Biol. 16:4117-4127.

Kreienbuhl P., Keller H.U., Zimmermann A. (1992) Protein phosphatase inhibitors okadaic acid and calyculin A alter cell shape and F-actin distribution and inhibit stimulus-dependent increases in cytoskeletal actin of human neutrophils. Blood 80:2911-2919.

Kureishi Y., Kobayashi S., Amano M., Kimura K., Kanaide H., Nakano T., Kaibuchi K., and Ito M. (1997) Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation. J. Biol. Chem. 272:12257-12260.

Leung T., Manser E., Tan L., and Lim L. (1995) A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. J Biol Chem. 256:29051-29054.

Maier G.D., Wright M.A., Lozano Y., Djordjevic A., Matthews J.P., Young M.R. (1995) Regulation of cytoskeletal organization in tumor cells by protein phosphatases-1 and -2A. Int. J. Cancer 61:54-61.

Matsui T., Amano M., Yamamoto T., Chihara K., Nakafuku M., Ito M., Kanano T., Okawa K., Iwamatsu A., and Kaibuchi K. (1996) Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP-binding protein Rho. EMBO J. 15:2208-2216.

Mills J.C., Stone N.L., Erhardt J., Pittman R.N. (1998) Apoptotic membrane blebbing is regulated by myosin light chain phosphorylation. J. Cell Biol. 140:627-636.

Niggli V. and H. Keller (1997) Low concentrations of the phosphatase inhibitor okadaic acid stop tumor cell locomotion. Eur J Pharmacol. 324:99-108.

Sebbagh M., C. Renvoize, J. Hamelin, N. Riche, J. Bertoglio, and J. Breard (2001) Caspase-3-mediated cleavage induces MLC phosphorylation and apoptotic membrane blebbing. Nature Cell Biol. 3:346-352.

Shimizu T., Ihara K., Maesaki R., Amano M., Kaibuchi K., and Hakoshima T. (2003) Parallel coiled-coil association of the RhoA-binding domain in Rho-kinase. J. Biol. Chem. 278:46046-46051.

Somlyo A.P., and Somlyo A.V. (2003) Ca^{2+} sensitivity of smooth muscle and non-muscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. Physiol. Rev. 83:1325-1358.

Wicki A., and Niggli V. (2001) The Rho/Rho-kinase and the phosphatidylinositol 3-kinase pathway are essential for spontaneous locomotion of Walker 256 carcinosarcoma cells. Int. J. Cancer 91:763-771.

Wilson A.K., Takai A., Ruegg J.C., de Lanerolle P. (1991) Okadaic acid, a phosphatase inhibitor, decreases macrophage motility. Am J Physiol. 260:105-112.

Wymann M.P., Bulgarelli-Leva G., Zvelebil M.J., Pirola L., Vanhaesebroeck B., Waterfield M.D., Panayotou G. (1996) Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. Mol Cell Biol. 16:1722-1733.

Zheng B., Woo C.F., Kuo J.F. (1991) Mitotic arrest and enhanced nuclear protein phosphorylation in human leukemia K562 cells by okadaic acid, a potent protein phosphatase inhibitor and tumor promoter. J. Biol. Chem. 266:10031-10034.

5.7. Legends to Figures

Fig. 1. Effects of okadaic acid on subcellular localization of (A) ROCK I and (B) ROCK II in Walker carcinosarcoma cells. Walker carcinosarcoma cells (2.4 x 10^6/ml) were incubated for 45 min at 37°C in Gey's solution in the presence of okadaic acid as indicated, followed by isolation of membrane- and cytosol-containing fractions. Membrane-containing (closed bars) and cytosolic (open bars) fractions from 1.2 x 10^6 cells were prepared and analyzed by SDS-PAGE and immunoblotting using antibodies against ROCK I (diluted 1:1500) and ROCK II (diluted 1:1000). Band intensities were quantified by densitometry. Mean ± SD of 3 independent experiments.

Fig. 2. Effects of okadaic acid on subcellular distribution of (A) PI3-Kinase subunits p85α and (B) p110γ in Walker carcinosarcoma cells. Walker carcinosarcoma cells (2.4 x 10^6/ml) were incubated for 45 min at 37°C in Gey's solution in the presence of okadaic acid as indicated followed by isolation of membrane- and cytosol-containing fractions. Membrane-containing (closed bars) and cytosolic (open bars) fractions from 1.2 x 10^6 cells were prepared and analyzed by SDS-PAGE and

immunoblotting using antibodies against p85α (diluted 1:1200) and p110γ (diluted 1:1000). Band intensities were quantified by densitometry. Mean ± SD of 3 independent experiments.

Fig. 3. Okadaic acid decreases Rho A activity in Walker carcinosarcoma cells. (A) Cells (20 x 10^6/ml) were incubated for 45 min at 37°C in Gey's solution in the absence or presence of 2.0 μM okadaic acid. Then cells (10 x 10^6 per lane) were lysed and the extent of RhoA activation was assessed by a pull-down assay as described in Materials and Methods. GTP-loaded proteins were analyzed by SDS-PAGE and immunoblotting using an antibody against RhoA (diluted 1:250). Band intensities were quantified by densitometry. Mean ± SD of 3 independent experiments. (B) Lysates from cells incubated in the absence of inhibitor were incubated either with 10 mM GTPγS (positive controls) or with 100 mM GDP (negative controls) followed by the pull-down assay.

Fig. 4. Effects of okadaic acid (OA) and Y-27632 on phosphorylation of MLC in Walker carcinosarcoma cells. Cells (1 x 10^6/ml) were incubated for 30 min at 37°C in Gey's solution in the absence or presence of 20 μM Y-27632 followed by incubation with increasing concentrations of okadaic acid as indicated. The reaction was stopped by the addition of ice-cold trichloroacetic acid (10% final concentration). The precipitated proteins of 0.5 x 10^6 cells were separated on a 15% SDS gel. Then immunoblotting using an antibody against phosphorylated MLC (diluted 1:500) was performed. The arrow indicates a band of 18 kDa. Quantitative evaluation of the experiment is shown in panel a. Band intensities were quantified by densitometry. Mean ± SD of 3 independent experiments.

Fig. 5. Effects of Y-27632, ML-7 or blebbistatin on okadaic acid-induced membrane blebbing. Walker carcinosarcoma cells were preincubated with 2 μM okadaic acid for 45 min at 37°C, followed by 30 min incubation with the indicated inhibitors. Cells were fixed with 1% glutaraldehyde. Morphology of cells was analyzed using DIC optics. Mean ± SD of 3 independent experiments.

Fig. 6. Effect of okadaic acid on phosphorylation of ERM in Walker carcinosarcoma cells. Cells (1 x 10^6/ml) were incubated for 45 min at 37°C in Gey's solution in the absence or presence of okadaic

acid as indicated. Cells then were precipitated using TCA and subjected to immuno-blotting using a polyclonal rabbit anti-phospho ERM(Ezrin (Thr567) Radixin (Thr564) Moesin (Thr558)) antibody (1:10'000). P-ERM indicates ERM phosphorylated on (Ezrin (Thr567) Radixin (Thr564) Moesin (Thr558)). Two bands of approximately 75 kDa (ezrin) and 70 kDa (radixin and/or moesin) were detected. Mean ± SD of 3 independent experiments.

5.8. Figures

Fig. 1

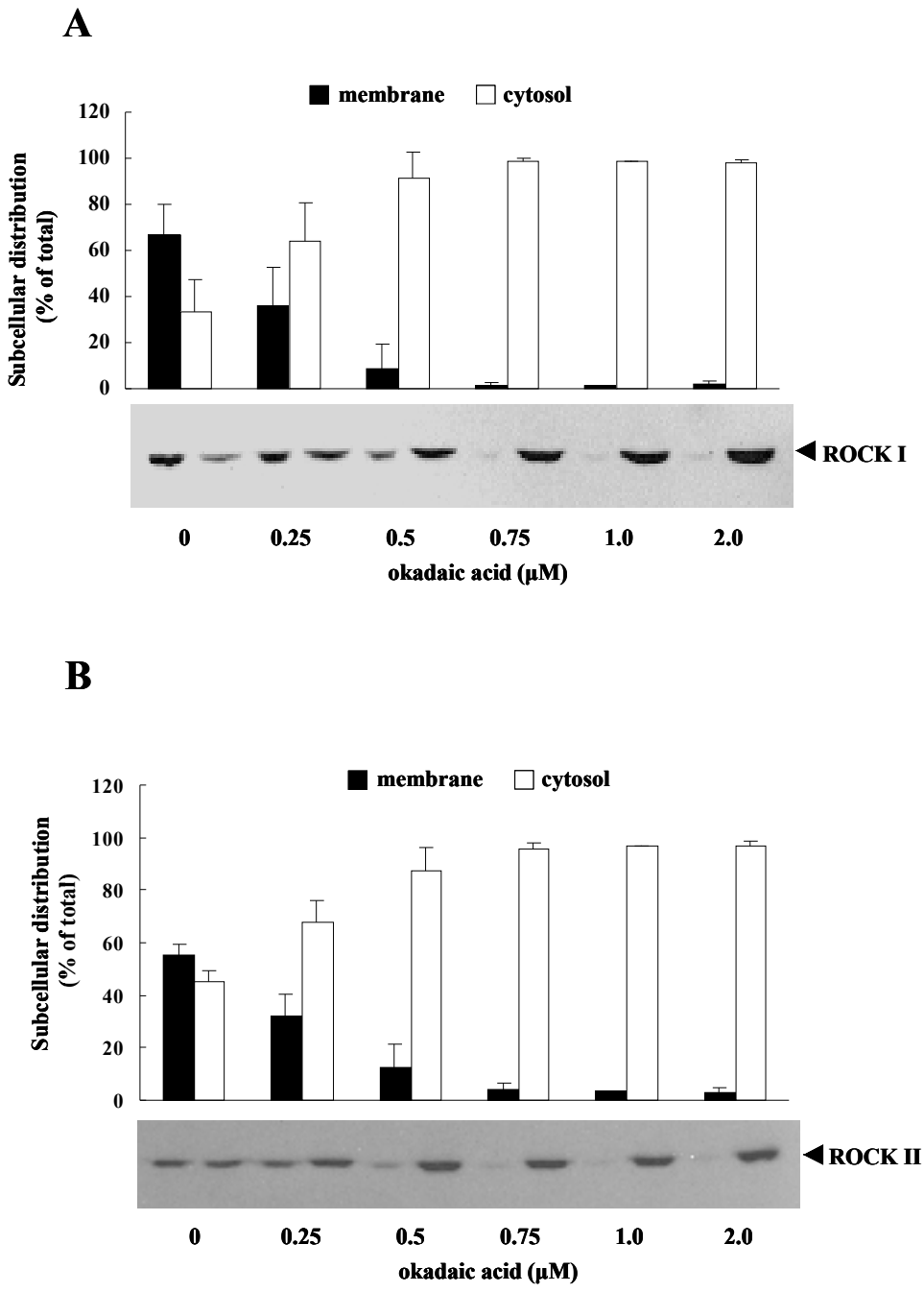


Fig. 2

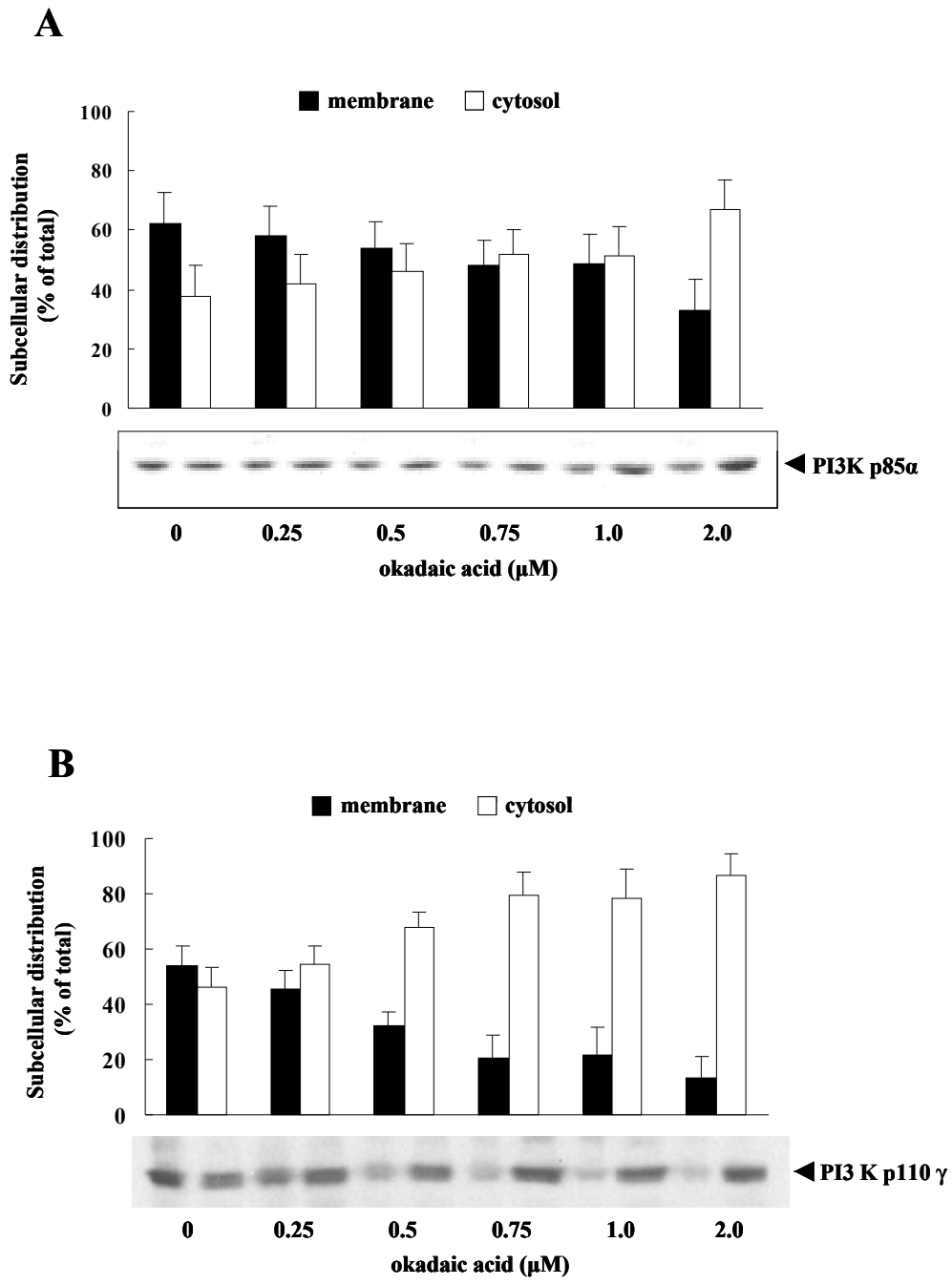

A
membrane
cytosol
Subcellular distribution (% of total)
100
80
60
40
20
0
PI3K p85α
0
0.25
0.5
0.75
1.0
2.0
okadaic acid (µM)
B
membrane
cytosol
Subcellular distribution (% of total)
100
80
60
40
20
0
PI3 K p110 γ
0
0.25
0.5
0.75
1.0
2.0
okadaic acid (µM)

Fig. 3

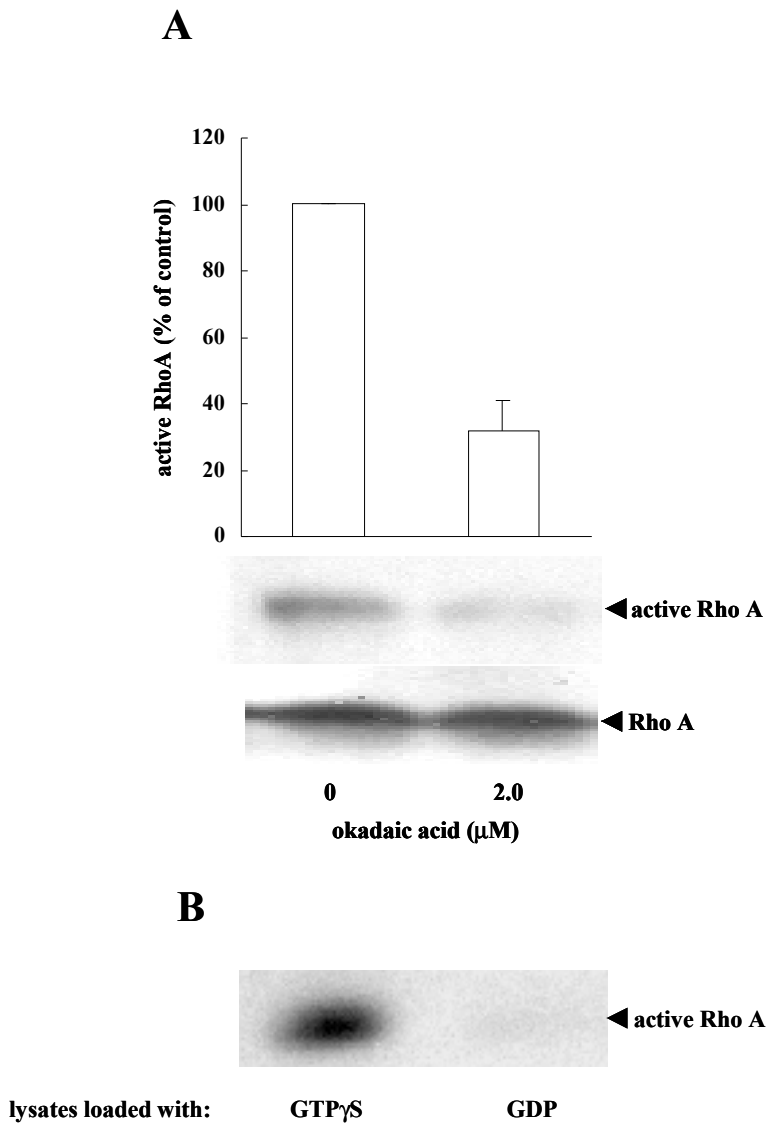

A
120
100
80
60
40
20
0
active RhoA (% of control)
active Rho A
Rho A
0
2.0
okadaic acid (μM)
B
active Rho A
lysates loaded with:
GTPγS
GDP

Fig. 4

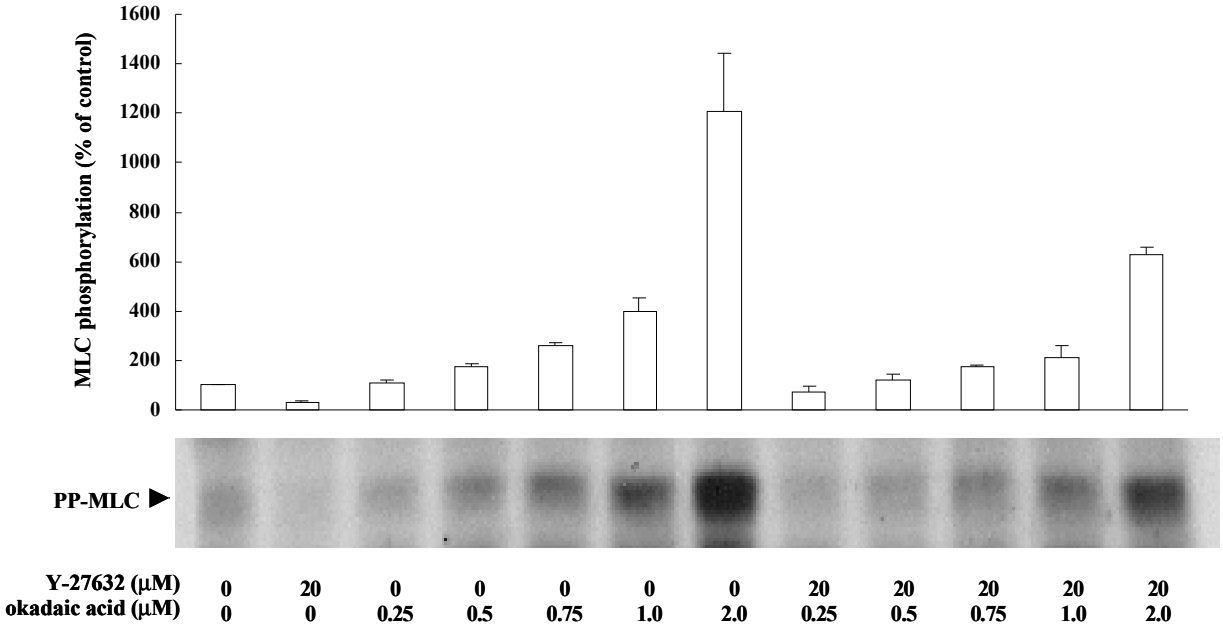

MLC phosphorylation (% of control)
1600
1400
1200
1000
800
600
400
200
0
PP-MLC
Y-27632 (μM) 0 20 0 0 0 0 0 20 20 20 20 20
okadaic acid (μM) 0 0 0.25 0.5 0.75 1.0 2.0 0.25 0.5 0.75 1.0 2.0

Fig. 5

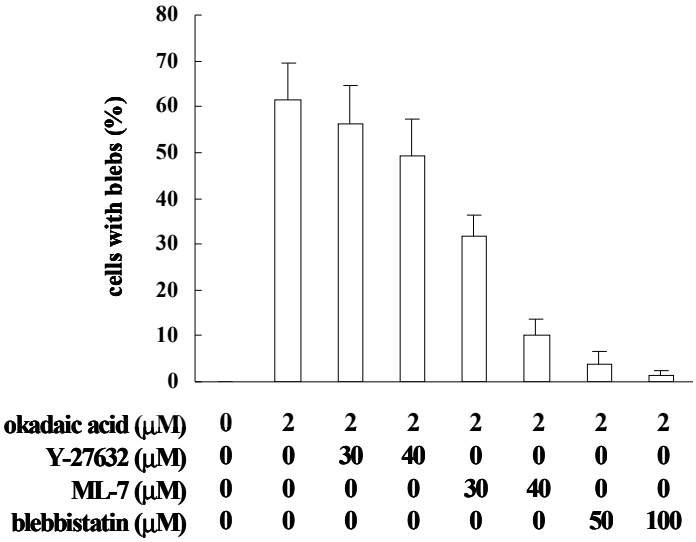
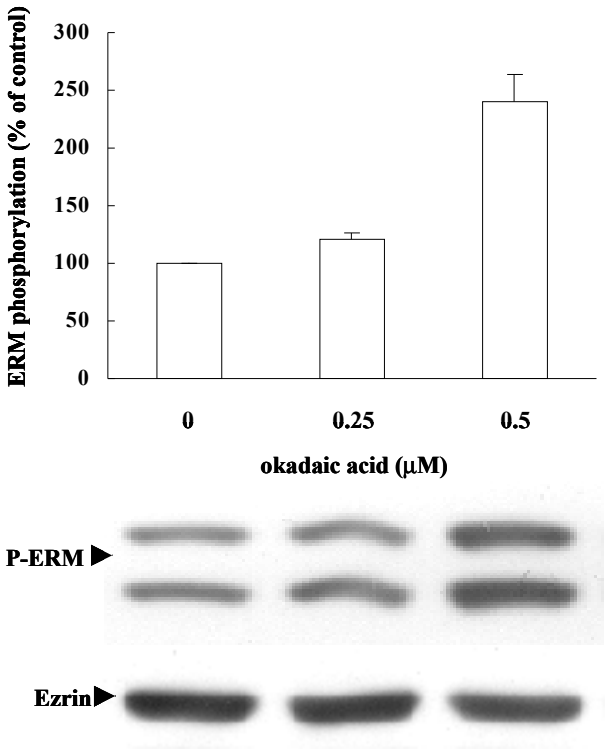

cells with blebs (%)
80
70
60
50
40
30
20
10
0
okadaic acid (μM) 0 2 2 2 2 2 2 2
Y-27632 (μM) 0 0 30 40 0 0 0 0
ML-7 (μM) 0 0 0 0 30 40 0 0
blebbistatin (μM) 0 0 0 0 0 0 50 100

Fig. 6


ERM phosphorylation (% of control)
300
250
200
150
100
50
0
0
0.25
0.5
okadaic acid (μM)
P-ERM
Ezrin

6. Conclusions

We have studied the regulation of cell migration by signalling pathways in Walker carcinosarcoma cells. These cells spontaneously polarize and migrate and are thus an excellent model system for investigating tumor cell locomotion. It has been shown recently that the proteins RhoA, Rho-kinase, and PI 3-kinase are constitutively activated in these cells and play key roles in the processes of polarization and migration.

In this study we have investigated downstream effectors of Rho/Rho-kinase. We could show that both Rho/Rho-kinase and MLCK control myosin activity in Walker carcinosarcoma cells, and that myosin activation is crucially required for development of polarity and migration. An appropriate level of Rho/Rho-kinase activity seems to be important for the regulation of MLC phosphorylation, cell polarity and migration. Rho/Rho-kinase inhibition as well as delocalised strong activation appear to prevent motile responses. Delocalized strong activation of ROCKI leads to increased MLC phosphorylation, membrane blebbing, and cell death. The decrease in MLC phosphorylation observed after introduction or expression of constitutively activated RhoA mutants indicates that high delocalised RhoA activity negatively regulates downstream signalling impacting on myosin regulation.

We also showed that phosphorylation and thus activation of ERM proteins requires activity of Rho but not Rho-kinase in Walker carcinosarcoma cells. Again, an appropriate level of Rho activity is required for maintaining cell polarity and constitutive ERM phosphorylation as inhibition and constitutive activation of Rho both decrease the level of ERM phosphorylation.

In a third part of the thesis we provided evidence that constitutive phosphatase activity might be required for membrane localization of Rho-kinase I and II and of PI 3-kinase 110γ and for maintaining constitutive levels of MLC and ERM phosphorylation and constitutive RhoA activity optimal for migration.

7. Curriculum Vitae

Name	Marc
Surname	Gutjahr
Date of Birth	December 2nd 1976
Place of Birth	La Chaux-de-Fonds
Nationality	Swiss
Marital Status	Single

Education

1991-1996	Matura Typus C, Gymnasium Langenthal, Switzerland
1996-1999	"Vordiplom" in Microbiology University of Berne, Switzerland
1999-2001	Lizentiat (Master of Science) Supervisor: Prof. Dr. E. Schweingruber Institute of Cell Biology, University of Bern, Switzerland.
2001-2005	Ph.D. Thesis Supervisor: Prof. Dr. V. Niggli Dept. of Pathology, University of Berne, Switzerland.

8. Acknowledgements

My special thanks go to Prof. Dr. Verena Niggli for giving me the opportunity to do my PhD thesis in her laboratory, for many interesting discussions and for her patience.

Many thanks to Christoph Oppliger for his continuous help with all the biochemical techniques, cell culturing and the good time in the laboratory.
I also thank the other lab members Patrick Lier, Alireza Dehghani-Zadeh, Alexandra Nievergelt, Anna Meszaros, Sophie Tornay, Jérémie Rossy, Manuela Schmid, and especially Silvia Gränicher for all the administrative work.

Furthermore, I would like to thank my family and my friends for supporting me during my studies.