p116Rip : a new player in RhoA signalling

Mulder, J.

$p116^{Rip}$: a new player in RhoA signalling

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Voor mijn ouders

Chapter 1

General Introduction

Introduction

Cells undergo morphological changes throughout the lifetime of organisms. The ability of cells to change shape is a necessity for key events such as cell division, attachment, and migration. As such, changes in cellular morphology are required during physiological processes such as immune responses, muscle contraction, neuritogenesis, wound healing, and angiogenesis. Similarly, pathological conditions, such as cancer, require changes in cellular morphology during disease progression.

The cytoskeleton, a dynamic, structural framework within the cell, mediates changes in morphology and provides mechanical support. Three types of filaments form the cytoskeleton: actin filaments, microtubules, and intermediate filaments (Fig. 1). The filaments and their associating proteins are essential for morphological changes and also communicate with one another. Each filament consists of joined single subunits. The assembly of single subunits into filaments (polymerisation) or disassembly of filaments into single subunits (depolymerisation) underlies the dynamic nature of morphological changes. This so-called remodelling of the cytoskeleton is tightly regulated by external and internal stimuli.

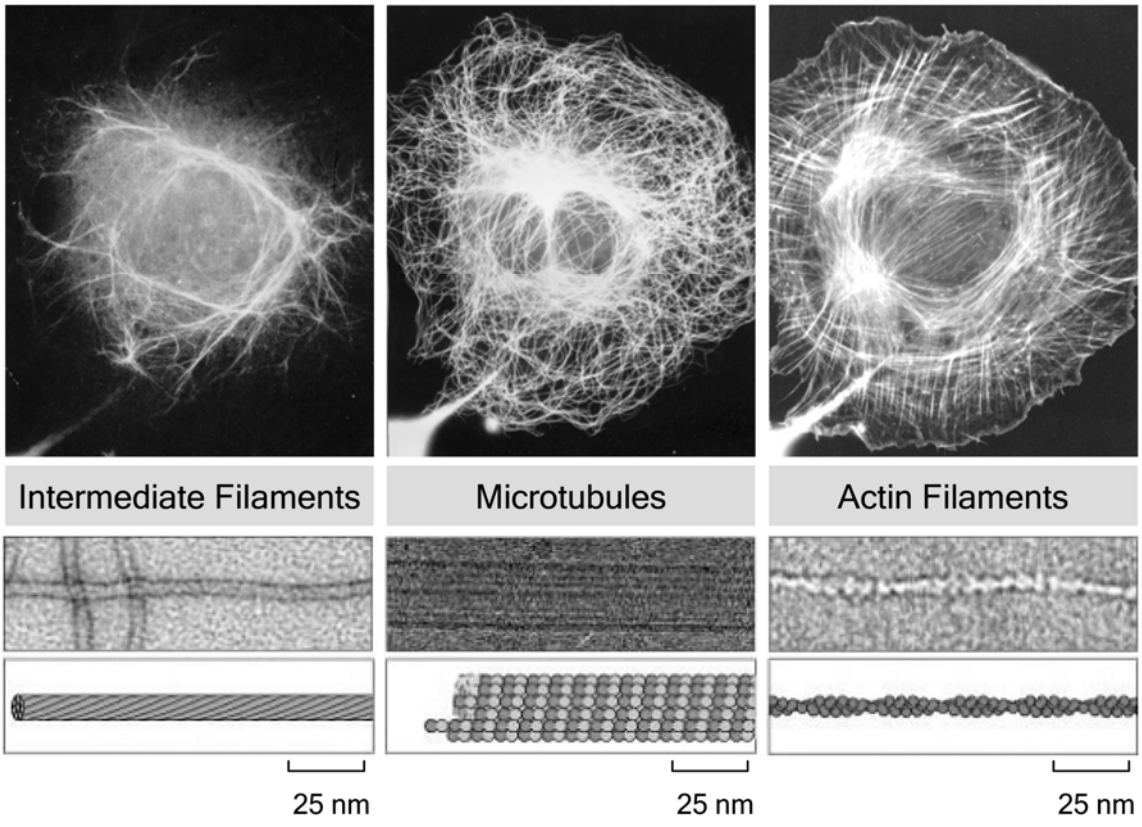


Figure 1. The cytoskeleton
The three types of cytoskeletal filaments in a fibroblast as detected by fluorescence microscopy using specific probes. The intermediate filaments (using vimentin antibodies); microtubules (using tubulin antibodies); and the actin cytoskeleton (using fluorescent phalloidin) (adapted from (Herzog et al., 1994)). The schematic diagram and electron micrographs in the lower panels show how these filaments are built from subunits (adapted from Essential Cell Biology (Alberts et al., 2003)).

The actin cytoskeleton consists of networks and bundles that extend throughout the cell and underneath the plasma membrane. Dynamic regulation of the actin cytoskeleton is required for changes in cell shape, anchorage and motility. The Rho family of small GTPases have a central role in linking signalling pathways to remodelling of the actin cytoskeleton (Burridge and Wennerberg, 2004; Ridley, 2001a). Specifically, the turnover rate and localisation of actin (de)-polymerisation is controlled by a wide range of actin-associated proteins that are the (in)direct targets of Rho GTPase signaling pathways. For instance, the GTPase RhoA signals to the actin-binding and motor protein myosin to exert force on actin filaments leading to mechanical tension within a cell. In this way, activation of the RhoA pathway leading to actomyosin contractility enables cells to undergo morphological changes in response to stimuli.

This thesis focuses on the function of $p116^{Rip}$, an actin-binding protein with scaffold function, in the RhoA pathway leading to contractility of the actin cytoskeleton.

Rho GTPases

Rho GTPases regulate a wide spectrum of cellular processes (Etienne-Manneville and Hall, 2002). Members of this family, including RhoA, Rac1 and Cdc42, were initially recognised as regulators of actin cytoskeleton and were subsequently shown to be involved in mechanical processes that require morphological changes such as cell polarity, division, adhesion, protrusion, and migration. However, subsequent research has demonstrated a role for Rho GTPases in a wide range of cellular activities besides actin remodelling including organisation of the microtubule cytoskeleton (Fukata et al., 2003; Wittmann and Waterman-Storer, 2001), vesicular trafficking (Ridley, 2001b; Qualmann and Mellor, 2003), cell-cycle progression (Ridley, 2004), cytokinesis (Matsumura et al., 2001), apoptosis (Coleman and Olson, 2002), and gene transcription (Sahai and Marshall, 2002). In addition to their physiological role, Rho GTPases influence key processes in cancer, including cell transformation, survival, invasion, metastasis and angiogenesis (Ridley, 2004; Malliri and Collard, 2003; Sahai and Marshall, 2002).

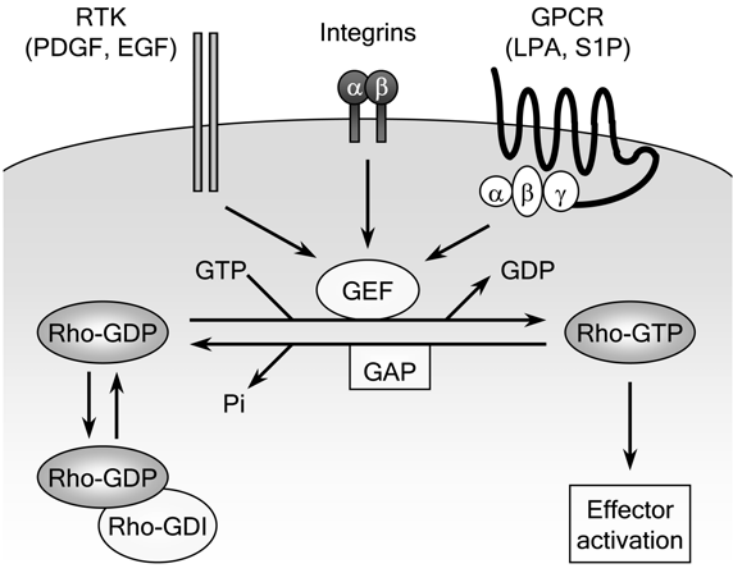


Figure 2. Rho GTPase activation
Rho-family GTPases are activated by various upstream signals including receptor tyrosine kinases (RTK) such as PDGF or EGF, integrins ($\alpha\beta$) and G-protein-coupled receptors (GPCR) such as LPA (Moolenaar et al., 2004) or S1P. They alternate between a GDP-bound (Rho-GDP) inactive state, and a GTP-bound (Rho-GTP) active state, which is regulated by GEFs, GAPs, and GDIs. S1P, sphingosine-1-phosphate; LPA, lysophosphatidic acid; PDGF, platelet-derived growth factor; EGF, epidermal growth factor.

Rho proteins were first identified by their homology to Ras hence the name Rho (Ras-homologous) (Madaule and Axel, 1985). Unlike Ras, Rho proteins themselves are not commonly mutated in tumours, instead their expression is often elevated in cancerous cells or mutations are found in proteins that regulate Rho protein activity. Constitutively active forms of RhoA and Rac1 induce an oncogenic phenotype in fibroblasts but to a much lesser degree than Ras. Moreover, the oncogenic properties of Ras have been shown to be critically dependent on RhoA, Rac1, and Cdc42 (Malliri and Collard, 2003; Ridley, 2004).

Similar to other members of the Ras superfamily, Rho proteins regulate signal transduction by acting as molecular switches that cycle between GDP and GTP-bound states (Fig. 2). Activation of Rho GTPases (exchange of GDP by GTP) is stimulated by guanine-nucleotide exchange factors (GEFs) and is often associated with translocation of Rho proteins to the cell membrane (Rossman et al., 2005). Most Rho GTPases have an intrinsic ability to hydrolyse GTP to GDP, which can be promoted by GTPase-activating proteins (GAPs) (Moon and Zheng, 2003; Peck et al., 2002). Rho proteins can also be sequestered in the cytoplasm in their GDP-bound form by guanine-nucleotide dissociation inhibitors (GDIs) (Olofsson, 1999). Cell-surface receptors, including integrins, growth factor receptors and G-protein-coupled receptors, act upon GEFs and GAPs to modulate Rho GTPase activity. In the GTP-bound, activated state, Rho proteins specifically bind to effector molecules that facilitate downstream signalling (Bishop and Hall, 2000; Aspenstrom, 1999). The effects of Rho proteins on actin remodelling are well established. Cdc42 induces cell polarisation and arranges actin polymerisation to form finger-like filopodial protrusions. Rac1 promotes the extension of lamellipodia at the leading edge of the cell and the formation of new focal contacts. RhoA promotes myosin-dependent contractility of actin filaments leading to the formation of actin stress fibres and focal adhesions. Additionally, Rho and Rac are mutually antagonistic since they suppress each other's activity, for example during cell migration, indicative of cross-talk between Rho GTPase signalling pathways (Burridge and Wennerberg, 2004).

RhoA-related subfamily

The mammalian Rho family of GTPases, consisting of over 20 family members, can be divided into 5 subfamilies (Burridge and Wennerberg, 2004; Wherlock and Mellor, 2002; Wennerberg and Der, 2004). Initially, it was assumed that other members of the Rho GTPase family were functional equivalents of their more-studied counterparts Rac1, Cdc42 and RhoA. In fact, they appear to have distinct effects on cell behaviour. For instance, RhoE/Rnd3, a member of the GTPase-deficient Rnd subfamily, inhibits Rho signalling. This occurs at two levels; by activating p190RhoGAP (Wennerberg et al., 2003) and inactivating the RhoA effector Rho kinase 1 (ROCK1) (Riento et al., 2003). Moreover, within the Rho-related subfamily, comprised of RhoA, RhoB, and RhoC, members are not functionally redundant. The clearest difference in functions between the Rho members becomes apparent in cancer (as reviewed in (Wheeler and Ridley, 2004)). However, the Rho-related members do not appear to differ in their ability to interact with upstream GEFs or downstream effectors. The differences in function are most likely caused by differential transcriptional regulation and subcellular localisation (Wennerberg and Der, 2004; Wheeler and Ridley, 2004). RhoA is the best-characterised member of the Rho-related protein family. This thesis will focus on the events that occur downstream of RhoA activation.

RhoA signalling: ROCK and mDia

The RhoA pathway is required for the generation of tension in non-muscle cells that eventually leads to cell rounding, retraction, or contraction. The underlying force of contractility is mediated through phosphorylation of the motor protein myosin II. Phosphorylation of the regulatory myosin light chain (MLC) of myosin II enhances the actin-binding and actin-induced ATPase activity of myosin. Together with changes in actin polymerisation and stabilisation, MLC phosphorylation triggers contractility (Amano et al., 1998). RhoA induces myosin-dependent contractile force on actin filaments through the recruitment and activation of two of its effectors, the mammalian ortholog of *Drosophila melanogaster* diaphanous (mDia) and Rho-kinase (ROCK) (Fig. 3).

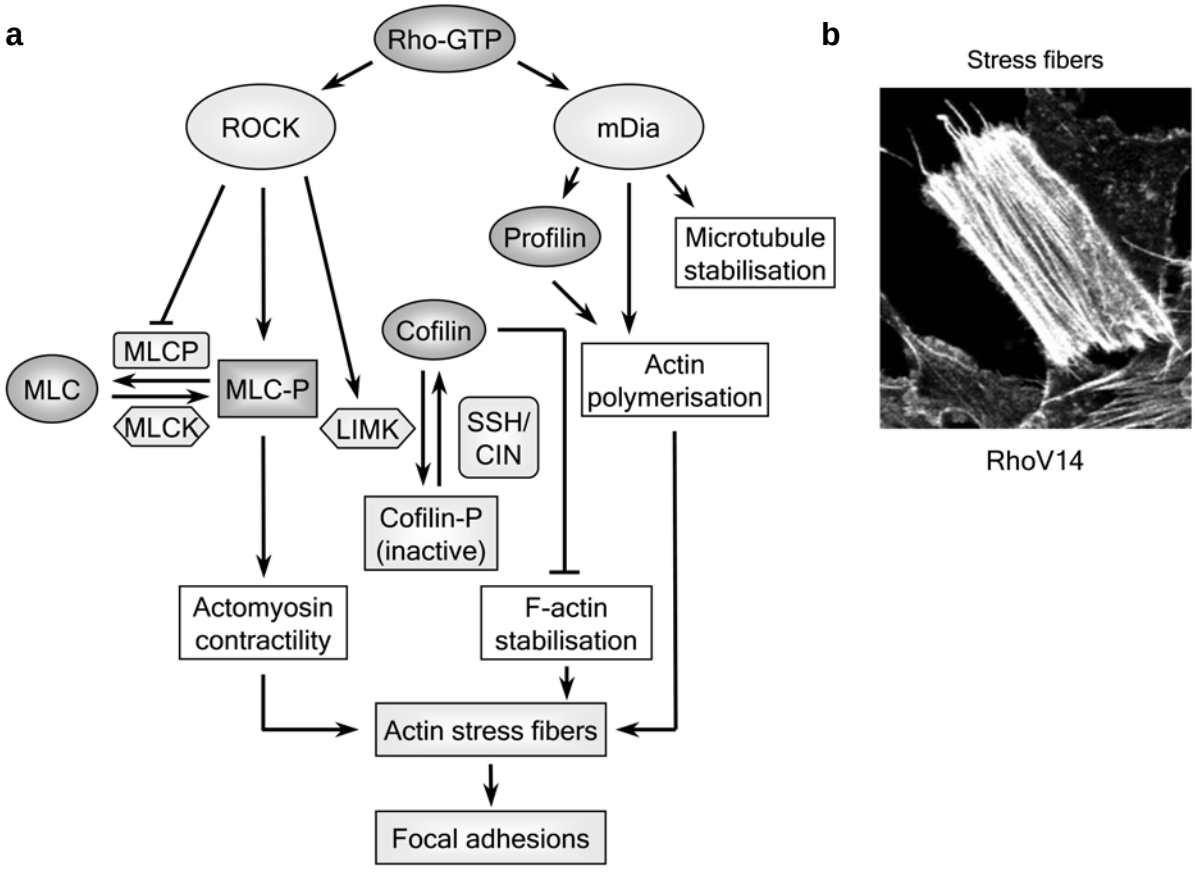


Figure 3. RhoA signalling
(**a**) Rho is a crucial modulator of remodelling of both the actin and microtubule cytoskeleton and cell motility, through the activation of its effector proteins ROCK and mDia. Activation of these effectors leads to the stabilisation of actin filaments, actin polymerisation, and increased actomyosin contractility, thereby promoting the formation of stress fibres, focal adhesion assembly and subsequent cell adhesion. See text for further details. ROCK, Rho-kinase; LIMK, LIM-kinase; MLC, myosin light chain; MLCP, MLC phosphatase; MLCK, MLC-kinase; SSH, slingshot; CIN, chronophin. (**b**) Actomyosin contractility induced by RhoA. The formation of actin stress fibres by transient expression of dominant active RhoV14 in a NIH3T3 fibroblast as visualised by phalloidin staining.

ROCK activation positively contributes to actin-myosin dependent force generation through the phosphorylation of a number of downstream target proteins (Riento and Ridley, 2003). ROCK induces an increase in MLC phosphorylation primarily by phosphorylation of the myosin-binding subunit (MBS) of myosin light chain phosphatase (MLCP), which leads to

inhibition of MLCP (Kimura et al., 1996; Kawano et al., 1999). Like MLC kinase (MLCK), ROCK can also phosphorylate MLC directly on residue Ser19 (Totsukawa et al., 2000; Amano et al., 1996). The antagonist of the Rho/ROCK pathway, MLCP, is a heterotrimer consisting of a catalytic subunit (PP1c), a 20-kDa protein of unknown function (M20), and MBS, which targets MLCP to its substrates including MLC (Ito et al., 2004). *Drosophila melanogaster* MBS mutants fail to complete dorsal closure, suggesting that this process requires spatially regulated myosin activation (Tan et al., 2003; Mizuno et al., 2002). Likewise, *Caenorhabditis elegans mel-11*, which encodes MBS, and *let-502*, which encodes ROCK, have opposite functions in embryonic elongation (Wissmann et al., 1999). Besides regulation of myosin phosphorylation, ROCK controls the stabilisation of F-actin by activating LIM kinases. LIM kinases phosphorylate and thereby inactivate the actin-severing protein cofilin (Maekawa et al., 1999), leading to stabilisation of actin filaments (Riento and Ridley, 2003). A specific phosphatase called slingshot activates ADF/cofilin by removing the inhibitory phosphate (Niwa et al., 2002), for example during axon growth (Ng and Luo, 2004). Another, unrelated cofilin phosphatase, chronophin, was identified recently (Gohla et al., 2005). Phosphorylation of other ROCK targets is also likely to play a role in actomyosin dependent contractility (Riento and Ridley, 2003). The Rho-ROCK pathway is essential for myosin contractility as shown by expression of dominant-negative forms of ROCK or by pharmacological inhibition of ROCK activity (Hirose et al., 1998; Ishizaki et al., 1997).

ROCK acts in concert with the other downstream target of RhoA, mDia, to induce the formation of stress fibers (Watanabe et al., 1999; Nakano et al., 1999). mDia belongs to the family of formin-homology containing family of proteins, which have been implicated in actin assembly. The binding of RhoA to mDia leads to the unfolding and subsequent activation of this scaffold protein. mDia facilitates actin nucleation and actin polymerisation by the actin-binding protein profilin (Li and Higgs, 2003). mDia also regulates the orientation and stabilisation of microtubules downstream of RhoA (Palazzo et al., 2001; Ishizaki et al., 2001), for example during cell migration (Watanabe et al., 2005). The mDia and ROCK pathways described here are involved in cell migration and neuritogenesis but also in RhoA-dependent signalling to the nucleus.

RhoA signalling to the nucleus

In addition to their effects on the actin cytoskeleton, Rho GTPases induce the expression of genes associated with cell proliferation and cell-cycle progression (Coleman et al., 2004). RhoA contributes to cell-cycle progression by increasing the levels of cyclin D1 through promoting sustained activation of ERK (Welsh et al., 2001) and repression of the CDK inhibitors p27Kip1 and p21Cip1 (Olson et al., 1998). Moreover, RhoA stimulates the expression of the c-fos and c-jun proto-oncogenes, which are members of the AP1 family of transcription factors that play a key role in normal and aberrant cell growth (Treisman et al., 1998). RhoA stimulates c-jun expression through ROCK-dependent JNK activation, that occurs independently from the ability of ROCK to promote actin polymerisation (Marinissen et al., 2004). In contrast, expression from the c-fos serum response element (SRE) can be regulated by either MAPK signalling pathways or RhoA-dependent changes in actin dynamics. Ultimately, the pathways leading to c-jun and c-fos expression converge in the nucleus to regulate AP1 activity.

Expression from the c-fos SRE regulatory element requires the binding of the serum response factor (SRF) and the association of SRF with different cofactors. The ternary

complex factor (TCF) family of Ets domain proteins are controlled through MAP kinase signal pathways (Treisman, 1996). In contrast, coactivators of the megakaryocytic acute leukemia (MAL) family are largely regulated through Rho GTPase signalling, as described in more detail below. Association of either TCF or MAL with the transcription factor SRF appears to be mutually exclusive (Miralles et al., 2003).

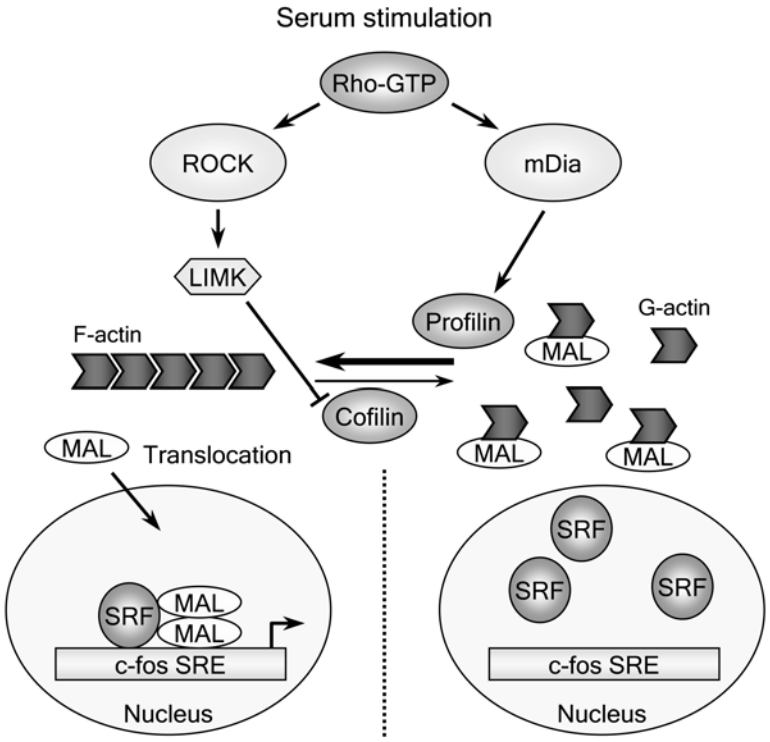


Figure 4. Model for the role of MAL in Rho-mediated activation of SRF
In serum-starved cells, MAL is predominantly cytoplasmic and confiscated by actin monomers. Upon serum stimulation, Rho is activated and causes an accumulation of F-actin and a corresponding decrease in the level of G-actin through the activation of the downstream effectors ROCK and mDia. As a consequence, MAL is no longer sequestered by G-actin and relocates to the nucleus where it associates with SRF as a dimer and activates SRE-mediated gene expression. SRF, serum response factor; SRE, serum response element; MAL, megakaryocytic acute leukaemia protein.

Downstream of RhoA, SRF activity is regulated as a consequence of actin reorganisation. Activation of RhoA induces actin polymerisation and subsequent SRF activation through two effectors, ROCK and mDia, that together induce F-actin assembly and stabilisation (see Figure 3). The contribution of either the Rho-mDia-profilin or the Rho-ROCK-LIMK-cofilin pathway to SRF activation appears to be cell type specific (Geneste et al., 2002). Recently, the SRF cofactor MAL, was shown to be a cellular sensor for the amount of unpolymerised, monomeric actin (G-actin) within cells (Miralles et al., 2003). Upon depletion of the G-actin pool, MAL redistributes to the nucleus where it mediates SRF activation (Fig. 4). MAL regulation thus provides a direct link between actin cytoskeletal dynamics in the cytoplasm and transcriptional activation in the nucleus.

The RhoA pathway and neuritogenesis

The organisation of the nervous system relies primarily on the morphological complexity of neurons. Neurons extend neurites, one of which differentiates into an axon, whereas the others become dendrites (Goldberg, 2003) (Fig. 5A). The growth cone, located at the tip of axons and dendrites, is a motile structure consisting of filopodia and lammellipodia that explores the environment (see Fig. 7B). The morphological changes that occur during the development of a neuron establish a high degree of polarity and depend on the regulation of the actin and microtubule cytoskeleton in response to extra-or intracellular cues (Luo, 2002; da Silva and Dotti, 2002). Accordingly, Rho GTPases are key regulators of neuronal polarity,

axon outgrowth and retraction, axon guidance, dendrite development, axon regeneration, and neuronal migration (Govek et al., 2005).

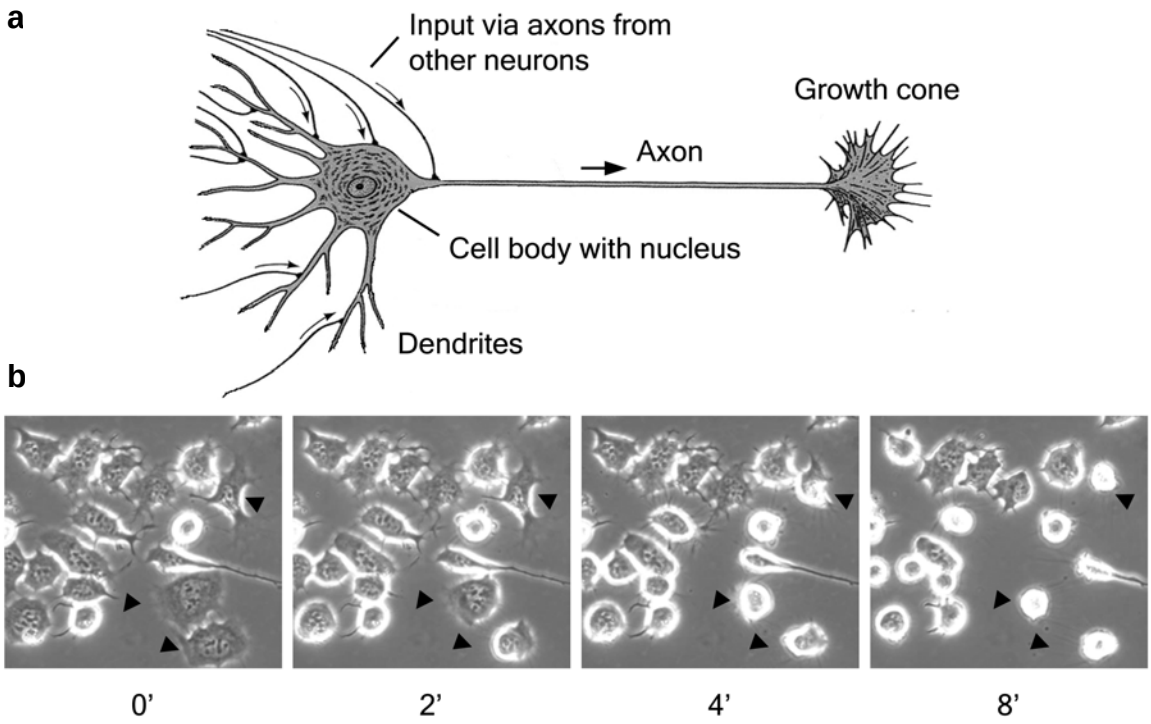


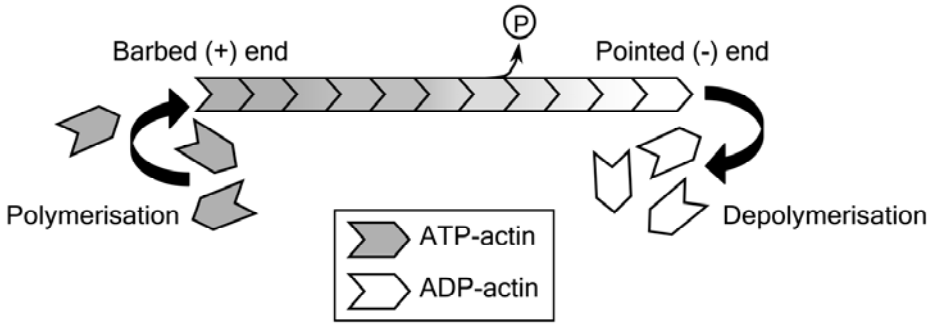
Figure 5. Neuronal morphology
(**a**) Schematic diagram of a typical vertebrate neuron. The arrows indicate the direction in which signals are transmitted. The axon conducts signals from the cell body, while the multiple dendrites receive signals from the axons of other neurons. The nerve terminals (growth cones) end on the dendrites or cell body of other neurons or on other cell types, such as muscle or gland cells. (**b**) LPA-induced neurite retraction of N1E-115 neuroblastoma cells. The LPA induced neurite retraction and cell rounding is dependent on the RhoA/ROCK pathway leading to actomyosin contractility. N1E-115 cells were serum starved for 4 hours and LPA (1 µM) was added, and subsequent changes in cell morphology were monitored by time-lapse microscopy (time-lapse kindly provided by P. Ruurs).

Whilst Rac1 and Cdc42 are positive regulators of neurite outgrowth, RhoA inhibits neurite extension (Luo, 2000; Govek et al., 2005). As is the case with migration in other cells, the force that underlies directional movement of the neuronal axon and growth cone is generated by myosin (Brown and Bridgman, 2004). In neuroblastoma cells and in primary neurons, regulation of myosin II activity by the RhoA-ROCK pathway (Fig. 3, and Fig. 5B) has been shown to be crucial for neurite elongation, guidance, and branching (Luo, 2000). Overexpression of constitutively active RhoA induces neurite retraction and arrest growth in neuronal cell lines (Jalink et al., 1994; Kozma et al., 1997) and in primary neurons (Bito et al., 2000). Conversely, inactivation of RhoA by ADP-ribosylation using the C3-exoenzyme (a specific RhoA inhibitor) promotes neurite extension and growth cone motility and abolishes lysophosphatidic acid (LPA)-induced actomyosin contractility (Jalink et al., 1994). Likewise, inactivation of ROCK produces a similar effect in neuroblastoma cells and cerebellar granule cells (Hirose et al., 1998; Bito et al., 2000) and blocks LPA-induced neurite retraction and myosin II phosphorylation (Hirose et al., 1998).

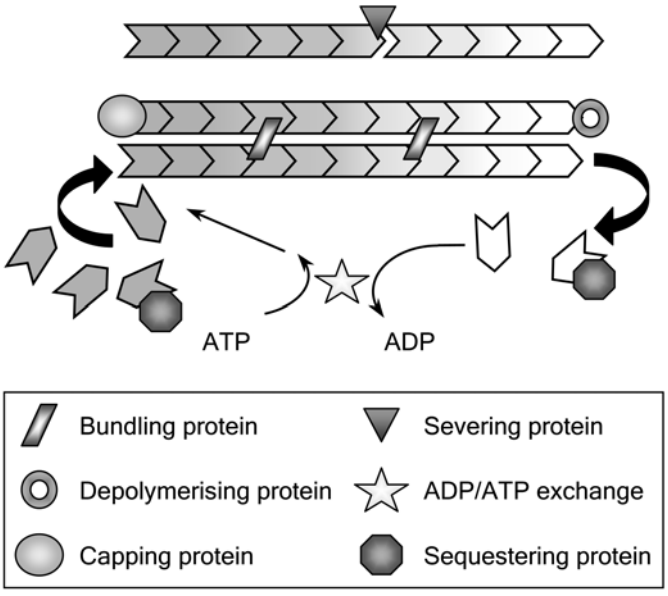
Antisense and knock-out strategies against the two different forms of the conventional myosin II present in neurons reveal that myosin IIA is required for neurite

retraction and myosin IIB induces growth cone motility and neurite outgrowth (Wylie and Chantler, 2003; Bridgman et al., 2001). Apart from myosin, neuronal development is regulated by signalling pathways that target other actin-binding proteins (Dent and Gertler, 2003). For instance, in mammalian hippocampal neurons it was recently shown that the regulation of actin stability during neuritogenesis occurs via RhoA/ROCK-dependent modulation of the downstream effector and actin-binding protein profilin IIa (da Silva et al., 2003). In addition, the actin depolymerisation factor cofilin, whose activity is regulated by the ROCK/LIMK pathway (Fig. 3), appears to be essential for axon growth in *Drosophila melanogaster* neurons (Ng and Luo, 2004).

BOX 1. Actin and actin-binding proteins



Treadmilling Filamentous (F)-actin is asymmetric and the two ends retain different kinetic characteristics. Actin monomers assemble much more rapidly at the 'barbed end' compared to the 'pointed end' (these names correspond to the arrowhead appearance of myosin heads bound to actin filaments). Subunit treadmilling occurs at a steady state in which no net increase in polymerised actin is observed. The critical concentration (of actin monomers) of the pointed end is higher than that of the barbed end. At a monomer concentration situated between the values of critical concentrations of the barbed and pointed ends, there is a net dissociation of monomers (bound to ADP) from the pointed end, balanced by the addition of monomers (bound to ATP) to the barbed end. This leads to treadmilling; the relocation of the filament in the direction of the barbed end without affecting the length of the filament. ATP hydrolysis in the filament is essential to maintain treadmilling. See text for further details.



Many *actin-binding proteins* (ABPs) bind to F- or G-actin and influence its dynamics or state. Among ABPs, some link actin filaments in a loose network (crosslinking proteins, e.g. filamin A), in tight bundles (bundling proteins, e.g. fascin and α-actinin) (see also Fig. 5), or anchor filaments to membranes (e.g. the members of the ERM (ezrin/radixin/moesin) family). Others bind to the barbed end of a filament and prevent further elongation (capping proteins, e.g. gelsolin), fragment filaments (severing proteins, e.g. ADF/cofilin), or might favour the depolymerisation of pointed ends (e.g. ADF/cofilin). ABPs also regulate the addition of monomers by sequestering them or favouring ADP/ATP exchange (e.g. profilin) (adapted from (Revenu et al., 2004)).

The actin cytoskeleton

The above paragraphs underline the significance of dynamic actin regulation for cell morphology, migration, neuritogenesis, and modulation of gene transcription. The dynamic nature of the actin cytoskeleton is enabled by the ability of actin to rapidly switch between the filamentous polymeric form (F-actin) and a monomeric globular (G-actin) form. This allows a cell to modify the structure of the actin cytoskeleton quickly and adequately in response to extra- and intracellular signals. Actin filaments are polar structures, with a pointed (minus) and a barbed (plus) end. ATP-bound monomers bind preferentially to the barbed end of a pre-existing filament. ATP is rapidly hydrolysed following polymerisation, resulting in ADP-bound monomers at the barbed end of the filament. The subsequent loss of ADP-bound monomers at the pointed ends leads to growth of the filament in the direction of the barbed end, a process known as actin filament treadmilling (Box 1).

The dynamics of the actin cytoskeleton are tightly regulated by a plethora of actin-binding proteins (ABPs) and the hydrolysis of ATP by actin (Pollard and Borisy, 2003; Revenu et al., 2004; Rafelski and Theriot, 2004). ABPs initiate or terminate polymerisation, link actin filaments to each other or to the membrane, whilst others additionally act as scaffolding proteins (Box 1) (Winder and Ayscough, 2005). A higher order structure of actin filaments is accomplished by the joining of actin filaments by cross-linking and bundling ABPs (Fig. 6). Upstream signalling pathways regulate the activity of ABPs through changes in intracellular pH, protein phosphorylation, cytosolic Ca^{2+} concentrations, as well as phosphoinositide levels (Revenu et al., 2004; Janmey and Lindberg, 2004; Yin and Janmey, 2003; Hilpela et al., 2004).

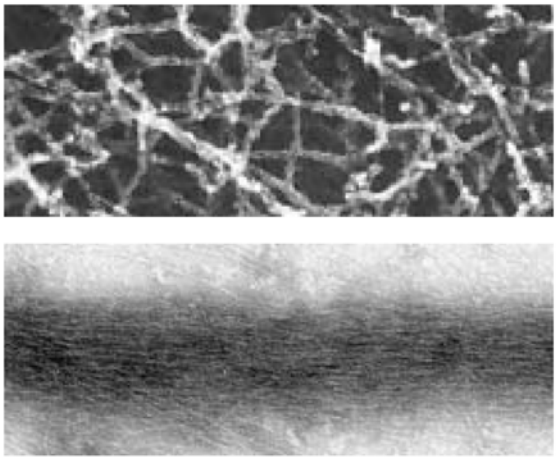


Figure 6. Cross-linking of actin filaments by ABPs
In vitro experiments using electron microscopy to visualise the structure of the actin filament network. In the presence of an actin cross-linking protein the actin network has a gel-like organisation (above, adapted from (Niederman et al., 1983)). The presence of an actin-bundling protein leads to the formation of actin bundles (below) (Mulder et al., 2003).

The interplay of distinct ABP activities can lead to the formation of specific actin-based structures. One example of this is the formation of filopodia and lamellipodia at the leading edge of cells during cell migration and in the neuronal growth cone (Fig. 7A, B). In lamellipodia, the actin filaments are short and highly branched with numerous barbed ends facing the leading edge. Branching occurs when the pointed end of one actin filament joins the side of another filament. This branching generally occurs at an angle of around 70° and is dependent on the action of distinct ABPs, the Arp2/3 (actin-related protein) complex. Capping proteins at the leading edge are responsible for the maintenance of short actin filaments in lamellipodia. Filopodia are thought to arise within the lamellipodial network when capping of actin filaments is prevented, resulting in the elongation and bundling of actin

filaments and subsequent protrusion of the cell membrane (Revenu et al., 2004; Pollard and Borisy, 2003) (Fig. 7C, D).

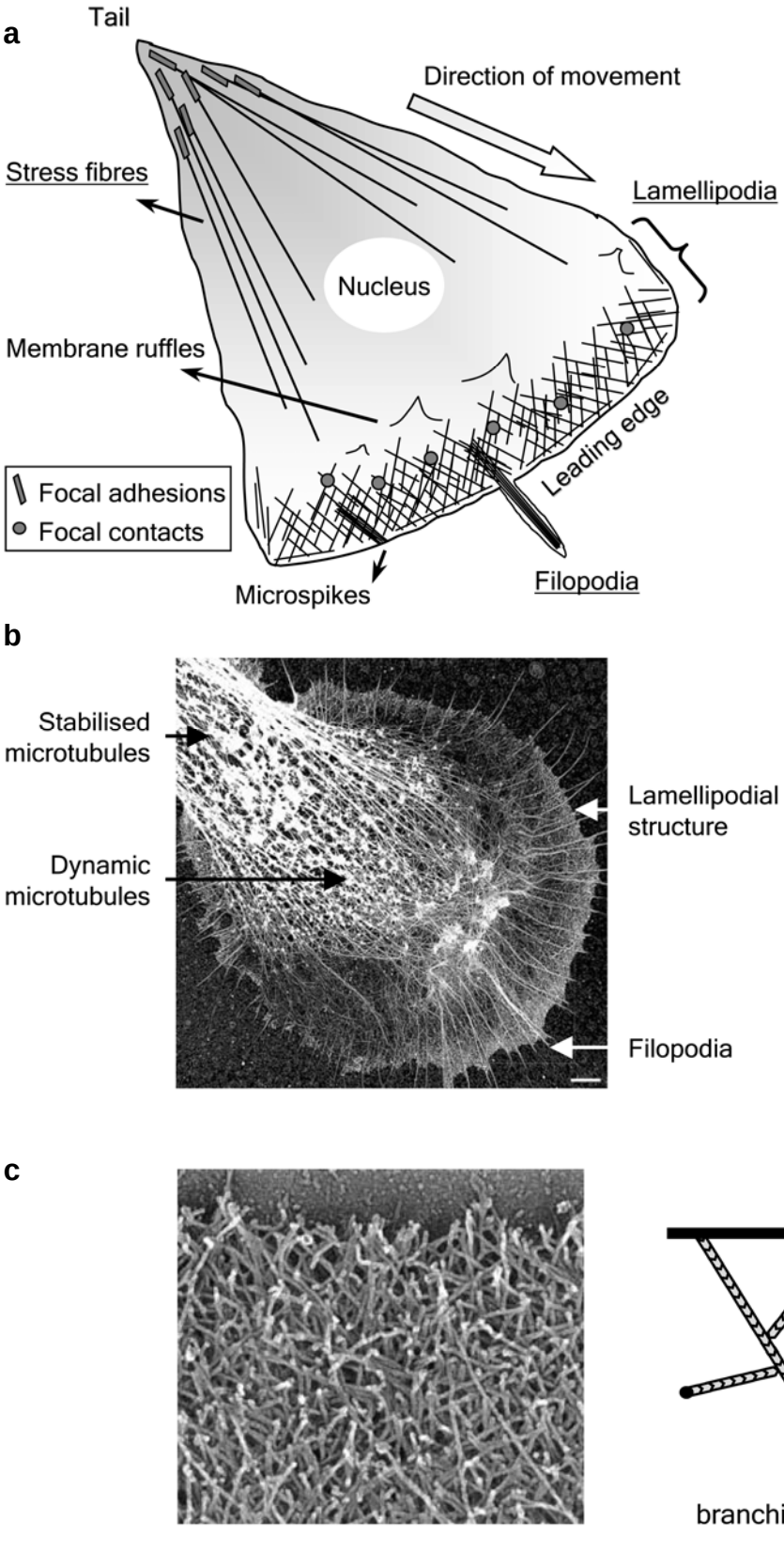


Figure 7. Actin-based structures
(**a**) Schematic diagram of the actin cytoskeleton in a migrating fibroblast. The different forms of actin filaments are illustrated. At the leading edge, branched actin filaments exist in lamellipodia and parallel F-actin bundles in filopodia and microspikes (which are filopodia that not protrude beyond the membrane). The rear of the cell, the tail, contains contractile bundles; the actin stress fibres. Substrate adhesion sites are also indicated: focal complexes in lamellipodia and filopodia and focal adhesions at the termini of stress fibres. (**b**) Electron micrograph showing the cytoskeletal ultrastructure of a growth cone from an *Aplysia* bag cell neuron. Filopodia with F-actin bundles and lamellipodial actin networks are visible. Also indicated are the stabilised and dynamic microtubules (adapted from (Schaefer et al., 2002)). Bar, 3.5 µm.

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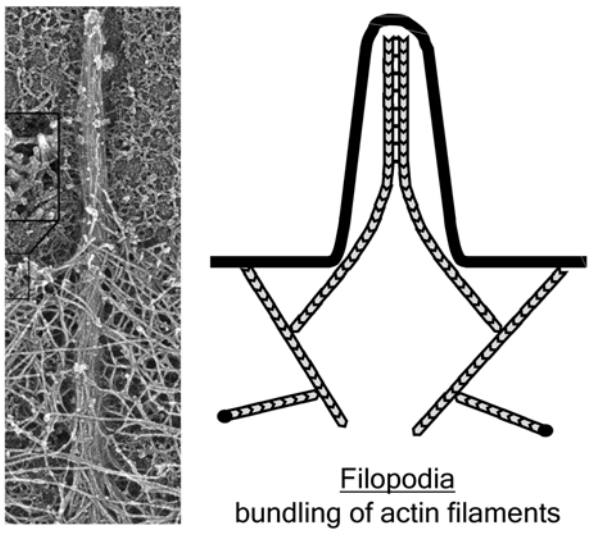

Figure 7-*continued*
Representation of the mechanisms of lamellipodia and filopodia formation at the leading edge of cells. The branched dendritic structure of a keratocyte lamellipodium and the parallel actin bundles in the filopodium of a B16F mouse melanoma cell are shown (adapted from (Svitkina et al., 2003; Svitkina et al., 1997). (**c**) The schematic diagrams indicate how lamellipodia are formed when capping activity predominates. (**d**) Filopodia arise from the lamellar network when elongation of actin filaments predominates and/or capping activity is reduced. See text for further details.

Actin and actin-binding proteins in the nucleus

Besides signalling to the nucleus through changes in actin dynamics in the cytoplasm as described above, recent evidence implicates a role for actin in several nuclear activities including transcription, chromatin remodelling and nucleocytoplasmic trafficking (Bettinger et al., 2004; Shumaker et al., 2003; Blessing et al., 2004; Olave et al., 2002). The nonmuscle isoform β-actin appears to be involved in transcription by all three classes of nuclear RNA polymerases. Firstly, actin appears to be essential for the initiation of transcription by RNA polymerase II (pol II) as the presence of β-actin in pre-initiation complexes was shown to be crucial for the formation of these complexes (Hofmann et al., 2004). Likewise, the association of β-actin with pol III is required for basal pol III transcription in a purified transcription system (Hu et al., 2004). Finally, β-actin associates with ribosomal RNA genes and is indispensable for transcription by pol I (Philimonenko et al., 2004). In the latter study it was also shown that the nuclear, actin-binding, and motor protein myosin I plays a positive role in pol I transcription.

Additionally, nuclear actin is an integral component of chromatin remodeling complexes (for review, see Bettinger et al. 2004 and Olave et al. 2002). Actin stimulates the ATPase activity of the Brg1 subunit in the SWI/SNF-like BAF complex, and is required for association of the complex with the nuclear matrix (Zhao et al., 1998). Moreover, actin filament binding of the complex is regulated in a phosphatidylinositol 4,5-biphosphate (PIP_2)-dependent manner (Rando et al., 2002). Actin may fulfil a structural role by anchoring chromatin remodelling complexes to the nuclear matrix. Alternatively, the ATPase activity of actin may be used to regulate cycles of configuration and formation of the complex (Bettinger et al., 2004; Olave et al., 2002).

Most of the nuclear actin is thought to be organised differently from its cytoplasmic filamentous form, as filamentous actin cannot be detected in the nucleus. Nuclear actin might exist in a monomeric form, as short filaments, or may adopt a novel oligomeric form (Bettinger et al., 2004). Therefore, it is tempting to assign a role for ABPs in nuclear actin regulation. Indeed, the list of ABPs that are localised in the nucleus is expanding (Rando et al., 2000; Bettinger et al., 2004). Nuclear localisation of ABPs is often specific and a target for regulation as illustrated for instance by the accumulation of the dephosphorylated, active form of the ABP cofilin in the nucleus (Olave et al., 2002). Furthermore, recent evidence

suggests that several members of the gelsolin family of ABPs have a role as transcriptional coactivators in the nucleus (Archer et al., 2005). Overall, the emerging evidence regarding the function of nuclear actin indicates a possible role for ABPs in nuclear events.

p116^{Rip}

Mouse p116^{Rip} (predicted size 116kDa, Rho-interacting protein) was first identified as a putative binding partner of RhoA in a yeast two-hybrid screen using dominant active RhoV14 as bait (Gebbink et al., 1997). The p116^{Rip} sequence contains several protein interaction domains, including two pleckstrin homology domains (PH), two proline-rich stretches and a C-terminal coiled-coil domain, but lacks any known catalytic motif.

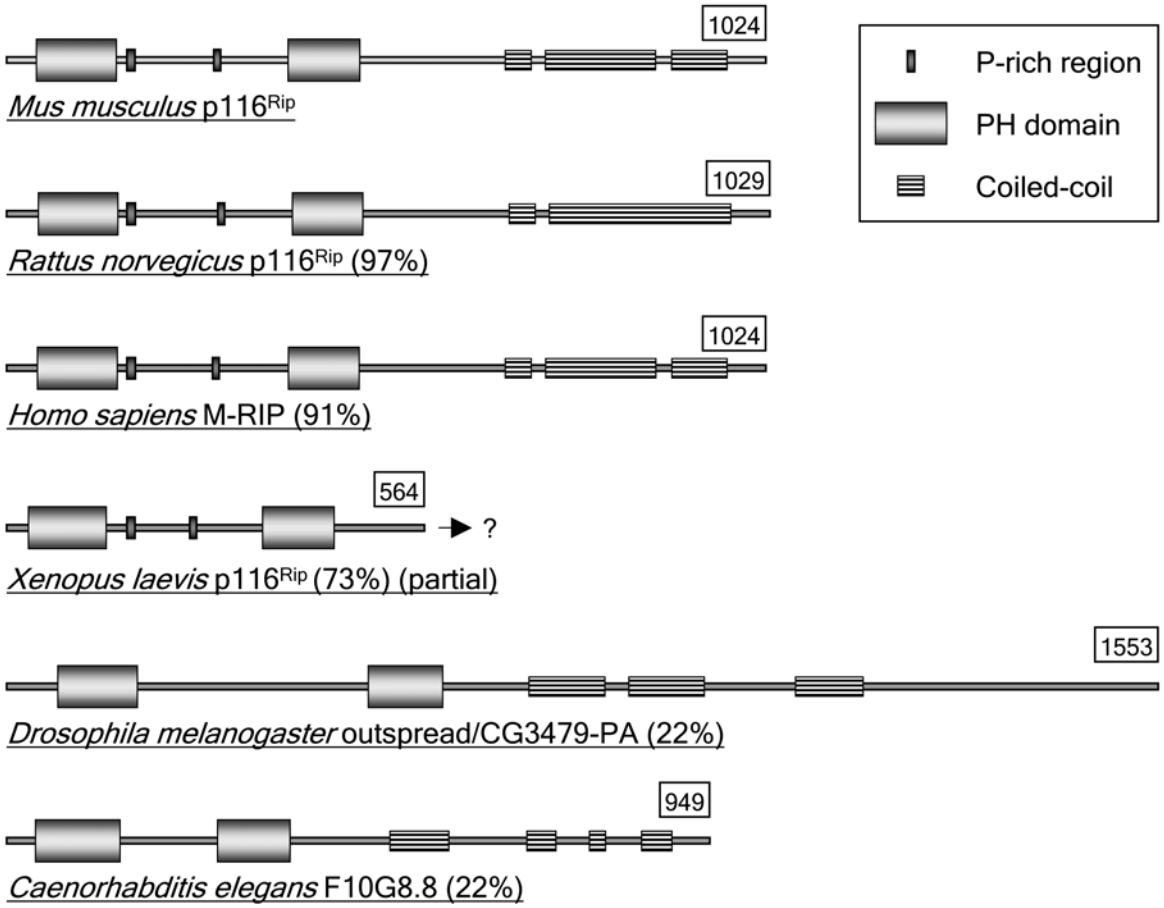

Figure 8. Structural alignment of p116^{Rip}
Schematic representation of the domain structure of p116^{Rip} orthologues as defined by the SMART program. The degree of homology to murine p116^{Rip} is indicated as the percentage of identity at amino acid level. The amino acid length of the p116^{Rip} orthologues is indicated on the right and boxed. GenBank^{TM} accession numbers: *Mus musculus* p116^{Rip}, U73200; *Rattus norvegicus* p116^{Rip}, AF311311; *Homo sapiens* M-RIP, AY296247; *Xenopus laevis* p116^{Rip}, BC073109; *Drosophila melanogaster* outspread or CG3479-PA, NP_723879; *Caenorhabditis elegans* F10G8.8, NP_492655. See text for further details.

The p116^{Rip} protein is widely expressed in a variety of tissues as determined by Gebbink et al. and data present in several expression databases such as Gene Expression Atlas (http://symatlas.gnf.org/SymAtlas/). Initial results indicated that p116^{Rip} expression in neuronal cells inhibited Rho- and LPA-induced contractility and promotes neurite outgrowth.

This thesis investigates p116^{Rip}'s cellular function with particular emphasis upon its interplay with RhoA, F-actin, and MBS.

p116^{Rip} orthologues

The p116^{Rip} protein is conserved throughout evolution. p116^{Rip} orthologues are present in organisms ranging from *Caenorhabditis elegans* to *Homo sapiens* (Fig. 8). These orthologues all possess a similar domain organisation: two PH domains and a C-terminal coiled-coil structure. The PH domains, in particular, display a high degree of conservation. The N-terminal PH domains of the *C.elegans* ("F10G8.8") and *D.melanogaster* ("outspread") orthologues are 37% and 36% identical at amino acid (aa) level to that of mouse p116^{Rip}, respectively. The second PH domains share 37% and 34% of identity with that of mouse p116^{Rip}, respectively. Two proline-rich regions located in between the two PH domains are conserved only amongst the vertebrate homologues of p116^{Rip}. Reduction of F10G8.8 expression using RNA interference does not lead to any overt phenotype in *C.elegans*. However, mutations in the *D.melanogaster* orthologue have been identified that affect the wing function, hence the name "outspread". Moreover, by yeast-two hybrid analysis, the F10G8.8 protein has been found to interact with several proteins whose function is unknown at present (for further information see www.flybase.org and www.wormbase.org).

More recently the full-length rat and human orthologues of mouse p116^{Rip} have been cloned (Lanson, Royals, and Claycomb, 2000, unpublished) (Koga and Ikebe, 2004; Surks et al., 2003). The mouse p116^{Rip} gene is located on chromosome 11-B1.3, human p116^{Rip} (M-RIP, myosin phosphatase-RhoA interacting protein) on chromosome 17-p11.2, and rat p116^{Rip} on chromosome 10-q22.

p116^{Rip} isoforms and related proteins

The p116^{Rip} protein family includes p116^{Rip} (splice variants isoform 1 and 2, see Fig. 9C), the splice variant KIAA0864 and a unique protein named Tara (Trio-associated repeat on actin) (Fig. 9A).

The KIAA0864 gene has an insertion of an unusual large alternative exon between exon 15 and 16 of p116^{Rip}. Consequently, KIAA0864 shares part of the C-terminal coiled-coil with p116^{Rip} and contains other coiled-coil structures upstream that are encoded for by the alternative exon. As the full-length gene of KIAA0864 has not been cloned it is so far unclear whether the KIAA0864 gene includes also the downstream exons (1-13) of p116^{Rip} that encode for the two PH domains. However, the splice variant KIAA0864 does exist at the transcriptional level as determined by rtPCR using primers specifically directed to the alternative exon (Fig. 9B). A recent study proposed a role for KIAA0864 in regulating neurite outgrowth since its expression is appears to be restricted to nervous tissue (Nakamura et al., 2005).

Additionally, two other splice variants of p116^{Rip} are found in the NCBI database that are conserved at least in human and mice; a longer protein, isoform 1 (1037 aa residues) and a shorter protein, isoform 2 (1024 aa residues). The two isoforms differ due to an alternative use of exon 23. Isoform 2 includes exon 23 wherein a stop codon is found. However, isoform 1 skips exon 23 and uses exon 24 that also encodes a stop codon but results in a longer protein isoform (Fig. 9C). Recently, it was shown by rtPCR and microarray analysis that the expression of exon 23 in the p116^{Rip} protein changes in a tissue specific

manner, indicating a tissue-specific expression of these two isoforms (Pan et al., 2004). This thesis deals with the shorter p116^{Rip} protein, isoform 2.

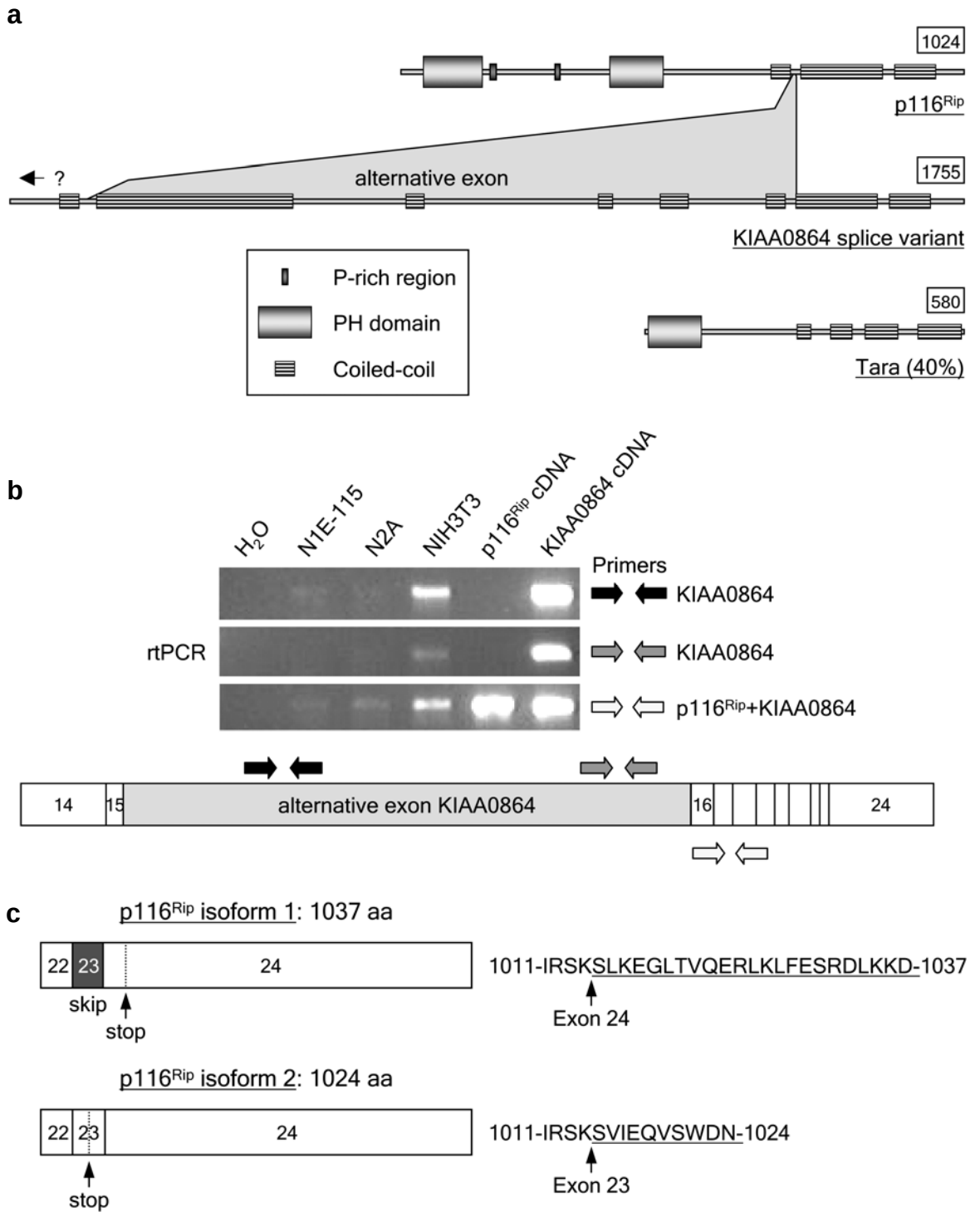

Figure 9. p116^{Rip} isoforms and related proteins
(**a**) The KIAA0864 protein is a splice variant of p116^{Rip} containing an alternative exon between exon 15 and 16 of p116^{Rip}. The Tara protein, a unique protein, is similar to the C-terminus of p116^{Rip} including the second PH domain and the coiled-coil structure. The degree of homology to p116^{Rip} is indicated as the percentage of identity at amino acid level. The amino acid length of the proteins is indicated on the right and boxed. Isoform 2 of p116^{Rip} is depicted (see Fig. 9C). The domain structures of murine homologues are shown as defined by the SMART program. GenBank^{TM} accession numbers: KIAA0864, BAC41453; Tara, NP_613045. See text for further details. (**b**) RT-PCR analysis of KIAA0864 and p116^{Rip} expression in N1E-115, Neuro-2A, and

NIH3T3 cells. The cDNAs of p116^{Rip} and KIAA0864 (partial) (provided by Koga H., Kazusa DNA Research Institute, Japan, http://www.kazusa.or.jp/en/) were taken along as controls. KIAA0864 expression is detected by two different primer sets that are directed to the alternative exon of KIAA0864. A primer set that is directed to an exon shared by p116^{Rip} and KIAA0864, the 16th exon of p116^{Rip}, was taken along as a control. (**c**) Murine p116^{Rip} isoforms 1 and 2 (GenBank^{TM} accession numbers NP_957697 and NP_036157) differ in the use of exon 23. While isoform 1 skips exon 23 and uses exon 24 wherein the encoded sequence terminates (stop), isoform 2 uses the sequence of exon 23 that also encodes for a stop codon. On the right the consequential difference in C-terminal amino acid sequence of the two isoforms is shown.

Tara is a unique protein located on the mouse chromosome at 15-E1 and in human at 22-q13.1 and appears to be conserved only in vertebrates. The Tara protein is shorter than p116^{Rip}, includes a PH domain and a C-terminal coiled-coil structure and shares a high degree of overall amino acid identity with the C-terminus of p116^{Rip}. The PH domain of Tara, in particular, is highly similar to the second PH domain of p116^{Rip}. Intriguingly, although the amino acid sequence and the domain organisation of p116^{Rip} and Tara are very similar, the proteins appear to have contrasting effects upon actin organisation (Seipel et al., 2001).

PH and coiled-coil domains of p116^{Rip}

The most striking features of p116^{Rip} are the two PH domains and the coiled-coil domain. The coiled-coil is one of the most abundant protein folding and assembly motifs consisting of α-helices wrapping around each other forming a supercoil. Although, coiled-coils have one of the simplest dimerisation interfaces they mediate highly selective protein associations. The coiled-coil domain of p116^{Rip} is moderately similar to the coiled-coil domains of myosin family members, including myosin heavy chain and paramyosin. The coiled-coil of p116^{Rip} was shown to mediate interactions with RhoA (*in vitro*), p116^{Rip} itself, and MBS (Chapter 3 and 4).

PH domains are commonly found in a wide range of proteins involved in intracellular signalling or cytoskeletal organisation. The best-characterised function of PH domains is the binding to phosphoinositides, yet some mediate protein-protein interactions. Thus far, phosphoinositide binding has not been reported for the PH domains of p116^{Rip}. Blast searches with the PH domains of p116^{Rip} using the swissprot database reveal that the two PH domains are not significantly homologues to one another. However, both PH domains show moderate homology to known 3-phosphoinositide binding proteins (Gab27/p97, PtdIns(3,4,5)P3 (Maroun et al., 1999); PEPP1, PtdIns3P (Dowler et al., 2000); myosin X, PtdIns(3,4,5)P3 and PtdIns(3,4)P2 (Axelrod, 1992)). The second PH domain in particular is similar to the PH domain of the PEPP1, 2, and 3 family members (33% identical, 46 % similar) the PH domain of AtPH (PtdIns3P (Dowler et al., 2000), 25% identical, 49% similar), the PH domain of DAPP1 (PtdIns(3,4,5)P3 and PtdIns(3,4)P2 (Dowler et al., 1999), 29% identical, 48% similar), and the PH domain of FAPP1 (PtdIns4P (Dowler et al., 2000), 27% identical, 45% similar). Both PH domains are also moderately similar to the PH domain of the tyrosine kinase Etk/BMX whose PH domain mediates the interaction with the FERM domain of focal adhesion kinase (Chen et al., 2001). The PH domain of another member of this family of tyrosine kinases, Btk, was shown to directly interact with F-actin (Yao et al., 1999). The latter finding is of particular interest since the N-terminal PH domain of p116^{Rip} is likely to be involved in F-actin binding (Chapter 2).

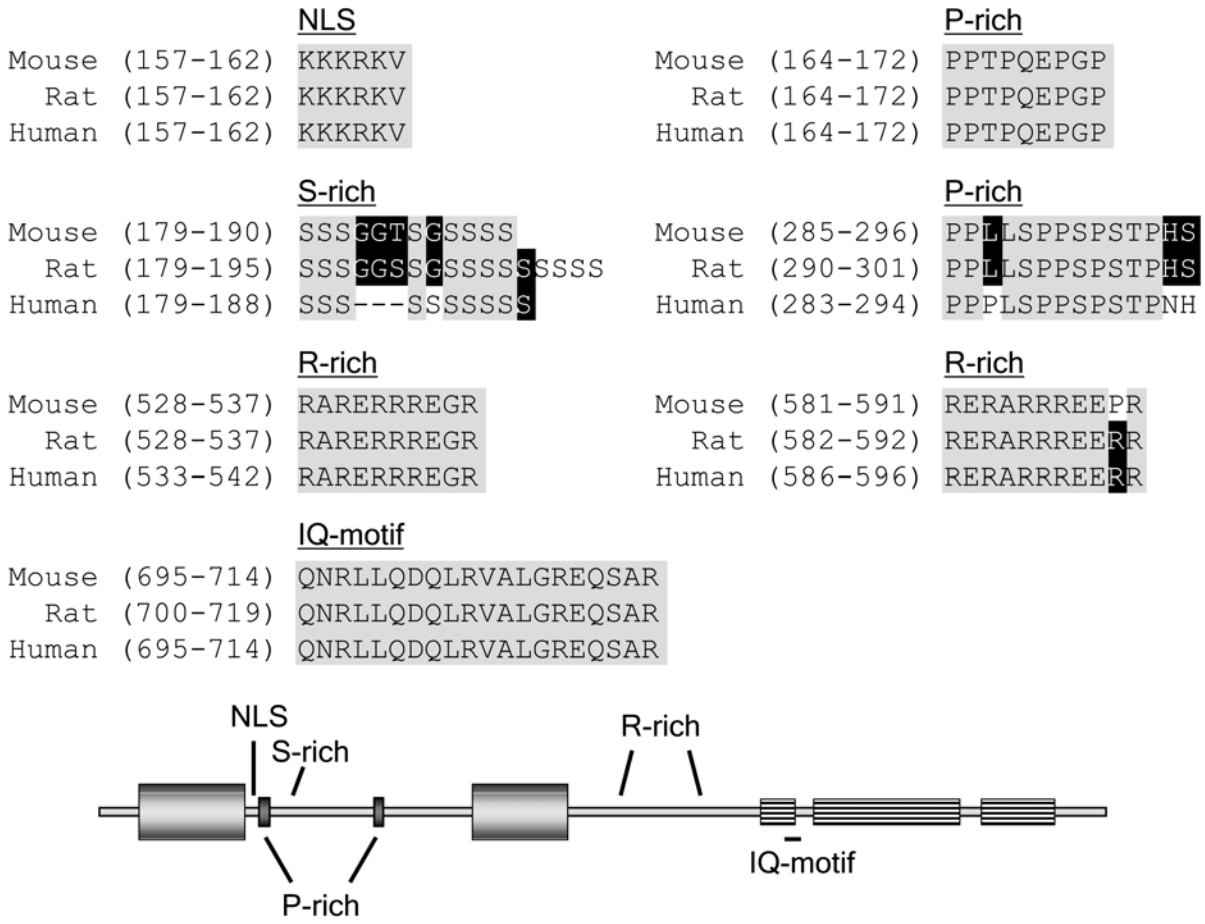

Figure 10. Conserved domains and putative interaction motifs of p116^{Rip}
Alignment of mouse, human, and rat p116^{Rip} using Vector NTI. Proline (P)-, serine (S)-, and arginine (R)-rich motifs, putative nuclear localisation sequence (NLS), and CaM binding site (IQ-motif) are indicated. Residues conserved in two of the three orthologues are boxed in black, and absolutely conserved residues are indicated in grey.

Putative motifs and interaction domains of p116^{Rip}

Besides the PH-, and coiled-coil domains, p116^{Rip} has several potential motifs that may mediate the interaction of p116^{Rip} with other proteins, regulate p116^{Rip}'s activity, or determine the localisation of p116^{Rip} (Fig. 10).

The two proline-rich regions (aa 164-172 and 285-296) match the consensus requirements for binding to SH3-domain (Cesareni et al., 2002) or WW-domain class IV containing proteins (Macias et al., 2002). Moreover, vertebrate p116^{Rip} has two conserved arginine-rich regions (aa 528-537, and aa 581-591) and a less conserved serine-rich region (aa 179-190) of unknown function. The amino acid sequence of p116^{Rip} contains one potential nuclear localisation signal that meets all the predicted requirements of an NLS (aa 157-162, see http://cubic.bioc.columbia.edu/predictNLS). Accordingly, p116^{Rip} displays partial nuclear localisation (Chapter 2). The coiled-coil domain of p116^{Rip} contains a potential IQ motif (aa 695-714) that is conserved among the vertebrate orthologues. The IQ motif is a calmodulin (CaM)-binding sequence (using the calmodulin target database at http://calcium.uhnres.utoronto.ca/ctdb) and was first characterised in myosins. The IQ motif refers to a group of sequences that share the IQ motif found in a number of mostly Ca^{2+}-independent CaM binding proteins. Changes in intracellular Ca^{2+} levels regulate myosin

function through the CaM-associated IQ motifs. In addition, potential kinase phosphorylation sites are present in the p116^{Rip} protein including sites for CaMKII, PKC, CKI, CKII, GSK3, p70S6K, PKG, and PKA.

Potential role of p116^{Rip} in disease

There are some indications for a role of p116^{Rip} in cancer. The human orthologue of p116^{Rip}, M-RIP, is amplified in 4 of 22 patients with osteosarcoma, a bone forming cancer (Atiye et al., 2005). Furthermore, using an *in vivo* invasion assay combined with cDNA microarray analysis, the p116^{Rip} gene was shown to be up-regulated by a ratio of 3.33 in invasive carcinoma cells of primary mammary tumours when compared to non-invasive cells derived from the same tumours (Wang et al., 2004). The genes that were identified in this study were generally involved in motility pathways and were co-ordinately up-regulated in invasive cells. This indicated that p116^{Rip}, in concert with the other genes identified, may facilitate the enhanced migratory behaviour of these cells. The p116^{Rip} gene is also up-regulated during the proliferation and self-renewal of pre-BI cells, indicating a role of p116^{Rip} in the development of B cells (Hoffmann et al., 2002). As such, p116^{Rip} expression might contribute to the specific homing, response of developing lymphocytes to a particular environment, proliferative expansion and the induction of V-DJ re-arrangements in early precursor B cells (Hoffmann and Melchers, 2003). There is also evidence of up-regulation of p116^{Rip} gene transcription during renal failure in the chronic disease diabetes mellitus. Microanalysis and northern blotting showed a late-onset induction of the p116^{Rip} gene in endothelin-induced mesangial cell hypertrophy, which is a model for diabetic renal failure (Goruppi et al., 2002). In diabetes, hypertrophy is associated with the progression to renal failure and is marked by an increase in overall protein synthesis, new gene transcription, and in some cases reorganisation of the actin cytoskeleton. Extraordinarily, p116^{Rip} gene transcription was markedly down-regulated in the muscles of space-flown rats (Nikawa et al., 2004). Space travel affects physiological functions in many ways and causes muscle atrophy, the loss of muscle tissue, in particular. A model was proposed in which loss of gravity would decrease specifically the expression of cytoskeletal proteins, resulting in a disturbance of mitochondrial localisation in cells. The latter will most likely cause oxidative stress and a lack of energy leading to muscle atrophy.

p116^{Rip} protein complexes

p116^{Rip} and related proteins have been identified in several protein complexes. Using a proteomics based approach, KIAA0864 was shown to be selectively recruited to the EGF-receptor complex upon receptor stimulation, suggesting a role for KIAA0864 in EGF signalling (Blagoev et al., 2003). p116^{Rip} itself was found to be in a complex consisting of citron-N, a brain-specific Rho-binding protein, ROCK2, profilin IIa, and LIMK (Camera et al., 2003). The protein complex was identified by passing mouse brain lysate through a citron-N C-terminal fragment affinity column and performing subsequent mass spectrometry on specific eluted proteins. Citron-N appeared to be enriched and associated with the Golgi apparatus of hippocampal neurons. Furthermore, citron-N was shown to be necessary for Golgi positioning and structural organisation. Therefore a model was proposed that involves a function of the protein complex in Golgi maintenance through local recruitment of Rho-dependent actin polymerisation and reorganisation of the actin cytoskeleton.

Outline of this thesis

Activation of the RhoA pathway leads to reorganisation of the actin cytoskeleton. Furthermore, changes in actin dynamics induced by the Rho pathway causes activation of the transcription factor SRF and subsequent SRE gene transcription. This thesis assesses the function of $p116^{Rip}$ in the RhoA pathway leading to contractility of the actin cytoskeleton and subsequent activation of SRF.

In Chapter 2 we identify $p116^{Rip}$ as an F-actin binding protein with the ability of bundling F-actin *in vitro* and report that expression of $p116^{Rip}$ leads to disruption of actin cytoskeletal structures. Chapter 3 describes the interaction of $p116^{Rip}$ with MBS and reveals an essential function of $p116^{Rip}$ in neurite outgrowth. Chapter 4 demonstrates that $p116^{Rip}$ inhibits RhoA-induced activation of the transcription factor SRF without affecting active RhoA levels. Additionally, we attest the ability of $p116^{Rip}$ to oligomerise and discuss the underlying mechanism of the inhibitory affect of $p116^{Rip}$ on RhoA-induced SRF activation.

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Chapter 2

p116^{Rip} is a novel filamentous actin-binding protein

Mulder J., Poland M., Gebbink M. F. B. G., Calafat J., Moolenaar W. H., and Kranenburg O. (2003) J. Biol. Chem. 278, 27216-27223

p116^{Rip} is a novel filamentous actin-binding protein

Abstract

p116^{Rip} is a ubiquitously expressed protein that was originally identified as a putative binding partner of RhoA in a yeast two-hybrid screen. Overexpression of p116^{Rip} in neuroblastoma cells inhibits RhoA-mediated cell contraction induced by lysophosphatidic acid (LPA); so far, however, the function of p116^{Rip} is unknown. Here we report that p116^{Rip} localises to filamentous actin (F-actin)-rich structures, including stress fibres and cortical microfilaments, in both serum-deprived and LPA-stimulated cells, with the N terminus (residues 1–382) dictating cytoskeletal localisation. In addition, p116^{Rip} is found in the nucleus. Direct interaction or colocalisation with RhoA was not detected. We find that p116^{Rip} binds tightly to F-actin (K_d ~0.5 μm) via its N-terminal region, while immunoprecipitation assays show that p116^{Rip} is complexed to both F-actin and myosin-II. Purified p116^{Rip} and the F-actin-binding region can bundle F-actin in vitro, as shown by electron microscopy. When overexpressed in NIH3T3 cells, p116^{Rip} disrupts stress fibres and promotes formation of dendrite-like extensions through its N-terminal actin-binding domain; furthermore, overexpressed p116^{Rip} inhibits growth factor-induced lamellipodia formation. Our results indicate that p116^{Rip} is an F-actin-binding protein with in vitro bundling activity and in vivo capability of disassembling the actomyosin-based cytoskeleton.

Introduction

Dynamic remodelling of the actin-based cytoskeleton drives cell shape changes, cell division, and motility. Cytoskeletal remodelling involves the assembly and disassembly of filamentous actin (F-actin) and is effectuated by cell-surface receptors that signal through small GTPases of the Rho family, notably RhoA, Rac and Cdc42 (Ridley, 2001; Etienne-Manneville and Hall, 2002). Many different actin-associated proteins participate in regulating actin dynamics in concert with Rho GTPases (Ayscough, 1998; Ridley, 1999; Svitkina and Borisy, 1999; Borisy and Svitkina, 2000; Wear et al., 2000; Higgs and Pollard, 2001; Janmey, 2001). Some actin-binding proteins promote organisation of actin into higher-order structures, whereas others control actin remodelling in response to extracellular stimuli such as growth factors, hormones, or cell adhesion cues. Although only few proteins bind actin monomers, there are more than 100 that bind polymeric F-actin, and many of them induce cross-linking or bundling of F-actin (dos Remedios and Thomas, 2001).

In our ongoing studies to delineate Rho signalling by the lipid growth factor lysophosphatidic acid (LPA) (Kranenburg et al., 1999; Moolenaar, 1999), we previously identified a ubiquitously expressed protein of 116 kDa, provisionally named p116^{Rip}, which binds relatively weakly to activated RhoA in a yeast two-hybrid assay (Gebbink et al., 1997). The p116^{Rip} sequence predicts several protein interaction domains, including at least one PH domain, two proline-rich stretches, and a C-terminal region predicted to form a coiled-coil domain. This suggests that p116^{Rip} may have a scaffolding role recruiting different proteins into a RhoA-regulated macromolecular complex. When overexpressed in N1E-115 neuroblastoma cells, p116^{Rip} promotes cell flattening and process extension and inhibits

cytoskeletal contraction in response to LPA (Gebbink et al., 1997). The p116^{Rip} phenotype was reminiscent of what is observed after RhoA inactivation (using dominant-negative RhoA or C3 toxin), which led to the suggestion that p116^{Rip} may negatively regulate RhoA signalling (Gebbink et al., 1997). However, the function of p116^{Rip} remains unknown; importantly, no evidence that p116^{Rip} binds directly to RhoA in mammalian cells has been discovered (Gebbink et al., 2001).

In the present study, we set out to characterise p116^{Rip} in further detail. We find that p116^{Rip}, rather than directly binding to RhoA, interacts with F-actin via its N-terminal region and colocalises with dynamic F-actin structures such as stress fibres, cortical microfilaments, filopodia, and lamellipodial ruffles. Furthermore, we show that p116^{Rip} induces bundling of F-actin *in vitro*, with the bundling activity residing in the N-terminal region. Yet overexpression of p116^{Rip} or its N-terminal actin-binding domain disrupts the actin cytoskeleton and thereby interferes with growth factor-induced contractility and lamellipodia formation. Our studies specify p116^{Rip} as a novel F-actin-binding protein and demonstrate that p116^{Rip} can affect, either directly or indirectly, the integrity of the actomyosin-based cytoskeleton.

Results

We originally isolated p116^{Rip} through its interaction with activated RhoA-V14 in yeast two-hybrid assays in which the RhoA isoprenylation site was mutated to prevent membrane targeting (Gebbink et al., 1997); no interaction was found with other small GTPases, notably activated RhoB, RhoE, Rac1, Cdc42, and Ras. The interaction between p116^{Rip} and RhoA-V14 was relatively weak, however, and in subsequent studies, we have been unable to confirm that p116^{Rip} interacts with RhoA in mammalian cells (Gebbink et al., 2001), supplementary data). Furthermore, overexpression of p116^{Rip} in COS-7 cells did not significantly influence the activation state of endogenous RhoA (Chapter 4). We therefore conclude that p116^{Rip} is unlikely to be a high-affinity binding partner and/or negative regulator of RhoA.

p116^{Rip} localises to dynamic actin-rich structures and the nucleus

As a first step in elucidating the function of p116^{Rip}, we examined its subcellular localisation in N1E-115 and NIH3T3 cells using a polyclonal anti-p116^{Rip} antibody raised against the putative "RhoA-binding domain" (RBD; (Gebbink et al., 1997)). Cells were simultaneously double-stained with rhodamine-phalloidin to visualise F-actin. In serum-deprived N1E-115 cells, endogenous p116^{Rip} colocalises with F-actin structures, especially the actin-rich microspikes (Fig. 1, top). After stimulation with LPA, a potent activator of RhoA, N1E-115 cells rapidly round up and neurites retract (Jalink et al., 1993; Jalink et al., 1994). In those contracted cells, p116^{Rip} is found relocalised to the contractile actomyosin ring at the cell cortex (Fig. 1). In NIH3T3 cells, maintained either in serum-free medium or stimulated with LPA, p116^{Rip} colocalises with F-actin-rich structures, particularly along stress fibres, at cortical microfilaments, and at the leading edge of lamellipodia (Fig. 1, bottom). Of note, p116^{Rip} staining is also detected in the cytoplasm and the nucleus (Fig. 1, bottom). Specificity of the observed immunostaining was confirmed by using the GST-RBD polypeptide antigen (previously termed Δ2; (Gebbink et al., 1997)), which blocked the

p116^{Rip} fluorescence signal. Furthermore, p116^{Rip} transfected into COS-7 or N1E-115 cells showed the same subcellular distribution pattern as endogenous p116^{Rip}: colocalisation with F-actin-rich structures as well as nuclear and cytoplasmic staining (Fig. 2B and results not shown). No colocalisation with endogenous RhoA was detected in either N1E-115 or NIH3T3 cells (results not shown).

The NT region of p116^{Rip} dictates subcellular localisation

The subcellular localisation of p116^{Rip} raises the possibility that p116^{Rip} is an F-actin-binding protein. To test this notion, we examined the intracellular localisation of distinct domains of p116^{Rip} and determined their detergent solubility. p116^{Rip} contains several putative protein and phospholipid interaction motifs, including a central PH domain, an N-terminal PH domain (aa 43–152; not noted previously (Gebbink et al., 1997)); two proline-rich regions, and a C-terminal coiled-coil region (Fig. 2A). The putative "RhoA-binding domain" (RBD) that was isolated in yeast two-hybrid screens (Gebbink et al., 1997) overlaps with the coiled-coil region, as indicated in Fig. 2A.

We generated HA-tagged p116^{Rip} and three truncated versions (HA-tagged) encoding the CT coiled-coil region, the RBD and the NT half (NT-p116^{Rip}; Fig. 2A). The various cDNA constructs were transiently transfected into N1E-115 cells and the subcellular localisation and detergent solubility of the resulting proteins were analysed. Transfected HA-p116^{Rip}, like endogenous p116^{Rip}, localises to cortical F-actin (and the nucleus; data not shown). In contrast, the p116^{Rip}-CT and RBD polypeptides display nuclear and cytoplasmic localisation (Fig. 2B). In keeping with these results, the CT and RBD truncation mutants are largely Triton-soluble, whereas full-length p116^{Rip} (transfected and endogenous) is about 50% insoluble (Fig. 2C and results not shown), consistent with association with the cytoskeleton.

Similar to full-length p116^{Rip}, the N terminus of p116^{Rip} (NT-p116^{Rip}) colocalises with F-actin and is also detectable in the nucleus (Fig. 2D and results not shown). When the NT-p116^{Rip}-expressing cells were analysed at >48 h after transfection, however, the F-actin cytoskeleton was largely disrupted (see below). Collectively, these results indicate that the N-terminal part of p116^{Rip} (aa 1–382) determines its subcellular localisation.

Binding of p116^{Rip} to F-actin

F-actin associates with the motor protein myosin-II to generate contractile forces in non-muscle cells. In metabolically labelled N1E-115 cells, we found that endogenous p116^{Rip} as well as the purified polypeptide NT-p116^{Rip} (fused to GST) coprecipitated with proteins of 43 and 200 kDa (Fig. 3A, lanes 2 and 4, respectively). Immunoblot analysis confirmed that the 43-kDa protein is actin (not shown), and revealed that the 200 kDa protein represents the heavy-chain of non-muscle myosin-II (Fig. 3B). Although the reciprocal precipitations yielded variable results, our data support the notion that p116^{Rip} associates with actomyosin *in vivo*.

We next investigated the actin-binding properties of NT-p116^{Rip}. As shown in Fig. 4A, NT-p116^{Rip} (fused to GST) cosediments with purified F-actin, as did α-actinin, whereas GST alone and BSA did not. Fusion proteins containing the C-terminal regions of p116^{Rip} (CT and RBD) failed to cosediment with F-actin (results not shown). It thus seems that the N-terminal region of p116^{Rip} contains an F-actin-binding domain. We next examined the binding affinity of full-length p116^{Rip} for F-actin. Increasing concentrations of purified F-actin (0–3.5 μm) were mixed with a fixed amount of p116^{Rip} (1 μm). After high-speed centrifugation, the

amount of p116^{Rip} cosedimenting with F-actin was determined. From the resulting binding curve we estimate that p116^{Rip} binds to F-actin with an apparent dissociation constant (K_d) of about 0.5 μm (Fig. 4B).

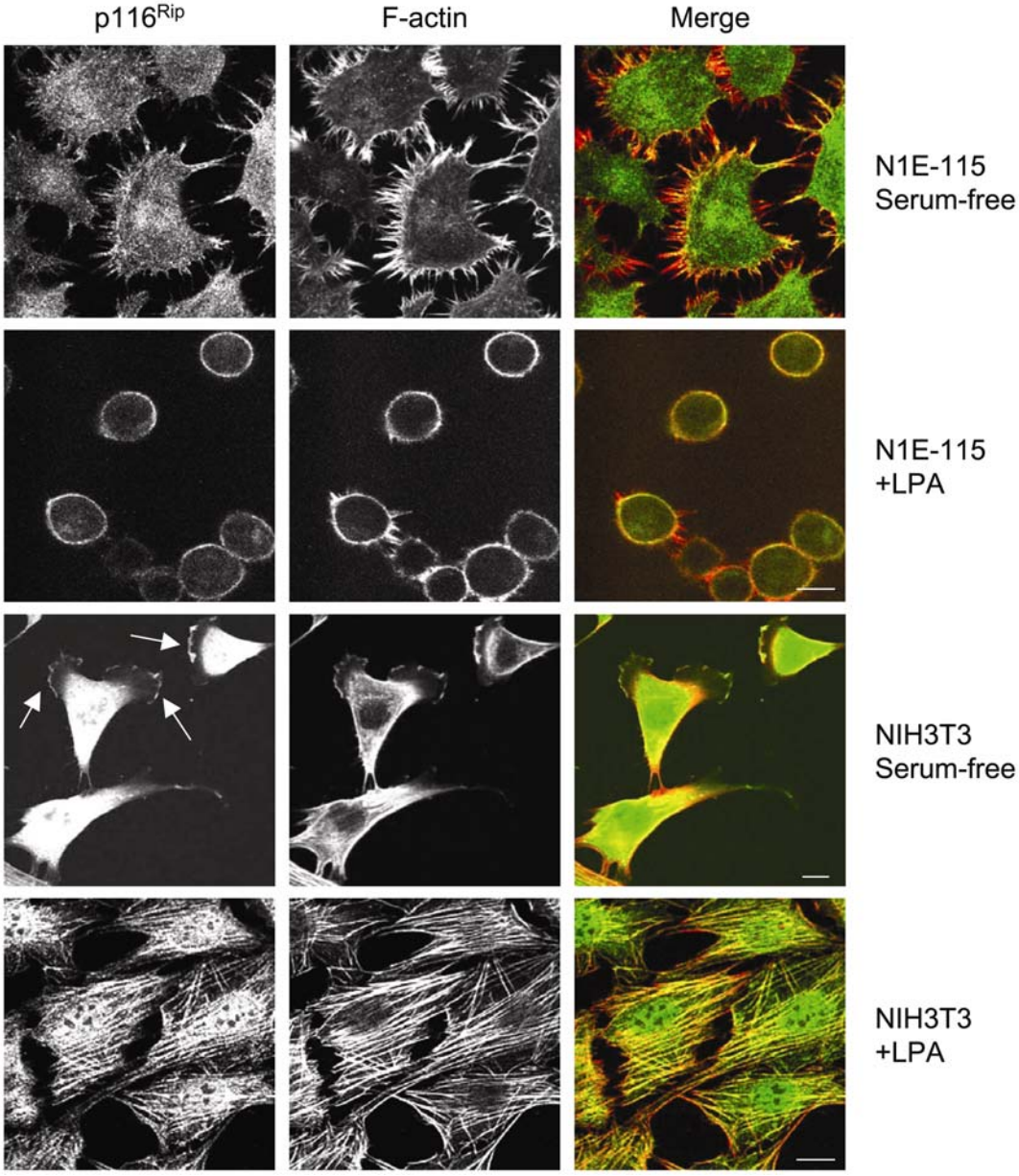

Figure 1. Immunofluorescence analysis of p116^{Rip} localisation
Cells were grown on glass coverslips and treated as follows: N1E-115 or NIH3T3 cells were cultured in serum-free medium and were either left untreated or were stimulated with LPA (1 μM) for 10 min to induce RhoA-mediated cytoskeletal contraction and cell rounding (N1E-115 cells) or stress fibres (3T3 cells). NIH3T3 cells were used to visualise lamellipodia and membrane ruffles. Abundant filopodia (microspikes) are observed in serum-starved N1E-115 cells. The distribution of endogenous p116^{Rip} was visualised by immunofluorescence using polyclonal anti-p116^{Rip} antibody (raised against part of the coiled-coil region, aa 545–823 (Gebbink et al., 1997)), whereas F-actin was detected by rhodamine-conjugated phalloidin. Merges are shown in green (p116^{Rip}) and red (F-actin). p116^{Rip} is seen to colocalise with filopodia, cortical actin, membrane ruffles, and stress fibres. In addition, p116^{Rip} is found in the nucleus (best visible in the two bottom rows) and the cytoplasm. Scale bars, 10 μm.

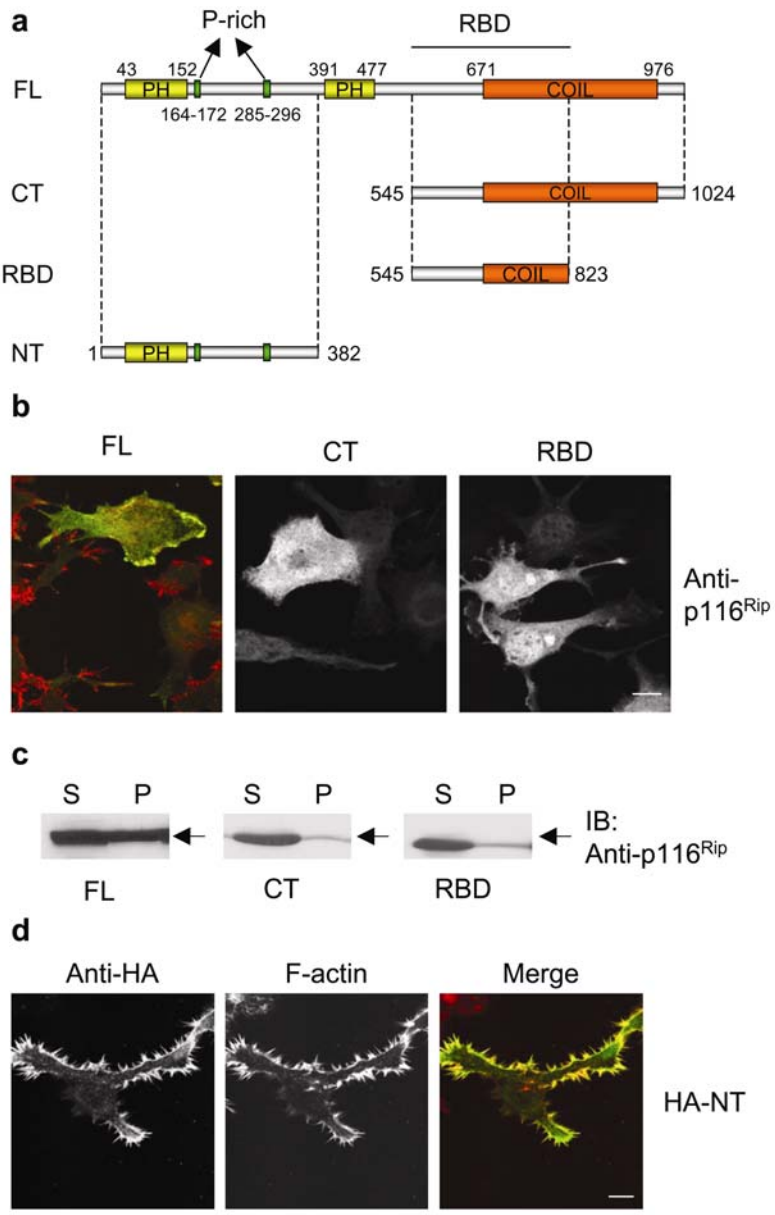

Figure 2. Association of p116^{Rip} with the actin cytoskeleton (**a**) Expression constructs used for transfection in N1E-115 cells encode FLp116^{Rip} and three deletion mutants encompassing the complete C terminus (construct CT; aa 545–1024) or part of the C terminus (construct RBD; aa 545–823) including the "RhoA-binding domain," as indicated, or the N-terminal PH domain of p116^{Rip} (construct NT; aa 1–382.). P-rich, proline-rich regions. The N-terminal PH domain was not recognised earlier (Gebbink et al., 1997). (**b**) Confocal analysis of transfected N1E-115 cells using anti-p116^{Rip} antibody and rhodamine conjugated to phalloidin (red staining only in left). Transfected full-length p116^{Rip} colocalises with cortical actin, whereas truncation mutants CT and RBD are found in the cytoplasm and in the nucleus. (**c**) Solubility of transfected full-length or truncated p116^{Rip} in a buffer containing 0.1% Triton-X-100, as examined by subcellular fractionation into a supernatant (s) and pellet (p) fraction. The p116^{Rip} antibody was used for detection on Western blot. Full-length p116^{Rip} is partially insoluble while the truncation mutants CT and RBD are largely soluble. IB, immunoblot. (**d**) N1E-115 cells were transfected with an HA-tagged construct encoding a truncated version of p116^{Rip} encompassing the N-terminal PH domain (construct HA-NT; aa 1–382). Cells were fixed 24 h after start of transfection, and the localisation of the expressed NT construct was analysed by immunofluorescence using anti-HA antibody. F-actin was stained with rhodamine-conjugated phalloidin (red staining). It is seen that the NT protein colocalises with F-actin.

To define the N-terminal regions mediating F-actin binding in further detail, we made various deletion mutants and determined their F-actin cosedimentation properties, as illustrated in Fig. 4C. Whereas p116^{Rip} has no obvious sequence homology to known actin-binding proteins, potential actin-binding motifs include the N-terminal PH domain (Yao et al., 1999) as well as a stretch of positively charged residues (KKKRK, aa 157–161) that could interact with the highly anionic actin filament (Tang and Janmey, 1996). We found that one deletion mutant (Δ6; aa 1–212), comprising both the PH domain and the positive stretch, can bind F-actin, whereas the other mutants cannot (Fig. 4C). Thus, the extreme N terminus (aa 1–43), the PH domain, and the adjacent cationic residues are all necessary to mediate F-actin binding. Further definition of the critical actin-binding motif(s) within the N-terminal region must await future studies.

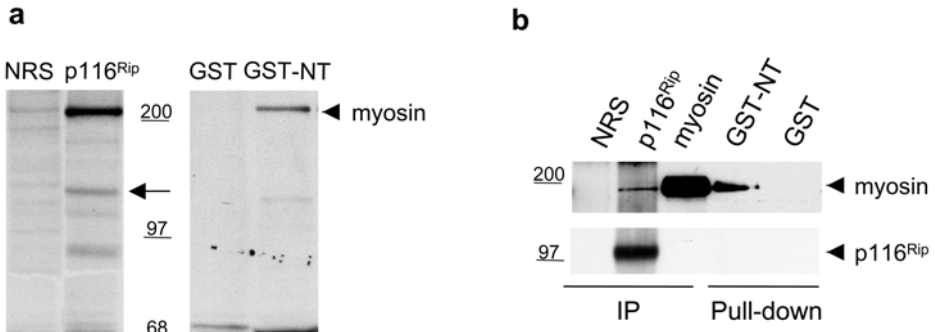

Figure 3. Association of p116^{Rip} with actomyosin complexes *in vivo*
(**a**) N1E-115 neuroblastoma cells were starved in methionine/cysteine-free medium for 30 min and then labeled for 4 h with [^{35}S]methionine/cysteine. Endogenous p116^{Rip} was immunoprecipitated (IP) using polyclonal anti-p116^{Rip} antibodies (arrow). Normal rabbit serum (NRS) was used as a control for immunoprecipitation (left). GST pull-down assays were performed with either GST alone or the GST fusion protein containing the isolated actin-binding domain (GST-NT-p116 ^{Rip}; right lanes). Precipitates were subjected to SDS-PAGE and analysed by autoradiography. (**b**) Western blot analysis of precipitates and GST pull-down assays using polyclonal anti-p116^{Rip} and anti-myosin-II antibodies, endogenous p116^{Rip}, and myosin-II were immunoprecipitated using polyclonal anti-p116^{Rip} antibodies and polyclonal anti-myosin-II antibodies, respectively (left lanes). Normal rabbit serum (NRS) was used as a control for immunoprecipitation. GST pull-down assays were performed with either GST alone or the GST fusion protein containing the isolated actin-binding domain (GST-NT-p116^{Rip}; right lanes). Myosin coprecipitates in p116^{Rip} immuno-complexes and in the GST-NT-p116^{Rip} pulldown assay.

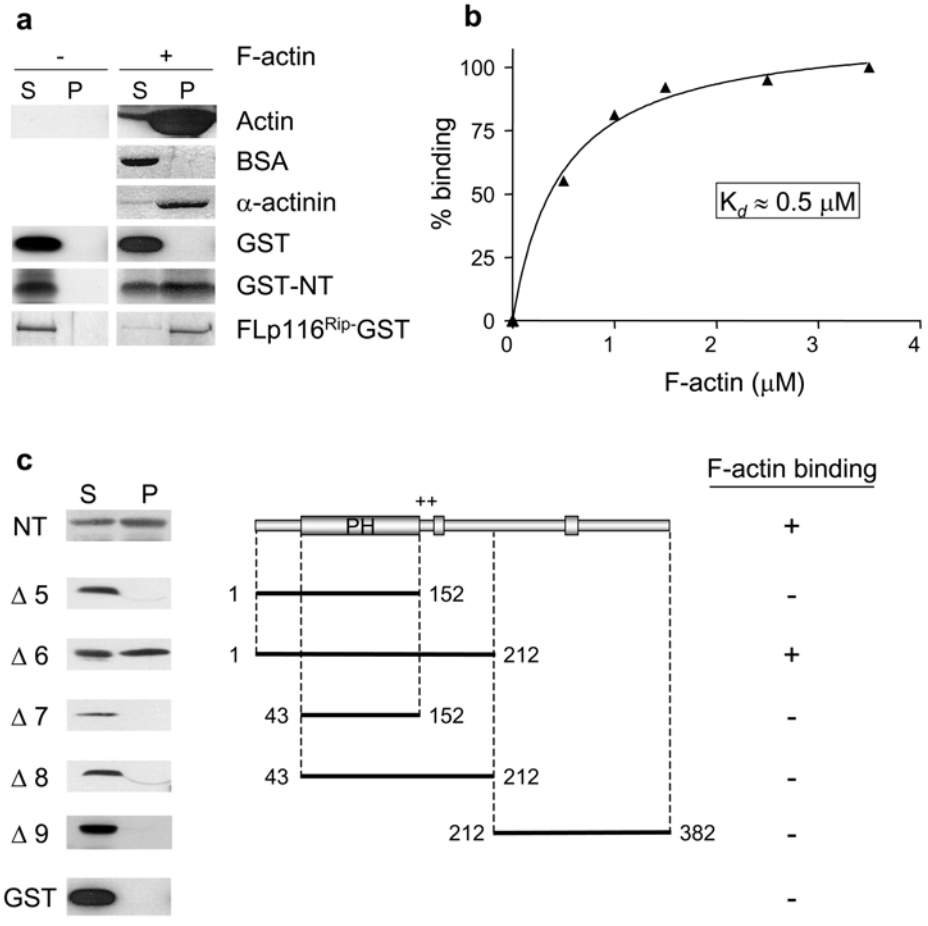

Figure 4. *(previous page)* Direct binding of p116^{Rip} to F-actin *in vitro*
(**a**) Purified proteins GST-NT, GST alone, α-actinin, p116^{Rip}-GST, p116^{Rip}-Myc, or BSA were incubated with (+) or without (-) *in vitro* prepared actin filaments. F-actin was subsequently pelleted by ultracentrifugation. Co-sedimentation of the various proteins with F-actin was analysed by SDS-PAGE followed by Coomassie staining of the gel. GST-NT, p116^{Rip}-GST, p116^{Rip}-Myc, and α-actinin, but not GST or BSA, cosediment with F-actin (right). None of the proteins tested was pelleted if F-actin was omitted form the reaction mixture (left; not shown for BSA and α-actinin). S, supernatant fraction; P, pellet fraction. (**b**) Direct plot of binding of p116^{Rip}-GST to F-actin. A fixed amount of p116^{Rip}-GST (1 µM) was mixed with various amounts of F-actin (0–3.5 µM), followed by ultracentrifugation. Amounts of the free and bound p116^{Rip} were quantified as described. The percentage of bound p116^{Rip}-GST was plotted against the concentration of F-actin. The curve was obtained by nonlinear fitting to a rectangular hyperbola. The apparent K_d was estimated to be ≈0.5 µM. (**c**) Diagram of purified recombinant p116^{Rip} proteins that have deletions in the actin-binding domain (NT) (Δ5, aa 1–152; Δ6; aa 1–212; Δ7, aa 43–152; Δ8, aa 43–212; Δ9, aa 212–390) and that are fused to GST (right). Cosedimentation of the deletion mutants with F-actin was determined by Western blot analysis using a GST antibody. ++, stretch of positive residues, KKKRK, at position 157–161.

p116^{Rip} induces bundling of F-actin in vitro via its N-terminal region

We next examined the ability of p116^{Rip} and NT-p116^{Rip} to induce actin cross-linking *in vitro*, using α-actinin as a positive control. Myc-p116^{Rip} and GST-p116^{Rip} were isolated from transfected COS-7 cells using affinity chromatography, and protein purity was determined by Coomassie Blue staining. Myc-p116^{Rip}, like GST-p116^{Rip}, binds F-actin, as shown by cosedimentation assays using lysates from transfected COS-7 cells (see Fig. 6A, left). Because the dimeric nature of GST could mediate artifactual actin cross-linking by GST-p116^{Rip}, we also used Myc-p116^{Rip}. Purified GST-p116^{Rip}, Myc-p116^{Rip}, α-actinin, or GST alone were incubated with F-actin, and the samples were subsequently analysed by electron microscopy. In the absence of p116^{Rip} or in the presence of GST alone, long actin filaments were randomly distributed all over the grid and no organised actin bundles were observed (Fig. 5A). In the presence of either GST-p116^{Rip} or Myc-p116^{Rip}, however, F-actin became organised into thick bundles similar to those formed by the actin-bundling protein α-actinin (Fig. 5, B, C, and D). The bundles consisted of many actin filaments closely aligned in juxtaposition, with no branching of filaments observed.

We also tested the isolated N-terminal actin-binding domain (NT-p116^{Rip}; aa 1–382) and the C-terminal coiled-coil region (CT-p116^{Rip}; aa 545–1024) for bundling activity. In these experiments, GST-fusion proteins were produced in bacteria followed by GST cleavage. As expected, the NT-p116^{Rip} protein induced actin bundling similar to full-length p116^{Rip}, whereas no actin bundles were observed after incubation of F-actin with the CT polypeptide (Fig. 5, E and F). Thus, p116^{Rip} induces bundling of F-actin *in vitro* through its N-terminal actin-binding domain.

Expression of p116^{Rip} or the N-terminal actin-binding domain promotes stress fiber disassembly and process outgrowth

We investigated the effects of overexpression of p116^{Rip} and its N-terminal region on cell morphology and cytoskeletal organisation in NIH3T3 cells. To this end, we used HA-tagged p116^{Rip} and a p116^{Rip}-GFP fusion protein (its direct binding to F-actin was confirmed; Fig. 6A). Contrary to expectations raised by the actin-bundling studies, overexpression of p116^{Rip} in NIH3T3 cells resulted in loss of stress fibres and outgrowth of long dendrite-like processes (Fig. 6B). This phenotype was observed with wild-type p116^{Rip}, Myc-, HA- and p116^{Rip}-GFP

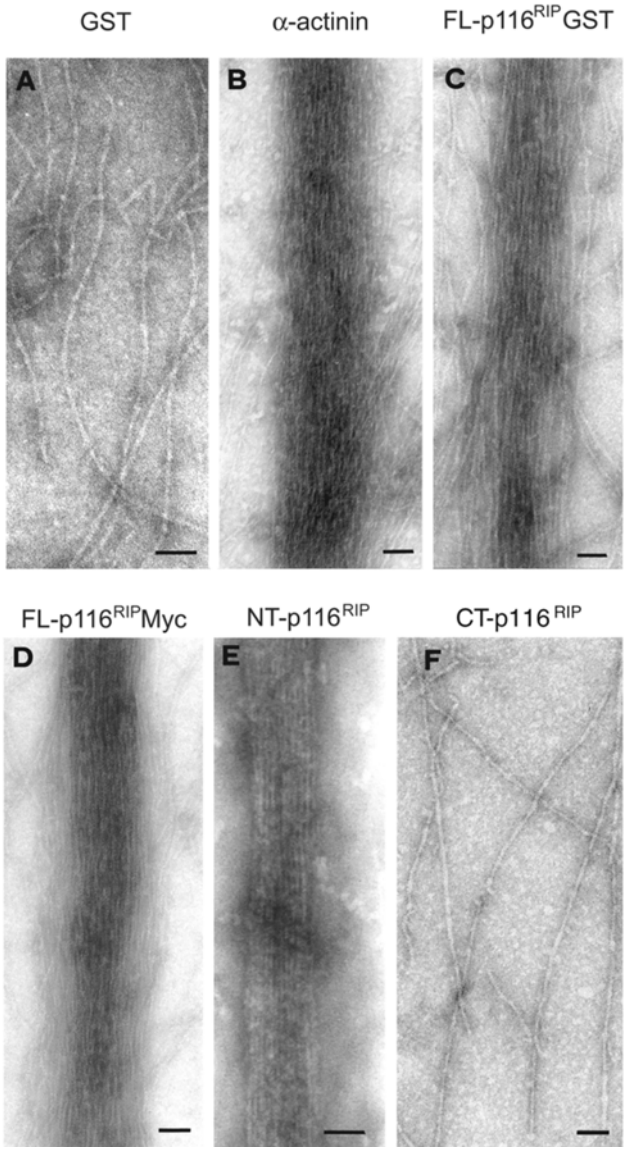

Figure 5. p116^{Rip} induces bundles of F-actin Electron micrographs showing negatively stained preparations of actin filaments incubated with GST (**a**), bundles formed by incubating F-actin with α-actinin (**b**), p116^{Rip}-GST (**c**), p116^{Rip}-Myc (**d**), NT-p116^{Rip} (**e**), and CT-p116^{Rip} (**f**). Scale bars, 50 nm.

(Fig. 6, B and C, and results not shown). Less than 10% of the p116^{Rip}-transfected NIH3T3 cells contained stress fibres, compared with >60% of the GFP-expressing control cells (Fig. 6D). LPA stimulation of NIH3T3 cells leads to rapid RhoA-mediated cell contraction (albeit less dramatic than in N1E-115 cells). However, no contractile response to LPA was seen in the p116^{Rip}-overexpressing NIH3T3 cells, similar to what we previously observed in p116^{Rip}-overexpressing N1E-115 cells (13). Loss of stress fibres was already detectable at 6 to 8 h after transfection, whereas process extension appeared at later time points (>12–16 h, when p116^{Rip} levels were more elevated).

Expression of the actin-binding region (HA-NT-p116^{Rip}) in NIH3T3 cells led to the same dramatic loss of stress fibres and induction of dendrite-like extensions. In contrast, cells expressing the C-terminal domain only (HA-CT-p116^{Rip}) displayed a normal stress fiber pattern (Fig. 7, A and B). Thus, the NT region of p116^{Rip} is necessary and sufficient for stress-fiber disruption and consequent loss of contractility in p116^{Rip}-overexpressing cells.

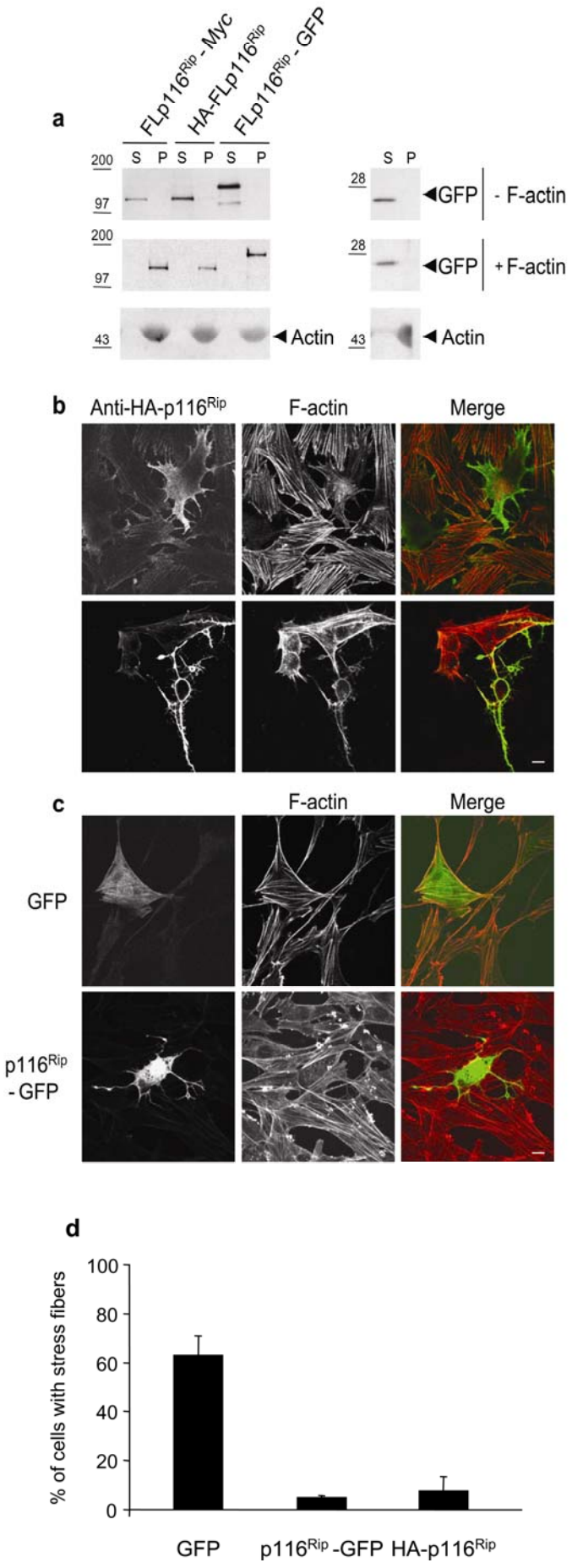

Figure 6. Expression of full-length p116^{Rip} or the isolated actin-binding domain induces a dendritic morphology and loss of stress fibres in NIH3T3 cells
(**a**) Binding of transfected p116^{Rip} to F-actin. COS-7 cells were transfected with the indicated plasmids. Supernatant aliquots were pelleted in either the presence (centre blot) or absence (top blot) of filamentous actin. Cosedimentation of the proteins with F-actin was tested by SDS-PAGE and Western blot using polyclonal anti-p116^{Rip} and anti-GFP antibodies. Actin was detected by PonceauS staining. Transfected FLp116^{Rip} (GFP fusion, Myc-, or HA-tagged), but not GFP alone, is seen to cosediment with F-actin *in vitro.* (**b**, and **c**) NIH3T3 fibroblasts were transfected with an HA-tagged p116^{Rip} construct, a p116^{Rip}-GFP fusion construct, or GFP alone. Cells were fixed and stained 24 h after transfection. Cells expressing GFP, p116^{Rip}-GFP, or HA-FLp116^{Rip} were visualised by immunofluorescence microscopy using either GFP fluorescence or an anti-HA antibody (left). Centre column shows staining with rhodamine-conjugated phalloidin (red in the merged pictures). Note the loss of stress fibres and the dendritic-like morphology of cells that overexpress HA-FLp116^{Rip} or p116^{Rip}-GFP, compared with GFP-expressing control cells. (**d**) Quantitative analysis of transfected cells containing stress fibres. At least 100 cells with similar expression levels of p116^{Rip} or control constructs were counted for each experiment. Cells containing four or more stress fibres were scored as "cells with stress fibres." To eliminate observer bias, cells were counted using a "blind" setup. Data are the means (± S.D.) of three independent experiments.

Finally, we examined the cytoskeletal response of p116^{Rip}-overexpressing NIH3T3 cells to platelet-derived growth factor (PDGF), which is a potent inducer of Rac-mediated lammelipodia formation and membrane ruffling. PDGF induced prominent lamellipodia formation in the control cells but not in the p116^{Rip}-GFP-expressing cells (Fig. 8, A and B). We conclude that although p116^{Rip} has actin-bundling activity *in vitro*, overexpression of p116^{Rip} in fibroblasts and neuronal cells disrupts F-actin assembly and thereby interferes with Rho/Rac-controlled cytoskeletal remodelling.

Discussion

We originally isolated p116^{Rip} as a RhoA-interacting protein in a yeast two-hybrid screen (Tang and Janmey, 1996; Gebbink et al., 1997). Binding to activated RhoA was relatively weak, however, and our initial conclusion that p116^{Rip} interacts directly with RhoA in mammalian cells turned out to be premature (Gebbink et al., 2001).
In fact, we have since found that p116^{Rip} is unlikely to be a direct binding partner of RhoA (supplementary data). In the present study, we provide the first insights into the function of p116^{Rip}. We show here that p116^{Rip} is an F-actin-binding protein that has bundling activity *in vitro*, with the actin-binding domain residing in the N-terminal region (aa 1–212; construct Δ6-p116^{Rip}; Fig. 4C). This conclusion is based on the following observations: 1) p116^{Rip} and NT-p116^{Rip} associate with actomyosin complexes *in vivo*; 2), the N-terminal region of p116^{Rip}, but not its C-terminal half, cosediments with F-actin *in vitro*; and 3), purified full-length p116^{Rip} and NT-p116^{Rip} induce the assembly of actin filaments into thick bundles *in vitro*. In addition, we show that endogenous p116^{Rip} localises to dynamic F- actin-rich structures that are normally under the control of Rho family GTPases, notably along stress fibres, in cortical microfilaments as well as in filopodia and lamellipodia. Furthermore, p116^{Rip} is also detected in the nucleus, consistent with p116^{Rip} containing several potential nuclear localisation signals (between residues 43–587; not shown). There is growing evidence for the presence of actin and actin-binding proteins in the nucleus, but very little is still known about their importance for normal cell function (Rando et al., 2000). One challenge for future studies is to determine how nuclear targeting of p116^{Rip} is normally regulated.

The N-terminal region p116^{Rip} shows no obvious sequence similarity to known F-actin-binding proteins. Therefore, p116^{Rip} does not classify as a member of the superfamily of actin-binding proteins that includes α-actinin/spectrin members, plectin, filamin, and dystrophin (McCann and Craig, 1997; Lappalainen et al., 1998; Van Troys et al., 1999). At least three distinct mechanisms could account for the actin-bundling activity of NT-p116^{Rip}. One possibility would be that NT-p116^{Rip} is able to dimerise and thereby induces actin bundling. However, using transfected COS cells, we did not detect an interaction between NT-p116^{Rip} and full-length p116^{Rip} (results not shown), which argues against the possibility that the NT domain can form dimers. The second possibility is that NT-p116^{Rip} might bundle F-actin through the polycationic KKKRK motif (residues 157–161), just after the first PH

domain (Tang and Janmey, 1996). However, mutational analysis reveals that neither the first PH domain nor the cationic motif is sufficient for F-actin binding (Fig. 4C). A third possibility is that NT-p116^{Rip} may harbor two actin-binding domains, each of which binds a separate actin filament; as yet, we have no evidence for or against this notion. Further studies are required to identify the N-terminal sequence motifs in p116^{Rip} that determine F-actin binding and bundling.

Contrary to what one would expect for a protein with actin-bundling activity, overexpression of p116^{Rip} in NIH3T3 cells causes loss of stress fibres and produces a dendrite-like morphology. This phenotype, which is reminiscent of cells expressing dominant-negative RhoA (Jalink et al., 1994; Brouns et al., 2001) requires the N-terminal actin-binding domain of p116^{Rip} but not the C-terminal coiled-coil region. The importance of the N terminus in determining cytoskeletal architecture can also be inferred from the observation that overexpressed p116^{Rip} causes cell flattening in N1E-115 cells, whereas an N-terminally truncated version does not (Gebbink et al., 1997). Loss of stress fibres and other actin-rich structures is a common feature of overexpressed actin-monomer (G-actin) sequestering proteins (Vartiainen et al., 2000; Lee et al., 2001; Mattila et al., 2003), but our efforts to test whether NT-p116^{Rip} can bind G-actin yielded negative results (not shown). However, there is precedent for actin cross-linking proteins to cause F-actin disassembly *in vivo*. In particular, overexpression of the actin-binding region of neurabin, an F-actin cross-linking protein, causes collapse of stress fibres and promotes filopodial outgrowth, apparently by recruiting protein phosphatase I to F-actin-rich structures (Oliver et al., 2002). Furthermore, overexpression of villin, a protein that can bundle, cap, nucleate, or sever actin *in vitro*, results in the disappearance of stress fibres and enhanced microvilli elongation, a phenotype that strictly correlates with the actin-bundling activity of villin (Friederich et al., 1999).

Overexpressed p116^{Rip} not only induces an inactive RhoA phenotype but also interferes with PDGF-induced lamellipodia formation, which is a typical Rac-mediated response. The present findings lead us to suggest that, rather than being a negative regulator of Rho/Rac, p116^{Rip} can destabilise F-actin-rich structures by competing with and displacing other actin-cross-linking proteins. An alternative or additional possibility is that p116^{Rip} may recruit regulatory proteins that disassemble the F-actin network (such as actin-severing proteins or protein phosphatases; see (Oliver et al., 2002)). As for the displacement model, the neuronal F-actin-binding protein drebrin induces the formation of highly branched processes, similar to that observed with p116^{Rip}. It does so by interfering with the actin binding and bundling activities of fascin, α-actinin, and tropomyosin (Ishikawa et al., 1994; Sasaki et al., 1996). A similar mechanism might underlie the p116^{Rip} overexpression phenotype.

Finally, we note that a recently identified actin-binding protein named Tara (593 residues) shows a high degree of similarity to p116^{Rip} (46% overall amino acid identity (Seipel et al., 2001)). In common with p116^{Rip}, Tara contains an N-terminal PH domain and a C-terminal coiled-coil region, but it lacks the N-terminal actin-binding region of p116^{Rip}. No actin cross-linking activity has been reported for Tara until now; nevertheless, overexpression of Tara in HeLa cells leads to enhanced formation of stress fibres and cortical F-actin (Seipel et al., 2001). Thus, despite their structural similarities, p116^{Rip} and Tara have opposing actions on F-actin organisation.

In conclusion, our studies specify p116^{Rip} as a novel F-actin-binding protein with bundling activity *in vitro* and demonstrate that p116^{Rip} can affect, directly or indirectly, the integrity and contractility of the actomyosin-based cytoskeleton. Further insight into the physiological role of p116^{Rip} in cytoskeletal regulation will rely on the identification of additional binding partners of p116^{Rip} as well as on interference approaches by using RNA interference-expressing vectors. These studies are currently in progress.

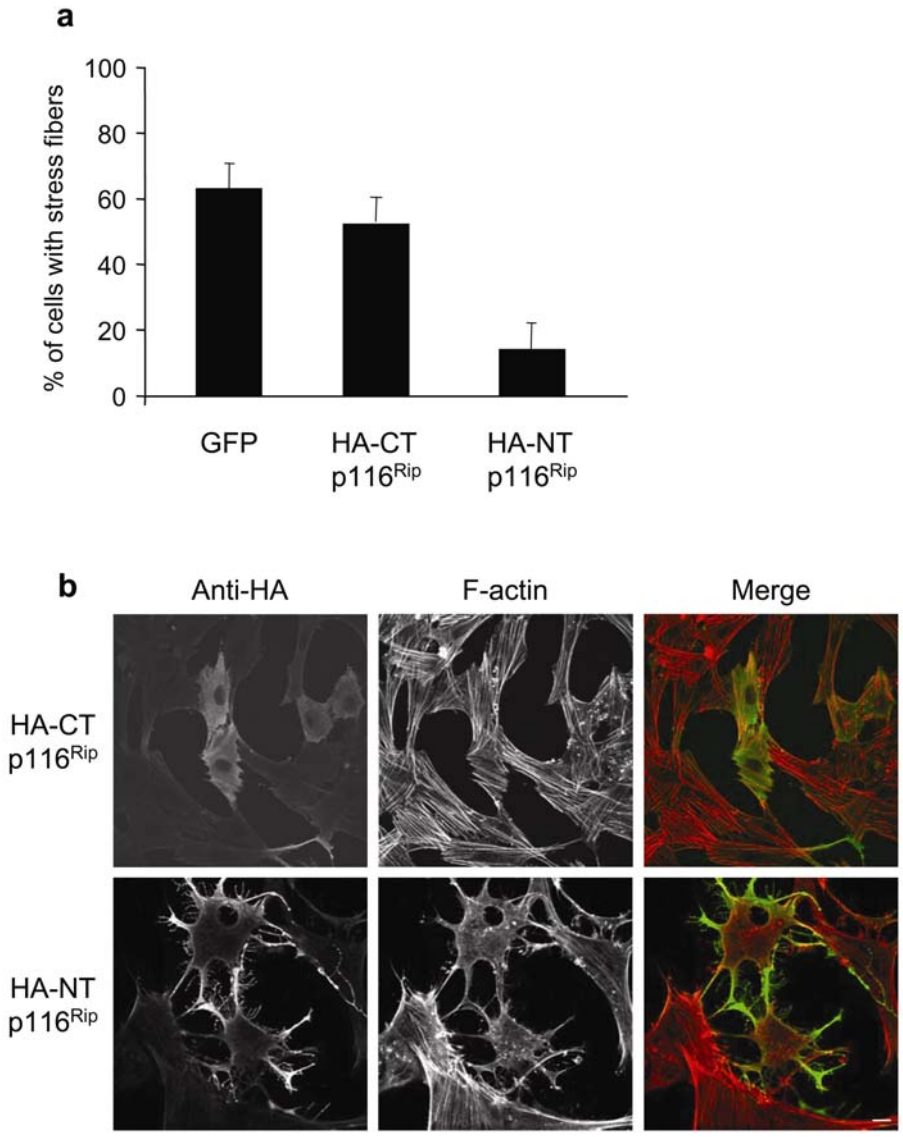

Figure 7. Loss of stress fibres induced by p116^{Rip} and its actin-binding domain
(**a**) NIH3T3 cells expressing the deletion constructs were analysed for the presence of stress fibres. At least 100 cells with similar expression levels of HA-NT-p116^{Rip} or HA-CT-p116^{Rip} were counted for each experiment. Cells containing four or more stress fibres were scored as "cells with stress fibres". To eliminate observer bias, cells were counted by using a blind setup. Data are the means (± S.D.) of three independent experiments. (**b**) NIH3T3 cells were transfected with the indicated deletion mutants, fixed, and immunostained 24 h after transfection. Cells expressing p116^{Rip} deletion mutants were visualised by immunofluorescence using anti-HA antibody (left). Centre column shows staining with rhodamine-conjugated phalloidin (red in the merged pictures).

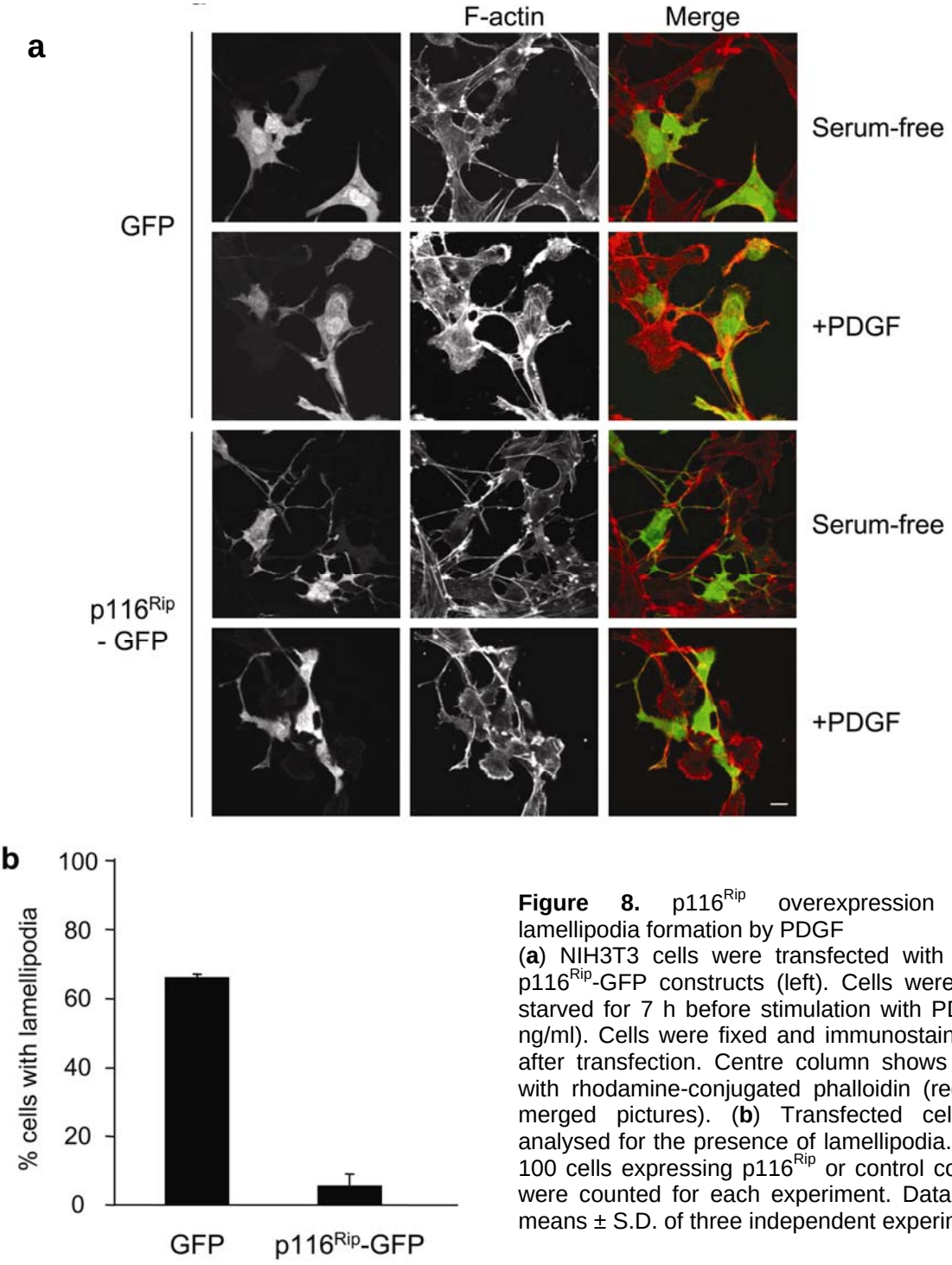

Figure 8. p116^{Rip} overexpression inhibits lamellipodia formation by PDGF
(**a**) NIH3T3 cells were transfected with GFP or p116^{Rip}-GFP constructs (left). Cells were serum-starved for 7 h before stimulation with PDGF (25 ng/ml). Cells were fixed and immunostained 24 h after transfection. Centre column shows staining with rhodamine-conjugated phalloidin (red in the merged pictures). (**b**) Transfected cells were analysed for the presence of lamellipodia. At least 100 cells expressing p116^{Rip} or control constructs were counted for each experiment. Data are the means ± S.D. of three independent experiments.

Materials and Methods

Cell culture and transfection

N1E-115 and COS-7 cells were grown in Dulbecco's modified Eagle's medium containing 7.5% fetal calf serum. NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium containing 10% newborn calf serum. N1E-115 cells were transiently transfected using the calcium phosphate precipitation method described previously (Kranenburg et al., 1995). NIH3T3 cells were transfected using Lipo-fectAMINE Plus (Invitrogen), and serum was added to the cells after the transfection

procedure as described by the manufacturer. COS-7 cells were transfected by the DEAE-dextran method as described previously (Zondag et al., 1996).

Expression constructs

Generation of full-length (FL) pcDNA3-p116^{Rip} (aa 1–1024), pcDNA3-HA-CTp116^{Rip} (aa 545–1024), pcDNA3-HA-RBDp116^{Rip} (aa 545–823), and prp261-RBDp116^{Rip} (aa 545–823) has been described previously (Gebbink et al., 1997). pcDNA3-HA-FLp116^{Rip} was engineered by use of a polylinker created by annealing primers 5'-ggatggcttacccatacgatgttccagattacgcgtgc-3' and 5'-acgcgtaatctggaacatcgtatgggtaagccatccgc-3' encoding the HA-tag sequence and a SacII restriction site. The polylinker was ligated into pcDNA3-FLp116^{Rip} via the SacII site. pcDNA3-HA-NTp116^{Rip} (aa 1–382) and GST-NT were generated by PCR using primers 5'-cggggtaccacatgtcggcggccaaggaa-3' (forward) and 5'-cggaattccggcgtcatggaggattctgt-3' (reverse) for NT. GST-CT was generated from pcDNA3-HA-CTp116^{Rip}. GST-Δ5 (aa 1–152), GST-Δ6 (aa 1–212), GST-Δ7 (aa 43–152), GST-Δ8 (aa 43–212); GST-Δ9 (aa 212–390) were generated similarly by PCR using specific primers. The PCR products were digested with KpNI-EcoRI and ligated into pcDNA3-HA and pRP265, a derivative of pGEX-1N. FLp116^{Rip}-peGFPN1-was constructed by ligation of a HindIII-ScaI fragment out of pcDNA3-FLp116^{Rip} and a PCR fragment using primers 5'-cagagcagtactcccaaaagtgcctgg-3' (forward) and 5'-cgcggtaccagtcgacagaattcgttatcccatgagac-3' (reverse), encoding ScaI and Asp718 restriction sites, into peGFPN1 (Clontech). pMT2sm-FLp116^{Rip}-GST was generated by ligation of a NotI-ScaI fragment of pcDNA3-FLp116^{Rip} and a PCR fragment using primers 5'-cagagcagtactcccaaaagtgcctgg-3' (forward) and 5'-cggggtaccggaattcgttatcccatgagacctg-3' (reverse), encoding ScaI and Asp718 restriction sites, into pMT2sm-GST. pMT2sm-FLp116^{Rip}-myc was generated by cloning FLp116^{Rip}-myc into pMT2sm. Sequence of all constructs was verified by automated sequencing.

Solubility assay

Cells were lysed in ice-cold lysis buffer (0.1% Triton X-100, 50 mM Tris, pH 7.4, 150 mM NaCl, and 1 mM EDTA, supplemented with protease inhibitors) and were left on ice for 15 min. Lysates were centrifuged for 30 min (13,000 rpm; Eppendorf table centrifuge, 4 °C). Pellet and supernatant fractions were collected, dissolved in sample buffer, and subjected to SDS-PAGE. Proteins were detected by Western blotting using the polyclonal anti-p116^{Rip} antibody (1:1000 dilution).

Expression and purification of recombinant fusion proteins

The *E. coli* strains BL21 or DH5α were transformed with plasmids encoding GST-NT, GST-CT, GST-Δ5, GST-Δ6, GSTΔ7, GSTΔ8, GSTΔ9, or GST, respectively. Colonies were obtained and used to inoculate Luria broth/ampicillin. Cultures were grown and isopropyl β-D-thiogalactoside was added overnight to induce expression of the fusion proteins when the cultures reached an OD between 0.4 and 0.6. Bacteria were harvested by centrifugation at 4000 x g, resuspended in cold lysis buffer (50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride), and lysed by sonification followed by the addition of 0.5% Nonidet P-40. Lysates were cleared by centrifugation at 4000 x *g*, and resulting supernatants were incubated with glutathione-Sepharose 4B beads (Amersham Biosciences). Beads with affinity-bound proteins were washed five times with lysis buffer, and bound proteins were eluted with 50 mM Tris-HCl, pH 8.0, containing 10 mM reduced glutathione. In some cases, GST was cleaved off by incubating beads with affinity-bound proteins with thrombin (Amersham Biosciences) according to manufacturer's protocol.

Purified full-length p116^{Rip} fused to GST or purified full-length p116^{Rip} fused to Myc were obtained by transfection of COS-7 cells with the constructs pMT2sm-FLp116^{Rip}-GST and pMT2sm-FLp116^{Rip}-myc, respectively. Cells were lysed in ice-cold lysis buffer (1% Nonidet P-40, 50 mM Tris, pH 7.4, 200 mM NaCl, 2.5 mM $MgCl_2$, and 10% glycerol, supplemented with protease inhibitors). Lysates were cleared by centrifugation (13,000 rpm; 10 min). Further purification of the full-length GST fusion proteins occurred in the same manner as the purification of GST fusion proteins produced

in bacteria. FLp116^{Rip}-myc fusion protein was purified using a column of monoclonal myc antibodies (9E10) chemically cross-linked to protein G-Sepharose beads (Amersham Biosciences). The protein was eluted off with 0.1 M glycine, pH 2.5, and fractions were collected in tubes containing 0.1 volume of 1 M Tris-HCl, pH 8.0, to make an end pH of 7.0. Eluted proteins were subjected to SDS-PAGE, followed by protein staining with Coomassie Blue to estimate the purity and concentration of the proteins in the fractions. Some of the proteins were concentrated using Centricon 10-kDa cutoff devices (Millipore). Protein concentration was also determined by the Bradford method using BSA as a standard. Purified proteins were stored in aliquots at -80 °C in 10% glycerol.

F-actin cosedimentation assay

COS-7 cells were transfected with the indicated expression vectors and lysed for 48 h after transfection in ice-cold lysis buffer (1% Nonidet P-40, 50 mM Tris, pH 7.4, 200 mM NaCl, 2.5 mM $MgCl_2$, and 10% glycerol, supplemented with protease inhibitors). Lysates were cleared by centrifugation (13,000 rpm; 10 min) and supernatant aliquots were run out on SDS-PAGE gels to check for expression (data not shown). 10-µl aliquots were used in the *in vitro* actin-binding assay.

F-actin cosedimentation assays were performed according to the manufacturer's protocol (Cytoskeleton, Denver, CO). Briefly, prespun aliquots of COS-7 cell lysates (100,000 x *g*, 30 min) or purified proteins GST-NTp116^{Rip}, GST, FLp116^{Rip}-GST, BSA, α-actinin (Cytoskeleton), or GST-Δ5p116^{Rip}; GST-Δ6p116^{Rip}; GST-Δ7p116^{Rip}; GST-Δ8p116^{Rip}; GST-Δ9p116^{Rip} were incubated for 1 h at room temperature with 40 µg of pure actin filaments. The final concentration of F-actin was 18 µM. Filaments were subsequently pelleted by centrifugation 100,000 x *g* (Beckman airfuge). As a control for actin-independent sedimentation, the various proteins were also centrifuged under conditions in which filamentous actin was omitted from the mix. Cosedimenting proteins were resolved by SDS-PAGE and detected by either Coomassie Blue staining or by Western blot analysis using anti-p116^{Rip} antibodies, anti-GFP rabbit polyclonal antibodies (van Ham et al., 1997), or an anti-actin mouse monoclonal antibody (Mab 1501R; Chemicon).

For quantitative analysis, a fixed concentration of FLp116^{Rip}-GST (1 µM) was mixed with increasing amounts of F-actin (0–3.5 µM) in polymerisation buffer and incubated at room temperature for 30 min. Proteins were centrifuged as above and total pellets and supernatants were loaded separately on SDS-polyacrylamide gels. Protein bands were detected by Coomassie Blue staining and were scanned and quantified using the software program TINA. The amount of p116^{Rip} bound to different concentrations of F-actin was fit to a single rectangular hyperbola using Prism (ver. 3; GraphPad Software, San Diego, CA). In all cases, entire pellet and supernatant fractions were loaded separately on SDS-polyacrylamide gels and detected by either Coomassie Blue staining or by Western blot (above).

Electron microscopy

To test for bundling activity, actin filaments (5 µM) were incubated for 30 min with purified proteins GST, α-actinin (both 2 µM), FL-p116^{Rip} (0.5 µM), NT-p116^{Rip} (0.5 µM), and CT-p116^{Rip} (0.5 µM) at room temperature. Samples were absorbed on to glow-discharged carbon-coated formvar film on a copper grid and negatively stained with 1% uranyl acetate and examined with a Philips CM10 electron microscope.

Metabolic Labeling

N1E-115 cells were incubated in methionine/cysteine-free media for 30 min and labeled for 4 h with medium containing [^{35}S]methionine/cysteine (200 µCi/ml; Amersham Biosciences). Labeled medium was aspirated and cells were washed once in ice-cold PBS. Cells were scraped in ice-cold lysis buffer (1% Nonidet P-40, 50 mM Tris pH 7.4, 200 mM NaCl, 2.5 mM $MgCl_2$, and 10% glycerol, supplemented with protease inhibitors). Lysates were cleared by centrifugation (13,000 rpm; 10 min) and supernatants were tumbled with protein A-Sepharose beads precoupled to either preimmune

rabbit serum, anti-p116^{Rip} antibodies (Gebbink et al., 1997), or anti-myosin IIA antibodies (BTI, Oklahoma City, OK) for 1h at 4 °C. GST pull-down assays were performed with 20 µl of GSH-Sepharose beads loaded with 20 µg of either GST alone or the GST-NT-fusion protein containing the isolated actin-binding domain. Beads were washed five times in ice-cold lysis buffer and resolved by SDS-PAGE. Proteins were detected by autoradiography. In some cases the gels were blotted and further analysed by Western blotting to assess the identity of labeled proteins using the polyclonal anti-p116^{Rip} antibody (1:1000 dilution) and anti-myosin II antibodies (1:500 dilution).

Antibodies and Confocal Microscopy

The FRA58 antibody directed against GST-RBD (amino acids 545–823) has been described previously (Gebbink et al., 1997). N1E-115 cells and NIH3T3 cells were grown on gelatin-coated glass coverslips in six-well plates. N1E-115 cells were serum-starved overnight and NIH3T3 cells for 7 h. Cells were fixed 24 h after transfection in 3.7% formaldehyde in PBS for 10 min, permeabilised (0.1% Triton X-100/PBS; 2 min), blocked (1% BSA/PBS; 30 min), and incubated with primary antibodies (FRA58 preimmune serum, polyclonal FRA58 anti-p116^{Rip}, 3F10 anti-HA rat monoclonal antibodies (Roche; 1 h). Subsequently, cells were washed and incubated with secondary antibodies (Goat-anti-rabbit-fluorescein isothiocyanate (DAKO) and Goat-anti-rat-fluorescein isothiocyanate (Rockland); 30 min)) together with rhodamine-conjugated phalloidin (Molecular Probes). Coverslips were mounted in Vectashield and analysed using a Leica TCS-NT confocal microscope.

Acknowledgments

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Supplementary data

Studies involving the interaction between p116^{Rip} and RhoA

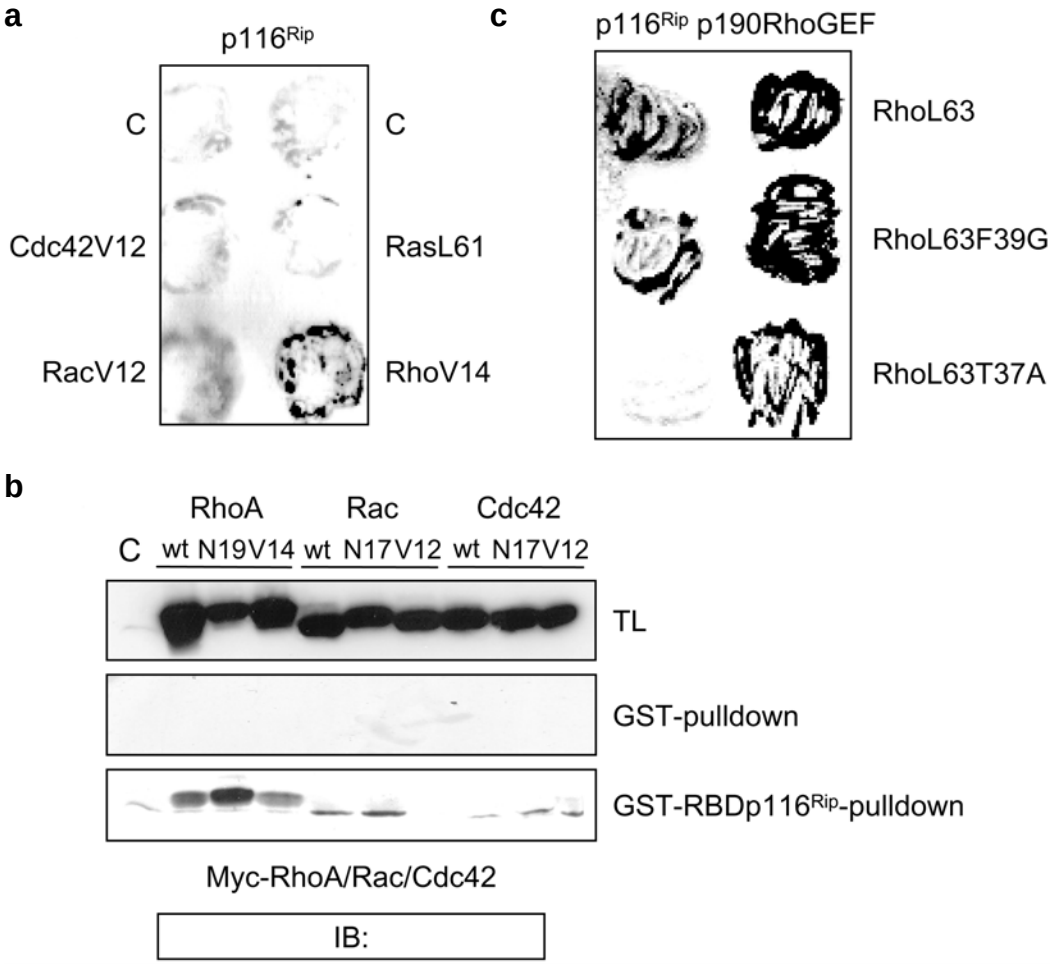

Figure 1. p116^{Rip} interacts specifically with RhoV14

(**a**) The cDNA encoding p116^{Rip} was isolated in a yeast two-hybrid screen using activated V14RhoA as bait. Using the yeast 2-hybrid system, it was determined that p116^{Rip} binds to activated RhoA, but not to the activated forms of other small GTPases Rac, Cdc42, or Ras. Empty vector controls are indicated (C). (**b**) COS-7 cells were transfected with plasmids encoding inactive (N), active (V), and wt forms of Rac, Cdc42, and RhoA. GST pull-downs were performed with either GST alone or GST-RBDp116^{Rip} (the putative RhoA binding domain of p116^{Rip} (RBD; aa 545-823)). Total cell lysates (TL) and pull-downs were subjected to SDS-PAGE and analysed by immunoblotting (IB) using α-myc and α-GST. Again, p116^{Rip} interacted mainly with RhoA and not with Rac, or Cdc42. Furthermore, the interaction of p116^{Rip} and RhoA is nucleotide independent. (**c**) p116^{Rip} binding to several effector loop mutants of RhoA using yeast two-hybrid analysis. While the control, p190RhoGEF binds to all effector loop mutants of RhoA, p116^{Rip} fails to interact with the L63A37RhoA mutant. This result indicates that, although weak, the yeast two-hybrid interaction between p116^{Rip} and RhoA is specific, mediated by specific residues in the effector loop of RhoA. Note that p116^{Rip} binding to RhoA is weak when compared to p190RhoGEF and RhoA binding.

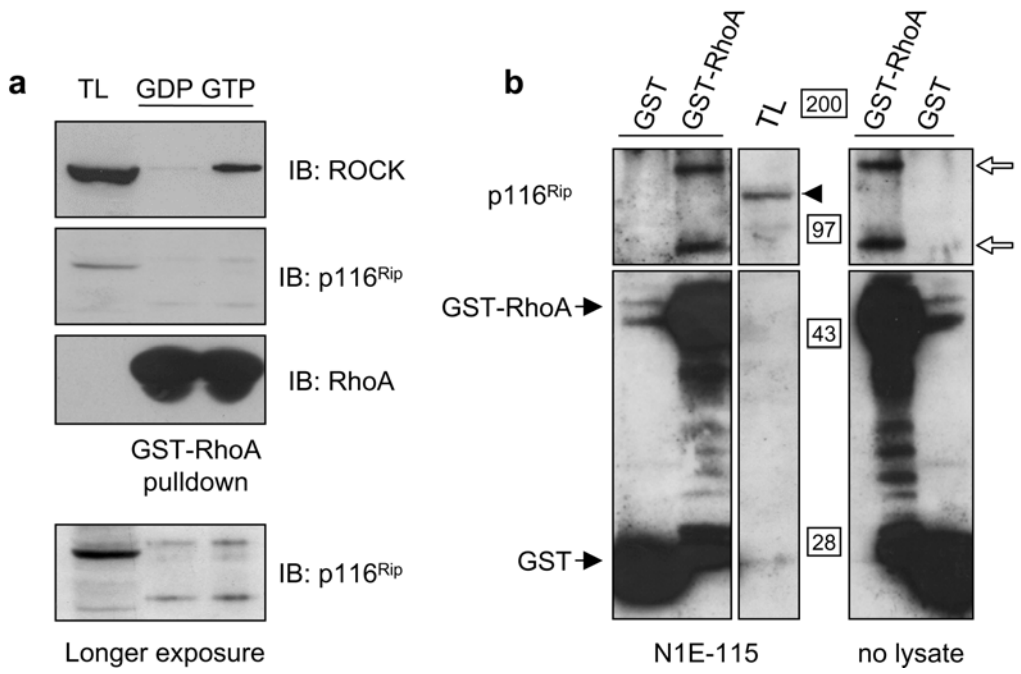

Figure 2. No detectable endogenous interaction between RhoA and p116^{Rip}
(**a**) GST pull-downs were performed in N1E-115 lysates using GST-RhoA fusion proteins loaded with either GDP or GTP, and precipitates were subjected to SDS-PAGE. Blots were incubated with α-RhoA, α-p116^{Rip}, or α-ROCK. Total cell lysates (TL) were analysed for ROCK and p116^{Rip} expression (left panel). Whereas endogenous ROCK associates with Rho in a GTP-dependent manner, p116^{Rip} does not co-precipitate with either RhoA-GDP or RhoA-GTP. (**b**) GST-RhoA or GST fusion proteins (arrows) were incubated with N1E-115 lysates (left panel) or lysis buffer (no lysate, right panel). Precipitates were subjected to SDS-PAGE and blots were incubated with α-p116^{Rip} or α-GST. TL analysed for p116^{Rip} expression are shown (arrow head, middle panel). This figure shows that the two proteins that are immunoreactive with the p116^{Rip} antibodies originate from the GST-RhoA fusion preparation in *E.coli* (open arrows).

Concluding remarks

Thus, despite the specific RhoA-p116^{Rip} interaction *in vitro* and in yeast two-hybrid analysis, the *in vivo* existence of a p116^{Rip}-RhoA complex remains ambiguous. Also in intact cells p116^{Rip} and RhoA do not obviously co-localise, as determined by confocal analysis (Chapter 2). There may be several reasons for the inability to detect an endogenous interaction between p116^{Rip} and RhoA. In addition to the possibility that the two proteins just do not interact in an *in vivo* situation, the interaction might be too transient to be detected by co-precipitation or pull-down assays. The RhoA binding domain of p116^{Rip} is not homologous to that of other Rho-binding proteins and SMART, Pfam, or MOTIF search programs do not identify a consensus Rho-binding domain in p116^{Rip}. Studying the affinity of the RhoA-p116^{Rip} interaction might be helpful to shed more light on the nature of this interaction and the likelihood of an endogenous p116^{Rip}-RhoA complex. As yet, we conclude that p116^{Rip} is unlikely to interact with RhoA.

Chapter 3

p116^{Rip} targets myosin phosphatase to the actin cytoskeleton and is essential for RhoA/ROCK-regulated neuritogenesis

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p116^{Rip} targets myosin phosphatase to the actin cytoskeleton and is essential for RhoA/ROCK-regulated neuritogenesis

Abstract

Activation of the RhoA-Rho kinase (ROCK) pathway stimulates actomyosin-driven contractility in many cell systems, largely through ROCK-mediated inhibition of myosin-II light-chain phosphatase. In neuronal cells, the RhoA-ROCK-actomyosin pathway signals cell rounding, growth cone collapse and neurite retraction; conversely, inhibition of RhoA/ROCK promotes cell spreading and neurite outgrowth. The actin-binding protein p116^{Rip}, whose N-terminal region bundles F-actin in vitro, has been implicated in Rho-dependent neurite remodelling; however, its function is largely unknown. Here we show that p116^{Rip}, through its C-terminal coiled-coil domain, interacts directly with the C-terminal leucine zipper of the regulatory myosin-binding subunits of myosin-II phosphatase, MBS85 and MBS130. RNAi-induced knockdown of p116^{Rip} inhibits cell spreading and neurite outgrowth in response to extracellular cues, without interfering with the regulation of myosin light-chain phosphorylation. We conclude that p116^{Rip} is essential for neurite outgrowth and may act as a scaffold to target the myosin phosphatase complex to the actin cytoskeleton.

Introduction

Small GTP-binding proteins of the Rho family, notably RhoA, Rac and Cdc42, are key regulators of the actin cytoskeleton in response to extracellular cues (Etienne-Manneville and Hall, 2002). In particular, RhoA regulates actomyosin-driven contractile events and morphological changes in many cell types, including those of the nervous system (Hall, 1998; Kaibuchi et al., 1999; Luo, 2000). It does so primarily by stimulating the activity of Rho-kinase (ROCK), which phosphorylates the regulatory myosin-binding subunit (MBS) of myosin light chain (MLC) phosphatase. When phosphorylated by ROCK, MBS inhibits the activity of MLC phosphatase and thereby promotes MLC phosphorylation and actomyosin contractility (Kimura et al., 1996; Kawano et al., 1999). In neuronal cells, activation of the RhoA-ROCK-actomoysin pathway is necessary and sufficient to induce growth cone collapse, retraction of developing neurites and transient rounding of the cell body in response to certain receptor agonists, such as lysophosphatidic acid (LPA), thrombin and sphingosine-1-phosphate (Jalink et al., 1994; Postma et al., 1996; Kozma et al., 1997; Amano et al., 1998; Hirose et al., 1998; Bito et al., 2000). Conversely, inactivation of the RhoA-ROCK pathway, using pharmacological inhibitors or dominant-negative constructs, is sufficient to promote neurite outgrowth and growth cone motility (Jalink et al., 1994; Kozma et al., 1997; Hirose et al., 1998; Bito et al., 2000). From these studies, it has emerged that RhoA-actin pathways are fundamental to neurite remodeling, guidance and branching not only in neuronal cell lines but also in primary neurons (Luo, 2000; Luo, 2002). Many questions remain, however, as it remains unclear how components of Rho signalling pathways are compartmentalised and assembled into functional signalling modules, and how the specificity of signal transduction is controlled.

Various actin-associated proteins participate in regulating cytoskeletal dynamics downstream of Rho GTPases, with some proteins arranging actin into higher-order structures and others controlling actin remodeling in response to physiological stimuli (Ayscough, 1998; Bamburg, 1999; Borisy and Svitkina, 2000; Wear et al., 2000; Higgs and Pollard, 2001; Da Silva et al., 2003). We previously identified a ubiquitously expressed protein of predicted size 116 kDa, named p116^{Rip}, which binds relatively weakly to constitutively active RhoA(V14) in a yeast two-hybrid assay (Gebbink et al., 1997). The p116^{Rip} sequence reveals several protein interaction domains, including two PH domains, two proline-rich stretches and a C-terminal coiled-coil domain; it lacks any known catalytic motif. When overexpressed in N1E-115 neuroblastoma cells, p116^{Rip} promotes cell flattening and neurite extension and inhibits LPA-induced neurite retraction, reminiscent of what is observed after RhoA inactivation using dominant-negative RhoA or the Rho-inactivating C3 toxin (Gebbink et al., 1997). This led us to hypothesize that p116^{Rip} may negatively regulate RhoA signalling. More recently, however, we found that p116^{Rip}, rather than directly binding to RhoA (Gebbink et al., 2001), interacts through its N-terminal region with F-actin and colocalises with dynamic actomyosin-rich structures, including stress fibers and cortical microfilaments in filopodia and lamellipodia (Mulder et al., 2003). Furthermore, we found that p116^{Rip} induces bundling of F-actin *in vitro*, with the bundling activity residing in the N-terminal region. Despite being an actin-bundling protein, overexpression of p116^{Rip} or its N-terminal actin-binding domain in neuroblastoma cells disrupts the actin cytoskeleton and thereby inhibits actomyosin contractility to promote neurite outgrowth (Mulder et al., 2003). Thus, p116^{Rip} is an actin-binding protein that may act as a scaffold for multiple protein interactions involved in neurite remodeling.

To understand the normal biological function of p116^{Rip} in neuritogenesis, we set out to identify binding partners of the C-terminal domain of p116^{Rip} and to undertake a loss-of-function analysis using RNA interference (RNAi). We report here that the coiled-coil domain of p116^{Rip} interacts directly with the regulatory myosin-binding subunits of MLC phosphatase, MBS85 and MBS130. Furthermore, our RNAi-studies show that p116^{Rip}-deficient cells fail to undergo neurite outgrowth in response to various extracellular cues (RhoA/ROCK inhibition, intracellular cAMP elevation, or growth factor deprivation), while the regulation of MLC phosphorylation remains intact. Thus, p116^{Rip} is an essential component in the RhoA/ROCK-actomyosin pathway that regulates neuritogenesis.

Results

Identification of myosin phosphatase targeting subunits, MBS85 and MBS130, as binding partners of p116^{Rip}

We previously reported that the N-terminal region of p116^{Rip} (residues 1-382) binds tightly to F-actin (K_d approx. 0.5 μM), shows F-actin-bundling activity *in vitro*, and dictates the subcellular localisation of p116^{Rip} to dynamic actin-rich structures such as stress fibers and cortical microfilaments (Mulder et al., 2003). To identify additional binding partners of p116^{Rip}, we performed a yeast two-hybrid screen with the C-terminal part of p116^{Rip} (aa 391-1024) as bait and a human testis cDNA library as prey. We detected a strong interaction with the very C-terminal leucine-zipper domain (aa 686-782) of the regulatory myosin-binding

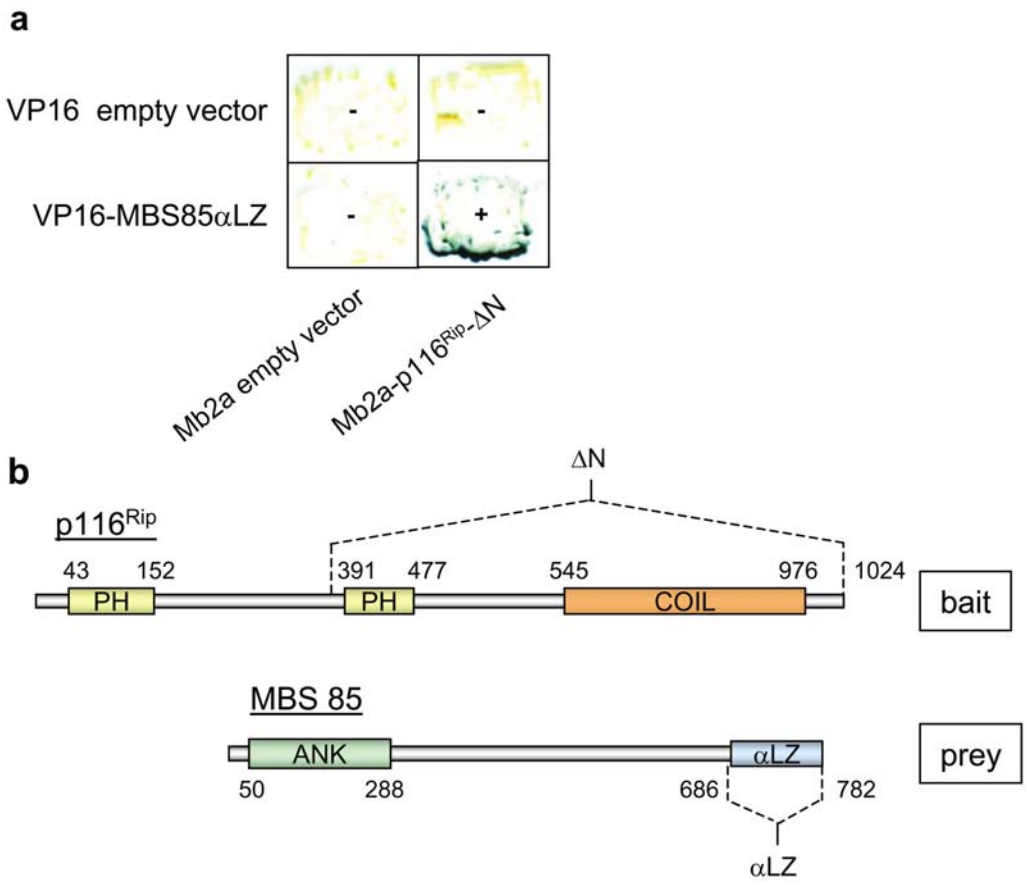


Figure 1. Yeast two-hybrid interaction between p116^{Rip} and MBS85
(**a**) MBS85 was identified by screening a human testis cDNA library using the C-terminal part of p116^{Rip} as bait (p116^{Rip}-ΔN). Binding of the α-helical leucine zipper of MBS85 to the C-terminus of p116^{Rip} was confirmed in retransformation assays, in which clones became positive for β-galactosidase activity within 1 hr. Yeast cells (Y190) were transformed with Mb2a encoding the GAL4 binding domain fused with the C-terminus of p116^{Rip} and VP16 encoding the GAL4 activation domain fused with the C-terminus of MBS85 and screened for β-galactosidase activity (+). Empty vector controls are indicated. (**b**) Schematic representation of the interacting domains (bait-prey) in p116^{Rip} and MBS85. Abbreviations: PH, pleckstrin homology domain; ANK, ankyrin repeats; Coil, coiled-coil domain; αLZ, α-helical leucine zipper domain. Numbers refer to amino acid residues.

subunit p85 (MBS85) (Fig. 1A). MBS85 is structurally and functionally related to its better-known and more widespread family member MBS130 (Tan et al., 2001), with both proteins containing N-terminal ankyrin repeats and an α-helical leucine zipper at the C-terminus. MBS85 is highly expressed in brain and heart, but unlike MBS130, not in smooth muscle (Tan et al., 2001). Through their N-terminal region, MBS85 and MBS130 bind to the catalytic subunit, protein phosphatase-I (PP1δ; 37-kDa), and thereby regulate phosphatase activity toward the myosin-II light-chain (MLC). MBS binds to the active, GTP-bound form of RhoA and is a direct target of ROCK (Kimura et al., 1996). MBS phosphorylation by ROCK results in inhibition of phosphatase activity with a consequent increase in MLC phosphorylation and actomyosin contractility.

A schematic representation of the two-hybrid interaction between the C-terminal parts of p116^{Rip} and MBS85 is shown in Fig. 1B. Given the high similarity between MBS85 and MBS130 in their α-helical leucine repeats (66% at the amino acid level) (Tan et al., 2001), we examined the interaction of p116^{Rip} with both MBS85 and MBS130 in COS-7 cells.

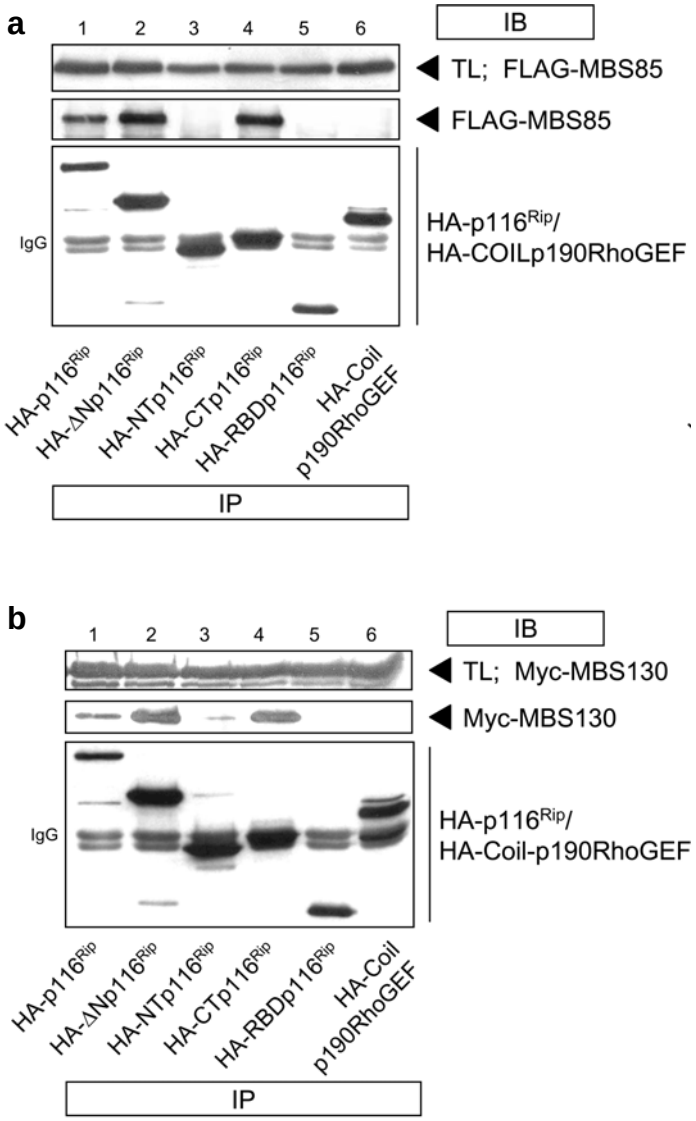
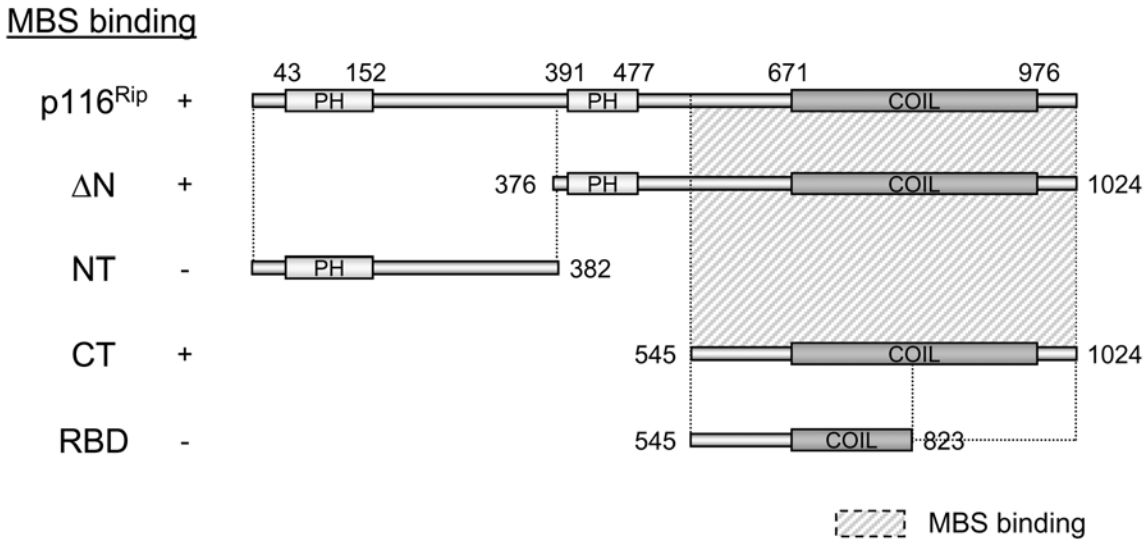


Figure 2. The coiled-coil domain of p116^{Rip} interacts with the C-terminal leucine zipper of MBS85 and MBS130 in mammalian cells (**a**, **b**) COS-7 cells were transfected with plasmids encoding FLAG-MBS85 or Myc-MBS130 and the indicated HA-tagged p116^{Rip} truncation mutants or HA-coilp190RhoGEF. Total cell lysates (TL) were analysed for MBS85 and MBS130 expression (upper panels). Immunoprecipitation (IP) of HA-tagged proteins and co-precipitation of MBS85 or MBS130 was analysed by immunoblotting (IB) using α-HA, α-FLAG, and α-Myc antibodies. (**c**) Analysis of the interaction between the coiled-coil domain of p116^{Rip} and the leucine zipper of MBS (MBS85 and MBS130), as determined in COS-7 cells; + denotes interaction with MBS. NT, N-terminal region; CT, C-terminal region; RBD, 'Rho-binding' domain (as defined in Gebbink et al., 1997).

c



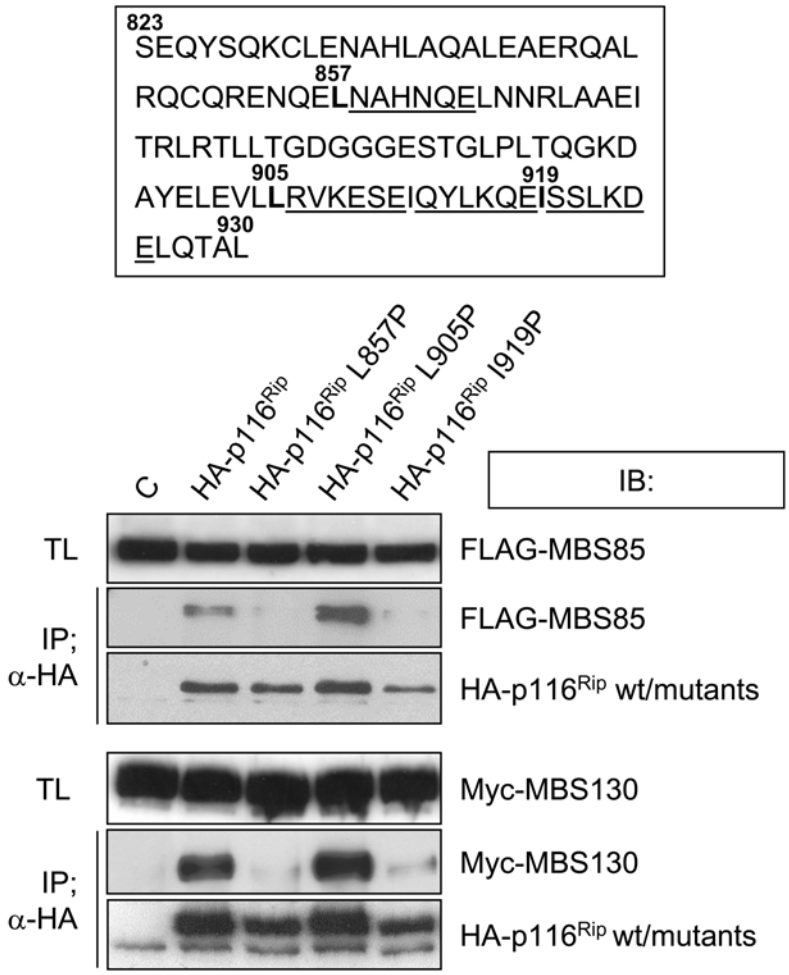


Figure 3. (Iso)leucine residues in the coiled-coil of p116^{Rip} mediate binding to the leucine zippers of MBS85 and MBS130

Representation of residues 823-930 of the coiled-coil domain of p116^{Rip}. Leucine zipper-like motifs are underlined. (Iso)leucines in bold were mutated to proline by site-directed mutagenesis. COS-7 cells were co-transfected with plasmids encoding either FLAG-MBS85 or Myc-MBS130 and indicated HA-tagged plasmids encoding p116^{Rip} (iso)leucine mutants or an empty HA-tagged plasmid as control. Total cell lysates (TL) analysed for MBS85 and MBS130 expression are shown (upper panels in both figures). Immunoprecipitation (IP) of HA-tagged proteins and co-precipitation of MBS85 or MBS130 was analysed by immunoblotting (IB) using α-HA, α-FLAG, and α-Myc antibodies.

We found that epitope-tagged forms of MBS85 and MBS130 both co-precipitate with HA-tagged p116^{Rip} (Fig. 2A, B), indicating that the p116^{Rip}-MBS85/130 interaction occurs *in vivo*.

To map the region in p116^{Rip} that mediates MBS binding, we generated HA-tagged truncation mutants of the C-terminal part of p116^{Rip} and expressed them in COS cells. As a negative control, we used the C-terminal coiled-coil domain of an unrelated protein, the Rho-specific exchange factor p190RhoGEF (van Horck et al., 2001). All p116^{Rip} truncation mutants containing the coiled-coil domain were found to precipitate both MBS130 and MBS85 from COS cell lysates (Fig. 2A-C). No MBS interaction was detected with a truncated coiled-coil mutant ('RBD'; aa 545-823) or with the isolated N-terminal part of p116^{Rip} (NT; aa 1-382), nor with the coiled-coil domain of p190RhoGEF (Fig. 2A, B). These results indicate that the coiled-coil region of p116^{Rip} (aa 545-1024) interacts specifically with the α-helical

leucine zippers of MBS85 and MBS130, and that the MBS-binding region of p116^{Rip} is located within the C-terminal half of the coiled-coil domain (aa 823-1024; Fig. 2C).

Leucine zippers are commonly regarded as regular coiled-coil structures (O'Shea et al., 1989), in which the (iso)leucine repeats form a hydrophobic region where side-chain interactions mediate protein-protein interaction; mutagenesis of selected (iso)leucine residues (to alanine, valine, or proline) abrogates the interaction (Turner and Tjian, 1989; Hu et al., 1990). To determine whether leucine-isoleucine motifs in the coiled-coil of p116^{Rip} are essential for binding to the MBS leucine zipper, we replaced residues Leu(857), Leu(950) and Ile(919) in p116^{Rip} with Pro residues (Fig. 3). Protein-protein interactions were examined by co-expression of these p116^{Rip} mutants with either MBS85 or MBS130. As shown in Fig. 3, the L857P and I919P mutants, but not the L905P mutant, failed to interact with MBS85 and MBS130. We conclude that residues Leu(857) and Ile(919) in p116^{Rip} are critical for direct interaction with the leucine-zipper domains of MBS85 and MBS130.

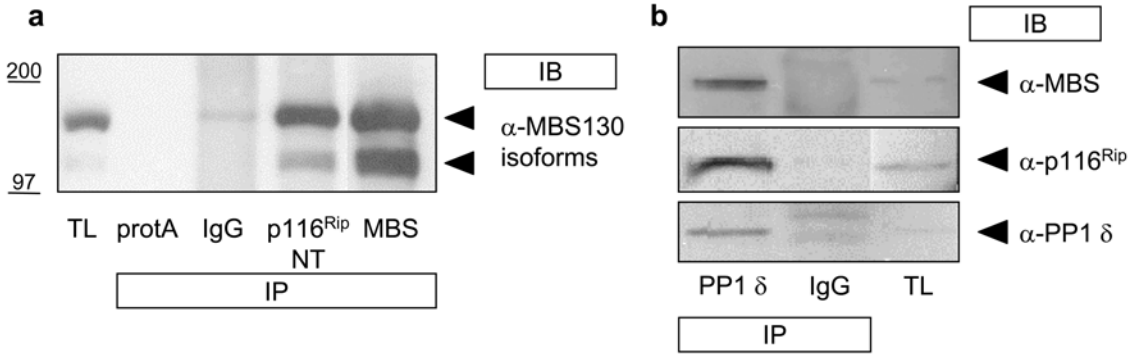


Figure 4. Association of endogenous p116^{Rip} with the MLC phosphatase complex
(**a**) p116^{Rip} and MBS130 were immunoprecipitated (IP) from N1E-115 cells, and subjected to western blotting (IB) using MBS130 antibodies. Control lysates were incubated with protein A-Sepharose 4B beads alone, or together with control IgG. Both isoforms of MBS130 are detected in p116^{Rip} immunoprecipitates. TL denotes total cell lysates. (**b**) PP1δ immunoprecipitates (IP) from N1E-115 cells and total lysate (TL) were subjected to western blotting (IB) using MBS130, p116^{Rip}, and PP1δ antibodies. MBS130 and p116^{Rip} are seen to co-immunoprecipitate with endogenous PP1δ.

Endogenous interaction of p116^{Rip} with the myosin phosphatase complex in neuronal cells

We next examined complex formation between endogenous p116^{Rip} and MBS130 in neuronal N1E-115 cells. As shown in Fig. 4, endogenous MBS130 and p116^{Rip} were co-immunoprecipitated from N1E-115 cell lysates using a polyclonal anti-p116^{Rip} antibody; endogenous MBS85 could not be detected due to lack of antibodies. Two separate MBS130-reactive bands are observed in p116^{Rip} precipitates from N1E-115 cells (Fig. 4A), consistent with MBS130 existing in two isoforms one of which contains a central insertion (Hartshorne, 1998). No MBS130 protein was detected in control immunoprecipitates. MBS associates with the catalytic subunit, PP1δ. Fig. 4B shows that p116^{Rip} and MBS130 are both present in PP1δ immunoprecipitates from N1E-115 cells. We conclude that p116^{Rip} is a component of the MBS-PP1δ complex in neuronal cells.

RNAi-induced knockdown of p116^{Rip} inhibits neurite outgrowth

Transient overexpression of p116^{Rip} results in disassembly of the actin cytoskeleton and promotes neurite extension in N1E-115 cells (Gebbink et al., 1997; Mulder et al., 2003). Expression of the actin-binding region alone (NT-p116^{Rip}) led to the same phenotype, whereas cells expressing the C-terminal domain alone displayed a seemingly normal F-actin pattern (Mulder et al., 2003). While these results suggest that the N-terminal half of p116^{Rip} is critical for mediating F-actin (dis)assembly, they do not allow any conclusion about the normal physiological function of p116^{Rip}. A more informative and elegant approach to assessing the importance of putative scaffold proteins like p116^{Rip} is to knockdown their endogenous expression levels by RNA interference (RNAi).

We used the expression vector pSUPER, which directs stable expression of small interfering RNAs (Brummelkamp et al., 2002a). Persistent suppression of gene expression by this vector allows the analysis of loss-of-function phenotypes that develop over longer periods of time. A green fluorescent protein (GFP)-containing construct was created that targets mouse p116^{Rip} mRNA (with GFP expressed from a distinct PGK promotor), termed pSUPER-GFP (pS-GFP). Targeting efficacy was tested in HEK293 cells by co-transfecting HA-p116^{Rip} and pS-GFPp116^{Rip}. HA-p190RhoGEF was used as a negative control (van Horck et al., 2001). At 4 days after transfection, HA-p116^{Rip} expression is knocked down by almost 100%, whereas HA-p190RhoGEF expression remains unaffected (Fig. 5A). The RNAi construct was then transfected into Neuro-2A and N1E-115 cells to study the phenotypic consequences of p116^{Rip} knockdown. Immunoblot analysis confirmed effective knockdown of endogenous p116^{Rip} in Neuro-2A cells (Fig. 5F).

Neurite outgrowth (with concomitant growth arrest) can be induced by various treatments, including pharmacological inhibition of the RhoA-ROCK pathway, elevation of intracellular cAMP levels, and growth factor deprivation. While control Neuro-2A and N1E-115 cells underwent flattening and neuritogenesis within a few hrs after serum withdrawal, we consistently observed that their p116^{Rip}-deficient counterparts (pS-GFP-p116^{Rip}-positive) maintained a rounded shape even after prolonged periods (>48 hrs) of serum starvation. Also after prolonged incubation with the membrane-permeable cAMP analogue, dibutyryl-cAMP (db-cAMP; 4 days), the p116^{Rip}-deficient cells (pS-GFPp116^{Rip}-positive) remain rounded with little or no sign of process extensions: less than 9% of the p116^{Rip}-depleted cells is capable of sending out neurites as compared to 54% of the GFP-expressing control cells (Fig. 5B, C). Finally, addition of the ROCK inhibitor Y-27632 resulted in rapid cell flattening and initiation of neurite outgrowth in both Neuro-2A and N1E-115 cells, whereas the p116^{Rip}-deficient cells failed to show any morphological response to Y-27632 (Fig. 5D, E). Similar results were obtained with the Rho-inactivating C3-exoenzyme (our unpublished results). Co-expression of RNAi-resistant p116^{Rip} (containing three silent mutations in the target sequence) with pS-GFPp116^{Rip} largely restored the morphological responses (our unpublished results), consistent with the observed effects being specific for p116^{Rip} loss-of-function (although introduction of RNAi-resistant p116^{Rip} is in fact “overexpression”).

Together, these results indicate that p116^{Rip} is essential for Neuro-2A and N1E-115 cells to send out neurites in response to various extracellular cues, notably RhoA/ROCK inhibition, growth factor withdrawal and intracellular cAMP elevation.

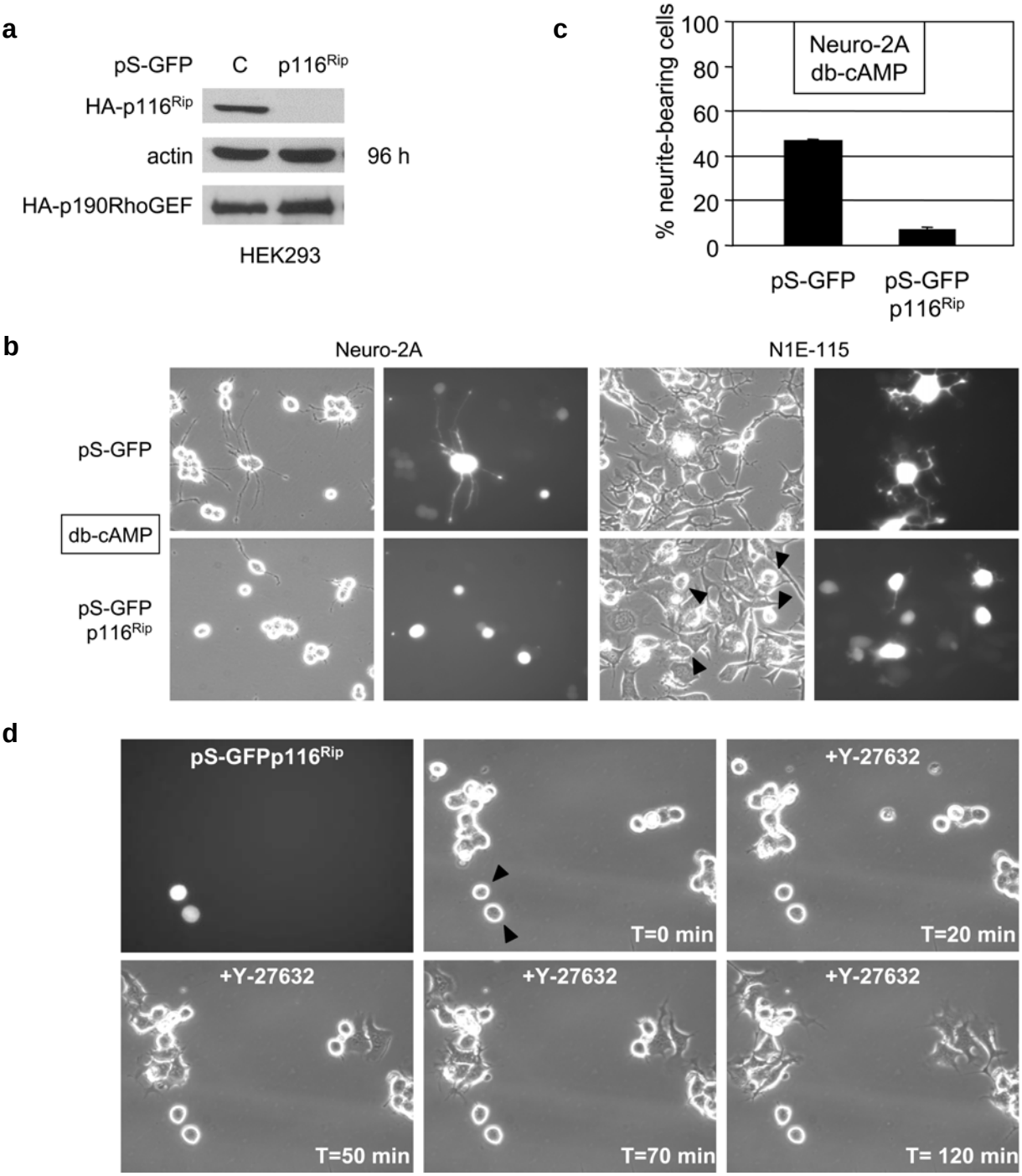
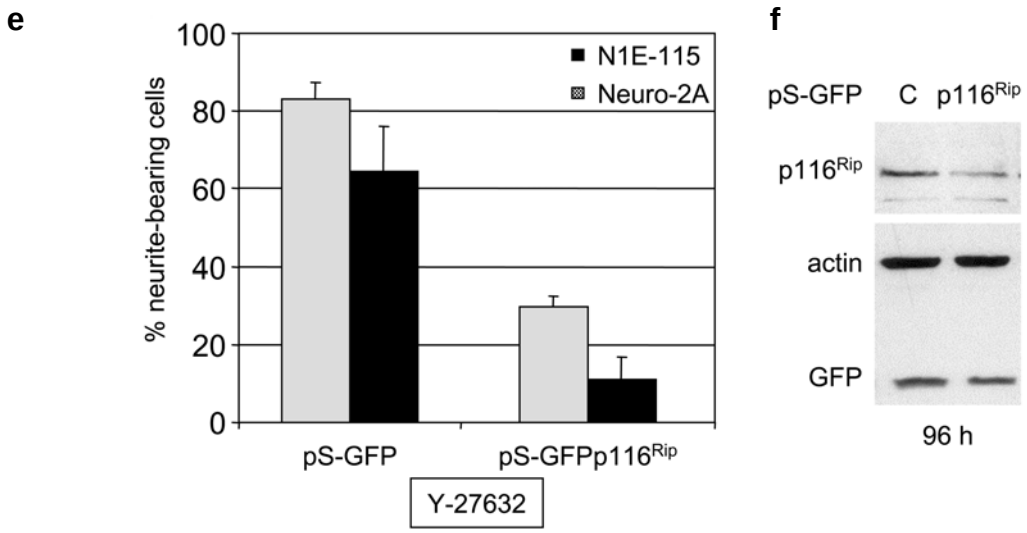


Figure 5. RNAi-mediated knockdown of p116^{Rip} prevents neurite outgrowth
(**a**) pS-GFP (Control, C) or pS-GFPp116^{Rip} plasmids were co-transfected with plasmids encoding HA-p116^{Rip} or HA-p190RhoGEF (van Horck et al., 2001) into HEK293 cells. Co-transfection with pS-GFPp116^{Rip} knocks down the expression of HA-p116^{Rip}, but not the expression of HA-p190RhoGEF or actin. (**b**) Neuro-2A and N1E-115 neuroblastoma cells were transfected with pS-GFP or pS-GFPp116^{Rip} plasmids. At 72 hrs after transfection, neurite outgrowth was induced by addition of 1 mM db-cAMP. After two days, cells were analysed by fluorescence (right panel) and phase-contrast (left panel) microscopy. Arrowheads point to pS-GFPp116^{Rip}-positive cells. (**c**) Neuro-2A cells were transfected with pS-GFP or pS-GFPp116^{Rip} plasmids. At 72 hrs after transfection, neurite outgrowth was induced by addition of db-cAMP (1 mM). The number of GFP-positive, neurite-bearing Neuro-2A cells was determined at 48 hrs after addition of db-cAMP. At least 100 GFP-positive cells were counted for each experiment. Bars denote the means (± s.d.) of three in-



dependent experiments. (**d**) N1E-115 cells were transfected with pS-GFPp116^{Rip} plasmids. At 9 hrs after transfection, the ROCK inhibitor Y-27632 (10 μM) was added and subsequent changes in cell morphology were monitored by time-lapse microscopy. Note that control cells start to flatten and initiate neurite outgrowth within approx. 30 min. of Y-27632 addition, whereas the p116^{Rip}-deficent cells (arrowheads) fail to change their morphology. (**e**) As in (b), the number of GFP-positive Neuro-2A or N1E-115 cells showing neurite outgrowth was determined at 8 hrs after addition of Y-27632 (96 hrs after transfection). At least 100 cells were counted for each experiment. Bars denote the means (± s.d.) of three independent experiments. (**f**) Neuro-2A cells were transfected with the indicated plasmids, lysed 96 hrs after transfection and analysed for p116^{Rip} expression. pS-GFPp116^{Rip} knocks down endogenous p116^{Rip} expression, taking into account a transfection efficiency of Neuro2A cells of about 30%.

p116^{Rip}-deficiency does not impair the regulation of MLC phosphorylation

Our observation that p116^{Rip} deficiency interferes with neuritogenesis is reminiscent of what is observed after constitutive activation of the RhoA-ROCK pathway eventually leading to phosphorylation of the 20-kDa MLC. We therefore examined whether p116^{Rip} deficiency affects the regulation of MLC phosphorylation. Phosphorylation of nonmuscle myosin-II on MLC residue Ser-19 regulates both its motor activity and filament assembly (for review see (Bresnick, 1999). MLC phophorylation is mediated mainly by myosin light-chain kinase with probably an additional but less established role for ROCK (Kawano et al., 1999; Bresnick, 1999). The MLC phosphorylation state can be determined using a phospho-specific antibody that recognises MLC only when Ser-19 is phosphorylated (Matsumura et al., 1998).

In agreement with findings by others (Amano et al., 1998), we found that differences in MLC phosphorylation on Ser-19 are hard to detect in neuroblastoma cells (our unpublished results); therefore, we turned to NIH3T3 cells. Cells were infected with control pS retrovirus (pRS) and pRS-p116^{Rip} retrovirus (Brummelkamp et al., 2002b). After three days, cells were serum starved and then stimulated with either LPA (as a RhoA-activating agonist) or with calyculin A (CA), a potent inhibitor of type-I phosphatases and as such a strong inducer of neurite retraction. In parallel experiments, the ROCK inhibitor Y-27632 was added to cells maintained in serum-containing medium. As shown in Fig. 6, p116^{Rip} expression levels are strongly reduced in pRS-p116^{Rip} cells. Yet pRS control and pRS-p116^{Rip} cells, maintained in serum-free medium, show comparable basal levels of phosphorylated MLC (as detected by anti-phospho-MLC-Thr18/Ser19 antibody), while LPA

and calyculin A are both capable of further enhancing MLC phosphorylation (Fig. 6, lower panel). Neuro-2A and N1E-115 cells underwent rapid neurite retraction and cell rounding in response to LPA and calyculin A, whereas the rounded pRS-p116^{Rip} cells did not show any further shape change in response to either stimulus. Finally, Fig. 6 shows that the ROCK inhibitor Y-27632 strongly reduces basal MLC phosphorylation in both control and p116^{Rip}-deficient cells maintained in serum-containing medium. From these results, we conclude that p116^{Rip} deficiency interferes with neurite outgrowth, but leaves RhoA/ROCK regulation of MLC phosphorylation intact.

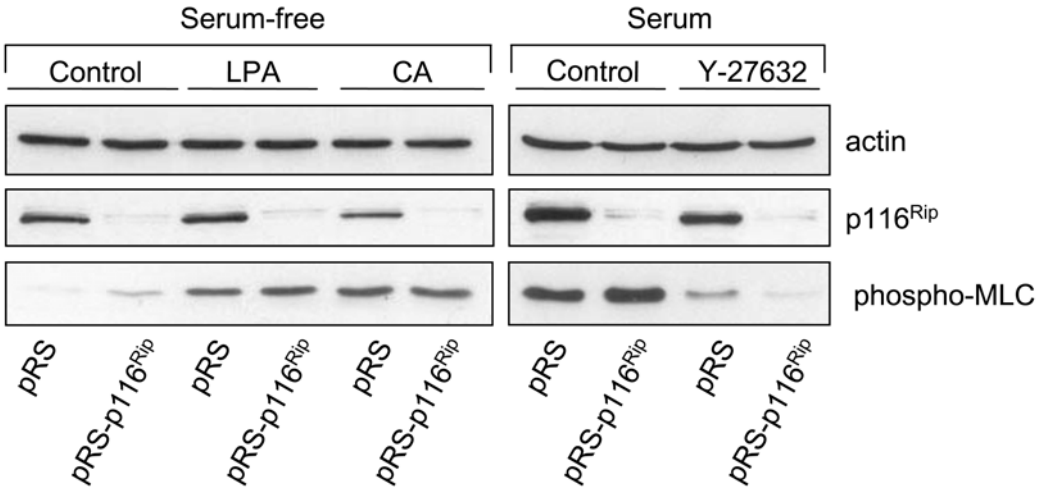


Figure 6. Knockdown of p116^{Rip} does not affect the regulation of MLC phosphorylation
NIH3T3 cells were infected with p116^{Rip} RNAi-encoding retrovirus (pRS- p116^{Rip}). At 72 hrs after infection, cells were either serum-starved (0.1% FCS for 24 hrs) or left in serum-containing media. Serum-starved cells were stimulated with 2.5 μM 1-oleoyl-LPA for 10 min or with 0.1 μM calyculin A (CA) for 5 min (left panels). Cells that were maintained in serum-containing medium were incubated with 10 μM Y-27632 for 45 min (right panels). Cells were analysed for p116^{Rip} expression (middle panels) and MLC phosphorylation (on Thr18/Ser19; lower panels). Actin expression was determined to test for equal loading.

Changes in p116^{Rip} levels affect detergent solubility of MBS

Because p116^{Rip} is an F-actin-bundling protein (Mulder et al., 2003), the present results suggest that p116^{Rip} links the MBS-phosphatase complex to the cytoskeleton. To obtain biochemical evidence for this notion, we examined the association of MBS with the Triton-insoluble fraction (loosely defined as the "cytoskeletal" fraction) as a function of p116^{Rip} expression levels. Since retroviral infections of N1E-115 and Neuro-2A were unsuccessful in our hands, we produced p116^{Rip} RNAi-encoding adenovirus. N1E-115 cells infected with either control pAS or pAS-p116^{Rip} adenovirus were lysed and the solubility of MBS was determined (Mulder et al., 2003). As shown in Fig. 7 (A, B), pAS-p116^{Rip} N1E-115 cells show complete knockdown of p116^{Rip}, with all cells having a rounded morphology. In serum-starved control cells, MBS is about 50% insoluble in 0.1% Triton. After treatment of the cells with LPA or CA, MBS moves to the soluble fraction (Fig. 7C, left panel), suggesting that MBS dissociates from the cytoskeleton. In p116^{Rip}-deficient cells (serum-starved), however, 80% of MBS is already found in the soluble fraction, and CA tends to shift MBS even more into the soluble fraction when compared to control cells (Fig. 7C, right panel).

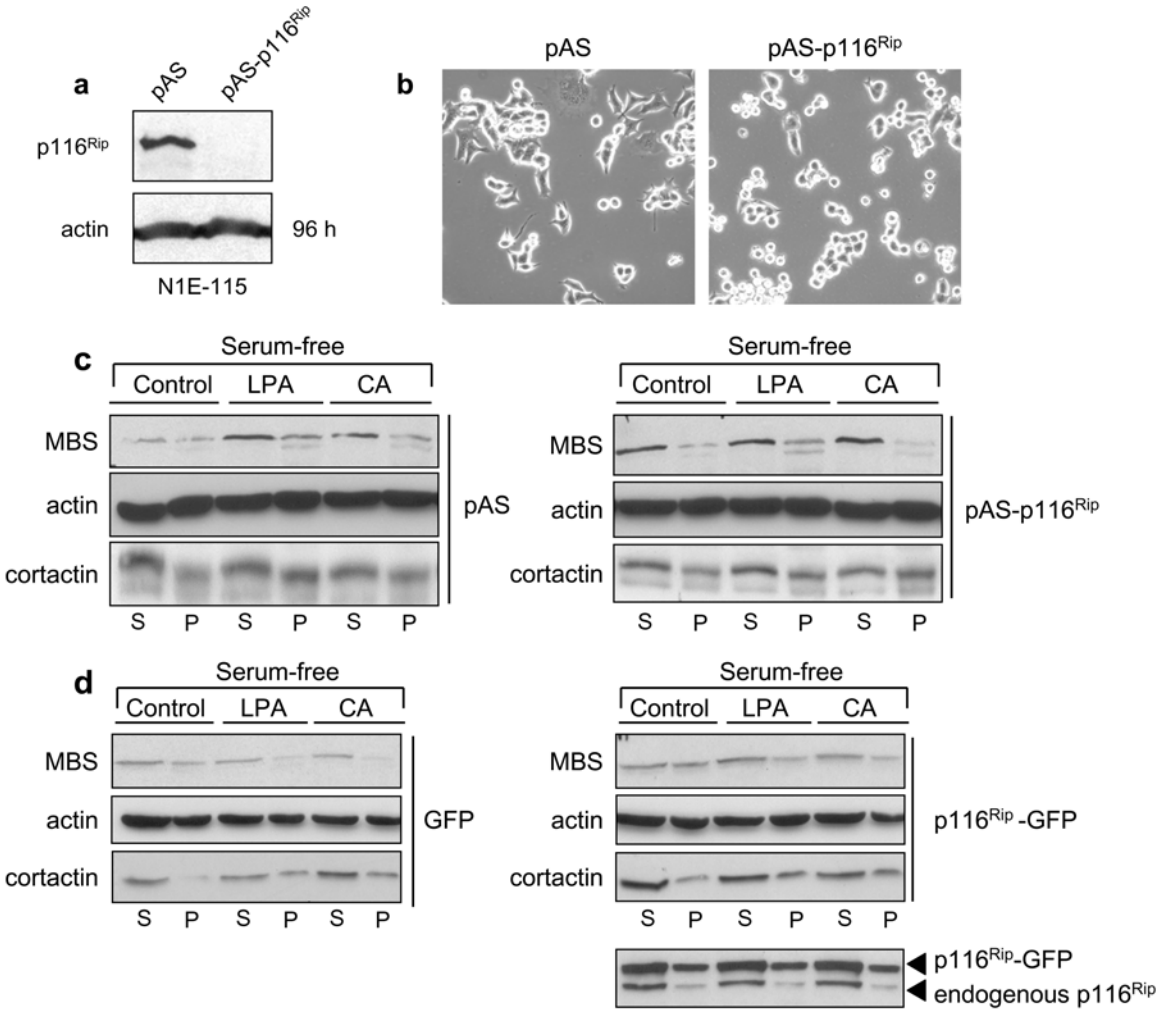


Figure 7. MBS associates with the Triton-insoluble cytoskeletal fraction in a p116^{Rip}-dependent manner
(**a**) N1E-115 cells were infected with control (pAS) or p116^{Rip}-RNAi-encoding (pAS-p116^{Rip}) adenovirus and lysed 96h after infection. pAS-p116^{Rip} downregulates the expression of endogenous p116^{Rip} very efficiently. (**b**) N1E-115 cells were infected with control (pAS) or p116^{Rip}-RNAi-encoding (pAS-p116^{Rip}) adenovirus and viewed by phase-contrast microscopy. (**c**, **d**) At 72 hrs after infection, cells were serum-starved. The next day, serum-starved cells were stimulated with 2.5 μM 1-oleoyl-LPA for 5 min or with 0.1 μM calyculin A (CA) for 4 min (left panels). The solubility of MBS was determined using a buffer containing 0.1% Triton-X-100, and subcellular fractionation was performed resulting in a supernatant (S) and pellet (P) fraction of control pAS or pAS-p116^{Rip} infected N1E-115 cells. Supernatant and pellet fractions were immunoblotted for MBS, cortactin, and actin. (**d**) N1E-115 cells were transfected with GFP or p116^{Rip}-GFP constructs. 48 hrs after transfection cells were serum starved overnight, stimulated and analysed as in c.

Overexpression of p116^{Rip} causes the opposite effect in that it inhibits the shift of MBS out of the pellet after treatment with LPA or CA (Fig. 7D). In control experiments, we monitored the solubility of cortactin, an F-actin-binding protein that does not interact with MBS. As shown in Fig. 7 (C, D), cortactin moves to the insoluble fraction (in response to LPA or CA) in a manner independent of p116^{Rip} expression levels. Note that, in growing cells, p116^{Rip} is equally distributed between the insoluble and soluble fractions (Mulder et al.,

2003), whereas in serum-starved cells, p116^{Rip} is found more in the soluble fraction. While no shift in p116^{Rip} was detected upon LPA or CA treatment (Fig. 7D, right panel), there is a clear correlation between the amounts of p116^{Rip} and MBS in the insoluble fraction. It thus appears that the association of MBS with the cytoskeleton depends on p116^{Rip}, in keeping with the notion that p116^{Rip} functions as a scaffold that links MBS to the actin cytoskeleton.

Discussion

Dynamic remodeling of the actomyosin-based cytoskeleton via Rho GTPases is fundamental to neurite retraction and outgrowth, axon guidance and dendritic branching ((Luo, 2000; Luo, 2002). Mutations in components of Rho GTPase signalling pathways have been documented in human neurological disorders (for references see (Luo, 2000)), which underscores the importance of Rho signalling in the development and function of the nervous system. While many details of Rho GTPases and their regulation have been elucidated in recent years, it remains unclear how the individual components of Rho-regulated pathways are assembled into functional signalling modules and how the specificity of signal transduction is determined, although scaffold proteins are likely to play an important role.

p116^{Rip} as a possible scaffold, linking myosin phosphatase to F-actin

The present study, together with our previous findings (Mulder et al., 2003), suggests that p116^{Rip} serves as a scaffold that links the RhoA/ROCK-regulated myosin phosphatase complex to the F-actin cytoskeleton. p116^{Rip} was recently characterised as an actin-binding protein that through its N-terminal domain bundles F-actin *in vitro* and dictates the localisation of p116^{Rip} to F-actin-rich structures, which are normally under the control of Rho GTPases, in both neuronal and non-neuronal cells (Mulder et al., 2003). Although p116^{Rip} was initially isolated as a RhoA-interacting protein in a yeast two-hybrid screen (using activated V14RhoA as bait; (Gebbink et al., 1997), our subsequent studies indicated that p116^{Rip} is unlikely to interact directly with either RhoA-GTP or RhoA-GDP under normal physiological conditions ((Gebbink et al., 2001; Mulder et al., 2003); and chapter 3). Overexpression studies showed that the N-terminal actin-binding region of p116^{Rip} can disrupt F-actin integrity and inhibit RhoA-regulated actomoysin contractility to promote neurite outgrowth in neuroblastoma cells and process extension in NIH3T3 cells (Gebbink et al., 1997; Mulder et al., 2003). Interestingly, database analysis reveals that p116^{Rip} is evolutionary conserved, as there are predicted orthologues in *Drosophila* and *Caenorhabditis elegans* (termed “outspread” and “F10G8.8” respectively) showing a similar domain arrangement. However, the normal function of p116^{Rip} in cytoskeletal regulation in general, and neuritogenesis in particular, has remained elusive.

Here, we find that the C-terminal coiled-coil domain of p116^{Rip} interacts directly with the myosin binding subunits of MLC phosphatase, MBS85 and MBS130, with selected (iso)leucine residues in the coiled-coil being essential for interaction with the C-terminal leucine zipper in MBS. Co-immunoprecipitation experiments show that the MBS130-p116^{Rip} interaction occurs endogenously in neuronal cells (Fig. 4). By modulating and targeting the catalytic phosphatase subunit (PP1-δ) to the myosin-II light chain, MBS130 regulates

contractile processes such as neurite retraction and/or outgrowth in response to RhoA-ROCK activation. The closely related MBS85 isoform, which is highly expressed in brain and heart (but not in smooth muscle), likely acts in a very similar manner (Tan et al., 2001). In vivo studies on MBS85 are hampered, however, by the fact that its endogenous levels in cultured cells are much lower than those of MBS130 and antibodies are not yet available (Tan et al., 2001).

In addition to binding to p116^{Rip}, the C-terminal leucine zipper of MBS also interacts directly with the GTP-bound, active form of RhoA (Kimura et al., 1996). Binding of MBS130 to both p116^{Rip} and RhoA-GTP implies the existence of a p116^{Rip}/MBS/RhoA-GTP complex *in vivo*. That the interaction between p116^{Rip} and RhoA-GTP is indirect, and likely very transient in nature, may explain why the p116^{Rip}-RhoA association escapes detection under physiological conditions. Aside from binding to p116^{Rip} and RhoA-GTP, the leucine zipper of MBS has also been reported to bind to a 20-kDa subunit of unknown function (Hartshorne, 1998), to the ROCK-substrate moesin (at least in epithelial cells; (Fukata et al., 1998) and to cGMP-dependent protein kinase, which mediates physiologic relaxation of vascular smooth muscle (Surks et al., 1999). This suggests that, depending on cell type or/and its subcellular localisation, MBS may exist in distinct signalling complexes with different cellular functions. In this respect it should be noted that ROCK and the myosin phosphatase complex can regulate the phosphorylation state of proteins other than MLC (Bauman and Scott, 2002). Examples include the actin-binding proteins adducin and moesin, which interact directly with MBS and are subject to dual regulation by ROCK and MBS (Fukata et al., 1998; Fukata et al., 1999; Nakamura et al., 2000). It is therefore conceivable that p116^{Rip} may also be a target of myosin phosphatase. Consistent with this possibility, we find that p116^{Rip} is a phosphoprotein (J. Mulder, unpublished results) and associates *in vivo* with the whole myosin phosphatase complex, consisting of MBS and the catalytic PP1δ subunit (Fig. 4B). How the phosphorylation of p116^{Rip} is regulated and may influence its function remains a challenge for future studies.

p116^{Rip} is essential for RhoA/ROCK-regulated neuritogenesis

Knockdown studies using RNAi in Neuro-2A cells indicate that p116^{Rip} is essential for neurite outgrowth induced by extrinsic cues that inhibit RhoA/ROCK activity, notably (i) the RhoA-inactivating C3 exo-enzyme; (ii) the ROCK inhibitor Y-27632; (iii) removal of serum (a rich source of the RhoA-activating agonist LPA); and (iii) treatment of the cells with db-cAMP, which raises intracellular cAMP levels to activate protein kinase A and thereby inhibits RhoA-mediated contractility at multiple levels leading to neurite outgrowth (Dong et al., 1998; Essler et al., 2000; Neumann et al., 2002; Snider et al., 2002) and references therein). It is of note, however, that p116^{Rip} knockdown does not interfere with MLC phosphorylation induced by either LPA or the phosphatase-I inhibitor calyculin A (at least in NIH3T3 cells), nor does it inhibit Y27632-induced MLC dephosphorylation (i.e. ROCK regulation of MLC phosphatase activity). In other words, it appears that p116^{Rip} loss-of-function prevents ROCK from remodeling the F-actin cytoskeleton, but not from regulating MLC (de)phosphorylation. Although MLC is dephosphorylated (by Y27632 treatment), p116^{Rip}-deficient cells maintain their “contracted” morphology. MLC-independent regulation of contractility is not without precedent, however (Seasholtz, 2003). Taken together with our previous data, the present results suggest a model in which p116^{Rip} acts as a scaffold to link the RhoA/ROCK-regulated myosin-II phosphatase complex to the actin cytoskeleton (Fig. 8).

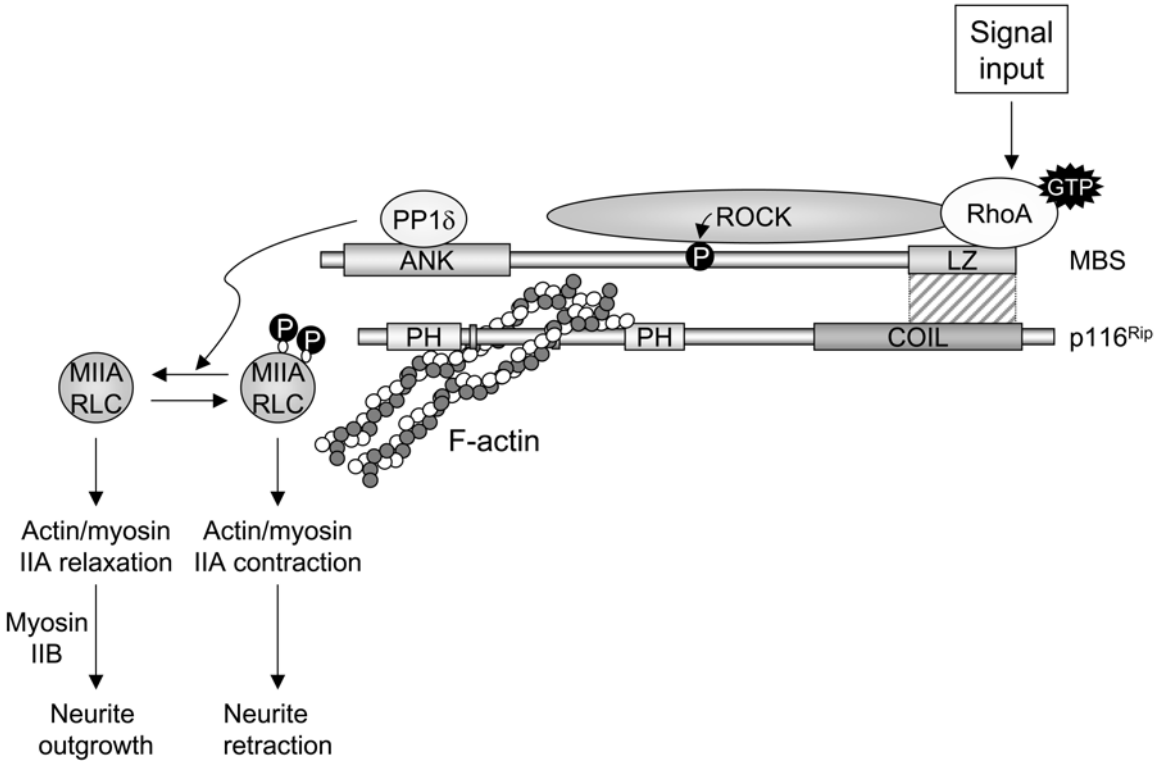


Figure 8. Model of p116^{Rip} in association with the ROCK-regulated myosin phosphatase complex and F-actin
We propose that p116^{Rip} acts as a scaffold whose C-terminal coiled-coil binds to the MBS subunits (MBS85 and MBS130) of nonmuscle myosin-II phosphatase, while its N-terminal domain binds to (and bundles) F-actin (Mulder et al., 2003). MBS is a direct target of ROCK and can also bind RhoA, as indicated. The location(s) of the myosin-II-binding sites on MBS is somewhat controversial, as binding of myosin-II (or its light-chain) to both N- and C-terminal regions has been reported (Hartshorne, 1998; Velasco et al., 2002). MBS phosphorylation inhibits PP-I activity and thereby promotes MLC phosphorylation (by one or more candidate kinases, including MLC kinase and ROCK) leading to increased myosin-II activity (the conventional IIA isoform; (Wylie and Chantler, 2003) and neurite retraction. Dephosphorylation of the light chain of myosin IIA may allow myosin IIB action to predominate, leading to enhanced growth cone motility and neurite outgrowth (Bridgman et al., 2001; Wylie and Chantler, 2003). ANK, ankyrin repeats; LZ, leucine zipper; MIIA, myosin IIA; RLC, regulatory light chain.

As such, p116^{Rip} is essential for actomyosin “relaxation”, which is thought to be initiated by the reduced activity of the conventional myosin IIA isoform (Bridgman et al., 2001; Wylie and Chantler, 2003). Reduced myosin IIA activity may allow myosin IIB action to predominate, leading to enhanced growth cone motility and neurite outgrowth (Bridgman et al., 2001; Wylie and Chantler, 2003), as illustrated Fig. 8.

In a recent study, the human homologue of p116^{Rip} was identified in a yeast-two hybrid screen using the C-terminus of MBS130 as bait (Surks et al., 2003). Similar to the present findings in neuronal cells, p116^{Rip} and MBS130 were shown to interact in vascular smooth muscle cells, which led the authors to hypothesise that p116^{Rip} serves to target RhoA to myosin phosphatase to regulate myosin phosphorylation. In this model, p116^{Rip} would be essential for RhoA/ROCK-regulated myosin phosphorylation. Our knockdown studies show, however, that such is not the case as RNAi-induced loss of p116^{Rip} inhibits RhoA/ROCK actomyosin activity but not the MLC (de)phophorylation machinery.

The identification of other components of the p116^{Rip} complex should provide further insight into how it participates in Rho/ROCK regulation of cytoskeletal contractility in general, and neuritogenesis in particular. Furthermore, given the importance of the RhoA-actin pathway in transcriptional regulation (Hill et al., 1995; Etienne-Manneville and Hall, 2002; Miralles et al., 2003), future studies should also examine the role of p116^{Rip} in RhoA signalling to the nucleus.

Materials and Methods

Cell culture, transfection and materials

All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 8% fetal calf serum. Human embryonic kidney (HEK)293 cells were transfected using calcium phosphate precipitation. N1E-115 and Neuro-2A cells were transfected using FuGENE 6 reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. COS-7 cells were transfected using DEAE–dextran as described previously (Zondag et al., 1996). The following materials were obtained from the designated sources: dibutyryl-cAMP (db-cAMP) (Sigma-Aldrich, St. Louis, MO), calyculin A (CA) and Y-27632 (Calbiochem, San Diego, CA), 1-oleoyl-LPA (Sigma-Aldrich), FLAG M2 mouse monoclonal antibody (mAb) (Sigma-Aldrich), rabbit anti-PP1δ and anti-cortactin 4F11 (Upstate Biotechnology, Lake Placid, NY), anti-actin mAb (Chemicon International, Temecula, CA), rabbit anti-green fluorescent protein (GFP) (van Ham et al., 1997), anti-phospho-MLC-Thr18/Ser19 (Cell Signaling Technology, Beverly, MA), anti-myc 9E10 mAb and anti-HA 12CA5 mAb from hybridoma supernatants (American Type Culture Collection, Manassas, VA), anti-MBS130 (CRP, Berkely, CA). Rabbit p116^{Rip} antibodies directed against GST-RBD (amino acids 545-823) have been described before (Gebbink et al., 1997). Rabbit anti-p116^{Rip} serum (p116^{Rip}NT) directed against the N-terminus of p116^{Rip} (amino acids 1-382) was made by immunising rabbits with purified GST-NT protein (Mulder et al., 2003). p116^{Rip}NT antibodies were used to immunoprecipitate p116^{Rip} from N1E-115 lysates because p116^{Rip} antibodies directed against RBD compete with MBS for the same binding site on p116^{Rip}.

RNAi and plasmids

The p116^{Rip} RNAi targeting vectors (pS-GFPp116^{Rip}) were based on a 19-mer sequence present in the coding sequence of human, rat and mouse p116^{Rip}: 5'-gagcaagtgtcagaactgc-3'. 64-mer synthetic oligonucleotides for cloning into pSuperGFP (pS-GFP) were synthesised, annealed and ligated into the pS-GFP plasmid or into the pS plasmid as described (Brummelkamp et al., 2002a). To obtain retroviral pSuper constructs (pRS), pS-p116^{Rip} was digested with *EcoR*I-*Xho*I and the insert containing the RNAi targeting sequence and promotor was ligated into pRS (Brummelkamp et al., 2002b). The pS-GFP plasmid consists of the pS plasmid including the GFP protein under control of a pGK promoter. Adenoviral pAS constructs were designed as follows: pS-p116^{Rip} or pS were digested with *Xho*I-*BamH*I and the insert containing the RNAi targeting sequence and promotor was ligated into pENTR1A of the virapower adenoviral expression system (Invitrogen, Carlsbad, CA). The p116^{Rip} point mutants (L857P, L905P, and I919P) were obtained using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) using pcDNA3-HA-FLp116^{Rip} (Mulder et al., 2003) as a template. pcDNA3-HA-ΔNp116^{Rip} was generated from Mb2a-p116^{Rip}-ΔN; pcDNA3-HA-NTp116^{Rip}, pcDNA3-HA-CTp116^{Rip} and pcDNA3-HA-RBDp116^{Rip} have been described elsewhere (Gebbink et al., 1997); pcDNA3-HA-FLp190RhoGEF and pcDNA3-HA-Coil-p190RhoGEF are described by (van Horck et al., 2001). pXJ40-FLAG-p85 (MBS) and pEFBOS-myc-MBS130 plasmids were kindly

provided by Dr. T. Leung (Institute of Molecular and Cell biology, Singapore) and Dr. K. Kaibuchi (Nagoya University, Nagoya, Japan), respectively.

Yeast two-hybrid analysis

Mb2a-p116^{Rip}-ΔN, in which p116^{Rip} lacking the N-terminus is fused to the GAL4 DNA-binding domain of Mb2a, was generated by polymerase chain reaction (PCR) using primers 5'-acgcgtcgacccggtaccactccacagaatcctccatga-3' (forward) and 5'-ataagaatgcggccgcaagctttcagttatcccatgagacctg-3' (reverse). The PCR product was digested with *Sal*I-*Not*I and ligated into Mb2a. Mb2a-p116^{Rip}-ΔN was integrated into the genome of yeast strain Y190 and used as bait in screening a human testis cDNA library in pVP16, according to earlier described procedures (Gebbink et al., 1997). Binding specificity of cDNAs obtained from positive yeast colonies was confirmed by retransformation assays.

Western blotting and immunoprecipitation

COS-7 cells co-expressing various constructs were scraped in ice-cold lysis buffer (1% NP-40, 50 mM Tris pH 7.4, 200 mM NaCl, 2.5 mM $MgCl_2$, 10% glycerol, supplemented with protease inhibitors). Extracts were clarified by centrifugation and precleared with 0.5% bovine serum albumin (BSA)-blocked protein-A–Sepharose beads for 1 hour at 4 °C. Precleared lysates were incubated overnight with anti-hemagglutinin (HA) at 4 °C and immunocomplexes were removed by incubation with protein-A-Sepharose beads for 30 min at 4 °C. Beads were washed, resuspended in Laemmli sample buffer and boiled. Samples were separated by SDS–PAGE and analysed by western blotting using anti-myc, anti-HA, or anti- FLAG antibodies. N1E-115 lysates were prepared similarly and supernatants were precleared with 0.5% BSA-blocked protein-A-Sepharose beads pre-coupled to either pre-immune rabbit serum (IgG), p116^{Rip}AB, PP1δ, or anti-MBS antibodies overnight at 4 °C. Beads were washed, resuspended in Laemmli sample buffer and boiled. Samples were separated by SDS–PAGE and analysed by western blotting using anti-MBS, anti-p116^{Rip}, or anti-PP1δ antibodies. HEK293 cells were co-transfected (1:1) with plasmids as indicated; 96 hrs after transfection, cells were lysed as before. Blots were incubated with anti-HA and/or anti-actin mAbs. Neuro2A cells were transfected with plasmids and lysed similarly 96 hrs after transfection. Blots were incubated with rabbit anti-p116^{Rip}, rabbit anti-GFP and anti-actin mAb. In all cases, Bradford protein assays were performed on the lysates to test for equal loading on SDS-PAGE.

Fluorescence microscopy

In RNAi studies, Neuro-2A cells were transfected with pS-GFP, pS-GFPp116^{Rip} or a combination of pS-GFP/pS-GFPp116^{Rip} and pcDNA3-HA-FLp116^{Rip} variants (ratio 1:8). To induce neurite outgrowth, N1E-115 and Neuro-2A cells were either exposed for 48 hrs to serum-free Neurobasal medium supplemented with B-27 (NB-B27, Invitrogen) containing db-cAMP (1 mM); alternatively, cells were incubated with Y-27632 (10 μM) for 8 hrs. Fluorescence and corresponding phase-contrast images pictures were taken on a Zeiss Axiovert microscope equipped with a CCD camera. GFP-positive cells were analysed for neurite outgrowth, defined as processes longer than one cell body diameter.

Retroviral infections and detection of myosin light chain phosphorylation

Phoenix-Eco package cells were transfected with pRS or pRS-p116^{Rip}. The supernatant containing viral particles was harvested at 48 hrs and 72 hrs after transfection. For infection of NIH3T3 cells, cells were incubated with 1 ml of viral stock in the presence of 10 μl Dotap (1 mg/ml; Roche Diagnostics). The following day cells were washed with phosphate-buffered saline (PBS) and fresh medium was added. 72 hrs after infection, NIH3T3 cells were serum starved (0.1% fetal calf serum (FCS)) for 24 hrs or left in serum-containing media and then stimulated for 10 min with 2.5 μM LPA, 5 min with 0.1 μM calyculin A (CA), or 45 min with 10 μM Y-27632. Cells were washed once with ice-cold PBS and

lysed in Laemmli's sample buffer. Cell lysates were subjected to immunoblotting using anti-phospho-MLC-Thr18/Ser19, rabbit p116^{Rip} antibodies and anti-actin mAb.

Adenoviral infections and solubility assay

Adenovirus was produced according to the manufacturer's protocol (virapower adenoviral expression system; Invitrogen). N1E-115 cells were infected with either pAS control or pAS-p116^{Rip} virus (by using equal amounts of virus particles per cell). At 72 hrs after infection, cells were serum starved. The following day cells were stimulated for 5 min with 2.5 μM LPA, or 4 min with 0.1 μM calyculin A (CA). The Triton solubility assay was performed as described (Mulder et al., 2003). In brief, detergent lysates were centrifuged for 30 min using an Eppendorf table centrifuge. Equal amounts of pellet and supernatant fractions were subjected to SDS-PAGE. Proteins were detected by immunoblotting using antibodies against MBS, cortactin, actin and p116^{Rip}.

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Chapter 4

p116^{Rip} inhibits RhoA-mediated SRF activation

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p116^{Rip} inhibits RhoA-mediated SRF activation

Abstract

The small GTPase RhoA regulates diverse cellular processes including cytoskeletal reorganisation and transcriptional activation. p116^{Rip} is an F-actin-bundling protein that binds to the regulatory myosin-binding subunit (MBS) of myosin-II phosphatase. Overexpression and knockdown studies suggest that p116^{Rip} interferes with Rho-mediated cytoskeletal contraction. Here we find that p116^{Rip} inhibits RhoA-induced activation of the transcription factor SRF without affecting the activation state of RhoA. In addition, we show that p116^{Rip} undergoes oligmerisation through its C-terminal coiled-coil domain. Abolition of p116^{Rip}'s ability to oligomerise or bind MBS did not affect p116^{Rip} inhibition of RhoA-induced transcription. These results, together with our previous findings, show that p116^{Rip} influences RhoA signalling at a point downstream of RhoA and Rho-kinase. We propose that p116^{Rip} interferes with RhoA-mediated transcription through its ability to disassemble the F-actin cytoskeleton, thereby altering the balance between F- and G-actin.

Introduction

Rho GTPases are involved in a wide range of cellular responses, ranging from changes in cell morphology to transcriptional activation and cell cycle progression (Malliri and Collard, 2003; Raftopoulou and Hall, 2004; Coleman et al., 2004; Burridge and Wennerberg, 2004). For example, activation of RhoA (by LPA or serum) leads not only to cytoskeletal contraction in diverse cell types (Ridley and Hall, 1992; Jalink et al., 1994), but also to activation of the SRE response element via activation of the transcription factor SRF (Hill et al., 1995). Changes in actin dynamics are both necessary and sufficient for SRF activation (Sotiropoulos et al., 1999; Posern et al., 2002). Like all GTPases, RhoA signals via downstream effectors that are activated upon binding to active, GTP-bound RhoA (Aspenstrom, 1999; Bishop and Hall, 2000; Ridley, 2001). SRF activation is mediated by two distinct RhoA effectors, ROCK and mDia, which act cooperatively to induce F-actin assembly and stabilisation (Copeland and Treisman, 2002; Geneste et al., 2002). Recently, the MAL protein was found to be a cellular sensor for the amount of G-actin. Upon depletion of the G-actin pool, MAL distributes to the nucleus where it mediates SRF activation (Miralles et al., 2003). RhoA-induced actomyosin contractility is driven by the action of the same downstream effectors, mDia and ROCK (Nakano et al., 1999; Watanabe et al., 1999). Next to stabilising F-actin, ROCK activation leads to inhibition of myosin light chain (MLC) phosphatase by phosphorylating the regulatory myosin-binding subunit (MBS), leading to enhanced MLC phosphorylation and actomyosin contractility (Kimura et al., 1996; Kawano et al., 1999).

Using a yeast two-hybrid screen to search for RhoA effectors we have previously identified a ubiquitously expressed protein of 116 kDa, named p116^{Rip}, that interacted with constitutively active RhoA (Gebbink et al., 1997). Subsequent studies revealed that p116^{Rip} is an F-actin binding protein with F-actin bundling properties *in vitro*. RNAi-induced knockdown of p116^{Rip} inhibits cell spreading and neurite outgrowth in response to signals that lead to inhibition of the RhoA pathway (Mulder et al., 2004). In addition, overexpression

of p116^{Rip} (or its N-terminal actin-binding domain) disrupts the actin cytoskeleton and thereby inhibits actomyosin contractility (Gebbink et al., 1997; Mulder et al., 2003). Furthermore, we and others reported that p116^{Rip} interacts with the regulatory myosin-binding subunit (MBS) of myosin II phosphatase (Surks et al., 2003; Mulder et al., 2004; Koga and Ikebe, 2005) and p116^{Rip} may target MBS to the actin cytoskeleton (Mulder et al., 2004). Thus, p116^{Rip} may interfere with RhoA signaling at multiple levels.

The p116^{Rip} sequence contains two proline-streches, two PH domains, and a C-terminal coiled-coil structure, which interacts with the leucine zipper of MBS (Surks et al., 2003; Mulder et al., 2004). Here, we show that the coiled-coil domain of p116^{Rip}, consisting of 6 coiled-coils, is both necessary and sufficient to mediate homo-oligomerisation of p116^{Rip}. Point mutations within the coiled-coil domain block the ability of p116^{Rip} to bind MBS or to oligomerise. Furthermore, we find that overexpression of p116^{Rip} inhibits RhoA-mediated SRF activation without affecting active RhoA levels. Mutational analysis reveals that this inhibition does not depend on p116^{Rip}-MBS interaction or on oligomerisation, but correlates with p116^{Rip}'s ability to disrupt the cytoskeleton. We conclude that p116^{Rip} acts downstream in the RhoA-ROCK pathway and may abrogate RhoA-induced SRF activation through its ability to interact with and disassemble the F-actin cytoskeleton.

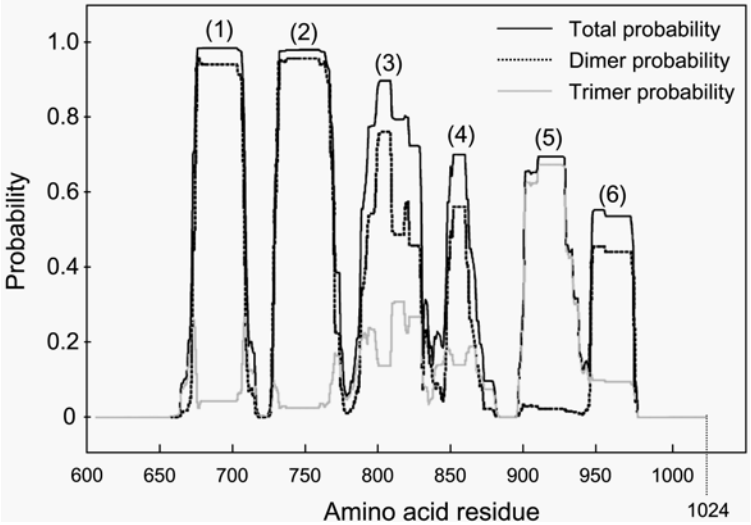


Figure 1. The coiled-coil domain of p116^{Rip}
The coiled-coil probabilities of p116^{Rip} (aa 600-1024) were detected by MULTICOIL (Wolf et al., 1997) using default settings; a probability cutoff of 0.5 and a window of 28 residues. Blue and red lines mark the location of the six predicted dimeric or trimeric coiled-coils, respectively. The sum of the two probabilities represents the propensity for the region forming any sort of coiled coil (black line).

Results and Discussion

The coiled-coil of p116^{Rip} mediates homo-oligomerisation

Aside from two PH domains, p116^{Rip} contains a coiled-coil structure, as predicted by the MULTICOIL program (Wolf et al., 1997). Coiled-coils consist of α-helices wrapping around each other forming a supercoil. The sequences of coiled-coils consist of a heptad repeat pattern, $(abcdefg)_n$, in which residues at the **a** and **d** positions are often hydrophobic, and residues at the **e** and **g** positions are predominantly charged (Burkhard et al., 2001; Mason and Arndt, 2004). As shown in Fig. 1, the C-terminus of p116^{Rip} (aa 600-1024) contains six coiled-coil regions with probabilities >0.5 or 1. For comparison, the extended coiled-coil domain of myosin heavy-chain, to which the coiled-coil of p116^{Rip} is similar, has a probability of 1. Furthermore, the MULTICOIL program predicted that coiled-coil regions 1 to 4 and 6 have a high probability to form dimers, while region 5 has a high probability to form trimers (Fig. 1).

Coiled-coil structures often mediate homo- or hetero-oligomerisation (Burkhard et al., 2001; Mason and Arndt, 2004; Marianayagam et al., 2004). To investigate whether p116^{Rip} oligomerises through its coiled-coil domain, we generated HA-tagged truncation mutants of the C-terminal part of p116^{Rip} and expressed them in COS-7 cells. Indeed, p116^{Rip} was found to oligomerise since full-length Myc-p116^{Rip} precipitated with full-length HA-p116^{Rip} (Fig. 2A, lanes 1-3). Furthermore, all p116^{Rip} truncation mutants containing the coiled-coil domain precipitated Myc-tagged p116^{Rip} from COS-7 cell lysates (Fig. 2A). No oligomerisation was detected with a truncated coiled-coil mutant ('RBD'; aa 545-823) or with the isolated actin-binding domain of p116^{Rip} ('NT'; aa 1-382) (Fig. 2A), indicating that the coiled-coil domain of p116^{Rip} mediates oligomerisation *in vitro*. To test the importance of the coiled-coil domain for oligomerisation, we made GST fusion proteins of p116^{Rip} lacking either the actin-binding domain or the coiled-coil domain, ΔNp116^{Rip}-GST (aa; 376-1024) and ΔCp116^{Rip}-GST (aa; 1-713), respectively (Fig. 2B). The ΔCp116^{Rip}-GST mutant, lacking most of the coiled-coil (713-1024) failed to precipitate myc-p116^{Rip} when co-expressed in COS-7 cells. Thus, the coiled-coil region of p116^{Rip} (aa 671-976) is necessary for oligomerisation of p116^{Rip} (Fig. 2C).

Coiled-coil proteins can be grouped into two classes. The first class is defined by long coiled-coil domains of several hundred amino acids. The second class is comprised of short coiled-coil domains of six or seven heptad repeats, also called leucine zippers. As coiled-coil sequences generally interact with other coiled-coil sequences, both classes are frequently found as homo- and hetero-dimerisation domains (Burkhard et al., 2001;

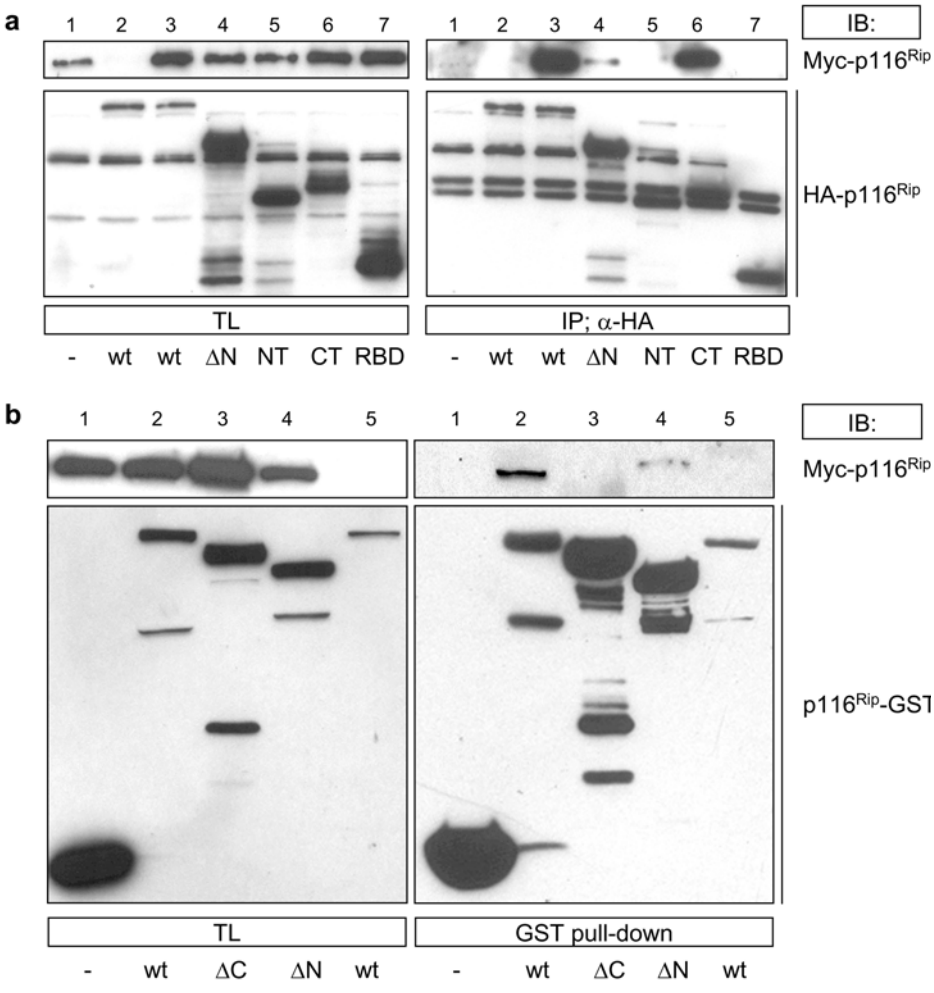


Figure 2. The coiled-coil domain of p116^{Rip} is necessary and sufficient for p116^{Rip} oligomerisation (**a**) COS-7 cells were transfected with plasmids encoding Myc-p116^{Rip} and indicated HA-tagged p116^{Rip} truncation mutants Total lysates (TL, left panel) and immunoprecipitates (IP, right panel) were analysed by immunoblotting (IB) using α-HA and α-Myc (**b**) COS-7 cells were transfected with plasmids encoding Myc-p116^{Rip} and indicated GST fusion proteins of p116^{Rip} truncation mutants. TL (left panel) and GST precipitates (right panel) were analysed using α-GST and α-Myc.

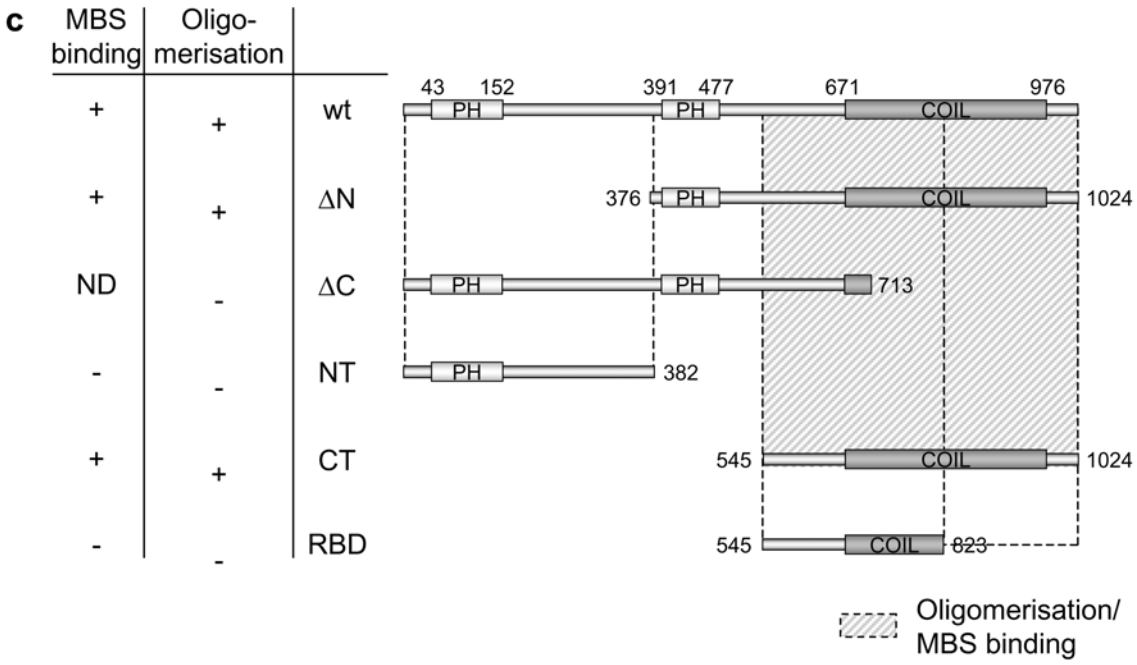


Figure 2-continued (**c**) Schematic representation of the oligomerisation via the coiled-coil domain of p116^{Rip} as determined in COS-7 cells; + denotes p116^{Rip} oligomerisation or MBS binding (Mulder et al., 2004). -, empty vector; wt, wild-type p116^{Rip}; NT, N-terminal actin-binding region domain (Mulder et al., 2003); CT, C-terminal region; RBD, 'Rho-binding' domain (Gebbink et al., 1997); ΔN, p116^{Rip} lacking the N-terminal region; ΔC, p116^{Rip} lacking the C-terminal region; ND, not done.

Marianayagam et al., 2004). Replacement of (iso)leucine residues at position **a** or **d** with alanine, valine or proline affects the stability of the coiled-coil structure and often abolishes coiled-coil based binding (Wagschal et al., 1999; Tripet et al., 2000).

Part of the coiled-coil domain of p116^{Rip} (aa 823-1024) (Fig. 2C) was recently shown to hetero-oligomerise with the leucine zipper of MBS (Surks et al., 2003; Mulder et al., 2004). In addition, substitution of residues Leu(857) and Ile(919) in p116^{Rip} with Pro residues showed that these residues were critical for the direct interaction with the leucine-zipper domain of MBS. Next, we asked which leucine-isoleucine motifs in the coiled-coil are necessary for homo-oligomerisation. The L857P, L905P, and I919P point mutants were used together with double mutants L857P/L864P, L905P/I912P and I919P/L926P. All residues are located at the **a** position of the heptad repeat, except for residues Leu(857) and Leu(864) which are located at the **d** position (Fig. 3A). Oligomerisation was examined by co-precipitation of Myc-p116^{Rip} with the HA-tagged p116^{Rip} mutants. As shown in Fig. 3B, only double mutants L905P/I912P and I919P/L926P failed to oligomerise. We conclude that residues Ile(912) and Leu(926) in the 5th coil of p116^{Rip} are critical for p116^{Rip} homo-oligomerisation.

To study a possible overlap of p116^{Rip} and MBS binding regions within the coiled-coil of p116^{Rip} (Fig. 2C), the double mutants were also tested for association with MBS130 and and MBS85. All double mutants failed to interact with either MBS85 or MBS130 (Fig. 4). This indicates that, in addition to residues Leu(857) and Ile(919), residue Ile(912) is critical for a direct interaction with the leucine-zipper domains of MBS85 and MBS130. These data are summarised in Table 1. In conclusion, p116^{Rip}-p116^{Rip} and p116^{Rip}-MBS oligomerisation is mediated by an overlapping region within the coiled-coil domain of p116^{Rip}. Furthermore, the

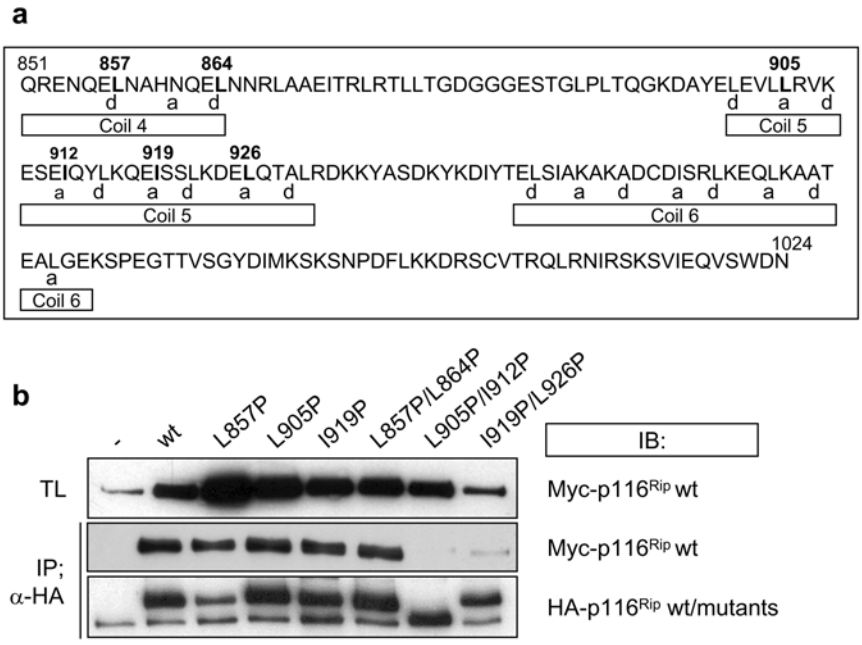


Figure 3. (Iso)leucine residues in the coiled-coil of p116^{Rip} mediate homo-oligomerisation

(**a**) Representation of residues 851-1024 of the coiled-coil domain of p116^{Rip}. Coil regions and hydrophobic residues at the **a** and **d** positions are indicated. (Iso)leucines in bold were mutated to proline by site-directed mutagenesis. (**b**) COS-7 cells were co-transfected with plasmids encoding either Myc-p116^{Rip}wt and indicated HA-tagged (iso)leucine mutants of p116^{Rip} or an empty HA-tagged plasmid as control (-). Total cell lysates (TL) analysed for p116^{Rip} expression are shown (upper panels). Immunoprecipitation (IP) of HA-tagged p116^{Rip} and co-precipitation of wild-type myc-p116^{Rip} was analysed by immunoblotting (IB) using α-HA and α-Myc. Note that the double mutant L905P/I912P has a higher mobility, indicating distortion of the coiled-coil structure (see also Fig. 4).

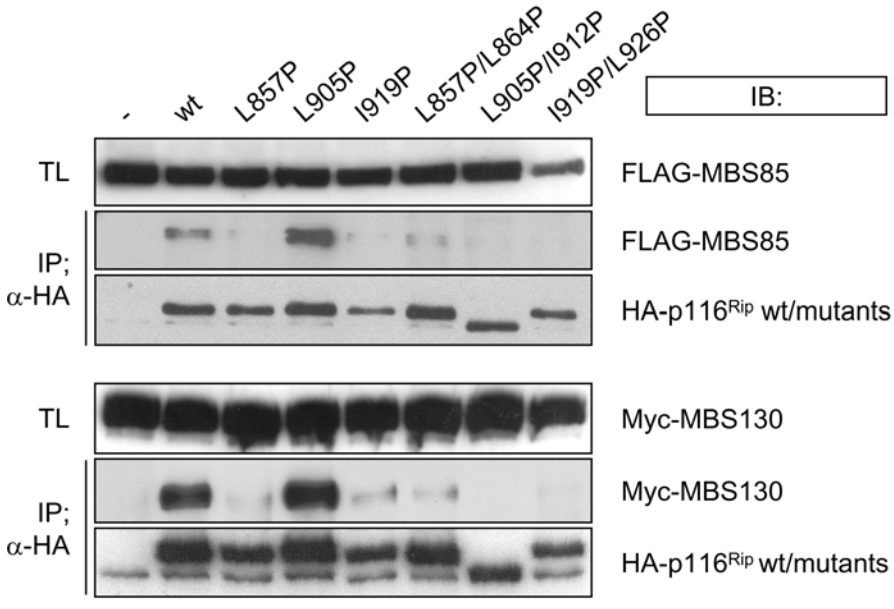


Figure 4. (Iso)leucine residues in the coiled-coil of p116^{Rip} mediate binding to MBS85 and MBS130.

COS-7 cells were co-transfected with plasmids encoding either FLAG-MBS85 or Myc-MBS130 and indicated HA-tagged plasmids encoding p116^{Rip} (iso)leucine mutants or an empty HA-tagged plasmid as control (-). Total cell lysates (TL) analysed for MBS85 and MBS130 expression are shown (first and fourth panels). Immunoprecipitation (IP) of HA-tagged proteins and co-precipitation of MBS85 or MBS130 was analysed by immunoblotting (IB) using α-HA, α-FLAG, and α-Myc.

single and double (iso)leucine mutants of p116^{Rip} can serve as a tool to study the importance of either p116^{Rip}-p116^{Rip} or p116^{Rip}-MBS oligomerisation for p116^{Rip} function.

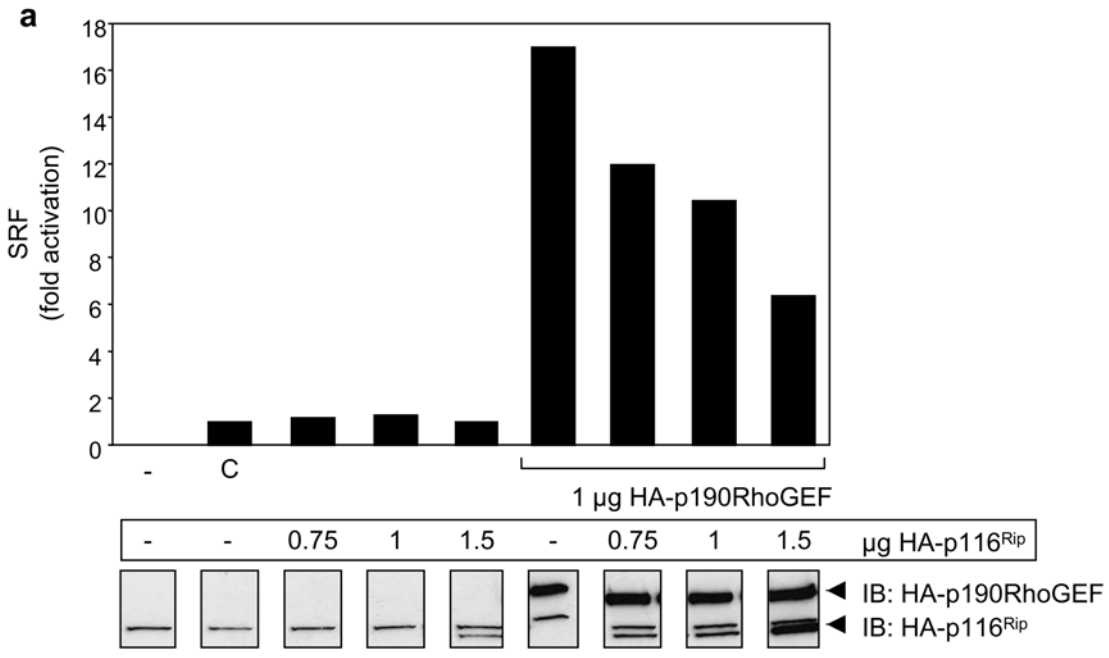
Table 1. Summary of results

p116^{Rip} mutants	p116^{Rip}	MBS
p116^{Rip} wt	+++	+++
p116^{Rip} L857P	+++	-/+
p116^{Rip} L905P	+++	+++
p116^{Rip} I919P	+++	-/+
p116^{Rip} 857P/L864P	+++	-/+
p116^{Rip} L905P/I912P	-	-
p116^{Rip} I919P/L926P	-	-

Interaction with p116^{Rip} in COS-7 cells
+++ = strong interaction
-/+ = weak interaction
- = no interaction

p116^{Rip} interferes with RhoA-induced activation of SRF

RhoA, Rac1, and Cdc42 can activate the serum response factor (SRF) (Hill et al., 1995) through their ability to induce actin polymerisation. It was recently shown that MAL, a G-actin-associated SRF coactivator, translocates from the cytoplasm to the nucleus in response to Rho-induced actin polymerisation (Miralles et al., 2003). Since overexpression of p116^{Rip} interferes with RhoA-induced cytoskeletal changes (Gebbink et al., 1997; Mulder et al., 2003), we examined how p116^{Rip} may affect RhoA-mediated SRF activation. We used a reporter assay in which the luciferase gene is under the control of a c-Fos serum-responsive promotor element (pSRE.L). Since this modified SRE element depends on SRF, it specifically reports Rho GTPase signalling (Mao et al., 1998). HEK293 cells were co-transfected with pSRE.L, RhoV14 or p190RhoGEF (van Horck et al., 2001) and full-length p116^{Rip}. As shown in Fig. 5 (A, B), expression of p116^{Rip} inhibits RhoV14- or p190RhoGEF-induced activation of SRF in a dose-dependent manner.



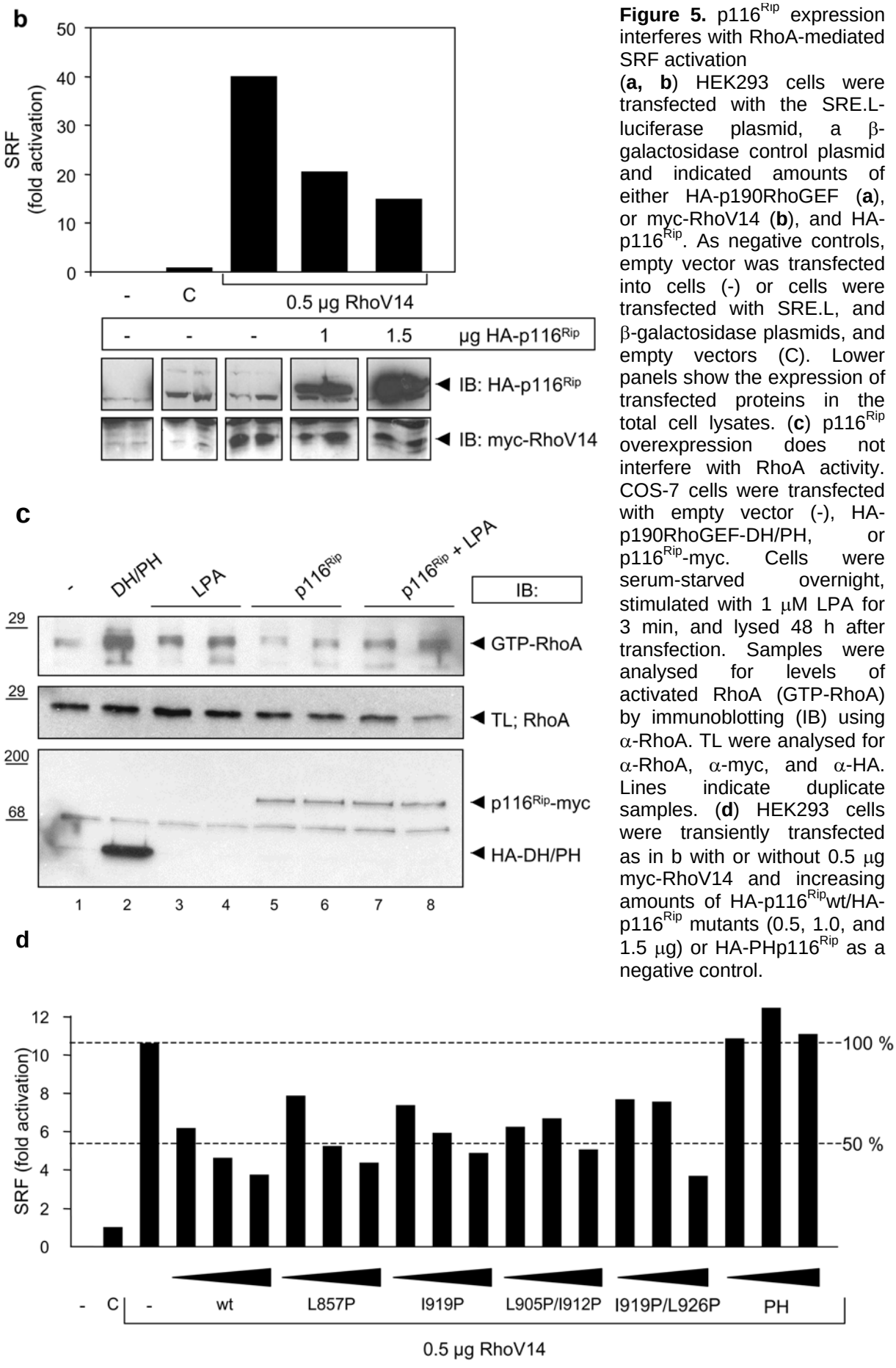


Figure 5. p116^{Rip} expression interferes with RhoA-mediated SRF activation

(**a, b**) HEK293 cells were transfected with the SRE.L-luciferase plasmid, a β-galactosidase control plasmid and indicated amounts of either HA-p190RhoGEF (**a**), or myc-RhoV14 (**b**), and HA-p116^{Rip}. As negative controls, empty vector was transfected into cells (-) or cells were transfected with SRE.L, and β-galactosidase plasmids, and empty vectors (C). Lower panels show the expression of transfected proteins in the total cell lysates. (**c**) p116^{Rip} overexpression does not interfere with RhoA activity. COS-7 cells were transfected with empty vector (-), HA-p190RhoGEF-DH/PH, or p116^{Rip}-myc. Cells were serum-starved overnight, stimulated with 1 µM LPA for 3 min, and lysed 48 h after transfection. Samples were analysed for levels of activated RhoA (GTP-RhoA) by immunoblotting (IB) using α-RhoA. TL were analysed for α-RhoA, α-myc, and α-HA. Lines indicate duplicate samples. (**d**) HEK293 cells were transiently transfected as in b with or without 0.5 µg myc-RhoV14 and increasing amounts of HA-p116^{Rip}wt/HA-p116^{Rip} mutants (0.5, 1.0, and 1.5 µg) or HA-PHp116^{Rip} as a negative control.

p116^{Rip} does not affect RhoA activation

Overexpression of p116^{Rip} induces a dn-RhoA phenotype (Gebbink et al., 1997; Mulder et al., 2003) and inhibits RhoA-dependent SRF activation (Fig. 5A, B). Therefore, we investigated whether p116^{Rip} affected RhoA signalling at the level of RhoA activation. p116^{Rip} was overexpressed in COS-7 cells and RhoA activation was analysed before and after LPA stimulation. As a positive control COS-7 cells were transfected with an activated mutant of p190RhoGEF (DH/PH) (van Horck et al., 2001). Basal Rho-GTP levels were unaffected by overexpression of p116^{Rip} (Fig. 5C, upper panel, compare lanes 1 and 5-6). LPA activates RhoA in both control and p116^{Rip} transfected cells (Fig. 5C, upper panel, compare lanes 1 to 3-4, and lanes 5-6, to 7-8). Thus, p116^{Rip} does not interfere with LPA-induced RhoA activation.

Inhibition of SRF activity by p116^{Rip} does not depend on its oligomerisation or interaction with MBS

We next examined whether inhibition of SRF activity depends on p116^{Rip}'s ability to homo-oligomerise or associate with MBS. Wild-type p116^{Rip}, (iso)leucine single mutants that no longer bind MBS, or (iso)leucine double mutants that fail to interact with both MBS and p116^{Rip}, were co-expressed with activated RhoA in HEK293 cells and SRF activation was determined. A deletion mutant of p116^{Rip} containing only the second PH domain was used as a negative control. Wild-type p116^{Rip} and (iso)leucine mutants are all equally able to inhibit the RhoA-induced SRF activation (Fig. 5D), indicating that the ability of p116^{Rip} to homo-oligomerise or interact with MBS does not contribute to its affect on SRF activity (Table 2).

Inhibition of SRF activity by p116^{Rip} correlates with its capacity to disrupt the cytoskeleton

p116^{Rip} might modulate Rho-induced SRF activation through its effect on F-actin containing structures. To investigate this, we studied the cytoskeletal organisation of NIH3T3 cells expressing the (iso)leucine mutants of p116^{Rip}. Expression of all mutants results in a dramatic loss of stress fibres similar to wild-type p116^{Rip} (Fig. 6). Less than 15% of the p116^{Rip} wild-type or (iso)leucine transfected NIH3T3 cells contain stress fibres, compared to >60% of GFP-expressing control cells. Thus, the effect of p116^{Rip} overexpression on SRF activation is associated with its ability to disrupt the cytoskeleton (Table 2).

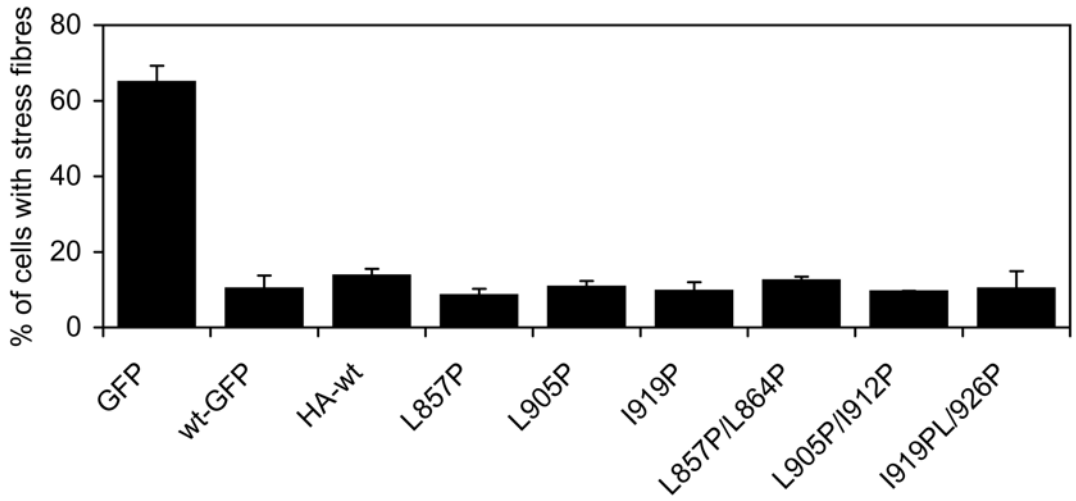


Figure 6. Expression p116^{Rip} (iso)leucine mutants induces a loss of stress fibres in NIH3T3 cells
NIH3T3 cells were transfected with HA-tagged p116^{Rip} wild-type (wt), HA-tagged p116^{Rip} (iso)leucine mutants, GFP, or p116^{Rip}-GFP fusion constructs. Transfected cells were analysed for the presence of stress fibres as previously described (Mulder et al., 2003).

Table 2. Summary of results

	SRF activity (%) [b]	MBS Interaction [a]	p116^{Rip} Interaction [a]	Loss of stress fibres [c]
RhoV14	100	NA	NA	NA
RhoV14 + p116^{Rip} wt	35	+++	+++	yes
RhoV14 + p116^{Rip} L857P	40	-/+	+++	yes
RhoV14 + p116^{Rip} I919P	46	-/+	+++	yes
RhoV14 + p116^{Rip} L905P/I912P	47	-	-	yes
RhoV14 + p116^{Rip} I919P/L926P	35	-	-	yes
RhoV14 + PH-p116^{Rip}	105	ND	ND	ND

[a] Interaction with p116^{Rip} in COS-7 cells:
+++ = strong interaction
-/+ = weak interaction
- = no interaction
[b] SRF activity induced by RhoV14 in the absence or presence of 1.5 μg p116^{Rip} wt or p116^{Rip} mutants
[c] As determined in NIH3T3 cells upon overexpression of p116^{Rip} wt or p116^{Rip} mutants, see Figure 6

Concluding remarks

We have shown that p116^{Rip}, through its C-terminal coiled-coil domain, homo-oligomerises and hetero-oligomerises with MBS. Moreover, the (iso)leucine mutants described here will allow to address the importance of the MBS-p116^{Rip} and p116^{Rip}-p116^{Rip} complexes. Future studies should investigate whether homo-oligmerisation occurs *in vivo* and how homo-oligomerisation contributes to the function of p116^{Rip}.

The present data show that p116^{Rip} expression abrogates RhoA signaling to the nucleus. This is in line with earlier observations showing that p116^{Rip} expression levels are correlated inversely with an active RhoA phenotype (Mulder et al., 2003; Mulder et al., 2004). Interestingly, p116^{Rip} was found to localise to the nucleus, although the function of this localisation remains unknown. However, our data exclude the possibility that the MBS-p116^{Rip} interaction or p116^{Rip} oligomerisation plays a role in the transcriptional inhibition by p116^{Rip}. Indeed, MBS is not directly involved in actin polymerisation but is primarily involved in the regulation of MLC phosphorylation downstream of ROCK. In addition, a role of MBS in SRF activation has not been reported. It has been suggested that p116^{Rip} may inhibit RhoA activity directly, acting as a GTPase-activating protein (GAP) (Koga and Ikebe, 2005). This seems quite unlikely, as p116^{Rip} overexpression does not affect active RhoA levels and p116^{Rip} does not contain a typical GAP domain. Furthermore, RhoA inactivation through an alleged GAP activity of p116^{Rip} can be excluded in our SRF-reporter assays, since we use constitutively active RhoA that cannot undergo GTP hydrolysis.

p116^{Rip} inhibition of RhoA signalling

We have shown that p116^{Rip} overexpression antagonises RhoA signalling, as evidenced by loss of stress fibres, induction of neurite outgrowth and inhibition of gene transcription (Fig. 5 and 6) (Gebbink et al., 1997; Mulder et al., 2003; Mulder et al., 2004), but it has been unclear at what level p116^{Rip} interferes with RhoA signalling. Collectively, our studies show

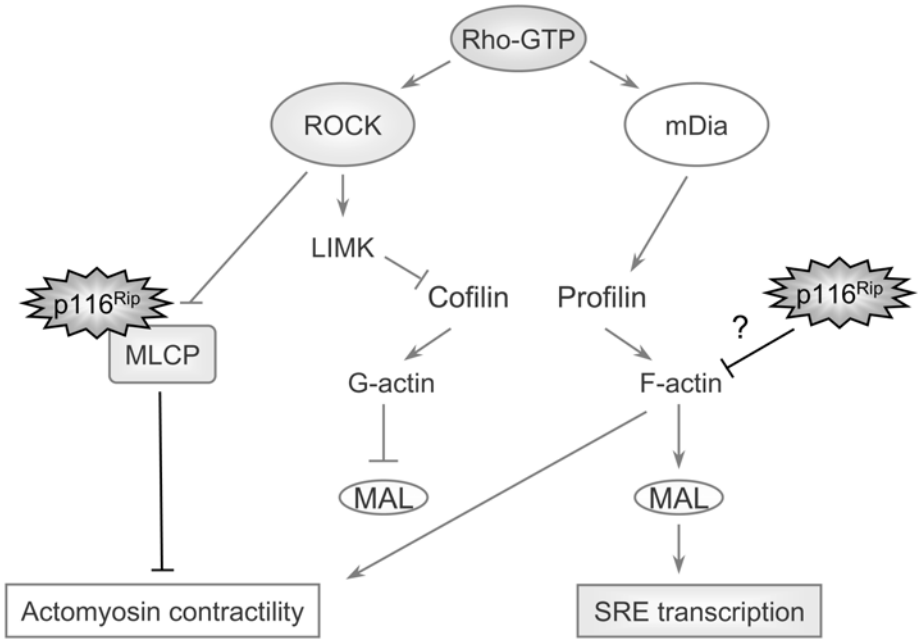


Figure 7. Model of p116^{Rip} counteracting the RhoA pathway. RhoA activation leads to reorganisation of the actin cytoskeleton through the activation of the RhoA effector proteins ROCK and mDia. Activation of these effectors leads to the stabilisation of actin filaments, accumulation of F-actin, and increased actomyosin contractility. As a consequence, MAL is no longer sequestered by G-actin and relocates to the nucleus where it associates with SRF and activates SRE-mediated gene expression. We propose that p116^{Rip} interferes with the RhoA pathway at multiple levels: 1) by its direct affect on F-actin leading to inhibition of RhoA-mediated gene transcription and actomyosin contractility, 2) by acting in parallel with MLCP resulting in reduced actomyosin contractility (Mulder et al., 2004)d}4). SRF, serum response factor; SRE, serum response element; MAL, megakaryocytic acute leukaemia protein, ROCK, Rho-kinase; LIMK, LIM-kinase; MLC, myosin light chain; MLCP, MLC phosphatase.

that p116^{Rip} acts downstream of the RhoA-ROCK pathway, because: (1) p116^{Rip} overexpression does not affect basal levels of activated RhoA or activation of RhoA by LPA (Fig. 5C); (2), knock-down of p116^{Rip} prevents neurite outgrowth induced by the ROCK inhibitor Y-27632 (Mulder et al., 2004); and (3), p116^{Rip} does not bind ROCK ((Koga and Ikebe, 2005) and our unpublished data).

Activation of the transcription factor SRF is regulated by changes in the G-actin pool (Sotiropoulos et al., 1999; Miralles et al., 2003). We have previously shown that p116^{Rip} is an F-actin-binding protein that, upon overexpression, leads to disassembly of the actomyosin cytoskeleton (Mulder et al., 2003). Here, we show that the inhibition of Rho-induced SRF activity by p116^{Rip} correlates with the ability of p116^{Rip} to disrupt the cytoskeleton. Therefore, our data suggest a model in which overexpression of p116^{Rip} disassembles the F-actin cytoskeleton, leading to an increase in the G-actin pool (downstream of RhoA) and decreased SRF activation (Fig. 7). Future studies should address how p116^{Rip} may affect G- and F-actin levels.

From our RNAi studies, we concluded that p116^{Rip} acts as a scaffold protein that targets MBS to the actin cytoskeleton (Mulder et al., 2004). Whilst the RhoA/ROCK pathway leads to phosphorylation of MLC, MBS antagonises the RhoA pathway through dephoshorylation of MLC and other cytoskeleton-associated proteins (Fukata et al., 1999; Nakamura et al., 2000; Amano et al., 2003). In conclusion, p116^{Rip} acts downstream of the Rho/ROCK pathway probably in tandem with MBS and F-actin to negatively influence the RhoA pathway and thereby induces relaxation of the actomyosin cytoskeleton (Fig. 7).

Materials and Methods

Cell culture, transfection and materials

All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 8% fetal calf serum. COS-7 cells were transfected using DEAE–dextran as described previously (Zondag et al., 1996). The following materials were obtained from the designated sources: FLAG M2 mouse monoclonal antibody (mAb), 1-oleoyl-LPA (Sigma), anti-GST 2F3 mAb, anti-myc 9E10 mAb and anti-HA 12CA5 mAb from hybridoma supernatants (ATCC); anti-RhoA 26C4 mAb (Santa Cruz Biotechnology), 3F10 anti-HA rat mAb (Roche).

Plasmids

pcDNA3-HA-ΔNp116^{Rip}, pcDNA3-HA-FLp116^{Rip}, pMT2sm-FLp116^{Rip}-GST, pcDNA3-HA-NTp116^{Rip}, pcDNA3-HA-CTp116^{Rip} and pcDNA3-HA-RBDp116^{Rip} have been described elsewhere (Gebbink et al., 1997; Mulder et al., 2003; Mulder et al., 2004). pcDNA3-HA-p190RhoGEF is described by (van Horck et al., 2001). pcDNA3-FLp116^{Rip}-myc was generated by digestion of pcDNA3-FLp116^{Rip} with *Not*I-*Not*I and ligation of the insert into pcDNA3-Myc. pMT2sm-HA-p190RhoGEF-DH/PH is described by (van Horck et al., 2001). pcDNA3-HA-PHp116^{Rip} (aa 383-488) was generated by PCR using primers 5'-cctgacctgctgaatttcaag-3' (forward) and 5'-atctggggcagatgctgg-3' (reverse). The PCR product was digested with *KpN*I-*EcoR*I and ligated into pcDNA3-HA. SRE.L-luciferase plasmids and GST-C21, are described elsewhere (Mao et al., 1998; Sander et al., 1999). The construction of dominant-active RhoA pMT2sm-myc construct has been described previously (Gebbink et al., 1997). pMT2sm-ΔNp116^{Rip}-GST was constructed by ligation of a *Bst*XI-*Sal*I, blunt-ended fragment out of FLp116^{Rip}-peGFPN1 into *Sma*I cut pMT2sm-GST. pMT2sm-ΔCp116^{Rip}-GST was generated by digestion of pMT2sm-FLp116^{Rip}-GST with *Sma*I and ligation the insert into *Sma*I-*Not*I, blunt-ended pMT2sm-GST. The p116^{Rip} double point mutants (L857P/L864P, L905P/I912P, and I919P/L926P) were made with the QuikChange Site-Directed Mutagenesis Kit (Stratagene) using the p116^{Rip} single point mutants (L857P, L905P, and I919P, (Mulder et al., 2004)) as a template. pXJ40-FLAG-p85 (MBS) and pEFBOS-myc-MBS130 plasmids were kindly provided by Dr. T. Leung and Dr. K. Kaibuchi, respectively.

Western blotting, immunoprecipitation, and pull-down assays

COS-7 cells co-expressing various constructs were scraped in ice-cold lysis buffer A (1% NP-40, 50 mM Tris pH 7.4, 200 mM NaCl, 2.5 mM $MgCl_2$, 10% glycerol, supplemented with protease inhibitors). Extracts were clarified by centrifugation and precleared with 0.5% BSA-blocked protein-A–Sepharose beads for 1 hour at 4 °C. Precleared lysates were incubated overnight with anti-HA at 4 °C and immunocomplexes were removed by incubation with protein-A-Sepharose beads for 30 min at 4 °C. Beads were washed, resuspended in Laemmli sample buffer and boiled. Samples were separated by SDS–PAGE and analyzed by western blotting using anti-myc, anti-HA, or anti- FLAG antibodies.

GST pulldowns were performed as follows. COS-7 cell lysates were prepared as above. GSH-sepharose was used to collect the p116^{Rip}-GST protein precipitates. Beads were washed, eluted

in Laemmli sample buffer and boiled. Samples were separated by SDS–PAGE and analysed by western blotting using myc or GST antibodies.

SRF-mediated luciferase gene transcription

Hek293 cells were seeded at a density of 1 x 10^5 cells per well in 12-well plates. Cells were transfected with pCMV-β-gal (0.2 ug), pSRE.luc (0.2 μg) and indicated plasmids. The total DNA per well was kept constant at 3 μg DNA per well using pcDNA3 and/or pMT2sm empty vectors. 24 h after transfection fresh DMEM containing 0.5 % FCS was added to the cells. 48 h after transfection, cells were lysed in 200 μl Reporter Lysis Buffer (25 mM Tris-HCl pH 7.8, 2 mM DTT, 2 mM EDTA, 10% glycerol and 1% Triton X-100). Luciferase activity was measured using 20 μl of lysate and 50 μl of Luciferase Substrate (Promega) at 495 nm. β-Galactosidase measurements were used to normalise transfection efficiency. Luciferase and β-Galactosidase activity were quantified using a Victor luminometer (Wallac, Turku, Finland). Equal amounts of lysate were subjected to SDS-PAGE and western blotting to determine the expression of transfected proteins. The experiments were repeated at least three times; representative experiments are shown.

RhoA activation assay

RhoA activity was analysed as described (van Horck et al., 2001), using a GST-fusion protein containing the RhoA binding domain of Rhotekin (GST-C21). In brief, COS-7 cells were transfected with indicated constructs, stimulated and lysed in Nonidet P-40 fish buffer. Cleared lysates were incubated for 45 min at 4 °C with 20 μg of GST-C21 coupled to GSH-Sepharose. The beads were washed three times using fish buffer and bound proteins were separated by SDS-PAGE and Western blotting.

Confocal Microscopy

NIH3T3 cells were grown on gelatin-coated glass coverslips in six-well plates. Cells were fixed 48 h after transfection in 3.7% formaldehyde in PBS for 10 min, permeabilised (0.1% Triton X-100/PBS; 2 min), blocked (1% BSA/PBS; 30 min), and incubated for 1 h with primary HA antibody 3F10. Subsequently, cells were washed and incubated with secondary antibodies (Goat-anti-rat-fluorescein isothiocyanate (Rockland); 30 min) together with rhodamine-conjugated phalloidin (Molecular Probes). Coverslips were mounted in Vectashield and analysed using a Leica TCS-NT confocal microscope.

Acknowledgments

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Summary

Cells sense changes in their environment via specific sensors, so-called receptors that are located on the outer surface, at the plasma membrane. Receptors are activated by binding of their cognate ligands, signal molecules such as growth factors, which leads to distinct cellular responses. Stimulated receptors pass on the message by triggering a wide range of signalling cascades involving successive activation of enzymes and generation of “second messengers” (signal transduction). Which cellular responses arise after stimulation of a receptor depends on the signal molecule (agonist), the type of receptor, and the combination of both. In this way, growth factors that bind to their specific receptor encourage cells to divide. Similarly, next to affecting cell growth, receptor agonists can promote alterations in cellular morphology and motility. Changes in cell morphology and polarity are fundamental for many physiological (and pathological) processes, and are brought about by the dynamic regulation and reorganisation of the cytoskeleton.

The cytoskeleton provides the strength and motility of a cell and consists of three types of networks: the actin cytoskeleton, the microtubule network and intermediate filaments. These networks are characterised by filaments, which arise through the joining of single subunits into large strands (polymerisation). A higher order structure of the actin cytoskeleton is accomplished by the organisation of actin filaments into networks by actin-crosslinking and -bundling proteins. Other actin-binding proteins (ABPs) function as scaffolds holding the actin network and associating proteins together. In addition, various ABPs stimulate or reduce polymerisation of actin, thereby affecting the length and amount of actin filaments. So, the organisation of the actin network is determined by the combined activities of ABPs.

The Rho family of small GTPases, in particular the members RhoA, Rac, and Cdc42, has a central role in the regulation of the actin cytoskeleton. Rho GTPases act as molecular switches that are inactive when complexed with GDP and active when bound to GTP. Receptors that induce remodelling of the actin cytoskeleton all converge on these Rho GTPases to elicit their effects. Activated Rho GTPases transmit the signal to proteins that directly affect the assembly and disassembly of the cytoskeleton, such as ABPs. For example, activation of RhoA leads to cell contraction by the actin-associated motor protein myosin, which exerts force on actin filaments.

Besides their effects on actin reorganisation, Rho GTPases are involved in many other cellular responses, such as gene transcription leading to changes in protein expression. Serum or its major constituent lysophosphatidic acid activate for instance the serum response factor (SRF), which controls many growth factor-regulated genes, through RhoA-dependent increases in actin polymerisation.

Deregulation of Rho GTPase activity can contribute to various aspects of tumourigenesis. Therefore, studying the Rho GTPase signal transduction pathway will increase our understanding of altered cell behaviour as occurs during the migration, invasion, and metastasis of cancer cells.

The $p116^{Rip}$ protein was first identified as a putative RhoA-interacting protein. It contains several protein interaction domains and is widely expressed throughout the body. Initial results suggested that $p116^{Rip}$ expression in neuronal cells inhibited RhoA-induced

contraction of the actin cytoskeleton. This thesis focuses on the function of p116^{Rip} with emphasis on its role in RhoA-induced remodelling of the actin cytoskeleton and induction of gene transcription.

Chapter 1 describes the major signal transduction pathways downstream of RhoA leading to gene transcription and remodelling of the actin cytoskeleton, especially in neuronal cells. Moreover, we characterise p116^{Rip}: the conservation of p116^{Rip} among diverse organisms, its chromosomal location, occurrence of splice variants, related proteins, domain structure, and potential protein-protein interaction and regulatory motifs. Furthermore, chapter 1 summarizes published data on the presence of p116^{Rip} in various protein complexes and its regulation under physiological conditions.

In **chapter 2** we show that p116^{Rip} is an F-actin binding protein that co-localises to, binds and bundles filamentous actin through actin-binding domain(s) located at its N-terminus. In addition, we show that overexpression of p116^{Rip} in NIH3T3 cells leads to disruption of RhoA-dependent stress fibres and Rac-mediated lamellipodia formation, as shown by the formation of dendrite-like extensions. These findings indicate that p116^{Rip} can affect, either directly or indirectly, the integrity of the actin cytoskeleton.

In **chapter 3** we show that p116^{Rip}, through its C-terminal coiled-coil domain, interacts directly with the regulatory myosin-binding subunits (MBS) of myosin light chain (MLC) phosphatase, MBS85 and MBS130. MLC-phosphatase is a crucial component in the RhoA pathway. The function of p116^{Rip} is investigated by blocking its expression using a recently developed technique, RNA interference (RNAi). This technique involves the production of small specific pieces of RNA that block expression of the corresponding protein. RNAi-induced knockdown of p116^{Rip} inhibits cell spreading and neurite outgrowth in response to extracellular stimuli, demonstrating that p116^{Rip} is essential for neurite outgrowth. RNAi-induced knockdown of p116^{Rip} does not affect MLC phosphorylation; the association of MBS with the cytoskeleton appears to depend on p116^{Rip}. We suggest a model in which p116^{Rip} acts as a scaffold to target the myosin phosphatase complex to the actin cytoskeleton.

Chapter 4 reports on the inhibition of RhoA-induced activation of the transcription factor SRF by p116^{Rip}. However, overexpression of p116^{Rip} does not directly affect RhoA activation. In addition, we demonstrate that p116^{Rip} can oligomerise through its C-terminal coiled-coil domain. Mutational analysis revealed that transcriptional inhibition by p116^{Rip} does not depend on the p116^{Rip}-MBS interaction (chapter 3) or on oligomerisation. Exactly how this inhibition occurs is as yet unclear, but we propose that p116^{Rip} abrogates RhoA-induced SRF activation due to its ability to disassemble the F-actin cytoskeleton (chapter 2). We conclude that p116^{Rip} counteracts RhoA signalling downstream of RhoA and Rho-kinase (ROCK).

In conclusion, we have shown that p116^{Rip} is essential for neurite outgrowth and counteracts RhoA action. This occurs downstream of RhoA and ROCK, probably at multiple levels: 1) by its direct effect on actin remodelling, 2) by acting as a scaffold linking MBS to the cytoskeleton. Further studies in primary neurons, should reveal how p116^{Rip} regulates neurite outgrowth. Since p116^{Rip} counteracts the RhoA pathway, it will be interesting to investigate the possible role of p116^{Rip} in RhoA-dependent tumour development.

Samenvatting

Cellen reageren op veranderingen in hun omgeving via sensoren, receptoren genaamd, die op de plasmamembraan liggen, aan de buitenkant van het celoppervlak. Prikkeling oftewel activering van receptoren door middel van binding van signaalstoffen aan een receptor, leidt tot specifieke reacties van een cel. Het signaal van de receptoren wordt vertaald en vanaf het celoppervlak doorgegeven naar het binnenste van de cel. Dit geschiedt via signaalwegen waarbij achtereenvolgende activatie van enzymen en generatie van nieuwe signalen optreedt (signaaltransductie). Welke veranderingen precies optreden in een cel na receptor stimulatie hangt af van de signaalstof, de receptor en de combinatie van beide. Zo zetten sommige signaalstoffen, zogenaamde groeifactoren, na binding aan de bijbehorende receptor, cellen aan tot delen. Op soortgelijke wijze kan activatie van receptoren naast verandering in celgroei leiden tot veranderingen in celmobiliteit en celmorfologie. Veranderingen in de celmorfologie en celpolariteit zijn fundamenteel voor vele fysiologische en tevens pathologische processen. Dynamische regulatie en organisatie van het cytoskelet liggen ten grondslag aan veranderingen in celmorfologie.

Het cytoskelet zorgt voor de stevigheid en mobiliteit van een cel en bestaat uit drie verschillende typen netwerken: het actine cytoskelet, het microtubuli netwerk en de intermediaire filamenten. De vorm van deze netwerken wordt gekenmerkt door filamenten die ontstaan door aaneenschakeling van subeenheden in lange strengen (polymerisatie). Een hogere orde van actine cytoskeletstructuur wordt bereikt door het organiseren van actine filamenten in netwerken door crosslinking of bundelings eiwitten. Andere actine bindende eiwitten (ABP's) fungeren als “brug-eiwitten,” die het actine netwerk en associërende eiwitten ondersteunen en bijeenhouden. Daarnaast zijn er vele ABP's die de polymerisatie van actine subeenheden stimuleren of reduceren en daardoor de lengte en de hoeveelheid aan actine filamenten in de cel beïnvloeden. De organisatie van het actine netwerk wordt dus bepaald door de gecombineerde activiteiten van ABP's.

De Rho familie van kleine GTPases, in het bijzonder de leden RhoA, Rac en Cdc42, spelen een centrale rol in de regulatie van het actine cytoskelet. Rho GTPases zijn eiwitten die zich gedragen als moleculaire schakelaars: ze zijn inactief wanneer ze een complex vormen met GDP en actief als ze GTP-gebonden zijn. Receptoren die na activatie leiden tot veranderingen in de organisatie van het actine cytoskelet seinen alle via Rho GTPases. De Rho GTPases geven op hun beurt het signaal door naar eiwitten die de status van het actine cytoskelet direct of indirect beïnvloeden, zoals de ABP's. Zo resulteert RhoA activatie bijvoorbeeld in de contractie van cellen door stimulatie van het motoreiwit myosine, dat kracht uitoefent op actine filamenten.

Daarnaast zijn Rho GTPases betrokken bij vele andere cellulaire reacties, zoals gentranscriptie, wat leidt tot specifieke veranderingen in eiwitproductie. Serum en het voornaamste bestanddeel van serum, lysophosphatidic acid, activeren de serum response factor (SRF) via een RhoA-afhankelijke toename van actine polymerisatie. SRF is een transcriptiefactor die veel groei-gerelateerde genen tot activiteit aanzet.

Een verkeerde regulatie van Rho GTPase activiteit kan diverse aspecten van tumorontwikkeling bevorderen. Het bestuderen van de RhoA signaaltransductie route zal

ons begrip vergroten met betrekking tot veranderingen in celgedrag zoals die optreden tijdens de migratie, invasie en metastasering van kankercellen.

p116^{Rip} werd oorspronkelijk geïdentificeerd als een mogelijk RhoA-bindend eiwit ("RhoA-interacting protein"). Het eiwit wordt overal in het lichaam geproduceerd en bevat verschillende domeinen die potentiële interacties met andere eiwitten mogelijk maken. De eerste resultaten suggereerden dat p116^{Rip} een remmende invloed had op de RhoA-geïnduceerde contractie van het actine cytoskelet . Dit proefschrift beschrijft de functie van p116^{Rip}, waarbij de nadruk ligt op de rol die p116^{Rip} speelt in de door RhoA veroorzaakte reorganisatie van het actine cytoskelet en activatie van gentranscriptie.

Hoofdstuk 1 beschrijft de voornaamste signaaltransductie routes die via RhoA leiden tot gentranscriptie en reorganistie van het actine cytoskelet, met name in neuronale cellen. Tevens wordt p116^{Rip} gekarakteriseerd: het voorkomen van het eiwit in diverse organismen, de chromosoomlokatie, splicevarianten, verwante eiwitten, de domeinstructuur en potentieel interessante eiwit-eiwit interacties. Daarnaast bevat hoofdstuk 1 een samenvatting van gegevens in de literatuur over de aanwezigheid van p116^{Rip} in eiwitcomplexen en de regulatie van p116^{Rip} tijdens fysiologische en pathologische processen.

In **hoofdstuk 2** tonen we aan dat p116^{Rip} een actine-bindend eiwit is dat colokaliseert met actine filamenten en actine filamenten bundelt via actine bindend(e) domein(en) die gelokaliseerd zijn in de "kop" van het eiwit. Bovendien vinden we dat verhoogde p116^{Rip} expressie leidt tot het verlies van (RhoA-afhankelijke) stress fibers en (Rac-geïnduceerde) lamellipodia, hetgeen zich manifesteert als neuriet-achtige uitgroei. Dit suggereert dat p116^{Rip} de integriteit van het actine cytoskelet kan beïnvloeden.

Hoofdstuk 3 beschrijft de binding van het coiled-coil domein van p116^{Rip} aan de myosine-bindende subeenheden (MBS) van myosine light chain (MLC) phosphatase, MBS85 en MBS130. MLC phosphatase is een essentieel enzym in de RhoA signaaltransductie route. Verder wordt de functie van p116^{Rip} bestudeerd door de expressie van het eiwit te blokkeren door middel van een recent ontwikkelde techniek, genaamd RNA-interferentie (RNAi). Deze techniek maakt gebruik van de productie van kleine specifieke stukjes RNA, die expressie van het betreffende eiwit beletten. Remming van p116^{Rip} eiwitproduktie leidt ertoe dat neuronale cellen zich niet meer goed kunnen uitspreiden en geen neurietuitgroei initiëren. Dit laat zien dat p116^{Rip} essentieel is voor neurietuitgroei. Vervolgens werd bekeken of dit effect te maken had met de p116^{Rip} partner MBS. Echter, verlaging van p116^{Rip} eiwitproduktie heeft geen effect op MLC activiteit, maar wel op de associatie van MBS met het actine cytoskelet. Deze experimenten suggereren dat p116^{Rip} als brug-eiwit fungeert om het MBS-complex te verbinden met het actine cytoskelet.

In **hoofdstuk 4** laten we zien dat overproduktie van p116^{Rip} de (RhoA-geïnduceerde) activatie van de transcriptiefactor SRF remt zonder een direct effect te hebben op de activiteit van RhoA zelf. Verder tonen we aan dat p116^{Rip} met zichzelf kan oligomeriseren via het coiled-coil domein. Analyse door middel van mutanten laat zien dat deze remming onafhankelijk is van oligomerisatie of een interactie met MBS (zie hoofdstuk 3). Hoe deze remming precies in zijn werk gaat is onduidelijk. We concluderen dat p116^{Rip} de RhoA signalering remt op een niveau dat zich beneden RhoA en Rho-kinase (ROCK) bevindt.

Tot besluit wordt in dit proefschrift aangetoond dat p116^{Rip} fundamenteel is voor neurietuitgroei en de werking van RhoA tenietdoet. Dit gebeurt waarschijnlijk op verschillende manieren: 1) door de reorganisatie van het actine cytoskelet direct te

beïnvloeden, 2) door te fungeren als een brug-eiwit om het MBS complex te verbinden aan het actine cytoskelet. Nader onderzoek in primaire neuronen zal moeten duidelijk maken hoe $p116^{Rip}$ precies betrokken is bij morfologische veranderingen van neuronale cellen. Aangezien $p116^{Rip}$ de normale RhoA signalering remt, is het bovendien interessant om de mogelijke rol van $p116^{Rip}$ in (RhoA-afhankelijke) tumorontwikkeling te bestuderen.

Curriculum Vitae

Jacqueline Mulder werd geboren op 13 februari 1974 te Sittard. Na het behalen van het VWO diploma aan het Stella Maris College in Meerssen in 1993, begon zij in datzelfde jaar aan de studie Biologie aan de Universiteit Utrecht. In 1996 werd, gedurende een stage van negen maanden (bij de vakgroep Moleculaire Biologie aan de Universiteit van Utrecht onder begeleiding van dr. G.C. Scheper), het mechanisme onderzocht waarmee eiwit synthese platgelegd wordt na heat shock. In 1997 doorliep zij een stage van zes maanden in Engeland (bij de afdeling Picornaviruses, Institute for Animal Health in Pirbright onder begeleiding van Dr. G.J. Belsham), waar zij onderzoek deed aan de translatie van picornavirussen en vacciniavirussen. In 1998 behaalde zij het doctoraal examen Biologie en begon zij met het promotieonderzoek, zoals beschreven wordt in dit proefschrift, onder begeleiding van Prof. W.H. Moolenaar bij de afdeling Cellulaire Biochemie op het Nederlands Kanker Instituut te Amsterdam. Daarna werd zij in dezelfde groep als post-doc aangesteld om onderzoek te doen naar LPA signalering. Met ingang van october 2005 zal zij werkzaam zijn als post-doc in de groep van Dr. Michael B. Kastan, St. Jude Children's Research Hospital, te Memphis, Tennessee, om onderzoek te doen naar DNA schade.

List of publications

Mulder J., Ariaens A., van den Boomen D., Moolenaar W.H. (2004). p116^{Rip} targets myosin phosphatase to the actin cytoskeleton and is essential for RhoA/ROCK-regulated neuritogenesis. Mol. Biol. Cell *15*, 5516-5527.

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