Characterization of functional domains of mDia1, a link between the small GTPase Rho and the actin cytoskeleton

Anja Krebs*, Martin Rothkegel, Martin Klar and Brigitte M. Jockusch‡

Cell Biology, Zoological Institute, Technical University of Braunschweig, D-38092 Braunschweig, Germany

*Present address: Medizinische Klinik I, University of Erlangen, D-91054 Erlangen

‡Author for correspondence (e-mail: bmj@tu-bs.de)

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SUMMARY

The widely expressed diaphanous proteins, a subclass of formins, comprise links between the Rho GTPases and the actin-based cytoskeleton. They contain several functional domains that are thought to be responsible for interaction with different ligands: the FH1 domain for binding the actin-associated protein profilin; the RBD for targeting activated Rho; and the C-terminal CIID module for autoregulation of the overall diaphanous activity. Using deletion constructs of the murine mDia1, we have analyzed the functional properties of these three domains separately in in vitro assays and in transiently and stably transfected cell lines. We show that the proline-rich FH1 domain effectively binds to profilins in vitro as well as in cells, that the RBD complexes with the CIID in a species-restricted manner and that overexpression of RBD causes spontaneous ruffling and loss of stress fibers, together with loss of directional motility. Supertransfection of cells stably expressing the RBD with dominant negative Rac effectively suppresses ruffling. Our data contribute to the understanding of the function of these domains in linking the actin cytoskeleton with the Rho-signaling cascade. Furthermore, they suggest that inactivation of Rho by exogenous RBD causes upregulation of Rac activity in the transfected cells.

Key words: Diaphanous proteins, Rho-like GTPases, Profilin, Actin-dependent cell motility, Ruffling

INTRODUCTION

The diaphanous proteins are members of the ubiquitous formin protein family that can physically link the actin filament system with the Rho GTPases. This connection is thought to be essential in the GTPase-mediated modulation of the actin organization, as required in cytokinesis, establishment and maintenance of cell polarity and directional transport (Frazier and Field, 1997; Wasserman, 1998). For the mammalian homologues, there is evidence that they contribute to nuclear activities (Frazier and Field, 1997) (see below). Diaphanous-related proteins have been identified in yeast, moulds, nematodes, insects and vertebrates (Castrillon and Wassermann, 1994; Frazier and Field, 1997; Wasserman, 1998). They are multidomain proteins of approximately 1000-2000 amino acid residues, comprising a Rho GTPase-binding domain (RBD), three 'formin homology' domains (FH1-3), two coiled-coil domains (CCD1-2) and an intramolecular interaction domain at their C-terminus (CIID; Watanabe et al., 1999), which can interact with the N-terminus (Alberts, 2001; Castrillon and Wassermann, 1994; Petersen et al., 1998; Watanabe et al., 1999). The importance of a functional Dia1 protein in man (hDia1) is stressed by the findings that a C-terminally truncated form of this protein is associated with an autosomal dominant form of progressive hearing loss (Lynch et al., 1997), and that hDia1 gene disruption correlates with premature ovarian failure (Bione et al., 1998). In mammalian cells, endogenous Dia1 is localized in ruffling peripheral membranes (Watanabe et al., 1997) and also in the mitotic spindle (Kato et al., 2001). However, activity, regulation, crosstalk and functional significance of ligand binding by the different domains are not completely understood.

The RBD of diaphanous proteins has been the focus of several studies. It is a target of activated Rho proteins (Imamura et al., 1997; Kohno et al., 1996; Watanabe et al., 1997). While the RBDs of mammalian diaphanous proteins bind only activated RhoA-C, one of the budding yeast diaphanous proteins also interacts with Cdc42 (Evangelista et al., 1997). The small GTPases of the Rho family, Rho, Rac and Cdc42, regulate actin filament organization (Hall, 1998; Machesky and Hall, 1996; Nobes and Hall, 1995; Ridley, 1996). In mouse fibroblasts, Rho has been shown to stimulate the formation of large, actin-based cell-matrix adhesion sites (focal contacts) and contractile microfilament bundles (stress fibers), and to induce and maintain the cleavage ring in cytokinesis (Chrzanowska-Wodnicka and Burridge, 1996; Narumiya, 1996; Ridley and Hall, 1992). Rac activity is required for membrane ruffling at the periphery of the cells and the formation of small focal complexes (Nobes and Hall, 1995; Ridley et al., 1992), and Cdc42 induces the formation of filopodia (Kozma et al., 1995; Nobes and Hall, 1995). Binding of activated Rho to the RBD of murine (m)Dia1 stimulates actin reorganization to prominent stress fibers, in cooperation with other GTPase effectors such as the Rho kinase ROCK

(Nakano et al., 1999; Watanabe et al., 1999), and depends on the presence of the FH1 module (Takaishi et al., 2000).

The highly conserved proline-rich FH1 domain is held responsible for the observed interaction between mDia1 and mDia2 and SH3-module proteins, which links both diaphanous proteins to tyrosine kinase signaling and serum response factor (SRF)-dependent transcriptional activity (Alberts, 2001; Tominaga et al., 2000). Furthermore, the FH1 domains in diaphanous proteins from budding yeast, fission yeast, insects and mouse are considered to mediate binding to profilin (Chang et al., 1997; Evangelista et al., 1997; Imamura et al., 1997; Manseau et al., 1996; Watanabe et al., 1997). Profilins are involved in the regulation of actin polymerization. They bind simultaneously to G-actin and to polyproline-sequences, as contained in the FH1 domain (Schlüter et al., 1997). Targeting profilin-actin complexes to the FH1 domains might thus effectively recruit actin to specific cellular sites, and stimulate filament polymerization. For subsequent organization of stress fibers, as induced by activated Rho, RBD and FH1 domains may act in concert. In addition, for yeast diaphanous proteins, binding to actin itself and to other actin-binding proteins has been described (Evangelista et al., 1997), indicating that in these cells the physical link between diaphanous proteins and the actin cytoskeleton may not be confined to profilin-binding.

At present, the information on ligand binding and cellular function of the FH2 and FH3 domains is rather scarce. The FH2 module of mDia1 has been implicated in the coordination of microtubule and actin filament networks (Ishizaki et al., 2000), but the molecular basis of this linkage is unclear. The FH3 domain, which is less well conserved among different species and overlaps with the RBD, is essential for targeting the relevant fission yeast diaphanous protein to the polar tips of mating cells (Petersen et al., 1998). This may also be related to cytoskeleton-mediated processes of generating cell polarity. In HeLa cells, the FH3 domain was found essentially responsible for mDia1 association with the mitotic spindle (Kato et al., 2001).

When truncated mDia1 constructs lacking the RBD are transiently transfected into cells, they induce SRF-dependent nuclear transcription (Tominaga et al., 2000) and stimulate stress fiber formation (Watanabe et al., 1999), suggesting that this domain acts as a negative regulator on several levels of diaphanous activity. Autoregulation of Dia1 and Dia2 proteins has been found to involve intramolecular bonding between N- and C-terminal motifs and its release by activated Rho (Alberts, 2001; Watanabe et al., 1999). Thus, RBD-Rho complex formation has a dual function: it opens the diaphanous polypeptide and activates the entire molecule, and it stimulates site-specific Rho-dependent actin reorganization.

To further elucidate the role of different diaphanous domains in signal-dependent actin organization and its consequences on cellular motility, we have generated a catalogue of deletion fragments of mDia1 and studied their properties in detail, in vitro, in transiently and in stably transfected cells. We show that in a physiological environment, the FH1 domain of mDia1 forms complexes with endogenous profilin, and the RBD with the C-terminal intramolecular interaction domain CIID, respectively. Furthermore, we demonstrate that the RBD interacts with the endogenous mDia1 in a species-restricted manner and that overexpression of this domain causes extensive, spontaneous ruffling, loss of stress fibers and loss of directed locomotion.

MATERIALS AND METHODS

DNA and vectors

The cDNA fragment encoding mouse mDia1 (EMBL accession number U96963) was subcloned into the expression plasmids pET28a(+) (Novagene, Madison, WI), pcDNA3-flag (Invitrogen, Leek, NL; modified in our own laboratory), pcDNA3-Bipro (Rüdiger et al., 1997) and pEGFP-C2 (Clontech, Heidelberg, Germany). The various truncations of mDia1 were designed either according to suitable restriction sites or by PCR and inserted into the same vectors, using synthetic oligodeoxynucleotides as linkers. For all constructs, the correct reading frames were confirmed by sequencing. pcDNA3/N17Rac1, coding for a dominant negative Rac GTPase (gift of A. Wittinghofer, Dortmund, Germany), was used in supertransfections.

Antibodies

For monitoring profilin in immunoblots and immunofluorescence, the monoclonal antibody 2H11 (Mayboroda et al., 1997) and our own polyclonal antibody against bovine profilin (Buss et al., 1992) were used. For the detection of RhoA, ezrin, endogenous and transfected mDia1, the transfected RBD-containing mDia1 fragment and transfected myc-Rac in immunoprecipitation, immunoblots and immunofluorescence experiments, respectively, the following antibodies were used: polyclonal antibodies against RhoA (Santa Cruz Biotechnology, Heidelberg, Germany) and human ezrin (gift of P. Mangeat, Montpellier, France), a polyclonal antibody against mDia1 (gift of S. Narumiya, Tokyo, Japan), a monoclonal antibody against mDia1 (Becton Dickinson, Heidelberg, Germany); and a monoclonal antibody against the myc tag (gift of B. Winter, Braunschweig, Germany). Immunoprecipitation of the epitope tagged proteins in lysates of transfected cells was performed with the monoclonal antibodies anti-flag M2 (Sigma, Deisenhofen, Germany) and 4A6, which recognizes the birch profilin tag (BiPro tag; Rüdiger et al., 1997).

Cell culture, transfection and selection procedures

CHO cells were grown in DMEM/Ham's F-12 medium (1:1 mixture), supplemented with 10% fetal bovine serum (FBS). HeLa cells were transfected by the calcium phosphate method, using 2 μg DNA/20-mm dish, whereas CHO cells were transfected using the FuGene 6 reagent (Roche Biochemicals, Mannheim, Germany) according to the manufacturer's protocol. They were then incubated for at least 12 hours, rinsed three times with phosphate buffered saline (PBS) and incubated for at least 6 hours. Stable CHO derivatives were obtained by culturing CHO cells (7×10^4/60 mm dish) for 24 hours and then transfecting them with the calcium phosphate method with 10 μg of either the control vector encoding the green fluorescent protein (pEGFP-C2), or the vector coding for the fusion protein of EGFP and deletion fragment 1-448mDia1 (pEGFP-1-448mDia). For each construct, clones were selected after culture in geneticin containing selective medium (0.6 mg G418/ml, Life Technologies, Karlsruhe, Germany). For expression of a dominant negative Rac GTPase, the stable CHO lines were supertransfected with pcDNA3/N17Rac1.

Wounding assay

Locomotory activity was assessed by following the closure of a wound set experimentally into a dense monolayer. CHO cells and their derivatives were seeded at a density of 1×10^5 cells in 60 mm dishes and grown to confluency. A wound approximately 1 mm wide was obtained by scratching the monolayer with a pipette tip. Cell debris was removed by rinsing the cultures extensively with PBS, and fresh medium with or without 0.1 μg/ml colcemide (Biochrom, Berlin,

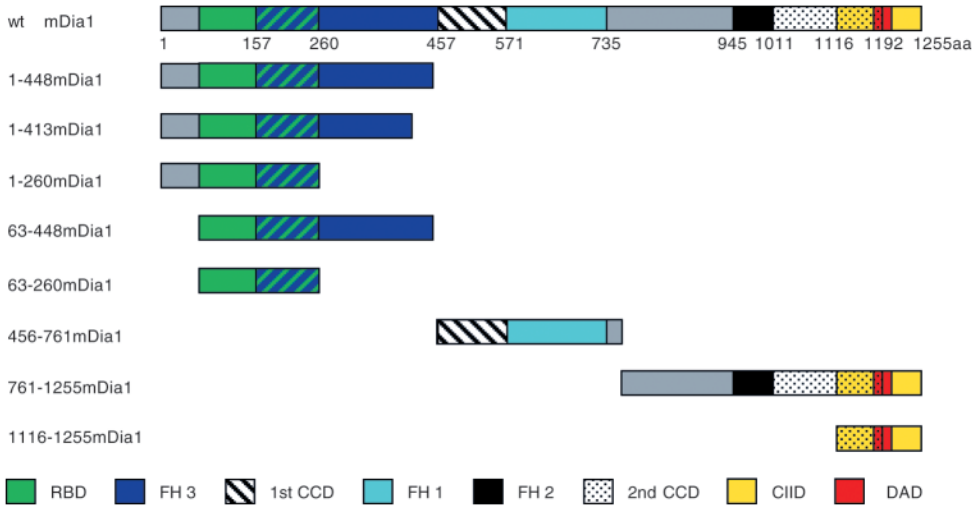

Fig. 1. Molecular anatomy of wild type (wt) mDia1 and the deletion fragments used in this study. Location and extension of the domains and motifs within the polypeptide, as compiled from several studies, are indicated and colour-coded. For references see text. RBD, Rho-binding domain, comprising amino acid residues 63-260; FH1 (aa residues 571-735), FH2 (aa 945-1010), FH3 (aa 157-456), formin homology domains 1, 2 and 3, respectively. The two coiled coil regions are 1st CCD (aa 457-570) and 2nd CCD (aa 1011-1192). Within the C-terminal intramolecular interaction domain (CIID; aa 1116-1255), the highly conserved diaphanous-related formin autoregulatory domain (DAD; aa 1177-1207) has been identified.

Germany) was applied. Wound closure was documented by photographing the cells under phase-contrast microscopy at time points 0, 2, 4, 6, 8 and 24 hours after wounding.

Immunofluorescence

Cells were grown on glass coverslips for one day prior to fixation in 4% formaldehyde and subsequent extraction with 0.2% Triton X-100 in PBS. The samples were then incubated for 30 minutes at 37°C with the respective antibody. Goat anti-mouse or anti-rabbit IgG coupled to tetramethylrhodamine B isothiocyanate (TRITC) (Dianova, Hamburg, Germany) served as secondary antibodies and phalloidin-TRITC (Sigma) for labelling of F-actin. The cells were examined in a conventional light microscope equipped with epifluorescence (Axiophot, Zeiss, Oberkochen, Germany).

Immunoprecipitation of protein complexes after in situ crosslinking

In situ crosslinking, precipitation and analysis of protein complexes were performed essentially as described (Hüttelmaier et al., 1997; Weiss et al., 1998) with slight modifications. Transfected HeLa and CHO cells were grown on 10 cm dishes (5×10^6/dish), rinsed in PBS and incubated at room temperature for 30 minutes with 0.5 mM of the membrane permeant crosslinker dithio-bis(succinimidylpropionate) (DSP; Pierce, Sankt Augustin, Germany). Excess crosslinker was quenched with 0.2 M glycine. After rinsing in PBS, cells were lysed in RIPA buffer (50 mM Tris, pH 7.2, 1% (v/v) Triton X-100, 0.25% desoxycholate, 1 mM EGTA, 150 mM NaCl, 2.5 mM sodium azide, 1 μM pepstatin A, 80 μM Pefabloc SC, 0.46 μM aprotinin; 30 minutes at 4°C). Cellular material was then scraped off the dish with a rubber policeman, homogenized by pipetting and centrifuged at 15,000 ***g*** at 4°C. Then, 40 μl of a 50% slurry of protein G sepharose preblocked with 2% (w/v) BSA in RIPA buffer was added to the samples, and incubation continued under stirring at 4°C for 2 hours. The sepharose beads were collected by centrifugation, washed three times with RIPA buffer and once with PBS before the samples were boiled in SDS sample buffer containing 20% (v/v) β-mercaptoethanol, to cleave the crosslinker. Finally, the samples were analyzed by SDS-PAGE and immunoblotting, using standard procedures.

In vitro transcription/translation

The coding regions of wt-mDia1 and its deletion fragments were cloned into the vector pET28a. The [^{35}S]-methionine labeled proteins were synthesized by in vitro transcription/translation using the 'TNT-coupled reticulocyte lysate system' (Promega, Heidelberg, Germany), according to the manufacturer's protocol.

Affinity precipitation

For the expression of recombinant mouse PFN I and II the T7 RNA polymerase expression system (Studier and Moffatt, 1986) was used. The *E. coli* strain BL21 (DE3) pLysS was transformed with the expression vectors pET21c/PFN I and pMW172/PFN IIa (gift of W. Witke, Monterotondo, Italy), containing the coding regions of either mouse profilin I or profilin IIa. The expressed proteins were purified by poly-(L-proline) affinity chromatography as described previously (Giehl et al., 1994). Eluted fractions were pooled and dialyzed against 10 mM Tris/HCl pH 7.6, 2 mM $CaCl_2$, 1.25 mM dithiothreitol (DTT). The purity of the protein preparations was judged by SDS-PAGE. Purified recombinant mouse profilins I and IIa, and BSA (control) were coupled to NHS-Sepharose 4B Fast Flow (Amersham-Pharmacia, Freiburg, Germany) with an efficiency of 10-15 μg bound protein per μl NHS-material. Free NHS-groups were blocked with ethanolamine. Aliquots of 25 μl in vitro transcription/translation reaction mixture were diluted in PBS and incubated with 10 μl conjugated sepharose for 4 hours at 4°C. After sedimentation, the beads were successively washed with PBS, PBS with 140 mM NaCl and PBS with 0.2% Triton X-100. The denatured proteins of the first supernatant and of the washed pellets were separated by SDS-PAGE and the gel was blotted onto nitrocellulose membrane. The [^{35}S]-labeled proteins were detected by autoradiography.

RESULTS

The molecular anatomy of mDia1 and the deletion fragments used in this study

Fig. 1 shows schematically the mDia1 sequence and the position and extension of the various functional domains, as revealed by sequence comparison among various diaphanous proteins from yeast to man. FH1 and FH2 modules were identified in the entire formin family (Castrillon and Wassermann, 1994; Wasserman, 1998), while the members of the diaphanous subfamily share the RBD, FH3, the first and second CCDs, and the C-terminal CIID (Petersen et al., 1998; Watanabe et al., 1999; Watanabe et al., 1997). The CIID comprises the diaphanous autoregulatory motif DAD, which in mDia2 has been shown to mediate the interaction with the RBD (Alberts, 2001). The various deletion constructs designed from the mDia1 sequence and used in this study are also depicted. For clarity, the colour code used in this diagram will

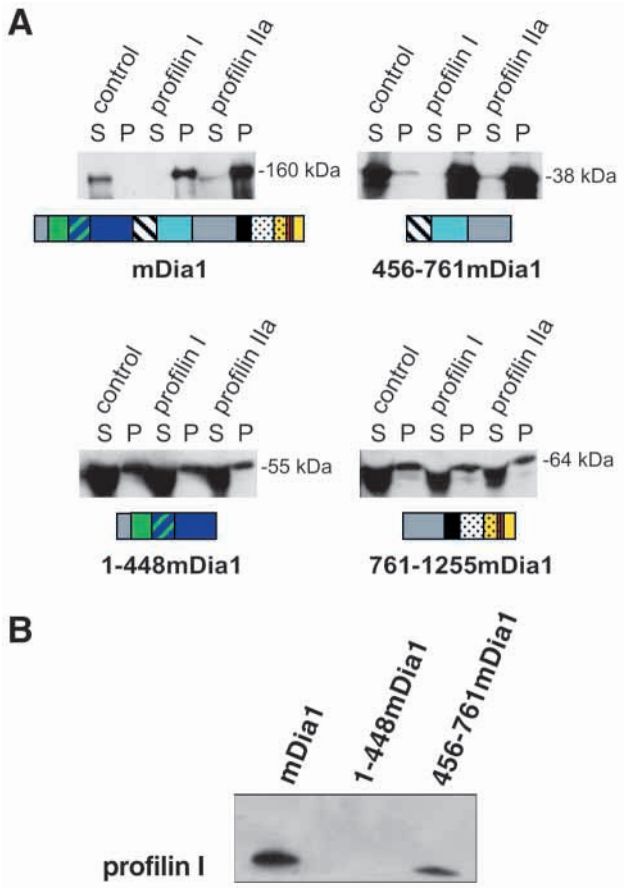

Fig. 2. Interaction of the mDia1 FH1 domain with profilins. (A) Autoradiograms of supernatants and pellets obtained after affinity precipitation of in vitro translated [^{35}S]-labeled mDia1 protein and its fragments with mouse profilins I and IIa. Profilins were coupled to NHS-HiTrap material, incubated with in vitro translated [^{35}S]-methione-labelled mDia1 polypeptides and subjected to centrifugation. Aliquots of supernatants (S) and pellets (P) were separated by SDS-PAGE, blotted and subjected to autoradiography. Control: BSA coupled to NHS-HiTrap material. Coprecipitation of the in vitro translated products depends on the presence of the FH1 domain, as in wt mDia1 and 456-761mDia1. (B) Immunoblots obtained by SDS-PAGE from immunoprecipitates of profilin I from extracts of HeLa cells transfected with BiPro-tagged mDia1 and two of its deletion fragments, containing (456-761mDia1) or lacking (1-448mDia1) the FH1 domain. HeLa cells were treated with the membrane permeant crosslinker DSP before lysis, mDia1 proteins were precipitated from the lysates with an antibody against the BiPro tag (4A6), centrifuged and subjected to SDS-PAGE and subsequent blotting. The presence of profilin I in the pellets was monitored with a monoclonal profilin antibody (2H11), which recognizes human profilin I. Note that profilin I only coprecipitates in the presence of the FH1.

be used throughout this study, although, for space reasons, the relative dimensions of the individual domains are not always taken into account.

The FH1 domain of mDia1 binds to profilins in vitro and in cells

Previously, in vitro binding assays showed that mDia1 is a ligand for the actin-modulating protein profilin (Watanabe et al., 1997). We investigated whether this interaction is mediated

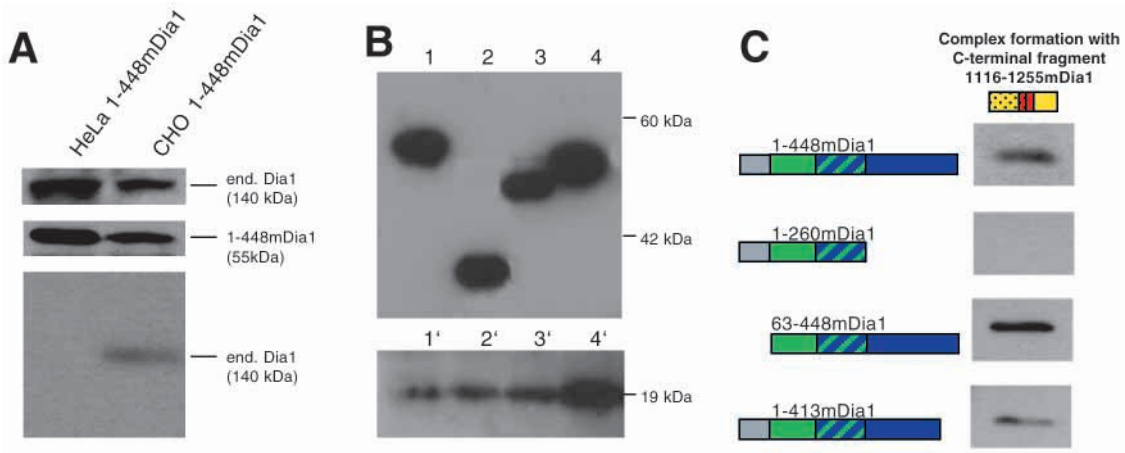

Fig. 3. Intramolecular interactions of the mDia1 domains. (A) Immunoblots obtained from BiPro-1-448mDia1-transfected HeLa and CHO cells. After transfection, cells were treated with the membrane permeant crosslinker DSP before lysis, centrifuged and subjected to SDS-PAGE and subsequent immunoblotting. The top panel shows the endogenous Dia1 (end. Dia1) in both cell lines, as detected with a monoclonal antibody against mDia1. The center panel shows the presence of the transfected fragment in both cell lines, as detected with the BiPro-tag antibody. The bottom panel shows an immunoblot analysis with the monoclonal mDia1 antibody of the precipitates obtained with the BiPro-tag. Note that the endogenous Dia1 protein is only precipitated from CHO cells, indicating species-restriction of recognition between the RBD and mDia1. (B) Immunoblots obtained from HeLa cells cotransfected with BiPro-tagged 1116-1255mDia1 and either the flag-tagged fragments 1-448mDia1 (lanes 1,1') 1-260 mDia1 (lanes 2,2'), 63-448mDia1 (lanes 3,3′) or 1-413mDia1 (lanes 4,4'). The top panel shows the expression of the N-terminal fragments, as obtained with the flag antibody, (lanes 1-4), the bottom panels show the expression of the C-terminal 1116-1255 fragment, as seen with the BiPro antibody. Note that all these fragments were expressed in the relevant cells. (C) Immunoblots obtained from immunoprecipitates of HeLa cells transfected as in (B). Cells were treated with the membrane permeant crosslinker DSP before lysis. Proteins were immunoprecipitated with the flag antibody, centrifuged and subjected to SDS-PAGE and blotting. 1116-1255mDia1 was monitored with the BiPro antibody. Note that coprecipitation of the CIID with the mDia1 fragments requires the presence of the RBD plus the FH3.

by the FH1 domain and asked whether both murine profilin isoforms, I and II (specifically IIa), can complex with the FH1. [^{35}S]-labelled proteins, as obtained in an in vitro transcription/translation assay from full length (wt) mDia1 and deletion constructs with (456-761mDia1) or without (1-448mDia1 and 761-1255mDia1) the FH1 domain were incubated with recombinant mouse profilin I and IIa coupled to sepharose beads. Fig. 2A shows the autoradiograms obtained after sedimentation of the sepharose beads and SDS-PAGE of pellets and supernatants. A minor proportion of the protein fragments appeared in the sediment even in the controls (which contained BSA- instead of profilin-coupled beads), which may be due to misfolding or aggregation of the in vitro translated products. However, substantial cosedimentation was only seen in cases that involved the FH1 domain (Fig. 2A). No difference was seen in this assay with respect to the profilin isoform used.

To corroborate the results obtained by affinity precipitation and to analyse the ability of these proteins to form complexes under in vivo conditions, HeLa cells were transiently transfected with cDNAs coding for either wt-mDia1, 1-448mDia1 or 456-761mDia1, equipped with an N-terminal epitope from birch profilin, specifically and selectively recognized by a corresponding monoclonal antibody (BiPro-tag; Rüdiger et al., 1997). This antibody (4A6) does not recognize mammalian profilins (Wiedemann et al., 1996). In order to stabilize protein complexes within the living cells, they were treated with the membrane-permeant crosslinker DSP before lysis (Hüttelmaier et al., 1998; Weiss et al., 1998). Immunoprecipitations were performed with these lysates with the 4A6 antibody and protein G sepharose and the precipitates were sedimented and processed for SDS-PAGE and immunoblotting with mammalian profilin-specific antibodies. As shown in Fig. 2B, endogenous profilin I, which is the isoform expressed in HeLa cells, coprecipitated with either the full-length mDia1 or the FH1-comprising 456-761mDia1, but not with the mDia1 fragment lacking the FH1 domain but containing the RBD and most of the FH3 module (1-448mDia1). These results confirm the in vitro data and demonstrate that mDia1 and endogenous profilin interact in living cells.

The RBD-CIID interaction is species-restricted and requires the FH3 fragment

Exogenous RBD, when transfected into cells, has been shown to bind to endogenous mDia1 and mDia2, thus activating them (Alberts, 2001; Watanabe et al., 1999). We analyzed the requirements for this interaction in two different cell lines. Human (HeLa) and hamster (CHO) cells were transiently transfected with the RBD-containing 1-448mDia1 fragment, fused to the BiPro tag. Immunoblots revealed that in both cell lines the endogenous Dia1 protein was detectable with an antibody against mammalian Dia1 (Fig. 3A, top panel) and expressed the exogenous construct, as revealed by the tag-specific antibody (Fig. 3A, center panel). Cells were then treated with DSP, and lysates were subjected to immunoprecipitation with the 4A6 antibody and SDS-PAGE. Blotting with the mDia1-specific antibody revealed that the endogenous Dia1 of

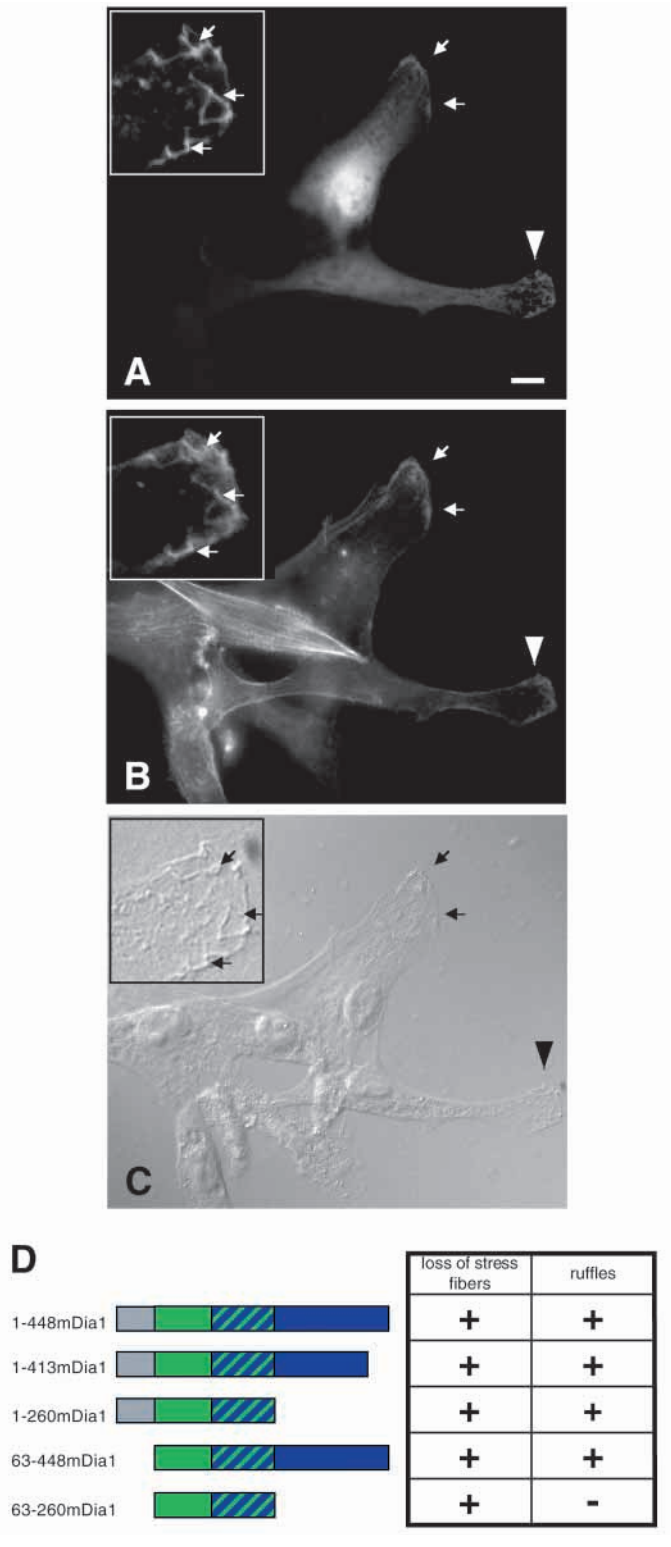

Fig. 4. Actin reorganization and altered morphology of NIH 3T3 cells overexpressing the RBD. (A-C) Cells transfected with EGFP-63-448mDia1. (A,B) Double fluorescence analysis showing EGFP fluorescence (A) and F-actin (B). (C) Nomarski optics. The mDia1-fragment-expressing cells are flatter, lack stress fibers and display prominent ruffles (arrowheads). Bar: 10 μm. Insets are enlargements of the area marked by the arrowhead. (D) Catalogue of the fragments (expressed as fusion proteins with EGFP) tested with respect to loss of stress fibers and ruffling activity. Note that the exogenous, isolated RBD induces loss of stress fibers, while ruffling also requires the presence of either the N-terminal extension or the complete FH3 domain.

the rodent (CHO) line coimmunoprecipitated with the exogenous RBD-containing fragment (Fig. 3A, bottom panel). Precipitation of CHO-Dia1 with this method was never quantitative, but consistently seen in several independent experiments, indicating that murine RBD-containing fragment can release the intramolecular bonding in hamster endogenous Dia1 and complex with its C-terminal part. However, this was strictly confined to rodent cells: the murine mDia1 fragment consistently failed to coprecipitate with hDia1 from HeLa cells (Fig. 3A, bottom panel).

This result enabled us to test complex formation of the relevant mDia1 fragments in a cellular environment without interference of endogenous Dia1 (Fig. 3B,C). HeLa cells were cotransfected with several flag-tagged N-terminal constructs and BiPro-tagged CIID. Expression of all proteins was verified in immunoblots, using the corresponding tag antibodies (Fig. 3B). Subsequently, transfected cells were treated with DSP to stabilize putative complexes in situ, and then lysed and subjected to immunoprecipitation with the flag antibody. After SDS-PAGE, the precipitates were analyzed for the presence of CIID in immunoblots, using the BiPro antibody. As shown in Fig. 3C, RBD-containing mDia1 fragments interacted and coprecipitated with the CIID. However, the isolated full length RBD (comprising aa residues 63-260), even when equipped with the N-terminal extension (residues 1-62), was unable to form stable complexes with the CIID. By contrast, all fragments containing the RBD plus the major part of the FH3 domain (up to residue 413) successfully bound CIID. These results show that the RBD alone is insufficient for this interaction and that the FH3 domain participates in RBD-CIID binding.

The RBD causes loss of stress fibers and induces ruffling

The effect of overexpressing RBD on the actin organization was tested in murine cells, to enable cooperation between the exogenous fragment with endogenous mDia1. NIH 3T3 cells were transiently transfected with various EGFP-equipped N-terminal deletion fragments of mDia1, and the localization of these fragments and the organization of the actin cytoskeleton were monitored by double fluorescence analysis after staining with TRITC-phalloidin. Fig. 4 shows that the fragment comprising the RBD and almost the entire FH3 domain (63-448mDia1) causes flattening of the expressing cells and loss of stress fibers. The fragment itself was found to colocalize with F-actin in prominent membrane ruffles (Fig. 4A-C). Ruffling activity was high in the transfected cells, but very low in nontransfected control cells in the same culture. By using different deletion fragments, we found that loss of stress fibers and ruffling were both dependent on the presence of RBD, but ruffling required additional RBD-flanking residues: either the N-terminal extension or the major portion of the FH3 (Fig. 4D). Identical results were obtained with either BiPro- or flag-tagged constructs and corresponding immunofluorescence to detect transfected cells. In all cases where ruffling was induced, the exogenous protein colocalized with F-actin within the ruffles.

Stable CHO clones expressing the RBD of mDia1 ruffle spontaneously and show disoriented motility

To study the effect of the RBD on the reorganization of actin and motility in a more quantitative manner, we constructed

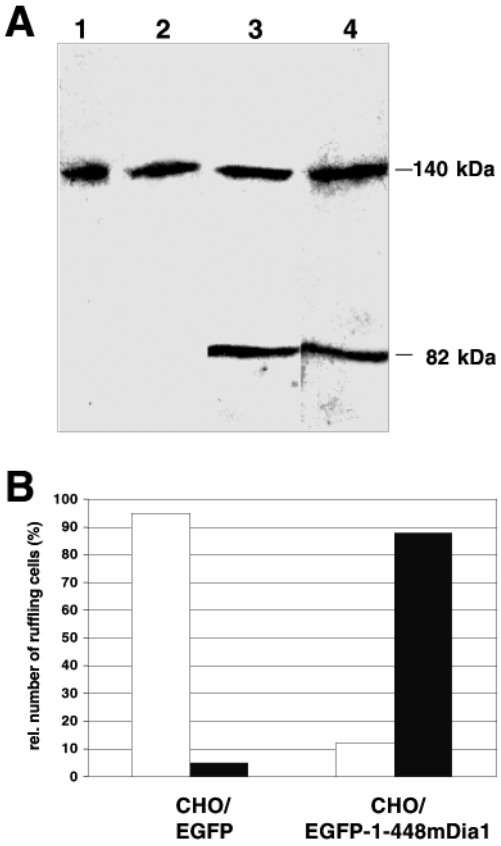

Fig. 5. Spontaneous ruffling activity of stable CHO clones expressing EGFP-1-448 mDia1. (A) Immunoblots of control CHO cells (lane 1), CHO transfected with the EGFP-containing vector (lane 2), and two clones (termed 1A and 1B) stably transfected with EGFP-1-448mDia1 (lanes 3 and 4), as obtained with the polyclonal mDia1 antibody. Note that these clones express the fragment in amounts approximately equal to the endogenous mDia1. (B) Proportion of spontaneously ruffling cells in the mock-transfected (CHO/EGFP) and clone 1A cells. Cells were fixed and examined by Nomarski optics and for EGFP-fluorescence, and classified according to the presence of ruffles (black bars) or no ruffles (white bars). Approximately 300 cells were counted for each class, in each of 4 independent experiments.

stable CHO cell clones, expressing either EGFP-1-448mDia1 or EGFP (controls). Cell clones were selected in geneticin-selective medium. By quantitating the expression of EGFP-1-448mDia1 in immunoblots with EGFP- and mammalian Dia1-specific antibodies, we selected for three lines that expressed equal amounts of the exogenous fragment, approximately to the same level as the endogenous Dia1. These cells were viable and proliferated like the wild type or mock-transfected CHO. When examined by light microscopy, it was seen that virtually all cells expressing the RBD-containing fragment showed spontaneous and continuous ruffling, while very few of the control cells ruffled. Fig. 5 shows the expression of endogenous Dia1 and the transfected EGFP-1-448 mDia1 fragment as seen with an mDia1 antibody for clones 1A and 1B (Fig. 5A) and the ruffling activity of clone 1A (Fig. 5B). Analogous to the findings with transiently transfected 3T3 cells, the EGFP-1-448mDia1-expressing CHO cells appeared flatter and showed much fewer stress fibers compared with

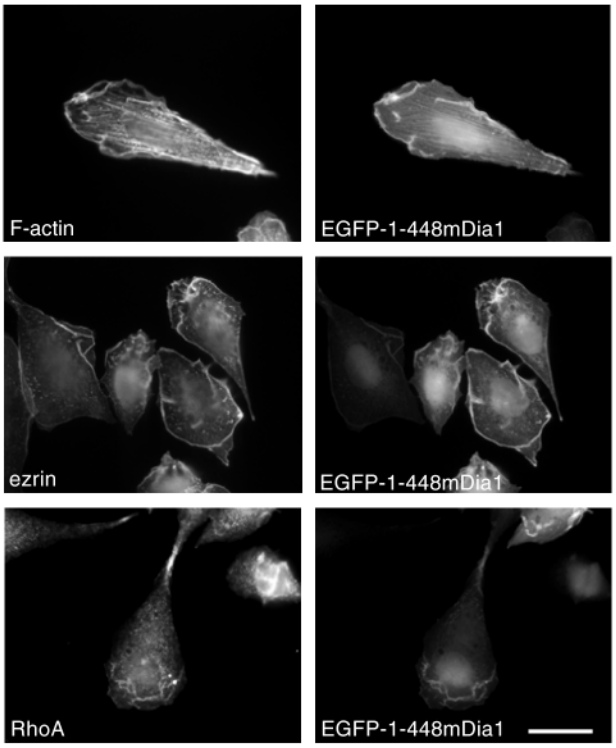

Fig. 6. Double fluorescence analysis of cells stably expressing EGFP-1-448mDia1. For each pair of images, the EGFP fluorescence is shown on the right, TRITC-phalloidin staining (indicative of F-actin) and immunostaining for ezrin and RhoA, respectively, are shown on the left. Note that these ruffles contain the cytoskeletal proteins actin and ezrin, which colocalize with the RBD (as contained in the 1-448mDia fragment) and its ligand RhoA. Bar, 20 μm.

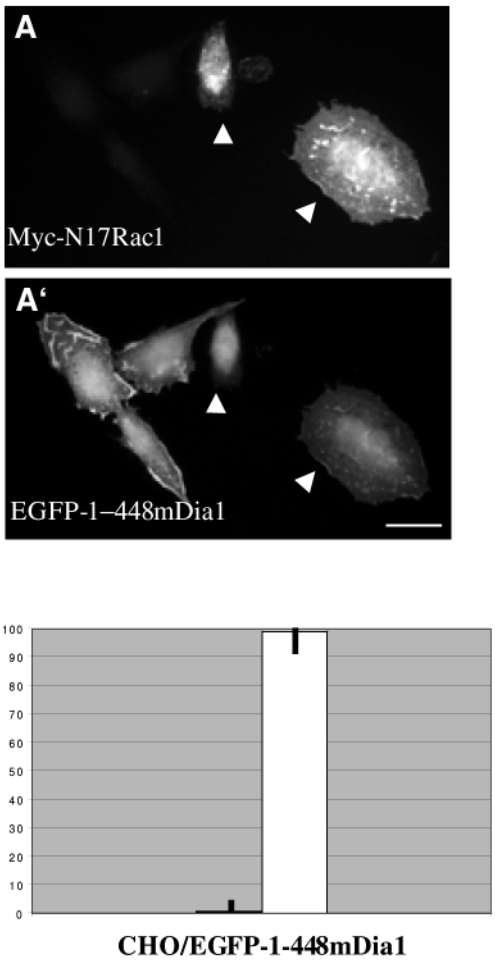

Fig. 7. Supertransfection of 1-448-mDia-expressing cells with dominant negative myc-tagged Rac1 (N17Rac1). (A,A') Double fluorescence analysis with antibody against the myc-tag (A) and EGFP fluorescence (A'), to detect the N17Rac1-expressing cells among the 1-448mDia-expressing clone 1A cells (arrowheads). Bar, 20 μm. (B) Quantitation of ruffling activity of clone 1A cells coexpressing N17Rac1, identified from images as shown in (A). Note that N17Rac1 is effectively suppressing ruffling. At least 300 cells were counted, in four independent experiments.

mock-transfected controls. In most cells, ruffles were not confined to a bona fide cell front but developed all around the cell periphery. Double fluorescence analysis of the ruffling cells, as shown in Fig. 6, revealed that within the ruffles, filamentous actin colocalized with ezrin, an established ruffle marker (Bretscher, 1991) and also with EGFP-1-448mDia1 and RhoA. Hence, both the expressed exogenous RBD and its ligand, the Rho GTPase, were identified as components of these ruffles.

Since ruffling is considered to require the activity of Rac, we tested the contribution of Rac in ruffle formation in these stable lines. Cells were supertransfected with cDNA coding for a dominant-negative Rac and the double transfectants were identified and examined by fluorescence microscopy. Fig. 7 shows that coexpression of myc-N17Rac1 and EGFP-1-448-mDia1 resulted in a drastic reduction of ruffling activity: less than 10% of these supertransfected cells showed ruffles (Fig. 7A,A',B). Hence, the extensive ruffling of RBD-overexpressing cell lines is Rac-dependent.

Extensive ruffle formation and loss of a defined cell polarity, as described here, might be expected to have consequences on the motile behaviour of the afflicted cells. In particular, such cells should be handicapped in directed locomotion. This hypothesis was tested in a wounding assay. A scratch wound was scraped into confluent cell layers, and the invasion of this cell free area was monitored by phase-contrast microscopy. As shown in Fig. 8, clonal CHO cells expressing EGFP-1-448mDia1 were much slower in wound closure in comparison to cells expressing the EGFP control vector alone. Obviously, they were markedly delayed in directed migration. The difference was slightly more pronounced when cell proliferation was blocked by the addition of colcemid, but was also clearly seen in the absence of the drug (compare Fig. 8A with B).

DISCUSSION

In this study, we present a functional analysis of several domains of mDia1, a protein linking Rho signaling with actin organization. We demonstrate that complex formation between this mammalian protein with the actin-binding protein profilin is indeed mediated by the proline-rich FH1 domain, in analogy to data described for the budding yeast diaphanous protein

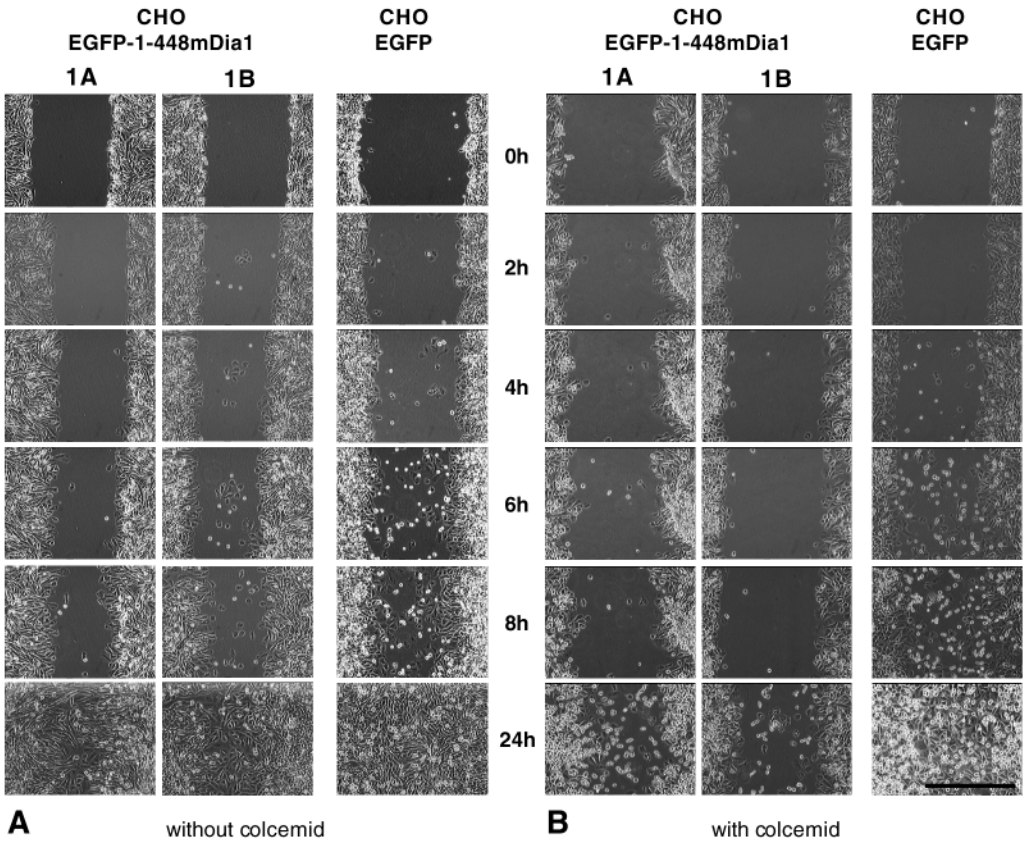

Fig. 8. Directed locomotion of CHO clones expressing EGFP-1-448mDia1 (clones, 1A and 1B) or EGFP only (control). Directed locomotory activity was recorded under phase-contrast as the velocity to close a wound set in the confluent culture, at the times indicated. Cells were kept and monitored either without (A) or with (B) colcemid in the medium. Note that wound closure is considerably faster in the control culture than in both RBD-expressing clones. Colcemid enhances this effect, possibly by inhibiting cell proliferation. Bar, 1 mm.

(Imamura et al., 1997), and proving previous predictions derived from experiments with full length recombinant mDia1 (Watanabe et al., 1997). The FH1, equipped N-terminally with the first CCD, is both essential and sufficient in binding profilin in vitro and in cells. We found that mDia1 reacts with both profilin isoforms I and IIa, but the nature of our experiments does not preclude a possible differential affinity of mDia1 for either isoform. Mammalian profilin IIa, the predominant neuronal isoform, has a higher affinity for proline-rich sequences than profilin I (Lambrechts et al., 1997; Wittenmayer et al., 2000). Hence, isoform preference leading to possible tissue- and cell-specific functions of these interactions might be envisioned, as has been implicated for other polyproline-ligands of profilins (Lambrechts et al., 1997).

Our results imply that the hereditary progressive hearing loss, described for a family expressing a C-terminally truncated form of hDia1 is probably not due to lack of interaction between this gene product and profilin in the hair cells of the inner ear (Lynch et al., 1997). This fragment still contains the FH1 domain, and our data showing in vitro complex formation between several deletion fragments of mDia1 and profilins suggest that FH1 is apparently properly folded even in very short fragments. The FH1 module is multifunctional, as it not only binds profilins but also several SH3 proteins involved in growth regulation (Tominaga et al., 2000). This may explain the fact that we could not obtain viable cells overexpressing this domain (data not shown), but renders any analysis on the biological function of the interaction between mDia1 and profilins difficult. Binding of profilins has been described for a variety of poly-proline-cluster proteins, ranging from microfilament-associated components (Reinhard et al., 1992) to proteins involved in RNA-processing (Giesemann et al., 1999) and mRNA-localization (Zhao et al., 2001). At present, the cellular significance of these interactions are far from understood but are thought to provide the cell with profilin-actin complexes in regions where rapid actin polymerization is required or at least advantageous. This view is supported by findings that profilins stimulate VASP-associated actin-based motility of the pathogen *Listeria monocytogenes* (Geese et al., 2000; Laurent et al., 1999), contribute to the dynamics of cortical actin filaments as executed by the profilin-binding, proline-cluster proteins WASP and WAVE (Takenawa and Miki, 2001) and optimize actin-dependent transcription of viral RNA (Burke et al., 2000). The importance of the poly-proline-binding site on profilin for cell viability has been unambiguously demonstrated in yeast (Lu and Pollard, 2001).

In contrast to the situation with the FH1 domain, overexpression of the Rho-binding domain was tolerated by a number of cell lines, including HeLa, CHO and 3T3 cells, and we show that the RBD can interact with the endogenous mDia1 protein in transfected cells. An intact RBD is essential for this interaction, as an N-terminally truncated RBD fragment was found ineffective (data not shown). Remarkably, we found that this interaction is species-restricted (i.e. complex formation could only be observed within rodents). This might be explained by sequence variance within the RBD-binding CIID. For example, while the overall sequences between hDia1 and mDia1 are highly homologous, the C-terminal part of the CIID of both proteins displays considerable differences: the aa residues 1228-1240 read **PG**P**V**KV**P**K**K**SE**GV** in mDia1

versus **AV**P**A**KV**S**K**N**SE**TF** in hDia1 (sequences for both proteins according to EMBL accession numbers U96963 and AF051782, respectively). On the other hand, the motif aa 1177-1207, N-terminally located, but still contained within the CIID and termed diaphanous autoregulatory domain, DAD, (Alberts, 2001) (Fig. 1), was found to interact with the RBD in vitro and in microinjection experiments (Alberts, 2001). This sequence is identical between mDia1 and hDia1. This apparent discrepancy might be due to the fact that in the study cited, mDia2 was examined, not mDia1. Taken together, these data and ours suggest that the DAD, a highly conserved motif, is essential for molecular recognition of the RBD, but may not be sufficient, depending on the Dia isoform or the relevant species.

Several groups have analyzed the effect of overexpressing or injecting isolated mDia domains on actin organization. An increase in RBD or RBD-containing fragments resulted in loss of stress fibers in HeLa cells (Watanabe et al., 1999), consistent with our data reported here for 3T3 and CHO lines. By contrast, an increase in DAD resulted in an increase of F-actin and stress fibers in NIH 3T3 (Alberts, 2001), and overexpression of various mDia1 fragments lacking the RBD induce stress fibers in HeLa cells (Watanabe et al., 1999) and cause a parallel arrangement of stress fibers in MDCK and HeLa cells (Ishizaki et al., 2000; Takaishi et al., 2000). Hence, it seems that unbalancing the level of the different Dia domains may shift the actin suprastructural organization to different sides. However, so far, nobody has reported the prominent ruffling activity that we see upon overexpressing the RBD in both NIH 3T3 and CHO cells. By examining three different, independently created stable clones, we found that this ruffling is indeed exhibited continuously by all cells. The ruffles are defined by their content in F-actin and ezrin, and they also contain the transfected RBD and RhoA. A colocalization of full-length mammalian Dia1, profilin and Rho has previously been described in ruffles of MDCK cells, induced by the application of 12-O-tetradecanoylphorbol-13-acetate (TPA) (Watanabe et al., 1997), but the cells described here ruffle spontaneously, without addition of external factors. An intact RBD is required for this phenomenon, as transfection with an N-terminally truncated RBD did not cause ruffling (data not shown). The ruffling cells, most of which were flatter and without a distinct morphological polarity, behaved disoriented and were handicapped in directed locomotion. Loss of stress fibers and ruffling activity were correlated in most cases, but a fragment comprising the RBD but lacking the C-terminal portion of the FH3 induced only the former. This suggests that the N-terminal part of the FH3 may positively influence ruffle formation, either by cooperating with or at least by stabilizing the RBD. By contrast, the extreme C-terminal part of the FH3 domain, in particular two leucine residues at positions 434 and 455, seem essential for targeting mDia1 to the mitotic spindle (Kato et al., 2001).

Ruffling activity is generally ascribed to the activity of Rac (Nobes and Hall, 1995; Ridley et al., 1992), and this was also confirmed for the stable clones described here: supertransfecting the stably RBD-expressing CHO cells with N17Rac1, a dominant negative Rac mutant that interferes with growth factor-dependent ruffling activity (Ridley et al., 1992), abolished ruffling completely. Consistent with this assumption, we observed that microinjection of the ADP-ribosyltransferase C3, which is a potent inhibitor of Rho (Aktories and Hall, 1989), induces ruffling in CHO control cells (data not shown). Hence, our data suggest that by overexpressing RBD, endogenous activated Rho is effectively downregulated in its activity, and, as a consequence, Rac activity is upregulated. Such feedback between Rho and Rac activity has been repeatedly reported, and while in most cases Rac was seen to regulate Rho (Zigmond, 1996), there are some recent observations that also postulate the reverse (Cox et al., 2001), corroborating our concept. Future research will have to clarify on a molecular basis the crosstalk between these two GTPases and the precise role of mDia1 in actin organization.

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