Phospholipids regulate localization and activity of mDia1 formin

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**Abstract**

Diaphanous-related formins (DRFs) are large multi-domain proteins that nucleate and assemble linear actin filaments. Binding of active Rho family proteins to the GTPase-binding domain (GBD) triggers localization at the membrane and the activation of most formins if not all. In recent years GTPase regulation of formins has been extensively studied, but other molecular mechanisms that determine subcellular distribution or regulate formin activity have remained poorly understood. Here, we provide evidence that the activity and localization of mouse formin mDia1 can be regulated through interactions with phospholipids. The phospholipid-binding sites of mDia1 are clusters of positively charged residues in the N-terminal basic domain (BD) and at the C-terminal region. Upon binding to the lipid bilayer the N-terminal region of mDia1 induces strong clustering of phosphatidylinositol-4,5-bisphosphate (PIP\textsubscript{2}) and subsequently inserts into the membrane bilayer thus anchoring mDia1 to the reconstituted plasma membrane. In addition, an interaction of phospholipids with the C-terminal region of mDia1 causes a drastic reduction of its actin filament assembly activity. Our data suggest that the N-terminal phospholipid-binding sites help to anchor formins at the plasma membrane, and the interaction with phospholipids in the C-terminal functions as a switch for transient inactivation.

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**Introduction**

Formins are ubiquitous and highly conserved multi-domain proteins that nucleate and elongate linear actin filaments by insertional incorporation of monomers to the filament barbed ends (Fai	extsc{x} and Grosse, 2006; Kovar and Pollard, 2004; Pollard, 2007). The proline-rich formin homology domain 1 (FH1) recruits profilin–actin complexes for filament elongation (Kovar et al., 2006; Paul and Pollard, 2008; Romero et al., 2004) which is accomplished by the adjacent FH2 domain (Higashida et al., 2004; Shimada et al., 2004; Xu et al., 2004). Members of the family of Diaphanous-related formins (DRF) fold on themselves and are thus intrinsically inactive by virtue of additional regulatory sequences located in the N- and C-terminal regions of these proteins (Alberts, 2001; Li and Higgs, 2005; Wallar et al., 2006). Binding of activated small Rho family GTPases such as RhoA to the GTPase-binding domain (GBD) releases this intra-molecular inhibition by disrupting the interaction between the C-terminal Diaphanous-auto-regulatory domain (DAD) and the N-terminal Diaphanous-inhibitory domain (DID) (Brandt et al., 2007; Nezami et al., 2006; Otomo et al., 2005; Rose et al., 2005; Wallar and Alberts, 2003). The dimerization domain (DD) is sufficient to dimerize the N-terminal region even without the adjacent coiled-coil (CC) region, while a short linker within the FH2 domain facilitates the dimerization of the C-terminus (Otomo et al., 2005; Xu et al., 2004). Although the auto-inhibition and the GTPase signaling in mammalian DRF’s regulation are well understood, other mechanisms that control e.g. their localization are largely unknown. Formins are often enriched at the plasma membrane (Seth et al., 2006) or in filopodial tips (Block et al., 2008; Schirenbeck et al., 2005). The molecular basis of this distribution is still not entirely understood and the interaction with a membrane-associated GTPase is apparently not the only mechanism (Copeland et al., 2007; Seth et al., 2006; Zaoui et al., 2008). IQGAP1 and CLIP170 have been described to recruit mDia1 to the phagocytic cup (Brandt et al., 2007; Lewkowicz et al., 2008), and FMNL1 inserts into membranes after being myristoylated at the N-terminus (Han et al., 2009). Additional types of formin regulation have been reported for yeast formins. Budding yeast Bud6 interacts directly with the DAD of Bni1 and stimulates its activity, whereas Bud14 inhibits the activity of...
the formin Bnr1 by displacing it from the growing filament barbed end (Chesarone et al., 2009; Moseley et al., 2004). Furthermore, the N-terminal region and the FH1FH2 domain of Cdc12p are obviously important for its localization to the contractile ring (Yonetani et al., 2008).

Here we report that the mouse DRF mDia1 can be anchored to the plasma membrane through an interaction of its N-terminal basic domain (BD) with phospholipids. Furthermore, the C-terminal region of mDia1 also binds PI2, and this interaction inhibits mDia1-induced actin filament assembly. Thus our observations suggest that the activity and localization of mDia1 are two distinct phenomena.

Materials and methods

Cell culture and transfection

NIH 3T3 fibroblasts were maintained in DMEM with 10% FBS and 2 mM glutamine. Cells were transfected with 2 μg plasmid DNA using LipofectAMINE 2000 (Invitrogen). Microscopy was performed essentially as described (Schirenbeck et al., 2005). Briefly, 10 h after transfection live cells expressing GFP-fusion proteins were imaged in phosphate buffer using a LSM 510 Meta (Zeiss, Germany) at 30 °C.

Plasmids

For cloning and expression of EGFP-mDia1, the entire gene and truncated fragments (ΔDAD-amino acids #1–1179 and ΔBDΔDAD #61–1179) were PCR amplified from mouse cDNA and inserted into the BglII/Sall sites of pEGFP-C1 (Clontech). For the expression of mDia1, mDia2, mDia3 and the RhoA(V14) constructs in NIH 3T3 fibroblasts were maintained in DMEM with 10% FBS (Alabaster, AL). Lipids in desired concentrations were mixed, dried under a stream of nitrogen and hydrated in 20 mM Hepes, pH 7.5, 100 mM NaCl to yield multilamellar vesicles in a lipid concentration of 1 mM. To obtain unilamellar vesicles, vesicles were extruded through a polycarbonate filter (100 nm pore size) using a mini-extruder (Avanti Polar Lipids). One should take into account that experiments with lipid vesicles cannot directly reflect the situation at a biological membrane. In a large PC vesicle dotted with many PI2 molecules the geometry of the vesicle will allow only a few acidic lipid molecules to interact with a target protein. Consequently, the given PI2 concentration for the preparation of LUVs is only a very crude approximation to physiological conditions and requires very detailed titration experiments for exact binding characteristics. The inner leaflet of the plasma membrane contains in a normal cell (of all phospholipids) only 0.5–1% PI2 but 25–35% of PS (Lemmon, 2008; McLaughlin and Murray, 2005). Therefore, the lipid-binding data in this study focus more on qualitative than quantitative analyses.

Fluorescence spectroscopy experiments

Phospholipid clustering and membrane insertion experiments were performed essentially as described (Saarikangas et al., 2009). Briefly, fluorescence spectra and DPH anisotropy were measured with a PerkinElmer LS 55 spectrometer with both emission and excitation band passes set at 10 nm. Spectra were corrected for the contribution of light scattering in the presence of vesicles. NBD-PS fluorescence was excited at 470 nm and the emission spectra were recorded from 490 nm to 560 nm with band passes set at 5 and 10 nm, respectively. Bodipy-TMR-PI2 fluorescence was excited at 547 nm and the emission spectra were recorded from 555 to 600 nm in the presence of different concentrations of proteins. The percentage of quenching was calculated using the following equation:

\[ \text{%quenching} = \left(1 - \frac{F}{F_0}\right) \times 100, \]

where F is the fluorescence intensity in the presence of protein or liposomes, and \( F_0 \) is the fluorescence intensity in the absence of protein or liposomes. Fluorescence anisotropy of DPH was measured by including DPH into liposomes at X = 0.002. Fluorescence anisotropy for DPH was measured with excitation at 360 nm and emission at 450 nm, using 10 nm bandwidths. The lipid concentration used was 40 μM for DPH anisotropy experiments. NBD-PS, and bodipy-TMR-PI2 fluorescence measurements.

In vitro actin polymerization assays

Actin from skeletal muscle was purified as described (Spudich and Watt, 1971). Actin polymerization was measured by fluorescence spectroscopy with pyrene-labeled actin (Schirenbeck et al., 2006, 2005) and performed in a buffer containing 10 mM imidazole, 2 mM MgCl2, 0.2 mM CaCl2, 1 mM Na2ATP, and 50 mM KCl (pH 7.2).

TIRF assays

Time-lapse evanescent wave fluorescence microscopy was performed as described (Breitsprecher et al., 2008). Briefly, the assembly of 1 μM ATP-actin and 0.3 μM Alexa-Fluor-488-labelled ATP-actin in TIRF buffer (10 mM imidazole (pH 7.4), 50 mM KCl, 1 mM MgCl2, 1 mM EGTA, 0.2 mM ATP, 50 mM DTT, 15 mM glucose,
20 μg/ml catalase, 100 μg/ml glucose oxidase and 0.5% methylcellulose) on cover slips coated with 10 mM N-ethylmaleimide (NEM) myosin II and formins (in the presence or absence of liposomes). Images from an Olympus IX-81 inverted microscope were captured every 10 or 15 s with exposures of 200 ms or 500 ms with a Hamamatsu ER C8484 CCD camera (Hamamatsu Corp., Bridgewater, NJ).

**Cosedimentation assay**

Small unilamellar liposomes were made and concentrations calculated as described (Prehoda et al., 2000) with minor modifications. Briefly, l-α-phosphatidylcholine (PC), l-α-phosphatidyleserine (PS) and PIP2 were dissolved separately in chloroform/methanol/water (20:9:1) and dried under nitrogen. The lipids were then resuspended in 20 mM HEPES pH 7.3 and 150 mM NaCl followed by sonication until the solution became clear. At indicated ratios the lipids were mixed and sonicated again just prior to usage. All lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA).

Cosedimentation assays were performed by mixing ~3 μM protein and ~50 μM phospholipid vesicles at indicated ratios in 20 mM HEPES pH 7.3, 150 mM NaCl, 2 mM EGTA (total assay volume 50 μl), incubation at 25 °C for 60 min, and centrifugation at 10,000 × g for 30 min at room temperature. The pellets were washed once with 100 μl of the reaction buffer. Subsequently, the volumes of the pellets and supernatant fractions were normalized and analyzed by SDS-PAGE and Coomassie blue staining (Eichinger and Schleicher, 1992).

**Results**

**The N-terminal basic region of mDia1 interacts directly with liposomes**

The first 60 residues of mDia1 referred to as the basic domain (BD) harbor three clusters of positively charged residues encompassed by the amino acids #12–21, #35–42, and #47–54 (Fig. 1A). A similar basic region is also present in mDia2 but not in mDia3. DAAM proteins contain only a single poly-basic cluster close to the N-terminus (Fig. 1B). To test whether the basic domain of mDia1 can interact with the negatively charged phospholipids such as PS and PIP2, several truncation and deletion constructs were expressed as GST-fusion proteins and tested for their ability to cosediment with lipid vesicles containing PC (phosphatidylcholine), PS (phosphatidyleserine) and PIP2. After cosedimentation, proteins from pellets (P) and supernatants (S) were analyzed by SDS-PAGE. The results revealed that the BD of mDia1 but not the other regions in the N-terminal region interacts with lipid vesicles containing both PS and PIP2 (Fig. 2A). Vesicles containing only PC showed no interaction with the BD, implying the requirement of negative charges for the binding of mDia1 with membranes (not shown).

To map the lipid-binding region within the BD (amino acids #1–46), the cosedimentation assays were repeated in the presence of the two mDia1-derived synthetic peptides A and B spanning either the first or the second basic amino acid clusters (Fig. 1A). Both peptides (amino acids #12–21 and #35–42) could compete with N46 for liposomes containing PS and PIP2. In a competition assay with the synthetic peptides the inhibitory effect of the second basic stretch turned out to be significantly higher than that of the first poly-basic cluster (Fig. 2B). The construct (Δ12-42N570) that contained only the third poly-basic cluster did not interact with phospholipids (Fig. 2A). Presumably due to the presence of glutamic acid residues within the third poly-basic cluster (amino acids #47–54) this region was dispensable for phospholipid interactions. We therefore assume that the first two poly-basic clusters act in a 'bipartite' manner.

![Fig. 1](image.png)

**The N-terminal region of mDia1 clusters PIP2 and inserts into the membrane**

The binding of mDia1 BD to membranes was further investigated by fluorescence spectroscopy. The protein fragments tested were mDia1N570 (amino acids #1–570), mDia1Δ12-42N570 (amino acids #1–570 with a deletion of residues #12–46) and mDia1N46 (amino acids #1–46). In these assays, the fluorescent probes NBD-PS and bodipy-TMR-PIP2 were applied for examining the binding of mDia1 N-terminus to PS and PIP2, respectively, in large unilamellar vesicles (LUV). mDia1 fragments induced quenching of NBD fluorescence, indicating binding to PS (Fig. 3A). Membrane-binding of mDia1 fragments also induced quenching of bodipy fluorescence. Since bodipy has highly superimposable absorption and emission spectra it exhibits self-quenching when two or more molecules are brought together in close proximity. Thus, quenching is caused by the static and/or dynamic interaction of bodipy-TMR PIP2 and indicates clustering of PIP2 molecules by mDia1N570 (Fig. 3B).
Fig. 2. The BD interacts with negatively charged phospholipids. (A) Cosedimentation assays (S: supernatant, P: pellet) revealed that GST-tagged N570, Δ12-21N570, N54 and N46 interact with lipid vesicles (48:48:4, PC/PS/PIP2) but not the constructs ΔBDN570 and Δ12-42N570. The GST control did not bind lipid vesicles containing PC, PS and PIP2. (B) The protein/lipid interaction of N46 requires both basic stretches because each of the synthetic peptides can completely block binding to the vesicles.

Although our results revealed that the mDia1N570 binds both to PS and PIP2, it appears to interact with higher affinity with PIP2 (Fig. 3A and B). Deletion of residues 12–42 from the N-terminal mDia1 fragment diminished binding to PS and PIP2 (Fig. 3A and B). The peptide consisting of the first 46 residues bound strongly to both PS and PIP2, suggesting that these amino acids are important for PS and PIP2 binding (Fig. 3A and B). However, the robust lipid-binding activity of N46 might not represent the actual situation as this fragment is likely to be structurally simpler compared to that of the construct mDia1N570.

Fig. 3. The mDia1 N-terminus binds PS/PIP2 and inserts into the membrane. Constructs containing the BD quench the signals of both fluorescently labeled lipids PS (A) and PIP2 (B). The clustering of PIP2 by the BD (N46) was the most efficient. A protein fragment lacking the crucial residues in the BD (Δ12-42N570) showed in both cases the least lipid interaction. (C) Changes in the DPH anisotropy implied that both N570 and Δ12–42N570 inserted into the plasma membrane independent of the presence of PIP2. N46 is apparently too short and did not show significant changes of anisotropy. (D) However, both PIP2 and the presence of the BD in N570 boosted its insertion into the membrane. Liposomes at indicated ratios were used.
To test if the BD-membrane interaction is just electrostatic or whether the N-terminus is capable of inserting into the membrane bilayer, we studied steady-state fluorescence anisotropy of 1,6-diphenyl 1,3,5-hexatriene (DPH). DPH readily diffuses into the hydrophobic core of a lipid bilayer without influencing the physical properties of the plasma membranes and can thus be used to monitor changes in the rotational diffusion of the fatty acyl chains in the lipid bilayer (Zaritsky et al., 1985). Interestingly, binding of mDia1N570 to the reconstituted plasma membrane caused a considerable increment in DPH anisotropy, indicating that this fragment inserts into the lipid bilayer. Consistent with the sedimentation and lipid clustering data, the protein variant lacking residues 12–42 (∆Δ12–42N570) with reduced PS and PIP2-binding affinity inserted less efficiently into the lipid bilayer as compared to the wild type N-terminus fragment. Moreover, insertion of the mDia1 fragment N570 into the lipid bilayer was enhanced in the presence of PIP2, suggesting that the interaction with PIP2 facilitates membrane insertion. The N-terminal 46 amino acid peptide (N46) did not cause detectable changes in DPH anisotropy which implies that the BD is not directly involved in membrane insertion (Fig. 3C and D). However, it could play a pivotal role in facilitating the insertion of other regions of mDia1 by first attaching the protein to the membrane through electrostatic interactions with negatively charged lipids of the plasma membrane. Essentially, our data indicate that there is a substantial plasma membrane-BD interaction beyond the attraction for negative charges.

The basic domain (BD) is essential for targeting mDia1 to the plasma membrane

In a previous report the authors showed that the complete N-terminus of mDia1 up to the CC domain was localized at the plasma membrane and a point mutation in the GBD that renders defective Rho binding abolished membrane interaction only partially (Seth et al., 2006). This triggered the claim for GTPase-independent membrane-binding activities of mDia1. Therefore, we dissected the mDia1 N-terminus also in vivo by expressing wild type and truncated mDia1 constructs as enhanced green fluorescent protein (EGFP) fusions (Fig. 4A) in mouse NIH 3T3 fibroblasts and found, as expected, auto-inhibited full-length mDia1 (EGFP-mDia1FL) distributed uniformly throughout the cell (Fig. 4B, left panel).

Consequently, expression of a constitutively active construct that still contained the BD but lacked the DAD (EGFP-mDia1ΔDAD) was recruited to the plasma membrane (Fig. 4B, middle panel), and another constitutively active construct without the BD (EGFP-mDia1ΔBDΔDAD) lost its pronounced association with the plasma membrane again (Fig. 4B, right panel). In actin polymerization assays both mDia1ΔΔDAD and mDia1ΔBDΔDAD showed comparable activities (Fig. S1). So the distinct cellular distribution is not the result of differences in biochemical activity but depends at least in part on the lipid-binding. A strong accumulation of both EGFP-mDia1ΔDAD and EGFP-mDia1ΔBDΔDAD constructs in filopodial tips (Fig. 4B) suggests that the localization to filopodial tips cannot be the result of an interaction with the tip membrane.

Given that the four independent constructs EGFP-mDia1BD, EGFP-mDia1GBD, EGFP-mDia1BDGBD and EGFP-mDia1ΔBDΔDAD (Fig. 4B and not shown) failed to localize to the plasma membrane an involvement of multiple signals is quite obvious. In less than 15% of all cells expressing EGFP-mDia1ΔBDΔDAD, the protein still accumulated at the plasma membrane (Fig. 4C, right column). The most reasonable explanations for this small subpopulation are membrane recruitment through other formin domains and/or the formation of heterodimers between the endogenous DID and the DAD of mutated versions of mDia1. Since, mDia2 contains a similar basic region in its N-terminus (Fig. 1B), this type of membrane recruitment employed by the first two basic clusters may also be used by other formins.

**PIP2-BD interaction does not enhance Rho-induced activity of mDia1 but PIP2 inhibits the activity of FH1FH2DAD**

The domain architecture of mDia1 is reminiscent of N-WASP whose auto-inhibition is completely relieved by both Cdc42 and PIP2 (Higgs and Pollard, 2000; Prehoda et al., 2000; Rohatgi et al., 2000). Therefore, in vitro actin polymerization assays were carried out to decipher the role of PIP2 in the regulation of mDia1 activity. The tool for this assay was the artificial inhibition of FH1FH2DAD by N570. The two proteins fragments bind to each other in the same fashion as it is the case in auto-inhibited full-length formin. This inter-molecular DID/DAD interaction was only partially abolished by an addition of constitutively active RhoA(V14). Assuming that PIP2 will synergistically activate formin along with RhoA(V14), liposomes containing PC/PS/PIP2 were added to the cocktail of protein mixtures containing FH1FH2DAD, N570 and RhoA(V14). In contrast to our expectations, an inhibition in the formin activity was evident (Fig. S2). This result clearly suggests that (i) the BD-PIP2 interaction did not influence the formin activity synergistically with RhoA, and (ii) another phospholipid-binding and inhibitory region in mDia1 must be present in the C-terminal half of the molecule encompassing the FH1FH2DAD.

**PIP2 negatively regulates the actin assembly mediated by mDia1FH1FH2DAD**

To get further insights into the effects of PIP2 on formin-mediated actin assembly in vitro total internal reflection fluorescence (TIRF) microscopy was employed to evaluate the impact of PIP2 on formin-mediated actin nucleation and elongation at the single filament level. mDia1-associated actin filaments were previously shown to grow indistinguishably from the actin control in the absence of profilin. Although profilin enhances mDia1-mediated actin assembly about five-fold (Kovar et al., 2006), we could not use profilin in our experimental set-up since PIP2 inhibits the formation of the profilin–actin complexes (Lassing and Lindberg, 1985). Therefore, glass slides were first coated with GST-formin constructs and low amounts of N-ethylmaleimide (NEM)-myosin II. After addition of actin monomers the formation of actin filaments was then analyzed in a 100 μm² area. Formin-mediated actin assembly was scored by one of the following three criteria:

(i) the appearance of “buckling” filaments due to the insertional assembly of monomers at the barbed ends while the filaments were also attached to the substrate by NEM-myosin II (see schematic representation in Fig. 5B),
(ii) the capture and subsequent elongation of spontaneously growing actin filament barbed ends by formins, also resulting in filament buckling, and
(iii) a bright fluorescence at the growing barbed ends bound to immobilized formins, paralleled by a fading signal towards the pointed end due to bleaching.

The regulation of mDia1 activity was tested in the presence of Rhoa and phospholipids (Fig. 5A and C). Auto-inhibited full-length mDia1 (mDia1-FL) alone exhibited only a basal activity accounting for less than about 5% of active formins. An addition of constitutively active Rhoa(V14) enhanced mDia1-mediated actin assembly up to a maximum of over 100 active formins in the area of interest. Incubation of PIP2 containing vesicles with Rhoa-activated mDia1, however, drastically impaired this activity. Rhoa-activated...
Fig. 4. The basic domain is essential for recruitment of mDia1 to the plasma membrane. (A) Schematic representation of the constructs used for the transfections. The full-length and truncated mDia1 versions were expressed as EGFP-fusion proteins in NIH 3T3 fibroblasts and the live cells were imaged using a LSM510 Zeiss confocal microscope. (B) Representative cells of each mutant, expressing EGFP-mDia1FL (left), EGFP-mDia1ΔDAD (middle) and EGFP-mDia1ΔBDΔDAD (right) are shown. The presence of the EGFP-mDia1ΔBDΔDAD in emerging filopodia (inset, right panel) suggests that functional formin at the barbed ends of filopodial actin filaments might be anchored in the tip complex by means of membrane independent interactions with a formin. The scale bar represents 20 μm. Depicted below are the corresponding plots showing the fluorescence intensity profiles across the plasma membrane (white boxes). (C) Percentage of cells with plasma membrane localization for each mutant is plotted as a bar chart. The error bars indicate mean standard deviation of four independent transfections. Overall EGFP-mDia1FL (n = 68), EGFP-mDia1ΔDAD (n = 88) and EGFP-mDia1ΔBDΔDAD (n = 125) cells were counted.

full-length mDia1 lost its actin assembly activity after the addition of 150 nM PIP2 in PC/PS/PIP2 vesicles. The constitutively active C-terminal fragment encompassing only mDia1FH1FH2DAD was similarly inhibited by PC/PS/PIP2 vesicles, i.e. the BD in mDia1-FL does not contribute to this inhibition. An examination of mDia1FH1FH2DAD (amino acids #702–1255) by TIRF microscopy revealed that induced filament growth was almost completely inhibited by the addition of PIP2 containing vesicles (Fig. 5D and E). Spontaneous actin assembly in the absence of formin was not altered by PIP2 (not shown).
Fig. 5. TIRF microscopy of actin filaments assembled by full-length or mDia1 FH1FH2DAD. (A) Micrographs of the assembly of 1 μM ATP-actin and 0.3 μM Alexa-Fluor-488-labeled ATP-actin (488 actin) in TIRF buffer on cover slips coated with 10 nM NEM-myosin II and 400 nM of GST-mDia1-FL. Addition of 5 μM GTP-bound RhoA(V14) resulted in activation of mDia1. Addition of 1 μM PIP2 inhibited formin-mediated actin assembly. Arrow heads indicate growing filament barbed ends, circles mark barbed ends captured by mDia1. The asterisk highlights a representative formin-induced buckling filament. (B) Scheme of insertional assembly of monomer to a filament bound to GST-mDia1 (left) and NEM-myosin-II (right). (C) Quantification of active GST-mDia1-FL molecules. For each experiment an area of 100 μm × 100 μm was analyzed, arrow bars show standard deviations. (D) Micrographs of the assembly of 1 μM ATP-actin and 0.3 μM 488 actin in TIRF buffer on cover slips coated with 400 nM GST-mDia1FH1FH2DAD. 1 μM PIP2 liposome (48:48:4, PC/PS/PIP2) inhibits GST-mDia1FH1FH2DAD. (E) Quantification of active GST-mDia1FH1FH2DAD in the presence or absence of PIP2. Error bars indicate standard deviations.

Actin assembly was also inhibited in the presence of PIP2 if one used the formin isoforms mDia2FH1FH2DAD and mDia3FH1FH2DAD (Fig. S3). This profound effect of PIP2 on mDia-mediated actin assembly renders PIP2 a potent inhibitor of mDia forms. Since neither filament nucleation nor the capture of spontaneously growing filaments was observed in the presence of PIP2, we hypothesize that this acidic lipid entirely blocks the interaction of the formins with actin. Inhibition of actin polymerization by

Fig. 6. PIP2 inhibits mDia1FH1FH2DAD-mediated actin assembly and induces strong clustering and membrane insertion. (A) Actin (3 μM) was polymerized in the presence of wild type mDia1FH1FH2DAD and LUVs containing increasing concentrations of PIP2 (70:30, PC/PIP2). The dotted grey line represents the actin control. (B) mDia1FH1FH2DAD effectively clusters PIP2 and PS as observed by the quenching of NBD-PS and bodipy-TMR-PIP2. (C) A profound increment in the DPH anisotropy indicates that mDia1FH1FH2DAD also inserts into the membrane with LUVs composed of PIP2 or PS. The presence of PIP2 in the liposome boosted the membrane insertion ability of mDia1FH1FH2DAD. Liposomes at indicated ratios were used.
PIP₂ was also tested by fluorescence spectroscopy using large unilamellar liposomes. Titrations with different ratios of PC/PIP₂ (96:4, 90:10, 80:20, and 70:30) further demonstrated that PIP₂ inhibited formin-induced actin assembly in a dose-dependent manner. Fig. 6A shows a representative experiment with PC/PIP₂ ratios of 70:30. An interaction of PIP₂ with certain actin-binding proteins was frequently described in recent years whereas actin itself was never a target.

**mDia1FH1FH2DAD clusters PIP₂ and inserts into reconstituted membranes**

To understand the mechanism of mDia1 negative regulation by phospholipids we investigated the mDia1FH1FH2DAD phospholipid interaction using fluorescent lipid probes. As described above for the N-terminal mDia1 fragments, bodipy-TMR-PIP₂ was used to explore the membrane-binding ability of mDia1FH1FH2DAD. Interestingly, our data revealed that FH1FH2DAD binds strongly to PIP₂-rich membranes (Fig. 6B) which is consistent with our previous observations that increasing amounts of PIP₂ in the liposome mixture resulted in better inhibition (Fig. 6A). The bodipy-TMR-PIP₂ assay demonstrated that FH1FH2DAD also clustered PIP₂ upon binding to reconstituted membranes. DPH anisotropy measurements unequivocally indicated that like N570, FH1FH2DAD also inserted into the membrane containing PIP₂ or PS (Fig. 6C). It is, however, not clear why PS-containing liposomes lacking PIP₂ did not inhibit the actin assembly mediated by FH1FH2DAD of mDia1 or mDia2 or mDia3 FH1FH2DAD (not shown and Fig. S3A and B). One possibility is that a lipid with a more specific head group and charge density is required for negative regulation but not for the electrostatic interactions per se. Remarkably, the C-terminal fragment encompassing the FH1FH2DAD inserted more efficiently than the N-terminus of mDia1.

**The C-terminus of mDia1 harbors more than one PIP₂-binding site**

The intra-molecular interaction of DID and DAD was previously shown to be crucial for auto-inhibition of formins in vitro and in vivo (Lammers et al., 2005; Wallar et al., 2006). The inhibitory activity of PIP₂, however, employs a different mechanism and we hypothesized that the extreme C-terminus of mDia1 encompassing the DID of mDia1 might at least in part be responsible for the interaction with PIP₂. In agreement with this assumption, addition of an excess of mDia1DAD (amino acids #1180–1255) relieved PIP₂-mediated inhibition of the mDia1FH1FH2DAD fragment in a dose-dependent manner (Fig. S4). This confirmed that the C-terminus spanning the DID is not only crucial for auto-inhibition of full-length formin but also for the negative regulation of an active mDia1 by PIP₂. First attempts to map motifs that are important for the interaction between phospholipids and mDia1FH1FH2DAD by mutating the conserved poly-basic residues to alanine showed that point mutations in the DID and in the FH2 domain reduced clustering of PIP₂. The nuclease activity of a construct lacking the DID (mDia1FH1FH2) was also reduced by PIP₂ suggesting that more than one lipid-binding site is present within the FH1FH2 domain and the adjacent C-terminal region (not shown).

**Discussion**

Formins have been reported to target themselves to specific subcellular compartments by various mechanisms (Brandt et al., 2007; Copeland et al., 2007; Lewkowicz et al., 2008; Seth et al., 2006; Yonetani et al., 2008; Zaoui et al., 2008). In the present study we have demonstrated that the BD of mDia1 directly interacted with membrane phospholipids and this interaction is required for the targeting of mDia1 to the plasma membrane. Presumably this type of membrane recruitment is adapted by other DRF family members including mDia2 that possesses a similar BD. Our steady-state fluorescence spectroscopy data imply that the binding of mDia1 BD to phospholipids is not just an electrostatic interaction between the positively charged BD and the negatively charged lipids but could involve an additional interaction between hydrophobic protein regions with the lipid acyl chains in the membrane. Since both the first and the second basic clusters of mDia1 directly interacted with phospholipids independent of each other, they might work in a bipartite manner. Accordingly, the subcellular distribution of constitutively active DAAM1 (ΔDAD), which contains only one basic stretch in its N-terminus (Fig. 1B) was recently shown to be entirely cytoplasmic (Liu et al., 2008). The finding corroborates our hypothesis that a single basic cluster such as the one present in DAAM1 may not be sufficient for phospholipid interaction and eventually membrane targeting. In addition, we also identified lipid-binding motifs at the C-terminus of mDia formins including mDia1, mDia2 and mDia3 as targets for membrane lipids. It is tempting to speculate that also these homologous motifs transiently inhibit formin activity upon binding to membrane lipids and thus circumvent the auto-inhibitory switch between inactive (closed) and active (open) conformations. The activities of mDia formins on the microtubule (MT) cytoskeleton are also mediated by FH2 domains. So it would be interesting to see if the regulation of MT is also affected by PIP₂, although the function of mDia on the actin and MT cytoskeleton can be segregated (Bartolini et al., 2008).

An important feature of actin dynamics in migrating cells is the need of filament elongation directly at the plasma membrane. Consequently, the majority of the filaments is not distributed randomly but rather in a polarized fashion with the fast growing barbed ends pointing towards the membrane and in parallel to the direction of movement. Formins are dimers, which can bind to the barbed end, add hand-over-hand actin monomers and, if they harbor the appropriate domain, stay bound to the plasma membrane. In a previous report, Rosen and coworkers showed that the complete N-terminus of mDia1 (amino acids # 1–570) was localized at the plasma membrane (Seth et al., 2006). However, the inactivation of GBD by a point mutation abolished membrane attachment only partially suggesting a more sophisticated mechanism and a membrane-binding activity beyond the GBD. The discovery of an N-terminal region rich in basic residues (the BD) indicated the importance of BD-phospholipid interactions not only for plasma membrane recruitment but also for membrane insertion of active mDia1.

Our study in relation to published data indicates the cooperation among GBD, BD and DID. First of all, a membrane-bound GTPase opens the closed formin conformation by interaction with GBD (Li and Higgs, 2005; Rose et al., 2005), secondly the BD accomplishes the actual interaction with the negatively charged phospholipids PS/PIP₂ in the plasma membrane and finally the contact of DID to the scaffolding protein IQGAP strengthens the interaction between plasma membrane and the N-terminus of formin (Brandt et al., 2007). Thus a failure in any one of the signals will result in mis-localized mDia1.

The activity of potent actin nucleators has to be regulated differentially at independent levels. The inhibition of an active full-length mDia1 by PIP₂ occurs obviously after the protein has been activated by the GTP-bound RhoA upon a stimulus at the plasma membrane. The binding of mDia1 by RhoA opens the auto-inhibited structure and mDia1 attaches to the plasma membrane via its BD, maintaining its open conformation and nucleating filament formation. Possibly, the actin filaments grow until the C-terminus of formin reaches the plasma membrane. The C-terminus of formin then clusters PIP₂ and inserts into the lipid bilayer which leads to inhibition of actin filament elongation. The hydrolysis of PIP₂, i.e.
the action of an entirely different signalling cascade, could reactivate the nucleating activity of the formin which is already present at the membrane in an open but inactivated conformation. The model in Fig. 7 represents a working hypothesis that is based (i) on well known formin activities (e.g. auto-inhibition, regulation by GTPases, domain characteristics), (ii) on our new findings (membrane association via interaction with lipids at the N-terminus, inhibition by lipids at the C-terminus), and (iii) predicted activities that have to be confirmed experimentally in the future (e.g. transient nucleating activities at the membrane).

The most prominent and best studied machineries driving the nucleation of actin filaments in vertebrate cells are formins and the Arp2/3-complex (Mattila and Lappalainen, 2008). The latter requires activation by so-called nucleation promoting factors such as neuronal Wiskott–Aldrich syndrome protein (N-WASP) (Stradal et al., 2004). Like formins, N-WASP or WASP are folded in an auto-inhibited stage which is already present upon signal transduction. This auto-inhibited stage can be triggered either by the formation of lamellipodial actin networks by Arp2/3 or the generation of linear actin structures by formins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejcb.2010.06.001.

References


Figure S1

mDia1ΔDAD and mDia1ΔBDΔDAD constructs are equally active in vitro. 2 μM actin was polymerized in the presence of recombinant mDia1 constructs GST-mDia1ΔDAD (green line) and GST-mDia1ΔBDΔDAD (magenta line). The actin assembly mediated by GST-mDia1ΔBDΔDAD is identical to that of the GST-mDia1ΔDAD at the respective nanomolar concentrations. Actin control is indicated by a grey line.
**Figure S2**

PIP2 inhibits RhoA induced mDia1 activity. 2 μM actin was polymerized in the presence of mDia1FH1FH2DAD (orange line). Addition of the N-terminus of mDia1 (N570) resulted in intermolecular DID/DAD inhibition (blue). Inhibition was relieved by adding constitutively active RhoA which removes N570-DID from mDia1FH1FH2DAD (green line) but this activation was inhibited in the presence (grey line) of PIP2 (48:48:4, PC/PS/PIP2). Actin control is indicated by a dotted grey line.
**Figure S3**

PIP2 inhibits mDia2 and mDia3 mediated actin assembly. (A) 2 μM actin was polymerized in the absence of mDia2 or mDia3 (dotted grey lines). Addition of 50 nM GST-mDia2FH1FH2DAD (A) or GST-mDia3FH1FH2DAD (B) enhanced actin polymerization (orange lines), whereas polymerization was inhibited by addition of PIP2 vesicles (76:20:4, PC/PS/PIP2) in a dose-dependent manner (green and blue lines). mDia2 and mDia3 FH1FH2DAD mediated actin assembly was inhibited by PIP2 at a concentration of 2 μM and 700 nM, respectively. Actin assembly mediated by both mDia fragments remained unaffected by phospholipid vesicles lacking PIP2 (black lines).
Figure S4

mDia1DAD relieves PIP2-mediated inhibition of FH1FH2DAD activity. The green line represents actin assembly mediated by GST-mDia1FH1FH2DAD. While PIP2 (48:48:4, PC/PS/PIP2) inhibited the actin assembly (blue line), the addition of increasing amounts of mDia1DAD relieved the inhibition by PIP2 in a dose-dependent manner (yellow and magenta). mDia1DAD alone did not influence the actin assembly (dotted grey line).