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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_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-terminus functions as a switch for transient inactivation.

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 (Faix 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

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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 PIP_2 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/SalI sites of pEGFP-C1 (Clontech). For the expression of mDia1, mDia2, mDia3 and the RhoA(V14) constructs in *E. coli*, appropriate inserts were amplified by PCR and cloned into pGEX-6P-1 or pGEX-4T-1(GE Healthcare). Hot-Start Phusion polymerase (Finnzymes) was used for the site directed mutagenesis experiments.

Protein expression and purification

ArcticExpress™ RP competent cells (Stratagene) were transformed with GST-mDia1 full-length, GST-mDia1ΔDAD (amino acids #1–1179), GST-mDia1ΔBDΔDAD (amino acids #61–1179), GST-mDia1FH1FH2DAD (amino acids #702–1255), GST-mDia2FH1FH2DAD (amino acids #521–1171), GST-mDia3FH1FH2DAD (amino acids #586–1102) and GST-mDia1DAD (amino acids #1180–1255). The cells were grown to mid-log at 30 °C and subsequently expression was induced with 0.5 mM IPTG for 15 h at 10 °C. The N-terminal constructs of mDia1 and GST-RhoA(V14) were expressed in BL21RIL cells (Stratagene) at 16 °C for 10 h after induction at an optical density of 0.6 at 600 nm. Purification of GST and fusion proteins was done as described (Faix et al., 1998). The GST tag was cleaved off by incubating GST-RhoA(V14), GST-mDia1DAD, GST-mDia1N570, GST-mDia1Δ12-42N570, GST-mDia1N46, GST-mDia1 FH1FH2DAD with PreScission protease (GE Healthcare) or thrombin (Novagen) in PBS supplemented with 1 mM DTT at 4 °C for 10 h. Free GST and PreScission protease were then removed by passing the solution over a glutathione-agarose column; thrombin was removed by benzamidine agarose resin. All other proteins were used as GST fusions in the biochemical assays.

Preparation of large unilamellar vesicles (LUVs)

Diphenylhexatriene (DPH) was from Invitrogen and bodipy-TMR-PIP_2 was purchased from Echelon (Salt Lake City, Utah). 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]-hexanoyl}-*sn*-glycero-3-phosphoserine (NBD-PS), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanol-amine (POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylserine (POPS), and L-α-phosphatidylinositol-4,5-bisphosphate (PIP_2) were purchased from Avanti Polar Lipids (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 PIP_2 molecules the geometry of the vesicle will allow only a few acidic lipid molecules to interact with a target protein. Consequently, the given PIP_2 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% PIP_2 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-PIP_2 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, NBD-PS, and bodipy-TMR-PIP_2 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 $MgCl_2$, 0.2 mM $CaCl_2$, 1 mM Na_2ATP, 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 $MgCl_2$, 1 mM EGTA, 0.2 mM ATP, 50 mM DTT, 15 mM glucose,