Homo-oligomerization Is Essential for F-actin Assembly by the Formin Family FH2 Domain

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Formin proteins regulate the actin and microtubule cytoskeletons and also control the activity of the SRF transcription factor through depletion of the G-actin pool. Although the conserved formin homology 2 (FH2) domains of the mDia1 and Bni1 formins can nucleate actin polymerization in vitro, the activity of other FH2 domains and the relationship between actin polymerization and microtubule reorganization have been controversial. We show that, similar to the mDia1 FH2 domain, the FH2 domains of dia2 and 1d are sufficient for SRF activation in vivo. We demonstrate that an mDia1 mutant defective for microtubule rearrangement in vivo is also defective in SRF activation in vivo as well as actin polymerization in vitro and that the mDia2 FH2 domain promotes actin polymerization in vitro. Using co-immunoprecipitation, we show that mDia1 is oligomeric in its inactive autoinhibited state in vivo, that the active mDia1 and mDia2 FH2 domains form homo- but not hetero-oligomers in vivo, and that oligomerization is abolished by inactivating FH2 deletion and point mutations. Nevertheless, inactive mDia1 FH2 domain mutants retain the ability to interfere with cellular mDia activity. Our results show that self-oligomerization is essential for SRF activation in vivo and F-actin assembly in vitro and provide strong support for recent structural models of the FH2 domain.

Formin proteins are involved in many cytoskeletal processes such as cytokinesis, actin cable and stress fiber formation, neurite outgrowth, and intracellular trafficking because of their ability to regulate F-actin assembly and microtubule organization (reviewed in Ref. 1). Their ability to promote F-actin assembly also allows them to control the transcription factor SRF whose coactivator MAL/MKL1 responds to G-actin level (2–4). Although the formin family is defined by two conserved formin homology (FH) domains, FH1 and FH2, additional conserved domains were found in a formin subfamily, the diaphanous-related formins (DRFs). In the DRFs, an N-terminal Rho-binding domain (RBD) and C-terminal diaphanous autoregulatory domain (DAD) inhibit the activity of the FH1 and FH2 domains through a physical interaction, which is disrupted by Rho binding, whereas a poorly defined FH3 domain appears important for DRF subcellular localization (5–7).

The function of the DRF proteins has remained unclear until recently. The bundling of F-actin filaments to form stress fibers requires cooperation between mDia proteins and the ROCK family of Rho effector kinases (5, 8, 9). The relative roles of the DRF FH1 and FH2 domains in this process, however, have been controversial. The expression of mDia proteins alone is sufficient to induce F-actin assembly (10), and cell-biological analysis suggested that the mDia1 and mDia2 FH1 domains were essential for this assembly, implicating Src homology 3-containing FH1 ligands such as IRSp53, Src family kinases, and profilin-actin in the process (8, 9, 11). Moreover, an analysis of mDia-induced cell polarization identified a clustered FH2 domain point mutation competent for F-actin formation but not microtubule reorganization, suggesting that these functions are independent (12). In contrast to these results, quantitative analysis of mDia function by fluorescence-activated cell sorter analysis of F-actin assembly or SRF reporter gene activity showed that the mDia1 FH2 domain is sufficient to induce F-actin assembly in vivo and that inactivating FH2 mutations also disabled derivatives containing the FH1 domain (13). Moreover, certain inactive mDia FH2 mutants retained the ability to interfere with signal-induced activation of endogenous or co-transfected mDia proteins, although the molecular basis for this has been unclear (13).

Recent in vitro analysis of the mDia and Bni1 DRFs showed that the FH2 domain was sufficient to nucleate F-actin assembly in vitro (14–16), although the definition of the minimal functional FH2 domain and contribution of the FH1 domain and profilin to filament assembly remain unclear (17–19). These studies also demonstrated that the recombinant FH2 domain is oligomeric in solution (20–22), and crystallographic analysis revealed a dimeric FH2 domain structure (23). The relationship between DRF oligomerization and function in vivo has remained unclear, although certain monomeric mDia1 derivatives failed to promote actin polymerization but retained the ability to cap filament-barbed ends (19).

In this study, we have extended our analysis of the FH2 domain using our quantitative SRF activation assay and actin polymerization assays in vitro. We show that the FH2 domain is the minimal functional unit of mDia2 and of formin itself and that the mDia1 point mutant defective in microtubule reorganization is both profoundly defective for F-actin assembly and exhibits significant filament capping activity. We demonstrate that mDia proteins form obligate homo-oligomers and that FH2 oligomerization is essential for F-actin assembly in vitro and SRF activation in vivo. The results show that the FH2 domain complexes previously observed in solution (14, 15, 20–22) are essential for its function and demonstrate directly that FH2 oligomerization and function are compromised by mutations in

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conserved residues located at critical subunit contacts in recent crystal structures of the FH2 domain (23). Finally, our results suggest that at least some interfering DRF derivatives work, not by forming inactive complexes with endogenous intact DRF proteins but by capping actin filaments.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Transfections, SRF Reporter Assays—**Transfections, SRF reporter assays, and proteins mDia1 F1F2, F1F2ΔC2, FH2, and mDia2 ΔRBDAFH1 were described previously (9, 13). Other FLAG- or Myc epitope-tagged mDia and formin derivatives (see figures) were expressed similarly. For protein expression, mDia fragments were expressed by pGEX-6p. mDia1 point mutations were: KA3, Lys989/Lys994/Lys999 → Ala; LRR762A, Leu762/Arg763/Arg764 → Ala; KK753A, Lys753/Lys754 → Ala; and W767A, Trp767 → Ala.

**Protein Purification and in Vitro Actin Assays—**Glutathione S-transferase-mDia derivatives were purified as described previously (16) and recovered following cleavage with Prescision Protease (Amersham Biosciences). Actin polymerization assays were performed with pyrene-labeled actin monomers (Cytoskeleton) according to the supplied protocol. The critical concentration experiments were performed as described previously (15). 10 μM 5% pyrene-labeled actin was allowed to polymerize for 2 h at 25 °C and then was diluted to the indicated concentrations and incubated with 100 nM indicated proteins for 16 h at 25 °C. Fluorescence was monitored on a Spectromax M2 fluorometer (Molecular Devices) (excitation 365 nm, emission 407 nm).

**Immunoprecipitations—**Transfected cells were maintained at 37 °C overnight in 10% fetal calf serum. Cells were washed in ice-cold phosphate-buffered saline, scraped in 300 μl of lysis buffer (50 mM Tris, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5% Triton X-100, 5 mM NaF, and protease inhibitors), and kept on ice for 10 min. The lysate was clarified by centrifugation (15 min, 13,000 rpm, 4 °C), and incubated with precoupled anti-FLAG beads (Sigma) for 1–2 h at 4 °C. The beads were washed three times with cold lysis buffer, and the immunoprecipitated proteins were eluted with 50 μl of SDS-PAGE loading buffer. Equal volumes of the lysate and precipitated samples were analyzed by immunoblotting.

**Quantification of Immunoblots—**Cell lysates were prepared for luciferase assays as per the supplied protocol (Promega). Samples of the
lysate were diluted in 4× Laemmli buffer, subjected to SDS-PAGE, and blotted following standard procedures. The blotted proteins were visualized by immunoblotting with polyclonal anti-Myc antibody (Sigma) and detected with peroxidase-coupled anti-Rabbit IgG secondary antibody (Sigma) and ECL (Amersham Biosciences). Quantification of the ECL signal was performed on a Kodak Image Station 440.

RESULTS

To study FH2 function in more detail, we examined mDia1 FH2 point mutants in which clusters of highly conserved FH2 residues were converted to alanine (Fig. 1A). Mutants KK753A, LRR762A, and W767A removed residues in the N-terminal region of the FH2 domain previously shown to be required for FH2 function (13), whereas mutant KA3 disrupted a conserved helix in its central region (12). These proteins were transiently expressed in NIH 3T3 cells, and their ability to activate the SRF-dependent reporter gene 3D.ALuc was evaluated. All of the point mutants were expressed at similar or higher levels than the parental construct (for example see Fig. 4, D and E). Each of the three N-terminal point mutations completely inactivated the parental mDia1 FH1/FH2 protein in the SRF activation assay (Fig. 1A). Surprisingly, the KA3 mutation also effectively abolished SRF activation by mDia1 FH1/FH2 (Fig. 1A), even though this mutant has been reported not to affect F-actin polymerization (12).

A previous study of mDia2, a protein highly related to mDia1, has suggested that both its FH1 and FH2 domains are required for SRF activation and F-actin reorganization (9). Therefore, we directly compared functional domains in the two proteins using a series of mDia2 derivatives analogous to our original mDia1 mutants (Fig. 1B). As with mDia1, mDia2 FH1/FH2, which contains the mDia2 FH1 and FH2 domains, strongly activated the SRF reporter and, as reported previously, an mDia2 derivative retaining the FH3 and FH2 domains but lacking FH1 was virtually inactive in the SRF assay (Fig. 1B) (9). Nevertheless, as with mDia1, the expression of the isolated mDia2 FH2 domain was sufficient to induce potent SRF activation (Fig. 1B). Moreover, as with mDia1, deletions that impinged upon the integrity of the mDia2 FH2 domain completely inactivated the protein, even when FH1 was present (Fig. 1B). Thus, the FH1 domain is not required for SRF activation in vivo by either mDia1 or mDia2, and the FH2 domain represents the minimal domain required for SRF activation. However, in the case of mDia2, sequences in FH3 act to constrain activity of the FH2 domain (see “Discussion”).

To further confirm the general applicability of our observations, we also examined the ability of the expression of the FH1 and FH2 domains of the prototype formin, the mouse limb deformity protein (ld), to activate SRF. The expression of the ld FH1/FH2 domains also activated SRF, albeit to a significantly lower level than the mDia proteins (Fig. 1C). We used the SRF assay to test the effect of an N-terminal deletion of the ld FH2 domain, analogous to the inactivating deletions of mDia1 and mDia2, and found it to be inactive in the SRF assay (Fig. 1C). To assess the requirement for ld FH2 C-terminal sequences, we examined two C-terminal deletion mutants corresponding to two of the original limb deformity mutations, the strong Tg/Bri/Hd allele and the weak In2 allele (24). Based on our analysis of mDia FH2 domains, the In2 deletion should leave the FH2 domain intact, whereas the Tg truncation should not. Indeed, in the SRF assay, the Tg derivative completely abolished function, whereas the less severe In2 mutation had no effect on FH2 activity (Fig. 1C). Thus, ld itself possesses an FH2 domain whose expression can activate SRF and it appears that the functional limits of this domain match those mapped in the mDia proteins.

The results presented above show that FH2 constitutes the minimal formin domain sufficient for SRF activation. Our previous studies of the Rho-controlled signaling pathway to SRF are consistent with a model in which SRF is activated by depletion of the cellular G-actin pool (see Ref. 4). Recent studies have shown that the entire FH2 regions of Bni1 and mDia1 are sufficient for actin filament nucleation (15, 16, 18–21). To confirm that this holds true for mDia2 and to investigate the nature of the defect in the KA3 mutant, we examined the ability of purified mDia derivatives to promote actin polymer-
ization in vitro. Recombinant mDia1 and mDia2 were purified following cleavage from glutathione-S-transferase fusion proteins and tested for their ability to promote polymerization of pyrene-actin monomers in vitro. Purified mDia1 F1F2 and FH2 effectively induced actin polymerization (Fig. 2, A and B), as did the purified mDia2 FH2 domain (Fig. 2B). In contrast, purified F1F2A1 exhibited only background levels of actin polymerization activity in vitro, as did the mDia1 F1F2 KK753A and LRR762A FH2 domain mutants (Fig. 2, A and C; we were unable to express W767A). The F1F2.KA3 mutant also failed to promote polymerization under the assay conditions (Fig. 2C). These data demonstrate a strict correlation between the ability to nucleate actin polymerization in vitro and the ability to activate SRF in vivo.

Recent studies have suggested that monomeric FH2 derivatives may cap actin filament barbed ends (19). Therefore, we measured whether the different mDia1 derivatives affected actin critical concentration. The inclusion of F1F2 or its inactive derivative F1F2A1 in the reactions had no effect on critical concentration, but F1F2.KA3 significantly increased it (Fig. 2D). We conclude that, under the conditions of our assay, the KA3 mutant possesses effective barbed end capping activity. Although F1F2A1 did not induce capping under our assay conditions, we note that others have observed capping by an analogous mDia derivative, albeit at much higher protein concentrations (>2 μM) (19).

A number of inactivating deletions at the N and C termini of the mDia1 FH2 domain render it able to interfere with DRF function in vivo (13). Therefore, we tested whether the different inactive mDia point mutations act as interfering mutants. The inactive mDia1 F1F2 derivatives KK753A, LRR762A, and W767A inhibited SRF activation by co-expressed mDia1 or mDia2 FH1/FH2 domains as effectively as FH2 N-terminal deletion derivatives (Fig. 3A). The F1F2.KA3 mutant also effectively interfered with SRF activation by mDia1 F1F2 (Fig. 3C). In contrast, mDia1 FH2 C-terminal deletion mutants only slightly affected SRF activation by mDia1 F1F2 and left mDia2 F1F2 unaffected (Fig. 3A). To map the boundary of the sequences responsible for interference, further mDia1 FH2 deletion mutants were analyzed. N-terminal truncations to residue 808 of mDia1 were inactive and interfering, but the deletion of an additional 50 amino acids greatly reduced interference with mDia1 function (Fig. 3A). Similar results were obtained when interference with mDia2 FH1/FH2 function was tested (Fig. 3A).

The experiments in Fig. 2D showed that the inactive interfering mDia1 mutant F1F2.KA3 was able to efficiently cap filament barbed ends. Intriguingly, the region of FH2 required for interference in vivo was similar to the "core" FH2 domain, which at higher protein concentrations has been shown to cap the barbed end of filaments (19). We did not observe capping of actin filaments by other inactive mDia1 derivatives under our assay conditions. Therefore, we performed titration experiments to compare the relative efficiency of interference by F1F2.KA3 and by the deletion mutant mDia1-(808–1181). F1F2.KA3 worked much more efficiently than mDia1-(808–1181) as assessed by the protein expression level required for effective inhibition of mDia-induced SRF activation (Fig. 3C), suggesting that interference may be due at least in part to filament capping (see "Discussion").

Previous studies have shown that purified formin derivatives containing the FH2 region are oligomeric in solution (15, 20).2 Therefore, we investigated the relationship between FH2 oligomerization in vivo and mDia2 activity. Derivatives of mDia1 bearing FLAG and Myc epitope tags were transiently co-expressed in NIH 3T3 cells, and their association was evaluated using a co-immunoprecipitation assay and visualized by immunoblotting. First we examined self-association of intact mDia1. Myc-mDia1 was readily detectable in the immunoprecipitates of FLAG-mDia1 (Fig. 4A, lanes 3 and 4). Its recovery was dependent on FLAG-mDia1 expression and was not affected by inactivation of Rho signaling by co-expression of C3 transferase (Fig. 4A, lanes 5 and 6). It was not observed in control experiments using cells cotransfected with empty FLAG expression plasmid (Fig. 4A, lanes 1 and 2).

We next examined oligomerization of the FH2 domain. Myc-mDia1 FH2 was readily detectable in the immunoprecipitates of FLAG-mDia1 FH2. Its recovery was dependent on FLAG-mDia1 FH2 expression and was not observed in control exper-

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2 J. W. Copeland and S. J. Copeland, unpublished observations.
ments using cells co-transfected with empty FLAG expression plasmid (Fig. 4, lanes 1 and 2; data not shown). Co-immunoprecipitation was not affected by pretreatment of the extracts with the actin-binding drug Latrunculin B, demonstrating that the FH2-FH2 interaction was not a consequence of simultaneous binding to F-actin (data not shown). Similar results were obtained with the mDia2 FH2 domain. Myc-FH2 mDia2 was efficiently recovered in anti-FLAG immunoprecipitates from cells expressing both FLAG- and Myc-tagged mDia2 FH2 (Fig. 4, lanes 3 and 4). Strikingly, however, the FH2 domains of mDia1 and mDia2 did not form heteromeric complexes in this assay (Fig. 4, B and C, compare lanes 1 and 2 with 3–10).

We used the co-immunoprecipitation assay to investigate whether inactivating FH2 domain mutations affected its oligomerization. FLAG-tagged mDia1 FH2 effectively bound Myc-mDia1 F1F2 but was severely deficient in binding inactive derivatives of this protein lacking sequences at the N or C termini of the FH2 domain (Fig. 4B, compare lanes 5 and 6 with 7 and 10). Analogous results were obtained with the mDia2 derivative (Fig. 4C, compare lanes 5 and 6 with 7–10). We next examined oligomerization by mDia1 point mutants. Mutations KA3, LRR762A, W767A, and KK753A all significantly reduced the ability of mDia1 F1F2 to form homo-oligomers (Fig. 4D, compare lanes 1 and 2 with 3–10) and also abolished its ability to interact with wild-type mDia1 FH2 (Fig. 4E, compare lanes 1 and 2 with 3–10).

Taken together, these results show that the mDia1 and mDia2 domains form homo-oligomers in vitro and that oligomerization is essential for actin-polymerization in vitro and in vivo. Formation of inactive mDia oligomers, however, cannot be the basis by which inactive mDia1 derivatives interfere with mDia function, because these mutants are also defective in oligomerization (see “Discussion”).

**DISCUSSION**

In this study, we have shown directly that the FH2 domains of mDia1 and mDia2 were sufficient to induce actin polymerization in vitro and to activate the transcription factor SRF in vivo. Oligomerization of the FH2 domain, which is disrupted by point mutants at the FH2 N terminus and central core, was essential for both functions. Indeed, while this study was in progress, it was reported that the DRFs mDia1 and Bni1 are multimeric in solution (15, 20–22). The intact mDia1 protein is also oligomeric in the absence of Rho signaling. Actin polymerization appeared to be the core activity of the FH2 domain, since id, the prototype formin, was also active in the SRF reporter assay. Our results provide direct support for our model that the ability of mDia1 to activate SRF results from a direct effect on F-actin assembly rather than from indirect effects on
cellular signal transduction cascades (2, 13). Our data strongly support the recent structural studies of the FH2 domain (19, 23) and suggest that interfering mDia derivatives act not by forming inactive complexes with the intact protein but by acting as monomers to prevent actin filament assembly.

The FH2 domain of mDia2 is both necessary and sufficient for SRF activation and actin polymerization, in apparent contrast to a previous report (9). We found that sequences N-terminal to the mDia2 FH1 domain acted to inhibit activity of its FH2 domain, suggesting an explanation for this apparent discrepancy. Our previous experiments provided no evidence that mDia1 behaved in this fashion (13), although it is intriguing that the mDia1 FH3 domain has been reported to interfere with DRF function in epithelial cells (8). We are currently investigating interactions among FH3, FH2, and other factors.

The mDia1 F1F2.KA3 mutation removes three highly conserved lysines in FH2 and substantially inactivates both actin polymerization and SRF transcription. It was previously reported that this mutant could induce actin polymerization in vivo but not polarization of microtubules (12). Our finding that this mutant is defective in vitro for actin polymerization cautions against use of this mutant to distinguish between microtubule and actin-dependent phenotypes induced by mDia proteins. Indeed, recent studies place the spectraplakin ACF7 downstream of mDia1 in coordination of F-actin and microtubule organization (25). As with our other inactive mDia1 mutants, F1F2.KA3 is also defective in its ability both to self-associate and to associate with the wild-type mDia1 FH2 domain.

Deletion or point mutations that disrupt the N or C terminus of the FH2 domains of mDia1, mDia2, or ld-formin block the ability of these proteins to induce SRF activation in vivo and, where tested, their ability to nucleate actin polymerization in vitro. These mutations and the KA3 derivative also greatly reduce the ability of the FH2 domain of mDia1 and mDia2 to form homo-oligomers, demonstrating that oligomerization is essential for FH2 function. However, it is unlikely that multimerization of FH2 domains underlies the interference by dominant negative mDia1 derivatives for two reasons. First, mDia proteins with mutant FH2 domains lack the ability to oligomerize, so it is unlikely that interference reflects formation of inactive oligomers. Second, the interfering forms of mDia1 block mDia2 activity, even though hetero-oligomerization between mDia1 and mDia2 does not occur.

One potential explanation for these observations is that the interfering mDia mutants block a step downstream of diaphanosum function in the F-actin assembly pathway. An attractive notion is that the mutants act by capping actin filament barbed ends. Consistent with this finding, the KA3 mutant exhibited capping activity under our assay conditions (Fig. 2D). Although other interfering mDia mutants failed to cap under our assay conditions, an analysis of the mDia FH2 sequences required for interference suggested that their N-terminal border approximates to that of the minimal protease-resistant “core” FH2 domain (19). We note that capping by such mDia derivatives has been observed, albeit at greater than 20-fold higher protein concentrations than those used in our assays (19), and that an N-terminal deletion mDia derivative of this type interfered 10-fold less effectively than the KA3 mutant (Fig. 3). Given these considerations, we favor the idea that all of the interfering mDia derivatives work by acting as actin filament barbed-end capping proteins, although direct proof of this awaits further in vitro studies. Whereas the capping hypothesis would explain why mDia interfering mutants are not isoform-specific, it remains possible that interfering mDia derivatives interact with another limiting factor required for FH2 function in vivo. One such factor is VASP, which appears to function with mDia1 in the Rho-linked SRF activation pathway in fibroblasts (26, 27).

Our results provide strong support at the functional level for recently determined structures of DRF FH2 domain fragments from yeast Bni1 (23) and mammalian mDia1 (19). The structure of a minimal, active Bni1 FH2 domain comprises a head-to-tail dimer in which the FH2 N terminus loops around a postlike structure formed by the core FH2 homology region, whereas the FH2 C terminus contacts the intervening sequence. Mutations at conserved residues at the contact points abolish actin polymerization (23). According to this structure, our inactivating mDia1 N-terminal point mutants and truncations and the KA3 mutation would disrupt the loop-post interaction, consistent with our finding that they greatly decrease the stability of the FH2 dimer. In contrast, the minimal proteolytically stable mDia1 FH2 fragment, which lacks the N-terminal sequences of the minimal functional FH2 domain as defined by our previous studies (13), is monomeric and inactive in actin polymerization (19). However, consistent with this work, upon the addition of N-terminal FH2 domain sequences, the molecule dimerizes and becomes active in actin polymerization assays (19).

Previous studies have shown that the N-terminal RBD and C-terminal DAD regulatory domains interact to inhibit activity of the core FH1 and FH2 domains (5–7). Thus, the question arises of whether in the autoinhibited state the FH2 domain is monomeric and dimerizes following activation or dimeric but occluded prior to Rho binding (Fig. 5). The latter possibility is strongly supported by our finding that unactivated full-length mDia1 is oligomeric in vivo and by a recent study, which demonstrates inhibition of FH2-DAD function in trans by the mDia1 N terminus (15). The mechanism by which relief of DRF autoinhibition exposes the active FH2 domain will be of great interest.

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