The Diaphanous Inhibitory Domain/Diaphanous Autoregulatory Domain Interaction Is Able to Mediate Heterodimerization between mDia1 and mDia2*

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Sarah J. Copeland‡, Brenda J. Green‡, Sarah Burchat‡, Giuseppe A. Papalia‡, David Banner‡, and John W. Copeland‡1

From the ‡Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada and †Centre for Biomolecular Interaction Analysis, School of Medicine, University of Utah, Salt Lake City, Utah 84312

Formins are multidomain proteins that regulate numerous cytoskeleton-dependent cellular processes. These effects are mediated by the presence of two regions of homology, formin homology 1 and FH2. The diaphanous-related formins (DRFs) are distinguished by the presence of interacting N- and C-terminal regulatory domains. The GTPase binding domain and diaphanous inhibitory domain (DID) are found in the N terminus and bind to the diaphanous autoregulatory domain (DAD) found in the C terminus. Adjacent to the DID is an N-terminal dimerization motif (DD) and coiled-coil region (CC). The N terminus of Dia1 is also proposed to contain a Rho-independent membrane-targeting motif. We undertook an extensive structure/function analysis of the mDia1 N terminus to further our understanding of its role in vivo. We show here that both DID and DD are required for efficient autoinhibition in the context of full-length mDia1 and that the DD of mDia1 and mDia2, like formin homology 2, mediates homo- but not heterodimerization with other DRF family members. In contrast, our results suggest that the DID/DAD interaction mediates heterodimerization of full-length mDia1 and mDia2 and that the auto-inhibited conformation of DRFs is oligomeric. In addition, we also show that the DD/CC region is required for the Rho-independent membrane targeting of the isolated N terminus.

Formin homology proteins (formins) are a highly conserved family of cytoskeletal regulatory proteins. More than 30 formins have been described to date with more than 15 family members found in vertebrates (1). Formin activity is required in vivo for a diverse array of cellular functions such as stress fiber formation, actin cable formation in yeast, endosome motility, cell motility, cytokinetic ring formation, cell-cell junction assembly, filopodia formation, induction of cell polarity, and activation of the MAL/SRF signaling pathway (2–13). At the core of all these activities is the ability of formins to regulate actin cytoskeletal dynamics. This is achieved through the activity of two conserved domains, formin homology 1 (FH1) and FH2. FH1 consists of proline-rich repeats of varying sizes that can serve as ligands for SH3 and WW domains as well as the small actin-binding protein profilin (14, 15). FH2 is more highly conserved and has been shown in vitro to nucleate directly de novo actin polymerization and to stimulate F-actin accumulation in vivo (7, 13, 16–20). The existence of a third formin homology domain, FH3, is less well established (see below).

Formins can be subdivided into families based on their associated regulatory domains. The diaphanous-related formins (DRFs) are distinguished by the presence of two interacting regulatory domains, an N-terminal GTPase binding domain (GBD) and a C-terminal diaphanous autoregulatory domain (DAD) (19, 21, 22). One of the best-characterized DRFs is the mammalian homologue of diaphanous, mDia1. Recent in vitro functional and structural studies of mDia1 have subdivided the N-terminal GBD/FH3 into 4 subdomains: GBD (residues 69–129); the diaphanous inhibitory domain (DID) (residues 129–369); N-terminal dimerization domain (DD) (residues 377–452); coiled-coil (residues 452–568) (23–26). In the autoinhibited conformation the FH1 domain is thought to serve as a flexible hinge that allows the N-terminal DID and C-terminal DAD to interact. In this conformation the core conserved DAD sequence (aa 1181–1191) makes contact with the concave DAD binding pocket in DID, but complete autoregulation is dependent upon a tight interaction between DID and a series of basic residues (aa 1192–1196) immediately C-terminal to the DAD core (19, 25, 27). Both DID and DAD are highly conserved among mDia1, -2, and -3 and the related proteins DAAM1 and -2 (25). A recent report has also suggested that the N terminus contains a Rho-independent membrane targeting domain that is also subject to DID/DAD autoregulation (28).

The presence of two dimerization domains in mDia1, the N-terminal DD and the C-terminal FH2, has called into question previous models which suggest that DRFs in the autoinhibited conformation are monomeric (15, 23, 25, 29). In addition, diaphanous autoregulatory domain; DID, diaphanous inhibitory domain; DD, dimerization motif; CC, coiled-coil region; aa, amino acids; DMEM, Dulbecco’s modified Eagle’s medium; GST, glutathione S-transferase; NLS, nuclear localization signal; LPA, lysophosphatidic acid; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; RBD, Rho binding domain; ROCK, Rho-associated coiled-coil forming kinase.

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the individual contributions of the N-terminal subdomains toward mDia1 autoregulation have not been addressed in the context of the full-length protein.

We report here the results of an in vivo structure/function analysis of the mDia1 N terminus using in part the MAL/SRF transactivation pathway as a quantitative functional assay. We showed previously that activation of the MAL/SRF pathway occurs in response to depletion of the cellular G-actin pool (11–13) and that activation of a MAL/SRF-dependent reporter gene serves as an accurate and quantitative measure of mDia1 activity (13, 30). Using this assay we show that the DD is required for autoregulation of mDia1 in vivo. We also found that DD is likely required for the GBD-independent targeting of the N terminus to the plasma membrane and that this domain forms homo- but not heterooligomers. In contrast, we show that the DID/DAD interaction is able to mediate interaction between closely related DRF proteins and is able to act in trans to inhibit their activity. Finally we provide data that are supportive of a model where autoinhibited DRFs form dimeric or higher order structures.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The constructs pMLV-LacZ, p3D.A-Luc, pEF-SRFVP16, RhoA.V14, rac1.V12, cdc42.V12, mDia1 F1F2+C, F1F2, ΔDAD, ΔRBD.DID and mDia2 F1F2+C, F1F2 were described previously (11, 13, 19, 30). The constructs mD1N (codons 1–568), mD1NΔRBD (129–568), mD1N.DD/CC (353–568), mD1NΔDID (1–369), mD1N.DID (129–369), mD1NΔΔDID (258–369), ΔRBD (129–1255), ΔDD (1–1255Δ377–452), ΔDDΔDAD (1–1192Δ377–452), and mD2N (1–531) and mD2NΔRBD (148–531) were generated by PCR using standard techniques and subcloned into the FLAG and Myc epitope-tagged vectors pEF-FLAG and pEFNRSS (31) as indicated. GST-DAD contains codons 1116–1255 of mDia1 cloned into pGEX.6P2. pEFN.Cherry is a mammalian expression vector derivative of pRSetb.Cherry (Dr. Roger Tsien).

**Immunofluorescence**—Immunocytochemistry was performed as described previously (13). Briefly, NIH3T3 cells were transfected with the plasmids indicated in the figure legends and maintained in 0.5% fetal calf serum in DMEM, fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS), and permeabilized in 0.3% Triton-X-100 in PBS. F-actin was labeled with fluorescein phalloidin (Molecular Probes F432), FLAG-tagged proteins were detected with anti-FLAG M2 (Sigma F3165), and Myc-tagged proteins with rabbit anti-Myc (Sigma C3956). Secondary antibodies used were Alexa350 anti-rabbit (Molecular Probes A21049) and Alexa594 anti-mouse (Molecular Probes A21207). Raw264.7 cells were cultured in 5% CO₂, 10% fetal calf serum in DMEM and transfected using Lipofectamine 2000 according to the supplied protocol. Optical sections in Figs. 3 and 4 were captured with a 63× oil immersion objective using the apotome on a Zeiss Axioimager.Z1.

**Immunoprecipitations**—Co-immunoprecipitation experiments were performed as previously described (30) using anti-FLAG beads (Sigma F2426). The precipitated proteins were detected by immunoblotting using M2 anti-FLAG peroxidase conjugate antibody (Sigma A8592) and rabbit anti-Myc (Sigma C3956 or Santa Cruz Sc-789) and anti-rabbit peroxidase conjugate antibodies (Sigma A6667).

**Reporter Gene Assays**—The SRF reporter gene assays were performed as previously described (13, 30). Activation induced by expression of the indicated proteins is presented as a percent of the activation induced by expression of a fixed amount of SRF-VP16 fusion protein included as a positive control standard in each experiment (50 ng of DNA/35-mm plate). For inhibition studies, activation of the SRF reporter gene in the absence of the dominant negative is set to 100% in each experiment. Transfection efficiency is standardized to expression of β-galactosidase from a co-transfected MLV-LacZ reporter (250 ng/35-mm plate).

**Actin Polymerization Assays**—The in vitro actin polymerization assays were performed as previously described (30). mDia1 F1F2+C and mDia2 F1F2+C were cloned into pET30a and purified as His-tag proteins from Escherichia coli strain Rosetta BL21.DE3 using Ni²⁺ affinity resin. mD1N was cloned into pGEX.6P2, expressed in BL21, and purified by glutathione affinity chromatography and cleavage of the GST moiety using prescission protease. Protein concentrations were determined using A₂₈₀ and molar extinction coefficients obtained from the ExPASy website (mD1N = 16,305; mD1.F1F2+C = 21,805; mD2.F1F2+C = 25,035). N- and C-terminal derivatives were preincubated for 5 min at room temperature before initiation of polymerization. Final concentrations are as noted in Fig. 9. IC₅₀ values were calculated by plotting the polymerization rate from the linear section of each curve and interpolating the concentration of mD1N that would yield half-maximal inhibition.

**RESULTS**

To study the organization of the mDia1 N-terminal regulatory domain in more detail, we examined mDia1 derivatives lacking only RBD (aa residues 1–129), DD (aa 377–452), or DD and DAD (1–1192Δ377–452) (Fig. 1C). The previously described constitutively active mDia1 derivatives ΔRBDΔDID (263–1255) and ΔDAD (1–1192) were included for comparison. These derivatives were expressed by transient transfection in NIH3T3 cells, and the effect on activation of the co-transfected SRF reporter gene 3D.Aluc was assayed. This reporter is activated via the actin/MAL/SRF pathway in response to changes in actin treadmilling dynamics and serves as a rapid and quantitative measure of mDia1 activity (13, 30). As expected, expression of mDia1 ΔRBD had no effect on reporter gene activation, consistent with the DID/DAD interaction remaining intact in this derivative. In contrast, expression of ΔRBDΔDID, ΔDAD, or ΔDADΔDAD all induced potent activation of the SRF reporter gene (Fig. 1A). Expression of ΔDD also induced activation of the SRF reporter even though this deletion does not remove any residues that are predicted to be involved in the DID/DAD regulatory interaction (23–25). To determine whether the ΔDD deletion disrupted the structure of the adjacent DID, we performed GST-DAD pulldown experiments using lysates from transiently transfected NIH3T3 cells. ΔDAD served as a positive control and was efficiently captured by GST-DAD. Full-length mDia1 was very weakly captured by GST-DAD and only visible on very long exposures. Similarly, ΔDD was also poorly captured by GST-DAD. To determine
whether this was due to competition with the intact DAD of ΔDD, we performed the same pulldown using ΔDDDΔDAD. In this case ΔDDDΔDAD was captured with similar efficiency to ΔDAD, suggesting that the ΔDD mutation has not disrupted the conformation of DID. The negative control ΔRBDΔDID was not detectable in the GST-DAD pulldown even upon long exposures of the blot.

We used immunofluorescence to extend these observations and show directly the effect of expression of these proteins on actin polymerization in vivo (Fig. 2). As in the SRF assay, expression of mDia1 ΔRBD, like full-length mDia1, had no effect on F-actin accumulation in transfected cells (Fig. 2). Expression of mDia1 ΔDD, ΔRBDΔDID, ΔDDDΔDAD, and ΔDAD all induced the formation of actin stress fibers but of differing appearance (Fig. 2). ΔRBDΔDID induced robust thin stress fibers that run from end to end of the cell (Fig. 2). ΔDDDΔDAD and ΔDAD induced thin stress fiber formation as well as numerous cellular extensions and filopodia (Fig. 2, D and F), whereas ΔDD induced the formation of thick stress fibers with an obvious long axis of polarity (Fig. 2E). We also noticed a distinctive subcellular localization for each of these derivatives. Full-length mDia1, ΔRBD, and ΔDD all showed diffuse cytoplasmic staining with some puncta of protein apparent in full-length and ΔDD-expressing cells similar to the endogenous protein (32). In contrast ΔRBDΔDID was concentrated at the ends of stress fibers at the periphery of the cell, whereas ΔDAD and ΔDDDΔDAD seemed to be localized over the entire surface of the cell.

To further explore the requirements for mDia1 membrane targeting, we created a series of deletion derivatives of the isolated N terminus. We found that the cytoplasm of NIH3T3 cells
lacked sufficient depth to easily distinguish between cytoplasmic localization and membrane targeting (data not shown). Instead, we expressed these derivatives by transient transfection in Raw264.7 cells whose morphology enables a more obvious distinction between cytoplasm and membrane (28). mD1N (aa 1–568) was efficiently targeted to the membrane in all transfected cells and can be seen in optical sections as a ring around the cytoplasm (Fig. 3A). Co-expression of the Rho inhibitor C3

FIGURE 3. mDia1 N terminus targeting to the membrane has a Rho-independent component. The mDia1 derivatives mD1N and mD1NΔRBD were expressed in Raw264.7 cells in the presence and absence of C3 transferase. Cells were maintained in 10% fetal bovine serum, DMEM after transfection. pEF-mCherry was co-expressed as a non-targeted marker and is distributed throughout the nucleus and cytoplasm. A, left panel, mDia1N is recruited to the plasma membrane in all cells. B, inhibition of endogenous Rho signaling by co-expression of C3 inhibits membrane targeting of mD1N by roughly 50%, the remaining 50% are still membrane targeted (data not shown). C, left panel, mD1NΔRBD is recruited to the membrane in roughly half of the transfected cells. D, left panel, membrane targeting of mD1NΔRBD is unaffected by co-expression of C3. E, quantification of percent of cells with mD1N derivatives targeted to the membrane in the presence and absence of C3 (mD1N n = 127; mD1N+C3 n = 321; mD1NΔRBD n = 342; mD1NΔRBD+C3 n = 485). See Fig. 5D for a schematic of mDia1 derivatives.

FIGURE 4. Rho-independent membrane targeting is mediated by DD/CC. The mDia1 derivatives mD1N.DD/CC, mD1NΔDD, and mD1N.DID were expressed in Raw264.7 cells in the presence and absence of C3 transferase. Cells were maintained in 10% fetal bovine serum DMEM after transfection. pEF-mCherry was co-expressed as a non-targeted marker and is distributed throughout the nucleus and cytoplasm. A, mD1N.DD/CC is localized to the membrane in about one-fifth of transfected cells. B, in the remaining cells mD1N.DD/CC exhibits diffuse cytoplasmic staining and is excluded from the nucleus. C, mD1NΔDD is recruited to the membrane in roughly one-fifth of transfected cells. D, in the remaining cells mD1NΔDD exhibits a punctate cytoplasmic staining and is largely excluded from the nucleus. E, mD1N.DID is distributed diffusely throughout the cytoplasm and nucleus in all cells. F, co-expression of C3 transferase inhibits membrane targeting of mD1NΔDD but not mD1N.DD/CC (mD1N.DD/CC n = 213; mD1N.DD/CC+C3 n = 233; mD1NΔDD n = 238; mD1NΔDD+C3 n = 200; mD1N.DID n = 202; mD1N.DID+C3 n = 210). See Fig. 5D for a schematic of mDia1 derivatives.
DID/DAD Interaction between DRFs

FIGURE 5. DID, DAD, and the N-terminal dimerization domain are all required for efficient inhibition in trans. A, activation of the SRF reporter gene 3D.Aluc was assayed after expression of the indicated mDia1 derivatives (F1F2+C 0.1 μg; N-terminal derivatives 1.0 μg; C transferase 0.1 μg). The N-terminal deletion derivatives are all expressed to similar levels as determined by immunoblotting (data not shown). Activation by F1F2+C is completely inhibited by co-expression of derivatives containing DID and DD but only partially inhibited by DID alone. B, this inhibition is dependent upon the presence of DAD in the C-terminal derivative (F1F2 0.1 μg; 129–568 1.0 μg). Expression of the N-terminal mDia1 derivatives alone did not activate the SRF reporter (data not shown). C, as in A and B, inhibition by mDia1ΔRBD of mDia1-induced SRF activation is dependent on DAD (ΔRBDΔDD 0.1 μg; ΔDD 0.1 μg; Δ63 0.1 μg). D, schematic of mDia1 derivatives.

transferase interferes with this localization, resulting in loss of membrane targeting in roughly 50% of transfected cells (Fig. 3, B and E). mD1NΔRBD (aa 129–568), which lacks the RBD, was targeted to the membrane in half of the transfected cells (Fig. 3, C and E), and this targeting was unaffected by co-expression of C3 (Fig. 3, D and E). The red fluorescent protein mCherry was co-expressed as a space-filling marker and was distributed throughout the cytoplasm and nucleus in all cells (Fig. 3, A–D).

To determine which subdomain of the N terminus is required for Rho-independent membrane targeting, we generated the additional deletion derivatives mD1N.DD/CC (aa 354–568), mD1NΔDD (aa 1–369), and mD1N.DID (aa 129–369). mD1N.DD/CC was targeted to the membrane in ~15% of transfected cells (Fig. 4A), whereas in the remaining cells it was found to be distributed diffusely throughout the cytoplasm and excluded from the nucleus (Fig. 4A). mD1NΔDD was also membrane targeted in ~20% of transfected cells (Fig. 4C) with the remainder exhibiting a punctate cytoplasmic distribution of the protein that was largely excluded from the nucleus (Fig. 4D). mD1N.DID was distributed diffusely throughout the cytoplasm and nucleus in all transfected cells. Co-expression of C3 had no effect on mD1N.DD/CC membrane targeting but completely eliminated membrane localization of mD1NΔDD (Fig. 4F). C). Expression of the N-terminal derivatives alone did not activate the SRF reporter gene (data not shown). mD1NΔRBD is also able to inhibit the activity of the longer constitutively active derivatives ΔRBDΔDD, ΔDD, and ΔDAD (Fig. 2C), demonstrating that ΔDD is competent for regulation by the DID/DAD interaction if DID-DD is supplied in trans.

The SRF reporter gene assay results were corroborated by immunofluorescence. mD1.F1F2+C was co-expressed with the N-terminal deletion derivatives by transient transfection in NIH3T3 cells. mD1.F1F2+C induces the formation of thin stress fibers and, surprisingly, accumulates in the nucleus of transfected cells (Fig. 6C, D, and N, and see “Discussion”). mD1.F1F2+C nuclear localization is dependent on a cryptic C-terminal nuclear localization sequence (NLS); the related mDia1 derivative F1F2 is found primarily in the cytoplasm (Fig. 6F). The DD/CC region acts as a cytoplasmic anchor to prevent mDia1 nuclear localization (Figs. 2C and 6, A and M). The activity of the C-terminal NLS is also subject to autoinhibition. Co-expression of mD1N or mD1NΔRBD strongly inhibits the formation of F1F2+C-induced stress fibers and also prevents F1F2+C nuclear accumulation (Fig. 6, G–I and N). Similarly, co-expression of any of the DID-containing N-terminal derivatives of mDia1 largely prevented F1F2+C nuclear localization (Fig. 6, J–L and G).

These results suggest that the DD/CC subdomain mediates Rho-independent membrane localization of the mDia1 N terminus. We also made use of this series of N-terminal deletion derivatives to further investigate which subdomains are required for efficient autoinhibition in trans. mDia1 F1F2+C was expressed in the absence or presence of mDia1 N-terminal derivatives mD1N, mD1NΔRBD, mD1N.DD/ CC, mD1NΔDD, mD1N.DID, and mD1NΔDID, and the effects on activation of SRF-dependent transcription were determined (Fig. 5A). Expression of mD1.F1F2+C alone induced potent activation of the SRF reporter gene. This activation is completely inhibited by co-expression of mD1N or mD1NΔRBD. However, derivatives lacking an intact DID or DD (mD1N.DD/ CC, mD1NΔDD, mD1N.DID, and mD1NΔDID) had a greatly reduced ability to inhibit F1F2+C activity (Fig. 5A), consistent with the results obtained in the context of full-length mDia1 (Fig. 1A). As expected, this inhibition is dependent on the presence of DAD as the activity of mDia1 derivatives that lack DAD are not affected by co-expression of 129–568 (Fig. 5, B and E).
RhoA activity. We previously proposed that mDia1 is an essential mediator of Rho-induced SRF activation (11, 13, 31). To provide further support for this model, we examined the effect on SRF activation of co-expression of mD1N and mD1N\_RBD with constitutively active RhoA.V14, rac1.V12, and cdc42.V12. For comparison we also co-expressed the equivalent regions of mDia2, mD2N (aa 1–531), and mD2N\_RBD (aa 148–531). As expected, expression of mD1N, mD1N\_RBD, mD2N, and mD2N\_RBD all strongly inhibited RhoA.V14-induced SRF activation (Fig. 7A), presumably by binding to and inactivating endogenous mDia1; mDia2 is reported not to be expressed in NIH3T3 cells (20). Also as expected, mD1N\_RBD and mD2N\_RBD expression had no effect on rac.V12-induced SRF activation (Fig. 7A). However, expression of mD1N or mD2N strongly inhibited rac.V12 activity in this assay. Similar results were obtained with cdc42: expression of mD1N or mD2N completely inhibited cdc42 activity, whereas expression of mD1N\_RBD or mD2N\_RBD had a more moderate inhibitory effect (Fig. 7A). The inhibitory effects of mD1N and mD2N are likely not caused by titration of endogenous RhoA activity as co-expression of the Rho-inhibitor C3 transferase did not have a significant effect on rac- or cdc42-induced SRF activation (see “Discussion”).

We wished to extend these observations and examine the effects of the inhibitory mDia1 N-terminal derivatives on the RhoA-dependent activation of SRF in response to lysophosphatidic acid (LPA) stimulation (11, 33). As expected, expression of mD1N completely abolished LPA-induced SRF activation (Fig. 7B). However, despite the ability of mD1N\_RBD to inhibit RhoA.V14-induced activation of SRF, its expression only reduced LPA-induced activation of the SRF reporter gene by ~40% (Fig. 7B). Pretreatment with ROCK inhibitor alone was also unable to completely inhibit LPA-induced activation of SRF and resulted in a ~60% reduction in activation of the SRF reporter gene. However, in combination with mD1N\_RBD expression, pretreatment with ROCK inhibitor resulted in complete inhibition of LPA-induced activation of SRF. Pretreatment with MEK

nuclear accumulation (Fig. 6N). Expression of mD1N or mD1N\_RBD has no effect on F1F2-induced stress fiber formation (data not shown).

We noted that expression of mD1N completely eliminated stress fiber formation (Fig. 6G), similar to the effects of expression of the Rho-inhibiting exoenzyme C3 transferase (13). This suggests that mD1N acts in part to titrate out endogenous RhoA activity. We previously proposed that mDia1 is an essential mediator of Rho-induced SRF activation (11, 13, 31). To provide further support for this model, we examined the effect on SRF activation of co-expression of mD1N and mD1N\_RBD with constitutively active RhoA.V14, rac1.V12, and cdc42.V12. For comparison we also co-expressed the equivalent regions of mDia2, mD2N (aa 1–531), and mD2N\_RBD (aa 148–531). As expected, expression of mD1N, mD1N\_RBD, mD2N, and mD2N\_RBD all strongly inhibited RhoA.V14-induced SRF activation (Fig. 7A), presumably by binding to and inactivating endogenous mDia1; mDia2 is reported not to be expressed in NIH3T3 cells (20). Also as expected, mD1N\_RBD and mD2N\_RBD expression had no effect on rac.V12-induced SRF activation (Fig. 7A). However, expression of mD1N or mD2N strongly inhibited rac.V12 activity in this assay. Similar results were obtained with cdc42: expression of mD1N or mD2N completely inhibited cdc42 activity, whereas expression of mD1N\_RBD or mD2N\_RBD had a more moderate inhibitory effect (Fig. 7A). The inhibitory effects of mD1N and mD2N are likely not caused by titration of endogenous RhoA activity as co-expression of the Rho-inhibitor C3 transferase did not have a significant effect on rac- or cdc42-induced SRF activation (see “Discussion”).

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The relatively inefficient recovery of endogenous mDia1 in the presence of mDiaN or mDiaN RBD inhibitor had no effect on LPA stimulation either on its own or for co-immunoprecipitation of endogenous mDia1 from total lysate; %.

We used in vitro actin polymerization assays to provide further confirmation of the cross-regulatory interaction between mDia1 and mDia2. Purified mD1N.F1F2+C protein strongly stimulated actin polymerization in vitro (Fig. 9A). Preincubation of mD1N.F1F2+C with mDiaN inhibited its ability to activate actin polymerization in a concentration-dependent manner. The interaction of mDiaN with mD1N.F1F2+C is not able to completely inhibit mD1N.F1F2+C activity (Fig. 9A). This was not the case for mD2N.F1F2+C, where preincubation with a 3:1 molar ratio of mDiaN to mD2N.F1F2+C reduced polymerization to the base line of actin alone (Fig. 9B).

Finally we wished to determine whether the DID/DAD interaction could mediate heterodimerization between full-length mDia1 and mDia2. Co-immunoprecipitation experiments were performed on lysates from NIH3T3 cells co-expressing FLAG-tagged mDia1 and Myc-tagged mDia1 or mDia2. C3 transferase was also co-expressed to down-regulate endogenous Rho-
signaling, thereby maximizing the potential for a DID/DAD interaction. As shown in Fig. 10, FLAG-tagged mDia1 is able to co-immunoprecipitate both mDia1 and mDia2, showing that the full-length proteins are able to associate as hetero-oligomers.

**DISCUSSION**

We investigate here for the first time the function of individual subdomains of the N terminus of mDia1 in the context of the full-length protein. Our data provide in vivo support for the subdivision of the mDia1 N terminus into four subdomains: GBD (aa 1–129), DID (aa 129–369), DD (aa 369–452), and CC (aa 452–568) as has previously been proposed based on results obtained in vitro. In addition, our results suggest that each of these domains plays an important role in subcellular localization and auto-regulation of mDia1.

**Subcellular Localization**—The deletion derivative mD1N contains both the Rho-dependent and Rho-independent components for membrane targeting (28) and is targeted to the membrane in 100% of transfected cells (Fig. 3E). Deletion of the GBD eliminates the Rho-dependent component of membrane targeting (Figs. 3D and 4B), whereas deletion of DD/CC eliminates Rho-independent targeting (Fig. 4D). Our results do not exclude the possibility that DD/CC membrane localization is mediated by dimerization with endogenous mDia1. However, if this were the case, then it might be expected that this localization would also be Rho-dependent; therefore, we favor a model where DD/CC is recruited to the membrane by an additional unknown factor. A potential candidate for this factor is IQGAP1 (34), although the reported minimal IQGAP1 binding domain of mDia1 (aa 256–346) (34) does not overlap with DD/CC (aa 369–568). In isolation both Rho-dependent and Rho-independent targeting is relatively inefficient. This can be attributable in part to misfolding of the smaller deletion derivatives, but efficient membrane targeting is also likely to be dependent upon a complex of RBD bound to Rho.GTP and DD/CC bound to membrane factor X.

We were also struck by the efficient targeting of C-terminal derivatives of mDia1 to the nucleus. This targeting is dependent upon an NLS C-terminal to the FH2 domain (Fig. 6F). The NLS can be localized between residues 1192–1255 as a similar construct, ΔN3 (residues 543–1192), is largely cytoplasmic (Ref. 19 and data not shown). The function of this NLS is subject to autoregulation (Fig. 6, G and M) and can also be masked by the presence of an N-terminal cytoplasmic anchor (Fig. 6, A and M). This anchor likely overlaps with the Rho-independent membrane targeting activity located in the DD/CC subdomain. ΔRBDΔDID (aa 263–1255) is excluded from the nucleus (Figs. 2C and 6M), whereas a shorter deletion derivative, ΔRBDΔDID2 (353–1255), is not as efficiently maintained in the cytoplasm (Fig. 6, A and M). Further deletion of DD/CC results in...
mDia1 nuclear localization in the majority of transfected cells (Fig. 6, D, M, and N).

Why mDia1 or other formins should be targeted to the nucleus is unclear. It is worth noting that other formins have been reported to be translocated to the nucleus in specific circumstances. Caspase cleavage products of the formin FHOD1 are targeted to the nucleus during apoptosis (35). The C-terminal region of Formin1 contains an NLS (36, 37), and we find that mDia2 also has a cryptic NLS C-terminal to the FH2 domain (data not shown). Fozi-1, a DNA binding transcription factor, contains a divergent FH2 domain (38). On this basis it has been proposed recently that formins may act to regulate transcriptional machinery (25). It should be noted, however, that mDia1 nuclear localization is not required for activation of SRF-dependent transcription (13).

**Autoregulation**—We show here that DD is required for efficient autoinhibition of the full-length protein (Figs. 1A and 2E). We also show that, in agreement with previous in vitro studies (26), the N-terminal DD is required in vivo for efficient inhibition of the isolated C terminus by the isolated N terminus (Figs. 5A and 6K). It is not clear, however, why N-terminal dimerization would be required in the context of the full-length protein. Our DD deletion does not disrupt the structure of the adjacent DID (Fig. 1B). Nor is it likely that deletion of DD disrupts the hinge region of the protein such that the C-terminal DAD is unable to reach the N-terminal DID. Current models of mDia1 structure suggest that it is FH1 that serves as the flexible hinge assembly rate by mD1.F1F2 + C and mD2.F1F2 + C strongly induces actin polymerization and is completely inhibited by a 3-fold excess of mD1N to the base line of actin alone. A.U., fluorescence. B, mDia2 F1F2 + C strongly induces actin polymerization and is completely inhibited by a 3-fold excess of mD1N to the base line of actin alone. C, maximum actin assembly rate by mD1.F1F2 + C and mD2.F1F2 + C versus the concentration of mD1N. The IC_{50} of mD1N for mD1.F1F2 + C is -37.5 nM, and for mD2.F1F2 + C the IC_{50} is ~56 nM.

The sensitivity of mDia1 autoregulation to N-terminal dimerization may also be reflected in the results of the in vitro actin polymerization assays shown in Fig. 9. mDia1.F1F2 + C activity is maximally inhibited by a 1:1 molar ratio of mD1N (Fig. 9A). However, even a 10-fold molar excess of mD1N is unable to completely inhibit mD1.F1F2 + C to the base line of actin alone, consistent with previous results (18). This is not an artifact of the assay conditions as a 3-fold molar excess of mD1N is able to drive mD2.F1F2 + C activity to the base line of mD1N (Fig. 9A). However, even a 10-fold molar excess of mD1N is unable to completely inhibit mD1.F1F2 + C to the base line of actin alone, consistent with previous results (18). This is not an artifact of the assay conditions as a 3-fold molar excess of mD1N is able to drive mD2.F1F2 + C activity to the base line of actin alone (Fig. 9B). These results are consistent with mD1N having a higher affinity for mD1.F1F2 + C than for mD2.F1F2 + C but with the mD1N:mD1.F1F2 + C complex having a faster on/off
rate. The transient nature of the DID/DAD interaction may also be reflected in the results obtained in Fig. 1B. In this case we found that ΔDD was not efficiently captured by GST-DAD and that this is apparently due to competition with the DAD of ΔDD. However, ΔDD is constitutively active (Figs. 1A and 2E). This may reflect a short-lived complex between the DID and DAD of ΔDD, which is sufficient to out-compete exogenous GST-DAD but sufficiently transient to render ΔDD constitutively active in our assays. If this is so, then even in the context of the full-length protein, the additional constraint provided by N-terminal dimerization may be required to stabilize interactions between DID and FH2-DAD to maintain the protein in an autoinhibited conformation.

**mDia1/mDia2 Dimerization**—A dimeric model for the auto-regulated complex is also consistent with our finding that full-length mDia1 is able to co-immunoprecipitate full-length mDia2 (Fig. 10). This interaction is most likely mediated by the DID of one protein binding to the DAD of the other as neither DD nor FH2 form heterodimers in our assays (Fig. 8) (30). We found that in trans the regulatory domains of mDia1 and mDia2 were able to interact and cross-regulate (Figs. 8 and 9), but this cross-regulation did not extend to the closely related DRF DAAM1 (Fig. 8). Thus, cross-regulation between DRFs is likely to be restricted to closely related family members. These results demonstrate the potential for generating interfering formin derivatives that only target the activity of specific formin subfamilies.

The *in vivo* significance of the mDia1/mDia2 interaction is not clear. We were unable to efficiently co-immunoprecipitate endogenous mDia1 with mDia2 from lysates of Raw264.7 cells (data not shown). It should be noted, however, that the anti-Dia2 antibody used in these experiments binds to the C-terminal DAD region and reportedly recognizes both mDia2 and mDia3 (39, 40). This may have had an effect on our ability to capture an endogenous DID/DAD complex of mDia1 and mDia2. Potentially the formation of heterodimers of closely related DRFs may allow for more stringent regulation of DRF activity (e.g. more than one input required for activation) and/or unique subcellular targeting of the DRF complex.

**N-terminal Interfering Derivatives of mDia1**—We used expression of mD1N and mD1NΔRBD to test our model that mDia1 is an essential effector of RhoA-induced activation of the MAL/SRF pathway in NIH3T3 cells (11–13, 31, 41). Consistent with our model, expression of mD1N or mD1NΔRBD is sufficient to inhibit Rho.V14-induced activation of a MAL/SRF reporter gene (Fig. 7A). The inhibitory effect of mD1NΔRBD is likely mediated through its interaction with endogenous mDia1, and mD1NΔRBD is able to co-immunoprecipitate endogenous mDia1 from lysates of cells expressing Rho.V14 (Fig. 7C). Surprisingly, mD1NΔRBD is unable to strongly repress LPA-induced SRF activation (Fig. 7B) even though this activation is entirely Rho-dependent (Fig. 7B) (33). mD1NΔRBD expression does, however, sensitize the pathway to the requirement for ROCK activity. ROCK inhibitor alone does not completely inhibit LPA stimulation, but ROCK inhibitor in combination with mD1NΔRBD expression completely blocks LPA-stimulated SRF activation (Fig. 7B). This, it appears that downstream of Rho.V14, the mDia1 effector pathway predominates, but when Rho is activated by extracellular stimuli then either the ROCK or mDia1 effector pathway is sufficient to induce SRF activation. In part this may represent the Rho-independent activation of ROCK in response to extracellular stimuli (42). It has also been noted that the coiled-coil domain of ROCK shares some homology with the yeast protein Bud6. The activities of ROCK and mDia1 are tightly linked (19); thus, it is intriguing to speculate that ROCK may also act to enhance mDia1 activity similar to the stimulation of Bni1 activity by Bud6 (43, 44).

To our surprise expression of mD1N or mD2N was sufficient to inhibit SRF activation induced by rac.V12 or cdc42.V12 (Fig. 7A). This effect is not due to inhibition of endogenous Rho as rac.V12- and cdc42.V12-induced SRF activation is much less sensitive to expression of the Rho inhibitor C3 transferase. We were able to co-immunoprecipitate Rho.V14, but not rac.V12 or cdc42.V12, with mD1N (data not shown). Still, in the case of rac.V12, the data is most consistent with the idea that a transient interaction between rac and the GBD of mDia1 or mDia2 is sufficient to block rac activity. Expression of mD1NΔRBD and mD2NΔRBD partially inhibits cdc42 activity to a similar degree as C3 expression. This is consistent with a model where mDia2 acts in part to activate RhoA, as reported in other cell types (45), although we cannot exclude that cdc42 may activate endogenous mDia1 or mDia3 (39, 46, 47).

In conclusion, our data provide *in vivo* support for subdivision of the mDia N terminus into four domains, GBD (1–129), DID (129–369), DD (369–452), and CC (452–568), and suggest that each of these domains plays an important role for mDia1 autoregulation and subcellular localization. In addition, our results suggest a more complex model of DRF regulation where autoinhibited DRFs are in dimeric or higher order configurations. The ability of DID and DAD to regulate the activity of closely related DRF family members suggests that DRF hetero-oligomerization may allow for differential targeting and activation of specific DRF pairs to unique sites of action within the cell.

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**REFERENCES**

1. Higgs, H. N., and Peterson, K. J. (2005) *Mol. Biol. Cell* **16**, 1–13
2. Chang, F., Drubin, D., and Nurse, P. (1997) *J. Cell Biol.* **137**, 169–182
3. Evangelista, M., Poyne, D., Amberg, D. C., Boone, C., and Bretscher, A. (2002) *Nat. Cell Biol.* **4**, 260–269
4. Feierbach, B., and Chang, F. (2001) *Curr. Biol.* **11**, 1656–1665
5. Kobielak, A., Pasolli, H. A., and Fuchs, E. (2004) *Nat. Cell Biol.* **6**, 21–30
6. Pellegrin, S., and Mellor, H. (2005) *Curr. Biol.* **15**, 129–133
7. Sagot, I., Rodal, A. A., Moseley, J., Goode, B. L., and Pellman, D. (2002) *Nat. Cell Biol.* **4**, 626–631
8. Schirenbeck, A., Brettschneider, J., Arasada, R., Schleicher, M., and Faix, J. (2005) *Nat. Cell Biol.* **7**, 619–625
9. Severson, A. F., Baillie, D. L., and Bowerman, B. (2002) *Curr. Biol.* **12**, 2066–2075
10. Ishizaki, T., Morishima, Y., Okamoto, M., Furuyashiki, T., Kato, T., and Narumiya, S. (2001) **Nat. Cell Biol.** **3**, 8–14
11. Sotiropoulos, A., Ginettiis, D., Copeland, J., and Treisman, R. (1999)
DID/DAD Interaction between DRFs

12. Miralles, F., Posern, G., Zaromytidou, A. I., and Treisman, R. (2003) *Cell* **113**, 329–342
13. Copeland, J. W., and Treisman, R. (2002) *Mol. Biol. Cell* **13**, 4088–4099
14. Wallar, B. J., and Alberts, A. S. (2003) *Trends Cell Biol.* **13**, 435–446
15. Kovar, D. R. (2006) *Curr. Opin. Cell Biol.* **18**, 11–17
16. Pruyne, D., Evangelista, M., Yang, C., Bi, E., Zigmond, S., Bretscher, A., and Boone, C. (2002) *Science* **297**, 612–615
17. Kovar, D. R., Kuhn, J. R., Tichy, A. L., and Pollard, T. D. (2003) *J. Cell Biol.* **161**, 875–887
18. Li, F., and Higgs, H. N. (2003) *Curr. Biol.* **13**, 1335–1340
19. Watanabe, N., Kato, T., Fujita, A., Ishizaki, T., and Narumiya, S. (1999) *Nat. Cell Biol.* **1**, 136–143
20. Tominaga, T., Sahai, E., Chardin, P., McCormick, F., Courtnidge, S. A., and Alberts, A. S. (2000) *Mol. Cell* **5**, 13–25
21. Watanabe, N., Madaule, P., Reid, T., Ishizaki, T., Watanabe, G., Kakizuka, A., Saito, Y., Nakao, K., Jockusch, B. M., and Narumiya, S. (1997) *EMBO J.* **16**, 3044–3056
22. Alberts, A. S. (2001) *J. Biol. Chem.* **276**, 2824–2830
23. Rose, R., Weyand, M., Lammers, M., Ishizaki, T., Ahmadian, M. R., and Wittinghofer, A. (2005) *Nature* **435**, 513–518
24. Otomo, T., Otomo, C., Tomchick, D. R., Machius, M., and Rosen, M. K. (2005) *Mol. Cell* **18**, 273–281
25. Nezami, A. G., Poy, F., and Eck, M. J. (2006) *Structure* **14**, 257–263
26. Li, F., and Higgs, H. N. (2005) *J. Biol. Chem.* **280**, 6986–6992
27. Wallar, B. J., Stropich, B. N., Schoenherr, J. A., Holman, H. A., Kitchen, S. M., and Alberts, A. S. (2006) *J. Biol. Chem.* **281**, 4300–4307
28. Seth, A., Otomo, C., and Rosen, M. K. (2006) *J. Cell Biol.* **174**, 701–713
29. Goode, B. L., and Eck, M. J. (2007) *Annu. Rev. Biochem.* **76**, 593–627
30. Copeland, J. W., Copeland, S. J., and Treisman, R. (2004) *J. Biol. Chem.* **279**, 50250–50256
31. Geneste, O., Copeland, J. W., and Treisman, R. (2002) *J. Cell Biol.* **157**, 831–838
32. Gomez, T. S., Kumar, K., Medeiros, R. B., Shimizu, Y., Leibson, P. J., and Billadeau, D. D. (2007) *Immunity* **26**, 177–190
33. Hill, C. S., Wynne, J., and Treisman, R. (1995) *Cell* **81**, 1159–1170
34. Brandt, D. T., Marion, S., Griffiths, G., Watanabe, T., Kaibuchi, K., and Grosse, R. (2007) *J. Cell Biol.* **178**, 193–200
35. Menard, I., Gervais, F. G., Nicholson, D. W., and Roy, S. (2006) *Apoptosis* **11**, 1863–1876
36. Chan, D. C., and Leder, P. (1996) *J. Biol. Chem.* **271**, 23472–23477
37. O’Rourke, D. A., Liu, Z. X., Sellin, L., Spokes, K., Zeller, R., and Cantley, L. G. (2000) *J. Am. Soc. Nephrol.* **11**, 2212–2221
38. Johnston, R. J., Jr., Copeland, J. W., Fasnacht, M., Etchberger, J. F., Liu, J., Honig, B., and Hobert, O. (2006) *Development* **133**, 3317–3328
39. Yasuda, S., Oceguera-Yanez, F., Kato, T., Okamoto, M., Yonemura, S., Terada, Y., Ishizaki, T., and Narumiya, S. (2004) *Nature* **428**, 767–771
40. Colucci-Guyon, E., Niedergang, F., Wallar, B. J., Peng, J., Alberts, A. S., and Chavrier, P. (2005) *Curr. Biol.* **15**, 2007–2012
41. Grosse, R., Copeland, J. W., Newsome, T. P., Way, M., and Treisman, R. (2003) *EMBO J.* **22**, 3050–3061
42. Feng, I., Ito, M., Kureishi, Y., Ichikawa, K., Amano, M., Isaka, N., Okawa, K., Iwamatsu, A., Kaibuchi, K., Hartshorne, D. J., and Nakano, T. (1999) *J. Biol. Chem.* **274**, 3744–3752
43. Glynn, J. M., Lustig, R. J., Berlin, A., and Chang, F. (2001) *Curr. Biol.* **11**, 836–845
44. Moseley, J. B., and Goode, B. L. (2005) *J. Biol. Chem.* **280**, 28023–28033
45. Li, Z., Dong, X., Wang, Z., Liu, W., Deng, N., Ding, Y., Tang, L., Hla, T., Zeng, R., Li, L., and Wu, D. (2005) *Nat. Cell Biol.* **7**, 399–404
46. Goulignari, P., Kitzing, T. M., Knieling, H., Brandt, D. T., Offermanns, S., and Grosse, R. (2005) *J. Biol. Chem.* **280**, 42242–42251
47. Faix, J., and Grosse, R. (2006) *Dev. Cell* **10**, 693–706