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The Diaphanous-related Formin mDia1 Controls Serum Response Factor Activity through its Effects on Actin Polymerization

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SRF-dependent transcription is regulated by the small GTPase RhoA via its effects on actin dynamics. The diaphanous-related formin (DRF) proteins have been identified as candidate RhoA effectors mediating signaling to SRF. Here we investigate the relationship between SRF activation and actin polymerization by the DRF mDia1. We show that the ability of mDia1 to potentiate SRF activity is strictly correlated with its ability to promote F-actin assembly. Both processes can occur independently of the mDia1 FH1 domain but require sequences in an extended C-terminal region encompassing the conserved FH2 domain. mDia-mediated SRF activation, but not F-actin assembly, can be blocked by a nonpolymerizable actin mutant, placing actin downstream of mDia in the signal pathway. The SRF activation assay was used to identify inactive mDia1 derivatives that inhibit serum- and LPA-induced signaling to SRF. We show that these interfering mutants also block F-actin assembly, whether induced by mDia proteins or extracellular signals. These results identify novel functional elements of mDia1 and show that it regulates SRF activity by inducing depletion of the cellular pool of G-actin.

INTRODUCTION

The formin proteins are involved in many actin-mediated processes controlling cell and tissue architecture, playing important roles in cell polarity, cell-cell interactions, gastrulation, and cytokinesis (Castrillon and Wasserman, 1994; Chang *et al.*, 1997). Formins are defined by two regions of homology to the mouse limb deformity proteins, FH1 and FH2 (Castrillon and Wasserman, 1994); many contain an additional triad of conserved motifs termed FH3 (Petersen *et al.*, 1998; for reviews see Wasserman, 1998; Zeller *et al.*, 1999). The proline-rich FH1 domain interacts with potential effector proteins including the actin-binding protein profilin (Evangelista *et al.*, 1997; Imamura *et al.*, 1997; Watanabe *et al.*, 1997), SH3 domain proteins such as the Src tyrosine kinase (Uetz *et al.*, 1996; Fujiwara *et al.*, 2000; Tominaga *et al.*, 2000; Satoh and Tominaga, 2001), and WW domain-proteins (Chan *et al.*, 1996). Similar domains are found in the WASP/Scar and Ena/VASP families of cytoskeletal regulators (Machesky and Insall, 1999). Both the FH1 and the FH2 domain, which is contained within a larger conserved region, appear involved in cytoskeletal reorganization, whereas the FH3 domain appears involved in subcellular localization (Petersen *et al.*, 1998; Ozaki-Kuroda *et al.*, 2001; Sharpless and Harris, 2002).

The Diaphanous-related formins (DRFs) constitute a subgroup of the formin family distinguished by the presence of two additional conserved domains: an N-terminal Rho GTPase-binding domain (RBD; Kohno *et al.*, 1996; Evangelista *et al.*, 1997; Watanabe *et al.*, 1997), and a C-terminal diaphanous autoregulatory domain (DAD; Watanabe *et al.*, 1999; Alberts, 2001). Rho GTPases regulate DRF activity by relieving an inhibitory interaction between these domains (Watanabe *et al.*, 1999; Alberts, 2001). The DRFs promote accumulation of F-actin structures in yeast (Feierbach and Chang, 2001; Evangelista *et al.*, 2002; Sagot *et al.*, 2002) and vertebrates (Watanabe *et al.*, 1997, 1999; Nakano *et al.*, 1999; Tominaga *et al.*, 2000). In vertebrate cells the mDia DRF family cooperate with the Rho effector kinase ROCK in stress fiber formation. The effect of mDia proteins on F-actin level has not been directly quantitated (see Ridley, 1999) but it is thought that mDia promotes F-actin accumulation, whereas ROCK controls filament bundling (Nakano *et al.*, 1999; Watanabe *et al.*, 1999; Tominaga *et al.*, 2000). It appears that in

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both yeast and vertebrates, the FH1 and FH2 domains are required for cytoskeletal function (Evangelista *et al.*, 1997; Watanabe *et al.*, 1999; Alberts, 2001). The mDia proteins also regulate the microtubule cytoskeleton via an unidentified mechanism (Ishizaki *et al.*, 2001; Palazzo *et al.*, 2001).

Recent studies have demonstrated a close link between the control of cytoskeletal organization and the activity of the transcription factor SRF, which regulates a large number of growth factor-inducible and muscle-specific genes (for overview see Arsenian *et al.*, 1998). Activation of SRF by serum mitogens such as LPA is RhoA dependent and requires alterations in actin dynamics (Hill *et al.*, 1995; Sotiropoulos *et al.*, 1999). Expression of the DRFs, and other proteins that regulate actin polymerization such as LIM kinase, can potentiate SRF activity, and antibodies against the mDia1 DRF can block serum-induced activity of SRF in NIH3T3 cells (Sotiropoulos *et al.*, 1999; Tominaga *et al.*, 2000; Mack *et al.*, 2001). These observations and the finding that overexpression of either wild-type actin or nonpolymerizable actin mutants can interfere with signaling to SRF led us to propose that SRF is somehow negatively regulated by the cellular G-actin pool (Sotiropoulos *et al.*, 1999; Posern *et al.*, 2003). However, studies of mDia2 mutants have suggested that SRF activation involves recruitment of Src and possibly other accessory proteins to the mDia2 FH1 domain (Tominaga *et al.*, 2000; Alberts, 2001).

To clarify the relationship between mDia activity, actin polymerization, and SRF activation, here we delineate the mDia1 domains required for SRF activation and compare them to those required for F-actin accumulation. Using the sensitive and quantitative SRF reporter assay, we show that sequences within the mDia1 C-terminal region are required for SRF activation. These sequences, which include both the core FH2 domain and two previously unidentified regions outside it, precisely colocalize with sequences required for induction of F-actin accumulation assessed using a FACS-based assay. We show that specific inactive mDia derivatives are capable of blocking SRF activation or cytoskeletal rearrangements induced by extracellular signals and that the activation of SRF by mDia1 is blocked by expression of a nonpolymerizable actin mutant. Our data show that the integrity of the mDia1 C-terminal sequences is required for F-actin assembly and strongly support a model in which DRF proteins control the activity of SRF through their ability to regulate actin polymerization.

MATERIALS AND METHODS

Plasmids

Expression plasmids encoding mDia1 ΔRBD1, ΔRBD2, ΔRBD3, ΔRBD3ΔC, FH3/M/FH1, F2ΔN1, F2+DAD, Δ39, and Δ63 (Watanabe *et al.*, 1999) were a generous gift from Shuh Narumiya. All other mDia1 plasmids were generated by standard procedures and expressed by derivatives of EFplink carrying N-terminal Flag, myc, or HA epitope tags (Sotiropoulos *et al.*, 1999). FH1/FH2 comprises mDia1 codons 567-1182; in F1F2Δ1 and F1F2Δ2, codons 750–770 or 946–989, respectively, are replaced with three alanine codons introducing a *Not*I site. FH2 is a truncated derivative of FH1/FH2 comprising mDia1 codons 736-1182; F2ΔN2 is an N-terminal truncation of FH2 encoding codons 771-1182; F2ΔC1 and F2ΔC2 are C-terminal truncations with stop codons at positions 1150 and 1130, respectively. In ΔRBDΔFH1 codons 567–737 in ΔRBD2 were replaced by a *Not*I site as above. FH3/M encodes mDia1 codons 258–567; FH1 encodes codons 567–738. The actin G13R mutation was introduced into the actin expression plasmid EF-Flag-Actin (Sotiropoulos *et al.*, 1999); other plasmids were as described: MLV-LacZ, (Sotiropoulos *et al.*, 1999); 3D.Aluc (Geneste *et al.*, 2002); pCSXH-CSK, pCXSH-SrcKD (Grosse *et al.*, 2000); NLex.ElkC (Marais *et al.*, 1993). LexOP-luc was as LexOP.tkCAT (Marais *et al.*, 1993) modified to express luciferase; pSG5-SrcY527F was a gift from Erik Sahai.

Transfections and Reporter Gene Assays

NIH3T3 cell were transfected using Lipofectamine (Invitrogen, Carlsbad, CA). For luciferase assays, cells were transfected with 0.1 μg 3DA.Luc, 0.5 μg reference plasmid MLV-LacZ, and expression plasmids as in the figure legends, and empty EFplink plasmid to make up a total of 3 μg DNA/6-cm dish. For activation experiments the transfected cells were maintained in 0.5% FCS and harvested 24 h later for standard luciferase assay (Promega, Madison, WI), with transfection efficiency standardized by β-galactosidase assay. Data were expressed relative to reporter activation by the constitutively active SRF derivative SRFVP16 (0.1 μg), included in every set of transfections. For interference assays, stimulation was 40 h after transfection; in these experiments reporter activity is presented as percent of activity in vector-only, stimulated controls.

Immunofluorescence

NIH3T3 cells were transfected as above, fixed in 4% formaldehyde/PBS, and permeabilized in 0.3% Triton X-100/PBS. Antibody binding was in 5% FCS/PBS for 1 h at 37°C. Primary antibodies were M2 anti-Flag (F3165; Sigma, Poole, Dorset, United Kingdom), and anti-9E10 (Cancer Research UK), at 1/100–1/1000 dilution. Secondary FITC- and TRITC-anti-mouse antibodies (F0479; DAKO, High Wycombe, United Kingdom; Sigma T2659) were used according to the manufacturer's recommendations. FITC- or TRITC-labeled phalloidin (Molecular Probes, Eugene, OR) was used at 33–66 nM.

F-Actin FACS Assay

For detergent extraction experiments transfected cells were fractionated as described (Lyubimova *et al.*, 1997), using M2 antibody to detect the transfected Flag-actin reporter. For FACS assay, transfected cells (2×10^6 cells, 16 μg DNA/15-cm dish) were trypsinized and fixed in 4% para-formaldehyde/PBS before permeabilization and staining for epitope tag as above using Cy3-conjugated secondary antibody (715–166-150; Jackson ImmunoResearch Laboratories, West Grove, PA); F-actin was detected with FITC-phalloidin as above. Mean cellular F-actin content, as determined by phalloidin staining, was quantified using the FACScan (Becton-Dickinson, Plymouth, United Kingdom), and plotted relative to that of nontransfected cells.

RESULTS

Activated Derivatives of mDia1

To identify regions of mDia1 required for activation of SRF, we used the SRF reporter gene 3D.ALuc, which contains a synthetic promoter consisting of three core SRF binding sites with an actin gene TATA box (Mohun *et al.*, 1987). This promoter is strongly activated by the Rho pathway but is unresponsive to stimuli that activate the Ternary Complex Factor family of SRF accessory proteins (Hill *et al.*, 1995). We first studied a number of mDia1 N- and C-terminal truncation mutants, some of which have been characterized previously in cytoskeletal assays (Watanabe *et al.*, 1999). Expression of full-length mDia1 did not activate SRF, but derivatives lacking the N-terminal RBD were highly active

both in the SRF reporter assay (Figure 1A, ΔRBD1; Sotiropoulos *et al.*, 1999; Tominaga *et al.*, 2000) and in assays for actin stress fiber formation (Watanabe *et al.*, 1999; Tominaga *et al.*, 2000). Removal of further N-terminal sequences, including the remaining FH3 motif and a coiled-coiled domain, had a small effect on SRF activation (Figure 1A, ΔRBD2, ΔRBD3 compare protein levels). Although these proteins contain the DAD domain, this was not required for SRF activation, which was unaffected by its removal (Figure 1A, compare ΔRBD3 with ΔRBD3ΔC, FH1/FH2). We also tested two mDia1 C-terminal truncation mutants. A mutant lacking the 39 C-terminal residues, which leaves the DAD region intact, did not significantly activate SRF; in contrast, truncation of the DAD by removal of the 63 C-terminal residues generated an activating form of the protein (Figure 1A, compare Δ39 and Δ63). Activated mDia1 did not potentiate transcriptional activity of the Elk-1 C-terminal transcriptional activation domain, which is regulated by MAP kinase phosphorylation (unpublished data).

We next investigated the roles of individual mDia1 protein domains. Expression of the FH1 domain, previously implicated in cytoskeletal reorganization (Nakano *et al.*, 1999; Watanabe *et al.*, 1999; Tominaga *et al.*, 2000), was not sufficient for SRF activation; other N-terminal segments of the protein, which are inactive in actin reorganization (Nakano *et al.*, 1999; Watanabe *et al.*, 1999), also failed to activate SRF at any concentration tested (Figure 1A, FH3/M, FH3/M/FH1, FH1; unpublished data). Surprisingly, precise excision of the FH1 domain from the N-terminal mDia1 truncation ΔRBD2 reduced but did not abolish SRF activation (Figure 1A, compare ΔRBD2 with ΔRBDΔFH1). Further truncation of the FH1+FH2 derivative, to the FH2 region alone, reduced but did not eliminate SRF activation (Figure 1A, compare FH1/FH2 with FH2): as expression plasmid inputs were titrated to equalize protein expression levels, SRF activation by FH2 approached nearly 50% of that achieved by FH1/FH2 (Figure 1B).

Next we tested whether the different activated mDia1 derivatives all activate SRF independently of functional RhoA by coexpressing them with C3 transferase, which ADP-ribosylates and inactivates RhoA. Expression of C3 transferase is sufficient to reduce serum- and LPA-induced activation of SRF to background levels (Hill *et al.*, 1995). C3 transferase did not inhibit SRF activation by either the N-terminal truncation ΔRBD2, a ΔRBD2 derivative lacking the FH1 domain, or FH1/FH2 (Figure 1C). However, C3 expression did partially inhibit activation of SRF by the FH2 region alone (Figure 1C), suggesting that some aspect of its function is dependent on Rho. Thus the FH1 domain or sequences N-terminal to it are required for fully Rho-independent SRF activation by the mDia1 FH2 region (see DISCUSSION).

The FH2 Region Contains Sequences Essential for SRF Activation

To analyze the function of the mDia1 FH2 region in more detail, we constructed further N- and C-terminal truncations of the mDia1 FH2 fragment and tested them in the SRF reporter assay. Deletion of residues 736–752 reduced activity by 50%, and further deletion of residues 752–771 reduced activation to background levels (Figure 2A, compare FH2, F2ΔN1, F2ΔN2). Similarly, removal of 31 C-terminal residues had little effect, but truncation by an additional 20

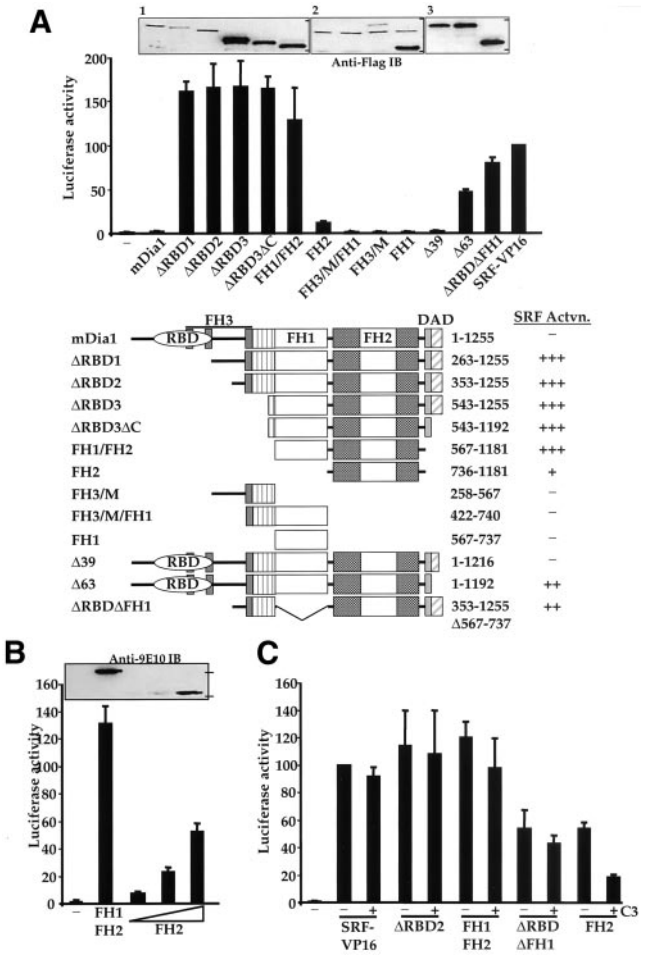

Figure 1. Activation of SRF by mDia1 does not require the FH1 domain. (A) Activity of mDia1 deletion constructs. The Rho binding domain (RBD), Formin Homology (FH) domains 1 and 2 (FH1 and FH2) domains, and the three FH3 motifs are indicated. A coiled-coil region homologous to the tail domain of myosin heavy chain (residues 449–550) is shown as a striped box. The minimal DAD domain (residues 1172–1255), predicted by functional analysis of mDia2, is shown as a striped box with a bar indicating the conserved core residues. Expression plasmids for each mDia1 deletion (0.1 μg/dish) were tested in the SRF reporter assay. Inset: relative expression of each protein determined by anti-Flag immunoblots (lines indicate 250, 75-kDa markers in panels 1 and 3, and 50, 25-kDa markers in panel 2). Reporter gene activity is expressed relative to activation by 0.1 μg of SRF-VP16 (see MATERIALS AND METHODS). Results are the mean ± SEM of three independent experiments. (B) SRF activation by the mDia1 FH2 region. Reporter activation by 0.1 μg of FH1/FH2 expression plasmid was compared with that by increasing amounts of expression plasmid encoding the mDia1 FH2 region (FH2; 0.1, 0.3, 1.0 μg). Inset: protein quantitation by anti-9E10 immunoblot. Results are the mean ± SEM of three independent experiments. (C) mDia1 activation of SRF is independent of Rho activity. Reporter activation by the indicated mDia1 derivatives was tested in the presence or absence of expression of the Rho-inactivating C3 transferase. Results are the mean ± SEM of three independent experiments. Relative to the activity observed in its absence, C3 expression reduced activity of the SRF reporter as follows: SRF-VP16, 91.4 ± 5.9%; ΔRBD2, 93 ± 5.7%; FH1FH2, 79.7 ± 12.0%; ΔRBDΔFH1, 87.0 ± 16.5%; FH2, 30.6 ± 1.1%; in parallel experiments C3 expression reduced RhoA. V14-induced SRF activity to 2.4 ± 1.3% (unpublished data).

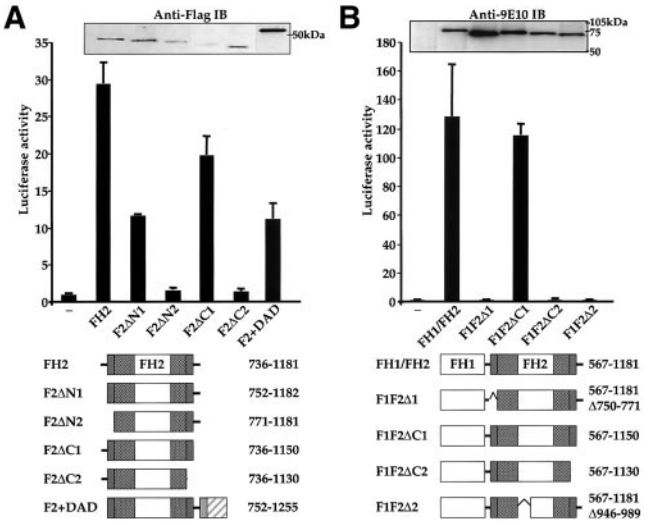

Figure 2. mDia1 C-terminal sequences required for SRF activation. (A) Deletions within the FH2 region. Expression plasmids encoding N- or C-terminal modifications of the FH2 region were transfected into NIH3T3 cells (0.3 μg/dish) and assayed as in Figure 1 for SRF activation. Proteins are shown schematically at the bottom of each panel, with sequences essential for activity indicated by the boxes. Results are the mean ± SEM of three independent experiments. Inset: relative protein expression (anti-Flag). (B) The extremities of the FH2 region are required in the presence of FH1. Expression plasmids encoding derivatives of FH1/FH2 lacking segments of the FH2 region were transfected into NIH3T3 cells (0.1 μg/dish) and assayed as in Figure 1 for SRF activation. Results are the mean ± SEM of three independent experiments. Inset: relative protein expression (anti-9E10).

abolished activity (Figure 2A, compare FH2, F2ΔC1, F2ΔC2). Larger deletions at either end of the FH2 region were also inactive (unpublished data). The presence or absence of the DAD domain had no effect on SRF activation by FH2 (Figure 2A, compare F2+DAD with F2ΔN1, F2ΔC1).

We showed above that activation of SRF by FH2 is partially dependent on functional Rho (Figure 1C, FH2). To exclude the possibility that deletion of residues 750–770 or 1130–1150 affect only the Rho-dependent activity of the FH2 region, we deleted these residues from FH1/FH2, which contains FH1 and can activate SRF independently of functional Rho. Deletion of amino acid residues 750–770 or 1130–1150 abolished SRF activation (Figure 2B, compare FH1/FH2 with F1F2Δ1, F1F2ΔC2). Together these results define the boundaries of the minimal SRF-activating fragment of the FH2 region as residues 752 and 1150. Deletion of residues 946–989 within the core FH2 domain at the center of this fragment also abolished SRF activation (Figure 2B, F1F2Δ2). SRF activation by mDia1 is thus dependent on a substantial region that is conserved throughout the formin family and encompasses the previously defined core FH2 domain (see DISCUSSION).

SRF Activation Defines mDia1 Regions Required for F-Actin Rearrangement

We previously proposed that activation of SRF by cytoskeletal remodeling proteins reflects their ability to promote actin polymerization (Sotiropoulos *et al.*, 1999). To test this hypothesis, we used our set of mDia1 mutants to investigate whether the mDia1 sequences required for SRF activation could be distinguished from those that promote actin rearrangements.

We used FITC-phalloidin immunofluorescence microscopy to investigate whether those mDia1 derivatives that activate SRF are also competent to induce F-actin rearrangements. Consistent with previous reports, in NIH3T3 cells the active mDia1 derivative FH1/FH2 caused a dramatic increase in parallel thin actin fibers, increased apparent phalloidin staining, and induced a characteristic elongated cellular morphology (Watanabe *et al.*, 1999; Ishizaki *et al.*, 2001). Expression of the minimal active FH2 fragment showed a similar phenotype, although the fibers were thicker and less well organized. As was the case with SRF activation, cytoskeletal rearrangements by these active mDia1 derivatives were not prevented by expression of C3 transferase (Figure 3A). In contrast, all the mDia1 deletions that were inactive in the SRF reporter assay had no obvious effect either on F-actin accumulation or on cell morphology in this assay (Figure 3B, F1F2Δ1, F1F2Δ2, and F1F2ΔC2, unpublished data). These results strongly suggest that mDia1 derivatives competent to activate SRF are also competent to induce cytoskeletal rearrangements.

SRF Activation Defines mDia1 Regions Required for Actin Polymerization

In the immunofluorescence assay apparent increases in phalloidin staining can result from the combination of rearrangement of preexisting F-actin and changes in cell morphology. We therefore sought to determine the effect of mDia1 on cellular F-actin content using methods unaffected by cell shape. We first used a qualitative assay for the F:G-actin ratio based on the differential extractability of F- and G-actin from cells by detergent (Lyubimova *et al.*, 1997). Transiently transfected Flag-actin was used as a reporter for the effects of transfected mDia proteins on actin (see MATERIALS AND METHODS). When expressed alone, Flag-actin was detected predominantly in the detergent-insoluble F-fraction (Figure 3C). Treatment with swinholide A, which sequesters G-actin, led to the recovery of Flag-actin entirely in the detergent soluble G-fraction, whereas treatment with jasplakinolide, which stabilizes F-actin, led to its recovery predominantly in the insoluble F-fraction (Figure 3C). Consistent with the notion that mDia1 acts to promote F-actin assembly, the two derivatives active in the SRF assay significantly reduced the ratio of G- to F-actin as determined by detergent solubility of Flag-actin, whereas the inactive derivatives had no effect (Figure 3C).

To quantify directly the effects of mDia1 on F-actin assembly, we compared the mean cellular F-actin content of cells expressing mDia1 derivatives with that of untransfected cells. NIH3T3 cells were transfected with mDia1 expression plasmids, fixed, and then stained both for the transfected mDia1 epitope tag and for F-actin using FITC-phalloidin. Stained cells were sorted using the FACS, and the mean amount of phalloidin staining per cell quantified for the transfected and untransfected populations in each sample (Howard and Meyer, 1984; Bleul *et al.*, 1996; Burger *et al.*, 1999). Untransfected cells contain a substantial amount of polymerized actin, as assessed by detergent extraction (Fig-

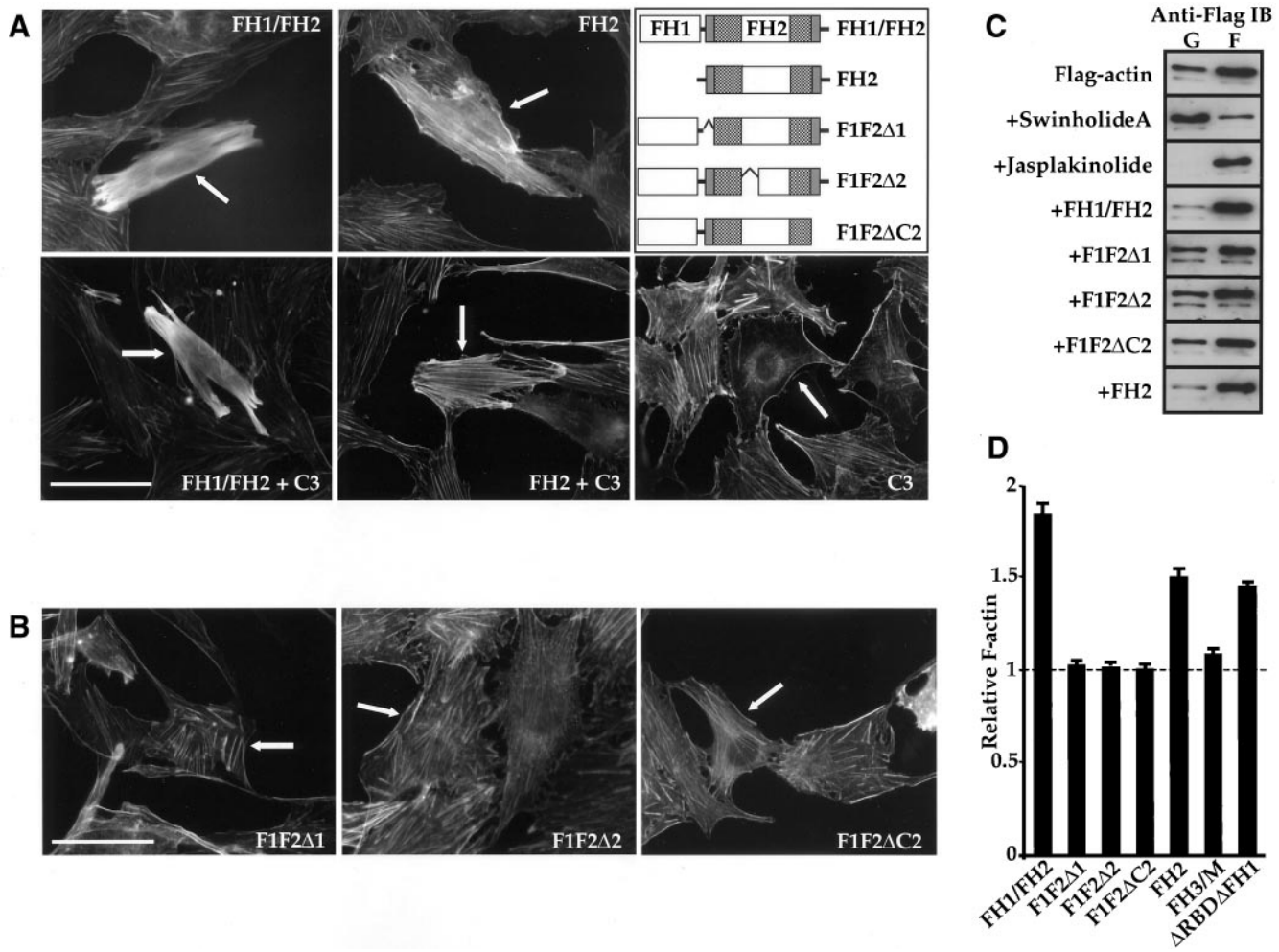

Figure 3. mDia1 mutants that activate SRF induce F-actin accumulation. (A) Active mDia1 mutants reorganize cellular F-actin structures. NIH3T3 cells were transfected with plasmids expressing active mDia1 derivatives (FH1/FH2, 0.1 μg; FH2, 1.0 μg) and C3 transferase (0.1 μg) as indicated. F-actin was visualized with FITC-phalloidin. The mDia proteins are shown schematically at the top right, with the essential FH2 sequences shown as boxes. Transfected cells were detected by mDia1 derivative epitope tag immunofluorescence. (B) Inactive mDia proteins do not reorganize cellular F-actin structures. NIH3T3 cells were transfected with plasmids expressing inactive mDia1 derivatives F1F2Δ1, F1F2Δ2, and F1F2ΔC2 (1.0 μg each) as indicated. F-actin was visualized with FITC-phalloidin. Transfected cells were detected by mDia1 derivative epitope tag immunofluorescence. (C) Active mDia1 proteins decrease G: F-actin ratio. NIH3T3 cells were transfected with plasmids expressing FH1/FH2 (0.1 μg), or FH2, F1F2Δ1, F1F2Δ2, and F1F2ΔC2 (1 μg) together with an expression plasmid encoding Flag-tagged wild-type β-actin (0.5 μg). Detergent-soluble and insoluble cell extract fractions were prepared as in MATERIALS AND METHODS, and the amount of Flag-actin in each evaluated by SDS-PAGE and immunoblotting with M2 anti-Flag antibodies. SwinholideA and jasplakinolide treated controls are shown in the bottom panel. A representative experiment is shown (N = 3). (D) SRF activation correlates with ability to promote F-actin assembly. NIH 3T3 cells were transfected with plasmids expressing the indicated mDia1 derivatives as above and the mean F-actin content of transfected cells determined, relative to untransfected cells in the same population, using the FACS. Data represent the mean ± SEM of three independent experiments.

ure 3C; Lyubimova *et al.*, 1997). Nevertheless, expression of an activated mDia1 derivative induced a significant increase in mean cellular F-actin content in the FACS assay (Figure 3D). As in the SRF activation assay, the ability of mDia1 derivatives to increase mean F-actin content was not dependent on the presence of the FH1 domain (Figure 3D; FH1/FH2, FH2, and ΔRBDΔFH1). In contrast, expression of mDia1 derivatives incapable of activating SRF did not detectably affect F-actin levels (Figure 3D: F1F2Δ1, F1F2Δ2, F1F2ΔC2, FH3/M). Thus, the ability of mDia1 derivatives to activate SRF precisely correlates with their ability to induce F-actin accumulation.

Actin Lies Downstream of mDia1 in the Signal Pathway to SRF

We showed previously that overexpression of wild-type actin inhibits signal-induced SRF activation but does not block activation of the SRF target gene Egr1, which is regulated independently of actin dynamics (Sotiropoulos *et al.*,

1999). Consistent with our previous proposal that SRF is activated in response to depletion of the cellular G-actin pool (Sotiropoulos *et al.*, 1999), SRF activity is also inhibited by expression of the mutant actin G13R, which is not polymerizable in vivo as judged by immunocytochemical and biochemical assays (Posern *et al.*, 2002). Because the ability of mDia1 to activate SRF correlates with its ability to promote actin polymerization, we next tested whether SRF activation by mDia1 could be inhibited by actin overexpression.

NIH3T3 cells were cotransfected with the activated mDia1 derivative FH1/FH2 and increasing amounts of wild-type actin or the polymerization-defective G13R mutant, and the effects on both reporter activity and cellular F-actin content measured as before. Expression of wild-type actin caused only a slight inhibitory effect on the activation of SRF by FH1/FH2 (Figure 4A), presumably because wild-type actin is incorporated into filaments by activated mDia1 before G-actin can accumulate to a level that inhibits SRF. Indeed, expression of wild-type actin substantially increased the mean cellular F-actin content of cells expressing FH1/FH2, presumably by providing an additional substrate for filament assembly (Figure 4B). In contrast, expression of actin G13R effectively inhibited SRF activation by FH1/FH2 (Figure 4A) and only slightly reduced its ability to promote cellular F-actin accumulation as measured in the FACS assay (Figure 4B). This decrease in F-actin is likely to be an indirect consequence of transcriptional repression of endogenous cytoskeletal actin genes, which are also SRF targets (Mohun *et al.*, 1987; Reuner *et al.*, 1995; Lyubimova *et al.*, 1997; Sotiropoulos *et al.*, 1999). Activation of the SRF reporter gene by SRFVP16, whose activity is independent of upstream signaling pathways, was not significantly affected by expression of wild-type or G13R actin (Figure 4A). These results provide strong evidence that actin lies downstream of mDia in the signal pathway to SRF; however, we were unable to detect interaction between the activated mDia1 and either wild-type actin or actin G13R in the yeast two-hybrid assay (unpublished data; see DISCUSSION).

We also investigated the role of the nonreceptor tyrosine kinase Src in SRF activation. The SH3 domain of Src is able to bind to both the mDia1 and mDia2 FH1 domains, and it has been proposed that Src acts as an essential mDia effector in SRF activation (Tominaga *et al.*, 2000). Expression of the constitutively active Src mutant Y527F induced SRF activation, but unlike the activation of SRF by mDia1, activation by Src was completely abolished by C3 coexpression (Figure 4C). This result places Src upstream of or parallel to Rho in our assays. To address the role of actin in activation of SRF by active Src, we coexpressed SrcY527F with wild-type or G13R mutant actin and observed that, as for mDia1, actin G13R inhibited Src-induced SRF activation. Taken together these data show that both mDia1 and Src activate SRF via effects on actin (see DISCUSSION).

Interfering mDia1 Proteins Inhibit SRF Activation and Actin Polymerization

Having identified a number of mDia derivatives inactive in both SRF activation and actin polymerization, we exploited the quantitative nature of the transcriptional assay to test whether any of them could interfere with signaling to SRF. Inactive mDia1 derivatives were coexpressed with activated mDia1, and reporter activity was measured. Mutants

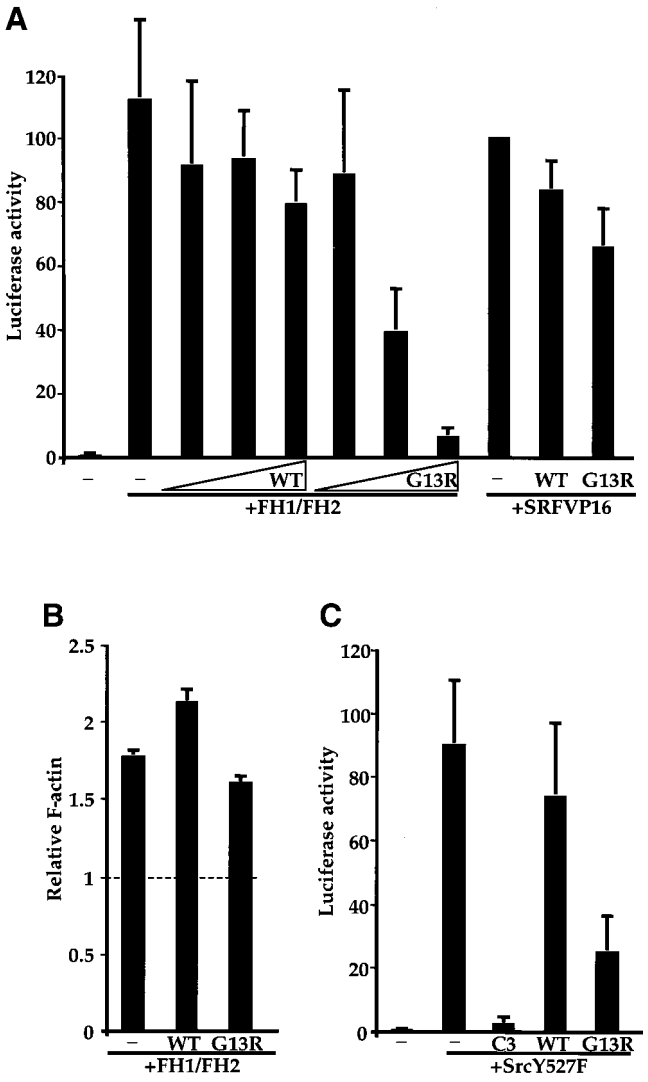

Figure 4. Actin acts downstream of mDia in SRF activation. (A) Activation of SRF is inhibited by nonpolymerizable actin. NIH3T3 cells were transfected with SRF reporter and either 0.1 μg of expression plasmid encoding mDia1 FH1/FH2, together with increasing amounts (0.1, 0.3, 1.0 μg) of plasmids expressing wild-type β-actin or the nonpolymerizable actin G13R, which contains a mutation in the ATP binding cleft; or 0.1 μg plasmid expressing the constitutively active SRF derivative SRF-VP16 with 1.0 μg of each actin expression plasmid. Reporter activation is presented as mean ± SEM of three independent experiments. (B) Expression of wild-type or nonpolymerizable actin does not inhibit mDia-induced F-actin accumulation. NIH 3T3 cells were transfected with expression plasmids encoding FH1/FH2 (0.1 μg) and either wild-type actin or actin G13R (1.0 μg each). Mean F-actin content of transfected cells was quantified relative to that of untransfected cells in the same population using the FACS. Results are the mean ± halfrange of two independent experiments. Wild-type actin expression alone increased mean cellular F-actin levels by up to 40%, whereas expression of G13R alone has no effect on mean cellular F-actin content (Posern *et al.*, 2002). (C) SRF activation by Src. NIH3T3 cells were transfected with SRF reporter and either 0.1 μg of expression plasmid encoding Src Y527F, together with plasmids expressing either C3 transferase (0.1 μg), wild-type or G13R actin (1.0 μg each). Reporter activation is presented as mean ± SEM of three independent experiments.

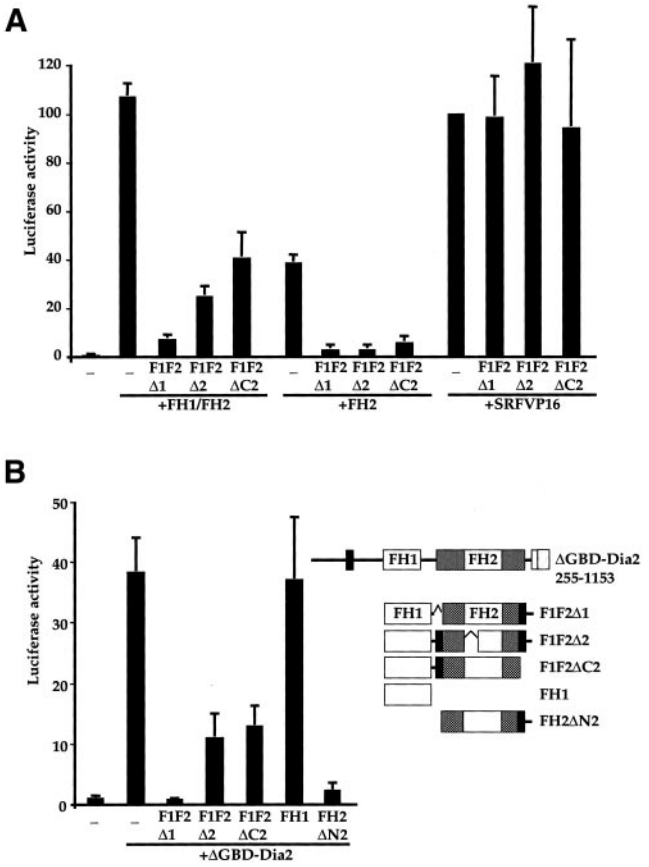

Figure 5. Interfering mutants of mDia1. (A) The inactive mDia1 mutants F1F2Δ1, F1F2Δ2, and F1F2ΔC2 inhibit SRF activation by activated mDia1. NIH3T3 cells were transfected with SRF reporter and plasmids expressing F1F2Δ1, F1F2Δ2, or F1F2ΔC2 (2.0 μg), together with activated mDia1 (FH1/FH2, 0.1 μg or FH2, 0.5 μg) or SRF-VP16 (0.1 μg). Reporter activation is presented as mean ± SEM of three independent experiments. (B) Activated ΔGBD-Dia2 is inhibited by mDia1 interfering mutants. NIH3T3 cells were transfected with SRF reporter and plasmids expressing F1F2Δ1, F1F2Δ2, F1F2ΔC2, FH1, or F2ΔN2 (2.0 μg each), together with ΔGBD-Dia2 (0.1 μg). Reporter activation is presented as mean ± SEM of three independent experiments. Interfering mDia1 mutants and ΔGBD-Dia2 are shown schematically at the right.

F1F2Δ1, F1F2Δ2, and F1F2ΔC2, each of which contains a short inactivating deletion within the FH2 region, inhibited SRF activation induced by the activated mDia1 derivatives FH1/FH2 and FH2 (Figure 5A). Mutant F1F2Δ1, which lacks sequences at the N-terminus of the FH2 region, had the largest effect on SRF activation reducing activation by FH1/FH2 and FH2 almost to background levels. None of the interfering mDia1 deletions affected reporter activation by the constitutively active SRF mutant SRFVP16 (Figure 5A). The mutants also blocked SRF activation by an activated derivative of mDia2, ΔGBD-Dia2 (Tominaga *et al.*, 2000), with similar relative efficacy (Figure 5B). Removal of the FH1 domain from F1F2Δ1 did not affect its ability to inhibit SRF activation by ΔGBD-Dia2 (Figure 5B). Moreover, expression of the isolated mDia1 FH1 domain had no effect (Figure 5B). Therefore, the observed inhibition does not reflect competition for factors binding to FH1. Because the interfering forms of mDia1 contain neither the RBD nor the DAD domain their inhibitory effect must arise from effects downstream of the FH2 region (see DISCUSSION).

Because the ability of mDia1 derivatives to activate SRF correlates with their ability to induce actin polymerization, we next tested the ability of the interfering mDia1 proteins to block mDia1- or mDia2-induced actin polymerization. We first used FITC-phalloidin immunofluorescence to examine the effect of the interfering mutant F1F2Δ1 on cytoskeletal reorganization induced by expressing activated mDia1 or mDia2 derivatives (Figure 6A). Expression of F1F2Δ1 completely blocked the ability of both mDia1 FH1/FH2 and ΔGBD-Dia2 to induce apparent F-actin accumulation, formation of thin F-actin fibers, and elongated cell morphology (Figure 6A, compare left and right panels). Expression of mDia1 F1F2Δ1 substantially reduced the ability of the activated mDia1 or mDia2 derivatives to increase mean cellular F-actin content as determined in the FACS assay (Figure 6B).

Interfering mDia1 Inhibits Signal-induced SRF Activation and Actin Reorganization

The results presented in the preceding section demonstrate that the inactive mDia1 derivatives F1F2Δ1, F1F2Δ2, and F1F2ΔC2 represent interfering mutants capable of inhibiting SRF activation and actin polymerization induced by activated mDia proteins. We next used these mutants to investigate the role of mDia proteins in signal-induced SRF activation. We also tested mutant FH3/M, comprising the region between the RBD and FH1, which is inactive in our assays (see Figures 1A and 3C) and can interfere with cytoskeletal integrity in MDCK epithelial cells (Nakano *et al.*, 1999). Expression of the interfering mDia1 proteins F1F2Δ1, F1F2Δ2, and F1F2ΔC2 significantly inhibited serum-induced activation of the SRF reporter gene, with F1F2Δ1 again being most effective; expression of FH3/M had no effect (Figure 7A). As previously reported, overexpression of Flag-actin also substantially inhibited signal-induced reporter activation (Figure 7A; Sotiropoulos *et al.*, 1999). The effect of interfering mDia1 was specific, because expression of F1F2Δ1 had no effect on serum-induced activation of a reporter controlled by the Elk-1 transcription factor, which is regulated by ERK phosphorylation (Figure 7B; Marais *et al.*, 1993). Expression of either the interfering mDia mutant F1F2Δ1, or actin, also completely inhibited SRF induction by LPA, a major serum mitogen which activates Rho (Figure 7C).

The effects of the interfering mutants upon serum- and LPA-induced SRF activation provide strong evidence that mDia proteins are essential components of these Rho-dependent signaling pathways in NIH3T3 cells. Because it has been proposed that Src activity is essential for signaling downstream of mDia, we also evaluated its significance in serum-induced SRF activation. Inhibition of Src, whether by coexpression of the Src-inactivating C-terminal Src Kinase (CSK), the kinase-inactive Src mutant Src K298 M, or by treatment of the cells with the Src inhibitor PP2, had no effect on serum-induced SRF activation (Figure 7D). In control experiments expression of CSK or Src K298 M completely blocked Src-dependent ERK activation (Grosse *et al.*, 2000).

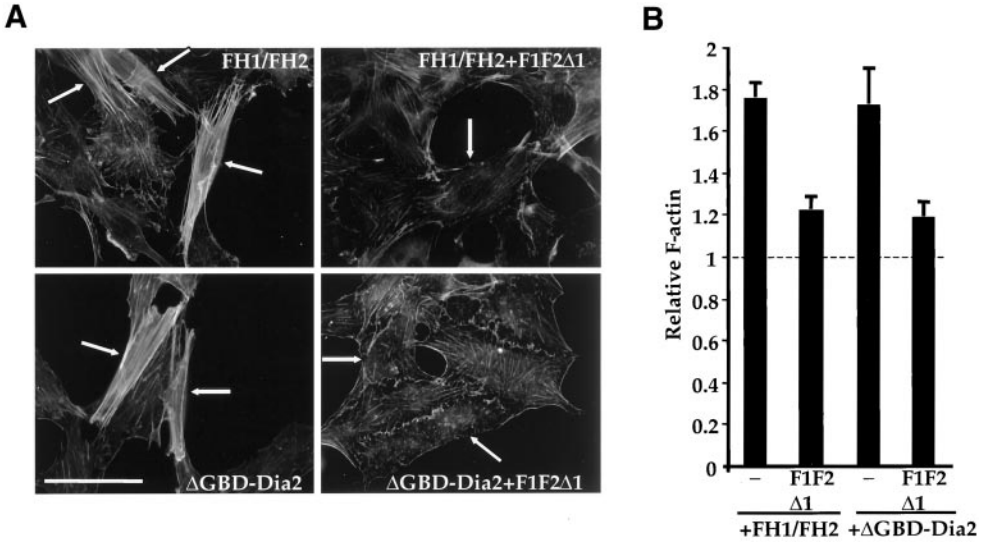

Figure 6. mDia1 interfering derivative F1F2Δ1 prevents reorganization of the F-actin cytoskeleton by activated mDia proteins. (A) NIH3T3 cells were transfected with plasmids expressing either the activated FH1/FH2 mDia1 derivative (0.1 μg; top panels) or the activated mDia2 derivative ΔGBD-Dia2 (bottom panels), together with a plasmid expressing F1F2Δ1 (2.0 μg) as indicated. F-actin was visualized with FITC-phalloidin. (B) NIH3T3 cells were transfected with expression plasmids encoding mDia1 and mDia2 derivatives as in A. Mean F-actin content of transfected cells was quantified relative to that of untransfected cells in the same population using FACS. Results are the mean ± SEM of three independent experiments. The inhibition by F1F2Δ1 is significant ($p < 0.05$) by Student's *t* test.

These results suggest that Src activity is not essential for serum-induced SRF activation.

Finally, we tested the effect of the interfering mDia1 protein F1F2Δ1 on signal-induced cytoskeletal rearrangements. LPA stimulation induces formation of thick parallel stress fibers in fibroblasts through activation of Rho (Ridley and Hall, 1992). Unstimulated NIH3T3 cells had relatively little F-actin and only a few short stress fibers, but LPA treatment stimulated formation of thick parallel stress fibers, many of which extended the length of the cell (Figure 7E). In contrast, upon LPA stimulation cells expressing F1F2Δ1 exhibited a marked reduction of F-actin staining and formed only short and poorly organized stress fibers (Figure 7E). These results contrast with those observed upon ablation of Rho activity by expression of C3 transferase, which results in the disappearance of F-actin bundles (Figure 3A): it is likely that this reflects the simultaneous inactivation of ROCK and mDia proteins, which cooperate in F-actin bundle formation (see DISCUSSION; Nakano *et al.*, 1999; Watanabe *et al.*, 1999; Tominaga *et al.*, 2000).

DISCUSSION

Activation of the transcription factor SRF by extracellular signals is mediated by Rho GTPases and requires alterations in actin dynamics (Hill *et al.*, 1995; Sotiropoulos *et al.*, 1999). The Diaphanous Related Formins (DRFs) are candidate effectors of RhoA in this signaling pathway (Sotiropoulos *et al.*, 1999; Tominaga *et al.*, 2000). In this study we performed a detailed analysis of the mDia1 DRF to investigate the relationship between mDia1-induced actin polymerization and SRF activation. Our results show that the ability of mDia1 derivatives to activate SRF strictly correlates with their ability to promote F-actin accumulation and reveal an important role for the mDia1 FH2 region in these processes. The quantitative transcription assay allowed the identification of inactive mDia1 derivatives whose expression can interfere with both SRF activation and F-actin assembly, whether induced by extracellular signals or expression of activated DRF proteins. Our findings suggest a model whereby SRF activation occurs as a consequence of mDia induced F-actin assembly (Figure 8A).

We previously proposed that SRF is activated in response to depletion of the cellular G-actin pool (Sotiropoulos *et al.*, 1999). Consistent with this model, mDia1-induced SRF activation, but not F-actin assembly, is inhibited by expression of the nonpolymerizable actin mutant G13R. This places actin downstream of mDia1 in the signal pathway to SRF and strongly suggests that it is the ability of mDia1 to regulate the level of G-actin, or a subpopulation of it, that controls SRF activity (Figure 8A). We have not detected direct interaction between activated mDia1 and G-actin, suggesting that SRF activation by mDia1 is likely to occur as a consequence of its effects on F-actin assembly rather than through direct interaction with actin. In contrast to a previous proposal (Tominaga *et al.*, 2000), our data indicate that the Src tyrosine kinase does not appear essential for SRF activation by serum-induced signals, instead appearing to act upstream or in parallel to mDia1.

Deletion of the N-terminal Rho binding domain (RBD) activates the mDia DRF proteins by relieving inhibitory interactions between the RBD and the C-terminal Diaphanous autoregulatory domain (DAD; Watanabe *et al.*, 1999; Alberts, 2001). In agreement with previous studies of DAD point mutations, we found that a C-terminal deletion that removes conserved sequences within the mDia1 DAD, while

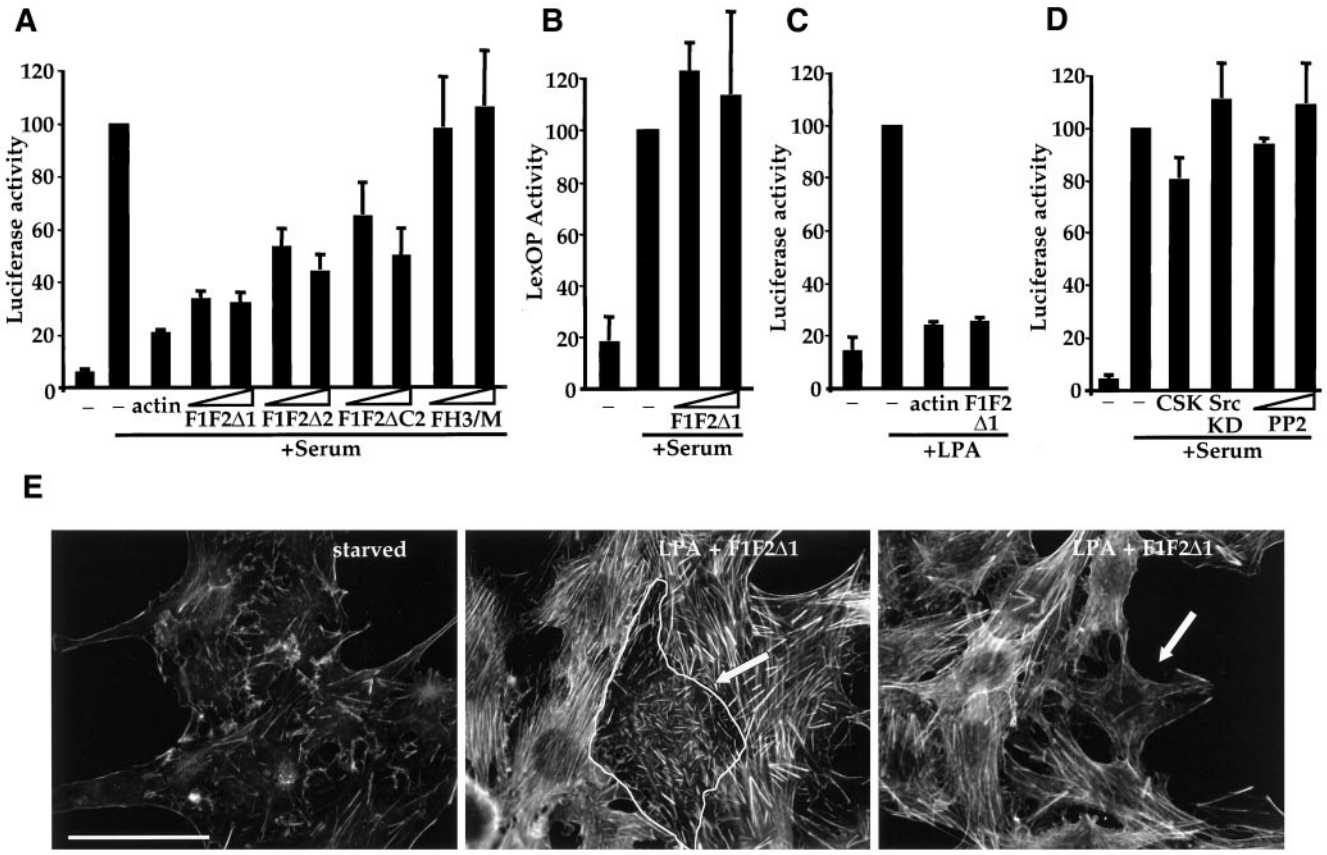

Figure 7. mDia1 interfering derivatives block SRF activation and cytoskeletal rearrangements. (A) Interfering derivatives block activation of SRF by serum stimulation. NIH3T3 cells were transfected with SRF reporter and plasmids expressing mDia1 derivatives F1F2Δ1, F1F2Δ2, F1F2ΔC2, or FH3/M (2.0 μg), or wild-type β-actin (1.0 μg), maintained in 0.5% FCS for 36 h, and then stimulated with 15% serum as indicated. Reporter activation is presented as mean ± SEM of three independent experiments. (B) F1F2Δ1 does not block activation of TCF Elk-1 by serum stimulation. NIH3T3 cells were transfected with the LexA operator controlled reporter LexOP-Luc (0.1 μg), an expression plasmids encoding NLex. ElkC (0.1 μg), a chimeric transcription factor comprising the C-terminal activation domain of Elk-1 fused to the bacterial LexA repressor (Marais *et al.*, 1993), and mDia1 derivative F1F2Δ1 (2.0 μg). Cells were serum-stimulated as in A. Reporter activation is presented as mean ± halfrange of two independent experiments. (C) F1F2Δ1 blocks activation of SRF by LPA stimulation. NIH3T3 cells were transfected with SRF reporter and plasmids expressing mDia1 derivative F1F2Δ1 (2.0 μg), or wild-type β-actin (1.0 μg), maintained in 0.5% FCS for 36 h, and then stimulated with 10 μM LPA as indicated. Reporter activation is presented as mean ± SEM of three independent experiments. (D) Src activity is not required for serum-stimulated SRF activation. NIH3T3 cells were transfected with SRF reporter and plasmids expressing C-terminal Src Kinase (CSK; 2.0 μg) or kinase inactive SrcK298 M (SrcKD; 2.0 μg) and processed as in A. Where indicated, cells were pretreated for 1 h before stimulation with the Src inhibitor PP2 (0.25 μM, 1.0 μM). Reporter activation is presented as mean ± SEM of three independent experiments. (E) F1F2Δ1 blocks stress fiber induction by LPA. NIH3T3 cells were transfected with a plasmid expressing mDia1 derivative F1F2Δ1 (2.0 μg), maintained in 0.5% FCS for 36 h, and then stimulated with 10 μM LPA for 2 min before fixation and staining for F-actin and the transfected protein epitope tag. Arrows indicate the transfected cells.

retaining the core homology, also strongly activates SRF. Our experiments revealed no requirement for the DAD domain in SRF activation or F-actin assembly suggesting that, like the RBD, DAD has primarily a regulatory function (see below).

SRF activation and cytoskeletal reorganization by mDia1 derivatives lacking either the FH1 domain itself, or sequences N-terminal to it, occurred independently of functional RhoA, placing the DRFs downstream of Rho in the signaling pathway. Our studies thus concur with previous findings (Nakano *et al.*, 1999; Watanabe *et al.*, 1999; Tominaga *et al.*, 2000) and do not support the recent proposal that formins function to activate Rho by recruiting GEFs (Habas *et al.*, 2001). Induction of SRF activity and F-actin accumulation by the mDia1 FH2 region alone was partially dependent on functional Rho, however, suggesting that either the FH3/coiled-coil domain or the FH1 domain is required to render mDia1 function completely independent of Rho activity. Functional Rho is required for subcellular localization of proteins such as Src (Fincham *et al.*, 1996), and it may be that in the absence of both FH1 and FH3 domains appropriate subcellular localization of the FH2 region becomes Rho dependent. Indeed, previous studies have shown that the FH3 domain mediates subcellular localization of DRFs (Petersen *et al.*, 1998; Ozaki-Kuroda *et al.*, 2001; Sharpless and Harris, 2002).

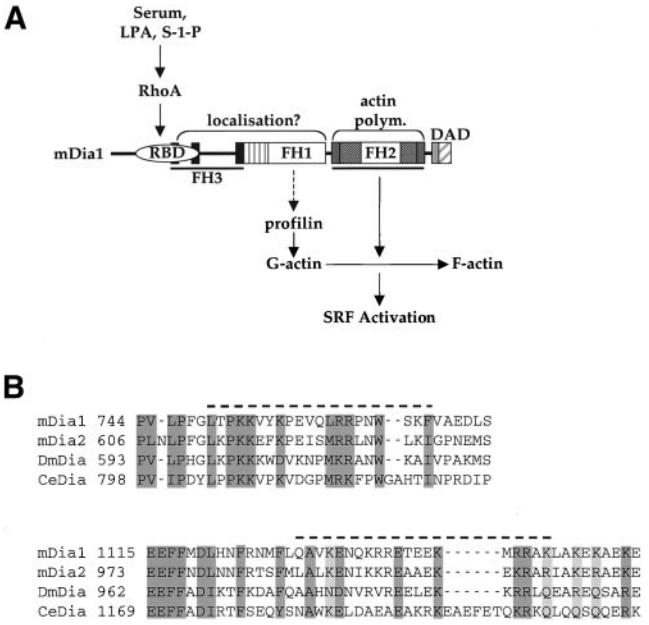

Figure 8. Functional domains in mDia1. (A) Speculative model for mDia1 functional domains. mDia1 is activated by Rho-GTP binding to the RBD, relieving interaction with the DAD and exposing the FH1 and FH2 domains (Watanabe *et al.*, 1999; Tominaga *et al.*, 2000). The figure shows functional regions within mDia1 as deduced from the studies of SRF activation and actin polymerization. The FH2 region, C-terminal to the FH1 and excluding the DAD homology region is sufficient to induce actin polymerization and thereby activate SRF by a mechanism involving depletion of the G-actin pool. The FH1 domain enhances this, possibly by its interaction with profilin or Src. The FH3 and myosin-tail domains, in addition to the FH1 domain, are likely involved in subcellular localization of mDia1. (B) The FH2 region N- and C-termini. Alignment with other Diaphanous Related Formins of the regions deleted from the N- and C-termini of the FH2 region in the interfering mutants F1F2Δ1 (top panel; mDia1 amino acids 750–770) and F1F2ΔC2 (mDia1 amino acids 1130–1150).

The integrity of virtually the entire FH2 region, which exhibits substantial sequence conservation throughout the formin family, is required for mDia1 function. Even in the presence of the FH1 domain, deletions that impinge on the N- and C-termini of the FH2 region, or the core FH2 motif, completely abolish both SRF activation and actin polymerization. The C terminus of the FH2, which is disrupted by the inactivating deletion of residues 1130–1150, contains a conserved EEFF motif reminiscent of the DDW motif mediating interaction of ActA, N-Wasp, and Cortactin with the Arp2/3 complex (Weed *et al.*, 2000; Uruno *et al.*, 2001). The possibility that the mDia1 FH2 region functions by recruiting Arp2/3 is made less likely, however, by the recent demonstration that genetically the yeast DRF Bni1 functions independently of the Arp2/3 complex (Evangelista *et al.*, 2002). Instead, we favor the notion that the FH2 region of mDia1 induces F-actin assembly directly by nucleating actin polymerization. Indeed, while this article was under review, it was shown that the FH2 domain of Bni1 is sufficient to nucleate actin polymerization in vitro (Pruyne *et al.*, 2002). The N-terminal inactivating deletion (amino acids 750–770) of mDia1 removes another conserved motif, corresponding to the binding site in Bni1 for the translation elongation factor EF1α (Umikawa *et al.*, 1998). EF1α interacts with actin (Demma *et al.*, 1990; Yang *et al.*, 1990) and binds to the ends of stress fibers, where it is thought to block actin polymerization (Murray *et al.*, 1996). We are currently addressing the possibility that mDia1 may function in part by relieving such inhibition in vivo.

Inactive derivatives of mDia1 containing deletions within the FH2 region strongly inhibit SRF activation and reorganization of the actin cytoskeleton, whether induced by extracellular signals such as serum or LPA or by expression of activated mDia1 and mDia2 derivatives. In contrast, an mDia1 derivative containing the FH3 domain, which interferes with F-actin structures in MDCK epithelial cells (Nakano *et al.*, 1999), was merely inactive in NIH3T3 fibroblasts, perhaps reflecting differences in mDia1 function in these different cell types. Our interfering mDia1 proteins act specifically, because they do not affect activity of the MAP kinase-regulated SRF accessory protein Elk-1. Moreover, experiments in PC12 cells indicate that they do not act as nonspecific Rho inhibitors because their expression does not affect Rho-dependent cofilin phosphorylation (Geneste *et al.*, 2002). The interfering mDia1 proteins must either interact nonproductively with downstream DRF effectors or interact with endogenous DRFs to generate nonfunctional complexes. Whatever the mechanism, the interactions involved must be mediated by the FH2-containing region of the mDia C terminus, because neither the DAD nor the FH1 domain is required for interference. Although interfering mDia1 derivatives interfere with DRF-induced F-actin accumulation, they do not abolish formation of F-actin bundles, which is likely mediated by ROCK (Nakano *et al.*, 1999; Watanabe *et al.*, 1999; Tominaga *et al.*, 2000), and their effect on cytoskeletal morphology is thus much less marked than that observed upon inactivation of Rho by C3 transferase expression. In keeping with our proposal that G-actin depletion and SRF activation are linked, our interfering mDia1 derivatives inhibit both LIM kinase-induced F-actin formation and SRF activation in PC12 cells (Geneste *et al.*, 2002).

In contrast to previous studies (Watanabe *et al.*, 1999; Alberts, 2001), our results indicate that the mDia1 FH1 domain is not required for SRF activation or F-actin assembly by overexpressed mDia1 derivatives, nor is it required for inhibition of SRF activation by interfering mDia1 proteins. This apparent discrepancy may reflect our use of fibroblast rather than epithelial cells and the sensitivity of our assays for SRF and F-actin. Our results suggest that mDia1 function is not strictly dependent on direct binding to poly-proline binding cofactors such as profilin and Src. Profilin binds to the FH1 domain of the formins Bni1, cdc12, mDia1, and mDia2 (Chang *et al.*, 1997; Evangelista *et al.*, 1997; Imamura *et al.*, 1997; Watanabe *et al.*, 1997) and in yeast profilin is required with the Bni1 FH1 domain, for Bni1-mediated assembly of actin cables (Evangelista *et al.*, 2002). We have confirmed that deletion of the mDia1 FH1 abolishes the interaction with profilin in two-hybrid assays (J.C., unpublished observations), in agreement with a biochemical study (Krebs *et al.*, 2001). It remains possible, however, that profilin might be recruited to mDia derivatives lacking FH1 through their interaction with other actin remodeling proteins. Alternatively, if profilin enhances actin polymerization with-

out being absolutely required for it, overexpression of mDia1 derivatives might be sufficient to bypass FH1-mediated profilin recruitment.

The mDia1 FH1 domain also binds the Src tyrosine kinase, and it has been proposed that Src mediates Dia-dependent signaling to SRF (Tominaga *et al.*, 2000; Alberts, 2001). Our findings, which suggest that activation by both mDia1 and Src involves alterations in actin dynamics, do not support this view. Activation of SRF by active Src is completely dependent on functional Rho, suggesting that the kinase either induces activation of Rho, perhaps via effects on p190RhoGAP (Chang *et al.*, 1995; Fincham *et al.*, 1999), or that functional Rho is required for its activity, perhaps through involvement of Rho in subcellular targetting of Src (Fincham *et al.*, 1996). Moreover, serum-induced SRF activation was not blocked upon inhibition of Src, whether by expression of the inactivating C-terminal Src kinase (CSK) or kinase-inactive Src, or by treatment of cells with Src inhibitor PP2, suggesting that Src is not required for SRF activation.

Two recent reports have implicated the mDia proteins in regulation of the microtubule cytoskeleton, both in its polarization (Ishizaki *et al.*, 2001) and in the generation of the stable glu-MT population (Palazzo *et al.*, 2001). It remains unclear whether these properties are controlled by the functional domains identified here. We have found that an FH2 domain mutation reported to selectively affect MT polarization (Ishizaki *et al.*, 2001) is also severely defective in SRF activation and F-actin assembly in NIH 3T3 cells (J.C. and R.T., unpublished data). Our preliminary data indicate that expression of our interfering mDia1 proteins does not inhibit serum- or LPA-induced glu-MT assembly. We are currently investigating the role of the DRFs in MT organization.

Our results establish a tight correlation between the ability of mDia1 derivatives to promote actin polymerization and to activate SRF. Both processes require the same sequences in and around the FH2 domain but do not require the FH1 domain, suggesting that they do not involve obligatory direct interaction of mDia1 with FH1 ligands such as profilin. We have obtained similar results with both mDia2 and mouse formin (J.C., unpublished data). We used the transcriptional assay to identify inactive mDia1 derivatives that interfere with signal-induced SRF activation and cytoskeletal reorganization and showed that actin itself appears to lie downstream of mDia in the Rho-SRF signaling pathway. Future work will focus on how mDia1 interacts with the actin polymerization machinery and how our deletions affect other known mDia functions.

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