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Mechanisms of Activation and Action of mDial in the Formation of Parallel Stress Fibers in MDCK Cells

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mDial is a downstream target molecule of Rho small G protein and regulates the formation of parallel stress fibers in MDCK cells. mDial consists of at least one Rho-binding domain (RBD), one FH3 domain (FH3D), one coiled-coil domain (CCD), one FH1 domain (FH1D), one FH2 domain (FH2D), and another CCD in this order from the N-terminus to the C-terminus. We constructed various deletion mutants of mDial and investigated the mechanisms of its activation and action by measuring the formation of parallel stress fibers in MDCK cells. We show here that at least FH1D and second CCD are essential for the formation of parallel stress fibers. Furthermore, we present the evidence suggesting that mDial has another domain which interacts with RBD, that this interaction masks FH1D and second CCD and keeps mDial inactive, and that the binding of Rho to RBD opens this folded structure, resulting in the activation of mDial.

Key Words: **Rho; mDial; stress fibers.**

Rho belongs to the Rho small G protein family and regulates the formation of stress fibers and focal adhesions in various types of cultured cells (reviewed in 1–3). Rho has at least two downstream target molecules, ROK-α/Rho-kinase/ROCK (4–6) and mDial (7), which have distinct actions but cooperatively exert the functions of Rho: mDial regulates the formation of parallel stress fibers, whereas ROCK regulates the formation of stellate stress fibers (8, 9).

mDial has one Rho-binding domain (RBD), one FH3 domain (FH3D), one coiled-coil domain (first CCD), one FH1 domain (FH1D), one FH2 domain (FH2D), and another CCD (second CCD) in this order from the N-terminus to the C-terminus (7, 9, 10). FH1D interacts with profilin and Src tyrosine kinase (7, 11).

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Profilin is an actin monomer-binding protein and stimulates its polymerization into actin filaments (reviewed in 12). The C-terminal region of mDial, containing FH1D, FH2D, and second CCD, induces the formation of parallel stress fibers, suggesting that the C-terminal region acts as a dominant active mutant of mDial (8, 9). The last 140 C-terminal residues of mDial interacts with the first 389 N-terminal residues of mDial, containing RBD, and this interaction is inhibited by the binding of Rho to RBD, suggesting that mDial shows Rho-regulated intramolecular interaction between the N- and C-termini (9). However, the functions of the domains other than FH1D and RBD have not been clarified.

In this study, we constructed various deletion mutants of mDial and investigated the mechanisms of its activation and action by measuring the formation of parallel stress fibers in MDCK cells.

MATERIALS AND METHODS

Materials and chemicals. MDCK cells were kindly supplied by Dr. W. Birchmeier (Max-Delbruck-Center for Molecular Medicine, Berlin, Germany). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) was obtained from Sigma Chemical (St. Louis, MO). The cDNA of mDial was provided by Dr. S. Narumiya (Kyoto University Faculty of Medicine, Kyoto, Japan). pEF-BOS-*myc*-mDial-ΔN1, -ΔN2, and -ΔRBDΔFH were constructed as described previously (8). Hybridoma cells expressing the anti-*myc* mouse mAb (9E10) were purchased from American Type Culture Collection (Rockville, MD). The anti-mDial-C pAb was raised in rabbits by standard procedures using glutathione-*S*-transferase-mDial (amino acids 946–1256) as an antigen (8).

Construction of expression plasmids of mDial mutants. mDial-ΔN1ΔC1 (amino acids 524–1192), -ΔN1ΔC2 (amino acids 524–1010), -ΔN1ΔC3 (amino acids 524–820), -ΔN2ΔC1 (amino acids 820–1192), -ΔN3 (amino acids 1011–1255), or -ΔN3ΔC1 (amino acids 1011–1192) coding sequence with the BamHI site upstream of the initiation methionine codon and downstream of the termination codon was synthesized by polymerase chain reaction (see Fig. 1). These fragments were digested by *Bam*HI and ligated into the *Bam*HI site of the pEF-BOS-*myc* plasmid.

Cell culture and microinjection. MDCK cells were maintained at 37°C in a humidified atmosphere of 10% CO_2 and 90% air in DME containing 10% FCS (Gibco Laboratories, Grand Island, NY), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. MDCK cells for the

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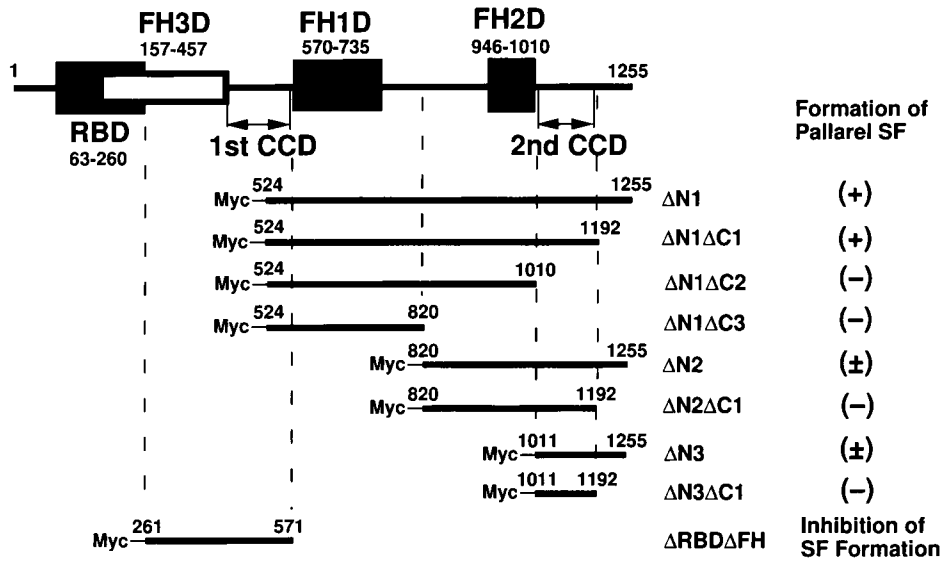

FIG. 1. Structures of mDial mutants and their effects on the actin cytoskeleton. Structural domains of mDial are schematically illustrated at the top and nine mutants of mDial are represented by the thick lines below. Effects of these mutants on the actin cytoskeleton are represented at the right. Numbers indicate amino acid residues of the N- and C-termini of each mutant. RBD, Rho-binding domain; FH1, formin homology domain 1; FH2, formin homology domain 2; FH3, formin homology domain 3; CCD, coiled-coil domain; SF, stress fibers.

microinjection experiments were seeded at a density of 3×10^4 cells per dish onto 35-mm grid dishes. At 24 h after seeding, the expression plasmids were microinjected into the nuclei of the cells at 0.05 mg/ml, and then returned to the incubator for 6 h before treatment with TPA or fixation.

Immunofluorescence microscopy. Cells were fixed in 3.7% paraformaldehyde in PBS for 20 min. The fixed cells were incubated for 10 min with 50 mM ammonium chloride in PBS and permeabilized with PBS containing 0.2% Triton X-100 for 10 min. After the cells were soaked in 10% FCS/PBS for 30 min, they were treated with the first antibodies in 10% FCS/PBS for 1 h. The cells were then washed with PBS three times, followed by incubation with the second antibodies in 10% FCS/PBS for 1 h. For the detection of actin filaments, rhodamine-phalloidin was mixed with the second antibody solution. After the cells were washed with PBS three times, they were examined using a LSM 410 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

RESULTS AND DISCUSSION

Requirement of Second CCD of mDial for the Formation of Parallel Stress Fibers

We first constructed various deletion mutants of mDial from the C-terminus. We have previously shown that mDial-ΔN1 induces the formation of parallel stress fibers, indicating that mDial-ΔN1 acts as a dominant active mutant of mDial (8) (Figs. 1, 2a, and 2b). mDial-ΔN1ΔC1, lacking the last 63 C-terminal residues, induced parallel stress fibers which were morphologically similar to those induced by mDial-ΔN1 (Figs. 1, 2c and 2d), indicating that mDial-ΔN1ΔC1 also acts as a dominant active mutant of mDial. This result is consistent with the result obtained using HeLa cells (9). However, either mDial-ΔN1ΔC2, lacking the last 245 C-terminal residues, but including second CCD, or mDial-ΔN1ΔC3, containing FH1D, but not FH2D, did not apparently affect the actin cytoskeleton (Figs. 1, 2e–2h).

These results suggest that second CCD of mDial is necessary for the formation of parallel stress fibers, although deletion of both FH2D and second CCD from mDial has been shown to abolish its function to form parallel stress fibers (9). Necessity of FH2D for the formation of parallel stress fibers is not known at present, because we did not construct mutants of the C-terminal region, which have mutations only at FH2D. Because Bni1, a yeast homologue of mDial,

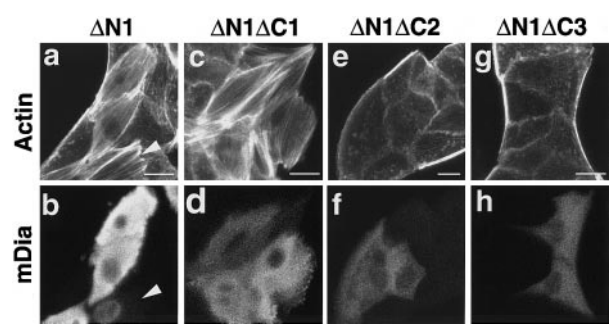

FIG. 2. Effect of deletion of the second coiled-coil domain on the actin cytoskeleton in MDCK cells. pEF-BOS-*myc*-mDial-ΔN1 (a, b), -ΔN1ΔC1 (c, d), -ΔN1ΔC2 (e, f), or -ΔN1ΔC3 (g, h) was microinjected into the nuclei of MDCK cells. At 6 h after the microinjection, the cells were fixed, double-stained with rhodamine-phalloidin (a, c, e, g) and the 9E10 anti-*myc* mAb (b, d, f, h), and analyzed by confocal microscopy at the basal levels. The results shown are representative of three independent experiments. Bars, 10 μm. Arrowheads in panel a and b indicate the cell in which the cytoplasmic staining of *myc*-mDial-ΔN1 is weak.

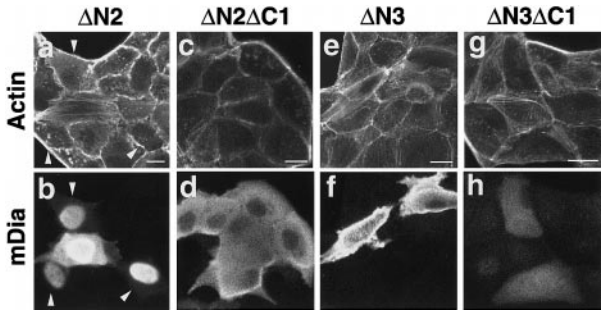

FIG. 3. Effects of deletion of the FH1 domain on the actin cytoskeleton in MDCK cells. pEF-BOS-*myc*-mDial-ΔN2 (a, b), -ΔN2ΔC1 (c, d), -ΔN3 (e, f), or -ΔN3ΔC1 (g, h) was microinjected into the nuclei of MDCK cells. At 6 h after the microinjection, the cells were fixed, double-stained with rhodamine-phalloidin (a, c, e, g) and the 9E10 anti-*myc* mAb (b, d, f, h), and analyzed by confocal microscopy at the basal levels. The results shown are representative of three independent experiments. Bars, 10 μm. Arrowheads in a and b indicate the cells in which the cytoplasmic staining of *myc*-mDial-ΔN2 is weak.

binds Aip3/Bud6, an actin-binding protein, at the last 306 C-terminal residues which do not contain FH2D (8), mDial may interact with a downstream target molecule at second CCD, and this interaction may be necessary for the formation of parallel stress fibers.

Requirement of FH1D of mDial for the Formation of Parallel Stress Fibers

We have previously shown that mDial-ΔN2, containing FH2D, but not FH1D, does not apparently affect the actin cytoskeleton (8), but we have noticed by further experiments that weak formation of parallel stress fibers were observed in about 30% of the cells expressing mDial-ΔN2. It was frequently observed in the cells expressing mDial-ΔN2 at higher levels, whereas obvious change in the actin cytoskeleton was not observed at lower levels (Figs. 1, 3a and 3b). *myc*-mDial-ΔN2 often showed the increased staining at the nuclei of the cells, and the formation of parallel stress fibers was not observed in the cells in which the cytoplasmic staining of *myc*-mDial-ΔN2 was weak (Figs. 3a and 3b). On the other hand, we have previously reported that *myc*-mDial-ΔN1 also often showed the increased staining at the nuclei of the cells, but the formation of parallel stress fibers was observed even in the cells in which the cytoplasmic staining of *myc*-mDial-ΔN1 was weak (4) (Figs. 1, 2a and 2b). mDial-ΔN2ΔC1, lacking the last 63 C-terminal residues, did not apparently affect the actin cytoskeleton (Figs. 1, 3c and 3d). *myc*-mDial-ΔN3, containing only the last 245 C-terminal residues, often showed the increased staining at the nuclei of the cells, and the formation of parallel stress fibers was not observed in the cells in which the cytoplasmic staining of *myc*-mDial-ΔN3 was weak (data not shown). However, weak formation of parallel stress fibers was frequently observed in the cells expressing mDial-ΔN3 at higher levels (Figs. 1, 3e and 3f). mDial-ΔN3 induced weak formation of parallel stress fibers in about 30% of the cells expressing mDial-ΔN3. mDial-ΔN3ΔC1, lacking the last 63 C-terminal residues, did not apparently affect the actin cytoskeleton (Figs. 1, 3g and 3h). The increased staining of either *myc*-mDial-ΔN1, -ΔN2, or -ΔN3 at the nuclei of the cells may be due to the presence of a putative nuclear-localization signal in the C-terminal region (amino acids 1196–1202).

These results, that mDial-ΔN1ΔC1 induced the formation of parallel stress fibers whereas mDial-ΔN2ΔC1 did not induce it, suggest that FH1D of mDial is furthermore necessary for the formation of parallel stress fibers. Because mDial interacts with profilin and Src tyrosine kinase at FH1D (7, 11), profilin and/or Src may be necessary for the mDial-induced formation of parallel stress fibers.

Formation of Parallel Stress Fibers by Overexpression of the Last 245 C-Terminal Residues of mDial

Because the parallel stress fibers induced by overexpression of mDial-ΔN2 or -ΔN3 were weak, we examined their effects in the cells after TPA stimulation. We have previously shown that TPA induces the formation of radial stress fibers at 2 h after the stimulation in wild-type MDCK cells (13, 14), whereas parallel stress fibers are formed in the mDial-ΔN1 expressing cells at 2 h after TPA stimulation (8). mDial-ΔN1ΔC1 also induced the formation of parallel stress fibers at 2 h after TPA stimulation (Figs. 1, 4a and 4b). Parallel stress fibers were also formed in about 30% of the cells expressing either mDial-ΔN2 or -ΔN3. The formation of parallel stress fibers was frequently observed in the cells expressing either mDial-ΔN2 or -ΔN3 at higher

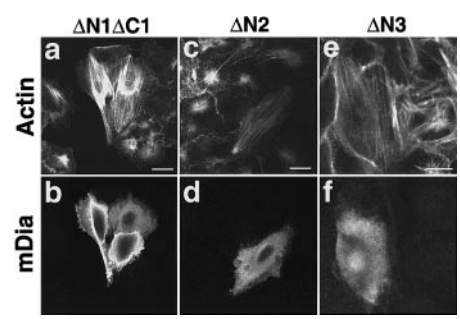

FIG. 4. Effects of mDial mutants on the TPA-induced reassembly of stress fibers in MDCK cells. pEF-BOS-*myc*-mDial-ΔN1ΔC1 (a, b), -ΔN2 (c, d), or -ΔN3 (e, f) was microinjected into the nuclei of MDCK cells. At 6 h after the microinjection the cells were stimulated with 100 nM TPA for 2 h and fixed. The cells were double-stained with rhodamine-phalloidin (a, c, e) and the 9E10 anti-*myc* mAb (b, d, f). The cells were analyzed by confocal microscopy at the basal levels. The results shown are representative of three independent experiments. Bars, 10 μm.

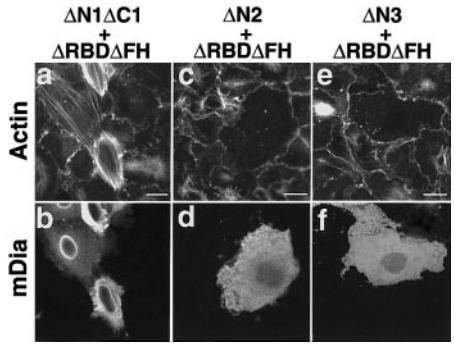

FIG. 5. Effects of coexpression of a dominant negative mutant of mDial with each mDial mutant on the TPA-induced reassembly of stress fibers in MDCK cells. pEF-BOS-*myc*-mDial-ΔN1ΔC1 plus -ΔRBDΔFH (a, b), pEF-BOS-*myc*-mDial-ΔN2 plus -ΔRBDΔFH (c, d), or pEF-BOS-*myc*-mDial-ΔN2 plus -ΔRBDΔFH (e, f) was microinjected into the nuclei of MDCK cells. At 6 h after the microinjection the cells were stimulated with 100 nM TPA for 2 h and fixed. The cells were double-stained with rhodamine-phalloidin (a, c, e) and the anti-mDial-C pAb (b, d, f). The cells were analyzed by confocal microscopy at the basal levels. The results shown are representative of three independent experiments. Bars, 10 μm.

levels (Figs. 1 and 4c–4f), but radial stress fibers were formed at lower levels (data not shown).

These results indicate that overexpression of either mDial-ΔN2 or -ΔN3 form parallel stress fibers. Because full-length mDial shows intramolecular interaction between the N- and C-termini, and binding of Rho to RBD in the N-terminus disrupts this intramolecular interaction (9), overexpression of either mDial-ΔN2 or -ΔN3 may interact with RBD of endogenous mDial, thereby inhibiting the intramolecular interaction and opening the inactive folded structure.

Involvement of the Region between RBD and FH1D of mDial in the Formation of Parallel Stress Fibers

We have previously shown that mDial-ΔRBDΔFH inhibits the formation of stress fibers and focal adhesions even 2 h after TPA stimulation, suggesting that mDial-ΔRBDΔFH acts as a dominant negative mutant of mDial, although the mechanism is not known (8). In the last set of experiments, we examined the effect of coexpression of either mDial-ΔN1ΔC1, -ΔN2, or -ΔN3 with mDial-ΔRBDΔFH on the actin cytoskeleton at 2 h after TPA stimulation. mDial-ΔN1ΔC1 induced the formation of parallel stress fibers even in the presence of mDial-ΔRBDΔFH, whereas stress fibers nearly disappeared in the cells coexpressing either mDial-ΔN2 or -ΔN3 with mDial-ΔRBDΔFH (Figs. 1 and 5).

These results indicate that the formation of parallel stress fibers induced by activated endogenous mDial is inhibited by mDial-ΔRBDΔFH, whereas that by the C-terminal region, containing FH1D, FH2D, and second CCD, is not inhibited. Because Bni1 interacts with Spa2 at the region between RBD and FH1D and this interaction is essential for the association of Bni1 with the plasma membrane (15), mDial may have also an additional domain that is associated with the plasma membrane through a Spa2-like protein, and mDial-ΔRBDΔFH may compete with endogenous mDial for the binding to the plasma membrane and inhibit the function of endogenous mDial. It may be noted, however, that this putative Spa2-like protein-binding domain is not essential for the function of mDial, because mDial-ΔN1 and mDial-ΔN1ΔC1 lacking this domain are able to exert the full function of mDial as described above.

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