mDia2 Induces the Actin Scaffold for the Contractile Ring and Stabilizes Its Position during Cytokinesis in NIH 3T3 Cells

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mDia proteins are mammalian homologues of *Drosophila* diaphanous and belong to the formin family proteins that catalyze actin nucleation and polymerization. Although formin family proteins of nonmammalian species such as *Drosophila* diaphanous are essential in cytokinesis, whether and how mDia proteins function in cytokinesis remain unknown. Here we depleted each of the three mDia isoforms in NIH 3T3 cells by RNA interference and examined this issue. Depletion of mDia2 selectively increased the number of binucleate cells, which was corrected by coexpression of RNAi-resistant full-length mDia2. mDia2 accumulates in the cleavage furrow during anaphase to telophase, and concentrates in the midbody at the end of cytokinesis. Depletion of mDia2 induced contraction at aberrant sites of dividing cells, where contractile ring components such as RhoA, myosin, anillin, and phosphorylated ERM accumulated. Treatment with blebbistatin suppressed abnormal contraction, corrected localization of the above components, and revealed that the amount of F-actin at the equatorial region during anaphase/telophase was significantly decreased with mDia2 RNAi. These results demonstrate that mDia2 is essential in mammalian cell cytokinesis and that mDia2-induced F-actin forms a scaffold for the contractile ring and maintains its position in the middle of a dividing cell.

INTRODUCTION

Cytokinesis is the final step in cell division that physically separates a dividing cell into two. In a somatic cell, separation, i.e., cleavage occurs in the middle of a dividing cell between the two spindle poles to ensure each set of segregated chromosomes inherited to each daughter cell. Although cytokinesis is a multistep process under coordinated control of cell cycle progression, cytoskeletal dynamics, and vesicle transport, the actomyosin-based constriction by the contractile ring that is constructed in the equatorial region of a dividing cell is recognized as a major driving force for physical separation till abscission (Balasubramanian *et al.*, 2004). However, how the position of the contractile ring, i.e., cleavage plane, is determined and maintained through cytokinesis and how and where actin filaments are produced and assembled with myosin and other molecules into the contractile ring in mammalian cells remain largely unknown.

The small GTPase Rho functions in several organisms and several lines of cultured mammalian cells as a molecular switch linking nuclear division and cytokinesis; Rho is activated in anaphase to telophase and induces the contractile ring in dividing cells (Mabuchi *et al.*, 1993; Piekny *et al.*, 2005;

Narumiya and Yasuda, 2006). In mammalian cells, the GTP-bound, activated form of Rho acts on two downstream effectors to induce actomyosin bundles; one is ROCK/Rho-kinase that activates myosin for cross-linking of anti-parallel actin filaments, and the other is mammalian homolog of *Drosophila* diaphanous (mDia) protein that induces actin filaments by catalyzing actin nucleation and polymerization (Watanabe *et al.*, 1997; Sagot *et al.*, 2002). mDia belongs to the formin family of proteins and there are three isoforms, mDia1-3 (Higgs, 2005). mDia has multiple domains, GBD (GTPase-binding domain) in the N-terminus, FH (formin homology) domains, FH1 and FH2, in the middle, and DAD (diaphanous auto-regulatory domain) in the C-terminus (Higgs, 2005; Rose *et al.*, 2005). The FH2 domain binds to the barbed end of an actin filament and catalyzes actin nucleation and polymerization. The FH1 domain accelerates actin elongation by the FH2 domain through binding to the actin monomer-binding protein, profilin (Watanabe *et al.*, 1997; Romero *et al.*, 2004; Kovar *et al.*, 2006). By this action, mDia induces long unbranched actin filaments in contrast to Arp2/3 complex that induces actin meshwork (Goode and Eck, 2007). In addition to the action on actin, mDia has been reported to stabilize and orient microtubules in interphase and mitotic cells (Ishizaki *et al.*, 2001; Palazzo *et al.*, 2001; Yasuda *et al.*, 2004). Intriguingly, although involvement of ROCK in cytokinesis has been examined previously (Kosako *et al.*, 2000), whether mDia protein is involved in cytokinesis of mammalian cells and if so, which mDia isoform functions in this process have not yet been examined thoroughly, though its nonmammalian orthologues such as Cdc12p in *Schizosaccharomyces pombe* and Diaphanous in *Drosophila*

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melanogaster have been shown essential for cytokinesis in each species (Castrillon and Wasserman, 1994; Tominaga *et al.*, 2000; Pelham and Chang, 2002; Dean *et al.*, 2005). To examine this issue, we have used RNA interference (RNAi) to deplete each mDia isoform in NIH 3T3 cells and identified that one of the mDia isoforms, mDia2, is essential for cytokinesis of this cell line. We have also performed fluorescence microscopy for contractile ring components such as RhoA, F-actin, myosin, anillin, and phosphorylated ERM (pERM), as well as live cell-imaging for myosin and mDia2, and examined localization and actions of mDia2 in cytokinesis. We now show that mDia2 localizes in the equatorial region of a dividing cell in anaphase and induces F-actin there to provide an actin scaffold for assembly of the contractile ring and stabilize its position during cytokinesis.

MATERIALS AND METHODS

Materials

Short interfering double-stranded RNA oligomers (siRNAs) K2 and A6 (Ara-kawa *et al.*, 2003; Yamana *et al.*, 2006) were used for RNAi for mDia1. Three different siRNAs, siRNA mDia2#1, #2 and #3, corresponding to nucleotide sequences of 289-313, 1889-1907, and 2150-2168, respectively, were used for RNAi for mDia2 (NM_019670). Two different siRNAs, siRNA mDia3#1 and #2, corresponding to nucleotide sequences of 967-991 and 1295-1319, respectively, were used for RNAi for mDia3 (AY312280). Stealth RNAi negative control duplexes (Invitrogen, Carlsbad, CA) were used for control RNAi. Block-iT Alexa Fluor Red fluorescent Oligo (Invitrogen) was used for determination of efficiency of siRNA transfection. pEGFP-MRLC was described previously (Miyachi *et al.*, 2006). pDsRed2-Histone H2BK has been described previously (Yasuda *et al.*, 2004). pEGFP-mDia2 was prepared as follows. cDNA for mDia2 (Yasuda *et al.*, 2004) was subcloned into pCR-Blunt vector (Invitrogen; pCR-Blunt-mDia2). The plasmid was digested with XhoI and KpnI and then subcloned into pEGFP-C1 (Clontech, San Jose, CA) to generate pEGFP-C1-mDia2 (XhoI-3'), lacking a fragment 5' of the XhoI site of the full-length mDia2. The 5'-XhoI fragment of mDia2 was obtained by PCR amplification using the pCR-Blunt-mDia2 as a template, with 5'-GAACTCGAGCTATG-GAGAGGCACCGGGC-3' as the forward primer and 5'-GTCCCTCTGCTC-GAGTTTCC-3' as the reverse primer. The resultant PCR fragment was digested with XhoI and then subcloned into pEGFP-C1-mDia2 (XhoI-3') to generate pEGFP-C1-mDia2. To prepare mDia2-RNAi-resistant pEGFP-mDia2, pEGFP-mDia2 r#1 and r#2, we introduced silent mutations into the region of pEGFP-C1-mDia2 targeted by mDia2 siRNA #1 using the QuikChange Site-Directed Mutagenesis Kit II (Stratagene, La Jolla, CA) with 5'-GAAAACAACCCAAAGCGCTGCCGGAAGCGAGGTGTGAAGCTTTT-TGAGAAGATG-3' as a template for pEGFP-mDia2 r#1 and 5'-CCCAAG-GCGCTGCCAGAGTCCGAAGTCTTGAAGCTTTTGTAG-3' for pEGFP-mDia2 r#2. To prepare pEGFP-mDia2 I704A, we introduced mutations into the codon of Ile 704 with 5'-GCTCAGAACCTTCAGCCTTCCTGAGCTCCTTCCG-3' as a template as described above.

Primary antibodies (Abs) used were mouse DM1A monoclonal Ab (mAb) to α -tubulin, fluorescein isothiocyanate (FITC)-conjugated DM1A, FITC-conjugated Ab to β -actin, and rabbit polyclonal Ab to myosin IIA (Sigma-Aldrich, St. Louis, MO); rat mAb to α -tubulin (Chemicon, Temecula, CA); goat Ab to mDia3 (N15), rabbit polyclonal Ab to green fluorescent protein (GFP; FL), and mouse 26C4 mAb to RhoA (Santa Cruz Biotechnology, Santa Cruz, CA); and rabbit polyclonal Ab to GFP from MBL (Nagoya, Japan). Rabbit polyclonal antibody to mDia1 was described previously (Watanabe *et al.*, 1997). Polyclonal C1 Ab to mDia2 was generated in rabbits against a glutathione S-transferase (GST) fusion protein of the C-terminal fragment of mDia2 (amino acid residues, 1056-1171). The fragment was subcloned into pGEX-6P-1 (GE Healthcare Life Science, Piscataway, NJ) to generate pGEX-mDia2-C, which was then used for transformation of *Escherichia coli* BL21 (Novagen, Madison, WI). After induction with 1 mM IPTG, the bacteria were lysed and the fusion protein was purified with a GSH-Sepharose column (GE Healthcare Life Science). The purified protein was injected into rabbits as antigen. The antibody to mDia2 was purified from antiserum by affinity chromatography using the antigen coupled with NHS-activated Sepharose (GE Healthcare Life Science). Polyclonal N1 Ab to mDia2 was also generated against a GST fusion protein of the N-terminal fragment of mDia2 (amino acid residues, 33-411) as described above and purified using the antigen coupled with CNBr-activated Sepharose (GE Healthcare Life Science). Rabbit anti-anillin and rat anti-phospho-ERM (pERM) Abs were kind gifts from Dr. Makoto Kinoshita (Kyoto University) and Professor Sachiko Tsukita (Osaka University, Japan).

Cell Culture and Transfection

NIH 3T3 cells and C2C12 cells were maintained in DMEM (GIBCO, Rockville, MD) supplemented with 10% fetal calf serum (FCS) at 37°C with an atmo-

sphere containing 10% CO₂. Transfection of plasmids was performed using Lipofectamine LTX Reagent (Invitrogen) according to the manufacturer's protocol. We diluted 1 μ g of each plasmid DNA and 2 μ l of PLUS reagent (Invitrogen) in 400 μ l of Opti-MEM, subsequently mixed with 5 μ l of Lipofectamine LTX. The lipofectamine solution was mixed with 2 ml of fresh medium and added to cells of 50–60% confluency in one well of a six-well plate. RNAi was performed using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer's reverse transfection protocol. We mixed 1.2 μ l of 20 μ M siRNA duplex and 4 μ l of Lipofectamine RNAiMAX in 400 μ l of Opti-MEM. NIH 3T3 cells or C2C12 cells of semiconfluency were washed and suspended with trypsin-EDTA. The siRNA mixture was added to 1.0×10^5 cells in 2 ml of the culture medium, and the cell suspension was then seeded in a well of a six-well plate. siRNA experiments in synchronized cells were performed as follows. NIH 3T3 cells were seeded and cultured for 16 h in a 100-mm dish with the culture medium containing 2 mM thymidine. The cells were then washed twice with phosphate buffered saline (PBS) and subjected to RNAi transfection as described above. The cells were then seeded and further cultured for 8 h. The medium was then replaced again with the culture medium containing 2 mM thymidine and the cells were cultured for another 16 h. After washing three times with PBS and once with fresh medium, the cells were incubated in the culture medium alone or in that containing 40 ng/ml nocodazole for 8 h. The cells in the latter procedure were then washed free of nocodazole as described above for thymidine removal. The cells were cultured in fresh medium for 2 h for time-lapse imaging for the statistical analysis of the phenotype induced by mDia2 RNAi or for 20 min either with or without 80 μ M blebbistatin (Tocris, Ballwin, MO) before being fixed for immunofluorescence.

Microinjection

NIH 3T3 cells were seeded and cultured for 6 h. The cells were microinjected with normal rabbit IgG (Santa Cruz) or affinity-purified N1 antibody to mDia2 (0.01 mg/ml) in PBS with 0.5 μ g/ml dextran-Alexa-fluor 594 (Molecular Probes, Eugene, OR) using a microinjection system (Eppendorf, Fremont, CA) with maintenance pressure of 400 hPa and injection pressure of 20 hPa for 0.1 s. The cells were then incubated for 10 h before fixation.

Fluorescence Microscopy

NIH 3T3 cells or C2C12 cells were plated onto a coverslip in a 35-mm culture dish for fluorescence microscopy. We used three different fixation protocols. For phalloidin staining for F-actin, cells were fixed with 4% paraformaldehyde in PBS at 37°C for 15 min. The cells were washed three times with PBS and permeabilized with 0.1% Triton X-100 in PBS for 5 min on ice, followed by three washes with PBS. For immunofluorescence for GFP, RhoA, anillin, myosin IIA, pERM, and mDia2, cells were fixed with 10% TCA on ice for 15 min (Yonemura *et al.*, 2004). The fixed cells were washed three times with PBS containing 30 mM glycine (G-PBS) and permeabilized with 0.2% Triton X-100 in G-PBS for 5 min on ice, followed by three washes with G-PBS. For staining for α -tubulin and for comparison of mDia1, mDia2, or mDia3 staining, the cells were fixed with methanol at -20°C for 5 min. After fixation and permeabilization as described above, the cells were incubated with 3% BSA in PBS for 1 h and were incubated at room temperature for 2 h or at 4°C overnight with following primary antibodies: rat anti-tubulin (1:1000 dilution), anti-pERM (1:1), anti-myosin (1:50), anti-anillin (1:200), anti-mDia1 (1:200), anti-mDia2 (1:200), and anti-mDia3 (1:50) Abs. After three washes with Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST), the cells were incubated with appropriate secondary antibodies coupled to Alexa Fluor 594 (Molecular Probes), and/or either Texas-Red phalloidin (1:200) or FITC-conjugated anti- α -tubulin (1:200). The samples were washed three times with TBST before mounting in Prolong Antifade DAPI-Gold (Molecular Probes) on glass slides. Staining was examined with a Leica SP5 confocal imaging system (Plan-Apo 63/1.40 NA; Deerfield, IL). Binucleate or multinucleate cells were identified on samples stained for tubulin and DNA. The percentage of binucleate or multinucleate cells was determined in a blinded manner by an observer without information of the identity of the samples. For the images of interphase cells in Figure 1 and Figure S1, build-up images were obtained from a collection of 10 Z sections of 0.5- μ m step intervals from the bottom to the top of the cells. For the images of dividing cells in Figure S3D and S4C and Figures 4–6, build-up images were obtained from a collection of 15–25 Z sections of 0.5- μ m step intervals around the middle section of the cells. The images were analyzed by the built-in software. Quantification of F-actin intensity in Figure 5A was performed using MetaMorph software (Universal Imaging, West Chester, PA) as follows. Images of 20 mitotic cells each in control and mDia2 RNAi groups were obtained from two different experiments, and the F-actin intensity in each cell was calculated by dividing the sum of Texas-Red phalloidin fluorescence intensity in each pixel by the total pixel counts in the cell area.

Immunoblotting

At indicated times of siRNA treatment, NIH 3T3 cells or C2C12 cells were washed twice with PBS and lysed in 100 μ l of Laemmli sample buffer. Immunoblotting was performed as described previously (Ando *et al.*, 2007). Primary antibodies were diluted in the blocking buffer and added: 1:1000

Figure 1. Effects of depletion of mDia isoforms in NIH 3T3 cells. (A) Depletion of mDia proteins (mDia1, mDia2 and mDia3) by siRNA treatment. NIH 3T3 cells were treated with siRNA specific for mDia1, 2, and 3 (A6, siRNAmDia2#1 and siRNAmDia3#2, respectively) or scrambled control siRNA. After 24 and 48 h, the cells were harvested and subjected to immunoblot for each protein. (B) Fluorescence staining for DNA (blue), F-actin (red), and tubulin (green) in NIH 3T3 cells treated with siRNAmDia2#1 for 48 h. All images show stacks of 10 different focal planes. Bar, 50 μ m. Arrows represent examples of the binucleate cells. (C) Frequency of binucleation in NIH 3T3 cells subjected to RNAi for mDia isoforms. NIH 3T3 cells were subjected to RNAi as indicated and the number of binucleate cells in each population was determined. The results are from three independent experiments, in each of which N > 200 cells were examined. Values are shown as percentage of binucleate cells. Error bars, SD. * p < 0.01 versus control RNAi, mDia1 RNAi, and mDia3 RNAi cells. (D) Rescue of the binucleate phenotype by expression of RNAi-resistant full-length mDia2 construct. NIH 3T3 cells were transfected with pEGFP or pEGFP-mDia2 r#1, and after 24 h, the cells were subjected to control or mDia2 RNAi (siRNAmDia2#1) for 48 h. The cells were either subjected to Western blotting with anti-mDia2 (top), GFP (middle), or tubulin (bottom, left) or subjected to fluorescence staining as described above for determining the number of binucleate cells in each population. Values are shown as percentage of binucleate cells expressing GFP per total cells expressing GFP (right). Error bars, SD. * p < 0.01. (E) Effects of microinjection of N1 antibody to mDia2. Control IgG or N1 antibody to mDia2 were microinjected into randomly growing NIH 3T3 cells along with Alexa Fluor 594 Dextran as a marker, and after 10 h the cells were fixed and stained for tubulin (green) and DNA (blue). Left, immunofluorescence images of control IgG- (top) or anti-mDia2 antibody- (bottom) injected NIH 3T3 cells. Bar, 50 μ m. Right, values are shown as percentage of binucleate cells per injected cells. Error bars, SD. * p < 0.01. The results are from three independent experiments, in each of which N > 100 cells were examined.

dilution for the Abs to α -tubulin, mDia1, mDia2, or GFP and 1:200 dilution for the Ab to mDia3. After overnight incubation at 4°C with these Abs except for incubation at room temperature for 2 h with the Abs to tubulin and GFP, the membranes were washed three times with TBS containing 0.05% Tween-20. The bound primary Abs were detected with corresponding horseradish peroxidase-conjugated secondary Abs (GE Healthcare Bio-Science, Piscataway, NJ; 1:3000 dilution in the blocking buffer) and ECL Western Blotting Detection System (GE Healthcare Bio-Sciences).

Time-Lapse Live Cell Imaging

NIH 3T3 cells were seeded on 35-mm glass-bottom dishes (MatTek, Ashland, MA) with or without siRNA transfection. The medium was replaced 18–24 h after siRNA treatment with DMEM containing 10% FCS and 300 nM Syto11 (Invitrogen), and the cells were incubated for 30 min at 37°C. The dish was then placed on a temperature-controlled stage maintained at 37°C with 5% CO₂. Live cell imaging was performed on an inverted microscope (model DMIRE2; Leica) as described previously (Oceguera-Yanez *et al.*, 2005). Sequential time-lapse images were acquired every 3 min for 120–225 min. For imaging of cells expressing either EGFP-mDia2 or MRLC-EGFP together with DsRed-histone-H2B, the cells were transfected at 8 h after seeding with indicated plasmid DNAs. Live cell imaging was performed on the confocal microscope (TCS-SP5; Leica) with 63 $\times$ /1.40 NA lenses. Sequential time-lapse images were acquired every 30 s or 1 min for 30 min.

Statistical Analysis

Data are presented as mean $\pm$ SD and were analyzed by Student's *t* test. *p* < 0.01 was considered statistically significant.

RESULTS

Effects of Depletion of Each mDia Isoform on Cytokinesis

To examine whether mDia isoforms are required for cytokinesis, and, if so, to identify which mDia isoform functions in this process, we transfected NIH 3T3 cells with siRNA specific to each mDia isoform, A6 for mDia1, siRNAmDia2#1 for mDia2, and siRNAmDia3#2 for mDia3. In these experiments, transfection efficiency of siRNA determined by Block-iT Red fluorescent Oligo was almost 100% (Figure S1A). The cells were collected 24 and 48 h after transfection and subjected to Western blotting. The treatment with each siRNA specifically and time-dependently suppressed the expression of corresponding mDia isoforms. After 48 h, only a negligible amount of each protein was detected by Western blotting; the percentage of depletion of endogenous proteins at 48 h were 75, 95, and 70% for mDia1, mDia2, and mDia3, respectively (Figure 1A). The siRNA treatment for each mDia isoform did not apparently change the cell shapes. Although RNAi for mDia1 and mDia3 did not affect cytokinesis (Figure S1, C and D), mDia2-RNAi increased the proportion of the cells with two nuclei (binucleate cells) as evidenced by immunofluorescence as well as flow cytometry (Figure 1B and Figure S1D). Failure of cytokinesis, consequently, increased the size of these cells (Figure S1B). Quantitative analysis revealed that the proportion of the binucleate cells significantly increased in the mDia2-depleted cell population compared with that in the control, mDia1-depleted, or mDia3-depleted population; the percentage of binucleate cells increased to 25.8 ± 4.9 and $39.8 \pm 1.2\%$ 24 and 48 h after transfection with mDia2 siRNA, respectively (Figure 1C). We confirmed these results by using additional nonoverlapping siRNAs for mDia1 and mDia3, K2 and siRNAmDia3#1, respectively, and two additional nonoverlapping siRNAs for mDia2, siRNAmDia2#2 and #3. Although all siRNAs sufficiently depleted respective mDia isoforms, only treatment with siRNAs specific for mDia2 significantly increased the rates of binucleate cells (data not shown). To further validate that binucleation is caused by mDia2 depletion, we attempted to rescue the phenotype by cotransfecting a RNAi-resistant full-length construct of mDia2 fused to enhanced GFP (EGFP) (EGFP-mDia2 r#1) with siRNAmDia2#1. Expression of EGFP was

used as a control in these experiments. Depletion of endogenous mDia2 and expression of EGFP-mDia2 r#1 were verified by immunoblotting (Figure 1D, left). Expression of the EGFP-mDia2 r#1 fully rescued the effects of mDia2 RNAi on cytokinesis; the rate of binucleate cells returned to the level in the control cells after the expression of this construct (Figure 1D, right). These results suggest that the generation of binucleate cells was specifically induced by reduction of mDia2 protein. We further microinjected the antibody N1 to mDia2 and evaluated its effect on cytokinesis. Ten hours after we microinjected the mDia2 antibody into randomly growing NIH 3T3 cells, we observed significant increase in the percentage of binucleate cells compared with control IgG injected cells (Figure 1E). We obtained similar results when we injected the mDia2 antibody into NIH 3T3 cells synchronized in G2 by double thymidine block and observed 5 h later (data not shown). These results that depletion of mDia2 induces cytokinesis failure demonstrate that an mDia isoform is required for cytokinesis of NIH 3T3 cells and that it is mDia2 that functions as an mDia isoform essential in this process. In contrast to these findings, Tominaga *et al.* (2000) previously microinjected antibodies to mDia1 or mDia2 into dividing NIH 3T3 cells and found that the injection of anti-mDia1 antibody and not the antibody to mDia2 interfered with cytokinesis. Curiously, they did not find significant expression of mDia2 in these cells, and yet they rescued cytokinesis failure induced by anti-mDia1 antibody by overexpression of full-length mDia2. Although the reason for such apparent discrepancy is currently unknown, we examined a possibility that mDia1 functions redundantly with mDia2 in cytokinesis. We therefore depleted mDia isoforms in every possible combination and examined cytokinesis failure. We found significant increases in the number of multi(bi)-nucleate cells upon depletion of mDia1 or mDia3 only combined with mDia2 depletion, i.e., combined depletion of mDia1 and mDia2, that of mDia2 and mDia3 or that of mDia1, mDia2 and mDia3, and not that of mDia1 and mDia3 (Figure S1E). Importantly, combination of mDia1 and mDia2 depletion did not enhance the rate of binucleate cells compared with that found on mDia2 depletion alone. These results suggest that at least in the NIH 3T3 cells we used, mDia2 is primarily important in cytokinesis among mDia isoforms.

Localization of mDia2 during Cell Division

To obtain insights into action mechanism of mDia2 in cytokinesis, we next investigated the localization of endogenous mDia2 in different phases of cell division by staining for mDia2, tubulin, and DNA in dividing NIH 3T3 cells using the C1 antibody to mDia2. This analysis revealed that signals of mDia2 localized around the basal part of cell cortex of rounding cells in prometaphase to metaphase, appeared at equatorial cell cortex in late anaphase, accumulated in the cleavage furrow in telophase, and finally concentrated in the intercellular bridge at the end of cytokinesis (Figure 2). The specificity of these signals was verified by their loss with mDia2 RNAi both on Western blot analysis and immunofluorescence (Figure 1A and Figure S2A). We observed similar localization of mDia2 by using a different mDia2 antibody N1 (data not shown). The localization of mDia2 described above was also confirmed by expressing pEGFP-mDia2 and monitoring the GFP signal. The GFP signals began to localize at the equatorial surface during late anaphase and concentrated in the cleavage furrow during cytokinesis (Movie S1). For comparison, we examined the localization of mDia1 and mDia3 during cell division. Weak diffuse signals for mDia1 were observed over the cell bodies and intercellular bridge at the end of telophase

Figure 2. Localization of mDia2 during cell division. NIH 3T3 cells in different phases of cell division were stained for mDia2 (red), tubulin (green), and DNA (blue). Each image shows a single focal plane; the image of a prometaphase cell represents that at the basal plane, and those of cells in anaphase, telophase and cytokinesis represent those at the middle plane. Bar, 5 μ m.

(Figure S2B); signals for mDia3 were found in association with the central spindle in anaphase, and the associated mDia3 signals were concentrated in the midbody as central spindle microtubules were bundled in cytokinesis (Figure S2C). Staining for neither mDia1 nor mDia3 yielded signals in the cleavage furrow as that for mDia2. These localization patterns of mDia1 and mDia3 are consistent with our previous results (Kato *et al.*, 2001; Yasuda *et al.*, 2004). These results suggest that mDia2 accumulates specifically in the cleavage furrow during cytokinesis.

We next wondered whether this function of mDia2 in cytokinesis is conserved in other cell lines. We observed that mDia2 RNAi also induced cytokinesis failure in C2C12 mouse myoblast cells and mDia2 localized to the cleavage furrow of these cells (Figure S3), supporting the conserved localization and function of mDia2 during cytokinesis.

Abnormal Contraction in mDia2-depleted Cells

To investigate how mDia2 depletion induces cytokinesis failure in NIH 3T3 cells, we monitored the progression of cell division of mDia2-RNAi cells by videomicroscopy. We first used randomly growing cells subjected to control and mDia2 RNAi. In control cells, the cleavage furrow appeared 6 min after anaphase onset and ingressed progressively thereafter to separate two daughter cells within several minutes, and the separated daughter cells linked by the intercellular bridge began to spread at 18 min (Figure 3A and Movie S2). In contrast, mDia2 RNAi cells exhibited abnormal cytokinesis behavior. In one group of the cells, the cleavage furrow appeared between two daughter cells in anaphase and began to ingress. However, the ingression was not properly maintained but followed by robust contraction at aberrant sites of daughter cells that prevented the ingression at the cleavage furrow and pushed separating chromosomes one end to the other, resulting in fusion of the daughter cells and production of a binucleate cell (Figure 3B and

Figure 3. Cytokinesis failure in mDia2-RNAi cells. (A–C) Selected frames from time-lapse movies of NIH 3T3 cells subjected to control RNAi (A and Movie S2) or RNAi for mDia2 (B and C and Movies S3 and S4). Phase contrast and green fluorescence images were monitored by videomicroscopy after DNA (green) was stained by Syto11. 0 min, anaphase onset. Insets show magnified views of the DNA in each merged image. Arrows in B and C indicate abnormal contraction in the mDia2-depleted cells. Bar, 10 μ m.

Figure 4. Abnormal localization of contractile ring components during anaphase/telophase in mDia2-depleted cells. (A) Immunofluorescence. NIH 3T3 cells were transfected with siRNA for mDia2 (siRNAmDia2#1) and synchronized with thymidine and nocodazole, and at 32 h after transfection the cells were released into DMEM containing 10% FCS for 20 min. The cells were fixed and stained for indicated molecules (red), tubulin (green), and DNA (blue). All images show stacks of focal planes as described in *Materials and Methods*. Bar, 5 μ m. Arrows indicate abnormal accumulation of indicated molecules. (B and C) Selected frames from time-lapse movies of NIH 3T3 cells subjected to control (B and Movies S5 and S6) or mDia2 RNAi (C and Movies S7 and S8), and labeled with DsRed-histone H2Bk (red) and MRLC-EGFP (green). Bar, 5 μ m. (D) Effects of combined depletion of mDia2 and other mDia isoforms. NIH 3T3 cells were transfected with siRNA for mDia isoforms in indicated combination for 48 h, and the cells were stained for F-actin (red), tubulin (green), and DNA (blue). A typical example is shown out of 20 cells examined. Arrows indicate abnormal accumulation of F-actin. Bar, 5 μ m.

Movie S3). In another group of mDia2 RNAi cells, contraction was frequently observed already at metaphase, and such contraction at abnormal sites continued in anaphase to telophase and apparently inhibited appearance and functioning of the cleavage furrow at the prospective site, leading to formation of a binucleate cell upon spreading (Figure 3C and Movie S4).

To statistically analyze the cytokinesis failure in mDia2-RNAi cells, we next enriched mitotic cells by using thymidine and nocodazole after RNAi treatment and tracked the cell division by videomicroscopy. We confirmed a similar phenotype of the cytokinesis failure in mDia2-RNAi cells (data not shown). Percentages of cells showed cytokinesis failure were 6.1 and 49.0% for control ($n = 33$) and mDia2-RNAi cells ($n = 51$), respectively. Percentages of cells showed the abnormal contraction were 3.0 and 76.5% for control ($n = 33$) and mDia2-RNAi cells ($n = 51$), respectively, suggesting that there is a link between the cytokinesis failure and the abnormal contraction induced by mDia2 RNAi. These results indicate that mDia2 is required for maintenance and functioning of the contractile ring between two daughter cells.

Effects of mDia2 Depletion on Distribution of the Contractile Ring Components

The contractile ring is composed of several components including F-actin, myosin, anillin, and pERM (Straight *et al.*, 2003; Yokoyama *et al.*, 2005). To examine whether these components accumulate and are maintained at the prospective cleavage site during cytokinesis of mDia2 RNAi cells, we stained for these contractile ring components by using Texas-Red phalloidin and antibodies to each molecule in cells depleted of mDia2. We stained for myosin by using antibody to myosin heavy chain IIA. mDia2-RNAi cells showed aberrant shapes (Figure 4), which probably reflected abnormal contraction observed in time-lapse imaging analysis (Movies S3 and S4). In these mDia2-RNAi cells, signals for each of F-actin, myosin, anillin, and pERM were not observed at the cleavage furrow as in control cells, but aberrantly localized around the cell cortex where abnormal

cell shape change was observed (Figure 4A). To examine dynamics of such localization of the contractile ring components, we expressed EGFP-fusion of myosin regulatory light chain (MRLC-EGFP; Miyauchi *et al.*, 2006) and followed its movement during cell division. Although myosin accumulated normally at the cleavage furrow from anaphase to telophase and concentrated there as the furrow ingressed in cytokinesis in control-RNAi cells (Figure 4B and Movies S5 and S6), significant accumulation of MRLC-EGFP was found at sites of abnormal contraction in the cell cortex of mDia2-RNAi cells. Intriguingly, such abnormal contraction was observed already in prometaphase/metaphase before chromosomes began to segregate and became more robust in anaphase to telophase, and MRLC-EGFP was found to accumulate at sites of each contraction (Figure 4C and Movies S7 and S8). This contraction-associated localization pattern of EGFP-MRLC appears similar to localization of other components of the contractile ring in fixed preparation of mDia2 RNAi cells, indicating that the components of the contractile ring in mDia2-depleted cells were not maintained at the cleavage furrow but moved together to the site of abnormal contraction. We next examined effects of combined depletion of mDia2 with other mDia isoforms in order to examine whether this abnormal contraction is a direct consequence of the loss of mDia2 or due to a shift in the balance of Rho effectors caused by the mDia2 depletion. Combined depletion of either mDia1 or mDia3 or both with mDia2 did not abolish abnormal contraction caused by the loss of mDia2 (Figure 4D). Immunofluorescence study also showed that there was no clear accumulation of mDia1 and mDia3 at the site of abnormal contraction (data not shown). These results suggest that mDia2 itself is important for proper positioning and maintenance of the contractile ring components during cell division.

Effects of Blebbistatin on Accumulation of the Contractile Ring Components in mDia2-depleted Cells

The above results demonstrate that the contractile ring components accumulate at various sites of mDia2-RNAi cells where aberrant contraction occurs. On the other hand, it has

Figure 4. (cont).

been shown that various contractile ring components utilize apparently different mechanisms and accumulate at the equatorial region of the dividing cell cortex in anaphase and are assembled there to the contractile ring (Straight *et al.*, 2003; Yokoyama *et al.*, 2005). We wondered whether mDia2 depletion interfered with such initial accumulation process of the components. We could not fully examine this issue in intact

mDia2-depleted cells because of strong contraction in these cells. We therefore used blebbistatin, a myosin II ATPase inhibitor, to suppress contraction and examined localization of each component (Straight *et al.*, 2003). Immunofluorescence analysis showed that the accumulation of signals for F-actin at the equatorial region appeared weaker in mDia2-RNAi cells compared with control-RNAi cells (Figure 5A,

Figure 5. Localization of contractile ring components in blebbistatin-treated cells. NIH 3T3 cells were subjected to control or mDia2 RNAi and synchronized with thymidine and nocodazole, and at 32 h after transfection the cells were released into the medium containing 80 μ M blebbistatin for 20 min. The cells were fixed and stained for indicated molecules (red), tubulin (green), and DNA (blue). All images show stacks of focal planes as described in *Materials and Methods*. Bar, 5 μ m. (A) Phalloidin staining for F-actin in blebbistatin-treated cells. Left, phalloidin staining of control or mDia2-RNAi cells are shown. Right, quantitative analysis of fluorescence intensity for F-actin in control or mDia2-RNAi cells. Fluorescence intensity of phalloidin staining for total area of each cell was quantified using MetaMorph in 20 cells of each group from two different experiments as described in *Materials and Methods*. * $p < 0.01$. (B) Immunofluorescence for MyosinIIA, anillin, and pERM in blebbistatin-treated cells.

left). Decrease of F-actin in mDia2-RNAi cells was verified by quantitative fluorescence intensity measurements of whole cells subjected to control and mDia2 RNAi (Figure 5A, right). Given that mDia proteins have actin-nucleating and -polymerizing activity, these results indicate that mDia2 is indeed responsible for formation of F-actin at this site. Therefore, we next examined whether this actin-nucleating/polymerizing activity of mDia2 is indeed required for the cytokinesis by attempting to rescue the mDia2 RNAi-induced cytokinesis failure by expressing an actin-polymerization-defective GFP-mDia2 mutant. Mutation into Ile⁷⁰⁴ of mDia2 has been shown to render mDia2 actin polymerization-defective (Xu *et al.*, 2004; Harris *et al.*, 2006). We found that GFP full-length mDia2 I704A could not rescue the mDia2 RNAi-induced cytokinesis failure, whereas this GFP full-length mDia2 I704A localized to the cleavage furrow (Figure S4, B and C). These data further support that the actin-nucleating/polymerizing activity of mDia2 is required for cytokinesis.

In contrast to the above findings on F-actin, we found signals for myosin, anillin, or pERM at equatorial cortex of both control and mDia2-RNAi cells in the presence of blebbistatin, but these components showed broader localization in mDia2-RNAi cells compared with control cells (Figure 5B). The percentages of cells showing the localization of each components wider than the 120° sector from the center of the cell are 20 and 62% for myosin for control and mDia2-RNAi cells ($n = 50$ for each), 16 and 58% for anillin for control and mDia2-RNAi cells ($n = 50$ for each), 29 and 40% for pERM for control and mDia2-RNAi cells, respectively ($n = 45$ for each). We also treated control-RNAi cells with latrunculin B in the presence of blebbistatin during anaphase/telophase to

test the contribution of F-actin in this effect, and found that latrunculin-treated cells showed dispersion of signals for anillin and myosin around the cell cortex (data not shown). Given that latrunculin-treated cells showed dispersed localization of the contractile ring components including anillin, the mDia2-driven F-actin production in the equatorial cortex appeared important for concentration and maintenance of the contractile ring components, myosin II, anillin, and pERM in the cleavage furrow during anaphase/telophase.

Effects of mDia2 Depletion on Rho Localization during Cytokinesis

The small GTPase Rho is activated in anaphase to telophase, localizes in the cleavage furrow, and induces assembly of the contractile ring there (Piekny *et al.*, 2005). It is also believed that Rho acts on downstream effectors such as ROCK and citron kinase and produces contraction of the contractile ring for cleavage (Madaule *et al.*, 1998; Kosako *et al.*, 2000; Ueda *et al.*, 2002; Yamashiro *et al.*, 2003; Piekny *et al.*, 2005). It is therefore interesting to know whether aberrant contraction seen in mDia2-depleted cells is associated with or dissociated from Rho activation. To examine this issue, we stained for RhoA during anaphase/telophase in mDia2-depleted cells in the presence or absence of blebbistatin (Figure 6, A and B). Although signals for RhoA accumulated in the cleavage furrow during anaphase/telophase in control cells, RhoA localized aberrantly at sites of the cell cortex of apparently abnormal contraction in mDia2-RNAi cells without blebbistatin (Figure 6A). On the other hand, in the presence of blebbistatin, almost all signals for RhoA were restricted to the equatorial region of mDia2-RNAi cells. The equatorial localization of RhoA was found in 18 of 20 cells in mDia2-

Figure 6. Effects of mDia2 depletion on RhoA localization during cytokinesis. (A) Aberrant localization of RhoA in mDia2-RNAi cells. NIH 3T3 cells were transfected and synchronized as described in the legend for Figure 4. The cells were released into the medium without blebbistatin. After 20 min, the cells were fixed and stained for RhoA (red), tubulin (green), and DNA (blue). All images show stacks of 15–25 focal planes. Bar, 5 μ m. (B) Effects of blebbistatin on RhoA localization in mDia2-RNAi cells. NIH 3T3 cells were subjected to mDia2 RNAi and synchronized with double thymidine block. The cells were treated with DMSO or 80 μ M blebbistatin for 30 min 6.5 h after thymidine removal and stained for RhoA (red), tubulin (green), and DNA (blue). All images show stacks of 15–25 focal planes. Bar, 5 μ m.

RNAi cells with blebbistatin compared with 8 of 20 in mDia2-RNAi cells without blebbistatin (Figure 6B). Given that blebbistatin was added upon mitosis in this experiment, these results taken together suggest that RhoA localizes in the prospective cleavage furrow and induces the contractile ring complex there in a manner independent of mDia2, but that mDia2-driven F-actin is important for maintenance of the RhoA-containing contractile ring complex at the site of the cleavage furrow.

DISCUSSION

In this work, using RNAi, we have examined the involvement of three mDia isoforms in cytokinesis and identified mDia2 as an essential mDia isoform required for cytokinesis in NIH 3T3 cells and C2C12 cells (Figure 1 and Figure S3). We have also analyzed localization of the mDia isoforms and found that mDia2 localizes specifically in the cleavage furrow during cytokinesis. We have found that depletion of mDia2 substantially reduced the amount of F-actin accumulating in the cleavage furrow but did not affect accumulation of other contractile ring components such as RhoA, myosin, anillin, and pERM in the equatorial region. However, without mDia2, these components could not be properly integrated into the contractile ring, but apparently formed incomplete complexes, which were shifted away from the

equatorial region and induced contraction at aberrant site of a dividing cell.

Our findings summarized above can thus not only suggest functions of mDia2 in cytokinesis but also address to some of the major questions regarding cytokinesis. First, given that mDia molecules can stabilize and align microtubules (Ishizaki *et al.*, 2001; Palazzo *et al.*, 2001; Yasuda *et al.*, 2004) and that the cleavage plane is suggested to be specified by spindle microtubules that are stabilized in the equatorial region (Canman *et al.*, 2003), it is possible that mDia isoforms are involved in determination of the cleavage plane. However, the above observation that depletion of mDia2 does not interfere with RhoA accumulation in the equator argues against this idea and rather suggests that RhoA accumulates first in the cleavage plane and recruit mDia2 there. This is consistent with the property of mDia2 to bind to members of the Rho GTPases such as RhoA, Rac1, and Cdc42 (Alberts *et al.*, 1998; Yasuda *et al.*, 2004). However, binding to RhoA cannot explain selective localization of mDia2 to the cleavage furrow, because other mDia isoforms can bind to RhoA as well. Further analysis is therefore required to elucidate a mechanism for selective localization of mDia2 to the cleavage furrow.

Second, it is intriguing that mDia2 localizes in the cleavage furrow and that its depletion reduced the F-actin amount there. Previously, it was argued whether F-actin is formed in the cleavage furrow or formed elsewhere and transported to the furrow (Wang, 2005; Eggert *et al.*, 2006). Given that mDia molecules are capable of catalyzing actin nucleation and polymerization, our results strongly suggest that the majority of F-actin is produced in situ in the cleavage furrow by the action of mDia2 and accumulate there. Then, what functions do actin filaments induced by mDia2 exert in cytokinesis? Abnormal contraction at aberrant sites apparently by the contractile ring components including RhoA, myosin, anillin, and pERM in mDia2-depleted cells suggests that with mDia2 depletion and/or with depletion of mDia2-induced F-actin, the contractile ring is not properly organized and is not maintained at the prospective site of the cleavage furrow, which indicates that mDia2 and mDia2-induced F-actin link these components together to form the contractile ring and stabilize its position in the equatorial region. Formins such as mDia2 can produce long, straight actin filaments. The structure of the contractile ring was studied by electron microscopy in fission yeast and newt eggs, and these studies revealed that it consists of anti-parallel bundles of straight actin filaments that are bound to the plasma membrane through barbed ends (Mabuchi *et al.*, 1988; Kamasaki *et al.*, 2007). Although it is argued whether such structure is applied also to the contractile ring in mammalian cells (Eggert *et al.*, 2006), it is tempting to speculate that mDia2 induces straight actin filaments of opposite directionality in the prospective site of the cleavage furrow, which provide an actin-based scaffold encircling the equatorial region of dividing cells and facilitate formation of the contractile ring complex by incorporating other components of the ring such as myosin, anillin, and pERM to this actin scaffold. By such actions, mDia2 may restrict the movement of the contractile ring and stabilize its position. mDia2 may also function in anchoring the actin filaments of the contractile ring to the plasma membrane, because it accumulates in the equatorial cell cortex and its binds to the barbed end of actin filaments. Our results are thus consistent with and have substantially extended the findings by Dean *et al.* (2005), who examined localization of myosin in dividing *Drosophila* S2 cells subjected to RNAi for diaphanous and showed that diaphanous is required for

maintenance of myosin II to the cleavage furrow. It has to be mentioned, however, that construction of the contractile ring may not be governed solely by mDia2 but by interdependent actions of the contractile ring components including mDia2. Recently, anillin has been reported to bind myosin, and RNAi of anillin induces a phenotype similar to that we have found in mDia2-depleted cells (Straight *et al.*, 2005).

Finally, in this study, we also noted that the mitotic cells depleted of mDia2 exhibited mild oscillatory contractions during prometaphase and metaphase and that the cortical localization of myosin was not uniform as typically seen in control cells (Movies S4, S7, and S8). Mitotic cell rounding is the process in which a flat interphase cell becomes spherical and is associated with rearrangement of the actin cytoskeleton, de-adhesion, and an increase in cortical rigidity (Maddox and Burridge, 2003; Thery and Bornens, 2006). Maddox and Burridge (2003) reported that mitotic cell rounding requires activation of RhoA. In this study, we observed that mDia2 localized to the cell margin in rounding NIH 3T3 cells and mDia2-depleted cells exhibit impaired mitotic rounding (Figures 2 and 3B). The oscillatory contractions of mDia2-depleted cells mentioned above may be caused by impaired rigidity of mitotic cells in the absence of mDia2. Eisenmann *et al.* (2007) showed that expression of Dip, which they claimed as an inhibitory binding protein for mDia2, induced nonapoptotic blebbing in HeLa cells, which is thought to be caused by breaks in cortical rigidity. These results suggest that, in addition to its action in cytokinesis, mDia2 also function in maintenance of cortical rigidity and rounding of mitotic cells. Given that cytokinesis is now recognized as a consequence of many events occurring globally in the cell cortex through cell division (Maddox and Burridge, 2003; Wang, 2005; Mukhina *et al.*, 2007), these results may suggest that mDia2 functions not only by inducing F-actin in the cleavage furrow but also by regulating the cortical rigidity globally. It may shift the balance of the contractility of mitotic cells by shifting its accumulation in the cell dependent on the phase of cell division. Elucidation of a mechanism how functions of mDia2 in different phases of cell division is regulated may unravel how cells execute mitosis and cytokinesis properly through adjusting cell morphogenesis with chromosome separation.

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