Localization of a mammalian homolog of diaphanous, mDia1, to the mitotic spindle in HeLa cells

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SUMMARY

mDia1 is a mammalian homolog of *Drosophila* diaphanous and works as an effector of the small GTPase Rho. It is a member of the formin homology (FH) proteins and contains the Rho-binding domain and an FH3 region in its N terminus, an FH1 region containing polyproline stretches in the middle and an FH2 region in the C terminus. Several lines of evidence indicate that mDia1 and diaphanous are essential in cytokinesis. mDia1 is present in a large amount in the cytoplasm of both interphase and mitotic cells. Using the instantaneous fixation method that preferentially extracts soluble components, we have analyzed localization of mDia1 in mitotic HeLa cells. Immunocytochemistry using polyclonal anti-mDia1 antibody revealed specific immunofluorescence localized to the mitotic spindle. This localization was seen from prophase to telophase. Western blot analysis also detected anti-mDia1 immunoreactivity in the mitotic spindle fraction isolated from mitotic HeLa cells. Consistently, expression of full-length mDia1 as a fusion protein with green fluorescence protein (GFP) revealed the GFP fluorescence again in the mitotic spindle in HeLa cells. Expression of GFP fusions of various truncated mutants of mDia1 identified that this localization is determined by a 173 amino acid-long sequence between the Rho-binding domain and the FH1 region, which contains the C-terminal part of the FH3 region. Point mutation analysis revealed that Leu^{434} and Leu^{455} in the FH3 region are essential in localization to the mitotic spindle. Neither electroporation of *botulinum* C3 exoenzyme nor microinjection of Val14RhoA into mitotic cells affected the localization of endogenous mDia1 to the mitotic spindle, suggesting that mDia1 localizes to the mitotic spindle independent of Rho activity. The present study has thus established the mDia1 localization in the mitotic spindle. This localization suggests a role of mDia1 in the spindle-cleavage furrow interaction during cell division.

Key words: Rho, Formin homology protein, mDia1, Cytokinesis, Mitotic spindle

INTRODUCTION

The small GTPase Rho regulates actin cytoskeleton by shuttling between the GDP-bound inactive form and the GTP-bound active form (Narumiya et al., 1997; Hall, 1998). Typically, a GTP-bound active form causes the organization of actin stress fibers, and inactivation of Rho leads to the dissolution of these F-actin structures as revealed by expression or microinjection of Val14RhoA and treatment of cells with C3 exoenzyme, respectively. Similar experiments using Val14RhoA and C3 exoenzyme have further revealed that Rho works as a molecular switch in various other cellular processes such as cell morphogenesis, cell motility and cytokinesis. Cytokinesis is carried out by contraction of the contractile ring, an actomyosin bundles anchored to the equatorial cell cortex between two divided nuclei, which results in production of two daughter cells (Rappaport, 1996; Robinson and Spudich, 2000). Treatment of cells with C3 exoenzyme inhibits cytokinesis and produces multinucleate cells (Mabuchi et al., 1993; Kishi et al., 1993; Drechsel et al., 1997). Moreover, the microinjection of C3 exoenzyme into cells undergoing cytokinesis causes the dissolution of the contractile ring and the regression of the cleavage furrow (Mabuchi et al., 1993). These findings indicate that activation of Rho is required for induction, maintenance and contraction of the contractile ring. Indeed, accumulation of GTP-bound Rho was noted at the end of metaphase and was maintained throughout cytokinesis (Kimura et al., 2000). However, it remains to be known how Rho induces the contractile ring and triggers cytokinesis.

Rho performs its cellular functions by binding to downstream effectors. Putative Rho effectors have been identified and isolated on the basis of their selective binding to the active form of Rho (Watanabe et al., 1996; Ishizaki et al., 1996). mDia1 was also isolated as such a binding molecule by a yeast two-hybrid system (Watanabe et al., 1997). It is a mammalian homolog of *Drosophila* diaphanous, and is a member of formin homology (FH) proteins that contains the Rho-binding domain and an FH3 region in its N terminus, an FH1 region containing polyproline stretches in the middle and an FH2 region in the C terminus (Wasserman, 1998). The FH1 region of mDia1 serves as a binding site for an actin-monomer binding protein, profilin (Watanabe et al., 1997). Profilin is known to induce actin polymerization in the presence of actin monomers and thymosin β4 (Pantaloni and Carlier, 1993; Kang et al., 1999). Indeed, overexpression of N-terminally truncation mutants containing the FH1-FH2 unit of mDia1 without the Rho-binding domain induced thin actin stress fibers in HeLa cells. This finding together with the intramolecular interaction

between the N terminus containing the Rho-binding domain and the C terminus of mDia1 suggests that active RhoA binds to the N terminus to expose the functional FH1 and FH2 regions for activation. It was shown previously that Rho-induced stress fibers is mediated also by another Rho effector, a Rho-associated kinase (ROCK/ROK/Rho-kinase; Leung et al., 1996; Ishizaki et al., 1997; Amano et al., 1997). This action of ROCK was proposed to be due to its actin bundling activity by virtue of an increased myosin contractility (Chrzanowska-Wodnicka and Burridge, 1996). Consistently, the thin stress fibers induced by active mDia1 were disorganized by treatment of the cells with a specific ROCK inhibitor, Y-27632 (Uehata et al., 1997). Moreover, co-expression of active mDia1 with active ROCK corrected excessive bundling and disorganized alignment of actin fibers induced by overexpression of active ROCK alone (Watanabe et al., 1999). These findings indicate that mDia1 and ROCK cooperate downstream of Rho in induction of stress fibers. In non-transfected cells, mDia1 is colocalized with Rho and profilin in membrane ruffles of rapidly spreading cells and in phagocytic cups around fibronectin-coated beads (Watanabe et al., 1997), indicating that mDia1 is involved in the organization of Rho-induced actin cytoskeleton at these sites in interphase cells. Then, how does mDia1 act in cytokinesis?

In *Drosophila*, mutations in *diaphanous* gene resulted in polyploidity of cells (Castrillon and Wasserman, 1994), and, in mammalian cells, microinjection of a specific anti-mDia1 antibody into NIH 3T3 cells produced binucleate cells (Tominaga et al., 2000), suggesting that a product of *diaphanous* and mDia1 are essential in cytokinesis in cells of respective species. The phenotypic analysis in *Drosophila* embryo deficient in *diaphanous* gene (Afshar et al., 2000) revealed that diaphanous is essential for formation of the metaphase furrow, cellularization and formation of the pole cells, the events dependent on actin reorganization in *Drosophila* embryonal cell cycle. Diaphanous is required for recruitment of anillin and Peanut, a *Drosophila* septin, to the metaphase furrow. During these processes, Diaphanous is localized to the cortical actin caps in interphase and prophase and to the growing tip of the metaphase and cellularization furrows. How diaphanous is targeted to these sites, however, remains unknown. Compared to *Drosophila*, much less is known on the localization and functions of mDia1 in mitotic mammalian cells, although mDia1 is occasionally seen enriched in the intercellular bridge in dividing cells (Watanabe et al., 1997; Tominaga et al., 2000). Involvement of other Rho effectors including ROCK and citron kinase in cytokinesis was also reported (Kosako et al., 1999; Madaule et al., 1998; Ishizaki et al., 2000). These two Rho effectors are concentrated in the cleavage furrow during cytokinesis, suggesting that mDia1 may also cooperate with these Rho effectors in organization of the contractile ring. The contractile ring is induced in equatorial cell cortex between the two divided nuclei. Previous studies indicate that the position of the ring is determined by central spindle microtubules in mammalian cells (Rappaport, 1996; Mastronarde et al., 1993; Cao and Wang, 1996). We have investigated localization of mDia1 during mitosis using the instantaneous fixation method that yields preferential preservation of the cytoskeletons. We have revealed that mDia1 localizes to the mitotic spindle during mitosis independent of the Rho activity.

MATERIALS AND METHODS

Cell culture and fixation

HeLa cells were grown on glass coverslips in DMEM supplemented with 10% FCS maintained at 37°C with an atmosphere containing 5% CO_2. The cells were fixed by an instantaneous fixation method described previously (Arcangeletti et al., 1997). Briefly, the cells were rinsed with CSK buffer (10 mM NaOH-PIPES, pH 6.9, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA and 1.2 mM PMSF) pre-warmed at 37°C, and the cells were then treated for 20 minutes with 1% paraformaldehyde and 10% Triton X-100 in CSK buffer at 37°C. The cells were washed with phosphate buffered saline (PBS) three times, and blocked with 5% BSA in Tris buffered saline (TBS) (the instantaneous fixation method). Alternatively, the washed cells were treated with 0.5% paraformaldehyde and 10% Triton X-100 in CSK buffer for 5 minutes, and then fixed with 4% paraformaldehyde in CSK buffer for 20 minutes at 37°C (the modified instantaneous fixation method).

Immunofluorescence

Fixed cells were incubated with rabbit polyclonal anti-mDia1 antibody which was affinity-purified as described (Watanabe et al., 1997) and mouse monoclonal anti-β-tubulin antibody (clone TUB 2.1, Sigma) as primary antibodies. Alexa Fluor™ 488-labeled anti-rabbit IgG and Alexa Fluor™ 568-labeled anti-mouse IgG (Molecular Probes) were used as secondary antibodies to detect anti-mDia1 antibody and anti-β-tubulin antibody, respectively. DNA was visualized with TOPRO-3 (Molecular Probes). For competitive experiment, the cl.50-encoded part of mDia1 containing the antigenic determinants was expressed as a GST fusion protein (GST-cl.50) in *Escherichia coli*, purified and added with primary antibodies at the final concentration of 0.2 μg/μl. For latrunculin A (LatA) treatment of mitotic cells, HeLa cells were synchronized at the beginning of S phase by thymidine double block (Madaule et al., 1998). After 10 hours recovery from the second thymidine block, latrunculin A was added to the culture medium at final concentration of 0.3 μg/ml. After 2 hours culture, the cells were subjected to the modified instantaneous fixation method and staining for mDia1, tubulin and DNA. Fluorescence images were acquired by an MRC1024 laser scanning confocal microscope imaging system (Bio-Rad) equipped with a Zeiss Axiovert 100TV microscope.

Preparation of the mitotic spindle fraction

Preparation of mitotic spindles from HeLa cells was performed as described (Kuriyama et al., 1984). HeLa cells were cultured in the medium containing 40 ng/ml nocodazole. After 12 hours, mitotic cells were collected by mechanical shaking and released from the nocodazole block by washing twice with a fresh culture medium without the drug. The cells were suspended in fresh culture medium and cultured for 1 hour. For preparation of the mitotic spindle fraction, half of the cell suspension was pelleted and resuspended at a density of 10^7 cells/ml in a preparation buffer (2 mM NaOH-PIPES, pH 6.8 and 0.05% Triton X-100) containing 20 μg/ml taxol and the cells were gently disrupted by vortex-mixing. The precipitates were recovered by centrifugation and washed with the same solution. The lysates were centrifuged at 1,000 ***g*** for 5 minutes and the pellet was recovered (the mitoic spindle fraction). As control fraction, the remaining half of the cell suspension was subjected to the same procedure using preparation buffer containing 50 μg/ml nocodazole instead of taxol. The pellets were lysed in Laemmli sample buffer, and SDS-PAGE was carried out as described (Laemmli, 1970) using a 10% acrylamide gel under reduced conditions. Separated proteins were transferred onto a nitrocellulose membrane (Schleicher & Schuell), and probed with polyclonal anti-mDia1 or monoclonal anti-β-tubulin antibody. For competitive experiment, HeLa lysate was subjected to western blot analysis with or without recombinant GST-cl.50 at a final concentration of 0.04 μg/μl. Reactive protein bands were visualized

using horseradish peroxidase-conjugated secondary antibodies and Enhanced Chemiluminescence Reagent (Amersham Pharmacia Biotech). For immunofluorescence, a portion of the preparations was suspended in PBS, placed on a glass coverslip and fixed with 3.7% paraformaldehyde in PBS for 20 minutes. The preparations were washed with PBS three times, and blocked with 5% BSA in TBS. Mitotic spindles and attached chromosomes were stained with monoclonal anti-β-tubulin antibody and TOPRO-3, respectively, as described above.

Construction and expression of GFP-tagged mDia1 mutants

pGEX 4T-1 constructs encoding the truncation mutants of mDia1 were described (Watanabe et al., 1999). The *Bgl*II-digested fragments of each truncated mDia1 were cloned into the *Bam*HI site of pEGFP-C1 (Clontech) (Fig. 3A). Glutamine^{431}, leucine^{434}, aspartic acid^{440} and leucine^{455} residues of H3 were individually mutated to alanine or glutamic acid. Q431A, L434A, D440A, L455A, L434E and L455E mutants of H3 were constructed by substitution of a single nucleotide or two nucleotides using QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions by using pEGFP-C1-H3 as a template and the mutagenic primer pairs listed in Table 1.

The pEGFP-C1 vectors encoding mDia1 mutants were transfected to HeLa cells using Lipofectamine Plus (Gibco/BRL) in OPTI-MEM as described (Fujita et al., 2000). After transfection, the cells were cultured in DMEM supplemented with 10% FCS for 12 hours. To enrich mitotic HeLa cells, the cells were cultured in the medium containing 40 ng/ml nocodazole for 12 hours. The cells were released from nocodazole block by washing with the fresh culture medium without the drug. After 1 hour recovery, the cells were subjected to instantaneous fixation and staining. DNA was labeled with TOPRO-3. Expression of these mutants was analyzed by western blot analysis using anti-GFP antibody (MBL, Nagoya, Japan).

Electroporation of C3 exoenzyme

HeLa cells were synchronized at the beginning of S phase by thymidine double block (Madaule et al., 1998). After 6 hours recovery from the second thymidine block, the cells were dissociated by trypsinization and collected by centrifugation as a pellet. The cells were washed with OPTI-MEM and suspended in OPTI-MEM at a density of 2×10^6 cells/ml. C3 exoenzyme prepared as described (Morii and Narumiya, 1995) was added to the cell suspension at a final concentration of 50 μg/ml. Two hundred fifty microliter of the suspension was transferred into a cuvette with an electrode distance of 0.4 cm and a single pulse (300 V, 960 mF, 100 Ω) was applied at room temperature with a Gene Pulser System (Bio-Rad). The cells were placed on fibronectin-coated glass coverslips and cultured in DMEM supplemented with 10% FCS. After 6 hours culture, the cells were subjected to the modified instantaneous fixation method and staining for mDia1, the mitotic spindle and DNA.

Table 1. Sequence of oligonucleotide pairs used for site-directed mutagenesis of pEGFP-C1-H3

Mutated residue	Oligonucleotide pair
Q431A	5′-GAGCTAGCTATGACTGCGATAGTTCTACACAAAAATG-3′ 5′-CATTTTTGTGTAGAACTATCGCAGTCATAGCTAGCTC-3′
L434A	5′-GCTATGACTCAGATAGTTGCACACAAAAATGGAAC-3′ 5′-GTTCCATTTTTGTGTGCAACTATCTGAGTCATAGC-3′
D440A	5′-CACAAAAATGGAACTGCTCCTGACTTCAAGTGC-3′ 5′-GCACTTGAAGTCAGGAGCAGTTCCATTTTTGTG-3′
L455A	5′-GATTGATATTGAGAGAGCGGTTGATCAAATGATTG-3′ 5′-CAATCATTTGATCAACCGCTCTCTCAATATCAATC-3′
L434E	5′-GCTATGACTCAGATAGTTGAACACAAAAATGGAAC-3′ 5′-GTTCCATTTTTGTGTTCAACTATCTGAGTCATAGC-3′
L455E	5′-GATTGATATTGAGAGAGAGGTTGATCAAATGATTG-3′ 5′-CAATCATTTGATCAACCTCTCTCTCAATATCAATC-3′

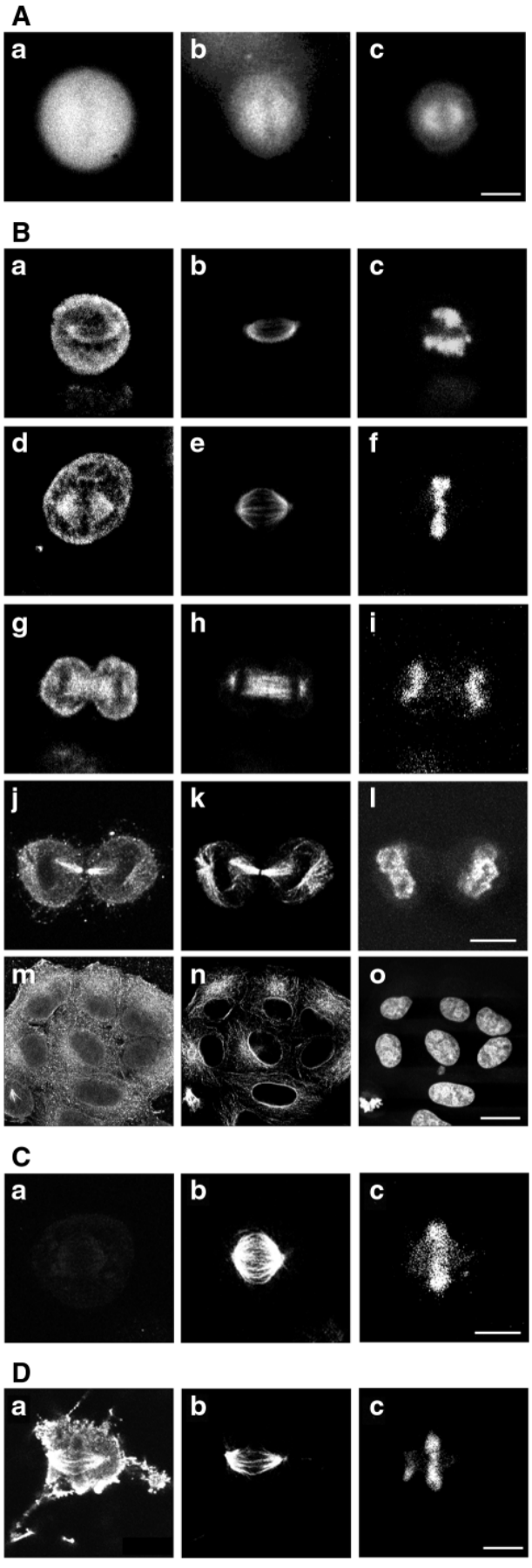
Microinjection of Val14RhoA

Recombinant Val14RhoA protein was prepared as described (Watanabe et al., 1999). For microinjection, Val14RhoA and tetramethylrhodamine-conjugated tubulin (Molecular Probes) were reconstituted at a concentration of 1 mg/ml each in PBS. HeLa cells were enriched in the mitotic phase by culture with 40 ng/ml nocodazole for 6 hours. Injections into mitotic HeLa cells were performed with an Eppendorf transinjector 5242 and micromanipurator 5171 attached to a Zeiss Axiovert 100 microscope. The pressure was 25 hectoPascal for 0.5 seconds using an Eppendorf femtotip needle. After injection, the cells were released from the nocodazole block by washing with the fresh culture medium. After 1 hour recovery, the cells were subjected to the modified instantaneous fixation method and staining for mDia1, the mitotic spindle and DNA.

RESULTS

Localization of mDia1 to the mitotic spindle in HeLa cells

Although mDia1 is essential in mitosis, its site of actions in mitotic cells is not known, though it is occasionally seen enriched in the intercellular bridge at the end of telophase (Watanabe et al., 1997; Tominaga et al., 2000). When cells are fixed prior to permeabilization and examined by immunofluorescence, mDia1 is seen diffusely in the cytoplasm (Fig. 1A, a). We wondered whether a small population of mDia1 associates with the cytoskeleton and this localization is masked by intense cytosolic staining. To examine this possibility, we used HeLa cells and subjected them to the instantaneous fixation method as developed previously (Arcangeletti et al., 1997) that simultaneously fixes and extracts cells to preferentially remove soluble components and preserve the cytoskeleton. Small amounts of components binding to the cytoskeleton are expected to be detected by this method. Although this method detected mDia1 staining enriched on some structures as shown in Fig. 1A, b, it did not fully extract cytosolic mDia1. We therefore modified the method by first extracting cells in the presence of a decreased paraformaldehyde concentration of 0.5%, followed by fixation of the cells with 4% paraformaldehyde (see Materials and Methods). Following this modified instantaneous fixation method, we stained fixed HeLa cells with anti-mDia1 antibody. We found positive immunofluorescence of the spindle shape in the middle of dividing cells (Fig. 1A, c). When the cells were counterstained with anti-β-tubulin antibody and TOPRO-3 and the mitotic spindle and chromosomes were visualized, this immunofluorescence overlapped exactly with the mitotic spindle (Fig. 1B). When the mitotic stages of cells were identified by morphology of the microtubules and the chromosomes, association with the spindle microtubules was first detected in cells in prophase (Fig. 1B, a-c), and the signal became intense when the spindles grew in metaphase (Fig. 1B, d-f). It was retained on the spindle when the spindle was elongated in anaphase to telophase (Fig. 1B, g-h) and made an intercellular bridge at the end of telophase (Fig. 1B, j-l). We detected the signal on the asters of cells in anaphase and telophase (Fig. 1B, g and j). In interphase cells subjected to the modified instantaneous fixation method, a part of immunofluorescence is



enriched on some filamentous structures, but the presence of the signal in other areas of the cells made difficult assignment of its localization to the microtubules (Fig. 1B, m-o). To verify specificity of this mDia1 immunofluorescence along the mitotic spindle, we employed competitive immunocytochemistry. Briefly, the fixed HeLa cells were incubated with the primary antibody together with recombinant GST-cl.50. The addition of GST-cl.50 to the primary antibody resulted in disappearance of the immunofluorescence signal of mDia1 (Fig. 1C). We next examined a possible participation of actin cytoskeleton in this mDia1 localization, because in *Drosophila* embryogenesis, the growth of microtubules accompanies the actin-associated membrane invagination and Diaphanous localizes to this site (Afshar et al., 2000). To this end, we treated mitotic HeLa cells with an actin filament depolymerizing drug, latrunculin A and studied mDia1 localization (Fig. 1D). Disruption of filamentous actin was confirmed by phalloidin staining (data not shown) and by a change in cell morphology. LatA-treated HeLa cells still formed the mitotic spindle normally (Fig. 1D, b) and mDia1 signal was detected along the mitotic spindle (Fig. 1D, a), indicating that mDia1 localization is independent of actin cytoskeleton.

The above immunofluorescence study indicates that mDai1 localizes to the spindle microtubules in mitotic cells. To confirm this, we prepared the mitotic spindle fraction of HeLa cells and examined the presence of mDia1 by western blot analysis. The mitotic spindle fraction (Tax) and the control fraction (Noc) were prepared as described (Kuriyama et al., 1984) from mitotic HeLa cells treated with taxol that stabilizes microtubules and nocodazole that depolymerizes microtubules, respectively (see Materials and Methods). Immunofluorescence analysis of these fractions with monoclonal anti-β-tubulin antibody revealed that spindle microtubules of the correct shape were present in the mitotic spindle fraction (Fig. 2A) and not in the control fraction (data not shown). Consistently, western blot analysis of these fractions with anti-β-tubulin antibody detected tubulin band in the mitotic spindle fraction and not in the control fraction (Fig. 2B, middle panel). When these fractions were subjected to western blot analysis with anti-mDia1 antibody, a positive

Fig. 1. Localization of mDia1 in HeLa cells during mitosis.
(A) Effects of fixation methods on mDia1 staining in mitotic cells. HeLa cells were either fixed prior to permeabilization (a) or subjected to the instantaneous fixation method (b) or to the modified instantaneous fixation method (c), and stained for mDia1. Bar, 10 μm. (B) mDia1 localization in cells during mitosis and in interphase. HeLa cells were fixed by the modified instantaneous fixation method and stained with polyclonal anti-mDia1 antibody (a,d,g,j,m), monoclonal anti-β-tubulin antibody (b,e,h,k,n) and TOPRO-3 (c,f,i,l,o). The cells at different mitotic phases, prophase (a-c), metaphase (d-f), anaphase (g-i), telophase (j-l) and interphase (m-o), are shown. Note that mDia1 localizes to the mitotic spindle in HeLa cells during mitosis. Bars: 10 μm (a-l); 20 μm (m-o).
(C) Competitive immunocytochemistry. The mitotic HeLa cell fixed as above was stained with polyclonal anti-mDia1 antibody (a), monoclonal anti-β-tubulin antibody (b) and TOPRO-3 (c) in the presence of GST-cl.50. Note specific disappearance of the anti-mDia1 immunofluorescence. Bar, 10 μm. (D) Effects of LatA on the mitotic spindle localization of mDia1. Mitotic HeLa cells were treated with LatA for 2 hours and were subjected to the modified instantaneous fixation method and staining with polyclonal anti-mDia1 antibody (a), monoclonal anti-β-tubulin antibody (b) and TOPRO-3 (c). Bar, 10 μm.

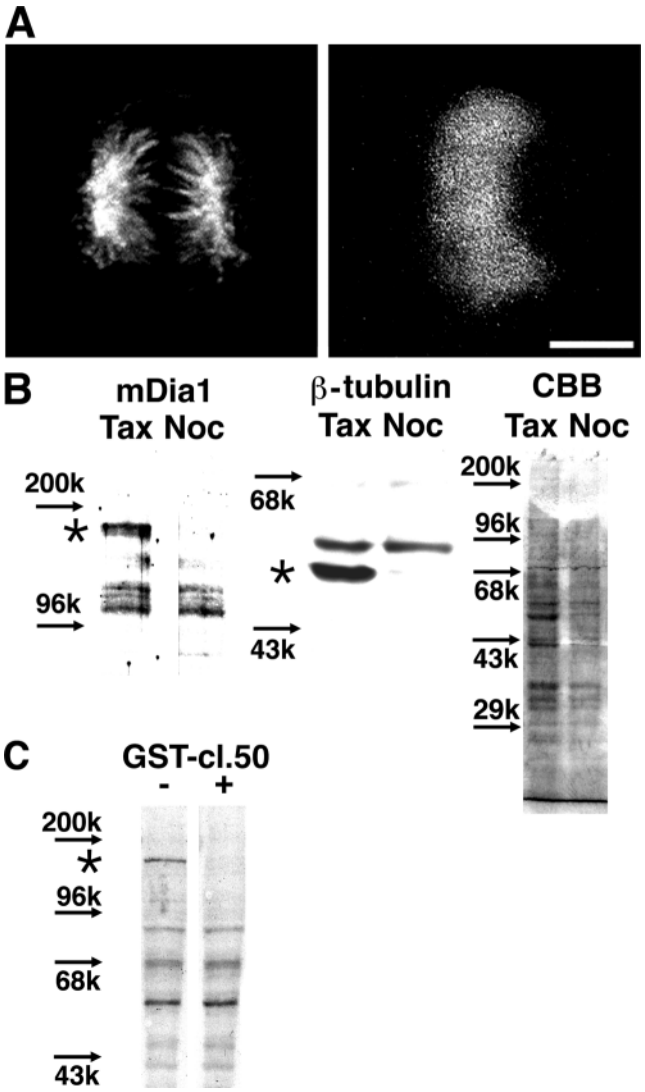


Fig. 2. Presence of mDia1 in the mitotic spindle fraction. The mitotic spindle fraction was prepared from HeLa cells treated with taxol (Tax), and the control fraction was obtained from the cells treated with nocodazole (Noc). (A) Immunofluorescence micrographs of the mitotic spindle preparation. The mitotic spindle fraction was placed on a glass coverslip, fixed with paraformaldehyde and stained with monoclonal anti-β-tubulin antibody (left) and TOPRO-3 (right). Bar, 10 μm. (B) The mitotic spindle fraction (Tax) and the control fraction (Noc) were subjected to immunoblotting with polyclonal anti-mDia1 antibody (left panel) or monoclonal anti-β-tubulin antibody (middle panel). Asterisks indicate band of mDia1 or β-tubulin, respectively. Protein staining of the two fractions by Coomassie Brilliant Blue is also shown (right panel). Note that the mitotic spindle fraction contained the correct shape of the mitotic spindles, and that mDia1 was detected in the mitotic spindle fraction and not in the control fraction. (C) Competitive western blot analysis with anti-mDia1 antibody using GST-cl.50. HeLa cell lysates were subjected to western blot analysis with anti-mDia1 antibody in the absence (left) and presence (right) of GST-cl.50.

immunoreactive band was detected at an expected molecular mass on the lane of the mitotic spindle fraction and not on that of the control fraction (Fig. 2B, left panel). We also performed competitive western blot analysis using GST-cl.50 (Fig. 2C). The addition of GST-cl.50 resulted in disappearance of the immunoreactive band of mDia1 (right panel), confirming the specificity of anti-mDia1 antibody. These results further suggested that mDia1 associates with the mitotic spindle. Finally, the spindle localization of mDia1 was confirmed by expressing full-length mDia1 as a GFP fusion protein. As shown in Fig. 3C, the GFP signal was detected on the spindle microtubules in mitotic cells when it was expressed as a fusion of full-length mDia1. The spindle localization was observed in all of the twenty four mitotic cells examined on one series of experiment (24/24), while no such signal was found with GFP alone (0/28).

Identification of a region of mDia1 important in the mitotic spindle localization

The above results clearly showed that mDia1 associates with the spindle microtubules in dividing cells and further indicates that we can use GFP-tagged mDia1 as a marker to analyze its localization to the spindle, as the background signal of GFP was quite low as revealed by expression of GFP alone. Using this method, we determined a region of mDia1 that is important in the spindle localization by constructing and expressing various mDia1 truncation mutants as GFP-fusions (Fig. 3A). HeLa cells were transfected with these vectors and the expression levels of the mutant proteins was first analyzed by western blot analysis using anti-GFP antibody. As shown in Fig. 3B, each mDia1 truncation mutants were expressed at almost the same level with expected molecular masses. HeLa cells expressing these proteins were then fixed by the instantaneous fixation method, and examined for GFP signals (Fig. 3C). mDia1 has the Rho-binding domain and an FH3 region in its N terminus, an FH1 region containing polyproline stretches in the middle and an FH2 region in the C terminus (Fig. 3A). GFP signals were detected on the mitotic spindle when the fluorescent protein was fused to the H1 (28/28), H2 (25/25), H3 (26/26) and H+P (25/25) fragments, all of which contained the C-terminal part of the FH3 and the following sequence prior to the FH1 region. On the other hand, little signal on the spindle was found when GFP fusions of the N1 (0/25) and F2 (0/30) fragments were expressed; these fragments did not contain the above region. This analysis has thus identified a 173 amino acid-long sequence containing the C-terminal part of FH3 as a critical region in determining the spindle localization of mDia1. A previous study (Petersen et al., 1998) identified an FH3 region as the conserved motif among FH proteins and showed that the FH3 regions of yeast formins, Fus1p and Cdc12p, are essential for their localization to the projection tip and the spindle pole body, respectively, in *Schizosaccharomyces pombe*. We aligned the amino acid sequence of the FH3 region in H3, the smallest fragment of mDia1 capable for localization to the spindle, with those of the corresponding regions of other FH proteins including Fus1p and Cdc12p. As shown in Fig. 4A, several amino acid residues are conserved among these proteins. Among them, we focused on four amino acid residues, Q431, L434, D440 and L455, of mDia1. We first changed these residues in H3 to alanine, expressed the point-mutants and examined their localization in mitotic HeLa cells. GFP signals on the mitotic spindle of Q431A (25/25) and D440A (21/21) were as strong and reproducible as that of wild-type H3, suggesting that Q431 and D440 were not important in spindle localization. On the other

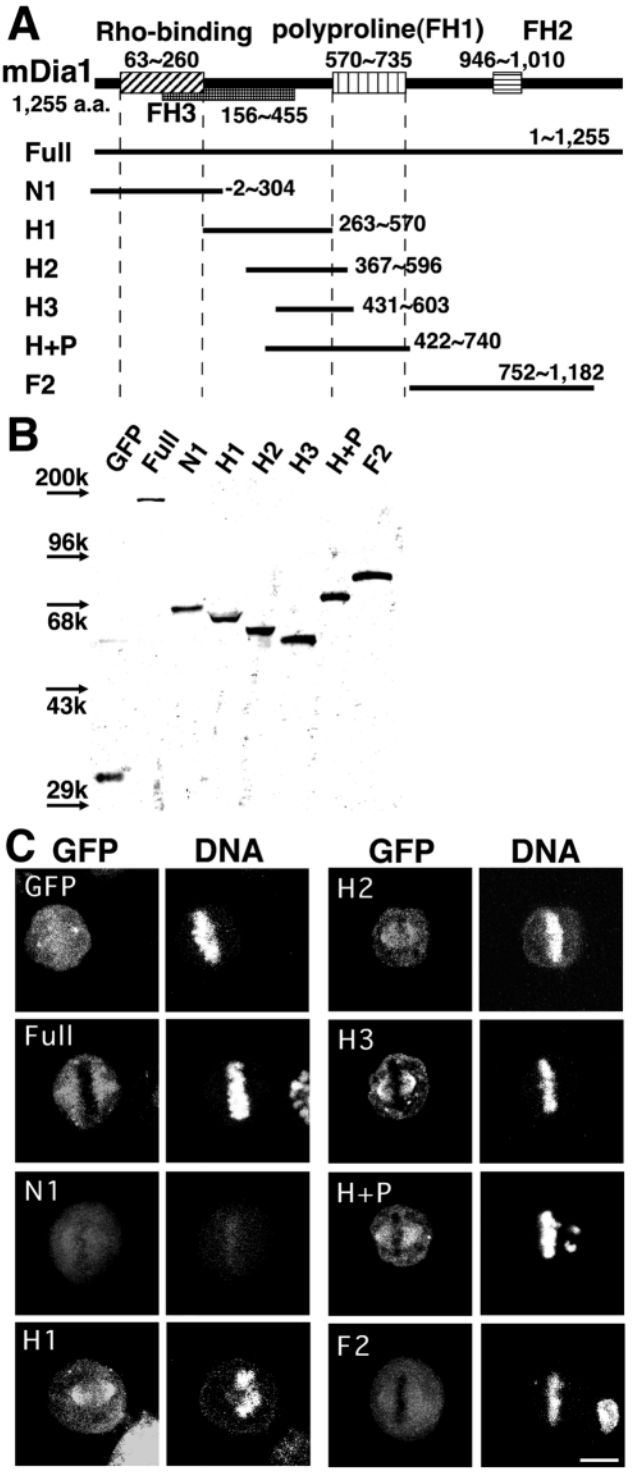


Fig. 3. Localization of GFP-mDia1 mutants in mitotic HeLa cells. (A) Schematic representation of mDia1 and its truncated mutants. Rho-binding domain and FH1, FH2, FH3 regions of mDia1 are indicated by boxes and amino acid numbers of the N- and C-termini are shown. The regions encoded by each mutant are indicated by thick lines. All mDia1 constructs were tagged with GFP at the N-termini. (B) Immunoblot analysis of GFP-mDia1 mutant proteins. HeLa cells were transfected with GFP-mDia1 constructs indicated above each lane and subjected to western blot analysis with anti-GFP antibody. (C) Immunofluorescence micrographs of the mitotic cells expressing each GFP-mDia1 construct. HeLa cells were transfected with indicated each GFP-mDia1 construct and subjected to the instantaneous fixation method and staining. Mitotic cells were identified by staining DNA with TOPRO-3, and their GFP fluorescence was examined. Note that the GFP fusions of Full, H1, H2, H3 and H+P localized to the mitotic spindle but no localization was observed with GFP alone or fusions of N1 and F2. Typical staining figures out of more than 100 observations in more than three independent experiments are shown. Bar, 10 μm.

hand, GFP signals of L434A (19/19) and L455A (21/21) were consistently present but reproducibly weaker than that of H3. We therefore next substituted glutamic acid for L434 and L455. GFP signal of L434E (8/20) at the mitotic spindle was as weak as and less consistent than that of L434A, and no specific GFP signal was found along the mitotic spindle in HeLa cells expressing L455E (0/20). The expression levels and sizes of these all point mutants were checked by western blot analysis using anti-GFP antibody (data not shown). We conclude that L434 and L455 of mDia1 are important for mDia1 to localize to the mitotic spindle.

Rho activity was not required for mDia1 localization to the mitotic spindle

We next examined whether the localization of mDia1 to the mitotic spindle depends on the Rho activity. We employed C3 exoenzyme that specifically ADP-ribosylates and inactivate Rho (Fig. 5A). To avoid inhibition of G_1-S transition by inactivation of Rho (Yamamoto et al., 1993), C3 exoenzyme was electroporated into HeLa cells after the cells were synchronized in the middle of S phase by double thymidine block. This procedure could successfully avoid inhibition of G_1-S progression. Control cells were subjected to the same procedure without C3 exoenzyme in the electroporation buffer (see Materials and Methods). After C3 loading, almost all cells spread with long, irregular protrusions at cell periphery, and showed much reduced phalloidin staining. The control cells showed normal actin cytoskeleton and cell morphology (data not shown). About 6 hours after electroporation, most of both C3-loaded and control cells entered into mitosis, forming the mitotic spindle with no apparent abnormality in their shapes. When the cells were fixed 3 hours later and observed, almost all C3-loaded cells became binucleate, while the ratio of binucleate cells was about 5% in control cells, confirming that this electroporation method using C3 exoenzyme effectively inactivates cellular Rho proteins. To focus on the localization of mDia1 during mitosis, we fixed the cells by the modified instantaneous fixation method 6 hours after the electroporation and subjected to immunocytochemistry using anti-mDia1 antibody. Immunofluorescence signal was detected along the mitotic spindle in C3-loaded cells as much as that in control cells. These results indicate that mDia1 localizes to the mitotic spindle independent of Rho.

We next wondered if Rho was involved in mobilization of mDia1 from the spindle microtubules. To examine this possibility, recombinant Val14RhoA was microinjected in mitotic HeLa cells. The activity of Val14RhoA used in this study was verified by microinjection into interphase cells and induction of stress fibers. HeLa cells were blocked at mitosis by treatment with nocodazole, and Val14RhoA was microinjected together with tetramethylrhodamine-conjugated

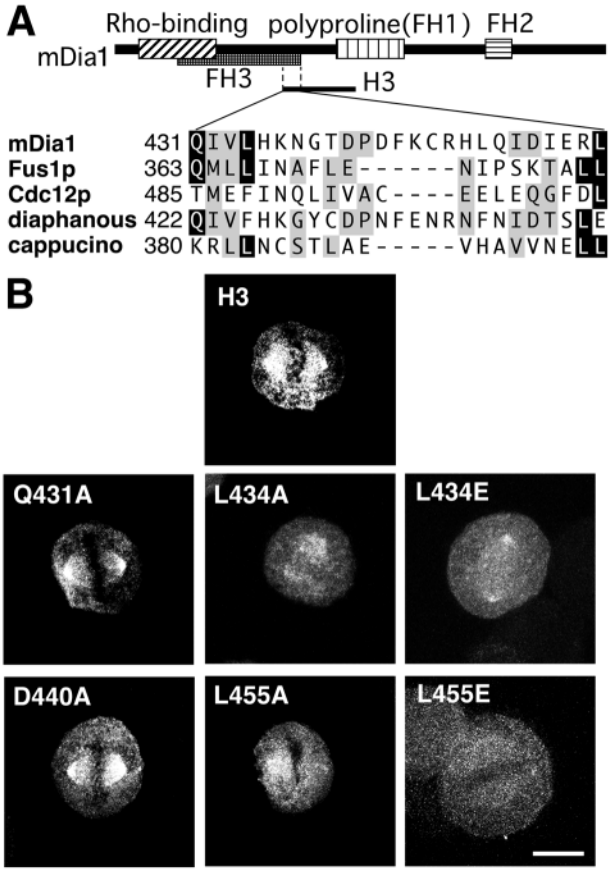


Fig. 4. Effects of point mutations on localization of GFP-H3 to the mitotic spindle. (A) The amino acid sequence of the FH3 region of mDia1 is aligned with those of Fus1p and Cdc12p of *Schizosaccharomyces pombe* and *Drosophila* diaphanous and cappucino (Petersen et al., 1998). Identical and homologous amino acids are shown in white letters in black background and by gray shadows, respectively. Q431, L434, D440 and L455 of H3 were replaced with either alanine or glutamic acid. Q431A, L434A, D440A, L455A, L434E and L455E were constructed with GFP-tag at the N-termini (see Materials and Methods). (B) HeLa cells were transfected with indicated GFP constructs and subjected to the instantaneous fixation. Signals of Q431A and D440A at the mitotic spindle were as strong as H3. L434A, L455A and L434E showed weaker signal than H3, and L455E did not localize to the mitotic spindle. Bar, 10 μm.

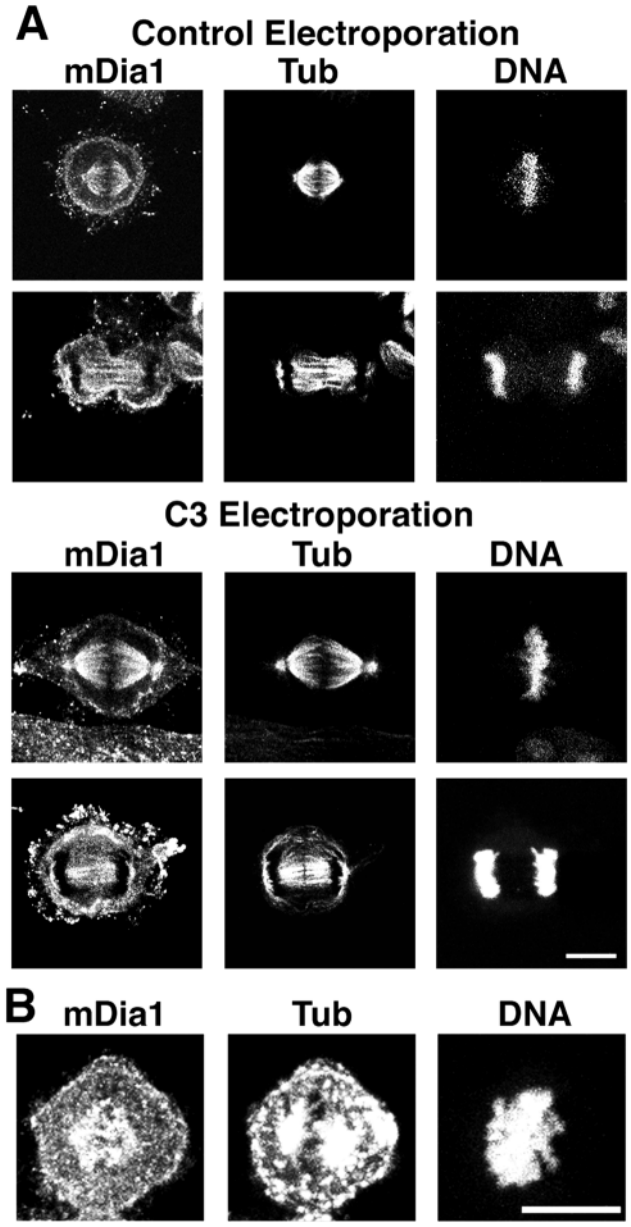


Fig. 5. Effects of C3 exoenzyme treatment (A) and Val14RhoA microinjection (B) on mDia1 localization in mitotic HeLa cells. (A) C3 exoenzyme was incorporated into synchronized HeLa cells at the middle of S phase by electroporation for Rho inactivation at mitosis. Mitotic cells were subjected to the modified instantaneous fixation, and then visualized for mDia1, the mitotic spindle and DNA. (B) Val14RhoA was microinjected with tetramethylrhodamine-conjugated tubulin into HeLa cells blocked at mitosis by nocodazole treatment. After recovery from nocodazole block, cells were subjected to the modified instantaneous fixation, and then visualized for mDia1 and DNA. Labeled tubulin was used as the marker for microinjected cells, and is shown. Note that neither inactivation of Rho nor introduction of active RhoA in excess had effect on mDia1 localization to the mitotic spindle in HeLa cells. Bar, 10 μm.

tubulin which was used as a marker for microinjected cells. At 1 hour after the nocodazole release, the cells were subjected to the modified instantaneous fixation method, and mDia1, the mitotic spindle and DNA were visualized. As shown in Fig. 5B, mDia1 was found along the mitotic spindle in the Val14RhoA-microinjected HeLa cells. These results indicate that activated Rho has no effect on the mitotic spindle localization of mDia1 at least under the present experimental conditions.

DISCUSSION

mDia1 localizes to the mitotic spindle

In this study we have obtained three lines of evidence to indicate that mDia1 localizes to the spindle microtubules during mitosis. Firstly, the immunofluorescence study using anti-mDia1 antibody showed the positive signal overlapping with the spindle microtubules in mitotic HeLa cells (Fig. 1).

Secondly, western blot analysis of the isolated mitotic spindle microtubules detected the anti-mDia1 immunoreactivity of the expected size in this fraction (Fig. 2). Thirdly, a GFP signal derived from GFP-tagged mDia1 expressed in mitotic HeLa cells localized to the mitotic spindle (Fig. 3). On the basis of these results, we conclude that mDia1 localizes to the mitotic spindle in dividing cells. This mDia1 localization has not been detected previously, probably due to strong cytosolic staining of this molecule. To conquer this problem, we modified the instantaneous fixation method developed by Arcangeletti et al. (1997). This modified method successfully removed most of the cytosolic staining, and yet preserved mDia1 binding to the microtubules (Fig. 1A). mDia1 appears to localize to all of the three types of spindle microtubules (Mastronarde et al., 1993), that is, the kinetochore microtubules, the nonkinetochore microtubules comprising the central spindle in the anaphase, and the astral microtubules, as shown in Fig. 1B, g to l. These findings clearly showed that mDia1 binds to microtubules of the mitotic spindle. To examine whether mDia1 can directly bind to microtubules, we prepared recombinant H3 protein in *E.coli* and added it to brain homogenates, which were then subjected to microtubule polymerization reaction. No significant association of H3 to microtubules was found in this reaction (data not shown), suggesting that the binding of mDia1 to microtubules may be limited to mitotic cells and that mDia1 associates with microtubules through another microtubules binding protein(s).

The FH3 region determines mDia1 localization

Our deletion analysis identified that localization of mDia1 to the spindle microtubules is determined by a small region of the molecule between the Rho-binding domain and the FH1 region (Fig. 3). Homology analysis revealed that this region contains a C-terminal part of the FH3 region, and mutations in Leu^{434} and Leu^{455} of this region verified that the FH3 region is indeed essential in targeting mDia1 to the spindle (Fig. 4). The FH3 domain was first identified as a domain determining localization of Fus1p of *Schizosaccharomyces pombe* to the projection tip of mating yeasts (Petersen et al., 1998). Fus1p is a member of FH proteins required for fusion of the fission yeast in conjugation process. The above study further showed that the FH3 region of Cdc12p, another FH protein of *S. pombe*, colocalizes with γ-tubulin of the spindle pole body in yeast. Our results together with these findings thus demonstrate that an FH3 region works as a targeting motif of FH proteins not only in yeasts but also in mammalian cells. Recently, Nakano et al. (Nakano et al., 1999) reported that expression of a fragment of mDia1 containing the FH3 region in MDCK cells interfered with the formation of actin bundles at the bottom of cells. These results indicate that the FH3 region of mDia1 may be involved also in targeting mDia1 to sites other than the mitotic spindle in interphase cells. The FH3 region consists of three clusters of homologous sequences, and the mDia1 targeting to the spindle microtubules requires only the third homologous region. It remains unclear at present whether the other two regions of the FH3 domain elicit other functions or they are dispensable for mDia1 function.

Possible functions of mDia1 localized to the mitotic spindle

mDia1 was isolated as a protein showing selective binding to the GTP-bound active form of Rho and is supposed to work as an effector downstream of Rho. We therefore examined if the mDia1 localization to the mitotic spindle is affected by the activity of Rho. To test this issue, it was crucial to develop an experimental conditions in which we can avoid that the C3 exoenzyme treatment modulates localization of molecules by affecting cell shapes. The electroporation method employed in this study has provided the most excellent condition to test the above issue at least in dividing cells. As shown in Fig. 5, neither inactivation of endogenous Rho nor introduction of an active Rho mutant into mitotic cells affected the mDia1 localization. These results raise two possibilities as to the function of mDia1 localized to the mitotic spindle. One possibility is that this localization of mDia1 represents the transition of mDia1 transportation to the cortical cytoskeleton. In mitosis, Rho is activated after nuclear division and acts as a link to cytokinesis by induction and maintenance of the contractile ring (Mabuchi et al., 1993; Kishi et al., 1993; Kimura et al., 2000). Indeed, in dividing oocytes of sea urchin, Rho is localized to the cleavage furrow and the midbody during cytokinesis (Nishimura et al., 1998). Mutation analysis of *Drosophila diaphanous* gene (Castrillon and Wasserman, 1994; Afshar et al., 2000) and neutralization of mDia1 in NIH 3T3 cells with the specific antibody (Tominaga et al., 2000) showed that diaphanous-related molecules including mDia1 are essential in cytokinesis. Previous experiments have indicated that the cleavage plane is determined by the mitotic spindle. In large echinoderm eggs, the stimulus for assembly of the contractile ring is suggested to be provided by the aster microtubules (Rappaport, 1996). On the other hand, in mammalian cells, the cleavage signal is suggested to come from the central interdigitating spindle microtubules (Cao and Wang, 1996). It is interesting in this respect that mDia1 is associated with the mitotic spindle (Fig. 1B). mDia1, which presumably binds to some component(s) of the mitotic spindle independent of Rho activation, could be delivered to the cortical region of the mitotic cells in a microtubules-dependent manner. Cdc12p, an FH protein of *S. pombe*, was reported to be recruited to the cleavage site by being transported as a particle on the microtubules (Chang, 1999). In *Drosophila* embryo, Diaphanous localizes to the growing tip of cellularization furrows and to contractile ring (Afshar et al., 2000). Though we did not detect enrichment of mDia1 in the cleavage furrow in this study, this failure may be due to weaker association of mDia1 to cell cortex that is disruptable by the instantaneous fixation method. Recently, a GDP-GTP exchange protein that is responsible for Rho activation in mitosis has been identified (Tatsumoto et al., 1999; Kimura et al., 2000). This protein, ECT2, was also suggested to localize to the mitotic spindle in metaphase, and to accumulate in the cleavage furrow and the midbody in telophase (Tatsumoto et al., 1999). The above mDia1 localization might be involved in the formation of cleavage signals derived from microtubules.

The other possibility for the function of mDia1 localized to the mitotic spindle not mutually exclusive each other is that mDia1 works on the regulation of the microtubules in mitosis. Giansanti et al. (Giansanti et al., 1998) examined the effects of mutations in *Drosophila* profilin, diaphanous and a kinesin related protein, KLP-3, on meiosis of spermatocytes, and found that mutations in all of these three genes affect not only the actin ring formation but also the organization of the central

spindle. Weak mutations caused less dense central spindle and strong mutations induced almost complete disappearance of the central spindle. Recently, a novel Rho GTPase activating protein, CYK-4, has been shown to be required for central spindle assembly and completion of cytokinesis in *Caenorhabditis elegans* (Jantsch-Plunger et al., 2000). In *Drosophila dia* embryos, there is variable defects in the organization of both actin- and microtubule-based structures (Afshar et al., 2000). These results suggest that the actin cytoskeleton and the microtubules interact during cell division and that diaphanous-related molecules work as a link of this interaction. Indeed, we have recently found that actin filaments and microtubules are aligned in parallel by overexpression of active mDia1 mutant in HeLa cells (Ishizaki et al., 2001). Moreover, in *Saccharomyces cerevisiae*, Bni1p, a yeast mDia1 homolog, makes a multiprotein complex in the cell cortex and serves as the connection of microtubules to the cortex to determine spindle movement and positioning (Heil-Chapdelaine et al., 1999; Lee et al., 1999; Miller et al., 1999). We attempted to identify functions of mDia1 localized to the mitotic spindle by overexpressing H3 protein. However, under the present experimental conditions, expressed H3 did not displace endogenous mDia1 binding to the mitotic spindle to the level detected by immunofluorescence and failed to act as a dominant interfering mutant possibly due to insufficient expression or weak affinity or both (data not shown).

There are now accumulating findings suggesting involvement of mDia1 in actin reorganization such as stress fiber formation and cytokinesis (Watanabe et al., 1997; Watanabe et al., 1999; Tominaga et al., 2000). However, how mDia1 regulates actin cytoskeleton in time and space remains elusive. Previous findings on accumulation of mDia1 in membrane ruffles and in phagocytic cups around fibronectin-coated beads are suggestive, but inconclusive in terms of targeting mechanism, motifs and associated molecules (Watanabe et al., 1997). Our present findings show the localization of mDia1 to the mitotic spindle microtubules without ambiguity, which forms a basis of future studies as to the action mechanism of this molecule. The next step to elucidate the function of mDia1 along this line is to identify a binding partner for mDia1 in the spindle microtubules. We have the point-mutants of mDia1 that do not localize to the mitotic spindle. These mutants can be used as negative controls to screen for molecule(s) which binds specifically to the FH3 motif of mDia1. Such identification is expected to give us a new insight into the molecular mechanisms of mDia1 actions in mitosis and other cellular processes induced by Rho.

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