mDia, one of the target proteins of the GTPase Rho, is known to be involved in cytoskeletal reorganization and cytokinesis. Here, we report that mDia enters the nucleus and binds to the transcription factor, Pax6. In cultured non-neuronal cells, overexpression of mDia with Pax6 causes redistribution of some Pax6 molecules from the nucleus to the cytosol and decreases Pax6 transcriptional activity. Because Pax6 functions in the early central nervous system morphogenesis, we also examined the effects of mDia on endogenous Pax6 localization and neurite extension in cerebellar granule cells. Here too, Pax6 was partially mislocalized to the cytosol, and its expression level was decreased by mDia overexpression. In addition, mDia overexpression in these cells led to increased neurite branching and length. These results strongly suggest that mDia influences Pax6-induced transcriptional activity and axonal pathfinding in a way opposite from ROCK (Rho kinase) and that it may act via Pax6 to modulate early neuronal development.

mDia (a mammalian homologue of Drosophila diaphanus) is a target of the small GTPase, Rho (1), and belongs to the formin homology (FH) protein family (2), which is required for cytokinesis. As a Rho partner, mDia has important roles in stress fiber formation, focal adhesion formation, and cell movement (3, 4). Using a yeast two-hybrid assay, we recently identified a novel mDia-interacting protein (5). We also identified a previously described protein, Pax6, as an mDia partner, using full-length mDia2 or truncated mDia2 (dominant active) as bait.

Pax6 is a transcription factor containing two DNA binding domains, a paired domain and a homeodomain, that play important roles in the developing central nervous system (6–8), especially in the cerebellum (9) and eyes (10). At these locations, Pax6, like Rho and mDia (11), is crucial for normal cell migration (9, 10). Mice or rats in which one copy of the Pax6 gene has been mutated exhibit small eyes (Sey) (12).

Both Rho and another of its downstream targets, p160ROCK (Rho kinase), have been proposed to negatively regulate the early steps of axon outgrowth in cultured cerebellar granule neurons (13). In the same system, it has been reported that Pax6 participates in the regulation of granule cell polarization independent of the Rho/ROCK pathway (14). Moreover, we have reported that signaling downstream of mDia is distinct from that downstream of ROCK and that the effects of mDia depend on the activity of Src-tyrosine kinase, which is known to promote neurite extension (15) and neuronal differentiation in the cerebellum and other brain regions (16). These findings led us to consider the possibility that the effects of mDia/Pax6 on neurite extension are distinct from those of Rho/ROCK (17, 18). In fact, opposing effects between ROCK and mDia on the maintenance of adherens junctions have been reported very recently (19). Here, we report that mDia enters the nucleus, binds to Pax6, and decreases Pax6 transcriptional activity in non-neuronal cells. In cerebellar granule cells, mDia also alters Pax6 subcellular distribution and enhances neurite branching and length.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen and Interaction—A HeLa cDNA library fused with the Gal4 activation domain (Clontech) was used, and screening was carried out using the increased amount of 3-aminotriazole as described previously (4).

Plasmids, Luciferase Assay—Recombinant cDNA expression vectors and constructs of GST fusion proteins were made by standard techniques and confirmed by sequencing. Leptomycin B was a gift from S. Narumiya (Kyoto University). For a luciferase assay, Pax6 was subcloned to pBIND vector (Promega) for expressing as GAL4-Pax6 fusion protein. Two days after transfection of Pax6/pBIND and mDia2/pEF (4) with pGL3Luc (Promega) into NIH3T3 cells using lipofectamine plus (Invitrogen), the cell lysates were collected and luciferase activities were examined using a Dual-Lucerase Assay System (Promega). Transcriptional activities of Pax6 were examined using a CD19.2-luc reporter plasmid (a kind gift from G. F. Saunders, University of Texas) as described previously (22). Pax6/pCDNA and/or mDia2/pEF were transfected with CD19.2/luc DNA, and luciferase activity was examined in the same way as above.

GST Pull-down Assay—GST pull-down assay was performed by mixing the GST fusion proteins and 35S-labeled in vitro translated proteins using a TNT kit (Promega) as reported previously (4). After 2 h incubation at 4 °C, the beads and bound materials were washed extensively and analyzed by SDS-PAGE followed by autoradiography.

Preparation of Cerebellar Granule Cells and Fluorescence Microscopy—Cerebellar granule cells were prepared as reported previously (14). Briefly, cerebella were collected from P2-P3 ICR mice, and thin slices were made in Ca2+-free and Mg2+-free Hanks’ balanced salt solution.
erslips coated with poly-L-lysine and laminin. After 8-h incubation, Eagle’s basal medium with 10% horse serum and plated on glass coverslips coated with poly-L-lysine and laminin. Analysis of these cultures was performed using an IP-Lab system (Solution Systems, Tokyo). or a confocal laser microscope (Bio-Rad) and an Olympus fluorescent microscope with a cooled CCD camera (Molecular Probes). Immunofluorescence images were obtained with mDia1 and mDia2 (4), followed by Allexa-labeled secondary antibody mouse Pax6 (kind gift from A. Kawakami, Tokyo University), anti-Pax6 (clone 63, kind gift from N. Osumi, Tohoku University), anti-mDia1 and mDia2 (4), followed by Allexa-labeled secondary antibody (Molecular Probes). Immunofluorescence images were obtained with an Olympus fluorescent microscope with a cooled CCD camera (Hamamatsu-photonics) or a confocal laser microscope (Bio-Rad) and analyzed using an IP-Lab system (Solution Systems, Tokyo).

RESULTS

A mouse homologue of formin has been reported to be localized in the nucleus (23), and inspection of the mDia protein sequence revealed a putative nuclear localization signal. We therefore examined whether mDia could enter the nucleus by using leptomycin B (24), an inhibitor of nuclear pore component, exportin. As shown in Fig. 1A, treatment of transfected NIH3T3 cells with leptomycin B caused GFP-mDia2 to translocate from cytosol to nucleus. After 8-h incubation, overexpression of mDia with Pax6 caused the change of Pax6 localization. GFP-Pax6 was localized in the nucleus of HeLa cells (left panel). Co-expression of GFP-Pax6 with mDia2 caused Pax6 to translocate from nucleus to cytosol (right panel). A color panel shows a merge image of co-localization of Pax6 and mDia2 (green image indicates GFP-Pax6 and red image indicates mDia2). C, DNA binding activity of Pax6. Transfected Pax6/pBIND in NIH3T3 bound to DNA and activated transcriptional activity. Co-transfection with mDia2/pEF blocked the binding of Pax6 to DNA and caused decreased activity of luciferase. Luciferase activity was expressed with Firefly/Renilla luciferase activities in two independent trials (data shows mean values calculated from triplicate samples). *, p < 0.001, unpaired t test. D, transcriptional activity of Pax6. Pax6/pDNA3 and/or mDia2/pEF were transfected with the CD19.2/luc reporter gene to NIH3T3 cells. The dual luciferase activities were examined the same as described above. mDia significantly blocked the transcriptional activity of Pax6. *, p < 0.001, unpaired t test.

Next, we attempted to confirm the in vitro interaction of mDia and Pax6 in a “pull-down” assay. A significantly greater degree of interaction was observed in the pairs of mDia1-B/Pax6-(1–131), mDia1-C/Pax6-(1–131), and mDia1-C/Pax6-(132–271) compared with each negative control (Fig. 2A, lane 1 in the lower panel). Fig. 2B shows a summary of the results from Fig. 2A and a model for the interaction of both proteins. A FH2 domain in mDia (mDia-C) appears to interact with the DNA-binding domains of Pax6 (Pax6-1–131 and Pax6-(132–271), providing a possible mechanistic explanation for the inhibition of DNA binding and transcriptional activity of Pax6 by mDia.

To explore the significance of the mDia-Pax6 interaction in vivo, we performed similar experiments using cultured cerebellar granule cells, which express Pax6 endogenously. We first demonstrated the expression of both mDia1 and mDia2 mRNAs in granule cells taken from 3-day-old mice (data not shown). Next, we examined the immunostaining of endogenous mDia1 and Pax6. Extraneuronal expression of endogenous Pax6 was observed in some cells and Pax6 expression overlapped with that of mDia1, suggesting their co-localization (Fig. 3). We examined neurite outgrowth in cerebellar granule cells (using Pax6 co-staining as a marker of neurons, not glia cells) within

![Image](https://example.com/image.png)
12–16 h after transfection with either GFP alone or GFP-tagged full-length mDia1. These experiments were performed in the presence of serum to permit activation of Rho, ROCK, and mDia. Short bipolar neurites arose in control GFP-transfected cells, a neurite extension pattern characteristic of cerebellar granule cells (Fig. 4A) (13). In cells transfected with mDia, we noted the appearance of endogenous Pax6 outside of the nucleus in some of the granule cells (Fig. 4, B and D, and Fig. 5C), consistent with the effect we observed in HeLa or NIH3T3 cells (Fig. 1B) and non-treated cerebellar granule cells (Fig. 3). Moreover, the immunoreactivity of Pax6 in the nucleus was weaker in the mDia-transfected cells than in the untransfected cells (Figs. 4C and 5, B and E). The expression of mDia1 also increased both the extent of neurite branching (Fig. 4, C and D) and neurite length (Fig. 4, B and D) compared with the GFP-transfected control cells (Fig. 4A). These effects are summarized in Fig. 4E. In brief, mDia expression led to decreased Pax6 immunoreactivity in more than 40% of the cells and Pax6 mislocalization in more than 30% of the cells (p < 0.05 and p < 0.05 versus control, respectively). In addition, elongation and multibranching of neurites upon mDia expression were observed in 67% (p < 0.05 versus control) and 55% (p < 0.05
versus control) of the cells, respectively. It is currently impossible to investigate the effect of endogenous mDia, because no reagents acting specifically on mDia are available. Therefore, we performed experiments on cerebellar granule cells in the presence of serum, which activates Rho, ROCK, and mDia. According to the reported results (17, 18), activation of Rho and ROCK causes neurite contraction. However, in our experiments, cerebellar granule cells retained the ability to extend their neurites in the context of mDia expression, suggesting that exogenous mDia can overcome the inhibitory effects of Rho and ROCK on neurite extension. Furthermore, a truncated form of mDia lacking both nuclear localization signal and FH2 domains, and therefore unable to bind Pax6 (Fig. 1C), was unable to rescue neurite extension in the presence of serum (data not shown). This finding suggests that the interaction of mDia with Pax6 is critical for its effects on neurites.

To further confirm the interaction of mDia effects and those of Rho or ROCK, we also examined the combined effects of mDia overexpression with either application of the ROCK inhibitor, Y-27632, co-transfection of RhoV14 (dominant active Rho), or co-transfection of ROCKΔ3 (dominant active ROCK) (Fig. 5). Application of Y-27632 alone changed the polarity of neurites and promoted their elongation (Fig. 5A) as reported previously (13, 14). mDia effects on multibranching or neurite length became more prominent in the presence of this drug in some neurites (Fig. 5, B and C). Neurite shortening was evoked by expression of RhoV14 or ROCKΔ3 as reported previously (Fig. 5, D and F), and the shortening was reversed by co-expression of mDia (Fig. 5, E and G). In Fig. 5G, the existence of mDia at the growth cone was observed (white circle). Furthermore, lamellipodia-like neurites were observed in some

**Fig. 3.** mDia expression and localization in the cerebellar granule cells. Co-localization of endogenous mDia and Pax6. The orange color indicates the expression of both mDia1 and Pax6 (merge). After plating the granule cells, cells were fixed after 20 h. For enhancing the neurite extension, ROCK was inhibited by addition of Y-27632 (ROCK inhibitor) to the medium at the same time of plating. Arrowheads show extranuclear localization of Pax6.

**Fig. 4.** Pax6 localization and expression in the cerebellar granule cells expressing mDia. A–D, Pax6 expression and neurite changes in the cerebellar granule cells expressed with GFP (control, A) or GFP-tagged mDia1 (B–D). Asterisks in A, B, and D show the Pax6 in the GFP or GFP-tagged mDia1-expressing cells. Arrowheads in B and D show extranuclear localization of Pax6. The arrow in C indicates the decreased Pax6 signal intensity in the nucleus with expression of GFP-mDia1. Red and green images indicate Pax6 and GFP-mDia, respectively. E, summary of the effects of mDia expression in the cerebellar granule cells. Decreased immunoreactivity was defined as more than 40% reduction of intensity of Pax6 using IP-Lab. Elongation indicates longer neurites by more than two times of mean length in the control cells. Multibranching means the neurites having more than three branches. Percent values indicate the cells showing positive effects by the above definition in the examined 50 cells from 15 to 20 different transfections. Statistical analyses were performed using the $\chi^2$ test and defined significant when the $p$ value was less than 0.05.
mDia Binds Pax6 and Enhances Neurite Extension

mDia, a formin homology protein and a target protein of the small GTPase, Rho, is known to be involved in cytoskeletal reorganization (stress fiber formation and microtubule organization (26)) and cytokinesis. The mechanistic basis for the effect of mDia on stress fiber formation is thought to include the de novo production of actin filaments (1, 5). This stress fiber formation occurs in cooperation with ROCK, which causes F-actin contraction through myosin light chain phosphorylation (25). It has also been reported that mDia regulates the transcription of focal adhesion molecules through the activation of serum response factor (4, 27). Mutations of formin homology proteins in all species examined are known to cause defects in cytokinesis (1). For example, mouse formin enters the nucleus, and mutations in this protein are reported to cause limb deformities and renal abnormalities (28), and the mutated human Dia2 is reported to be responsible for primary ovarian failure (29). However, a comprehensive picture of how mDia influences cellular morphology and how it interacts with other components downstream of Rho has not been totally clear.

A Rho/ROCK pathway is known to be involved in the negative regulation of axon outgrowth in cultured cerebellar granule neurons (13). In the present study, we showed that mDia caused neurite extension and multibranched in cerebellar granule neurons in the pathway distinct from that of Rho/ROCK. This represents the first description of positive effects of mDia on neurite extension and multibranched and suggests that mDia and ROCK might regulate the axon outgrowth in balance in the downstream of Rho activation.

The data presented here clearly demonstrate that mDia can localize to the nucleus, bind directly to Pax6, and alter the expression level and localization pattern of Pax6 in heterologous expression systems. Moreover, some of these mDia effects were also observed in native neurons. These findings represent the first example of Pax6 binding to an FH2 domain in mammalian cells.

Pax6 is thought to participate in neuronal differentiation and migration, a conclusion drawn largely from morphological analyses of Sye mice or rats (9, 14) and reported defects in specification of hindbrain motor neuron subtypes (7, 30). Rho plays an important role in cell migration in non-neuronal cells (31), and mDia, a protein downstream of Rho, interacts with Src, which in turn has been reported to be involved in the turnover of focal adhesions in cooperation with focal adhesion kinase (32). These facts, together with our studies, suggest that mDia, too, might play a role in neurite outgrowth and cell migration by inhibiting the Pax6-dependent transcriptional regulation of molecules such as F-actin, microtubule, cadherin, and other guidance cues. The neurite multibranched and lamellipodia-like neurites observed in cerebellar granule cells of Sey rat (14) and mDia-induced neurite changes might share similar origins. Rho GTPases (Rac and Cdc42) have been shown to be involved in neuronal growth cone morphology, neurite extension, and axonal pathfinding during early central nervous system development (33, 34). On the other hand, Rho kinase causes neurite contraction. This apparent importance of Rho GTPases to the morphology of neurites is consistent with the proposed effects of mDia, which might act to some extent through its interaction with Pax6. However, our data did not

**Fig. 5.** Effects of ROCK inhibitor and co-transfection of RhoV14 or ROCK3 with mDia on neurites extension and Pax6 expression in the cerebellar granule cells. A–G, combined effects of ROCK inhibitor, Y-27632, and mDia. Y-27632 was applied at the time of transfection of GFP (A) or GFP-tagged mDia (GFP-mDia). B and C, Y-27632 alone was found to change the neurite polarity and length (A). mDia effects became more prominent in elongation (B) and multibranching (C) of neurites. The two highlighted lower panels in B show shorter exposure of the same field in the upper panel and indicate that the cell is a granule cell implicated by staining of Pax6. D and E, effects of co-expression of RhoV14 and mDia. RhoV14 produced a very short neurite (D). mDia co-expression reversed the effect of RhoV14 (E). F and G, effects of co-expression of ROCK3 and mDia. ROCK3 produced very short neurites (F) like in D. mDia co-expression reversed the effect of ROCK3 (G) like in E. Red and green images indicate Pax6 and GFP-mDia, respectively. The open circle in G (green) shows the mDia at the growth cone. The reverse effects by mDia in E and G were confirmed to be significant (p < 0.05 for both settings versus without mDia) when χ² analyses were performed (n = 12). The markers used (asterisk, arrowhead, and arrow) in Pax6 stainings in Fig. 5 indicate the same as those described in the legends to Figs. 3 and 4.

**DISCUSSION**

mDia binding to a formin homology protein and a target protein of the small GTPase, Rho, is known to be involved in cytoskeletal reorganization (stress fiber formation and microtubule organization (26)) and cytokinesis. The mechanistic basis for the effect of mDia on stress fiber formation is thought to include the de novo production of actin filaments (1, 5). This stress fiber formation occurs in cooperation with ROCK, which causes F-actin contraction through myosin light chain phosphorylation (25). It has also been reported that mDia regulates the transcription of focal adhesion molecules through the activation of serum response factor (4, 27). Mutations of formin homology proteins in all species examined are known to cause defects in cytokinesis (1). For example, mouse formin enters the nucleus, and mutations in this protein are reported to cause limb deformities and renal abnormalities (28), and the mutated human Dia2 is reported to be responsible for primary ovarian failure (29). However, a comprehensive picture of how mDia influences cellular morphology and how it interacts with other components downstream of Rho has not been totally clear.

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exclude the possibility that mDia alone can cause neurite extension and morphological changes. Examining the effects of mDia on the neurons lacking Pax6 expression could provide more information about the role of mDia and its interaction with Rho GTPases.

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Additions and Corrections

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