mDia2 Shuttles between the Nucleus and the Cytoplasm through the Importin-α/β- and CRM1-mediated Nuclear Transport Mechanism

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Mammalian homolog of Drosophila diaphanous (mDia) consisting of three isoforms, mDia1, mDia2, and mDia3, is an effector of Rho GTPases that catalyzes actin nucleation and polymerization. Although the mDia actions on actin dynamics in the cytoplasm have been well studied, whether mDia accumulates and functions in the nucleus remains largely unknown. Given the presence of actin and actin-associated proteins in the nucleus, we have examined nuclear localization of mDia isoforms. We expressed each of mDia isoforms as a green fluorescent protein fusion protein and examined their localization. Although all the mDia isoforms were localized predominantly in the cytoplasm under the steady-state conditions, mDia2 and not mDia1 or mDia3 accumulated extensively in the nucleus upon treatment with leptomycin B (LMB), an inhibitor of CRM1-dependent nuclear export. The LMB-induced nuclear accumulation was confirmed for endogenous mDia2 by using an antibody specific to mDia2. Studies using green fluorescent protein fusions of various truncation mDia2 mutants and point mutants of some of these proteins identified a functional nuclear localization signal in the N terminus of mDia2 and at least one functional nuclear export signal in the C terminus. The nuclear localization signal of mDia2 bound to importin-α and was imported into the nucleus by importin-α/β complex in an in vitro transport assay. Consistently, depletion of importin-β with RNA interference suppressed the LMB-induced nuclear localization of endogenous mDia2. These results suggest that mDia2 continuously shuttles between the nucleus and the cytoplasm using specific nuclear transport machinery composing of importin-α/β and CRM1.

Subcellular localization of a protein must be accurately regulated for the protein to function in a proper compartment of a cell. As for the nucleus, some proteins such as transcription factors and those associated with chromatin are translocated to the nucleus after they are synthesized in the cytoplasm. Other proteins enter the nucleus and then come back to the cytoplasm. Ribosomal proteins, for example, are integrated into large (60 S) and small (40 S) ribosomal subunits in the nucleus and then transported back to the cytoplasm to function as translation machineries (1–3). There are also a group of proteins named nucleocytoplasmic shuttling proteins that shuttle between the nucleus and the cytoplasm. The nucleus and the cytoplasm are separated by the nuclear membrane containing nuclear pores. Molecules less than ~50 kDa can diffuse passively through the nuclear pore, whereas larger molecules require selective transport mechanisms to pass through the pore (4). This selective nuclear transport mechanism depends on specific amino acid sequences of substrate proteins named the nuclear localization signal (NLS) for import to the nucleus and the nuclear export signal (NES) for export to the cytoplasm, both of which are recognized typically by the importin-β family of receptors (5).

Rho GTPases regulate actin cytoskeleton through actions of their downstream effectors (6). mDia is an effector of Rho and catalyzes actin nucleation and polymerization to produce straight actin filaments (7), which are cross-linked by myosin to become stress fibers by the action of another Rho effector, ROCK (8). mDia consists of several conserved domains: the GTPase binding domain (GBD) at the N terminus, the formin homology (FH) domains, FH3, FH1, and FH2, in this order, in the middle, and the diaphanous autoregulatory domain (DAD) at the C terminus (Ref. 9 and Fig. 2). The FH2 domain makes a dimmer, binds to the barbed end of filamentous actin (F-actin), and catalyzes actin nucleation and polymerization there, whereas the neighboring FH1 domain binds profilin, a globular/monomer actin (G-actin)-binding protein, and promotes actin polymerization by FH2 domain possibly by raising local G-actin concentration (8, 10). Without activation by Rho, the GBD and the DAD physically interact, making the FH1 and FH2...
domains structurally unavailable for actin nucleation and polymerization (11). Binding of the GTP-bound form of Rho to the GBD disrupts the intramolecular interaction and makes mDia in a structurally open active form. There are three isoforms for mDia, i.e. mDia1, mDia2, and mDia3 (7, 12, 13). All of them are capable of actin nucleation and polymerization.

One of the reported functions of an mDia isoform is regulation of serum response factor activity (14). MAL, a coactivator of serum response factor, interacts with G-actin both in the cytoplasm and the nucleus, and its interaction with G-actin favors its cytoplasmic localization and suppresses transcription activation (15, 16). Rho signaling regulates the nuclear localization of MAL through the control of actin dynamics. mDia1 has been shown to activate serum response factor by decreasing the G-actin level by actin polymerization (14), implying the involvement of mDia1 in MAL localization. These results indicate that alteration in actin dynamics by mDia isoforms can influence some of the nuclear functions. Whether mDia1 or other mDia isoforms exert such an action by acting solely in the cytoplasm or acting also in the nucleus remains unknown. Some actin-polymerizing proteins such as FHOD1 (17) and N-WASP (18) have been reported to enter the nucleus. Here we have examined whether and, if so, how mDia isoforms enter the nucleus. We show that mDia2 continuously shuttles between the nucleus and the cytoplasm in a manner dependent on the NLS and the NES.

EXPERIMENTAL PROCEDURES

Plasmid Construction—For construction of a pEGFP-NLS (SV40 T-antigen) vector, two oligo DNAs (top, 5′-TCGAGCT-TATGGAGGTTCAAAAAGAAGAAGGTAAGAAGA-CCCCG-3′; bottom, 5′-CTTGTACAAGATGTCGAAGCCCCCATAGTGAAAGCCG-3′; reverse, 5′-GTACCAGGCTTCTCTACTACCTTC-TCTTCCTTTTGGACCTCTCCATAAGC-3′) were annealed and inserted into the XhoI-KpnI site of a pEGFP-C1 vector (Clontech).

A pEGFP-PK vector was constructed as follows. A cDNA encoding human pyruvate kinase (GenBank™ accession no. NM_002654) was amplified from total RNAs purified from HeLa cells using the SuperScript III One-Step RT-PCR system (Invitrogen). The primers used were as follows: forward, 5′-ATTATTGCTGGACATTTTGGAA-3′; reverse, 5′-ATTATTGCTGGACATTTTGGAA-3′; reverse, 5′-GTTCCTGAGCAGCAGAAGCGACCACACAC-ACGATGG-3′. The PCR product was digested with BsrGI and XhoI and inserted into the EcoRI-BamHI site of a pGEX-hGFP vector.

The expression plasmids of GFP-mDia1, GFP-mDia2, GFP-mDia2–(1–411), and GFP-mDia3 were constructed by insertion of the BglII-BglII fragment of a GFP-mDia2 plasmid into the BglII site of a pEGFP-C1 vector. A GFP-mDia2–(1–411) expression plasmid was constructed by insertion of the XhoI-XhoI fragment of a GFP-mDia2 plasmid into the XhoI site of a pEGFP-C3 vector. For construction of a GFP-mDia2–(1028–1156) expression plasmid, a GFP-mDia2–(1028–1171) expression plasmid was digested with SmaI and XmnI, and the larger fragment was self-ligated. A GFP-NLS-mDia2–(411–1171) expression vector was constructed by insertion of the BglII-MluI fragment of a GFP-mDia2–(411–1171) expression plasmid into the BamHI-MluI site of a pEGFP-NLS vector. For construction of a GFP-NLS-mDia2–(411–966) expression plasmid, a GFP-NLS-mDia2–(411–1171) expression plasmid was digested with EcoRI and Sall, and the larger fragment was self-ligated. GFP-PK-mDia2–(1–60), GFP-PK-mDia2–(1–41), and GFP-PK-mDia2–(1–32) expression plasmids were constructed by insertion of the XhoI-MluI fragment of GFP-mDia2–(1–60), GFP-mDia2–(1–41), and GFP-mDia2–(1–32) expression plasmids, respectively, into the XhoI-MluI site of a pEGFP-PK vector. For construction of a GFP-PK-mDia2(16–39) expression plasmid, two oligo DNAs (top, 5′-ATTATTGCTGGACATTTTGGAA-3′; bottom, 5′-ATTATTGCTGGACATTTTGGAA-3′) were annealed and inserted into the EcoRI-BamHI site of a pEGFP-PK vector. For construction of For construction of a GFP-PK-mDia2–(1–411) expression plasmid, two oligo DNAs (top, 5′-ATTATTGCTGGACATTTTGGAA-3′; bottom, 5′-ATTATTGCTGGACATTTTGGAA-3′) were annealed and inserted into the EcoRI-BamHI site of a pEGFP-PK vector.

The expression plasmids of GFP-mDia1, GFP-mDia2, GFP-mDia2–(411–1171), and GFP-mDia3 were constructed as described previously (7, 19, 20). A GFP-mDia2–(1–411) expression plasmid was constructed by insertion of the XhoI-XhoI fragment of a GFP-mDia2 plasmid into the XhoI site of a pEGFP-C1 vector. A GFP-mDia2–(1–153) expression plasmid was constructed by insertion of the BglII-BglII fragment of a GFP-mDia2 expression plasmid into the BglII site of a pEGFP-C1 vector. For construction of a GFP-mDia2–(1–60) expression plasmid, a GFP-mDia2 expression plasmid was digested with SmaI, and the larger fragment was self-ligated. For construction of a GFP-mDia2–(1–41) expression plasmid, a GFP-mDia2 expression plasmid was digested with Swal and Hpal, and the larger fragment was self-ligated. For construction of a GFP-mDia2–(1–32) expression plasmid, a GFP-mDia2 expression plasmid was digested with Smal, and the larger fragment was self-ligated. A GFP-mDia2–(1–1171) expression vector was constructed by insertion of the Xmal-Xmal fragment of a GFP-mDia2 expression plasmid into the BspEI site of a pEGFP-C1 vector. A GFP-mDia2–(1028–1171) expression plasmid was constructed by insertion of the Afel-SfiI fragment of a GFP-mDia2 expression plasmid into the Sma1-SfiI site of a pEGFP-C3 vector. For construction of a GFP-mDia2–(1028–1156) expression plasmid, a GFP-mDia2–(1028–1171) expression plasmid was digested with SmaI and XmnI, and the larger fragment was self-ligated. A GFP-NLS-mDia2–(411–1171) expression vector was constructed by insertion of the BglII-MluI fragment of a GFP-mDia2–(411–1171) expression plasmid into the BamHI-MluI site of a pEGFP-NLS vector. For construction of a GFP-NLS-mDia2–(411–966) expression plasmid, a GFP-NLS-mDia2–(411–1171) expression plasmid was digested with EcoRI and Sall, and the larger fragment was self-ligated. GFP-PK-mDia2–(1–60), GFP-PK-mDia2–(1–41), and GFP-PK-mDia2–(1–32) expression plasmids were constructed by insertion of the XhoI-MluI fragment of GFP-mDia2–(1–60), GFP-mDia2–(1–41), and GFP-mDia2–(1–32) expression plasmids, respectively, into the XhoI-MluI site of a pEGFP-PK vector. For construction of a GFP-PK-mDia2(16–39) expression plasmid, two oligo DNAs (top, 5′-ATTATTGCTGGACATTTTGGAA-3′; bottom, 5′-ATTATTGCTGGACATTTTGGAA-3′) were annealed and inserted into the EcoRI-BamHI site of a pEGFP-PK vector. For construction of a GFP-PK-mDia2–(1–411) expression plasmid, two oligo DNAs (top, 5′-ATTATTGCTGGACATTTTGGAA-3′; bottom, 5′-ATTATTGCTGGACATTTTGGAA-3′) were annealed and inserted into the EcoRI-BamHI site of a pEGFP-PK vector.
Expression and Purification of Recombinant Proteins—A GST-GFP or GST-GFP-mDia2-(16–39) expression plasmid was introduced into an Escherichia coli strain BL21. Expression was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside and incubated for 24 h at 37°C. Cells were harvested by centrifugation and resuspended in a high salt buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol) containing Protease Inhibitor mixture (Nacalai Tesque). After sonication, the extract was clarified by centrifugation (5000 g). The supernatant was incubated with glutathione-Sepharose 4B beads (Amersham Biosciences) in 10 ml of the high salt buffer containing 0.05% Triton X-100 for 2 h at 4°C with gentle agitation. After washing with the high salt buffer, the protein was eluted from the beads with elution buffer (100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 20 mM glutathione). The protein fraction was subjected to a PD-10 Desalting column (GE Healthcare) for further purification. Expression plasmids for GFP-fused proteins were transfected using the Effectene Transfection reagent (Qiagen) according to the manufacturer’s protocol. At 20 h after the transfection, leptomycin B (LMB, LC Laboratories) was added to medium to a final concentration of 10 nM for 30–90 min, depending on experiments. The cells were observed under a Leica AS MDW fluorescence microscope.

Cell Culture and Transfection—HeLa cells were incubated in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum at 37°C in an atmosphere containing 5% CO2. Expression plasmids for GFP-fused proteins were transfected using the Effectene Transfection reagent (Qiagen) according to the manufacturer’s protocol. At 20 h after the transfection, leptomycin B (LMB, LC Laboratories) was added to medium to a final concentration of 10 nM for 30–90 min, depending on experiments. The cells were observed under a Leica AS MDW fluorescence microscope.

RNA Interference—siRNAs for depletion of importin-β1 were obtained from Invitrogen. siImpβ1-1, siImpβ1-2, and siImpβ3-3 were designed to target the nucleotide number 2497–2521, 2638–2662, and 497–521 of human importin-β1, respectively. As a negative control siRNA, Stealth Negative Control Medium GC Duplex 2 (Invitrogen) was used.

siRNAs were transfected into HeLa cells using the Lipofectamine RNAiMax reagent (Invitrogen) according to the manufacturer’s protocol. Cells were incubated for 48 h with exchange of the medium once at 24 h after transfection. Cells were then harvested and subjected to Western blot analysis using a goat anti-importin-β antibody (Santa Cruz) and a mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase antibody (Ambion).

Immunofluorescence—All procedures were performed at room temperature. After the LMB treatment, HeLa or NIH3T3 cells were fixed with 4% formaldehyde in PBS for 15 min and then permeabilized with 0.2% Triton X-100 in PBS for 10 min. The cells were blocked with 3% skim milk in PBS for 1 h followed by incubation in the same solution containing rabbit anti-mDia2 antibody (20) for 1 h. After washing 3 times with PBS, the cells were incubated with Alexa 488-labeled donkey anti-mouse IgG (Molecular Probes) in the blocking solution for 1 h. The cells were then washed 5 times with PBS and observed using a ZEISS LSM 510 microscope equipped with a 40× NA1.4 objective lens at a wavelength of 488 nm in conjunction with a filter of BP505–530.

Microinjection—Two mg/ml purified GST-GFP or GST-GFP-mDia2-(16–39) protein with 0.5 mg/ml of Alexa594-labeled donkey anti-mouse IgG (Molecular Probes) was microinjected into the cytoplasm of HeLa cells. After incubation at 37°C for 60 min, cells were observed under a fluorescence microscope.

In Vitro Transport Assay—An in vitro transport assay was performed as described previously (22). Briefly, HeLa cells were plated on a slide glass. Cells were rinsed twice in an ice-cold transport buffer (20 mM HEPES, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 5 mM sodium acetate, 0.5 mM EGTA, 2 mM dithiothreitol) and permeabilized for 5 min in ice-cold transport buffer containing 40 mg/ml digitonin and washed. A recombinant GST-GFP-mDia2-(16–39) protein was expressed and purified as described previously (21).

RESULTS

Selective Nuclear Accumulation of mDia2 upon Leptomycin B Treatment—To examine subcellular localization of each mDia isoform, we expressed them as a GFP fusion protein in HeLa cells and examined their localization by GFP fluorescence. All the GFP-fused mDia isoforms are localized predominantly in the cytoplasm in cells at steady state (Fig. 1A). Two mechanisms underlie the predominant cytoplasmic localization of proteins; one is the size limitation by nuclear pores, which prohibits the passage of greater than 50-kDa molecules through the pore by passive diffusion (4), and the other is the selective nuclear export system, which recognizes a NES in substrate proteins and carries them out of the nucleus to the cytoplasm. The majority of proteins to be exported from the nucleus are exported by a nuclear export factor, CRM1, which recognizes the NES of these proteins (23). Because the molecular mass of mDia isoforms is more than 100 kDa, both mechanisms are possible. To examine this point, we used LMB, which specifically inhibits CRM1. We treated HeLa cells expressing each GFP-mDia isoform with LMB and examined their localization. Upon LMB treatment, mDia2, and not mDia1 or mDia3, accumulated in the nucleus (Fig. 1A). We also used an antibody specific to mDia2 and examined whether endogenous mDia2 also accumulates in the nucleus upon LMB treatment. We found nuclear accumulation of endogenous mDia2 upon LMB treatment in both HeLa and NIH3T3 cells (Fig. 1B), suggesting...
that the LMB-induced nuclear accumulation of mDia2 is not an artifact of GFP-fusion proteins and is not restricted to a specific cell type or species. We also wondered whether the LMB-induced nuclear accumulation reflects the active or inactive state of each mDia isoform. To address this question, we constructed structurally open active mutants of mDia isoforms by substitution of a specific Met residue, Met-1182 in mDia1, Met-1041 in mDia2, and Met-1053 in mDia3, in DAD for Ala and expressed these mutants as GFP fusion in HeLa cells (24). GFP-mDia2-(M1041A) again showed nuclear accumulation upon LMB treatment, whereas mDia1-(M1182A) did not show nuclear accumulation with or without LMB treatment. mDia3-(M1053A) was localized in the cytoplasm in most of the cells like mDia1 mutant but localized in a small population of cells in the nucleus with or without LMB treatment (supplemental Fig. S1). These results that mDia2 selectively accumulates in the nucleus upon LMB treatment indicate that mDia2 and not mDia1 or mDia 3 somehow enters the nucleus and is then exported by the CRM1-dependent mechanism.

Analysis of mDia2 Regions Involved in Nuclear Localization and Nuclear Export—The above results suggest that mDia2 actively shuttles between the nucleus and the cytoplasm whether or not it is structurally open. Most nucleocyttoplasmic shuttling proteins have NLS and NES in their own sequence. To identify NLS and NES in mDia2, we produced various truncation mutants of mouse mDia2 as GFP fusion proteins and analyzed their subcellular localization and their behavior upon LMB treatment. Each of truncation mutants of mDia2 was expressed in HeLa cells, and their localization was observed (Fig. 2). The N terminus deletion mutant, mDia2-(33–1171), was localized in the cytoplasm even after LMB treatment, indicating that the N terminus of mDia2 is responsible for the nuclear localization. Consistently, mDia2-(1–60), mDia2-(1–41), and mDia2-(1–32) were localized exclusively in the nucleus. Notably, mDia2-(1–153) was also predominantly localized in the nucleus, whereas mDia2-(1–411) was localized in both the cytoplasm and the nucleus, and LMB treatment induced the nuclear accumulation of mDia2-(1–411). The behaviors of the latter two truncation mutants suggest that the region spanning from the middle of GBD to the middle of FH3 is involved in the CRM1-dependent nuclear export.

We also examined in other regions for NES. The mutant complementary to mDia2-(1–411), mDia2-(411–1171), was not localized in the nucleus even in the presence of LMB, indicating that this protein cannot enter the nucleus. This is probably because it lacks NLS and is of a large size (87 kDa) and prevented us from examining whether this mutant contains NES for CRM1. We, therefore, attached the NLS of SV40 T-antigen to the N terminus of mDia2-(411–1171) (NLS-mDia2-(411–1171)) (25). We found that NLS-mDia2-(411–1171) was localized in the cytoplasm and accumulated in the nucleus upon LMB treatment. A shorter mutant, NLS-mDia2-(411–966), was localized in...
the nucleus without LMB treatment, suggesting the involvement of the C-terminal region of mDia2 in the CRM1-dependent nuclear export. Consistently, a short C-terminal fragment, mDia2-(1028–1171), changed the localization from the cytoplasm to the nucleus upon LMB treatment. These results indicate that the C terminus of mDia2 is the second region responsible for the nuclear export by CRM1.

We also noted that another C-terminal fragment, mDia2-(1028–1156), was localized predominantly in the nucleus without LMB treatment. Given that mDia2-(1028–1156) does not contain NLS and GFP-fused mDia2-(1028–1156) was small (44 kDa), it is likely that this protein enters the nucleus by passive diffusion and is retained in the nucleus by interaction with some nuclear structure or protein.

**mDia2 Has at Least One Functional NES Recognized by CRM1**—It is well known that NESs recognized by CRM1 are composed of a stretch of characteristically spaced hydrophobic amino acids such as \( \Phi X_2 \Phi X_2 \Phi X \) (\( \Phi = \text{Leu, Ile, Val, Phe, or Met; } X \) is any amino acid) (26), as was first reported for the human immunodeficiency virus type 1 Rev (LPPLERLTL) (27) and PKI (LALKLAGLDI) (28) proteins. Four NES-like sequences are present in the regions that were shown above to be involved in the nuclear export of mDia2, three in GBD and one at the C terminus, designated as NES-like 1–3 and NES-like 4, respectively (Fig. 3A). To examine whether these sequences function as NES recognized by CRM1, each of them was fused to GFP and expressed in HeLa cells. As shown in Fig. 3B, although these fusion proteins were small and expected to enter the nucleus by passive diffusion, fusion proteins of NES-like 3 and 4, not those of NES-like 1 and 2, were localized predominantly in the cytoplasm. The LMB treatment converted their predominant cytoplasmic localization to diffuse intracellular distribution (Fig. 3B) as shown for GFP alone (see for example Fig. 2), indicating NES-like 3 and 4 are recognized, and the GFP carrying these sequences are exported from the nucleus by CRM1. To confirm that these sequence function as NES in mDia2, we introduced point mutations into the hydrophobic amino acids in these sequences of GFP-fused mDia2-(1–411) and NLS-mDia2-(411–1171) and examined the localization (Fig. 3C). GFP-NLS-mDia2-(411–1171) (L1168G) accumulated in the nucleus, suggesting that NES-like 4 functions as NES of mDia2. On the other hand, GFP-mDia2-(1–411)(V268A/V269A) and GFP-mDia2-(1–411)(V274G/V276G)
were still localized in both the nucleus and the cytoplasm and accumulated in the nucleus upon LMB treatment. These findings suggest that the NES-like 3 does not function as a NES in intact protein and/or that there is an additional CRM1-dependent mechanism in this fragment. Finally, to examine contribution of the NES at the C terminus to nuclear export of the full-length protein, we introduced a L1168G mutation into full-length mDia2 (mDia2(L1168G)) and examined its behavior. GFP-fused mDia2(L1168G) was localized in the cytoplasm and accumulated in the nucleus upon LMB treatment (Fig. 3C). These results suggest that without NES-like 4, mDia2 can be exported by the CRM1-dependent manner possibly using its N-terminal region.

mDia2 Has Bipartite Basic NLS at the N Terminus—In the mDia2 truncation mutant analysis shown in Fig. 2, GFP fusion of short N-terminal fragments of mDia2 such as mDia2(1–60), mDia2(1–41), and mDia2(1–32) accumulated in the nucleus. To exclude a possibility that these fragments passively diffuse into the nucleus followed by retention, they were fused to pyruvate kinase (PK), a cytoplasmic protein without NLS that cannot enter the nucleus by diffusion (29). As shown in Fig. 4A, GFP-PK-fused mDia2(1–60) and mDia2(1–41) accumulated in the nucleus, indicating that these fragments contain NLS involved in selective nuclear import. On the other hand, GFP-PK-mDia2(1–32) remained in the cytoplasm as GFP-PK. These results together with the inability of mDia2(33–1171) to enter the nucleus (Fig. 2) suggest that NLS is present in mDia2(1–41), of which a sequence of amino acid 33–41 is critical but not enough.

In mDia2 (1–41) we found that the sequence of amino acid 16–39 contains two basic amino acid clusters separated by 15 non-basic amino acids, a motif resembling well known bipartite basic NLS (30). When this sequence was fused to GFP-PK (GFP-PK-mDia2(16–39)) and expressed, the protein accumulated in the nucleus (Fig. 4B), suggesting the above sequence functions as NLS. We then investigated whether this sequence functions as NLS in full-length mDia2. Amino acids substitution of first basic cluster (R18A/K19A) partially inhibited, whereas that of second one (K35A/R36A) completely abolished the nuclear accumulation of GFP-mDia2 upon LMB treatment (Fig. 4C). The K35A/R36A mutation also inhibited the nuclear accumulation of mDia2(M1041A), a structurally open mutant of mDia2, upon LMB treatment. These results

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**FIGURE 3.** mDia2 has at least one functional NES recognized by CRM1. A, the amino acid sequences of mouse, rat, and human mDia2 are aligned. Numbers denote the amino acid position relative to the N terminus. NES-like sequences are boxed by a black line. B, HeLa cells were transfected with each of GFP-fused NES-like sequences and incubated for 20 h. After treatment with or without 10 nM LMB for 1 h, the GFP fluorescence was observed under a fluorescence microscope. Scale bar, 20 μm. C, HeLa cells were transfected with each of GFP-fused mDia2 constructs with or without point mutations as indicated. At 20 h after transfection, cells were treated with or without 10 nM LMB for 1 h and then observed. Scale bar, 20 μm.
indicate that this bipartite basic sequence functions as the sole NLS.

The NLS of mDia2 Is Recognized and Used for Import into the Nucleus by the Importin-β/H9251/H9252 Complex—During the nuclear import of proteins, the NLS is recognized by nuclear import receptors such as importin-β. To investigate whether the nuclear import of mDia2 is mediated by importin-β, we depleted importin-β in HeLa cells by RNAi. We used three different siRNAs for importin-β (siImp-β-1, -2, and -3), all of which decreased the amount of importin-β in HeLa cells effectively (Fig. 5A). HeLa cells depleted of importin-β with these siRNAs were examined for localization of endogenous mDia2 after LMB treatment. We found that the nuclear accumulation of mDia2 by LMB treatment was inhibited markedly by importin-β depletion (Fig. 5B). A siRNA for negative control (siNC) neither depleted importin-β nor affected nuclear localization of mDia2. These results indicate that mDia2 is imported into the nucleus in an importin-β-dependent manner.

Importin-β recognizes NLS in two ways either by direct binding or by indirect interaction using an adaptor protein such as importin-α. To examine in which way the NLS of mDia2 is recognized by importin-β, we performed an in vitro reconstituted transport assay using recombinant importin-α and -β proteins in digitonin-permeabilized HeLa cells. A recombinant GST-GFP-mDia2-(16–39) protein, which was confirmed to be selectively imported into the nucleus when it was microinjected into the cytoplasm of HeLa cells (Fig. 6A), was used as a substrate. When Ehrlich total cytosol was used, GST-GFP-mDia2-(16–39) was effectively transported into the nucleus, whereas GTP-bound Ran (Q69L), which can prevent importin-β from recognizing NLS, inhibited the transport. In the ATP regeneration system, importin-β, when combined with importin-α, transported GST-GFP-mDia2-(16–39) into the nucleus as effectively as cytosol but not without importin-β (Fig. 6B). As reported before (21), Snail was transported into the nucleus by importin-β without importin-α in this assay (supplemental Fig. S2). These results indicate that importin-β uses importin-α as an adaptor to recognize the NLS of mDia2. Consistent with this, importin-α was shown to bind directly to mDia2-(16–39) (Fig. 6C).

DISCUSSION

We report here that mDia2 is a nucleocytoplasmic shuttling protein. This property of mDia2 is confirmed in human HeLa cells and mouse NIH3T3 cells, suggesting that it is a universal property of mDia2 over species and cell types. This idea is supported by the findings that the nuclear transport of mDia2 is mediated by ubiquitously expressed transport receptors: importin-α/β for import and CRM1 for export.
mDia2 is Exported by the CRM1-dependent Mechanism—We identified two regions in mDia2 that are involved in the nuclear export by CRM1. The first is in the region spanning from the middle of GBD to the middle of FH3. We found a NES-like sequence (NES-like 3) in this region, which behaved as a cargo of CRM1 when it was isolated and fused to GFP (Fig. 3, A and B). However, the mutation of this sequence in mDia2-(1–411) did not interfere with the CRM1-dependent nuclear export of this protein (Fig. 3C). Similarly, although the NES-like 4 at the C terminus also functions as a NES for CRM1 in GFP-NLS-mDia2-(411–1171), the mutation of this sequence did not interfere with the CRM1-dependent export of the full-length mDia2. These results indicate either that there is an unrecognized NES in the N-terminal region (1–411) or that mDia2 binds through this N-terminal region to another protein that is exported by the CRM1-dependent mechanism. Thus, it is likely that the full-length mDia2 can be exported by two different CRM1-dependent mechanisms; one is the C-terminal NES-dependent mechanism, and the other is the N-terminal region-dependent mechanism discussed above.

mDia2 Is Imported into the Nucleus without Activation—The bulk of mDia2 is thought to be inactive in a cell at steady state by the intramolecular binding between GBD and DAD domains (11). This supposition is supported by the fact that DAD overexpressed in NIH3T3 cells activates endogenous mDia2, leading to remarkable F-actin formation (31). Therefore, if only the activated form of mDia2 is imported to the nucleus, only the small population of mDia2 in the cell should accumulate in the nucleus and the bulk should stay in the cytoplasm. Upon LMB treatment, however, both endogenous and exogenously overexpressed mDia2 mostly accumulated in the nucleus, and little was found in the cytoplasm (Fig. 1), suggesting that mDia2 does not have to be activated to be imported into the nucleus. It is, therefore, likely that the NLS at the N terminus is accessible even when mDia2 is structurally closed. Although Copeland et al. (32) mentioned the presence of a cryptic NLS C-terminal to the FH2 domain of mDia2, we showed here that the NLS at the N terminus is the only NLS functioning in full-length mDia2 (Fig. 4C) and that the fragments lacking the N-terminal NLS did not enter the nucleus (Fig. 2). Besides, our findings suggest that even if mDia2 is structurally open, the region C-terminal to FH2 is not functional as NLS because both mDia2-(411–1171), a constitutively active form, and mDia2(K35A/R36A/M1041A), a form constitutively open and defective in the NLS at the N terminus, cannot accumulate in the nucleus upon LMB treatment (Figs. 3 and 4C).

Does mDia2 Bind to Rho GTPases in the Nucleus?—mDia isoforms have two functions; one is to bind to the active form of Rho GTPases, and the other is to catalyze actin nucleation and polymerization under the direction of Rho GTPases. It is well established that all of mDia isoforms bind to Rho. In addition, mDia2 has been reported to interact also with other Rho GTPases, Rac1, Rac2 (33), Cdc42 (34), and Rif (35), suggesting these GTPases can activate mDia2 according to circumstances. Among them, Rac1 was recently reported to accumulate in the nucleus specifically during the G2 phase of the cell cycle using the NLS at the C terminus (36). The authors discussed that Rac1 is sequestered from the cytoplasm into the nucleus in G2 so that the Rac1 actions on cells such as ruffling, spreading, and adhesion is shut off to facilitate rounding up of cells during cell division. Their findings together with our present findings suggest a possibility that a population of mDia2 makes a complex with Rac 1 in G2 and M and functions as an effector for Rac1 during cell division. Because Rho GTPases (<21 kDa) are small enough to enter the nucleus without selective nuclear import, some of them other than Rac1 may be able to activate mDia2 in the nucleus. We have recently reported that mDia2 plays a criti-
Is mDia2 Involved in Actin Dynamics in the Nucleus?—Emerging evidences suggest that actin is involved in several processes in the nucleus. Actin has been found as a component with all three eukaryotic RNA polymerases, RNA polymerase I, II, and III, and is required for transcription by these polymerases in vitro (39). Recently, Ye et al. (40) have presented convincing evidence for involvement of polymeric actin in this process. Using actin mutants and actin-depolymerizing drugs, they demonstrated that polymeric actin, in concert with nuclear myosin I, promotes the transcription of ribosomal genes by RNA polymerase I. Actin polymerization might also be involved in intranuclear chromatin repositioning. Dundr et al. (41) used live-cell imaging and showed that the movement of a U2 small nuclear RNA gene locus toward Cajal bodies during transcriptional activation was markedly inhibited by overexpression of a nonpolymerizable mutant of actin. Thus, actin polymerization seems to be important in some nuclear processes. Some actin-associated proteins are also present in the nucleus. For example, profilin is in the nucleus (42). Because profilin facilitates actin polymerization by mDia isoforms (10), mDia2 may associate with profilin in the nucleus and induce actin polymerization. However, if mDia2 induces polymerized actin in the nucleus, it may not be produced in comparable amounts or of comparable length to that in the cytoplasm or it may not be the same in structure as F-actin in the cytoplasm because we have not detected phalloidin-positive F-actin in the nucleus when we overexpressed a nuclear-targeting, constitutively active mutant of mDia2, GFP-NLS-mDia2-(411–1171) (L1168G) (Fig. 3C), in HeLa cells (data not shown). Indeed, it has been controversial whether actin in the nucleus can exist in the same filamentous form as we see in the cytoplasm (43, 44). Although phalloidin staining usually detected little signal in the nucleus, evidences accumulate to suggest that there exist polymeric forms of actin in the nucleus, which seem to be structurally different from classical F-actin (45, 46).

Is mDia2 an Only Nucleocytoplasmic Shuttling Protein among mDia Isoforms?—We have identified a bipartite basic NLS at the N terminus of mDia2. No similar sequence is found in the corresponding region of mDia1 or mDia3. However, we and others reported a sequence that may function as an NLS at the C terminus of mDia1 (8, 32). Although mDia1 and mDia3 do not accumulate in the nucleus of HeLa cells upon LMB treatment (Fig. 1A), this finding merely indicates that they are not exported from the nucleus exclusively by CRM1. There is a possibility that mDia1 and mDia3 are exported from the nucleus by another nuclear export receptor(s) exclusively or in addition to CRM1. The pulldown experiment of exportin-6 precipitated mDia1 from HeLa cell lysate together with the actin profilin complex, which is exported by this exporter (47). Whether mDia1 is exported from the nucleus by exportin-6 remains unknown. Here we also have found mDia3-(M1053A), an open mutant of mDia3 in the nucleus of a small population of HeLa cells (supplemental Fig. S1), and that LMB treatment promoted the nuclear accumulation of a GBD-deleted constitutively active mutant of mDia3 in HeLa cells. These results imply that mDia3 can enter the nucleus in some situation, and its nuclear export is mediated by CRM1 at least in part. We previously found that endogenous mDia3 associates with heterochromatin protein-1 in the nucleus of some populations of HeLa cells of randomly growing culture. Thus, nuclear translocation is possibly a common feature for mDia isoforms.

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5 T. Miki and S. Narumiya, unpublished data.
Nuclear Transport of mDia2

In summary, our present study has revealed that specific nuclear transport machinery composed of importin-α/β and CRM1 translocates mDia2 between the cytoplasm and nucleus. What function mDia2 exerts in the nucleus remains to be elucidated by future studies.

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