Mouse MIM, a Tissue-specific Regulator of Cytoskeletal Dynamics, Interacts with ATP-Actin Monomers through Its C-terminal WH2 Domain*

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The WH2 (WASP homology domain-2) is a small actin monomer-binding motif and is found in many proteins that regulate the actin cytoskeleton, including the β-thymosins, ciboulot, WASP, and verprolin/WIP (WASP-interacting protein). In sequence database searches we identified a novel mouse protein containing a WH2 domain in its C-terminal region. This mouse gene also shows strong sequence homology to human MIM (Missing In Metastasis), a cDNA fragment that is present in non-metastatic but absent in metastatic bladder cancer cell lines. Northern blot and in situ hybridizations show that MIM is strongly expressed in the developing neurons and skeletal and cardiac muscles in mouse embryos. In adult mice, the strongest expression of MIM mRNA is in liver, outer layers of the kidney, and in the Purkinje cells of the brain. Recombinant MIM protein interacts with actin monomers and inhibits actin filament nucleation in vitro. However, the MIM/ATP-G-actin complex can participate in actin filament assembly at the barbed end. MIM binds ATP-G-actin with a higher affinity (K_D = 0.06 μM) than ADP-G-actin (K_D = 0.3 μM) and inhibits the nucleotide exchange on actin monomers. Site-directed mutagenesis demonstrates that the actin monomer-binding site resides in the C-terminal WH2 domain of MIM. Overexpression of mouse MIM in NIH 3T3 cells results in the disappearance of actin stress fibers and appearance of abnormal actin filament structures. These data show that MIM is an ATP-G-actin binding protein that regulates cytoskeletal dynamics in specialized mammalian cell-types.

The actin cytoskeleton is central in a number of cellular processes such as motility, morphogenesis, cytokinesis, and endocytosis. The structure and dynamics of the actin cytoskeleton are spatially and temporally regulated by a large number of actin-binding proteins, whose own activities and localities are precisely regulated by various signaling pathways (1). Sequence and structural data on actin-binding proteins has revealed that many of these proteins interact with actin through a relatively small number of protein motifs. These include the calponin homology domain, the gelsolin homology domain, the actin-depolymerizing-factor homology domain, and the WASP homology 2 (WH2) domain (for reviews see 2–5).

The WH2 domain is a small (~35 residue) protein motif that interacts only with monomeric actin (5, 6). WH2 domains are found in many regulators of actin dynamics, including β-thymosins and ciboulot, which bind actin monomers and regulate filament assembly. β-thymosins are actin monomer-sequestering proteins, whereas ciboulot promotes actin assembly at the barbed end of the filaments (7, 8). WH2 domains are also present in more complex proteins such as WASP/Scar, verprolin/WIP, and Srv2/CAP. These are multifunctional regulators of actin dynamics that link intracellular signaling pathways to actin dynamics (5). For example, WASP and Scar mediate signals from PI(3)P and the small GTPases Cdc42 and Rac to the actin cytoskeleton by inducing actin assembly through activation of the Arp2/3 complex (9–11). The WH2 domain is essential for the activity of WASP and Scar and is believed to facilitate the assembly of actin monomers to the newly formed filament ends (12, 13). Also verprolin/WIP proteins interact with actin monomers through their WH2 domains, but the biological role of their actin monomer-binding activity is still unclear (14).

We searched sequence databases for WH2 domain-containing proteins in mammals, Drosophila melanogaster, Caenorhabditis elegans, and Saccharomyces cerevisiae. In addition to the previously characterized WH2 domain proteins (β-thymosins, ciboulot, WASP, verprolin/WIP, Scar, Srv2/CAP) we identified several previously unknown WH2 domain-containing proteins (5). One of these proteins is particularly interesting because it is highly homologous to a human cDNA fragment named MIM (Missing In Metastasis) that was recently identified in a differential screen for mRNAs specifically expressed in non-metastatic bladder cancer cells (15).

We show that mouse MIM is an actin monomer-binding protein that efficiently inhibits the nucleotide exchange on actin monomers and actin filament nucleation in vitro. The strong actin-modulating activities reside in the C-terminal WH2 domain of MIM. We found that MIM is strongly expressed in the developing heart, skeletal muscle, and central nervous system. In adult mouse tissues, strong expression levels are detected in liver and in certain regions of brain and kidney. Overexpression of MIM results in the disappearance of stress fibers and formation of abnormal F-actin structures in NIH 3T3 cells.
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Experimental Procedures

Plasmid Construction and Site-directed Mutagenesis—A DNA fragment corresponding to the full-length mouse MIM cDNA was amplified from I.M.A.G.E. Consortium mouse EST clone plasmid ID:5101888 (UK HGMP Resource Centre). The C-terminal half of mouse MIM was amplified from mouse E10 cDNA and I.M.A.G.E. Consortium mouse EST clone plasmid ID:2648606. Two different splice variants were obtained (see Fig. 1A), from which the shorter and more abundant form was used for the biochemical studies and is referred as MIM-CT in the text. The sfps used in the amplification of the full-length mouse MIM created BamHI and HindIII sites at the 5′ and 3′ ends of the PCR fragment. These fragments were digested and ligated into the pGAT2 vector (16) to create plasmids pPL149 (full-length mouse MIM) and pPL106 (MIM-CT). The site-directed mutation, which replaced lysines 746 and 747 into alanines and deleted the last 11 amino acids 749–759, was introduced into the MIM-CT by PCR, and the amplification product was cloned into NcoI-HindIII-digested pGAT2 vector to create a plasmid pPL141. For generation of in situ probes MIM-CT cDNA fragment was cloned into the BamHI (5′)-HindIII (3′) sites of pBluescript to create a plasmid pPL139. The plasmids for overexpressing green fluorescence protein (GFP) fusions of MIM were constructed by cloning sites of pEGFP-N1 and pEGFP-C1A (Clontech) to create plasmids pPL151 and pPL153. Site-directed mutations of pEGFP-N1 and pEGFP-C1A (Clontech) to create plasmids pPL149 and pEGFP-C1A (Clontech) to create plasmids pPL151 and pPL153.

Northern Blotting—A mouse MIM cDNA probe was prepared from the plasmid pPL106 as described for mouse twinfoalin (17). The MIM-CT cDNA probe was then hybridized to complementary mouse multiple tissue mRNA blot (Clontech) according to the manufacturer’s instructions. The Northern blot filters were exposed on a phosphorimaging screen overnight. β-Actin controls were used to ensure equal amounts of RNA in each lane.

Radioactive in Situ Hybridizations—The antisense probe was obtained by linearizing the plasmid pPL139 with BamHI and transcribing it with T3 RNA polymerase. For the sense probe HindIII and T7 RNA polymerase were used. The in situ hybridizations on the mouse tissues sections were performed using 35S-UTP-labeled riboprobes as previously described (18).

Protein Expression and Purification—Mouse MIM-CT was expressed as a glutathione S-transferase (GST) fusion protein in Escherichia coli BL21 (DE3) cells under the control of T7 lac promoter. Cells were grown in 12 liters of Luria broth medium to an optical density of 0.5 at 600 nm, and the expression was induced with 0.2 mM isopropyl-β-D-1-thiogalactoside. Cells were harvested 3 h after induction, washed with 180 ml of 20 mM Tris (pH 7.5), resuspended in 40 ml of phosphate-buffered saline, 0.2 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, lyzed by sonication, and centrifuged for 15 min at 15,000 × g. GST fusion proteins were enriched from the supernatant by glutathione-agarose beads (Sigma). The beads were then incubated overnight with thrombin (5 units/ml) at +4 °C to cleave MIM-CT away from GST. The beads were washed five times with 50 ml Tris-HCl (pH 7.5) and 150 mM NaCl, and the supernatant was diluted into 30 ml with 20 mM Tris-HCl (pH 7.5) and 25 mM NaCl. The solution was loaded onto an equilibrated Q-Sepharose High Performance Anion-exchange chromatography column (Amersham Biosciences) and eluted with a linear 0.25–1 M NaCl gradient. The peak fractions containing MIM-CT that eluted at ~0.35 M NaCl were pooled, and the buffer was exchanged to 10 mM Tris-HCl (pH 7.5) and 50 mM NaCl with a PD-10 desalting column (Amersham Biosciences). The protein solution was then concentrated in 10-kDa cutoff tubes (Centricon) into a final concentration of 100–200 μg/ml divided into small aliquots, frozen in liquid N2, and stored at −70 °C.

Human platelet actin and pyrene-labeled actin (human platelet actin) were also estimated from Coomassie Blue-stained SDS-PAGE. Fluorescence of pyrene-labeled actin (Cytoskeleton) was measured with BioLogic MOS-250 fluorometer at each concentration of MIM-CT with an excitation at 482 nm and emission at 535 nm. The data were analyzed using SigmaPlot software and fitted using the following equation

\[
E = \frac{1}{2} \left( 1 + \frac{z}{c} \right) \left( 1 - \frac{1}{z} \right) \left( \frac{z}{z + 2} - \frac{4}{2z} \right)
\]

where z and c are described in the two following equations.

\[
x = \frac{[MIM]_{tot}}{[Act]_{tot}}
\]

\[
c = 1 + \frac{K_d}{[Act]}
\]

Actin Filament Sedimentation Assays—Forty-μl aliquots of human platelet actin at desired concentration in G-buffer (20 mM Tris, pH 7.5, 0.2 mM ATP, 0.2 mM DTT, 0.2 mM CaCl2) were polymerized for 30 min by the addition of 5 μl of 10× initiation mix (20 mM MgCl2, 10 mM ATP, 1 mM KCl). Five μl of MIM-CT (0, 10, 20, or 40 μM) in G-buffer was added to the actin filaments formed by a 30-min incubation. The actin filaments were sedimented by centrifuging at 217,000 × g for 30 min in a Beckman Optima MAX Ultracentrifuge and a TLA 100 rotor. All steps were performed at room temperature. Equal proportions of supernatant and pellet fractions were loaded onto 10% SDS-polyacrylamide gels; the gels were stained with Coomassie Blue and scanned with FluorS-Imager (Bio-Rad, Hercules, CA).

Pyrene Actin Filament Assembly Assays—We followed actin polymerization in the presence of MIM by determining the increase in the fluorescence of pyrene-labeled actin (Cytoskeleton). Three μl actin (1.5 μg/ml actin) was mixed with 1 μl pyrene-labeled rabbit muscle actin (human platelet actin) with 0, 0.75, or 3 μM MIM-CT or MIM-CTAH2H in G-buffer (5 mM Tris-HCl, pH 7.5, 0.2 mM ATP, 0.2 mM DTT, 0.2 mM CaCl2) was polymerized by adding 1/10 reaction volumes of 10X initiation mix (20 mM MgCl2, 10 mM ATP, 1 mM KCl). Kinetics of actin filament assembly was followed by a BioLogic MOS-250 fluorescence spectrophotometer at an excitation wavelength of 365 nm and an emission wavelength of 407 nm. To examine the effect of MIM-CT for pointed end and barbed end filament assembly, gelsolin-actin seeds (10 μM) were added to a mixture of 3 μM actin and desired concentration of 0–6 μM MIM-CT in G-buffer. The phallolin-actin seeds were prepared as described (23) and gelsolin-actin seeds as described (24). The rate of actin filament assembly was monitored as described above.

Nucleotide Exchange Assay—The rate of nucleotide exchange was measured from the fluorescent signal provided by e-ATP bound to G-actin. Human platelet actin was dialyzed against e-ATP-G-buffer (20 mM Tris, pH 8.0, 1 mM DTT, 0.4 mM CaCl2, 0.2 mM e-ATP) overnight at +4 °C. An 80-μl mixture of e-ATP-actin and MIM-CT was prepared in G-buffer with final concentrations of 0.5 μM for actin and 0, 0.5, 1.5, 2, or 4 μM for MIM-CT. Twenty μl of 5 mM ATP was added to the mixture, and the reaction was immediately monitored by a BioLogic MOS-250 fluorescence spectrophotometer using an excitation wavelength of 360 nm and an emission wavelength of 410 nm.

Cell Culture and Immunofluorescence—NH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen), 2 μg/ml t-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were transfected with 1.5 μg of the desired plasmid using the FuGENE6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Immuno-fluorescence was carried out as described in Vartiainen et al. (17). F-actin was visualized by incubation of mouse MIM-CT (ε280 = 17,780 M−1 cm−1) and for actin (ε280 = 26,600 M−1 cm−1). Protein concentrations were also estimated from Coomassie Blue-stained SDS-PAGE. Fluorescence-monitored urea denaturation assays were carried out as described in (26).
RESULTS

Identification of a Novel Mouse WH2 Domain Protein—In database searches for novel WH2 domain-containing proteins, we identified several independent mouse ESTs that encoded a putative protein with a C-terminal WH2 domain. A full-length sequence of the coding region of this cDNA was obtained by sequencing I.M.A.G.E. consortium mouse EST clone plasmid ID: 5101888. The predicted protein is composed of 759 residues with an N-terminal sequence (MEAVI ... ). There is an in-frame stop codon 85 nucleotides upstream to the methionine (AUG) codon, and the nucleotides immediately 5’ to this AUG codon fulfill the rules for translational initiation sequences (27), suggesting that translation is initiated at this methionine. Our database searches and sequencing of PCR fragments from this mouse cDNA showed that a shorter form of this protein also exists. The shorter form lacks amino acids 646–681 and contains Leu-to-Pro substitutions at residues 483 and 487 (Fig. 1A). Because the nucleotide sequences of these two isoforms are otherwise identical, they are most likely splice variants of the same gene.

Our database searches revealed a human homologue that is 96% identical at the amino acid level. While the putative coding regions of the human and mouse cDNA clones were nearly identical at the nucleotide level, the regions 5’ from the putative translational initiation codons showed significantly lower homology. This provides further support that translation starts at this AUG codon. Interestingly, a region in the human cDNA is identical to a 300-bp cDNA fragment that was recently identified by differential display as a gene that was specifically expressed in non-metastatic bladder cancer cells and consequently named MIM (15). Both mouse and human MIM contain a WH2 domain in their C termini (Fig. 1A). The residues that are important for actin monomer-binding in the WH2 domain of thymosin-β4 (28) are well conserved in human and murine MIM, suggesting that these proteins interact with monomeric actin (Fig. 1B). The human and mouse MIMs also contain a
proline-rich region but otherwise do not share any detectable sequence homology to other known proteins (Fig. 1C).

Expression of MIM in Mouse Embryos and Adult Mouse Tissues—To examine the expression patterns of MIM, we carried out Northern blot and in situ hybridization analyses on embryonic and adult mouse tissues. A multiple-tissue Northern blot analysis showed that MIM mRNA is expressed in several adult mouse tissues at variable levels (Fig. 2, left panel). Particularly strong expression was detected in liver. The kidney, heart, lung, spleen, and brain showed only moderate or weak expression of MIM mRNA. No expression could be detected in skeletal muscle and testes. A Northern blot analysis of mouse embryos at different stages showed that the relative MIM expression increases during development (Fig. 2, right panel). The MIM cDNA probe used in this study recognized only a single ~6-kb mRNA, indicating that the probe was specific. The two MIM splice variants are very close to each other in size, and therefore cannot be distinguished from each other in this Northern blot. The blots were also hybridized with a β-actin control probe to ensure that each tissue samples contained equal amounts of mRNA (data not shown).

The expression pattern of MIM in developing mouse embryos was studied in more detail by RNA in situ hybridizations. During development (E10.5–18.5) the strongest MIM expression was in the developing heart, skeletal muscle, and central nervous system (Fig. 3A and data not shown). Weak expression was detected in the liver, kidney, and lung (Fig. 3A and data not shown). A control hybridization carried out with the sense probe showed that the signal was specific for the MIM mRNA (Fig. 3B). Additional hybridizations on mouse embryo sections from various developmental stages demonstrated that MIM is highly expressed in the postmitotic neurons of the spinal cord and brain (Fig. 3, C and E). Strong expression could also be detected in somitic and migrating myoblasts (Fig. 3C), in the developing muscles of the limbs and the body (Fig. 3, C and F), and in the developing cardiomyocytes (Fig. 3D). Weak expression was detected in the developing sensory systems including the inner ear and eye (Fig. 3E and data not shown).

We also examined MIM expression in adult mouse tissues by in situ hybridizations. Whereas MIM is strongly and widely expressed in the developing central nervous system until birth, in the adult brain MIM is expressed at high levels only in one special neuronal cell type: the Purkinje cells of the cerebellum (Fig. 3G). Weak expression was detected in other parts of the brain such as the septum, the piriform cortex, and the ependymal cells lining the ventricles (data not shown). In the kidney, MIM was highly expressed in the outer medullary zone and weakly in the cortex. MIM expression could not be detected in the medullary inner zone (Fig. 3H). Taken together, these hybridizations show that MIM is strongly expressed in the developing neurons and muscle cells but is down-regulated during the maturation of these cells. In adult mice MIM is highly expressed only in kidney, liver, and in specialized neuronal cells such as Purkinje cells.

**MIM Binds ATP-Actin Monomers through its C-terminal WH2 Domain**—The mouse and human MIM proteins contain a putative actin monomer-binding motif, WH2 domain, in their C termini. To examine whether mouse MIM is an actin-binding protein, we carried out a biochemical characterization of this protein. Because our attempts to express and purify the full-length mouse MIM were unsuccessful, we decided to express the WH2 domain containing C-terminal half (residues 404–759) of the more abundant splice variant of MIM (Fig. 1A). This fragment (MIM-CT) was produced as a GST fusion protein in *E. coli*. The GST was removed by thrombin digestion, and recombinant MIM-CT was further purified by anion exchange chromatography.

The fluorescence of the NBD-labeled actin monomers is affected upon interaction with many proteins, including ADF/cofilin, twinfilin, thymosin-β4, and ciboulot, thereby providing...
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a method to determine the affinities of these proteins for actin monomers (29–31). MIM-CT induced a 20–25% decrease in the fluorescence of NBD-G-actin (Fig. 4). The extent of the NBD-actin fluorescence decrease displayed a saturating behavior enabling us to calculate the $K_D$ values for MIM-CT/actin monomer complexes. MIM-CT bound to both ADP- (Fig. 4A) and ATP-actin monomers (Fig. 4B) with high affinity. However, the affinity of MIM-CT for ATP-actin monomers was 5-fold higher, ($K_D = 0.06 \mu M$) than for ADP-actin monomers ($K_D = 0.3 \mu M$). The assay was carried out in physiological ionic conditions (0.1 M KCl, 2 mM MgCl$_2$).

To examine the role of the C-terminal WH2 domain for actin monomer-binding, we designed a mutant protein, MIM-CT\(\Delta\)WH2, in which the WH2 domain was inactivated. The actin-binding site in the WH2 domain of thymosin-\(\beta_4\) has been mapped by mutational studies (28). We constructed a mutant protein in which the corresponding residues (Lys-746 and Lys-747) that have been shown to be most critical for G-actin binding in thymosin-\(\beta_4\) were replaced by alanines, and the last 11 amino acids (749–759) were deleted. The mutant protein was purified as described for wild-type MIM-CT. It showed similar stability than the wild-type MIM-CT in a fluorescence-monitored urea denaturation assay, suggesting that these mutations do not affect the protein stability (Fig. 5). MIM-CT\(\Delta\)WH2 did not affect the fluorescence of NBD-G-actin, suggesting that this mutant protein does not bind actin monomers with a detectable affinity (Fig. 4C).

Mouse MIM Inhibits the Nucleation of Actin Filaments, but Its Complex with ATP-G-actin Can Participate in Filament Barbed End Growth—The effect of MIM-CT on actin dynamics was examined by steady-state and kinetic assembly assays. The steady-state assay was an actin filament co-sedimentation assay where the actin concentration was 2 \(\mu\)M and the concentration of MIM-CT was varied from 0 to 4 \(\mu\)M. In the absence of MIM, a majority of actin was present in the pellet fraction, whereas in the presence of 2 and 4 \(\mu\)M MIM-CT \(\sim75\%\) of the actin was shifted to the supernatant fraction. This shows that mouse MIM efficiently decreases actin filament assembly (Fig. 6A, upper panel). In a similar assay MIM-CT\(\Delta\)WH2 did not increase the amount of monomeric actin, demonstrating that the C-terminal WH2 domain is essential for the ability of MIM to inhibit steady-state F-actin assembly (Fig. 6A, lower panel).

We also carried out an actin filament co-sedimentation assay to examine the interaction of MIM with F-actin. In this assay we used a constant concentration of MIM-CT (2 \(\mu\)M) and varied the concentration of actin from 0 to 8 \(\mu\)M. MIM-CT did not co-sediment with actin filaments, suggesting that MIM does not interact with F-actin with a detectable affinity (data not shown).

Pyrene actin assembly assays were carried out to directly analyze the effects of MIM on actin filament polymerization and nucleation. We first followed the polymerization of 3 \(\mu\)M actin (in the presence of 0/0.75/3 \(\mu\)M MIM-CT or MIM-CT\(\Delta\)WH2) for 10 min after addition of polymerization salts. The data showed that wild-type MIM-CT efficiently inhibits

![Fig. 4. Interaction of MIM-CT and MIM-CT\(\Delta\)WH2 with actin monomers.](image-url)

![Fig. 5. The stability of wild-type and mutant MIM-CT proteins measured by fluorescence monitored urea denaturation assay.](image-url)
the nucleation and assembly of the actin filaments, whereas MIM-CTΔWH2 did not affect the nucleation/assembly of actin filaments (Fig. 6B). We next examined the effects of MIM-CT on filament pointed end and barbed end assembly by using gelsolin-actin and phalloidin-stabilized actin seeds, respectively. MIM-CT efficiently inhibits actin assembly on 10 nM gelsolin-actin seeds demonstrating that MIM-CT blocks the pointed end filament growth (data not shown). MIM-CT also decreases the rate of assembly on phalloidin-actin seeds, but even high concentrations of MIM-CT (2-fold molar excess to ATP-G-actin) do not completely block the assembly. Instead, the assembly rate of MIM-CT/ATP-G-actin complex appears to saturate to ~40% level of that of ATP-G-actin alone (Fig. 6C). In conclusion, these assays demonstrate that mouse MIM efficiently inhibits the nucleation and pointed end assembly of actin filaments, but its complex with ATP-G-actin can participate in filament barbed end growth. The participation of MIM-CT in barbed end elongation is also in agreement with the co-sedimentation assay (Fig. 6A) where MIM-CT, despite its very high affinity for ATP-G-actin (0.06 μM; Fig. 4B), was unable to shift all actin to the monomeric fraction even when present in 2-fold molar excess to actin.

**MIM Inhibits the Nucleotide Exchange on G-actin**—Many actin monomer-binding proteins, such as ADF/cofilin, twinfilin, ciboulot, and thymosin-β4, decrease the rate of nucleotide exchange on actin monomers (31–34). On the other hand small actin monomer-binding protein, profilin, catalyzes the nucleotide exchange on actin monomers (35). We examined the effects of mouse MIM on the nucleotide exchange by following the rate of replacement of ε-ATP by ATP on actin monomers in the absence and presence of MIM-CT. MIM inhibits the rate of nucleotide exchange on actin monomers in a concentration-dependent manner (Fig. 7). The inhibition of the nucleotide exchange of 0.5 μM actin is almost saturated already at 0.5 μM MIM-CT. This result is in good agreement with the very high affinity of MIM-CT for ATP-G-actin (0.06 μM; Fig. 4B).

**Effects of MIM on Actin Dynamics in Vivo**—To study the effects of MIM on actin dynamics in vivo, we overexpressed mouse MIM as a GFP fusion protein in NIH 3T3 fibroblasts. In the fusion protein constructs GFP was located either at the N terminus or at the C terminus of MIM. In both cases the localization of MIM and the induced phenotypes in cells were
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similar to each other, indicating that the GFP did not affect the activity of MIM in cells (data not shown). MIM-GFP fusion proteins were uniformly cytoplasmic in NIH 3T3 cells and not present in the nucleus. Cells over-expressing MIM-GFP showed a clear decrease in the amount of stress fibers and accumulation of abnormal worm-like cytoplasmic actin filament structures (Fig. 8, A and B). These abnormalities did not occur in untransfected cells or in the cells overexpressing GFP alone (Fig. 8, C and D). Furthermore, overexpressing MIM-GFP fusion protein in HeLa and NRK cells induced similar abnormalities in the actin cytoskeleton (data not shown). These results suggest that the actin monomer-binding protein MIM is able to regulate actin dynamics also in living cells.

**DISCUSSION**

We show here that mouse MIM is an actin monomer-binding protein that inhibits the nucleation of actin filaments in vitro. These activities reside in the C-terminal WH2 domain of MIM. WH2 domains are also found in many other actin monomer-interacting proteins, such as WASP, Scar, thymosin-β4, ciboulot, actobindin, and WIP/verprolin. All available data indicate that these proteins also interact with actin monomers through their WH2 domains (for recent review see Ref. 5). Furthermore, at least thymosin-β4, ciboulot, and actobindin have significantly higher affinity for ATP-G-actin than for ADP-G-actin (31, 36). Our data shows that mouse MIM affinity for ATP-G-actin is ~5-fold higher than its affinity for ADP-G-actin, suggesting that WH2 domain has evolved as a motif that preferentially binds ATP-G-actin. It is important to note that mouse MIM binds ATP-G-actin with ~20-fold higher affinity (K_D = 0.06 μM, Fig. 4) than thymosin-β4, ciboulot, and actobindin (K_D = 0.7–2 μM) (31). For that reason, we would expect MIM to more drastically affect actin dynamics in cells than for example thymosin-β4.

Most actin monomer-binding proteins, including profilin, ADF/cofilin, twinfilin and WH2 domain proteins bind to the same site on the actin monomer, and thus compete with each other in actin monomer-binding (30, 37–39). Our data show that mouse MIM has a higher affinity for ATP-G-actin (K_D = 0.06 μM) than other actin monomer-binding proteins such as profilin (K_D = 0.1–0.15 μM), ADF/cofilin (K_D = 4–8 μM) and twinfilin (K_D = 0.5 μM) (1, 30). This suggests that in the cells where MIM is expressed, the majority of active MIM is associated with ATP-G-actin. On the other hand, the affinity of mouse MIM for ADP-G-actin (K_D = 0.3 μM), is significantly lower than the ones of ADF/cofilin (K_D = 0.1–0.15 μM) and twinfilin (K_D = 0.05 μM), indicating that MIM would not significantly affect the localization and dynamics of the cellular ADP-actin monomer pool (1, 31).

Some WH2 domain proteins such as thymosin-β4 inhibit actin filament assembly, whereas others such as ciboulot, actobindin and WASP do not sequester actin monomers when the barbed ends of filaments are available (8, 12, 31, 36, 40). Mouse MIM increases the amount of actin monomers in steady-state assembly assays and inhibits actin filament nucleation in kinetic assays (Fig. 6, A and B). MIM also blocks the pointed end filament assembly, but MIM-CT/ATP-G-actin complexes can participate in barbed end filament elongation (Fig. 6C). Therefore, our data suggests that MIM has similar effects on actin assembly as previously described for small WH2 domain proteins ciboulot and actobindin (8, 31). However, because MIM is a large, multidomain protein, it is likely to have additional activities that could affect or regulate the actin-modulating activities of the C-terminal WH2 domain.

Our Northern blot and in situ hybridization assays demonstrated that MIM mRNA is strongly expressed in developing neurons and myoblasts but absent in most mature neurons and muscle cells. In adult mice, MIM is present in high levels in liver, kidney, and in the cerebellar Purkinje cells (Fig. 3). Similarly, the small actin monomer-sequestering protein, thymosin-β10, is expressed in several developing neuronal cell-types, but only in Purkinje cells in adult animals (41). The transient expression of MIM in developing neurons and myoblasts suggests a role in polarized growth or motility: both processes are actin-dependent and typical for differentiating neurons and myoblasts. Mature muscle cells and neurons lack MIM and have a less dynamic actin cytoskeleton and consequently do not undergo similar morphogenetic changes than their embryonic precursors. The presence of MIM in adult Purkinje cells may be related to the growth and refinement of Purkinje cell dendrites, a phenomenon that probably also takes place in adult animal brains.

It is interesting to note, that the human homologue of MIM was identified as a gene that was present in non-metastatic but absent in metastatic bladder cancer cells (15). Our in situ hybridization data show that MIM is strongly expressed in developing neurons and myoblasts (Fig. 3). These are highly polar cell types, suggesting that MIM may help promote and maintain cell polarity. Polarity is essential for the growth and motility of myoblasts and neurons, whereas the loss of polarity in epithelial cells may affect adhesion and precede increased motility, invasiveness, and metastatic capability. This may provide an explanation for the lack of MIM in metastatic bladder cancer cell lines (15). Alternatively, MIM may negatively regulate cytoskeletal dynamics by restricting actin polymerization only to certain regions of migrating cells and in cells that undergo polarized growth. This negative regulatory role is supported by our biochemical (Fig. 6) and cell biological studies, where overexpressing MIM disrupted stress fibers and resulted in formation of abnormal actin filament structures (Fig. 8). In the future it will be important to examine the localization of endogenous MIM protein in various cell types. Unfortunately, our attempts to generate antibodies against mouse MIM have so far been unsuccessful. We would also like to use small interfering RNA and traditional mouse knockout technology to examine the importance of MIM during development and in adult tissues.

Taken together, our data show that mouse MIM binds actin monomers and regulates actin filament assembly in specific polarized mammalian cell-types. Because MIM is a relatively large, multidomain protein, it may have other activities and regulate actin dynamics in a complex fashion. Other large WH2 domain proteins (WASP, Scar, WIP/verprolin) are involved in signaling to the actin cytoskeleton, and the activities of these proteins are regulated by relatively complex mechanisms (1). Therefore, MIM may also be a link between cellular signaling pathways and actin filament assembly. In this respect, it is important to note that MIM contains also several proline-rich motifs that may interact with Src homology 3 or WW domains of certain signaling proteins. In the future it will be important to identify the interaction partners of MIM and learn how they regulate the activity and localization of MIM in specialized mammalian cells.

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