The ERM protein family members ezrin, radixin, and moesin are cytoskeletal effector proteins linking actin to membrane-bound proteins at the cell surface. Here we report on the cloning of myosin regulatory light chain interacting protein (MIR), a protein with an ERM-homology domain and a carboxyl-terminal RING finger, that is expressed, among other tissues, in brain. MIR is distributed in cultured COS cells, in a punctated manner as shown using enhanced green fluorescent protein (EGFP)-tagged MIR and by staining with a specific antibody for MIR. In the yeast two-hybrid system and in transfected COS cells, MIR interacts with myosin regulatory light chain B, which in turn regulates the activity of the actomyosin complex. Overexpression of MIR cDNA in PC12 cells abrogated neurite outgrowth induced by nerve growth factor (NGF) without affecting TrkA signaling. The results show that MIR, a novel ERM-like protein, affects cytoskeleton interactions regulating cell motility, such as neurite outgrowth.

Dynamic changes in cell shape and movements involve interactions between proteins in the cytoskeleton and the plasma membrane. Ezrin, radixin, moesin, and the related tumor suppressor merlin link the actin cytoskeleton to membrane-bound proteins located at membrane extension sites, such as microvilli, membrane rufflings, and at cell-cell contacts (1–3). ERM1 proteins are also involved in cell adhesion, influencing the redistribution of various cell adhesion molecules, such as ICAM-1 (4, 5). The amino-terminal domain of ERM proteins, comprising about 300 amino acids, is conserved among the different proteins and merlin and exhibits a high degree of sequence similarity (1–3). This amino terminus is related to sequences, was amplified by PCR and used for screening a gt10 human fetal brain library (CLONTECH). Hybridization was carried out overnight at 42 °C in 50% formamide, 6× SSC, 5× Denhardt’s, and 0.5% SDS, followed by washing for 10 min in 2× SSC, 0.5% SDS, for 15 min in 0.2× SSC, 0.5% SDS, and finally for 15 min in 0.2× SSC, 0.5% SDS at 50 °C. A positive phage of 1650 base pairs was cloned into the Bluescript KS vector and sequenced using an automated DNA Sequencer (Applied Biosystems). The nucleotide sequence encodes a putative protein with an open reading frame of 445 amino acids and was named MIR. The MIR sequence has been submitted to the GenBank™ database.

Experimental Procedures

Cloning of MIR—A search in GenBank™ for expression sequence tag cDNAs (EST) of novel members of the inhibitory of apoptosis protein (IAP) gene family (15) resulted in an EST sequence from human lung (accession number T63512) with a RING finger similar to those found in IAP proteins. A 375-base pair probe, corresponding to the EST sequence, was amplified by PCR and used for screening a Agt10 human fetal brain library (CLONTECH). Hybridization was carried out overnight at 42 °C in 50% formamide, 6× SSC, 5× Denhardt’s, and 0.5% SDS, followed by washing for 10 min in 2× SSC, 0.5% SDS, for 15 min in 0.2× SSC, 0.5% SDS, and finally for 15 min in 0.2× SSC, 0.5% SDS at 50 °C. A positive phage of 1650 base pairs was cloned into the Bluescript KS vector and sequenced using an automated DNA Sequencer (Applied Biosystems). The nucleotide sequence encodes a putative protein with an open reading frame of 445 amino acids and was named MIR. The MIR sequence has been submitted to the GenBank™ database.

Northern Blot Analysis—Multiple tissue Northern blots (CLONTECH) were hybridized for 2 h at 37 °C using the Express hybridization solution (CLONTECH) and the MIR cDNA probe labeled with [32P]dCTP (Rediprime, Amersham Pharmacia Biotech). Filters were washed in 2× SSC, 0.05% SDS for 30 min and in 0.1× SSC, 0.1% SDS for 40 min at room temperature. The levels of MIR mRNA were analyzed using a PhosphorImager (Molecular Dynamics), and compared with those of β-actin mRNA.

Yeast Two-hybrid System—The full-length coding sequence of MIR was fused to the GAL4 DNA-binding domain (GALDB) in the pYTH6 bait vector and integrated into the genome of the yeast strain, Y190. The resulting Y190-pYTH6-MIR strain was transformed with an expression cDNA library from HeLa cells (CLONTECH), fused to the GAL4 activation domain (GAL4AD) in the pGAD-GH vector (16). 4×10^6 transformants were screened for activation of the His-3 reporter gene on a medium deficient in the amino acids His, Trp, and Leu and supplemented with 25 μg 3-aminotriazole. Colonies appearing within 3–5 days were subjected to a β-galactosidase filter lift assay (16), and the positive clones obtained were rescued into Escherichia coli and retransformed into the Y190-pYTH6-MIR strain to confirm the interaction.
action. The clones were sequenced, and two were identified as the myosin regulatory light chain B (MRLC). Deletions of MIR cDNA were subcloned into the pYTH6 vector using a polymerase chain reaction-based strategy. These constructs were sequenced and studied for the interaction with MRLC.

Cell Culture and Assay for Neurite Outgrowth—Rat pheochromocytoma PC12 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% horse serum and 5% fetal bovine serum. Cells were transfected with 10 μg of pcDNA3.1 (Invitrogen) expressing MIR cDNA by the calcium phosphate method (17), and stable clones were selected using G418 (600 μg/ml, Life Technologies). PC12 cells were transiently transfected with a control vector, encoding the enhanced green fluorescence protein (EGFP, CLONTECH), and anti-HA antibodies (Boehringer-Mannheim). The signals were visualized using the enhanced chemiluminescence method.

Immunoprecipitation and Western Blotting—Monkey kidney COS-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum. Cells were co-transfected with histidine_{6} (His)-tagged MRLC expression vector (Invitrogen) and with the hemagglutinin (HA)-tagged MIR, pJ3H vector. After 24 h, cells were lysed in ice-cold RIPA lysis buffer (phosphate-buffered saline, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonident P-40) supplemented with a protein inhibitor mixture (Boehringer-Mannheim) and 100 μM sodium orthovanadate (Sigma). The lysate was preincubated at 4 °C, and the antibody to MIR (amino acid sequence, QQTRVLQEKLRKLKEAMLC) was raised in rabbits using standard procedures. The purified antibody recognized recombinant MIR in Western blots (data not shown). Cells were fixed for 10 min using 4% paraformaldehyde, washed with phosphate-buffered saline, and blocked overnight with skim milk (18). The MIR antibody, diluted 1:100, was added and incubated overnight at 4 °C. After washing, a secondary biotinylated anti-rabbit antibody (diluted 1:200, Dako) was added and incubated for 2 h at room temperature, followed by addition of the avidin-biotin complex (Vector Laboratories). The signals were visualized using diaminobenzidine (Sigma) as a chromogen.

Immunocytochemistry and MIR Antibody—A specific antipeptide antibody to MIR (amino acid sequence, QQTRVLQEKLRKLKEAMLC) was raised in rabbits using standard procedures. The purified antibody to MIR (amino acid sequence, QQTRVLQEKLRKLKEAMLC) was used as an antibody to MIR. The antibody was affinity purified on a Sepharose-derivatized MIR column (Amersham Pharmacia Biotech) and incubated with anti-HA antibody (Boehringer-Mannheim). The antibody-protein complex formed was bound to protein A-Sepharose (Amersham Pharmacia Biotech, Uppsala) and washed with a mixture (Boehringer-Mannheim) and 100 μM sodium orthovanadate.

**Fig. 1.** Structure of human MIR and sequence comparison. **a,** the deduced amino acid sequence of human MIR. Numbers on the right indicate the amino acid residue position. **b,** sequence alignment of amino acids at positions 1–282 of MIR with ERM proteins and merlin using the GCG Pile-Up program. **Gray boxes**, identical residues; **black boxes,** similar residues to MIR.

**Fig. 2.** Tissue distribution of human MIR mRNA. Northern blot analysis was done using Multiple Tissue Northern blots (CLONTECH) as described under "Experimental Procedures." Expression of β-actin was used as a standard. **a,** fetal, **b,** adult tissues.
**Results**

**MIR Is a Novel ERM-homology Protein**—Using a human fetal brain cDNA library to screen for novel genes, we cloned a cDNA encoding a novel protein with an open reading frame of 445 amino acids, and two distinct protein homology domains: an amino-terminal ERM-homology domain, and a RING finger at the carboxyl-terminal end. The complete amino acid sequence of human MIR is depicted in Fig. 1, together with an alignment of the ERM-homology region of the ERM proteins and merlin. The amino-terminal region of MIR exhibits the highest degree of similarity to ezrin and merlin, 25 and 28% identity, respectively (Fig. 1b). The RING finger in MIR resembles that of other proteins (Fig. 1) involved in regulation of cell growth and apoptosis, including proteins of the IAP family such as XIAP and c-IAP2.

**MIR mRNA Is Expressed in Various Human Fetal and Adult Tissues**—Previous studies have shown that the ERM proteins are expressed in many different tissues. Fig. 2 shows that MIR mRNA, with a size of about 3 kilobases, is present in various human tissues, including fetal and adult brain. The most prominent expression was seen in placenta and fetal lung, with approximately equal levels of MIR mRNA in other tissues.

**Distribution of MIR in Cultured COS Cells**—COS-7 cells were transiently transfected with MIR cDNA expressing EGFP at the amino-terminal end for visualization. The EGFP-MIR fusion protein showed a punctated pattern of GFP fluorescence throughout the cytoplasm (Fig. 3). The distribution of fluorescence of EGFP-MIR in COS cells is similar to that observed previously for ERM proteins fused to GFP (5). To study the localization of endogenous MIR, we stained COS cells with an antibody specific for MIR, which gave rise to a pattern similar to that obtained with EGFP-MIR (Fig. 3c). These results demonstrate that MIR distributes in a distinct punctate pattern within COS-7 cells, indicative of an association with the cytoskeleton.

**MIR Interacts with MRLC**—To reveal the function of MIR, we screened a HeLa cell cDNA library using the yeast two-hybrid system and identified MRLC (14) as an interactor for MIR. A qualitative assay for β-galactosidase showed that MRLC exhibited a strong binding to MIR, whereas other proteins such as merlin or XIAP did not interact (Table I). To characterize the binding site of MIR, deletions were made and analyzed further within the yeast system. None of the deletion constructs interacted with MIR, indicating that the whole molecule is required for binding (Table I). To confirm the interaction of MIR with MRLC, we co-transfected COS-7 cells with expression vectors carrying the cDNAs with a recognition sequence tag. Using an anti-HA antibody, we were able to show co-immunoprecipitation of MRLC with MIR (Fig. 4a). This data confirms that an interaction between these proteins occurs also in mammalian cells.

**MIR Inhibits Neurite Outgrowth of PC12 Cells but Does Not Affect TrkA Phosphorylation**—MRLC is part of the protein complex controlling the driving force for myosin molecules which control the actomyosin complex (21). We were therefore interested in studying whether MIR affects cell motility. PC12 cells were treated with NGF (17), and whereas control PC12 cells exhibited a profuse outgrowth of neurites after 50 ng/ml NGF, MIR stably transfected cells did not (Fig. 4b). The inhibition of neurite outgrowth by MIR overexpression could not be overcome by higher NGF concentrations (data not shown).

To study whether the PC12 cells overexpressing MIR are defective in their response to NGF, we investigated the potential involvement of the NGF receptor, TrkA, using a phosphorylation assay (19). Fig. 4c shows that TrkA is tyrosine phosphorylated by NGF in both control and MIR overexpressing cells, excluding an alteration in receptor activation in these cells. Likewise, the immediate early gene, cFos was equally stimulated by NGF in both types of cells (data not shown). These results lend credence to the view that NGF acts normally...
in MIR overexpressing cells and that the absence of an appreciable degree of neurite outgrowth by NGF in MIR transfected cells occurs downstream of TrkA. To substantiate the results obtained using stable cell lines, PC12 cells were transiently transfected with the EGFP-MIR expression vector. The results showed that overexpression of MIR significantly inhibited neurite outgrowth stimulated by NGF (Fig. 4d). Quantification of the data showed that MIR transfected cells had significantly less neurites compared with control PC12 cells (Fig. 4e).

**DISCUSSION**

In this study we have cloned MIR, a novel member of the ERM protein family which is expressed in a variety of different human tissues. In addition to the amino-terminal ERM homology domain, MIR has a carboxyl-terminal RING finger similar to other proteins involved in regulating growth and survival of cells (20). The ERM and the RING finger domains constitute structural motifs which probably mediate important protein-protein interactions. Using the yeast two-hybrid system, we identified MRLC as a protein interacting with MIR, suggesting a possible role of MIR in the actomyosin complex.

ERM proteins associate with proteins both in the plasma membrane and in the cytoskeleton, taking part in the co-ordination of cell motility. According to the present results, an essential function for MIR in transfected PC12 is to influence neurite outgrowth, probably by influencing cytoskeletal components involved in cell motility. MIR interacts with MRLC, which regulates the activity of the actomyosin complex in muscle as well as in nonmuscle cells (21). Two isoforms of MRLC are present in brain tissue and in cultured neuroblasts (22). Evidence has recently been provided for the involvement of myosin in neurite outgrowth (23). Neuronal cells express different forms of myosin including conventional myosin II, present at the leading edge of the growth cones (24). Mouse neuroblastoma cells express both myosin IIA and myosin IIB isoforms, but only the latter seems to be involved in neurite extension (24). In view of the present finding, it is reasonable to assume that MIR, via its interaction with MRLC, can inhibit the driving force of myosin, which controls the actomyosin complex. Previous studies have shown an effect of radixin and moesin on growth cone motility and process formation in neurons (11, 12). Radixin was shown to redistribute to the growth cones following NGF stimulation (11). These results suggest an important function for different ERM-like proteins in the control of neurite outgrowth, which may involve an interaction with MIR.
The activity of actomyosin complex is known to be regulated by the phosphorylation state of MRLC, which is influenced by the myosin light chain kinase and by the corresponding phosphatase (25). Increased MRLC phosphorylation leads to enhanced cell contractility and to increased calcium sensitivity in smooth muscle cells (26). A similar regulation of myosin activity probably occurs in neurons. Recent evidence suggests a critical role for Rho, belonging to the small GTPase Rho family of proteins (27) and for its associated Rho p160 kinase (ROCK) in mediating MRLC phosphorylation (26). It was recently shown that agonist-induced or enhanced Rho/ROCK activity is sufficient for mediating neurite retraction and cell rounding of neuroblastoma cells in culture (28). It was suggested that Rho/ROCK activity tonically inhibits cell process extensions via activation of actomyosin contractility and suppression of the assembly of intermediate filaments and microtubules (28).

Previous studies have suggested a role for ERM proteins in the activation cascade induced by Rho (5) via a conserved threonine in the carboxyl-terminal region of ERM proteins, which is phosphorylated by ROCK (29). On the other hand, ERM proteins are thought to contribute to the activation of Rho by sequestering the Rho GDP dissociation inhibitor, which in turn binds the inactive form of Rho (30).

In view of these mutual interactions between the Rho family members and ERM proteins, it is possible that the function of MIR is linked to alterations in Rho activity. Indeed, microinjection of active ROCK kinase in PC12 cells leads to neurite retraction (31). This is analogous to the effect observed for MIR in transfected PC12 cells, and suggests that MIR functions in mediating MRLC phosphorylation thereby hindering cytoskeleton interactions promoting neurite outgrowth.

MIR may have additional functions in cells, acting as a stabilizing factor for the cytoskeleton by cross-linking various protein components important for the attachment/detachment reactions required for cell motility. In keeping with such a notion, we have recently obtained evidence for the interaction of MIR with other regulatory proteins affecting the cytoskeleton. The nature of these proteins and the role of MIR in regulating cell motility in other cell types than neurons are currently under investigation.

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