MIR
a Novel ERM-like Protein in the Nervous System

BY
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ABSTRACT


Proteins of the band 4.1 superfamily are characterized by their sequence similarity to the ERM proteins ezrin, radixin and moesin, which are involved in cell motility, adhesion of cells, and signal transduction events. Little is however known of the function of ERM proteins in the nervous system, though an essential role for radixin and moesin in neuronal growth cone motility has been suggested.

This thesis is focused on the cloning, functional characterization and description of the tissue distribution in rat brain of MIR, a novel member of the band 4.1 superfamily. The cDNA of MIR encodes a protein of 445 amino acids which is composed of an ERM-homology domain and a RING finger, separated by an interregion. To reveal the cellular function of MIR, PC12 cell lines overexpressing MIR was generated and observed to inhibit NGF stimulated neurite outgrowth.

To elucidate the signal transduction of MIR by which it exerts its physiological activity, the yeast two-hybrid system was employed to screen for proteins that interact with MIR. A number of interactors known to regulate the cytoskeleton was obtained – among them myosin regulatory light chain-B which controls the actomyosin complex – and a novel type 2 membrane protein denoted NSAP for its similarity to saposin A-D. Overexpressed NSAP induced neurite outgrowth in PC12 cells and enhanced cell adhesion in fibroblasts.

The tissue distribution of MIR in rat brain, as determined by immunohistochemistry studies, showed that MIR is localized especially to neurons in hippocampus and cerebellum. The chromosomal localization of the MIR gene was assessed to 6p22.3-23, a region lost in the 6p23 deletion syndrome.

These results suggest that MIR is expressed in neurons in discrete regions of rat brain where it may regulate neurite outgrowth by modulating the cytoskeleton.

Key words: MIR, ERM, band 4.1 superfamily, MRLC, NSAP, neurite outgrowth, nervous system.

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The only mistake that can be done is never to try

Cindy Crawford
This theses is based on the following papers referred to in the text by their roman numerals:


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**ABBREVIATIONS**

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<th>Description</th>
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<tr>
<td>AKAP</td>
<td>A-kinase anchoring protein</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>NHE</td>
<td>Na-H exchanger</td>
</tr>
<tr>
<td>NHE-RF</td>
<td>Na-H exchanger releasing factor</td>
</tr>
<tr>
<td>PC12</td>
<td>Phaeochromocytoma 12</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CDS</td>
<td>Coding sequence</td>
</tr>
<tr>
<td>DAL-1</td>
<td>Differentially expressed in adenocarcinoma, 4.1B</td>
</tr>
<tr>
<td>E3KARP</td>
<td>NHE3 kinase-A regulatory protein</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescence protein</td>
</tr>
<tr>
<td>ERM</td>
<td>Ezrin, radixin and moesin</td>
</tr>
<tr>
<td>FERM</td>
<td>Four point one, ezrin, radixin and moesin</td>
</tr>
<tr>
<td>GDI</td>
<td>GDP dissociation factor</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanine diphosphate</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>Merlin</td>
<td>Moesin, ezrin, radixin-like protein</td>
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<tr>
<td>MIR</td>
<td>Myosin regulatory light chain-B interacting protein</td>
</tr>
<tr>
<td>MRLC-B</td>
<td>Myosin regulatory light chain-B</td>
</tr>
<tr>
<td>NF2</td>
<td>Neurofibromatosis 2</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NSAP</td>
<td>Neuronal saposin-like protein</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>RING</td>
<td>Really interesting new gene</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho associated kinase</td>
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INTRODUCTION

**Band 4.1 superfamily**

Members of the band 4.1 superfamily are homologous to the N-terminal part of the prototype band 4.1 or protein 4.1, which was originally isolated as a protein essential for the maintenance of shape and integrity of red blood cells (Leto, T.L. and Marchesi, V.T., 1984). The cytoskeleton linker-proteins ezrin, radixin and moesin (ERM) were subsequently found to contain a similar N-terminal domain, now collectively called the FERM domain (F for 4.1, E for ezrin, R for radixin and M for moesin) (Chisti, A.H. et al. 1998), or ERM homology domain. The relatively large FERM domain of about 300 amino acids contains several structural blocks enabling each FERM domain to interact with several different proteins. Binding of FERM domains to the cytoplasmic tail of transmembrane proteins allow many FERM domains to act in membrane targeting of cytoplasmic proteins (Girault, J-A. 1998). The FERM domains also interacts with a wide variety of cytoplasmic proteins and in a few cases with lipids (Tsukita, S., 1997; Bretscher, A. 1999). Members of this superfamily are usually localised beneath the plasma membrane at cell surface extension areas such as membrane rufflings, filopodias, lamellipodias and neurite extensions - participating in the regulation of these cell motility events by modulating cytoskeleton dynamics. The FERM domain proteins are also found at cell-cell and cell-matrix contact points, where they influence signaling pathways for cell growth and differentiation (Louvet-Vallée, S. 2000). The pleiotropic functions of members of the superfamily FERM domain proteins – from band 4.1 to the most recently identified distant members Janus activated kinases (JAKs) and Focal adhesion kinase (FAK) is just beginning to emerge.

The FERM domain is found in the N-terminal part of most proteins in the superfamily, including DAL-1, the ERMs and their closest relative neurofibromatosis 2/merlin; a group of cytoplasmic protein tyrosine phosphatases (PTPs), except PTP-BAS where it is centrally located; the JAKs and FAK; Rho GNEF, a guanine nucleotide exchange factor for the small GTPases Rho; in talin, a component of focal adhesions; in NBL4 and in open reading frame KIAA 0338. The FERM domain is also present in the unconventional myosin VIIa and myosin X, but is located in the C-terminal of these proteins (Fig.1). The FERM domain forms a clover-shaped globular structure according to the crystal structure analysis of moesin and radixin (Pearson, M.A. et al., 2000; Hamada, K., et al., 2000) and it is composed by three modules, F1-F3. The structure of module F1 is shared by ubiquitin, F2 resembles that of acyl-CoA binding protein and F3 is similar to ligand binding modules of phosphotyrosine binding, pleckstrin homology and Enabled/VASP Homology 1 domains which are implicated in signalling events mediated by protein-protein interactions.
Band 4.1 and related proteins

The erythrocyte protein 4.1R is crucial for the mechanical strength and plasticity of the red cell membrane - as has been revealed by studies in band 4.1R deficient patients suffering from congenital hemolytic anemias (Delaunay, J., 1995). This function of band 4.1R is performed by linking the skeletal network to the overlying cell membrane by anchoring spectrin/actin, through a C-terminal spectrin binding site in protein 4.1R, to integral membrane proteins - mainly glycophorin C (Schischmanoff, P.O., et al., 1995). The FERM domain of band 4.1R is responsible for the interaction with glycophorin C and this domain is also capable of binding the cytoplasmic tail of other transmembrane proteins, such as the erythrocyte anion exchanger band 3, CD44 and disc large (Marfatia, S.M., et al., 1995; Lombardo, C.R., et al., 1992; Nunomura, W., et al. 1997). The formation of these ternary complexes between spectrin/actin, protein 4.1R and transmembrane proteins might be regulated since the affinity of glycophorin C to band 4.1R is enhanced by phosphatidylinositol 4,5-bisphosphate (PIP2), in contrast to the reduced interaction of CD44 to band 4.1R by Ca$^{2+}$/calmodulin (Anderson, R.A and Marchesi, V.T., 1985; Nunomura, W., 1997). The function of protein 4.1R in nonerythroid cells is largely unknown, but transgenic mice with insertion of a lacZ transcriptional reporter into the band 4.1R locus revealed lacZ reporter activity in select cells of major organs and unexpectedly in specific neurons of the brain: the granular cells of the cerebellum and in the dentate gyrus within the hippocampus (Shi, Z-T., et al. 1999). In accordance with this expression pattern in the brain, transgenic band 4.1R-null mice exhibit impaired balance and coordination consistent with cerebellar dysfunction and deficits in spatial learning and memory, that might involve the hippocampus (Walensky, L.D., et al., 1998). A binding partner of protein 4.1R has been identified in neurons – the cytoplasmic tail of the transmembrane protein paranodin, which is related to the neurexin family and may be an important component for the tight interactions between axons and myelinating glial cells, typical of the paranodal region (Menegoz, M., et al., 1997). Paranodin could anchor cytoskeleton-associated proteins to the axonal membrane via band 4.1 at paranodal regions. In Drosophila, the FERM domain of 4.1 related coracle interact with neurexin IV (Ward IV, R.E., et al., 1998). This site of the axon is involved in several types of neuropathies (Maxwell, W.L., et al., 1991; Griffin, J.W., et al., 1996), but the relevance of protein 4.1 in these paranodal abnormalities has not been studies. Nor is it known if alterations of the paranodal regions is responsible for the behavioral dysfunctions observed in the transgenic band 4.1R-null mice described above (Shi, Z-T., et al., 1999; Walensky, L.D., et al., 1998).
**Figure 1.** Domain organization of the superfamily of 4.1 proteins. Members of the 4.1 superfamily are characterized by a ~300 amino acid segment similar to the conserved globular N-terminal region of protein 4.1, ezrin, radixin and moesin - the FERM domain, or ERM homology domain. Isoforms of 4.1R (red blood cell) have recently been cloned: 4.1G (general), 4.1N (neuronal) and the putative timor suppressor DAL-1 (differentially expressed in adenocarcinoma of the lung, or 4.1B). Merlin, the neurofibromatosis 2 protein, is a second tumor suppressor of the superfamily, but most closely related to the ERM proteins. The protein tyrosine phosphatases PTP’s, may contain PSD95-Dlg-ZO1-homology (PDZ) domains. CDEP is a novel member with a Dbl homology and a pleckstrin homology (PH) domain, which are found in the Rho guanine nucleotide exchange factors. Myosin VIIa and X differ in their centrally and C-terminally localized FERM domains. The RING finger domain in the C-terminal of MIR is unique among the 4.1 proteins.

Two novel neuronal homologues to band 4.1R have recently been discovered, 4.1 N and 4.1B (Peters, L., et al., 1998). mRNA expression of both of these homologues are strong in the brain – 4.1N is found in all neurons of the brain except Purkinje cells and most thalamic neurons, whereas 4.1B mRNA is localized in neurons that lack 4.1N. (Parra, M., et al., 2000). Protein 4.1N is enriched at synaptic contacts and colocalize with postsynaptic density protein of 95 kDa and the excitatory glutamate receptor type 1 (Walensky, L.D., et al., 1999). The functional importance of 4.1N and 4.1B in neurons are not known. A third new 4.1 homologue, protein 4.1G, which is widely expressed among human tissues including the brain (Parra, M., et al., 1998). 4.1 G was found to interact with the putative endoplasmatic reticulum chaperon FK506 binding protein FKBP13, which has high affinity for the immunosuppressive drug FK506 (Walensky, L.D., et al., 1998).

The erythroid and nonerythroid protein 4.1 homologues present a variety of different isoforms ranging in size from 30 kDa to 210 kDa mainly due to complex alternative splicing, but also by the use of two different initiation codons (4.1R)

**The ERM proteins**

Ezrin, radixin and moesin are effector proteins in the formation of actin dependent cell membrane protusions such as microvilli, filopodia and lamellipodia, which are important for exploration and migration of cells. The specialized cell membrane extentions of axon-like neurites, driven by growth cone motility, has in addition recently been shown to be dependent of the expression of radixin and moesin in primary cultured neurons (Paglini, G. et al., 1998; Castelo, L., and Jay, D.G., 1999). The ERM proteins are thought to mediate these cell motility events by anchoring F-actin to the cytoplasmic membrane, through the conserved C-terminal actin binding
motif (Turunen, O., et al., 1994) and the N-terminal ERM homology domain that is capable of interacting with different transmembrane proteins (Bretscher, A. 1999). Furthermore, cell adhesion, transformation of cells and malignancy, as well as signal transduction events may be mediated by the ERM proteins (Louvet-Vallée, S. 2000).

**Cell adhesion of ERM proteins**

Immunofluorescence studies of cultured epithelial and fibroblast cells have demonstrated that ERM proteins are enriched at cell-cell and cell-matrix adhesion sites (Sato, N., et al., 1992; Takeuchi, K., et al., 1994). Since the ERM proteins are not concentrated to established cell adhesion sites, it has been suggested that they play a role in the initial formation of these contacts (Sato, N., et al., 1992). Application of antisense technology to inhibit expression of all ERM proteins in mouse epithelial cells resulted in decreased cell-cell and cell-substrate adhesions in this cell line, providing evidence for the involvement of ERM proteins in cell adhesion events (Takeuchi, K., et al., 1994). This is further supported by the observation that antisense oligonucleotides to ezrin introduced into human colorectal epithelial tumor cell lines displayed a reduced cell-cell adhesiveness together with an increased motility and invasiveness. In contrast, cell-matrix adhesion was increased by antisense treatment to ezrin of these tumor cells (Hiscox, S., and Jiang, W.G., 1999). The molecular mechanisms involved in the effects of ERM proteins on cell-cell adhesion remains to be elucidated, but several cell type specific cell-cell adhesion receptors have been shown to interact or associate with the ERM proteins. Intracellular adhesion molecules (ICAM)-1 and -2, expressed on lymphocytes, binds to ezrin (Heiska, L., et al., 1998), whereas immunoprecipitates of ICAM-3 contain moesin, but not ezrin or radixin in T cell protrusions called uropods (Serrador, J.M., et al., 1997). The complement binding protein CD46 associates with moesin (Schneider-Schaulies, J., 1995) and the adhesion molecule CD43 may also bind to the ERM proteins (Yonemura, S., et al., 1993). Moreover, the ubiquitously expressed CD44 hyaluronan receptor that mediates cell-cell and cell-matrix adhesions binds directly to the ERM proteins in a variety of cell types (Tsukita, S., et al., 1994). The ERM proteins link CD44 to the cytoskeleton via an interaction of the N-terminal region to the cytoplasmic tail of CD44 and a C-terminal binding to F-actin. The functions of ERM proteins, in interacting with cell adhesion receptors, may be to recruit the cell adhesion molecules to specific cell membrane extension regions, where they become anchored to the cytoskeleton via the ERM-link. In accordance with this model, overexpressed ezrin targets ICAM-2 to uropods of thymoma cells that become sensitive to natural killer cell lysis (Helander, T.S., et al., 1996).

The mechanisms mediating the effects of ERM proteins on cell attachment to substrates are presently less well defined. However, the ERM proteins have been implicated in the signalling pathway of the small GTPase Rho that leads to the formation of focal adhesions, in a study of permeabilized fibroblasts (Mackay, D.J., et al., 1997). Focal adhesions are specialized contact sites
that cultured cells develop to adhere to the extracellular matrix via integrin receptor clustering and their assembly involves Rho stimulated contractility, and phosphatidylinositol-4,5-bisphosphate (PIP2) activation (Sechi, A.S. and Wehland, J. 2000), which is thought to activate the ERM proteins by a conformational alteration (Hirao, M., et al., 1996). The relation between Rho and the ERM proteins is bidirectional since the ERM proteins is capable of activating Rho by displacing the Rho inhibitor Rho-GDI (Takahashi, K., et al., 1997). These findings suggest that ERM proteins may modulate cell-matrix adhesion by effecting the signal transduction of Rho-driven formation of focal adhesions, which are associated with filopodia and lamellipodia (Nobes, C.D. and Hall, A., 1995). The evidence for this function of the ERM proteins was further supported by the discovery that ERM interacts directly with hamartin, a tumor suppressor implicated in cell-matrix adhesion upstream of the small GTPase Rho, to promote focal adhesions and stress fibers in Swiss 3T3 fibroblasts (Lamb, R.F., et al., 2000).

Regulation of ERM proteins

The morphogenic effects of the ERM proteins to induce cell surface protrusions and their ability to interact with some binding partners as well as the cytoskeleton, are tightly regulated by the phosphorylation status of ERM proteins and by PIP2. The ERM members are phosphoproteins as revealed by numerous studies demonstrating that phosphorylation of serine/threonine or tyrosine residues occur in response to different stimuli in many cell types. The phosphorylation event preceds relocation of ERM proteins from the cytoplasm to the cell membrane and leads to enhanced binding to actin fibers. Activation of RhoA in NIH3T3 fibroblasts relocalize ERM proteins to apical microvilli-like structures and for radixin, this translocation was preceded by phosphorylation of its C-terminal proposed to be mediated by a Rho dependent kinase, such as Rho kinase (ROCK), protein kinase N or PRK2 (Shaw, R.J., et al., 1998). Indeed, Thr568 of radixin is phosphorylated in Swiss 3T3 cells in a Rho dependent manner by ROCK, as are the corresponding threonine residues in ezrin (Thr567) and moesin (Thr558) without effecting actin binding (Matsui, T., et al., 1998). The same C-terminal threonine residue (Thr558) in moesin is phosphorylated during platelet activation and the protein kinase C Θ is able to perform this reaction in vitro (Berryman, M., et al., 1995). The phosphorylated moesin co-localize with actin filaments in extremely long filopodia formed in response to activation of the platelets. Tyrosine phosphorylation of transfected ezrin has been detected in hepatocyte growth factor (HGF) stimulated LLC-PK1 kidney-derived epithelial cells (Crepaldi, T., et al., 1997). The HGF induced cell motility observed in these cells was suppressed by site directed mutagenesis of the tyrosine residues 145 and 353 to phenylalanine. These two tyrosine residues are conserved among the ERM proteins and phosphorylated in ezrin as a consequence of stimulation of the EGF receptor kinase, that is followed by the translocation of ezrin to microvilli in A431 carcinoma cells.
In activated T lymphocytes, ezrin is phosphorylated on a tyrosine residue by the tyrosine kinase p56lck (Thuillier, L., et al., 1994). Interestingly, C-terminal Thr568 phosphorylation of radixin in vitro suppressed its capacity to interact with the N-terminal FERM domain in head-to-tail associations (Matsui, T., et al., 1998). Based on the observation that ERM proteins are retained in an inactive closed conformation in the cytoplasm, by C- to N-terminal interactions, a model has been presented for the activation of ERM proteins by phosphorylation induced conformational change, that would open up the molecules and expose masked binding sites for interacting proteins (Matsui, T., et al., 1998; Mangeat, P et al., 1999). In support of this model, C-terminal threonine phosphorylation of ezrin and moesin by PKCΘ, unmask the dormant binding sites to both ERM-binding phosphoprotein 50 (EBP50) and F-actin (Simons, P.S., et al., 1998). However, the ERM proteins form oligomers at the plasma membrane by engaging in C- to N-terminal self-associations (Berryman, M., et al., 1995) which would require dephosphorylation of the conserved C-terminal threonine. In fact, dephosphorylation of the ERM members is expected to be equally important in regulation of the activity of ERM proteins, as phosphorylation is. The phosphatases involved have not been identified, though one candidate is myosin light chain phosphatase which interact with moesin through its myosin binding subunit (Fukata, Y., et al., 1997). Furthermore, inactivation of ERM proteins may occur by calpain mediated proteolysis as indicated by increased levels of actin associated ezrin, but not moesin and radixin, due to calpain inhibition in NIH 3T3 cells (Potter, D.A., et al., 1998).

The membrane localized phosphatidylinositol-4,5-bisphosphate (PIP2) has been implicated in the regulation of ERM proteins activated by Rho and also to enhance the interaction of ERM to CD44 (Hirao, M., et al., 1996), ICAM-1 and ICAM-2 (Heiska, L., et al., 1998). In the paper of Hirao et al, activation of Rho induced the binding of ERM proteins to CD44 and this was suggested to be mediated by PIP2, which binds the FERM domain of ERM proteins (Niggli, V., et al., 1995), since the presence of PIP2 increased the ERM-CD44 interaction in an in vitro binding assay (Hirao, M., et al., 1996). In contradiction with these results, a truncated CD44 with the juxtamembrane cluster of positively charged amino acids retains the ability of binding the ERM proteins in vitro, even in the absence of PIP2 (Yonemura, S., et al., 1998) and PIP2 containing liposomes did not disassociate N- to C-terminal dimers of ezrin formed in vitro (Niggli, V., et al., 1995). However, PIP2 is synthesized by the Rho effector enzyme phosphatidylinositol 4-phosphate 5 kinase (PI4P5K) (Ren, X.D. and Schwartz, M.A., 1998) and in a recent in vivo study with mutants of PI4P5K it was demonstrate that Rho activated PI4P5K is essential for recruitment of ezrin to the cell membrane, where it induces the formation microvilli protusions in NIH3T3 cells (Matsui, T et al., 1999). These results show that PIP2 is involved in Rho induced activation of ERM proteins, though the role played by PIP2 in the binding of ERM to CD44 is still controversial.
Interactors of ERM proteins

The identification of binding partners of the ERM members by application of the two-hybrid system (Bhatur, S.G. and Goldenring, J.R., 1998) and by other methods has been hampered by their ability to form intra- and intermolecular selfassociations. Most of the interactors bind to the functional FERM domain in the N-terminal region of ERM and show redundant interactions with all members of the ERM family, including the closely related tumor suppressor merlin. The binding sites for many interactors are believed to be masked by selfassociation of ERM molecules and have to be activated by Rho induced phosphorylation or PIP2 synthesis, for the interaction to occur. The N-terminal of activated ezrin binds to EBP50, NHE3 kinase A regulatory protein (E3KARP) and Rho-GDI, whereas the activated C-terminal interacts with F-actin. The N-terminal of the dormant form of ezrin binds to CD44, ICAM-1,2 and MBS, the α-helical region downstream of the FERM domain interacts with the RII subunit of protein kinase A, whereas the binding domain of ezrin to CD43, ICAM-3 and GDP/GTP exchange factor (Dbl) are unknown.

EBP50 and E3KARP are PDZ containing proteins that mediate cAMP dependent inhibition of the transmembrane Na+/H+ exchanger 3 (NHE3) (Reczek, D., et al., 1997; Yun, C-H., et al., 1998) and EBP50 interacts via one of its PDZ domain with the cystic fibrosis transmembrane conductance regulator (Short, D.B., et al., 1998) and the β2 adrenergic receptor (Hall, R.A., et al., 1998). These three transmembrane proteins are regulated by protein kinase A (PKA) and it has been proposed that ezrin may participate in the regulation of their activity by recruiting PKA, via its PKA binding site, to EBP50 and E3KARP at microvilli extensions (Bretscher, A., 1999). ERM proteins may also modulate the activity of these transmembrane proteins by anchoring them to the cytoskeleton at the apical surface of the cell and thereby inhibiting their downregulation by endocytosis (Ibid).

The Rho dissociation inhibitor (GDI) inhibits the Rho family of small G proteins by locking them in the inactive GDP-bound form (Sasaki, T. and Takai, Y., 1998). This inactivation is counteracted by the binding of ERM proteins to Rho GDI which sensitizes Rho to be activated by Rho GDP/GTP exchange factors (Takahashi, K., et al., 1997). Rho is a key initiator of stress fibers and focal adhesions in fibroblasts mediated by Rho dependent activation of ERM proteins (Mackay, D.J., et al., 1997) and the ERM-Rho GDI interaction would therefore present a possible autoregulatory amplification pathway in this activation of ERM by Rho.

The most well known interactor to ERM proteins is the cytoskeleton component filamentous actin, which binds to a conserved F-actin binding site in the C-terminal 34 amino acids of the ERM proteins (Turunen, O., et al., 1994). The C-terminal F-actin binding site enables the ERM molecules to act as membrane-to-cytoskeleton linkers by crosslinking actin to transmembrane proteins through their N-terminal FERM domain. An additional F-actin binding site has been
suggested to reside in amino acid residues 279-332 (Roy, C., et al., 1997), but this remains to be unequivocally confirmed.

The integral membrane proteins CD43, CD44, ICAM-1, -2 and-3 are cell adhesion receptors and their interactions with ERM proteins, in addition to that of hamartin, are described in the subsection Cell adhesion of ERM proteins.

An interaction between ERM proteins and the type II protein A kinase regulatory subunit (R\(_\text{II}\)) was detected in parietal cells by affinity chromatography and in blot overlays and the binding site was mapped in ezrin to the \(\alpha\)-helical region between amino acids 373 and 439 (Dransfield, D.T., et al., 1997). Binding to the regulatory subunit R\(_\text{II}\) of cAMP-dependent PKA characterize a group of proteins referred to as A-kinase anchoring proteins (AKAPs), which target the kinase to specific intracellular localizations where it may phosphorylate its substrates (Colledge, M, and Scott, J.D., 1999). The importance of the ERM proteins in localizing PKA, in the capacity as AKAPs, to dynamic cytoskeletal structures has not yet been explored. Recently, important results were presented which implicates ezrin in the activation of the regulatory subunit p85 of phosphatidylinositol 3-kinase (PI 3-kinase), by directly binding to p85 in a kidney-derived epithelial cell line (Gautreau, A. et al. 1999). This results suggested that ezrin may participate in the survival signalling of PI 3-kinase in respons to growth factor stimulation and cell-matrix adhesion.

The results described above have been obtained predominantly by studies of ERM proteins in cultured fibroblasts and epithelial cells with limited knowledge gained of their function in neuronal cells. An essential role in neuronal growth cone morphology and motility has however been suggested for radixin and moesin, in contrast to ezrin, by selective inactivation of these two proteins by microscale chromophore-assisted laser inactivation in cultured chick dorsal root ganglion and by antisense experiments in rat hippocampal pyramidal cells. Ablation of radixin and moesin proteins in these cell types inhibited neurite outgrowth and lead to a reduction of growth cone size and advance rate, effected by retraction of growth cone lamellipodial veils and disorganization of actin filaments (Paglini, G. et al., 1998; Castelo, L. and Jay, D.G. 1999). Since alteration of cytoskeleton structures of motile regions at the cell surface by ERM is well documented in fibroblasts and other cell types, these results indicates that the function of ERM proteins may be similar in nonneuronal and neuronal cells. The distribution of individual ERM proteins in neuronal tissue responsible for these activites has not been thoroughly studied, but the available data indicate that they are partially overlapping. Radixin and moesin expression has been detected in developing rat cerebral cortex, hippocampus and some regions (Paglini, G. et al., 1998), whereas immunoreactivity to ezrin has only been found in neuronal cell bodies of chicken spinal cord, dorsal root ganglion and the optic tectum (Takahashi, M. et al, 1999; Everett, A. and Nichol, K.A., 1990). Immunohistochemical or in situ studies of mRNA expression of radixin and ezrin in brain has however not been performed in any species.
Differentiating neurons extend neurites during their development in response to stimulation by growth factors such as NGF. The neurite is organized at the distal end into a bulbous highly dynamic organelle called the growth cone, which regulate the rate and direction of the growing neurite in response to extracellular cues such as contacts with adhesive substratum and other cells and by diffusable chemoattractants and repellants, e.g., netrins and collapsin, respectively. The leading edge of the growth cone extend filopodia which are stabilized in the direction of guidance molecules by focal adhesions (Varnum-Finney, B. and Reichardt, L.F., 1994) and by lamellipodia that grow between the filopodia. The body of the growth cone will subsequently move by enlargement of the proximal end of lamellipodia (Mackay, D.J., et al., 1995). The cell membrane protrusions of filopodia and lamellipodia are actin rich structures induced by activation of the small GTPases Cdc42 and Rac, respectively, and their involvement in neuritogenesis have recently been demonstrated by a number of studies. Inactivating mutants of Rac and Cdc42 in Drosophila display severe defects in axon outgrowth (Luo, L., et al., 1994) and neurite extension induced by NGF in PC12 cells is inhibited by dominant negative Rac and Cdc42.

**Merlin**

The proteins of the superfamily 4.1 are localized by their ERM homology domain to submembrane structures at cell-cell and cell-matrix contact points and thus ideally positioned to interfere with growth regulatory signal events. Two members of the 4.1 family have so far been shown to inhibit cell growth by acting as tumor suppressors: the neurofibromatosis 2 (NF2) gene product merlin or schwannomin and DAL-1, or protein 4.1 (Tran, Y.K., et al., 1999). The neurofibromatosis 2 gene belongs to a group of less then fifteen tumor suppressors defined as genes that sustain loss-of-function mutations in the development of cancers (Haber, D. and Harlow, E., 1997). The NF2 gene is mutated in the germ line of patients with neurofibromatosis type 2 (Trofatter, J., et al., 1993) - an autosomal inherited disease characterized by bilateral vestibular schwannomas and meningiomas, but also by spinal schwannomas and less frequently other tumors of neuroectodermal origin. Somatic loss-of-function mutations in the NF2 gene is also a major cause of sporadic schwann cell tumors and meningiomas (Merel, P., et al., 1995). The slowly growing benign central nervous system tumors of NF2, affecting 1 in 40,000 individuals, typically cause loss of hearing and balance due to compression of schwannomas of the acoustic nerve (Eldridge, R., et al., 1981). The NF2 gene transcript is alternatively spliced in the hydrophilic C-terminal to produce two major isoforms of merlin, differing in the selective inclusion of exon 16. Isoform 1 lacks the exon 16 insertion and codes for 595 amino acids in humans, whereas isoform 2 contains the exon 16 insertion and produces a protein of 590 amino...
acids due to a premature stop codon in exon 16 that prevents translation of exon 17 (Haase, V., et al., 1994; Bianchi, A., et al., 1994)

The high sequence similarity of the FERM domain of merlin and the ERM proteins (about 63% identity) predict a predominant localization of merlin to the plasma membrane. This has indeed been confirmed by the application of immunofluorescence techniques for endogenous and overexpressed merlin in a number of cells and cell lines using a variety of different antibodies to merlin. The endogenous 66 kD merlin protein is mainly localized to membrane ruffles in human primary meningioma cells and adult fibroblasts where it co-localize with F-actin, but its distribution is nevertheless distinct from that of ezrin or moesin which are found at filopodia and microvilli, respectively (Gonzalez-Agosti, C., et al., 1996). A similar enrichment of endogenous merlin at ruffling membranes and leading edges of cells was detected in the rat Schwann cell line SCL4.1/F7 (Claudio, J.O., et al., 1997) and in the human schwannoma cell line STS88-14 (Scherer, S.S. and Gutmann, D.H., 1996), where it co-localized with F-actin. Transfected merlin was also concentrated at membrane ruffles in NIH3T3 and HeLa cells (Shaw, R.J., et al., 1998) and furthermore in COS cells (den Bakker, M.A., et al., 1995). In cultured epithelial MTD-1A cells however, transiently expressed, as well as endogenous merlin was targeted to lateral membranes and co-localized at these sites with E-cadherin (Maeda, M., et al., 1999). The localization of merlin to the plasma membrane is dependent on the N-terminal FERM domain, as has been revealed by studies of overexpressed deletion mutants in cell lines. Naturally occurring exon deletions of the FERM domain isolated from patients, e.g. ∆E2, ∆E3, ∆E2-E3, ∆E6, ∆E7, or deletion of the entire FERM domain, all dislocate to the cytoplasm and perinuclear cytoplasm, whereas the intact FERM domain sustain a plasma membrane location (Koga, H., et al., 1998; Deguen, B., et al., 1998). These mutants were not associated with organelles, endocytic compartments or the nuclear membrane (Deguen, B., et al., 1998).

Functional studies of merlin

Transgenic NF2 mice have been generated in an effort to model the human neurofibromatosis 2 disease. McClatchey et al. disrupted the mouse NF2 gene in embryonic stem cells, but the NF2+/− embryos failed to gastrulate and exhibited collapsed extraembryonic tissue around embryonic day seven (McClatchey, A.I., et al., 1997). The heterozygously mutated NF2 mice developed normally and displayed an unexpected variety of aggressive highly metastatic osteosarcomas, fibrosarcomas and hepatocellular carcinomas, but Schwann cell derived tumors were not observed (McClatchey, A.I., et al., 1998). However, transgenic mice were engineered to overexpress human merlin with an exon 2-3 deletion or a C-terminal truncation, controlled by a Schwann cell specific myelin promoter (Giovannini, M., et al., 1999). Mice expressing Sch-∆(E2-3) show a high prevalence of schwannomas and may accordingly serve as an animal model for the human NF2 disease, whereas
transgenic expression of C-terminally truncated merlin did not effect tumorigenesis. The schwannomas were induced by Sch-Δ(E2-3) expression in heterozygous mice with one wild type NF2 allele, which argues for a dominant function of the mutant protein. Interestingly, the results obtained by Giovannini et al. (1999) suggest a dominant gain of function by Sch-Δ(E2-3, not a loss of function, which would have implicated exon 2-3 of the FERM domain in the tumor suppressive effect of merlin. A loss of function mechanism for Sch-Δ(E2-3), as demonstrated for an interstitial N-terminal deletion of Drosophila merlin (LaJeunesse, D.R., et al., 1998), can nevertheless not be excluded on the basis of these experiments. The dominant effect may be explained by an aberrant influence on intermolecular interactions by the mutant protein.

Another line of evidence has linked the FERM domain of merlin to regulation of cell proliferation. Overexpressed full-length merlin and the FERM domain singularly, were both able to reverse the Ras induced phenotype with anchorage independent growth in soft agar of v-Ha-Ras transformed NIH3T3 cells (Tikoo, A., et al., 1994) and transfected wild type NF2 reduced the growth rate of cultured 3T3 fibroblasts a 3-fold, in contrast to a mutated form of merlin lacking 111 amino acids at the N-terminal of the protein and effect on cell growth (Lutchman, M, and Rouleau, G.A., 1995).

The rat mRNA of merlin is widely expressed in most tissues during embryogenesis and early postnatal life, but become restricted mainly to neurons of the central- and peripheral nervous systems in adults, eg. Purkinje cells of the cerebellum, cortical neurons and spinal motor neurons (Claudio, J.O., et al., 1995). In spite of embryonic expression of the NF2 message in tissues of the nervous system, the human disease does not involve neurons. Developmental analysis of the two isoform transcripts has revealed tissue specific and temporally distinct expression patterns in rat brain which may imply a functional difference between the two isoforms (Gutmann, D.H., et al., 1995). This view is supported in vivo by the observation that tumor growth is inhibited in nude mice subcutaneously injected by rat schwannoma cells that overexpress isoform 1 of merlin, while isoform 2 failed to influence schwannoma cell growth (Sherman, L., et al., 1997). Similar differential effects on cell growth were obtained in vitro by overexpressing the two isoforms in cultured schwannoma cells (Ibid). The tumor suppressor activity specific for isoform 1 of merlin demonstrated in these experiments may be explained by the ability of this isoform to engage in C-to N-terminal interdomain interactions in cis or in trans, in contrast to isoform 2, which does not self-associate (Sherman, L., et al., 1997; Huang, L., et al., 1998). The conformational change of the molecule and masking of the C-terminal of merlin-1 induced by self-association, may alter the affinity for interacting proteins important for tumor suppression. The Rho GDP dissociation inhibitor (Maeda, M., et al., 1999) and βII spectrin (Scoles, D.R., et al., 1998) bind exclusively isoform 2 of merlin, whereas interactors that only bind to the closed form of merlin-1, or to the unique exon 17 of isoform 1 have not yet been identified. The C-terminal of merlin-1 is capable of inducing cell death in NIH3T3 cells - an effect not observed for this region of isoform 2, or for the full length molecule of isoform 1 (Shaw, R.J., et al., 1998). An additional three splice isoforms
with multiple exon exclusions have recently been cloned: Mer150, Mer151 and Mer162 (Schmucker, B., et al., 1999). Functional studies of these new splice variants remains to be done.

The modus operandi of the tumor suppressor effect of merlin is still an enigma, six years after the cloning of the gene, but the signal transduction pathways leading to growth inhibition of cells can be elucidated by identifying interacting proteins to merlin. A number of interactors have been found to bind directly to merlin: actin, CD44, NHE-RF, Rho-GDI, ezrin and βII spectrin (Fig 2). All of these proteins also interact or associate with members of the ERM family, with the singular exception of βII spectrin. Merlin lacks the conserved F-actin binding site present in the C-terminal of the ERM proteins (Turunen, O., et al., 1994), but is able to associate with polymerized actin through exon 10 in the ERM homology domain of the full length clones of both isoforms of merlin (Xu, H-M. and Gutmann, D.H., 1998). The merlin-2 protein can also bind F-actin indirectly via its C-terminal interaction with βII spectrin and thereby forming the trimer complex merlin-βII spectrin-actin (Scoles, R.S., et al., 1998). These interactors do not offer an obvious explanation to the tumor suppressor activity of merlin. However, merlin was recently shown to interact with the hepatocyte growth factor regulated tyrosine kinase substrate HRS (Scoles, D.R. et al., 2000). HRS is a FYVE RING finger protein suggested to be involved in trafficking of endosomes and in signal transduction of the Janus activated kinases - functions that regulate growth factor induced proliferation of cells (Ibid). The preliminary results from this study is therefore of interest with respect to the mechanism of tumor suppression by merlin, but the specific transformation of Schwann cells by the loss of merlin function remains to be elucidated.

<table>
<thead>
<tr>
<th>FERM domain</th>
<th>a-helix</th>
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<th>C-terminal</th>
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<tr>
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<td></td>
<td>βII Spectrin</td>
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<td>Rho-GDI</td>
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<td>ezrin</td>
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</tbody>
</table>

**Figure 2.** Proteins that binds directly to merlin are listed below the corresponding interacting region of merlin. The functional FERM domain of merlin is most frequently involved in protein-protein interactions, most of which are shared with the ERM proteins.
THE PRESENT STUDY

Aims of the study

The work presented in this thesis is focused on cloning of MIR - a novel gene with an ERM homology domain, distinguishing members of the superfamily of 4.1 proteins and to elucidate the function of MIR by identifying its interactors and by characterizing its activities in cell lines. In addition, an effort is made to demonstrate the distribution pattern of the MIR protein within cells and in tissues of developing rat brain. To achieve these objectives, the following specific aims were defined:

- To clone the full-length cDNA of MIR by screening a human cDNA library with radiolabelled fragments of MIR.

- To identify interacting proteins in the signalling pathway of MIR by performing yeast two-hybrid system hunts with different region of MIR used as baits and co-immunoprecipite putative interactors in COS cells to demonstrate that specific binding of a prey to MIR may occur in mammalian cells.

- To study cytoskeleton dependent activities such as neurite outgrowth by overexpressing the MIR protein and its interactors in neuron-like PC12 cells.

- To detect the tissue distribution of MIR mRNA expression by northern blot analysis and of MIR protein expression in rat brain during development by immunohistochemistry techniques.
**Materials and methods**

**cDNA cloning of MIR (paper I)**

A BLAST search of the expression sequence tag cDNAs (EST) subsection of GenBank with the RING finger domains of inhibitory of apoptosis protein (IAP) gene family as the query sequence, resulted in an EST sequence from human lung (GenBank accession no. T63512) with a RING finger domain similar to those found in the IAP proteins. A 375 bp probe corresponding to the C-terminal of the EST sequence was amplified by PCR, labelled with $^{32}$PdCTP (Rediprime, Amersham) and used for screening a $\lambda$gt10 human fetal brain cDNA library (Clontech).

Hybridization was carried out overnight at 42°C in 50% formamide, 6xSSC, 5xDenharts and 0.5% SDS, followed by washing for 10 min in 2xSSC, 0.5% SDS, for 15 min in 0.2%SSC, 0.5% SDS and finally for 15 min in 0.2xSSc, 0.5% SDS at 50°C. A positive phage of 1650 bp was cloned into the Bluescript pKS vector and sequenced using an automated DNA sequencer (Applied Biosystems). The nucleotide sequence encodes a putative open reading frame of 445 amino acids that was named MIR. The sequence has been submitted to GenBank (accession no. AF 187016).

**Northern blot analysis (paper I and III)**

cDNA was labelled with $^{32}$PdCTP (Rediprime, Amersham) and used to probe Multiple Tissue Northern Blots filters (Clontech). Hybridisation was carried out for 2 h at 37°C in the Express hybridisation solution (Clontech) and the filters were washed in 2xSSC, 0.05% SDS for 30 min followed by 0.1xSSC, 0.1% SDS for 40 min at room temperature. The amounts of mRNA were analysed with a PhosphoImager (Molecular Dynamics) and compared to those of β-actin mRNA. A 375 bp C-terminal fragment of MIR and the cds of NSAP were used as probes (paper I and III, respectively).

**Yeast two-hybrid system screen (paper I and III)**

Different portions of MIR cDNA –the cds, the FERM domain and the RING finger plus a portion of the inter region – were cloned into the DNA binding domain (BD) of the bait vector pYTH6 and integrated into the yeast genome according to the procedure described by Aspenström, P., et al. (Aspenström, P. and Olson, M.F., 1995). An overnight culture of the yeast strain Y190:pYTH6-cDNA was diluted to OD 0.3 in 800 ml of SD/-trp media and rotated at 220 rpm at 30°C to OD 0.9. The culture was portioned into Falcon tubes and pelleted at 1850 rpm for 5 min at room temperature and washed by resuspending the cells in water by vortexing, pooled into 4 Falcon tubes and centrifuged as above. The cells were resuspended in 2x3.5 ml of 0.1M LiAc pH 7.5 in TE-buffer and pooled to 2 tubes. A mixture of 2x (100-250) µg of activation domain (AD) HeLa cell cDNA library and 20 mg of herring testes carrier DNA (Clontech) prebolied for 5 min and snap cooled in ice water and 2x30 ml PEG/TE/LiAc solution (40% PEG 4000, 0.1 M LiAc pH 7.5 in TE) were added. The mixture were incubated for 30 min at 30°C with gentle shaking and
2x3.5 ml DMSO added followed by a heat shock for 15 min at 42°C. The cells were chilled on ice and pelleted by centrifugation as above, resuspend in 8 ml of TE pH 7.5 and spread on 45 SD/-trp-leu-his plates supplemented with 25 mM 3-aminotriazole and incubated for 4-8 days at 30°C. Pink colonies indicative of HIS3 reporter gene activation were streaked onto SD/-trp-leu plates, grown for two days and assayed for the β-galactosidase reporter gene activation using a filter lift procedure.

Filter lift assay for β-galactosidase reporter gene activation (paper I and III)

Yeast cells co-transformed with DNA BD-bait and AD-prey plasmids on -Trp, -Leu plates were grown for 3-5 days and transferred to a nitrocellulose filter and lyzed by repeated freeze-thaw cycles by immersing the filter for 30 s in liquid nitrogen and letting it thaw in room temperature. The nitrocellulose filter were placed on a second filter soaked with 6 ml of buffer with X-gal substrate (6 ml Z-buffer, 16 ml β-mercaptoethanol and 100 µl of 20 mg/ml of X-gal) in a petri dish and incubate at 30°C until blue color appears (1-8 hr).

Yeast two-hybrid interaction assay (paper I and III)

A yeast two-hybrid interaction assay was employed to map the binding regions between domains of the MIR protein and MRLC or NSAP. Different portions of MIR were ligated into the DNA BD pYTH6 vector (paper I), or into the episomal DNA BD pAS2 vector (manuscript). The pYTH6-MIR plasmids were integrated into the genome of yeast strain Y190 and the AD pGAD-GH MRLC plasmid was finally transformed into the integrated yeast strains and transformants spread on -Trp and -Leu plates. The pAS2-MIR were cotransfected with deletion mutants of NSAP cloned into the AD pGAD-GH vector. Colonies were assayed for β-galactosidase reporter gene activation in the filter lift assay.

Co-immunoprecipitation and Western blot analysis (paper I)

COS-7 cells were cultured in DMEM with 10% fetal bovine serum and co-transfected with 1µg of 6xhistidine-tagged MRLC-pCDNA3.1 expression vector (Invitrogen) and 1µg of hemagglutinin (HA)-tagged MIR-pJ3H vector at 75% confluence by the FuGene (Boehringer) vehicle. After 24 h, cells were lysed for 20 min in ice cold RIPA lysis buffer (PBS, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, 100 µM sodium orthovanadate (Sigma), protease inhibitors (Boehringer)). The lysate was cleared by centrifugation for 10 min at 14,000 rpm and monoclonal anti-HA epitope antibody (5 µg) was added and rotated for 2 hr at 4°C. The antibody complexes formed were immunoprecipitated with 30 µl of protein A-Sepharose (Pharmacia-Biotech) for 1 hr at 4°C and washed 3 times in lysis buffer. Proteins were eluted by boiling in SDS-PAGE sample buffer for 10 min and the supernatant retrieved by centrifugation. Samples were resolved by SDS-PAGE and tranferred to nitrocellulose membranes for Western blot analysis. For analysis
monoclonal anti-HA epitope (Boehringer) and monoclonal anti-His (Clontech) were used. Bound antibodies were detected by enhanced chemiluminescence.

**Assay for neurite outgrowth and subcellular localization of MIR (paper I and III)**

Rat phaeochromocytoma PC12 cells were grown in Dulbecco´s modified Eagle´s medium supplemented with 5% horse serum and 5% fetal bovine serum. Cells were transfected with 10 µg of MIR-pCDNA3.1 vector (Invitrogen) by the calcium phosphate method (Inagaki, N., et al., 1995) and stable clones were selected using G418 (600 µg/ml, Life Technologies). PC12 cells were transiently transfected with EGFP-MIR expressing enhanced green fluorescence protein fused to the N-terminal of MIR and with the empty EGFP vector as control, using the FuGene reagent (Boehringer). To study neurite outgrowth, PC12 cells were plated onto collagen coated (Sigma) culture plates and 50 ng/ml of nerve growth factor (NGF) was added for two days. Neurite outgrowth was estimated by counting the number of positive PC12 cells exhibiting neurites longer than two times the cell diameter (Inagaki, N., et al., ibid). Statistical analysis was done according to Student´s t-test. The subcellular localisation of EGFP- tagged MIR in living cells was analysed with a Zeiss Axiovert microscope and an attached Hamamatsu camera.

**MIR antibody and immunocytochemistry (paper I and II)**

The Peptidestructure programme (GCG Wisconsin) was used to calculate an antigenic index profile of the inter-region of MIR. The amino acid sequence QQTRVLQEKLRKLKEAMLC was predicted to be antigenic and a specific antibody to this peptide was generated in rabbits according to standard procedures. The purified antibody recognized human and rat MIR in Western blot analysis. Immunocytochemistry was performed on COS-7 cells (paper I) and on hippocampal neurons and PC12 cells (paper II), fixed for 10 min using 4% paraformaldehyde, washed with PBS and blocked overnight by skimmed milk. The MIR antibody, diluted 1:100, was added and incubated overnight at 4°C. After washing the cells with PBS, a secondary biotinylated anti-rabbit antibody (diluted 1:200, Dako) was added and incubated for 2 hr at room temperature, followed by the addition of the avidin-biotin complex (Vector Laboratories). The antibody complex bound were visualised using diaminobenzidine (Sigma) as a chromogen.

**TrkA phosphorylation assay (paper I)**

PC12 cells overexpressing HA-tagged MIR or the pCDNA3.1 vector as control, were stimulated with NGF for 5 min. Cells were lysed in buffer A (20 mM HEPES pH 7.4, 125 mM NaCl, 1% Triton X-100, 10 mM EDTA, 2 mM EGTA, 2mM sodium orthovanadate, 50 mM NaF, 20 mM ZnCl₂, 10 mM sodium pyrophosphate, 1mM PMSF and 5 µg/ml leupeptin). An anti-Trk antibody was added to the cleared lysates and rotated for 2 hr at 4°C, followed by incubation for 1hr at 4°C with 30 µl of protein A-Sepharose (Pharmacia). The Sepharose beads were washed three times in buffer A, once with 0.5 M LiCl, 0.1 M Tris, pH 7.5 and once with 0.1 M NaCl, 1 mM EDTA, 2
mM Tris, Ph 7.5. Samples were boiled in SDS-PAGE loading buffer and the proteins resolved by SDS-PAGE and transferred to nitrocellulosa membrane for Western blot analysis using an anti-phosphotyrosine antibody (Transduction Laboratories) for the TrkA phosphorylation.

**Immunohistochemistry (paper II)**

Wistar rat brains were cut on a cryostat and mounted on slides. The sections were fixed with aceton/methanol for 10 min at –20°C, incubated with 0.5% H₂O₂ for 15 min and blocked for 2h with TNT-buffer. The slides were incubated for 24 h at +4°C with the anti-MIR antibody (1:200) described above, washed with TNT-buffer and a secondary biotinylated anti-rabbit antibody (1:200, Vector Laboratories) was added for 2 h at RT. The ABC solution with diaminobenzidine (Vectastain ABC kit, Vector Laboratories) as a chromogen was used to detect the signals obtained. For the double staining studies, the anti-MIR antibody was used and detected as above with the addition of a monoclonal neuronal nuclei marker (NeuN) antibody (1:150, Chemicon), or with a monoclonal glial fibrillary acidic protein (GFAP) antibody (1:200, Roche). The NeuN and GFAP signals were visualized by using a fluorescein isothiocyanate (FITC)-coupled anti-mouse antibody (1:200, Dako).

**Chromosomal localization (paper I and III)**

To assess the chromosomal localization of the human MIR gene, cds´s were used to search the STS database of NCBI for matching sequences mapped by PCR on human/rodent somatic cell hybrids, or physically by STS markers. The GeneMap’99 of the RH Mapping Consortium and OMIM gene map was used to obtain transcripts and known genes in the vicinity of the loci of MIR.

**Results**

**Cloning and structure of MIR (paper I and unpublished results)**

The nucleotide sequence of MIR was obtained by screening a human fetal brain cDNA library with a radiolabelled C-terminal fragment of the EST clone T63512 as a probe. A 1650 bp phage cDNA was sequenced revealing an open reading frame of 1338 bp with a stop codon in the GC-rich 5’ untranslated region in frame with the putative ATG start codon (GenBank accession number NM 013262). The sequence adjacent to the putative ATG of MIR, CAGCCATG, partially comply to Kozac’s rule of a strong translational initiator sequence CC(A/G)CCATGG. (Kozak, M., 1999). The open reading frame of MIR encodes a putative protein of 445 amino acids with two distinct protein homology regions: an N-terminal ERM homology domain characteristic for members of the protein 4.1 super family and a RING finger motif at the C-terminal end, a
unique feature among proteins of this super family. The sequence identity of the N-terminal region of MIR to that of the cytoskeleton protein merlin and ezrin is 28% and 25%, respectively. The most similar RING finger regions to the RING finger of MIR was obtained by a BLAST search of GenBank, resulting in a number of proteins involved in the regulation of cell growth and apoptosis, such as c-IAP2, XIAP, c-CBL, CGR19, PML and RFP.

Further support for the presence of a FERM domain in the MIR protein is given by a secondary structure prediction of the FERM domain of MIR and merlin using the PHD program (Rost, B. and Sander, C., 1994). The sequence similarity of the FERM domain of MIR and merlin (28% identity) is superceded at the level of secondary structure, as predicted by the PHD program. The order of the major structural elements, helices H3-H6 and H8-H9, interrupted by a number of short strands in MIR is clearly repeated in merlin according to these results (Fig. 3).

![Diagram showing secondary structure of MIR and merlin FERM domains]

**Figure 3.** PHD prediction of the secondary structure of the FERM domains of MIR and merlin. Black boxes denote extended sheats (E1-E7) and diagonally hatched boxes are helices (H1-H9).

**Genomic sequence and chromosomal localisation of the MIR gene (unpublished results and paper II)**

The genomic structure of the human MIR gene was elucidated by comparing the coding sequence of MIR with the PAC clone 13D10 (GenBank accession no. AL021407). The coding region of the MIR gene that spans 17.5 kb is composed of seven exons ranged in length of 90 bp to 421 bp and six introns between 0.5 and 10.9 kb long (Fig 4). Splice junction sequences comply to the GT-AG rule at all splice sites except the fifth splice donor sequence in which the
Figure 4. (A). The overall structure of the 17.5 kb MIR gene with the intron sizes in kilobases. The seven exons are shown in black bars. (B). The domain structure of the MIR protein with the start (ATG) and stop (TAA) codons. (C). The exon composition of the coding sequence of the MIR gene with the exon length given in bp. The color of the exons corresponds to similarly shaded amino acid domains of the MIR protein. A linear representation of the current transcript in human fetal brain is shown below.

Table 1

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intron starts with the dinucleotide GC (Table 1). A splice variant was detected in human fetal brain that exclude exon 1 and 2 for the alternatively used exon X - present in the large third intron. The exon structure of the MIR gene is not conserved in ezrin or merlin, whereas three exon/intron junctions are of the same codon phase in ezrin and merlin (Majander-Nordenswan, P., et al., 1998). The majority of the exons of the MIR gene (6/7) are part of the ERM homology domain. In ezrin, merlin and 4.1R (Huang, J.-P., et al., 1993), the ERM domains are constructed of 8,10 and 10 exons, respectively. The RING finger of MIR is encoded by exon 6 and 7 - not by a single exon - as is true for the closely related RZF domain of the murine homologue of XIAP (Farahani, R., et al., 1997). The localization on the human chromosome of MIR was determined by using the cDNA of MIR as a query sequence in an electronic PCR approach on the STS database of NCBI. A single PCR product (GenBank accession no. G21201, STS marker name WI-14205), corresponding to the 3'UTR region of MIR, was identified. The WI-14205 marker has been physically mapped to the DS1640-DS422 region on chromosome 6p22.3 close to the position of spinocerebellar ataxia type-1 gene (Zoghbi, H.Y. and Orr, H.T., 1995).

Interstitial deletions of 6p22.1-p23 (~5-6 Mbp) give rise to abnormalities typical of patients the 6p deletion syndrome, which is characterized by mental retardation, heart and kidney defects, craniofacial malformations and hydrocephalus (Davies, A.F., et al., 1996). Loss of MIR may contribute to these defects though a number of other genes are likely to be involved in the 6p deletion syndrome (Olavesen, M.G., et al., 1977; Olavesen, M.G., et al., 1995). The study of MIR in other disease conditions by genetic screens for mutations and detection of chromosomal aberrations of the MIR gene will benefit from the knowledge of the genomic structure and chromosomal localization of MIR.

**Tissue distribution of the MIR protein in rat brain (paper II)**

To study the expression pattern of the MIR protein in neonatal and adult rat brain tissue, immunohistochemistry experiments were performed with an anti-MIR antibody designed to recognize an epitope of the interregion between the FERM domain and the RING finger of human MIR. Western blot analysis on developing and adult rat brain demonstrated the specificity of this antibody in rat tissue, with the 53 kDa band of MIR present on blots from various regions, such as hippocampus, cerebral cortex and cerebellum. These regions, in addition to thalamus and substantia nigra (data not shown), did also stain positive to MIR in immunohistochemistry experiments. In hippocampus, immunoreactivity to MIR was detected in the CA1 and CA3 sublayers, in addition to the dentate gyrus and some other regions. In the cerebellum, both the external and internal granule layers containing the immature and mature granule cells, respectively, as well as Purkinje cells, stained positive for MIR.

To reveal the cell type expressing MIR in adult rat brain, double labelling experiments were performed with markers for neurons (NeuN) and glia cells (glial fibrillary acidic protein, GFAP). MIR and NeuN positive neurons were detected in the dentate gyrus and some other regions of
hippocampus, in the cerebellar cortex and in the Purkinje cells of cerebellum. These regions of the brain were generally not double stained with MIR and GFAP, indicating that the MIR protein is predominantly expressed by neurons in adult rat brain.

**Distribution of MIR in cultured cells (paper I and II)**

COS-7 cells were transiently transfected with a plasmid encoding the enhanced green fluorescent protein (EGFP) fused to the N-terminal of MIR. The fluorescence pattern of the EGFP-MIR protein displayed a punctated distribution, indicative of an association to the cytoskeleton of the fusion protein. Endogenously expressed MIR stained with an anti-MIR antibody showed a similar pattern. Immunocytochemistry for MIR was furthermore performed on embryonal hippocampal neurons, cultured for a week and on PC12 cells, demonstrating immunoreactivity to MIR in the cell bodies of the hippocampal neurons and in neurites and growth cones of differentiated PC12 cells.

**Interacting proteins to MIR (paper I, III and unpublished results)**

To elucidate the signal transduction pathway of MIR, yeast two-hybrid system screens were performed of a HeLa cell expression library with different regions of MIR as baits: the cds, the FERM domain and the RING finger with a part of the upstream inter-region. The identified interactors to the corresponding domains of MIR are depicted in Fig 5. The screen with full length MIR as bait resulted in two species of prey molecules: myosin regulatory light chain B (MRLC-B, paper I) and a novel protein denoted NSAP for its putative saposin domain (GenBank accession no. AY032624, paper III). The β-galactosidase filter lift assay gave further support for the binding in yeast of MIR to both preys. To demonstrate that the interaction between MIR and MRLC-B may occur in mammalian cells as well, co-immunoprecipitation of HA-tagged MIR and His-tagged MRLC-B was successfully performed in co-transfected COS-7 cells (paper I). Moreover, direct interaction of MIR to MRLC-B and NSAP was shown by applying a binding assay with GST-linked MIR and radiolabelled MRLC-B and NSAP generated in vitro (paper III). To map the binding site in MIR to MRLC-B and NSAP, C-terminal deletion mutants of MIR were constructed and analyzed in β-galactosidase filter lift assay of the two-hybrid system. No specific region of MIR was sufficient for binding indicating that full-length MIR is required for the interaction with both MRLC-B and NSAP (paper I and III, respectively).

NSAP is a novel protein of 182 amino acids with similarity to the saposin domain found in the family of saposin-like proteins represented by saposin A-D, natural killer cell lysin, surfactant protein B, acid sphingomyelinase and some other proteins (Munford, R.S., et al., 1995; Ponting, R.B, and Russell, R.B., 1995; paper III). The position of the six cysteins and most of the conserved lipophilic residues are present in NSAP. It has however two inserts of amino acids marked with slashes that corresponds to loop regions in the structure of pig NK-lysin (Liepinsh, E., et al., 1997). The N-terminal of NSAP encodes a putative type II membrane signal and the C-
terminal tertrapeptide HDEL is similar to the KDEL endoplasmatic reticulum retention motif ( ). To investigate a possible involvment of NSAP in membrane related effects, stable Swiss 3T3 cell lines expressing the fusion protein NSAP-DsRed were generated. An MTT assay was used to assess the adhesion properties of these cells to different substrates. The results show an enhanced cell-to-substrate adhesion of these NSAP-DsRed expressing cells as compared to controls.

The yeast two-hybrid screen with the FERM domain of MIR as bait (unpublished results) identified two proteins involved in the regulation of the microtuble system: colonic and hepatic overexpressed gene (ch-TOG) and bicaudal-D (BIC-D). The Ch-TOG protein binds to and promotes microtuble assembly in vitro at the mitotic spindle of rapidly dividing cells, but it also binds to the endoplasmatic reticulum localized microtubule system. The mRNA of ch-TOG is most abundantly expressed in brain tumors and in adult brain, particularly in the Purkinje cell bodies of the cerebellum (Charrasse S., et al., 1998; Ibid 1996). Bic-D is a cytoskeleton-like coiled coil protein with five α-helix domains, or heptad repeats, with similarity to the tail domain of myosin heavy chain. Mutations of Drosophila Bic-D disrupt the microtubule cytoskeleton and thereby blocks transport of mRNA for axis determinants in the oogenesis, causing the bicaudal or double abdomen phenotype (Baens, M. and Marynen, P., 1997; Suter, B., et al., 1989). The highest mRNA expression is found in human adult brain (Baens, M., ibid.). Drosophila Bic-D is moreover part of a protein complex with Lissencephaly-1, which is required for nuclear localization in the developing nervous system, indicating that Bic-D may be involved in vertebrate neural migration and that its absence may cause a Miller-Dieker-like lissencephaly (Swan A., 1999). Two novel prey clones were also obtained in this screen: Rabip4, a FYVE-finger coiled-coil domain containing effector protein of endosomal GTPase Rab4 (GenBank accession number MMU250024), and the PD2 protein, encoded by the PD2 gene which is amplified and overexpressed in human pancreatic adenocarcinoma (GenBank accession number HSA401156).

The screen with the RING finger domain of MIR as bait resulted in two identical prey clones (unpublished results) with high similarity to the tumor specific antigen MAGE-3b/6 (Itoh, K., et al., 1996). MAGE proteins are expressed in more than half of all cancers studied so far, including many tumors of the nervous system, but not in normal tissues with the exception of testicular cells in their undifferentiated proliferative phase (Scarcella, D.L., et al., 1999; Corrias, M.V., et al., 1996). The normal function of the MAGE family members in testes is not known, nor has any interactors to the MAGE proteins been identified.
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<th>FERM domain</th>
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<td>ch-TOG</td>
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<td>Bicaudal-D</td>
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<td>Rabip4</td>
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**Figure 5.** The domain structure of the MIR protein is shown above the different interactors to the respective domains, obtained in yeast two-hybrid system screens of a HeLa cDNA expression library. MRLC-B and NSAP bind to the full length protein of MIR.

**Effect on neurite outgrowth by MIR and NSAP.**

Cytoskeletal dynamics are important for cell motility phenomenon such as neurite outgrowth and primarily regulated by the contractility of myosin molecules controlled by MRLC (Howard, J., 1997). The presence of an ERM domain in MIR suggested a study of the effect of MIR on neurite outgrowth in PC12 cells stimulated by nerve growth factor (NGF) (Inagaki, N., et al., 1995). PC12 cells were treated with 50 ng/ml of NGF to induce a profuse outgrowth of neurites in control cells, whereas stable cell lines overexpressing MIR did exhibit reduced neurite process formation. This effect was not caused by defective response of NGF receptor TrkA receptor - the TrkA phosphorylation and expression of the immediate early response gene fos was intact in the MIR expressing cells. The inhibitory influence of neurite outgrowth of overexpressed MIR was repeated for PC12 cells transiently transfected with the EGFP-MIR vector. Analogous experiments were performed with stable PC12 cell lines overexpressing NSAP. An increased neurite outgrowth of these PC12 cells was measured both with and without stimulation with NGF, indicating that NSAP acted on NGF independent pathway to induce neuritogenesis in PC12 cells.

**Discussion**

The ERM proteins ezrin, radixin and moesin act as molecular linkers between the plasma membrane and cortical F-actin by binding transmembrane proteins to their N-terminal region and F-actin to a conserved actin binding motif of the C-terminal. The pleiotropic functions mediated by this linking capacity involves cell motility, migration and adhesion of cells (Bretscher, A., etal., 2000). Furthermore, ERM proteins participate in the regulation of cell proliferation and other cellular events which may not be directly related to their interaction with the cytoskeleton (Louvet-Vallée, S., 2000). The ERM are part of the larger band 4.1 superfamily of proteins.
which are defined by their sequence similarity to the N-terminal region of ERM proteins – the ERM-homology domain (Girault, E-A., et al., 1999).

This thesis presents a novel member of the band 4.1 superfamily, myosin regulatory light chain interacting protein MIR. The cDNA of MIR, which was isolated by screening a human fetal brain cDNA library, encodes a putative protein of 445 amino acids with two distinct protein homology regions: an N-terminal ERM-homology domain 28% and 25% similar to merlin and ezrin, respectively and a C-terminal RING finger (Borden, K.L. and Freemont, P.S., 1996; paper I) similar to other proteins involved in regulating proliferation and survival of cells, but unique among members of the band 4.1 superfamily.

To elucidate the cellular function of MIR, stable PC12 cell lines overexpressing the MIR protein were generated and assayed for neurite outgrowth. MIR expressing cells exhibited reduced NGF stimulated neurite extensions as compared to controls – an effect that could not be accounted for by defective NGF receptor TrkA signaling, measured as TrkA phosphorylation and activation of the immediate early gene cFOS. A similar result was observed in PC12 cells transiently transfected with a plasmid expressing an EGP-MIR fusion protein, indicating that the effect on neuritogenesis of MIR is not related to the chromosomal integrating site of the PC12 cells.

The presence of an ERM-homology domain in MIR suggests that the inhibitory effect on neurite outgrowth of MIR in PC12 cells may involve cytoskeleton dependent cell motility. In support of this view, yeast two-hybrid system screens with different regions of MIR used as baits, identified a number of interacting proteins that regulate both F-actin and the microtubule components of the cytoskeleton; MRLC-B (paper I), ch-TOG and BIC-D (unpublished results). MRLC controls the activity of the actomyosin complex in muscle and nonmuscle cells (Howard, J., 1997) and evidence for the involvement of myosin in neurite outgrowth has recently been provided (Wylie, S.R., et al., 1998; Hasson, T., et al., 1997). The activity of MRLC is regulated by the phosphorylation state of MRLC, which is influenced by myosin light chain kinase and the corresponding phosphatase (Somlyo, A.P. and Somlyo, A.V., 1994). Increased MRLC phosphorylation leads to enhanced cell contractility in smooth muscle cells (Uehata, M., et al., 1997), an effect likely to occur also in neurons. In addition, the Rho associated p160 kinase (ROCK) was recently shown to phosphorylate MRLC (Uehata, M., et al., ibid). Agonist-induced or enhanced Rho/ROCK activity was later demonstrated to be sufficient for mediating neurite retraction and cell rounding of neuroblastoma cells in culture and it was accordingly suggested that Rho/ROCK activity represses neurite extension by activation of actomyosin contractility and suppression of microtubule assembly (Hirose, M., et al., 1998). Previous studies have implicated the ERM proteins in the activation cascade induced by Rho (Matsui, T., et al., 1998) and conversely, ERM proteins may contribute to activation of Rho by sequestering the Rho inactivator Rho GDI (Takahashi, K., et al., 1997). These results raise the possibility of an involvement of ERM in the regulation of neurite morphology and process extension and this has
indeed been demonstrated for radixin and moesin. Double suppression of radixin and moesin in hippocampal rat neurons by an antisense approach inhibited neurite outgrowth and altered growth cone structure and motility (Paglini, G. et al., 1998).

Considering the important role played by Rho in neurite retraction and the mutual interaction of Rho and ERM proteins, it is possible that the function of MIR is linked to modulation of Rho signalling by interfering with the Rho/ROCK/MRLC pathway and to alteration of ERM activities. MIR does however not bind directly to constitutively active Rho (Pontus Aspenström, personal communication). Interestingly, MIR co-immunoprecipitate with ezrin in COS-7 cells and MIR and ezrin both bind to NSAP (unpublished results and paper III). NSAP is a novel saposin-like protein of 182 amino acids isolated in a yeast two-hybrid screen with the full-length protein of MIR used as a bait (paper III). A direct interaction between MIR and NSAP was furthermore demonstrated by application of the GST in vitro binding assay with GST-tagged MIR and $^{35}$S-labelled in vitro translated NSAP. The sequence similarity of NSAP to saposin A-D is of interest with respect to the ability of saposin C to induce neurite outgrowth in PC12 and neuroblastoma cells (O’Brian, J.S., et al., 1994). Likewise, stable PC12 cell lines overexpressing NSAP were shown to induce a profuse outgrowth of neurites, with, but also without the stimulation of NGF, indicating that a NGF receptor TrkA independent pathway is mediating this effect (paper III). In the previous study saposin C was given in the medium of PC12 cells, whereas in our case NSAP was expressed by the PC12 cells. NSAP does however encode a type 2 membrane signal in the N-terminal region of the molecule, typical for proteins targeted to membranes or secreted to the extracellular space. It is also possible that the stimulation of neurite outgrowth in PC12 cells by NSAP reflects increased adhesion of the cells to the substratum. In accordance with this view, an enhanced adhesion of Swiss 3T3 cells was observed after NSAP overexpression (paper III). Regardless of the mechanism involved in the stimulation of neurite extension by NSAP, these results raises the possibility that MIR inhibits neuritogenesis by a direct interaction with NSAP, as well as by its binding to MRLC.

To argue for an effect on neurite outgrowth of MIR in neurons, endogenous expression of MIR needed to be demonstrated in nerve cells. This was accomplished by immunohistochemistry experiments in rat brain tissue, doubled stained with a specific anti-MIR antibody and the neuronal marker NeuN (paper II). Immunoreactivity to both MIR and NeuN was detected in neurons of the hippocampus and cerebellum and to MIR in dissected embryonic rat neurons in short time cultures.

Further studies are however needed to firmly establish the mechanism by which MIR inhibits, and NSAP induces neurite outgrowth in NGF stimulated PC12 cells.
Figure 6. A flowchart representation of hypothesized mechanisms for the effect of MIR on neurite outgrowth. The interaction of MIR with regulatory proteins of both components of the cytoskeleton may be involved - BIC-D and TOG of the microtubule system and MRLC-B of F-actin fibers. The mechanism of the effect on neurite outgrowth of NSAP is currently unknown.
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