Homotypic and heterotypic interaction of the neurofibromatosis 2 tumor suppressor protein merlin and the ERM protein ezrin

Mikaela Grönholm1,*, Markku Sainio1, Fang Zhao1, Leena Heiska1, Antti Vaheri2 and Olli Carpén1

Departments of 1Pathology and 2Virology, University of Helsinki, Haartman Institute, PO Box 21 (Haartmaninkatu 3), FIN-00014 Helsinki

*Author for correspondence (e-mail: mikaela.gronholm@helsinki.fi)

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SUMMARY

Ezrin, radixin and moesin (ERM) are homologous proteins, which are linkers between plasma membrane components and the actin-containing cytoskeleton. The ERM protein family members associate with each other in a homotypic and heterotypic manner. The neurofibromatosis 2 (NF2) tumor suppressor protein merlin (schwannomin) is structurally related to ERM members. Merlin is involved in tumorigenesis of NF2-associated and sporadic schwannomas and meningiomas, but the tumor suppressor mechanism is poorly understood. We have studied the ability of merlin to self-associate and bind ezrin. Ezrin was coimmunoprecipitated with merlin from lysates of human U251 glioma cells and from COS-1 cells transfected with cDNA encoding for merlin isoform I. The interaction was further studied and the association domains were mapped with the yeast two-hybrid system and with blot overlay and affinity precipitation experiments. The heterotypic binding of merlin and ezrin and the homotypic association of merlin involves interaction between the amino- and carboxy-termini. The amino-terminal association domain of merlin involves residues 1-339 and has similar features with the amino-terminal association domain of ezrin. The carboxy-terminal association domain cannot be mapped as precisely as in ezrin, but it requires residues 585-595 and a more amino-terminal segment. Unlike ezrin, merlin does not require activation for self-association but native merlin molecules can interact with each other. Heterodimerization between merlin and ezrin, however, occurs only following conformational alterations in both proteins. These results biochemically connect merlin to the cortical cytoskeleton and indicate differential regulation of merlin from ERM proteins.

Key words: Cytoskeleton, Merlin, Neurofibromatosis 2, ERM protein, Ezrin

INTRODUCTION

ERM (ezrin-radixin-moesin) proteins are components of the cortical cytoskeleton and play a role in linking the actin-containing cytoskeleton to cell membrane molecules. The ERM family consists of three closely related proteins: ezrin (Gould et al., 1989; Turunen et al., 1989), radixin (Funayama et al., 1991) and moesin (Lankes and Furthmayr, 1991). They share 75-80% sequence homology and consist of three domains: a globular amino-terminal domain, an extended α-helical domain and a charged carboxy-terminal domain (Vaheri et al., 1997). The amino-terminal domain has considerable homology with members of the band 4.1 family of membrane-organizing proteins.

The amino-terminal domain of ERM proteins interacts with plasma membrane molecules such as CD43, CD44, intercellular adhesion molecule-1 (ICAM-1) and ICAM-2 (Helander et al., 1996; Hirao et al., 1996; Heiska et al., 1998; Yonemura et al., 1998), whereas binding sites for actin are contained both in the amino- and carboxy-terminal domain (Turunen et al., 1994; Pestonjamasp et al., 1995; Roy et al., 1997). ERM proteins regulate the cell surface distribution of adhesion molecules, organization of cell membrane structures and maintenance of cell shape (Lamb et al., 1997; Vaheri et al., 1997). These functions are partially redundant within the ERM family members. When the expression of all three ERM proteins is blocked by antisense oligonucleotides microvilli disappear and cellular adhesion is disrupted (Takeuchi et al., 1994).

Homotypic and heterotypic association of ERM proteins has been described (Gary and Bretschger, 1993, 1995; Andreoli et al., 1994; Magendantz et al., 1995; Pestonjamasp et al., 1995). The N-ERMAD (ezrin-radixin-moesin association domain) in ezrin has been mapped to amino acids 1-296 and the C-ERMAD to amino acids 479-585 (Gary and Bretschger, 1995). The carboxy-terminal functional domain, including the F-actin binding site, is masked in the native monomer but can be exposed by sodium dodecyl sulphate or if the domain is expressed as a truncated protein (Gary and Bretschger, 1995). According to this model, the folded state...
of the monomers and the homotypic/heterotypic oligomers in the cytoplasm represent the inactive form of the protein. Signals disrupting this intramolecular or intermolecular interaction may expose masked functional sites, such as membrane-binding and actin-binding domains, allowing ERM proteins to bind to other partners and function as crosslinkers.

The neurofibromatosis 2 (NF2) tumor suppressor protein merlin (schwannomin) is structurally related to ERM proteins (Rouleau et al., 1993; Trofatter et al., 1993). Inactivation of the NF2 gene, which encodes for merlin, leads to the development of schwannomas and meningiomas in the dominantly inherited NF2 disease. NF2 gene mutations are also found in sporadic schwannomas and a proportion of meningiomas (Louis et al., 1995). In addition to the overall ERM-like domain structure, merlin possesses some functional properties of ERM family members. The amino acid identity between merlin and ezrin is 61% in the amino-terminal domain, but the carboxy-terminal domain shares only 22% identity with ezrin (Turunen et al., 1998).

Two major alternatively spliced NF2 variants are expressed in vivo. The isoform I, lacking exon 16, encodes for a 595 amino acid protein with a predicted molecular mass of 66 kDa (Rouleau et al., 1993; Trofatter et al., 1993). Isoform II contains exon 16 which inserts 11 unique carboxy-terminal amino acids followed by a termination codon that prevents translation of exon 17 (Bianchi et al., 1994). In cultured cells, merlin is localized underneath the plasma membrane in a pattern typical of ERM proteins (Gonzalez-Agosti et al., 1996; Sainio et al., 1997). Overexpression of full-length isoform I induces morphogenetic changes, such as cell surface protrusions and elongation of cell body (Sainio et al., 1997). Transfected and endogenous merlin colocalizes with ezrin, although in cells with a poorly developed actin cytoskeleton merlin replaces ezrin in filopodia and ruffling edges (Sainio et al., 1997). Based on these findings, merlin, similar to ERM proteins, is a cytoskeleton-associated membrane organizing protein and thus a unique type of tumor suppressor. The tumor suppressor mechanism of merlin is, however, poorly understood. Overexpression of isoform I in rat schwannoma cells and NIH 3T3 cells inhibits cell proliferation, whereas truncated constructs or isoform II fails to influence schwannoma growth (Lutchman and Rouleau, 1995; Sherman et al., 1997).

So far, only few binding partners for merlin have been characterized. Merlin has been shown to associate with CD44 but it is not known whether the interaction is direct or indirect through other molecules (Sainio et al., 1997). In addition, merlin, as well as ezrin, binds to the regulatory cofactor for Na⁺/H⁺ exchange, hNHE-RF or EBP50 (Reczek et al., 1997; Murthy et al., 1998) and merlin has also been shown to interact with β-spectrin (Scoles et al., 1998) and associate with actin and microtubules (Xu and Gutmann, 1998). Whether these interactions are relevant to merlin’s antiproliferative effect is, however, unresolved. Since the carboxy terminus of merlin is not as conserved as in other ERM proteins, it is also unclear if the activity of merlin is regulated by head-to-tail association as is the case with other ERM family members. In this paper we have tested whether merlin is able to form homotypic interactions and to interact with the prototypic ERM family member, ezrin.

MATERIALS AND METHODS

Antibodies

All antibodies have been characterized. Ezrin was detected with 3C12 mAb (Böhlöing et al., 1996). Two merlin-specific rabbit antisera raised against synthetic peptides were used. Anti-schwannomin detects isoform I (Lutchman and Rouleau, 1995), whereas 1398NF2 detects both isoform I and II (den Bakker et al., 1995). Polyclonal LexA antibody was kindly provided by Dr E. Golemis, Fox Chase Cancer Center, Philadelphia, PA (Samson et al., 1989). 12CAS mAb, which reacts with the influenza virus hemagglutinin epitope (HA), was from Boehringer-Mannheim, GmbH, Mannheim, Germany. As controls, preimmune serum of 1398NF2 rabbit (pre) and X63 mAb (ATCC, Maryland, USA) were used.

Confocal microscopy

Human U251 glioma cells (Westermark, 1973), expressing endogenous merlin and ezrin, were grown on glass coverslips in MEM/10% fetal bovine serum and serum starved for 20 hours before fixation in −20°C methanol. For double staining of merlin and ezrin, fixed cells were incubated simultaneously with 1398NF2 antiserum (1:100 dilution) and 3C12 mAb (1:200 dilution), followed by TRITC-conjugated goat anti-rabbit IgG (Dako A/S, Copenhagen, Denmark) and FITC-conjugated goat anti-mouse IgG (Dako). Specimens were viewed with a confocal 410 Invert Laser Scan Microscope (Carl Zeiss, Oberkochen, Germany).

Coimmunoprecipitation of ezrin with merlin in U251 glioma cells and transfected COS-1 cells

U251 glioma cells from a 10 cm plate were lysed in 500 μl ELB-buffer (50 mM Heps, pH 7.4, 150 mM NaCl, 5 mM EDTA), 1% NP-40 and protease inhibitors, and centrifuged at 15,000 g for 1 hour at 4°C. The supernatant was incubated with 3C12 mAb, anti-schwannomin antisera or pre-immune rabbit serum and Protein G-Sepharose beads (Pharmacia, Uppsala, Sweden) for 4 hours at 4°C. Immunoprecipitates were washed with ELB-0.1% NP-40 and bound proteins were eluted from the beads by boiling in non-reducing Laemmli sample buffer. Samples were separated on SDS-PAGE, transferred to nitrocellulose filters (Schleicher & Schuell, Dassel, Germany) and immunoblotted with 3C12 mAb (1:3000 dilution) and 1398NF2 antibody (pre) as a control. Immunoprecipitation was done as described for U251 glioma cells but merlin was immunoprecipitated with 1398NF2 rabbit antisera.

Chemical cross-linking

U251 glioma cells from a 10 cm plate were rinsed in PBS and incubated for 30 minutes at room temperature in PBS containing a thiol-cleavable cross-linker dithiobis-succinimidy1-propionate (DSP, 0.2 mM) (Pierce Chemical Co., Rockford, IL), protease inhibitors and phosphatase inhibitors (200 μM Na3VO4, 50 mM NaF and 50 mM β-glycerophosphate). The reaction was quenched for 15 minutes by addition of 1 M Tris-Cl, pH 7.5, to obtain a final concentration of 50 mM. The cells were lysed in non-reducing Laemmli sample buffer. Samples were separated on SDS-PAGE under non-reducing and reducing conditions and transferred to nitrocellulose filters. The filters were subsequently immunoblotted for merlin with anti-schwannomin.
antiserum (1:2000 dilution), and after stripping according to
manufacturer’s instructions, for ezrin with 3C12 mAb (1:3000
dilution).

Recombinant DNA constructs
The merlin fusion protein constructs were generated from the cDNA
encoding for merlin isoform I. For the yeast two-hybrid method, inserts
were subcloned into the bait vector, EG202, which contains a LexA-
DNA binding domain (Gyuris et al., 1993) or the prey vector JG 4-5,
which contains the HA epitope tag (Gyuris et al., 1993). Merlin 1-100,
1-167, 1-546, 1-585, 1-595, 339-585, 252-595 and 339-595 were
directed by digestion with restriction endonucleases and ligation into the
bait and prey vector directly or via subcloning into pGEM expression
vector (Promega, Madison, WI). Merlin 1-339 was amplified by the
PCR method and subcloned into the two-hybrid vectors. Ezrin 1-309
and 1-585 were amplified by PCR, using the pCv6 clone (Turunen et
al., 1989) as a template, and subcloned into the two-hybrid vectors. Ezrin 1-170 and 278-585 were generated by digestion with restriction
endonucleases and ligation into the two-hybrid vectors. The authenticity
of all constructs was verified by sequencing.

Yeast two-hybrid system
The genotype of the Saccharomyces cerevisiae strain BOY1, kindly
provided by P. Ljungdahl, Ludwig Institute for Cancer Research,
Stockholm, Sweden, is MATα his3 trp1 leu2::6LexAop-LEU2
URA3::8LexAop-Gal1-LacZ. BOY1 mating type a was made using the
YCpHO CUT4 plasmid (Raghuraman et al., 1994). Yeast strains
were grown at 30°C in rich medium or in synthetic minimal medium
with appropriate amino acid supplements. Bait and prey constructs
were transformed into BOY1 yeast of both mating types by electroporation.
Yeast strains variation was less than 15%. The final values were the result of four independent determinations.

Production of recombinant proteins
The baculovirally expressed GST-merlin fusion protein, GST-m 1-595
was produced as described (Sainio et al., 1997). Additional carboxy-
terminal deletion constructs, GST-m 1-339 and GST-m 1-546, were
created by digestion with restriction endonucleases and ligation into pAcG2T vector (Pharmingen, San Diego, CA). The proteins were expressed using the BaculoGold (Pharmingen) baculovirus expression system and purified by glutathione agarose beads (Pharmingen). The ezrin baculovirus expression construct, a kind gift of P. Mangeat (Université Montpellier II, Montpellier, France) (Andreoli et al., 1994), was purified as described (Hirao et al., 1996). GST-ez 279-531
and GST-ez 477-585 in pGEX vectors (Pharmacia) were expressed in
Escherichia coli DH5α cells and purified by glutathione-Sepharose
beads as described (Turunen et al., 1994).

Blot overlay
Recombinant wild-type ezrin (ez 1-585) and GST-ez 477-585 were biotinylated as described (Gary and Bretscher, 1993). Ezrin, GST-
merlin and GST-ezrin constructs, GST and BSA (1-2 μg/lane) were run in 10% SDS-PAGE, blotted onto nitrocellulose filters and blocked overnight using 3% BSA in TBS-0.1% Tween-20. The blots were
incubated with biotin-labeled ezrin (0.1 μg/ml) in 1% BSA in TBS-
0.1% Tween-20 for 4 hours. Peroxidase-conjugated avidin (1:10000 dilution) (Extravidin, Sigma) was detected using enhanced chemiluminescence.

RESULTS
Distribution of both merlin and ezrin in human U251 glioma cells
depends on confluence
We compared the distribution of merlin and ezrin in U251 glioma
cells, which express these proteins endogenously, in sparse and confluent growth conditions. Previously, an increase in
cell density has been reported to upregulate merlin expression (Shaw et al., 1998). The subcellular distribution of both merlin and ezrin was affected by an increase in
confluency. In subconfluent U251 glioma cells, double staining
of merlin and ezrin revealed a highly overlapping subcellular
distribution at cell surface projections resembling ruffling
edges (Fig. 1A-C). In confluent cell cultures, only few cells with
accumulation of merlin and ezrin at the cell periphery were present. Instead, most of the cells had lost the
submembranous staining pattern of both proteins, and staining
showed a diffuse or punctate cytoplasmic pattern (Fig. 1D-F).

The results demonstrate a concomitant regulation for merlin
and ezrin distribution under different growth conditions.
Association of merlin and ezrin in vivo
The colocalization of merlin and ezrin in U251 glioma cells and in COS-1 cells (Sainio et al., 1997) and the previously reported heterodimerization between ERM proteins suggested that an interaction could occur between merlin and ezrin. The possible interaction was tested by coimmunoprecipitation experiments. The cDNA encoding for merlin isoform I or β-galactosidase was transfected into COS-1 cells that do not express detectable amounts of endogenous merlin. After 70 hours, merlin was detected by western blotting from lysates of cells transfected with specific but not the control DNA (Fig. 2A,B). Cell lysates were immunoprecipitated with merlin, ezrin (as a positive control) or an irrelevant control antiserum and coprecipitating ezrin was detected by western blotting. Fig. 2A shows that ezrin was present in the precipitate obtained by ezrin or merlin antibodies but not with control antiserum. The specificity was confirmed by lack of ezrin reactivity in precipitates obtained from β-galactosidase transfectants with merlin antiserum (Fig. 2B). In additional coimmunoprecipitation experiments from U251 glioma cell lysates ezrin could be detected in precipitates by ezrin and merlin antiserum but not by control antiserum (Fig. 2C). These results indicate that merlin and ezrin form a complex in vivo. This is not, however, a proof for a direct interaction between the proteins.

In further experiments, we tested whether merlin and ezrin exist as dimers in U251 cells. After treatment with a chemical cross-linker, novel bands, at sizes between 140-170 kDa, appeared in merlin and ezrin immunoblots of cell lysates (Fig. 2D). The bands were not present in lysates from cells that were not cross-linked or in lysates separated under reducing conditions. One of the separated bands was identically blotted with both merlin and ezrin antibodies, raising the possibility that it represents the merlin-ezrin heterodimer. The size of the lower band in the merlin immunoblot is consistent with a merlin homodimer. However, the possibility that merlin and ezrin would form a complex with other partners cannot be ruled out.

General comments of the in vitro interaction studies
To further study the interaction between merlin and ezrin and to map the interacting domains, several different approaches...
Homo- and heterotypic interaction of merlin and ezrin were used. A set of amino- and carboxy-terminal deletion constructs of merlin and ezrin was generated and expressed using the yeast two-hybrid system (Fig. 3). By the yeast mating system, combinations between all bait and prey constructs were analyzed, expressing the baits in the yeast mating type α and the preys in α mating type or vice versa. Interactions were detected by the survival of yeast in leucine-deficient medium (not shown) and by induction of β-galactosidase production.

The yeast coexpressing a bait (LexA-fusion) and a prey (HA-fusion protein) were also used for coimmunoprecipitation. Yeast lysates were coimmunoprecipitated using an anti-HA mAb and coprecipitating bait constructs were immunoblotted with the anti-LexA antibody. The yeast two-hybrid system showed slight variation in the expression levels of the proteins, when expressed in bait or prey vectors (Fig. 4). Regardless of whether the constructs were expressed as baits or preys similar binding results were obtained. Merlin constructs 1-585 and 339-595 were transactivating as baits in the two-hybrid system and therefore not suitable for scoring of β-galactosidase values. They could, however, be used in the coimmunoprecipitation experiment. Shorter carboxy-terminal constructs of merlin than m 339-595 could not be introduced into yeast cells since the cells failed to grow after transformation. The constructs may be toxic to the yeast or they may affect the cell proliferation.

Two additional independent methods were used to confirm the yeast two-hybrid results. We used the blot overlay experiments with purified recombinant protein constructs, as it has been successful in mapping the association domains of ezrin (Gary and Bretscher, 1995). In these experiments immobilized full-length ezrin is denatured and therefore the C-ERMAD is active (Gary and Bretscher, 1995). We also tested, using the affinity precipitation method, whether carboxy-terminal domains of merlin and ezrin from total cell lysates bind to GST-merlin.
Homotypic binding of merlin in vitro

An interaction between merlin molecules (m 1-595) was detected in the two-hybrid system by the activation of the β-galactosidase receptor gene (Fig. 5). This self-association could be further confirmed by coimmunoprecipitation of the expressed proteins from the yeast lysates (Fig. 6). If the last 10 or 49 carboxy-terminal amino acids were removed from one of the binding partners, the interaction was retained (Fig. 5). However, after removal of the residues from both proteins no binding was seen. This is very evident in Fig. 6, in which m 1-595 coimmunoprecipitates m 1-595 and m 1-585 whereas no interaction is seen between m 1-585 and m 1-585.

Mapping of the interaction domains indicated that m 1-595 interacts with the amino-terminal part of merlin. The first 339 amino acids, which contain the amino-terminal globular domain, are sufficient for binding, while amino acids 1-167 did not mediate the interaction. The carboxy-terminal domain (m 252-595), which contains the last 343 amino acids, bound to all four constructs (1-339, 1-546, 1-585, 1-595) that contain the intact amino-terminal domain (Figs 5, 6). A shorter carboxy-terminal construct, m 339-585, however, did not bind m 1-595 (Fig. 6) or m 1-339 (not shown). The merlin carboxy-terminal construct m 339-585, with the last 10 amino acids deleted, bound the short merlin amino-terminal construct m 1-167, but not constructs with an intact amino-terminal domain (Fig. 5).

M 339-585 also bound merlin carboxy-terminal construct m 252-595, whereas carboxy-terminal constructs with an intact carboxy terminus, m 252-595 (Fig. 5) and m 339-585 (not shown) did not bind m 252-595.

Based on the β-galactosidase activity, the strongest binding was detected between two full-length proteins (Fig. 5B). High β-galactosidase values were also seen in interactions between m 1-595 and m 1-339, between m 1-595 and m 1-546, and between m 1-167 and m 339-585.

The homotypic amino-terminal to carboxy-terminal association of merlin was also demonstrated by an independent assay, in which affinity precipitation was performed with recombinant proteins (Fig. 7). In these experiments, total lysates of yeast cells expressing merlin HA-fusion proteins, were allowed to bind beads containing GST-merlin constructs. GST-fusion proteins expressing merlin amino acids 1-595, 1-546 and 1-339, but not GST alone, bound to merlin 252-595 present in a cell lysate. These results further support an amino-to carboxy-terminal binding of merlin.

Homotypic interactions of ezrin in vitro

The yeast two-hybrid experiments with ezrin constructs provided results that were in accordance with previous studies (Gary and Bretscher, 1995). Unlike the results with merlin, two full-length ezrin molecules did not interact, if the proteins were in a native form (Figs 5, 6). In the blot overlay experiments (Fig. 8), where the immobilized protein had been exposed to...
Homo- and heterotypic interaction of merlin and ezrin

SDS, an interaction was detected as described earlier (Gary and Bretsch, 1995). Ez 1-585 and a carboxy-terminal construct ez 278-585 bound strongly in the blot overlay (Fig. 5) and coimmunoprecipitation (Fig. 6), and ez 1-585 bound to GST-ez 477-585 in the blot overlay (Fig. 8). The amino-terminal residues ez 1-309 did not interact with ez 1-585. Apparently, the C-ERMAD of the ez 1-585 protein is masked and cannot interact with ez 1-309 that contains the N-ERMAD. The amino-terminal residues 1-309 but not 1-170 bound the carboxy-terminal residues 278-585 in which the C-ERMAD is exposed (Figs 5, 6). In the blot overlay experiment the carboxy-terminal biotin-labeled probe (GST-ez 477-585) also bound the α-helical domain of ezrin (GST-ez 279-531), which could be a result of the two α-helices binding to each other. It also shows a weak band with GST-ez 477-585, which does not fit the model of head-to-tail binding. However, this band is clearly not as strong as the other ezrin interactions, and could represent a background signal (Fig. 8).

Heterotypic interactions of merlin and ezrin in vitro

The full-length ezrin and merlin did not bind to each other in the yeast two-hybrid system (Figs 5, 6). The result is thus analogous with findings for two ezrin monomers. However, constructs of merlin or ezrin with amino-terminal deletions (m 252-595, ez 278-585) heterodimerized with amino-terminal domains of reciprocal proteins. Very strong interaction was seen between ez 1-309 and m 252-595 (Figs 5, 6). M 1-339 and m 1-585 showed weak binding while m 1-546 bound stronger to ez 278-585 (Fig. 5). These results indicate that association sites in ezrin and merlin must be unmasked for heterodimerization. The regulation and/or binding affinity might slightly differ between ezrin and merlin, since full-length merlin did not bind ez 278-585 whereas full-length ezrin weakly bound m 252-595. However, full length ezrin (ez 1-585) did not bind a shorter carboxy-terminal merlin construct, m 339-585 which lacked the 10 most amino-terminal residues (Fig. 5).

The affinity precipitation results show that the GST-fusion m 1-546 and 1-339 but not m 1-595 binds ez 278-585 in a yeast
total lysate (Fig. 7). The results are in line with the two-hybrid experiments. In the blot overlay experiment, GST-m 1-339 as well as GST-m 1-546 bound to the biotin-labelled GST-ez 477-585 probe (Fig. 8). The result extends the two-hybrid experiments by demonstrating that the last 109 carboxy-terminal residues of ezrin are sufficient for heterodimerization with merlin.

**DISCUSSION**

The understanding of the biological functions and tumor suppressor mechanism of merlin has been hampered by insufficient knowledge of its molecular interactions. So far, only a few direct interaction partners to merlin have been reported. We now show that merlin interacts with an ERM family member, ezrin, and that merlin shows homotypic binding. The evidence for an interaction between merlin and ezrin in vivo includes subcellular colocalization, concomitant redistribution, and most importantly, communoprecipitation of endogenous or transfected merlin and endogenous ezrin from cell lysates and the presence of a band consistent with a merlin-ezrin heterodimer after chemical cross-linking. A heterodimerization between the two proteins could, in addition, be detected by several in vitro techniques. While merlin appears to be involved in regulation of ERM-dependent events, it may also have separate functions from ERM proteins and different means for functional regulation. This is suggested by our evidence that merlin, in contrast to ezrin, does not require exposure of the association domains for homotypic binding.

The homotypic binding of merlin and heterotypic binding between merlin and ezrin, in analogy with ERM proteins, occurred via amino-terminal interaction with the carboxy terminus. This is indicated by the fact that the carboxy-terminal deletions m 1-339 and m 1-546 did not show binding unless the partner contained an intact carboxy terminus. In ezrin, N-ERMAD contains residues 1-296 and further deletion of a few carboxy-terminal amino acids results in loss of activity (Gary and Bretscher, 1995) (Fig. 9). Homotypic binding of merlin occurred via residues 1-339, while 1-167 was inactive. Thus, the amino-terminal association domain of merlin seems to be very similar to ezrin.

Based on the fact that the residues of the carboxy-terminal association domain of ezrin are poorly conserved in merlin, it has been suggested that the carboxy terminus of merlin would not allow self-association (Gary and Bretscher, 1995). Our results indicate that this is not the case. In fact, m 252-595 not only bound to the amino terminus of merlin but also to ezrin. The carboxy-terminal binding region of merlin could not be mapped as precisely as the C-ERMAD of ezrin which is contained in residues 479-585. The C-ERMAD of ezrin mediates not only intramolecular binding to N-ERMAD of ezrin, but also to moesin (Gary and Bretscher, 1995), and as shown here, binding to the amino-terminal association domain of merlin. This result may reflect the conservation of the amino-terminal domain between ERM proteins and merlin, which apparently includes the residues critical for C-ERMAD binding. While the construct m 252-595 binds to the amino-terminal domain of merlin, m 339-595 did not mediate the interaction. This construct, m 339-595, disrupts the α-helical domain which could abolish a conformation needed for the interaction. However, overlapping carboxy-terminal constructs of ezrin, GST-ezrin fusion proteins 325-585 and 368-585, interact with the full length ezrin molecule (Gary and Bretscher, 1995). Alternatively, the result indicates that merlin needs a longer carboxy-terminal domain for binding than ezrin. A similar feature for the binding domain of merlin and ezrin is the absolute requirement of the most carboxy-terminal residues. In ezrin, deletion of the last two amino acids resulted in loss of activity (Gary and Bretscher, 1995), in merlin the last 10 residues were needed for interaction with the amino-terminal association domain. However, these ten residues are not sufficient for the interaction, which apparently involves an interplay between two or more different regions. If the last 10 amino acids in the merlin carboxy-terminal construct were removed the binding characteristics became strikingly different. This construct, m 339-585, did not bind the amino-terminal association domain in merlin, m 1-339, but did bind a shorter amino-terminal construct, m 1-167, and surprisingly the intact carboxy-terminal construct, m 252-595. This could indicate that the properties of the interactions are more complex than portrayed here, or that the constructs, m 339-585 and m 1-167, that disrupt the α-helix and the amino-terminal globular domain, respectively, do not present the folding patterns and charge of the intact association domains or intact molecule. This could allow the deviant binding to take place.

Our study indicates that the regulation of homotypic head to tail association of merlin is different from ezrin. This difference may have functional consequences. Based on the current model, an intramolecular association between N-ERMAD and C-ERMAD retains ERM proteins in a dormant state, in which binding sites for cell membrane components and actin are masked (Gary and Bretscher, 1995; Martin et al., 1997; Tsukita et al., 1997). In line with this idea, full length ezrin, when overexpressed in SF9 cells, does not induce morphogenetic cell surface alterations (Martin et al., 1995, 1997). Unmasking of these binding sites by conformational activation (Bretscher, 1989) or by amino- or carboxy-terminal deletions (Martin et al., 1995, 1997), results in prominent cell surface alterations. In contrast to ezrin, expression of merlin in mammalian cells (Sainio et al., 1997) or SF9 cells (F. Zhao,
MERLIN AND EZRIN

M. Sainio and O. Carpén, unpublished) results in cell surface alterations reminiscent of those caused by ‘active’ ezrin constructs. It is possible that this difference reflects the differential regulation of homotypic association between merlin and ezrin. In the yeast two-hybrid experiments, in which the proteins should retain a native conformation, full-length merlin (1-595) can bind another full-length molecule, while full-length ezrin (1-585) cannot bind another native ezrin or merlin molecule. A possible explanation for this is that the intramolecular association in ezrin is so stable that dimerization between expressed proteins does not occur. In merlin, the intramolecular association is not of high affinity and dimerization can occur. Dimerization or oligomerization has previously been shown to correlate with functional activity of ERM proteins (Berryman et al., 1995).

Full-length merlin binds amino- and carboxy-terminal constructs of merlin, but not amino- or carboxy-terminal constructs of ezrin. For the heterotypic interaction between merlin and ezrin, merlin has to be expressed as a truncated protein. Our interpretation of the results is the following. In the native merlin, the intramolecular binding sites, which allow homodimerization are exposed, whereas the binding sites for ezrin are masked. Heterodimerization between merlin and ezrin occurs only following conformational alterations in both proteins. Thus, also merlin apparently undergoes conformational activation, which is a prerequisite for heterodimerization but not for homodimerization. The differential regulation of homodimerization versus heterodimerization of merlin suggests that the rank of order for binding partners depends on cellular signals that affect activation of merlin and ezrin. The in vivo coimmunoprecipitation experiments strongly suggest that in cells at least a fraction of merlin and ezrin are in an active conformation and can associate heterotypically.

Merlin's tumor suppressor mechanism involves an ability to suppress cell proliferation with an unknown mechanism. Studies of the expression of merlin and ERM proteins in schwannomas, the tumors associated with NF2 gene defects, showed loss of merlin in all tumors, whereas the expression of ezrin, radixin and moesin is retained (Stemmer-Rachamimov et al., 1997). The ERM proteins are associated with cell growth, as they are downstream targets of the Rho family of small G proteins, that are involved in regulation of cell growth (Mackay et al., 1997). Moreover, abnormal regulation or high expression of ezrin has been associated with cell transformation and increased proliferation (Jooss and Muller, 1995; Kaul et al., 1996; Lamb et al., 1997). In this regard, the possible opposite effects of merlin and ezrin on cell proliferation may be linked to differential regulation of their association ability. Sherman et al. (1997) suggested that tumor growth inhibition by merlin depends on an interdomain association that occurs either in cis or in trans between the amino- and the carboxy-terminal domains. An alternative explanation is that the intact amino- and carboxy-terminal domains of merlin are required for heterotypic association with ezrin and other ERM proteins. Analysis of functional consequences of heterotypic binding between merlin and ezrin could provide novel information of merlin’s tumor suppressor function.

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Note added in proof
While this manuscript was under review, another publication demonstrating a homodimeric association of Merlin appeared (Huang et al., 1998).