Hierarchy of Merlin and Ezrin N- and C-terminal Domain Interactions in Homo- and Heterotypic Associations and Their Relationship to Binding of Scaffolding Proteins EBP50 and E3KARP*

Rachel Nguyen, David Reczek, and Anthony Bretscher†

From the Department of Molecular Biology and Genetics, Cornell University, Ithaca, New York 14853

The neurofibromatosis 2 tumor suppressor gene product merlin has strong sequence identity to the ezrin-radixin-moesin (ERM) family over its ~300-residue N-terminal domain. ERM proteins are membrane cytoskeletal linkers that are negatively regulated by an intramolecular association between domains known as NH2- and COOH-ERM association domains (N- and C-ERMADs) that mask sites for binding membrane-associated proteins, such as EBP50 and E3KARP, and F-actin. Here we show that merlin has self-association regions analogous to the N- and C-ERMADs. Moreover, the N/C-ERMAD interaction in merlin is relatively weak and dynamic, and this property is reflected by the ability of full-length recombinant merlin to form homooligomers. Remarkably, the merlin C-ERMAD has a higher affinity for the N-ERMAD of ezrin than the N-ERMAD of merlin. Both the ezrin and merlin N-ERMAD bind EBP50. This interaction with the ezrin N-ERMAD can be inhibited by the presence of the ezrin C-ERMAD, whereas interaction with the merlin N-ERMAD is not inhibited by either C-ERMAD. E3KARP binds tightly to the ezrin N-ERMAD but has little affinity for the merlin N-ERMAD. The implications of these associations and the hierarchies of binding for the function and regulation of merlin and ERM proteins are discussed.

The disease neurofibromatosis 2 (NF2) is a genetic disorder in which loss of heterozygosity of the NFZ gene leads to bilateral schwannomas of the auditory nerve and other tumors of the central nervous system (1). Genetic analysis and positional cloning mapped the human NFZ gene to chromosome 22 and led to the identification of the affected gene. The NF2 gene product, a protein referred to as either merlin or schwannomin (2, 3), shows sequence similarity to the ezrin-radixin-moesin (ERM) family of cytoskeleton-plasma membrane linking proteins, sharing ~65% sequence identity with the ERM family over the first 300 residues with ~45% identity overall. Two major isoforms of human merlin have been documented. Isoform I has 595 residues, resulting from translation of exons 1–15 and 17, and isoform II has 590 residues and is a translation of exons 1–16, replacing the last 16 residues from exon 17 of isoform I with 11 residues from exon 16 (4, 5).

Insight into the possible roles of merlin have come in part from studies of mice and flies lacking merlin, as well as from the identification of merlin-binding proteins. NFZ knockout mice are embryonic lethal as they do not develop proper extraembryonic tissues and fail to implant (7) and NFZ+/− heterozygous mice develop a wide range of metastatic tumors (8). Loss of merlin in Drosophila melanogaster results in overproliferation of cells (9). Interestingly, merlin shares many biochemical and cell biological similarities with the ERM family. In addition to their partly overlapping subcellular distributions (10–13), they bind to several common proteins, such as CD44, EBP50/human Na+/H+ exchanger regulatory factor, and RhoGDI (13–19). Moreover, merlin can associate with ERM family members (15, 20, 21). However, the nature of these interactions have so far not been examined in detail, especially in comparison with the better studied ERM proteins.

The three ERM family members are ~580 residues long and share ~75% sequence identity. They provide a regulated linkage between membrane-associated proteins and the cortical actin cytoskeleton, and they also participate in and are regulated by signal transduction pathways (for recent reviews, see Refs. 22–25). Biochemical and structural studies have revealed the nature of the conformational regulation of the ERM proteins. The NH2-terminal ~300-residue domain of any ERM member can form a tight association with the COOH-terminal ~100 residues of any member, leading these regions to be called NH2- and COOH-ERM association domains (N- and C-ERMADs) (26, 27). In their dormant monomeric state, the intramolecular interaction between the N- and C-ERMADs masks the C-terminal F-actin binding site (28, 29) and N-terminal binding sites for some membrane-associated proteins and Rho-GDI (19, 26, 30). Among the proteins known to bind the N-terminal domain of ezrin in a regulated fashion is the PSD-95/DigAZO-1-like domain-containing scaffolding protein EBP50 (an ERM-binding phosphoprotein of 50 kDa) (17, 30). Interestingly, merlin also binds EBP50 (also called human Na+/H+ exchanger regulatory factor (16)). Activation of ERM proteins to unmask binding sites involves dissociation of the intramolecular N/C-ERMAD, which can be achieved by phosphorylation of a threonine 20 residues from the C terminus in combination with specific inositol phospholipids (31–37).

The possibility that merlin might be subject to a similar regulatory mechanism has been investigated in a number of studies. In support of this possibility is the finding that the N-terminal half of merlin can associate in vitro with the C-terminal half of isoform I but not isoform II (6, 15, 20, 21), and

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† To whom correspondence should be addressed: Dept. of Molecular Biology and Genetics, Biotechnology Building, Cornell University, NY 14853. Tel.: 607-255-5713; E-mail: apb5@cornell.edu.

‡ The abbreviations used are: NF2, neurofibromatosis 2; ERM, ezrin-radixin-moesin; ERMAD, ERM association domain; GST, glutathione S-transferase; MBP, maltose-binding protein; Ez, ezrin; Mr, merlin; MES, 2-(N-Morpholino)ethanesulfonic acid; HA, hydroxyapatite.
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Ezrin and merlin have been shown to exist as heterodimers in vivo (15). Moreover, EBP50/human Na⁺/H⁺ exchanger regulatory factor binds better to merlin isoform II than to isoform I, suggesting that the C terminus is important in this intramolecular association (20). However, the idea that merlin might be regulated by a mechanism equivalent to the N-/C-ERMAD associations was initially considered unlikely, as isoform I shares only 24% sequence identity with the ERMs over the region corresponding to the C-ERMAD.

Therefore, we set out to determine whether the regions in the N- and C-terminal halves of merlin described above might be functionally analogous to the N- and C-ERMADs of the ERM proteins. Concurrently, the availability of the atomic structure of the moesin N-/C-ERMAD complex allowed us to use sequence alignments with merlin to examine whether isoform I was likely to have N- and C-ERMADs. Remarkably, the alignment showed that residues potentially on the N- and C-ERMAD interface were strongly conserved between merlin and the ERM proteins, thereby predicting a similar interaction for merlin (38). During the completion of the present work, affinity coelectrophoresis studies reported a high affinity interaction between the N- and C-terminal halves of merlin and between the N-terminal half of merlin and the C-terminal half of moesin (20). Analyses of homo- and heterotypic associations, as well as interactions involving the scaffolding proteins EBP50 and E3KARP, identify hierarchies of binding reactions between domains of these proteins. These studies allow us to demonstrate regulated interactions between merlin, ezrin, and their ligands that need to be integrated into any model for tumor suppression by merlin.

MATERIALS AND METHODS

Antibodies and Reagents—Rabbit antisera to ezrin have been described (39). Antiserum to N-terminal domain (residues 1-313) of merlin was raised against purified recombinant protein in rabbits at Cornell University (Ithaca, NY). cDNA for full-length merlin was a kind gift from Dr. J. F. Gusella. MBP-E3KARP was a kind gift from Dr. C. H. Yun. Restriction enzymes were purchased from Life Technologies, Inc., and fast protein liquid chromatography instrumentation was from Amersham Pharmacia Biotech. cDNA for full-length merlin was a kind gift from Dr. C. H. Yun. Restriction enzymes were purchased from Life Technologies, Inc., and fast protein liquid chromatography instrumentation was from Amersham Pharmacia Biotech.

Cloning of Ezrin and Merlin Constructs, EBP50, and E3KARP—The cloning of full-length ezrin (residues 1-586) and Ez-1-297 has been described (30). The cDNA for merlin N-terminal domain was purchased from Genome Systems Inc. (St. Louis, MO). This cDNA was subcloned into expression vector pQE50 (Qiagen Inc., Chatsworth, CA) by polymerase chain reaction amplification of JJRR-4 plasmid at appropriate sites with 5'- and 3'-end backbone of BSII/SK- plasmid from Dr. J. F. Gusella was used to generate the full-length subclone of full-length merlin for in vitro expression in E. coli. Briefly, the 1.2-kilobase pair fragment from the partial digest of clone JRR-1 with BamHI and HindIII, which contains the C-terminal half of merlin, was spliced to the N-terminal half of merlin in pQE50 at the 865 HindIII site of the coding sequence of merlin, and the 3'-end backbone of BSII/SK- was spliced to pQE50 at the BamHI site. The subclone, named JRR-4 (JRR-1 recloned, colony 4), was checked by restriction digest and found to be free of errors by DNA sequencing. All pQE50-derived plasmids were propagated in JM110 strain of E. coli (Qiagen). The cloning and expression of the COOH-terminal GST fusion constructs of ezrin, GST- Ez-475-586 and GST-Ez-475-584, have been described (28). The COOH-terminal GST fusion constructs of merlin, GST-Mr-359-595ci (isoform I), GST-Mr-359-595ci (isoform II), GST-Mr-477-595, GST-Mr-477-593, GST-Mr-477-588, and GST-Mr-502-595 were made by polymerase chain reaction amplification of JRR-4 plasmid at appropriate sites with 5'-BamHI and 3'-EcoRI overhangs and cloned into vector pGEX-3X (Amersham Pharmacia Biotech). Polymerase chain reaction products were inserted into pCRII Topo TA vector (Invitrogen Inc., Carlsbad, CA) and fidelity was confirmed by sequencing. These clones were propagated in JM110 strain of E. coli and EcoRI sites. All pGEX-derived plasmids were propagated in DH5α. The cloning of GST-EBP50 (30) and MBP-E3KARP (40) has been described.

Expression and Purification of Recombinant Proteins—For expression, untagged full-length and N-terminal constructs of ezrin and merlin, Ez-1-586, Ez-1-297, Mr-1-595, and Mr-1-313 were propagated in E. coli strain M15[pREP4] (Qiagen). Bacteria were grown in LB medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. Freshly shaken overnight cultures were diluted 1:20 or 1:30 and grown with vigorous shaking at 37 °C for 90-120 min until A 600 nm reached 0.4. At this point the culture, which point the bacteria were induced with 1.0 mM IPTG, was incubated with 3 M β-mercaptoethanol, and washed 4–5 times in 0.5% physiologic saline, and pellets were quick-frozen on dry ice/ethanol for later use.

Full-length ezrin (Ez-1-586), Ez-1-297, Mr-1-595 and Mr-1-313 were purified from total bacterial extracts by fast protein liquid chromatography (Amersham Pharmacia Biotech) over hydroxypatite (HA-Ultragal; Amersham Pharmacia Biotech) then S (Amersham Pharmacia Biotech) or Q (Amersham Pharmacia Biotech) Sepharose columns. Methods for purifying full-length ezrin and Ez-1-297 have been described (30). Because expression level is ~1/2 lower for full-length merlin and Mr-1-313, two bacteria pellets each from a 3-liter culture were used per preparation. One 3-liter pellet weighing ~10 g was resuspended in 30 ml of lysozyme solution (50 ml Tris-HCl, pH 7.4, 300 mM NaCl, 1 mM EDTA, 2 mg/ml lysozyme) by vortexing and sonication. The resulting supernatant was loaded over a 10-ml bed volume of HA resin. The two pellets were processed and loaded sequentially. The culture was developed using a 50-ml linear gradient of 0.3–0.8 M KHP04, Full-length merlin eluted at ~0.6 M KHP04, and Mr-1-313 at ~0.5 M. Peak HA fractions of full-length merlin or Mr-1-313 were pooled and dialyzed for 5 h against 60 volumes of Q (20 mM Bis-Tris-propane, pH 6.7, 40 mM NaCl, 0.5 mM dithiothreitol) or S (20 mM MES, pH 6.7, 150 mM NaCl) buffer, respectively. Full-length merlin was eluted from the Q Sepharose column with a linear gradient of Q buffer containing 40–1000 mM NaCl, coming off at ~0.5 M NaCl, and Mr-1-313 was eluted from an S Sepharose column with a linear gradient of S buffer containing 150–1000 mM NaCl, coming off at ~0.7 M NaCl.

Binding Assays—Proteins purified off the ion exchange columns were pooled, concentrated into coupling buffer (0.1 M NaCl, pH 8.0, 0.5 μM EBP50, 80 μM E3KARP) and loaded to a 1 ml Ni-NTA column (Qiagen) for covalent coupling to CNBr-activated Sepharose 4B beads (Sigma) according to the manufacturer’s protocol and as described in (17). GST fusion proteins were purified according to the manufacturer’s protocol (Amersham Pharmacia Biotech). Binding assays were performed in BA buffer (50 mM Tris-HCl, pH 7.4, 100 or 150 mM NaCl) for 2 h at 4 °C. 1 ml of beads, with 1–2 μg of protein coupled per ml, were used per reaction. The beads were washed 4–5 times in 0.5 M NaCl. 1 ml of samples was added to the reaction tube to normalize the final volume to 0.5 ml. The beads were incubated in extract for 30–60 min, pelleted by centrifugation at 16,000 × g, and washed 4–5 times in BA buffer. Bound proteins were eluted by boiling in 30–50 μl of 2× Laemmli buffer for 2 min, and 10–20 μl were loaded for analysis by gel electrophoresis.

For the mixing competition assays, bacterial extracts of the proteins of interest, normalized to contain equal amount of the induced proteins, were incubated together with protein coupled beads for 30–60 min. The beads were then pelleted and washed 4–5 times in BA buffer. One-third of the bound fraction was analyzed by SDS-polyacrylamide gel electrophoresis. For the challenge competition assays, beads were first incubated with a saturating amount of the first protein for 30–60 min, washed 4–5 times in BA buffer, and then challenged with the same amount of a second protein.

Gel Electrophoresis and Western Blots—All samples were boiled in Laemmli buffer and analyzed on 10 or 12% gels following standard protocols (41). For Western blots, proteins were transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore, Bedford, MA) using semidry transfer system (Integrated Separation Systems, Hyde Park, MA). Blots were blocked with 10% nonfat dry milk in rinse buffer containing 150 mM NaCl, 0.1% Tween-20, 50 mM Tris-HCl, pH 8.0, incubated with 1:5,000 rabbit antiserum in 1% milk followed by 1:10,000 peroxidase-conjugated goat anti-rabbit IgG (Sigma) in 1% milk. Blots were developed using enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech).

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Gel Filtration—Full-length merlin or ezrin was loaded onto a Superose 6 HR 10/30 (Amersham Pharmacia Biotech) gel filtration column equilibrated with 50 mM Tris-HCl, pH 7.4, 150 or 500 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol at a flow rate of 0.2 ml/min; 0.5-ml fractions were collected. The column was calibrated with conventional gel filtration standards (Sigma). Blue dextran was used to determine the void volume.

RESULTS

Expression of Recombinant Ezrin and Merlin Constructs—Ezrin is regulated by an intramolecular association between the N- and C-ERMADs, and these domains have been mapped to residues 1–297 and 475–586, respectively (Fig. 1A) (26). To determine whether merlin has analogous domains, and whether domains of ezrin can interact with domains of merlin, a number of ezrin and merlin constructs were utilized (Fig. 1B).

We have described several ezrin constructs that were used to define the N- and C-ERMADs as well as to demonstrate that the intramolecular interaction masks the F-actin and EBP50 binding sites in the full-length dormant molecule (26, 30). Among these (Fig. 1B) were untagged ezrin N-ERMAD (Ez-1–297) and GST fusion proteins containing the ezrin C-ERMAD (GST-Ez-475–586) and the mutated C-ERMAD (GST-Ez-475–584) missing the last two residues.

Equivalent and additional constructs for merlin were generated based on an alignment of the ERM and merlin protein sequences (38). Constructs were made to express untagged full-length merlin isoform I (Mr-1–595) and the N-terminal domain equivalent to the ezrin N-ERMAD, Mr-1–313. In addition, we generated constructs to express GST fusion proteins with the C-terminal regions of merlin isoforms I and II in which similarity to the ERMs significantly declines (GST-Mr-359–595i and GST-Mr-359–590ci), as well as regions of merlin isoform I corresponding to the ezrin C-ERMAD (GST-Mr-477–595), ones lacking the last two residues (GST-Mr-477–593) or seven residues (GST-Mr-477–588), and a shorter C-terminal construct (GST-Mr-502–595).

Expression of all of these constructs in bacteria resulted in readily identifiable bands corresponding to the induced proteins (Fig. 1C, asterisks), except for expression of full-length merlin (lane 5) and Mr-1–313 (lane 6). Expression of full-length merlin and Mr-1–313 can be detected by immunoblot with antibodies raised against merlin 1–313. Extracts were prepared from uninduced (U) and induced (I) bacteria. In C and D, molecular masses in kDa are shown at the left.

**Fig. 1. Summary of recombinant proteins used.** A, diagram of ezrin and merlin isoform I. B, diagram of recombinant proteins used: 1, full-length ezrin (residues 1–586); 2, Ez-1–297; 3, GST-Ez-475–586; 4, GST-Ez-475–584; 5, full-length merlin isoform I (1–595); 6, Mr-1–313; 7, GST-Mr-359–595ci isoform I; 8, GST-Mr-359–590ci isoform II; 9, GST-Mr-477–595; 10, GST-Mr-477–593; 11, GST-Mr-477–588; 12, GST-Mr-502–595; 13, GST; 14, GST-EBP50; 15, MBP-E3KARP. C, induced bands (*) of appropriate sizes can be seen after Coomassie staining of total induced bacterial extracts, except for full-length merlin (lane 5) and Mr-1–313 (lane 6). D, expression of full-length merlin and Mr-1–313 can be detected by immunoblot with antibodies raised against merlin 1–313. Extracts were prepared from uninduced (U) and induced (I) bacteria. In C and D, molecular masses in kDa are shown at the left.
is seven residues shorter than GST-Mr-477–595, it runs with a much slower mobility in SDS-polyacrylamide gel electrophoresis (compare Fig. 1C, lanes 3 and 9, respectively).

Purification of Full-length Merlin and Its NH₂-terminal Domain—The purification of full-length natural or recombinant merlin has not been reported, and therefore the intact protein has not yet been characterized biochemically. To aid our studies, we developed methods for the purification of both untagged recombinant full-length merlin and its free NH₂-terminal domain (Fig. 2).

Like ezrin (42, 43), merlin and its NH₂-terminal domain bind to hydroxyapatite resins with high affinity. Immunoblot analysis revealed that full-length merlin in crude bacterial extracts bound quantitatively to this resin (data not shown) and could be eluted using a potassium phosphate gradient. Subsequent chromatography on Q Sepharose yielded the purified full-length protein (Fig. 2A). Likewise, the NH₂-terminal domain (Mr-1–313) bound tightly to hydroxyapatite and could be obtained in a highly purified form after chromatography over S Sepharose (Fig. 2B), in a manner previously described for the ezrin N-ERMAD (17) and shown in Fig. 2C. The yield for each merlin construct was ~0.5 mg/liter of bacterial culture, whereas for the ezrin N-ERMAD it was ~10 mg/liter bacterial culture.

Merlin Isoform I Has Functional Domains Corresponding to the N- and C-ERMADs of Ezrin—To determine whether the C-terminal domain of merlin binds either the merlin or ezrin N-ERMAD, purified Ez-1–297 and Mr-1–313 were coupled to beads and used in pull-down assays. Beads were incubated with total soluble bacterial extracts expressing various constructs (shown in the summary diagram in Fig. 3D) and washed extensively, and bound proteins were eluted and analyzed by gel electrophoresis. In all of these experiments, the same molar concentration of the ezrin or merlin N-terminal domain was immobilized on the beads and the bacterial extracts contained a large excess of recombinant protein to allow for the recovery of both tight and relatively weak interactions as a starting point for our analysis (Fig. 3A).

Both sets of beads bound the ezrin C-ERMAD (Fig. 3A, lanes 1, GST-Ez-475–586), the C-terminal half of merlin isoform I (lanes 3, GST-Mr-359–595ci), and the C-terminal region of merlin corresponding to the ezrin C-ERMAD (GST-Mr-477–595). The C-terminal half of merlin isoform II (lanes 4, GST-Mr-359–590cii) and the merlin constructs lacking the last two or seven residues (GST-Mr-477–593 and GST-Mr-477–588) did not bind either set of beads (lanes 6 and 7). Thus, the C-terminal region of merlin isoform I, but not of isoform II, binds the N-terminal domains of ezrin or merlin, and efficient binding is dependent on the last two residues, as is the binding of the C-ERMAD of ezrin to the N-ERMAD of ezrin (26). We conclude that merlin isoform I has functional N- and C-ERMADs, and we use this nomenclature henceforth.

To compare the binding between homotypic and heterotypic N- and C-ERMADs, the sensitivity of the interactions to increasing salt concentrations was determined (Fig. 3B). Whereas the binding of either C-ERMAD to the ezrin N-ERMAD was relatively unaffected by increasing NaCl washes up to 2 M, the binding of either C-ERMAD to the merlin N-ERMAD was much more salt-sensitive, being significantly reduced by 0.5 M NaCl.

During studies of the various deletion constructs, we were surprised to find that the ezrin C-ERMAD construct lacking

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**Fig. 2.** Purification of full-length merlin (1–595), merlin 1–313, and ezrin 1–297. A, purification of full-length recombinant merlin over hydroxyapatite and Q Sepharose columns. B and C, final elution profiles over S Sepharose of merlin 1–313 (B) and ezrin 1–297 (C). Merlin 1–313 migrates with an apparent molecular mass of ~34 kDa and ezrin 1–297 with an apparent molecular mass of 32 kDa.

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**A**

- 0.3-0.8 M KH₂PO₄
- HA: Mr-1-595
- Q: Mr-1-595

**B**

- 200-
- 92-
- 66-
- 66-
- 31-
- 29-

**C**

- 205-
- 97-
- 66-
- 45-

**S:** Mr-1-313

**S:** Ez-1-297

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**Association of Merlin with Ezrin and EBP50**
the last two residues (GST-Ez-475–584) showed significant binding to the ezrin N-ERMAD (Fig. 3C, top panel) under physiological salt conditions. Previously, we had reported that the presence of these two residues were critical for the homo- 

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Merlin exists as oligomers under conditions of physiological salt. Full-length ezrin or full-length merlin were chromatographed on a Superose 6HR gel filtration column in either 0.15 or 0.5 M NaCl. The migration of standards (bovine serum albumin (BSA) at 66 kDa, and carbonic anhydrase (CA) at 29 kDa) and of ezrin monomers (M) and dimers (D), as described (43), are indicated.

**Fig. 4.** Merlin exists as oligomers under conditions of physiological salt. Full-length ezrin or full-length merlin were chromatographed on a Superose 6HR gel filtration column in either 0.15 or 0.5 M NaCl. The migration of standards (bovine serum albumin (BSA) at 66 kDa, and carbonic anhydrase (CA) at 29 kDa) and of ezrin monomers (M) and dimers (D), as described (43), are indicated.

**Fig. 5.** Competitive binding between N- and C-ERMADs of ezrin and merlin. A, competition between homo- and heterotypic C-ERMADs. Ez-1–297 (EzN), Mr-1–313 (MrN), or bovine serum albumin (BSA) beads were incubated for 30 min with a saturating amount of bacterial extract containing an equal mixture of Ez-475–586 and Mr-477–595 (Load). After washing, bound protein was eluted and analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie staining. B, sequential competition between the two C-ERMADs. Ez-1–297 or Mr-1–313 beads were incubated first with saturating amounts of Ez-475–586 or Mr-477–595, washed, and then challenged with a second round of the alternative C-ERMAD ligand. C, competition between N-ERMADs for the ezrin C-ERMAD. Left panel, purified Ez-1–297 and Mr-1–313 were incubated with GST-Ez-475–586 beads alone (lanes 1 and 2) or together (lane 3), the beads were washed and eluted, and released proteins were analyzed. Right panel, the two N-ERMADs were incubated with GST-Ez-475–586 beads (GST-EzC) and then challenged with buffer alone (lanes 4 and 5) or with the alternate N-ERMAD (lanes 6 and 7). L, load (mixture of the N-ERMADs used in these experiments).

Because both N-ERMADs showed a preference for the ezrin C-ERMAD, we wished to determine which of the two N-ERMADs bound preferentially to the ezrin C-ERMAD. Therefore, the ability of immobilized ezrin C-ERMAD (GST-Ez-475–586) to retain the two N-ERMADs was assessed (Fig. 5C). Both N-ERMADs bound to the ezrin C-ERMAD, whether added alone or together (Fig. 5C, left panel). When one N-ERMAD was prebound and then challenged with the other, the resin still bound both, but with a small preference for the ezrin N-ERMAD.

Because our results indicate that the merlin C-ERMAD has a higher affinity for the ezrin over the merlin N-ERMAD, it should be possible to bind full-length merlin to immobilized ezrin N-ERMAD. In fact, when a bacterial extract containing a very low level of full-length merlin (Fig. 1C, lane 5) is passed over a resin containing immobilized ezrin N-ERMAD, full-length merlin is retained (Fig. 3A, left panel, lane 2). Moreover, full-length merlin can also be affinity purified on immobilized merlin N-ERMAD (Fig. 3A, right panel, lane 2). A similar experiment done with full-length ezrin over immobilized ezrin N-ERMAD does not retain significant amounts of ezrin (17), revealing how much more dynamic the N/C-ERMAD association is in merlin than in ezrin.

**Relative Binding of EBP50 and E3KARP to N-terminal Domains of Ezrin and Merlin**—Human EBP50 was discovered by its ability to be retained from placental extracts bound on immobilized ezrin N-ERMAD (17). It was also discovered in a two-hybrid screen with merlin and called human NHE-RF (16), as it is the human homologue of rabbit NHE-RF (45, 46). EBP50 binds ezrin through its C-terminal 30 residues (30), as does the related protein E3KARP (30, 40). We were therefore...
interested in determining the relative affinities of these proteins for the ezrin and merlin N-ERMADs.

Bacterial extracts expressing GST-EBP50 or MBP-E3KARP were applied to beads containing the immobilized ezrin or merlin N-ERMAD and subjected to washes with increasing salt concentrations, and the retained protein was eluted and analyzed (Fig. 6, A and B). The binding of EBP50 to the ezrin N-ERMAD is highly resistant to salt washes, whereas the interaction with the merlin N-ERMAD is a bit more sensitive. Ezrin N-ERMAD beads retained E3KARP efficiently up to 1M salt, whereas binding to the merlin N-ERMAD was inefficient and very salt-sensitive. Ezrin N-ERMAD beads retained E3KARP efficiently up to 1M salt, whereas binding to the merlin N-ERMAD was inefficient and very salt-sensitive. To determine which ligand bound the N-ERMADs preferentially, extracts expressing GST-EBP50 and MBP-E3KARP were either mixed and applied to the N-ERMAD beads (Fig. 6C, left panel) or applied sequentially in excess (Fig. 6C, right panel). In either case, ezrin N-ERMAD beads bound equivalent amounts of EBP50 and E3KARP, whereas merlin N-ERMAD beads had a strong preference for EBP50.

We next examined whether EBP50 exhibits a preference for the ezrin or merlin N-ERMAD by incubating beads containing immobilized GST-EBP50 tail with the two N-ERMADs simultaneously or sequentially and then analyzed the retained proteins (Fig. 7A). Although both N-ERMADs were retained on the beads, there was some preference for retention of the ezrin N-ERMAD.

We have shown that EBP50 bound to the ezrin N-ERMAD can be largely displaced by subsequent addition of the ezrin C-ERMAD (30). We wished to examine whether the same situation existed with EBP50 bound to the merlin N-ERMAD as well as the ability of either C-ERMAD to displace bound EBP50. These experiments were performed with the two ligands added competitively or sequentially and similar results were obtained. Interestingly, although the ezrin C-ERMAD can displace EBP50 from the ezrin N-ERMAD, the merlin C-ERMAD cannot do this (Fig. 7B). Moreover, EBP50 bound to the merlin N-ERMAD is not displaced by either the ezrin or merlin C-ERMAD (Fig. 7C). Competitive binding between E3KARP with the C-ERMADs on ezrin N-ERMAD beads shows results similar to those for EBP50 (data not shown).

**DISCUSSION**

The studies reported here establish that merlin has functional domains equivalent to the N- and C-ERMADs of ezrin, refining the earlier interaction results (6, 15, 20, 21) and confirming the prediction, based on conserved residues expected to lie on the N-/C-ERMAD interface, that merlin is likely to have functional N- and C-ERMADs (38). The fact that the last two residues of the merlin C-ERMAD are required for this interaction and the lack of interaction between the equivalent C-terminal region of merlin isoform II are consistent with the N-/C-ERMAD model developed for ERM proteins. The questions naturally arose as to how robust this interaction is in merlin, whether ERMAD interactions might exist between merlin and ERM proteins, and how these interactions might affect binding of EBP50 and E3KARP to the N-ERMADs.

By analyzing isolated domains in solution, we have been able to establish the following binding hierarchy for the association domains of merlin, ezrin, and EBP50/E3KARP, from low to high: Mr-N-ERMAD/E3KARP < Mr-N-ERMAD/Mr-C-
FIG. 7. Binding of EBP50 to the ezrin and merlin N-ERMADs, and the effect of competition by the C-ERMADs. A, left panel, equal amounts of Ez-1–297 (EzN) and Mr-1–313 (MrN) were incubated either alone (lanes 1 and 2) or together (lane 3), with the GST fusion construct of the ERM binding domain of EBP50 (GST-EBP50-C) immobilized on glutathione beads. The beads were washed and eluted, and recovered proteins were analyzed. Because the GST-EBP50-C was not covalently bound to the beads, it is also recovered in the SDS elution. Right panel, GST-EBP50-C beads were incubated with one N-ERMAD and then challenged with either buffer or an extract expressing an excess of the alternate N-ERMAD. A mixture of the two N-ERMADs used is shown in lane L. B, competitive binding of GST-EBP50 (EBP) and ezrin C-ERMAD (EzC) or merlin C-ERMAD (MrC) to ezrin N-ERMAD beads. Binding competition between ezrin C-ERMAD and GST-EBP50 to Ez-1–297 (left panel) or Mr-1–313 (right panel) was done both competitively and sequentially. C, competitive binding of GST-EBP50 (EBP) and ezrin C-ERMAD (EzC) or merlin C-ERMAD (MrC) to merlin N-ERMAD beads. The experiment was similar to that shown in B except that merlin N-ERMAD beads were used. Bacterial extracts showing the mixture of recombinant proteins used are shown in lane L.

ERMAD < Mr-N-ERMAD/Ez-C-ERMAD ≤ Ez-N-ERMAD/Mr-C-ERMAD < Mr-N-ERMAD/EBP50 ≤ Ez-N-ERMAD/E3KARP < Ez-N-ERMAD/Ez-C-ERMAD. The competitive binding studies have provided a demonstration of the potential dynamic exchange between binding partners, even though associations may appear to be relatively stable in the absence of a competing species. In a broader sense, this establishes how proteins can select between multiple binding partners on the simple basis of thermodynamic favorability.

The merlin N-C-ERMAD interaction is of lower affinity than is seen for ezrin and is quite dynamic. This is revealed both by studying the interaction between the two separated domains in vitro, as well as the ability of recombinant full-length merlin to be retained on immobilized merlin N-ERMAD. It is also consistent with the finding that full-length merlin migrates as a monomer by gel filtration in 0.5 M NaCl under conditions that inhibit the N-C-ERMAD interaction, but as oligomeric species under physiological conditions, in which the N- and C-ERMADs can interact. Although merlin oligomers can be prepared in vitro, there is so far no evidence for their existence in vivo. Moreover, because merlin is a relatively minor protein, the interaction of merlin with the much more abundant ERM proteins may be more physiologically relevant.

If the homotypic N-C-ERMAD of merlin interaction had been of higher affinity than the heterotypic interaction with the domains of ezrin, this would have suggested that the self-association of merlin may simply be for self-regulation rather than participating in another pathway. Thus, we were very surprised and interested to find that the N-ERMAD of merlin has a strong preference for binding the C-ERMAD of ezrin over its own, and the C-ERMAD of merlin has a slight preference for the N-ERMAD of ezrin over its own. This has interesting implications for cells that express both ezrin and merlin. The bulk of ezrin in the cytoplasm is present in a dormant state, i.e. with its N- and C-ERMADs associated. Activation of ezrin by inactivating its C-ERMAD, perhaps involving phosphorylation and phosphatidylinositol 4,5-bisphosphate binding (32–34, 36, 37), will unmask the N-ERMAD. Because the N-C-ERMAD association in merlin is dynamic and the C-ERMAD has a preference for the N-ERMAD of ezrin over its own, a relatively stable heterodimer is expected to form in which the C-ERMAD of merlin binds to the N-ERMAD ezrin.

A well documented ligand for the N-terminal domains of ezrin and merlin is EBP50/human Na⁺/H⁺ exchanger regulatory factor (16, 17). Interestingly, we find that the closely related protein E3KARP, which binds with high affinity to ezrin (30, 40), has little affinity for merlin and is therefore probably not physiologically relevant. The region of EBP50 involved in binding to the ezrin N-ERMAD resides in the C-terminal 30 residues, a region that is especially well conserved (55% identity) between EBP50 and E3KARP. It will be very interesting to identify the residues in this region that contribute to the binding discrimination and examine the complementary surface residues on the ezrin and merlin N-ERMADs to which they bind. Structural studies, currently under way, that reveal the ERM/EBP50 interface should provide insight into this question.

How might the presence of EBP50 affect the molecular forms of merlin and ezrin present in a cell? EBP50 binds to the merlin N-ERMAD, and this interaction cannot be readily displaced by the merlin C-ERMAD, implying that the association of merlin with EBP50 is not as completely blocked as in ezrin. Merlin
isform II is expected to have a constitutively active N-ERMAD because it does not have an active C-ERMAD, and this difference in EBP50 retention between isoform I and isoform II has been reported (20). By contrast, ezrin can only bind EBP50 when it is activated. Our binding hierarchy indicates that activation of ezrin could lead to EBP50 switching its binding partner from merlin to activated ezrin. However, the complexes that form in vivo will be highly dependent on the local concentrations and activation states of ezrin, merlin, and EBP50.

Our results provide compelling biochemical evidence for an interrelationship between the functions of ezrin and merlin. It is interesting to note that whereas merlin is a tumor suppressor protein, overexpression of ezrin is correlated with uncontrolled growth (47–49). How the functions and regulation of these proteins are intertwined remains to be elucidated, but any model must account for the biochemical interactions documented here. Moreover, this represents an important step in an analysis of binding hierarchies, as merlin and ERM proteins have several additional common ligands, and more are sure to follow. Finally, we have presented a method for expressing and purifying full-length untagged recombinant merlin. As far as we are aware, this is the first report on the purification of the native protein and will provide a valuable reagent for future detailed biochemical and biophysical studies of this tumor suppressor gene product.

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