Interdomain Interaction of Merlin Isoforms and Its Influence on Intermolecular Binding to NHE-RF*

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Merlin, the neurofibromatosis 2 tumor suppressor protein, has two major isoforms with alternate C termini and is related to the ERM (ezrin, radixin, moesin) proteins. Regulation of the ERM proteins involves intramolecular and/or intermolecular head-to-tail associations between family members. We have determined whether merlin undergoes similar interactions, and our findings indicate that the C terminus of merlin isoform 1 is able to associate with its N-terminal domain in a head-to-tail fashion. However, the C terminus of isoform 2 lacks this property. Similarly, the N terminus of merlin can also associate with C terminus of moesin. We have also explored the effect of merlin self-association on binding to the regulatory cofactor of Na⁺/H⁺ exchanger (NHE-RF), an interacting protein for merlin and the ERM proteins. Merlin isoform 2 captures more NHE-RF than merlin isoform 1 in affinity binding assays, suggesting that in full-length merlin isoform 1, the NHE-RF binding site is masked because of the self-interactions of merlin. Treatment with a phospholipid known to decrease self-association of ERM proteins enhances the binding of merlin isoform 1 to NHE-RF. Thus, although isoform 1 resembles the ERM proteins, which transition between inactive (closed) and active (open) states, isoform 2 is distinct, existing only in the active (open) state and presumably constitutively more available for interaction with other protein partners.

Merlin is the tumor suppressor protein deficient in neurofibromatosis 2 (NF2), a dominantly inherited disorder characterized by bilateral vestibular schwannomas and other brain tumors (1, 2). Merlin has a striking similarity in sequence and structure with ezrin, radixin, and moesin, commonly referred to as the ERM proteins. The ERM proteins share ~78% amino acid identity with each other, and all three are 45–47% identical to merlin (3). Like the ERM proteins and protein 4.1, merlin possesses a FERM (protein 4.1, ezrin, radixin, moesin) domain (~270 amino acids defining membership in the protein 4.1 superfamily) in its N-terminal half, followed by a long a-helical segment and a charged C-terminal domain (4). The NF2 gene comprises 17 exons with alternative splicing of the penultimate exon producing two major merlin isoforms. Isoform 1 is a 595-amino acid protein produced from exons 1–15 and exon 17. Isoform 2 results from the presence of the alternatively spliced exon 16, which alters the C terminus of the protein to produce a 590-amino acid protein identical to isoform 1 over the first 579 residues (5–7). Mutational analysis has revealed a wide variety of mutations in the germine and tumors of NF2 patients as well as in sporadic schwannomas and meningiomas, confirming merlin’s tumor suppressor function (8).

ERM proteins act as linkers between integral membrane proteins and the actin cytoskeleton (9). Proteins identified as ligands for ERM proteins include CD44, CD43, ICAM1, ICAM2, and actin (9–13). We and others have recently identified the human homologue of a regulatory cofactor for Na⁺/H⁺ exchanger (NHE-RF) as a novel interactor for the conserved N terminus of merlin and ERM proteins (14, 15). In addition to interacting with many binding partners, the ERM proteins are capable of forming homo- and heterotypic associations between family members (16, 17). Indeed, several recent studies performed on the regulation of ERM proteins suggest that the availability of ERM domains to binding partners is controlled by self-association of the N-terminal and C-terminal regions (13, 18, 19). Thus the ERM proteins can exist in the “closed” state, where the N- and C-terminal regions undergo an intramolecular interaction, masking the respective ligand-binding site. This closed state can be converted to the “open” state in which intramolecular interaction is disrupted by a variety of cellular signals, including Rho-mediated signaling and the phospholipid PIP₂. In vitro binding studies performed with merlin isoforms suggested that the C terminus of isoform 1 can interact with its N terminus, and the C terminus of isoform 2 lacked this property (20). Homotypic interaction of merlin isoform 1 and heterotypic interaction between merlin and the ERM proteins have also been reported recently by yeast two hybrid and blot overlay assays (21, 22).

In view of the importance of self-association in regulation of the ERM proteins, we have used affinity co-electrophoresis (ACE) assays to explore the capacity of the two major merlin isoforms to self-associate and to interact with a representative ERM protein. The uniqueness of this assay is the ability to determine the dissociation constants of the observed interactions. Although, like the ERM proteins, the C terminus of merlin isoform 1 interacts in a head-to-tail fashion with its N-terminal domain, the C terminus of isoform 2 lacks this property. The N terminus of merlin is also able to associate heterotypically with the C terminus of moesin. Furthermore, in affinity binding experiments we observe that full-length merlin isoform 2 is
able to capture greater quantities of NHE-RF than full-length merlin isoform 1, consistent with the notion that the ligand binding is suppressed by the self-interaction of merlin isoform 1. In addition, the interaction between merlin isoform 1 and NHE-RF is enhanced in the presence of the phosphoinositide PIP₂. Thus, merlin isoform 1 behaves like the ERM proteins in its interdomain interaction and its regulation by phospholipids, but merlin isoform 2 does not exhibit this property and is always available for interaction with other ligands. The different regulation of inter- and intramolecular domain interactions of the isoforms of merlin could play an essential role in its tumor suppressor function.

**EXPERIMENTAL PROCEDURES**

**Expression Constructs**—Full-length isoform 1 (aa 1–595), full-length isoform 2 (aa 1–590), N-terminal (aa 1–332), and C-terminal (isoform 1 aa 340–595; isoform 2 aa 340–590; common to both isoforms aa 340–579) portions of merlin were expressed as glutathione S-transferase (GST) fusion proteins in pGEX2T. Also, full-length moesin (aa 1–577), N-terminal (aa 1–332), and C-terminal (aa 307–577) portions of moesin were expressed as GST fusion proteins in pGEX1T. Similarly, full-length NHE-RF (aa 338) was expressed as a GST fusion protein. Expression and purification of the GST fusion proteins were performed as described previously for merlin (23) and using standard methods for moesin and NHE-RF. In addition, full-length merlin isoform 1 and 2 were cloned into the mammalian expression vector pcDNA 3 engineered to have a FLAG tag at the N terminus. These constructs were transiently expressed in Cos-7 cells as described previously (24).

**Antibodies**—The polyclonal anti-merlin antibody (N21) and the polyclonal anti-GST antibody have been described previously (23, 25). A monoclonal anti-GST antibody have been described previously (23, 25). A rabbit polyclonal antibody IC270 was raised against the GST-NHE-RF fusion protein (aa 270–358). The anti-FLAG antibody M2 was commercially obtained (Kodak, IB).**

**Affinity Co-electrophoresis**—For affinity co-electrophoresis (ACE), purified GST fusion protein products of merlin and moesin were thrombin cleaved. ACE gels were prepared using 1% low melting point agarose in 125 mM potassium acetate, 50 mM Hepes, pH 7.5, and carried out as described above (25). Gels were run at 60 volts for 4 h, and the proteins were then transferred to nitrocellulose by capillary action and analyzed by immunoblotting using an affinity eluted N terminus–specific anti-merlin antibody (N21) at a dilution of 1:100 and protein A conjugated to 125I. Retardation coefficients were calculated as described previously (27). Dissociation constants were calculated from nonlinear, least squares fitting of plots of corrected retardation coefficient versus concentration of retarding protein (27, 28). Data were then fit, using a nonlinear least squares approach (Kaleidagraph, Synergy Software), to the equation $R = R_m/\left(1 + \left[P_0/k_{assoc}\right]\right)$, where $R$ is retardation coefficient and $P_0$ is protein concentration in a given lane of an ACE gel. The variables that were fit simultaneously were $K_d$, the dissociation constant, and $R_m$, the maximum value of $R$. Data from two independent experiments were used for calculating dissociation constants, and the $K_d$ value is shown with S.E.

**Affinity Precipitation Assays**—ZR-75-B cells were lysed in Brij lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 8.0, 30% glycerol, 1% Brij 96) containing a 1× protease inhibitor mixture (Roche Molecular Biochemicals), and the lysate was incubated with 600 pmol of GST-merlin immobilized on glutathione (GSH)-Sepharose 4B beads. The beads were washed extensively with phosphate-buffered saline containing Pefabloc, resuspended in Laemmli loading buffer, subjected to 10% SDS-PAGE, and immobilobilized with IC270 antisera (1:1000). In some experiments, GST-merlin immobilized on beads was incubated with ZR-75-B cell lysates along with 50 µM phosphatidylinositol 4-phosphate (Sigma). Each phospholipid was dissolved in distilled water to a final concentration of 1 mg/mL and sonicated three times each for 10 s.

Cos-7 cells expressing merlin isoforms 1 and 2 as FLAG-tagged proteins were lysed in Nonident P-40 lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 0.5% Nonident P-40) containing a 1× protease inhibitor mixture as described above. The lysate was incubated with 600 pmol of GST-NHE-RF full-length fusion protein or GST protein alone coupled to GSH-Sepharose 4B beads. The beads were washed as described above, and the separated proteins were immunoblotted with the anti-FLAG antibody M2.

**RESULTS**

**Interdomain Interaction of Merlin Isoforms**—To determine whether merlin as an ERM family member is capable of self-association and to ascertain whether the two major isoforms of merlin differ from each other in this property, we used the technique of ACE. Using this technique we were able to demonstrate direct binding between the N- and C terminal of merlin isoforms in solution and to measure the strength of the binding. Briefly, the thrombin cleaved N-term (aa 1–332) and C-terminal (iso 1, aa 340–595 or iso 2, aa 340–590) polypeptides were subjected to affinity electrophoresis in 1% agarose gel in physiological buffer. The N terminus of merlin (at 125 nM) was loaded into a long gel slot perpendicular to the direction of electrophoresis. Thrombin-cleaved GST fusion proteins of merlin C-domains isoform 1 (A), isoform 2 (B), domain common for both isoforms (C), or moesin (D) were loaded at 0, 750, 375, 188, 94, 47, 24, 12, and 6 μM (lanes 1–9, respectively) into multiple wells parallel to the direction of electrophoresis. After electrophoresis, during which time the migrating front of N-domain transversed the zones containing the C-domain, the contents of the gels were transferred to nitrocellulose and visualized by immunoblotting with an antibody against the N-domain of merlin. In E and F, electrophoresis was carried out as in A–D, except the purified GST protein was used as a control in place of the N-domain of merlin in the long slot, and C-domain of merlin isoform 1 (E) or C-domain of moesin (F) was loaded into the multiple wells. The migration of the control protein was detected using a specific antibody against GST. The arrowhead represents the origin where the C termini of various proteins were loaded.

**Fig. 1. Affinity co-electrophoresis of N- and C-domain of merlin (homotypic) and N-domain of merlin and C-domain of moesin (heterotypic).** Thrombin-cleaved GST fusion protein of merlin N-domain (125 nM) was loaded into a long gel slot perpendicular to the direction of electrophoresis. Thrombin-cleaved GST fusion proteins of merlin C-domains isoform 1 (A), isoform 2 (B), domain common for both isoforms (C), or moesin (D) were loaded at 0, 750, 375, 188, 94, 47, 24, 12, and 6 μM (lanes 1–9, respectively) into multiple wells parallel to the direction of electrophoresis. After electrophoresis, during which time the migrating front of N-domain transversed the zones containing the C-domain, the contents of the gels were transferred to nitrocellulose and visualized by immunoblotting with an antibody against the N-domain of merlin. In E and F, electrophoresis was carried out as in A–D, except the purified GST protein was used as a control in place of the N-domain of merlin in the long slot, and C-domain of merlin isoform 1 (E) or C-domain of moesin (F) was loaded into the multiple wells. The migration of the control protein was detected using a specific antibody against GST. The arrowhead represents the origin where the C termini of various proteins were loaded.
Self-association of Merlin Isoforms and NHE-RF Binding

To evaluate whether merlin can interact in a heterotypic fashion with other ERM family members, we performed ACE experiments where the N-domain of merlin was used as the faster migrating protein passing through the zones containing the C-domain of moesin (aa 305–557). Fig. 1D shows the interaction of merlin and moesin by the retardation of migration of the N terminus of merlin. The migration of purified GST used as a control protein was not affected by the merlin C-domain isoform 1 and moesin C-domain over the same range of concentrations (Fig. 1, E and F). From measurements of mobility retardation in Fig. 1A–F, we can calculate the dissociation constant for the interaction of the N- and C-terminal polypeptides of merlin isoforms 1 and 2 (homotypic) and for the interaction of merlin to moesin (heterotypic). To avoid problems arising from the saturation of the films, ImageQuant software (Molecular Dynamics) was used to determine the true midpoint of each of the bands (28). Fig. 2 shows the analyses of the six representative experiments. For every gel, retardation coefficients (R) were determined for each C-terminal protein concentration tested for the C termini. Dissociation constants were measurable for N-mer/C-mer iso 1 and N-mer/C-moesin gels by fitting the data to the equation $r = R_s/[1 + (K_{d,app}/[protein_{tot}])]$ (see “Experimental Procedures”). The remaining gels showed no detectable binding. Data for these gels could not be fit to the equation above and are instead represented as linear fits.

Evaluating Binding to NHE-RF—Using the retardation coefficient, we can calculate the dissociation constant for the interaction of the N- and C-terminal polypeptides of merlin isoforms 1 and 2 (homotypic) and for the interaction of merlin to moesin (heterotypic). To avoid problems arising from the saturation of the films, ImageQuant software (Molecular Dynamics) was used to determine the true midpoint of each of the bands (28).

### Experimental Procedures

**NHE-RF Binds Differentially to Merlin Isoforms**—We recently identified NHE-RF, a regulatory factor for the Na⁺−H⁺ exchanger isoform 3 (NHE3), as an interacting protein for merlin in a two-hybrid screen and demonstrated that NHE-RF can bind to merlin, moesin, and radixin via their conserved N-terminal regions (14). It is well documented that the binding of ERM proteins to their ligands is suppressed in the native full-length protein (13, 18, 19), a phenomenon explained by the interdomain interactions of the ERM proteins that could compete with the ligand binding (29). We therefore investigated whether NHE-RF displays differential binding with isoforms 1 and 2 of merlin by performing affinity precipitation experiments. Briefly, equal quantities of merlin full-length isoforms 1 and 2, N-domain, C-domain of both isoforms 1 and 2, expressed as GST fusion proteins, were bound to Sepharose beads and incubated with equal amount of ZR-75-B cell lysates. After extensive washes, the coupled proteins were removed from the beads by boiling and were detected on Western blots using a specific polyclonal antibody against NHE-RF. The results shown in Fig. 3 demonstrate that the N-domain and full-length isoform 2 of merlin exhibit a greater affinity for NHE-RF than merlin isoform 1. As expected NHE-RF did not bind to either the C-domain of both isoforms of merlin or the GST control protein. These results were further confirmed by at least three independent experiments.

To confirm the differential binding of merlin isoforms to NHE-RF, affinity binding assays were performed utilizing merlin isoforms expressed as FLAG-tagged proteins in Cos-7 cells and GST fusion protein of NHE-RF. The expression of FLAG-tagged merlin isoforms in Cos-7 cells were examined with an anti-FLAG antibody (M2) and found to be equally expressed (Fig. 4, lanes 1 and 2). Cos-7 cell lysates expressing approximately the same amount of the isoforms were incubated with 600 pmol of GST-NHE-RF beads. The bound proteins were separated on a 7.5% SDS-PAGE and probed with M2 antibody (Fig. 4). Merlin isoforms expressed in mammalian cells also revealed a differential binding to NHE-RF, and the analysis of the supernatants that were not bound to the beads clearly showed that NHE-RF beads capture 3–5-fold more merlin isoform 2 than isoform 1 in duplicate set of experiments. These data are consistent with the ACE results supporting that merlin isoform 2 exists constitutively in an open conformation that allows its ligand, NHE-RF, to interact without being hindered by the interdomain interaction that occurs in merlin isoform 1.
Phospholipids Enhance the Binding of NHE-RF to Merlin Isoform 1—Phosphatidylinositol 4-phosphate and PIP2 enhance the interaction of ERM proteins to its ligands CD44, ICAM-1, and ICAM-2 (13, 18). ERM proteins bind to phosphatidylinositol 4-phosphate and PIP2 (30), and it is believed that this binding evokes conformational changes in these proteins in a manner similar to the regulation of vinculin activity by PIP2 (31). We therefore examined whether PIP2 influences the binding of NHE-RF to merlin isoforms. For this, PIP2 or phosphatidylinositol serine was included in the affinity precipitation assays. In the presence of PIP2, the amount of NHE-RF bound to merlin isoform 1 significantly increased (Fig. 5). The intensity of the bands was further analyzed by densitometric scanning of the autorads using transmittance analysis (Fluor-S, Multiimager, Bio-Rad). Results from three independent experiments revealed a 3-fold increase (3.10 ± 1.04) in binding of NHE-RF to merlin isoform 1 in the presence of PIP2. The binding of NHE-RF to either the N-domain of merlin or merlin isoform 2 (1.07 ± 0.45) was not influenced by PIP2 (Fig. 5). The control phospholipid phosphatidyl serine did not enhance the NHE-RF binding to merlin isoform 1 (Fig. 5).

**DISCUSSION**

Despite an overall structural similarity to the ERM proteins, merlin differs from these relatives in having two isoforms with alternative C termini and in having a demonstrated tumor suppressor function. Both isoforms are expressed at the RNA and protein level in a variety of cell lines examined including NF2 target cells such as Schwann and meningial cells (Refs. 6 and 7 and our unpublished data).2 Work from other laboratories has demonstrated the interdomain interaction of merlin using yeast two hybrid, blot overlay, co-immunoprecipitation, and in vitro binding assays (20–22). Employing the technique of ACE, we not only demonstrate the difference between merlin isoforms in their interdomain interaction but also define the affinities of these interactions. The affinities that we have observed for merlin self-interaction and for the interaction of merlin with moesin are quite comparable with that reported for radixin using similar analysis (25). Because the C-terminal construct of merlin containing the common region (aa 340–579) did not exhibit the self-interaction, we believe that the isoform 1-specific C-terminal residues (aa 580–595) are critical for the interdomain binding. This is in agreement with a previous report suggesting that extreme C-terminal protein sequences encoded by exon 17 is critical for the interdomain interaction (20).

It is well established that the interaction of ERM proteins with their membrane partners, as well as with the actin cytoskeleton, is suppressed in the full-length molecule, a phenomenon explained by intramolecular self-association that masks the binding sites for other ligands (32, 33). In the presence of phospholipids such as phosphatidylinositol 4-phosphate or PIP2, the interdomain interaction is disrupted, thus exposing the ligand binding sites (13, 18). Interdomain interaction also blocks the binding of full-length ezrin and radixin to NHE-RF compared with their N termini (29). In our earlier studies (14), we failed to note a similar difference in binding to NHE-RF between the full-length merlin isoform 1 and the N-domain of merlin (aa 1–332). This apparent discrepancy could be explained by the fact that the previous studies were designed to answer qualitatively whether merlin binds to NHE-RF. However, the present studies were done in a quantitative fashion to address the differences in binding between the two isoforms of merlin. Our results demonstrate that the binding of merlin isoform 1 to its ligand NHE-RF is suppressed in the full-length molecule, and in the presence of PIP2 this suppression is relieved. Merlin isoforms expressed in mammalian cells show the same difference in their binding to NHE-RF as the bacterially expressed proteins. The fact that merlin isoforms expressed in mammalian cells show differential binding to NHE-RF suggests that this may have functional significance in vivo.

The studies performed here illustrate the differences in the ability of the two alternatively spliced isoforms of merlin to interact with NHE-RF and further show that a phospholipid such as PIP2 can regulate the interaction of merlin isoform 1 to NHE-RF. Thus these results document that merlin isoform 1 behaves in a manner similar to its ERM relatives, whereas merlin isoform 2 behaves distinctly and binds to NHE-RF more efficiently. Similarly, betaII-spectrin, a C-terminal interactor of merlin, has been shown to interact to a greater extent with the C terminus of merlin isoform 2 than with the C terminus of isoform 1 (34). Phosphorylation of a critical Thr residue at the C terminus of the ERM proteins has been implicated in stabilizing the open conformation of these proteins (19). This Thr residue is conserved in both isoforms of merlin; however, further studies are required to understand whether the phosphorylation of this residue is involved in regulating the intramolecular interaction of merlin isoform 1 and what role it might play in the function of merlin isoform 2.

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2 Solomon, F., personal communication.
Both NHE-RF and the related NHE-RF2 possess two PDZ domains known to mediate protein-protein interactions. The interaction of NHE-RF and NHE-RF2 with merlin and the ERMs is not mediated by the PDZ domains (14, 29, 35). However, the PDZ domains of both NHE-RF and NHE-RF2 can interact with several other membrane proteins, such as Na\(^+\)-H\(^+\) exchanger isoform 3, the β2-adrenergic receptor, the purinergic P2Y1 receptor, and the cystic fibrosis transmembrane conductance regulator, which functions as a Cl\(^-\) channel (35, 36). Thus, NHE-RF and NHE-RF2 appear to act as multifunctional adaptor proteins that may link merlin and the ERMs to different ion channels and receptors, providing many new possibilities for effects on intracellular signaling. Because merlin isoform 2 exists always in the open state, its interaction with NHE-RF and potentially with other merlin interactors may in fact occur when the equivalent sites in merlin isoform 1 and in the ERM proteins are masked in the closed state.

The strategy of comparing merlin with the related ERM proteins can be expected to produce similarities that are instructive concerning the overall function of these types of proteins and differences that could reveal the special tumor suppressor activity of merlin. In this study, we have observed both. The behavior of merlin isoform 1 with respect to interdomain interactions suggests that its regulation is similar to the regulation of ERM protein interactions. Moreover, the interaction of merlin with the ERM proteins suggests that these proteins could also be involved in mutual regulation of each other's activities. Although the full range of merlin interactions with other proteins remains to be delineated, it is likely that merlin sits within a web of interactions comprising multiple partners and signaling pathways, some of which are shared with the ERM family members. Interestingly, studies of the Drosophila homologue of merlin suggest that its growth suppression properties reside within the conserved N-terminal domain of the protein (37). Thus, the distinct tumor suppressor role of merlin could lie either in the distinct regulation of isoform 2, which differs from that of isoform 1 and the ERM proteins, or in the participation of merlin but not the ERM proteins in a signaling pathway that is uniquely important to NF2 target cells such as Schwann and meningeal cells.

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