The neurofibromatosis 2 protein product merlin selectively binds F-actin but not G-actin, and stabilizes the filaments through a lateral association

Marianne F. JAMES*, Nitasha MANCHANDA*, Charo GONZALEZ-AGOSTI^{*1}, John H. HARTWIG† and Vijaya RAMESH^{*2}

*Molecular Neurogenetics Unit, Massachusetts General Hospital, Building 149, 13th Street, Charlestown, MA 02129, U.S.A., and †Division of Experimental Medicine, Brigham and Women's Hospital, Longwood Medical Research Center, 221 Longwood Avenue, Boston, MA 02115, U.S.A.

The neurofibromatosis 2 protein product merlin, named for its relatedness to the ezrin, radixin and moesin (ERM) family of proteins, is a tumour suppressor whose absence results in the occurrence of multiple tumours of the nervous system, particularly schwannomas and meningiomas. Merlin's similarity to ERMs suggests that it might share functions, acting as a link between cytoskeletal components and the cell membrane. The N-terminus of merlin has strong sequence identity to the N-terminal actin-binding region of ezrin; here we describe in detail the merlin–actin interaction. Employing standard actin co-sedimentation assays, we have determined that merlin isoform 2 binds F-actin with an apparent binding constant of 3.6 μ M and a stoichiometry of 1 mol of merlin per 11.5 mol of actin in filaments at saturation. Further, solid-phase binding assays reveal that

merlin isoforms 1 and 2 bind actin filaments differentially, suggesting that the intramolecular interactions in isoform 1 might hinder its ability to bind actin. However, merlin does not bind G-actin. Studies of actin filament dynamics show that merlin slows filament disassembly with no influence on the assembly rate, indicating that merlin binds along actin filament lengths. This conclusion is supported by electron microscopy, which demonstrates that merlin binds periodically along cytoskeletal actin filaments. Comparison of these findings with those reported for ERM proteins reveal a distinct role for merlin in actin filament dynamics.

Key words: actin cytoskeleton, ezrin/radixin/moesin family, merlin, neurofibromatosis 2.

INTRODUCTION

Merlin, the tumour suppressor protein deficient in neurofibromatosis 2 (NF2), has high structural and sequence similarity to the ezrin, radixin and moesin (ERM) family of cytoskeleton–plasma-membrane linker proteins [1]. Merlin is 63% identical with the ERM proteins at the N-terminus, a region designated as the FERM (4.1 and ERM) domain [2]. In ERM proteins, the FERM domain binds directly to the cytoplasmic domains of several integral membrane proteins such as CD44, CD43, intercellular cell-adhesion molecule (ICAM)-1, ICAM-2 and ICAM-3 [3,4]. In addition, these domains in merlin and ERM (MERM) proteins bind the C-termini of the cytoplasmic adaptor protein, Na⁺/H⁺ exchanger regulatory factor (NHE-RF) [5,6], and its related family member NHE-RF2 [7]. The interaction of NHE-RF and NHE-RF2, via their PDZ domains, with multiple ion channels and receptors, might potentially associate the MERM proteins to the Na⁺/H⁺ exchanger type 3, β_2 -adrenergic receptor, purinergic P2Y1 receptor, cystic fibrosis transmembrane conductance regulator, the Na⁺/HCO₃[−] cotransporter and the vacuolar proton pump H⁺-ATPase [7–13].

In MERM proteins, the FERM domain is followed by an extended α -helical region and a charged C-terminus, although merlin maintains only respectively 21% and 35% sequence similarity with each region to the ERM proteins [2]. Moreover, unlike the ERM proteins, merlin exists as two major isoforms distinguished by alternative C-termini. Merlin isoform 1 is a 595-residue protein, derived from 16 constitutive exons, 1–15 and 17,

whereas isoform 2 is a 590-residue protein resulting from the alternatively spliced exon 16, replacing 16 C-terminal residues with 11 novel residues [1]. The C-termini of both isoforms of merlin bind β II-spectrin; isoform 2 binds more effectively [14].

In view of the multiple ligands identified for ERM proteins, it has been proposed that the ability to associate homotypically and heterotypically with each other might regulate their activities [3,4]. The intramolecular or intermolecular interaction of specific N- and C-ERM association domains ('ERMADs') results in folded ('closed') and inactive ERM proteins owing to the masking of their N- and C-terminal ligand-binding sites [3,4]. Similarly, merlin isoform 1 is capable of these 'head-to-tail' interactions, such that its N-terminus can associate with the C-termini of both isoform 1 and moesin, but not with the C-terminus of isoform 2. Merlin isoform 2 therefore seems to exist in a constitutively 'open' or active conformation owing to its exposed ligand-binding sites [15–18]. Phosphorylation and/or the binding of PtdIns(4,5)P₂ seem to disrupt these intramolecular and intermolecular interactions, exposing the N- and C-terminal binding sites and thereby activating their respective plasma-membrane–cytoskeletal cross-linking functions [4,15,19].

The C-termini of both ezrin and moesin bind directly to actin *in vitro* via a conserved actin-binding site contained in the last 34 residues [20–23]. Merlin lacks this domain owing to its partly divergent C-terminal sequence. Despite the lack of a conserved C-terminal actin-binding domain, merlin localizes to actin-rich structures in cultured cells [24–27] and associates with the detergent-insoluble cytoskeletal fraction of cell lysates [26,28,29].

Abbreviations used: ERM, ezrin, radixin and moesin family; FERM, 4.1 and ERM; GST, glutathione S-transferase; MERM, merlin and ERM; NF2, neurofibromatosis 2; NHE-RF, regulatory factor of Na⁺/H⁺ exchanger; WIP, Wiscott–Aldrich syndrome interacting protein.

¹ Present address: Psychiatry Research, University of Zurich, August-Forel-Strasse 1, 8008, Zurich, Switzerland.

² To whom correspondence should be addressed (e-mail ramesh@helix.mgh.harvard.edu).

Recently, binding studies *in vitro* in ezrin revealed two additional actin-binding sites at the N-terminus, residues 13–30 and 281–310, which bind F-actin and both F- and G-actin respectively [30–32]. This actin-binding region is highly conserved in merlin; the ability of merlin to bind actin through this domain was reported, although the influence of the interdomain self-association of merlin isoform 1 on actin binding was not addressed [33]. Another study did not observe this binding site in merlin isoform 1 by ^{125}I -labelled F-actin blot overlays [18].

The present study was undertaken to characterize, in depth, merlin's association with actin, incorporating isoform specificity as well as quantitative and functional analyses. Using standard co-sedimentation and solid-phase binding assays, we show that merlin's ability to self-interact in an isoform-specific fashion alters its ability to bind actin and that the binding affinity for actin is distinct from that determined for ezrin. Pyrene-labelled actin disassembly assays provide evidence that merlin isoform 2 stabilizes actin filaments by binding to their sides and slowing disassembly. These results offer a more complete understanding of merlin's normal role as a cytoskeleton-associated membrane organizing protein, which ultimately might aid in revealing how disruption of this function results in the loss of merlin's tumour-suppressing capacity.

MATERIALS AND METHODS

Materials and antibodies

Actin isoforms were detected by Western blot analyses with a mouse monoclonal actin antibody (Sigma); glutathione S-transferase (GST) protein was detected with monoclonal anti-GST (Santa Cruz Biotechnology). Merlin was detected by using affinity-eluted rabbit polyclonal antibody N21, whereas electron microscopy used the mouse monoclonal antibody 1C4. These antibodies recognize both isoforms of merlin [24]. Purified actin was isolated from rabbit skeletal muscle (α -actin) and rabbit brain (β/γ -actin) as described previously [34]. All experiments *in vitro* were conducted with purified rabbit skeletal muscle α -actin unless specified otherwise. Monomeric pyrene-labelled rabbit skeletal muscle α -actin was prepared as described previously [34].

Preparation of recombinant merlin proteins

Human merlin full-length and truncated fusion proteins were expressed and purified from *Escherichia coli* [24]. Merlin deletion constructs with residues 1–332, 1–345, 340–595 and full-length isoform 1 (residues 1–595) and isoform 2 (residues 1–590) were expressed as GST fusion proteins in pGEX2T. GST fusion proteins were purified with glutathione-Sepharose 4B (Pharmacia) as described previously [24]. For assays in which the GST moiety was removed, fusion proteins were treated with bovine thrombin (Sigma) for 2 h at room temperature.

Actin co-sedimentation

All samples were precleared by centrifugation at 200 000 g (TLA 100 rotor; Beckman Instruments) for 20 min at 4 °C. F-actin (α or β/γ), polymerized at 40 μM in buffer B [10 mM Hepes (pH 7.0)/100 mM KCl/2 mM MgCl_2 /0.1 mM EGTA/0.5 mM dithiothreitol/0.5 mM ATP] with 40 μM phalloidin (Sigma) [34] for 30 min at 37 °C, was added to variable concentrations of merlin full-length or deletion constructs (0.05–3.0 μM), to a final concentration of 4 μM . Samples were incubated for 30 min at room temperature before centrifugation at 200 000 g for 20 min. Supernatant and pelleted samples were adjusted to 150 μl in SDS/PAGE sample buffer. Equal volumes of supernatant and

pelleted samples, in the presence and absence of F-actin, were analysed by SDS/PAGE [10% (w/v) gel] and transferred electrophoretically to nitrocellulose membranes (Bio-Rad). The integrity of F-actin in pelleted samples was revealed with filters treated with 0.2% Ponceau S. Filters were immunoblotted with the N21 anti-merlin antibody (1:100), and proteins were detected with horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence SuperSignal detection systems (Pierce). Assays were conducted in parallel in the absence of F-actin (self-pelleting) and standards for each concentration were run on separate gels to quantify the total protein added (standard curves).

Solid-phase binding assays

GST-cleaved merlin isoforms 1 and 2, as well as N- and C-terminal deletion constructs, were assayed for F- and G-actin binding with a solid-phase protein–protein binding assay essentially as described [31]. Constant (2 μM) or titrated (0.5, 1 and 2 μM) recombinant merlin constructs or controls (BSA or GST) were coated in 96-well microtitre plates (Nunc) in 75 μl of buffer B (F-actin-promoting buffer) overnight at 37 °C. Wells were rinsed and residual binding sites were blocked in 2% (w/v) BSA/coating buffer. After wells had been rinsed with the appropriate F- or G-actin buffer [buffer A; 2 mM Hepes (pH 7.0)/0.2 mM MgCl_2 /0.2 mM CaCl_2 /0.5 mM dithiothreitol/0.5 mM ATP], F- or G-actin (5 μM and 50 nM respectively) was added to each merlin-coated well for 1 h at room temperature. Wells were rinsed and protein complexes were eluted by adding 50 μl of SDS/PAGE sample buffer to each well for 20 min at 65 °C. Samples were resolved by SDS/PAGE, transferred to nitrocellulose and immunoblotted with appropriate anti-merlin and anti-actin antibodies. Signals were detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence detection systems (Pierce). For quantification experiments, constant concentrations of merlin isoforms (1 or 2 μM in 50 μl) were coated on microtitre plates as described above and incubated with 50 μl of 0.25–10 μM F-actin. Actin standards (0.03–4 μM) were run on separate gels to quantify the actin protein bound (standard curves).

Quantification of merlin–actin binding

The relative protein values used to determine the stoichiometry of merlin–actin binding were obtained from Western blots of sedimenting merlin, with or without actin, and known quantities of merlin, by scanning autoradiographs and immunoblots directly with a Fluor-S Multiimager Analyzer (Bio-Rad). The value of merlin full-length isoform 2 and N-terminal construct 1–332 binding to actin was obtained by calculating the difference between the values of pelleted merlin in the presence and in the absence of F-actin. These values were converted to molar concentrations on the basis of the standard curve for each known merlin concentration. Actin saturation was determined by comparing the amount of merlin added into the amount of merlin bound. Linear transformation of these plots (Scatchard plot) provided a K_d and a stoichiometric molar ratio of merlin–actin binding. These results are representative of three independent sedimentation assays. Similarly, the solid-phase binding assay also was used to determine the dissociation constant of merlin isoform 2 binding to F-actin. The binding of actin to coated merlin isoforms was calculated from actin standard curves as described above.

G-actin pull-down assays

GST pull-down assays were performed as described by Rohatgi et al. [35], with minor modifications. Equal amounts of GST fusion proteins were immobilized on glutathione–Sephadex beads at a concentration of approx. 5 mg/ml of packed beads. G-actin was thawed and precleared at 120 000 *g* to remove any polymerized actin. Approximately 5–10 μ l of beads in 100 μ l of XB buffer [10 mM Hepes (pH 7.6)/100 mM KCl/1 mM MgCl_2 /0.1 mM EDTA/1 mM dithiothreitol], plus 0.1% (v/v) Tween 20, 0.2 mM ATP and 0.5 mg/ml ovalbumin, was incubated with 0.05 μ M G-actin, a concentration below the barbed-end critical concentration. After gentle tumbling for 1 h at 4 °C, the beads were washed twice in 100 μ l of XB buffer plus 0.1% (v/v) Tween 20 and 0.2 mM ATP. Proteins bound to the beads were eluted with SDS/PAGE sample buffer, resolved on a Tris/HCl minigel [10% (w/v) gel] (Bio-Rad) and immunoblotted with a monoclonal anti-actin antibody. Purified NWIP (N-terminal WASP-interacting protein) was kindly provided by Dr N. Ramesh (Children's Hospital, Boston, MA, U.S.A.).

Fluorescent measurement of actin assembly/disassembly

The effect of full-length isoform 2 and N-terminal merlin 1–332 on the rate and extent of polymerization and depolymerization of pyrene-labelled rabbit skeletal muscle actin was determined [34]. For actin polymerization assays, monomeric pyrene-labelled α -actin was polymerized to a final concentration of 1.5 μ M in the presence or absence of either 0.03–1.5 μ M GST–merlin isoform 2, N-terminal 1–332 or GST (control) proteins in buffer B. For depolymerization assays, pyrene-labelled F-actin (10 μ M) was diluted to 0.2 μ M in the presence or absence of 0.004–0.2 μ M merlin proteins in buffer A [36]. The increase or decrease in the rate of fluorescence was monitored in a PerkinElmer model LS-50 fluorimeter with excitation and emission at 366 and 386 nm respectively, over approx. 10 min. The final fluorescent readings for each sample were obtained at a single time point on the next day.

Electron microscopy

Immunogold labelling was conducted with the anti-merlin monoclonal antibody, 1C4, on Triton X-100-permeabilized meningioma cells. Meningioma cells were plated on coverslips and permeabilized with PHEM buffer containing 0.75% (v/v) Triton X-100 [34] plus 5 μ M phalloidin and protease inhibitors for 2 min. Soluble proteins were removed with a PHEM buffer wash. Before labelling glass-adherent cytoskeletons with the anti-merlin IgG, residual fixative was blocked with 0.1% NaBH_4 in PHEM buffer and the coverslips containing the cytoskeletons were washed twice in PBS and twice in PBS containing 1% (w/v) BSA, pH 8.2. Coverslips were covered with 25 μ l of primary antibodies at a final concentration of 10 μ g/ml, incubated for 1 h at 25 °C and washed three times with PBS/BSA. Coverslips were then incubated for 1.5 h with a 1:20 dilution of goat anti-mouse IgG-coated 10 nm colloidal gold particles, washed three times in PBS/BSA and three times in PBS, and fixed with 1% (w/v) glutaraldehyde in PHEM buffer for 10 min. Fixed specimens were washed extensively with distilled water, frozen rapidly, freeze-dried and rotary-coated with 1.2 nm of tantalum–tungsten at 45° and 3.0 mm of carbon at 90° without rotation, as described previously [37]. Cytoskeletons were examined and photographed in a Jeol 1200-EX electron microscope with a 100 kV accelerating voltage. No gold labelling was found when primary IgG was omitted or when preimmune IgG was used as a control.

RESULTS

Co-sedimentation of merlin with actin filaments

Using F-actin co-sedimentation assays, we examined the ability of recombinant merlin isoforms as well as N- and C-terminal deletion constructs to bind F-actin. Variable concentrations of GST–merlin fusion proteins were incubated with a constant amount of polymerized F-actin, followed by high-speed centrifugation to pellet F-actin. Equal volumes of pelleted and supernatant material were solubilized in SDS/PAGE sample buffer and analysed by Western blotting. Because ERM and merlin proteins characteristically aggregate and self-pellet [38], control assays were conducted in the absence of F-actin. The merlin N-terminal segment, encompassing residues 1–332, and the alternatively spliced full-length isoform 2, bound F-actin in a dose-dependent manner, whereas full-length merlin isoform 1 did not bind to F-actin by this method (Figure 1). Merlin also bound to β/γ -F-actin in co-sedimentation assays performed with the N-terminus. Although a significant increase in merlin

Figure 1 Actin co-sedimentation with GST–merlin fusion constructs

Top panels: GST–merlin N-terminus 1–332, full-length isoform 1 (residues 1–595) or isoform 2 (residues 1–590), at concentrations ranging from 0.1–3.0 μ M, were incubated with or without 4 μ M F-actin and subjected to high-speed centrifugation at 200 000 *g* to pellet F-actin. Pelleted proteins were resuspended in SDS/PAGE sample buffer, separated by SDS/PAGE [10% (w/v) gel] and transferred to nitrocellulose for immunoblot detection of merlin with the N21 antibody. GST control samples did not pellet in the absence or presence of F-actin (results not shown). The integrity of the F-actin in the pellet was detected by Ponceau S staining of the nitrocellulose. Each assay is representative of three separate experiments for each merlin construct. Bottom panel: schematic representation of merlin isoforms 1 and 2, identifying the major structural domains. The FERM domain, formally referred to as the protein 4.1 domain, precedes a long α -helical stretch interrupted by a proline-rich region and is followed by a charged C-terminus. Isoform 2 differs from isoform 1 only at the C-terminus. The insertion of exon 16 results in merlin isoform 2, a 590-residue protein that is identical with isoform 1 over the first 579 residues [1]. The N-terminal fragment used in these studies encompassed residues 1–332. Abbreviation: aa, amino acid residues.

Figure 2 Saturation and Scatchard analyses of GST–merlin isoform 2

Upper panel: variable concentrations of GST–merlin isoform 2 (0.05–3.0 μM) were incubated with a constant concentration of F-actin and assayed as described in Figure 1. Merlin co-sedimenting with F-actin was quantified by Scanning Fluor-S analysis of immunoblots after subtracting values obtained from self-pelleting (without actin) and compared with total merlin added at each concentration. GST–merlin isoform 2 saturates actin at approx. 2 μM total merlin added. A theoretical saturation curve (◇) representing K_d and B_{max} is included as an overlay on the original results (□). Lower panel: a linear transformation (Scatchard plot) yields a K_d of 3.6 ± 1.7 μM with approx. 12 actin molecules bound per molecule of merlin isoform 2. Results are means $\pm$ S.D. for three independent experiments. Abbreviations: B, bound; F, free.

N-terminus and isoform 2 pelleting was demonstrated in the presence of actin in comparison with pelleting in the absence of actin (self-pelleting), isoform 1 pelleting in the presence and in the absence of actin was almost indistinguishable. Similarly, we were unable to demonstrate actin binding activity with the C-terminal segment of merlin isoform 1, spanning residues 340–595, in co-sedimentation assays owing to the comparable levels of pelleted protein in the absence of actin (results not shown).

The interaction of merlin isoform 2 with actin was quantified with data from F-actin co-sedimentation assays. As increasing concentrations of GST–merlin isoform 2 (0.05–3.0 μM) were added to a constant concentration of F-actin (4 μM), the proportion of merlin sedimenting with the F-actin pellet increased until it saturated at approx. 2 μM of total merlin added (Figure 2). Merlin sedimenting in the presence and in the absence of actin

Figure 3 Solid-phase protein–protein binding assay

(A) The interaction of GST-cleaved merlin isoform 1 and F-actin was examined by coating titrated merlin concentrations on 96-well microtitre plates and overlaying with a constant concentration of F-actin (5 μM). Unbound proteins were washed and merlin–actin binding was determined after the elution of bound proteins with SDS/PAGE sample buffer, separating by SDS/PAGE and Western blotting with merlin N21 and anti-actin antibodies. (B) A constant concentration of GST-cleaved merlin isoform 1, N-terminus 1–332 and C-terminus 340–595 (2 μM) were coated on microtitre plates and examined for F-actin binding. BSA (2 μM) was coated as a control for non-specific F-actin binding. Reproducible results were obtained in five independent binding assays. (C) GST–merlin isoforms 1 and 2 were coated at a constant concentration of 2 μM (50 μl) and incubated with various concentrations of F-actin (0.5, 2 and 8 μM in 50 μl). Coated merlin and bound actin proteins were detected with anti-N21 and anti-actin antibodies respectively. The values of bound actin were calculated from actin standard curves (0.03–4 μM). GST-coated surfaces showed no actin binding (results not shown). Similar results were obtained from three independent experiments.

was quantified, and the difference compared with total merlin added was regarded as specifically bound. Scatchard analyses of these data yielded a K_d of 3.6 ± 1.7 μM (mean $\pm$ S.D.) for merlin isoform 2 to actin binding and a saturated stoichiometric molar ratio of 1:11.5 (merlin to actin).

Previous reports have suggested that the presence of N-terminal tags on fusion proteins hinders the ability of recombinant ERM proteins to bind actin [31,32,39]. Although our results indicate that full-length isoform 2 and the N-terminal 1–332 GST fusion proteins bound F-actin efficiently, we examined whether the inability of merlin isoform 1 to bind F-actin in co-sedimentation assays was related to the presence of the GST tag. GST-cleaved merlin isoform 1 also did not demonstrate F-actin binding by co-sedimentation assays. Similarly, we did not observe a significant difference in merlin N-terminus and isoform 2 binding to actin in the absence of GST (results not shown). Multiple co-sedimentation assays showed consistently that merlin in the presence or absence of GST behaved similarly.

Merlin isoform 1 binds actin less effectively than the free N-terminus

To examine further whether merlin isoform 1 is capable of binding F-actin, a solid-phase protein–protein binding assay was employed as an alternative approach [31]. This method, which was used to demonstrate the existence of the middle ezrin F- and

Figure 4 G-actin affinity pull-down assay

GST–merlin fusion proteins were immobilized on glutathione–Sepharose beads and tested for their ability to pull down G-actin *in vitro*. The presence of G-actin binding to merlin–glutathione–Sepharose was assayed by immunoblotting with an actin-specific antibody. The N-terminus of WIP was used as a positive control for G-actin binding. Purified actin was added to the final lane as an actin antibody control. These assays were performed three times with similar results. The positions of molecular mass markers are indicated at the left.

G-actin binding sites, alleviates the aggregation problem of merlin. Titrated amounts of GST–cleaved merlin fusion proteins were coated in 96-well microtitre plates and incubated with constant amounts of F- or G-actin. Bound proteins were eluted in SDS/PAGE sample buffer and analysed by immunoblotting. Merlin isoform 1 bound F-actin, although less effectively than the N-terminus (Figures 3A and 3B). The C-terminus of isoform 1 (residues 340–595) failed to bind F-actin; the BSA-coated surface showed no F-actin binding (Figure 3B). Similarly, GST–merlin isoform 2 bound F-actin more effectively than isoform 1 in this assay (Figure 3C). The solid-phase binding assay was used to quantify merlin isoform 2 binding to actin to provide independent confirmation of the co-sedimentation data. Merlin isoform 2 bound actin with a K_d of $2.08 \pm 0.74 \mu\text{M}$, a value in agreement with that determined by actin co-sedimentation assays. However, we could not quantitatively determine merlin isoform 1 binding to actin by this method, because we were unable to obtain actin binding data reproducibly in the linear range (Figure 3C).

Merlin does not bind G-actin

One advantage of the solid-phase binding assay is that it can be used to detect binding of monomeric actin. Although a distinct G-actin-binding site has been identified in the ezrin middle region (residues 281–310) [31,32], we were unable to detect G-actin binding to merlin (results not shown). Concurrently with solid-phase binding assays, GST pull-down assays were performed as an alternative approach to examine merlin's ability to bind G-actin *in vitro* [35]. In these studies, equal amounts of GST–merlin fusion proteins were immobilized on glutathione–Sepharose beads and incubated at $0.05 \mu\text{M}$ (below the critical concentration) with G-actin. Bound proteins were resolved by SDS/PAGE and detected by Western blotting. As shown in Figure 4, GST pull-down assays did not reveal G-actin binding to merlin, confirming the results of the solid-phase binding assay. As controls, the G-actin-binding protein, Wiscott–Aldrich syndrome interacting

Figure 5 Merlin slows F-actin disassembly in pyrene-labelled assays

(A) No effect on the kinetics of actin polymerization was detected when pyrene–G-actin ($1.5 \mu\text{M}$) was incubated without (□) or with merlin isoforms 1 (△), 2 (○) or the N-terminus ($0.15 \mu\text{M}$) (◇) in polymerization-inducing buffer. (B) Pyrene–F-actin ($10 \mu\text{M}$), in the absence (□) or presence of GST–merlin fusion proteins (◇, $0.2 \mu\text{M}$ N-terminus; ▤, $0.02 \mu\text{M}$ isoform 2; △, $0.02 \mu\text{M}$ isoform 2), was diluted to $0.2 \mu\text{M}$ with monomer buffer and the decrease in fluorescence intensity was followed. Representative assembly and disassembly assays are shown. Assays were performed at least five times with various concentrations of merlin proteins, with similar results.

protein (WIP), bound G-actin strongly, whereas GST–Cdc42 and GST did not.

Merlin slows actin filament disassembly

To assess the role of merlin in actin filament dynamics, actin polymerization and depolymerization assays were conducted multiple times. Pyrene-labelled G-actin was polymerized to $1.5 \mu\text{M}$ by the addition of KCl and MgCl_2 to 0.1 M and 2 mM respectively in the presence of increasing amounts of GST–merlin fusion protein isoform 2, N-terminal 1–332 or GST (0.03 – $1.5 \mu\text{M}$). We did not detect any alteration in the rate of actin polymerization in the presence of merlin, indicating that merlin does not nucleate actin assembly (Figure 5A).

Next, to determine the kinetics of actin disassembly, pyrene F-actin ($10 \mu\text{M}$) was diluted to $0.2 \mu\text{M}$ in the absence or presence of 0.002 – $1.0 \mu\text{M}$ GST–merlin isoform 2, N-terminal 1–332 or GST (control). F-actin alone, as expected, rapidly disassembled after dilution. The addition of either merlin isoform 2 or the N-terminus consistently slowed the rate of actin depolymerization in a dose-dependent manner (Figure 5B). Together with the binding results, this suggests that merlin might be binding to the sides of the actin filaments, thereby stabilizing their lengths. Fluorescence readings taken at extended time points indicated

Figure 6 Localization of merlin in the cortex of a meningioma cell determined in the electron microscope

Merlin was detected in the Triton X-100-insoluble cytoskeleton from a meningioma cell by using the merlin-specific monoclonal antibody 1C4 followed by 10 nm colloidal gold particles conjugated with goat anti-mouse IgG. Immunogold-labelled specimens were photographed after rapid freezing, freeze-drying, and tantalum–tungsten and carbon coating in a Jeol 1200-EX electron microscope. Gold particles of merlin on the cytoskeleton were highlighted in gold with Adobe PhotoShop software to add the colour to the micrograph. Scale bar, 100 nm. Inset: a negative control for the electron microscope image shows no gold labelling when the primary antibody 1C4 was omitted.

that merlin prevented the disassembly of filaments. These results are further supported by the cytoskeletal localization of merlin by immuno-electron microscopy (Figure 6). Immunogold staining shows that merlin resides on cortical actin filaments, particularly near their ends where filaments intersect one another. This distribution suggested that merlin was targeted to these cytoskeletal locations by both its actin-binding and membrane-protein-binding properties. Negative controls omitting the primary antibody did not reveal any gold particle (Figure 6, inset).

DISCUSSION

Merlin, as a tumour suppressor protein, represents a distinct member of the ERM protein family. Merlin differs from the ERM family members in being expressed as two major isoforms with alternative C-termini. Both endogenous and exogenous merlin in cultured cells concentrate in cell surface structures such as membrane ruffles, microvilli and filopodia, where actin filaments associate with plasma membranes [24,27]. A high-affinity F-actin-binding site present in the last 34 residues of ERM proteins is not conserved in merlin isoforms [1]. This is consistent with the observation that the C-terminal half of either merlin isoform, when expressed in cell culture, is not associated with stress fibres [25]. Recent studies document two actin-binding domains within the N-terminal and middle regions of the ERM proteins, which bind to F-actin and to F- and G-actin respectively [30,31]. In comparison with ERM proteins, merlin binding to actin is not well characterized. Blot overlay experiments performed with ^{125}I -labelled F-actin and merlin isoform 1 revealed no detectable binding to F-actin [18]. Actin co-sedimentation assays performed with products of merlin isoforms transcribed and translated *in vitro* revealed binding to F-actin via the N-terminus with no difference in the ability of both isoforms to bind F-actin [33]. The actin cytoskeleton mediates a variety of dynamic functions, including maintaining cell shape and regulating cell polarity, motility and division. Changes in the actin cytoskeleton of transformed cells, the role of adherence in transformation and metastasis, and the ability of some transforming genes to affect primarily cytoskeletal proteins all emphasize the significance of the actin cytoskeleton in cancer [40]. Defining merlin's normal function as a cytoskeleton-membrane linker protein and also its role in actin assembly might be critical for understanding the basis for NF2 and NF2-related tumours. We therefore undertook a detailed biochemical characterization of the merlin-actin interaction and demonstrated differences between the two major merlin isoforms as well as between merlin and the ERM proteins.

Although sharing several properties with ERM proteins, merlin differs from these proteins in its mode of interaction with the actin cytoskeleton. The subcellular distribution of merlin with detergent extraction methods demonstrates that merlin is recovered exclusively in the insoluble/cytoskeletal-associated fraction of cells ([29]; M. F. James and V. Ramesh, unpublished work), thus differing from ERM proteins, which distribute similarly to both the soluble and the insoluble fractions [23,28]. Employing actin co-sedimentation assays, we have demonstrated that the free N-terminus of merlin and full-length merlin isoform 2 are capable of binding to actin through a N-terminal actin-binding region that was identified in ezrin [31]. We have also shown that merlin isoform 2 to actin binding is saturable, with a K_d of approx. $3.6\ \mu\text{M}$ and a stoichiometric association of 12 actin molecules bound per merlin molecule. From the stoichiometry of 1:12 (merlin to actin), we can envisage the N-terminal half of merlin as contacting both strands of an actin filament composed of 12 actin molecules spanning a length of 37 nm. With co-

sedimentation assays we were unable to observe merlin isoform 1 binding to actin, which is contrary to a previous paper [33]. However, we have shown with the solid-phase binding assay that merlin isoform 1 binds actin, although to a lesser extent than the free N-terminus and isoform 2. Other studies have shown that ezrin binds to actin in a saturable manner with a molar ratio of approx. 1:10 (ezrin to actin) and with K_d values of 50–500 nM [31,41]. Another recent study elegantly demonstrated that phosphorylated moesin interacts with F-actin with a relatively high affinity ($K_d \approx 1.5\ \text{nM}$) and a maximum stoichiometry of 1:1 [23]. Thus merlin seems to bind actin with a lower affinity than that reported for ERM proteins.

Merlin's influence on the kinetics of actin disassembly differs from that of ezrin, which has been reported to promote pyrene-labelled actin assembly, with predominant effects on the filament elongation of β -actin [41]. Merlin has no effect on actin polymerization but can inhibit actin depolymerization in a dose-dependent manner. In addition, merlin does not bind G-actin. These observations indicate that the inhibitory effect of merlin on F-actin depolymerization is not due to capping of the ends of actin filaments or to nucleating activity, and suggests that merlin probably stabilizes F-actin through a lateral association. The latter possibility is further supported by electron microscopy data, which illustrate merlin as residing along filament lengths, most highly concentrated at cortical actin-rich regions. Among the ERM proteins, radixin exhibits actin barbed-end binding activity, whereas moesin and ezrin seem to bind to the sides of the filaments [4,22,41–43].

Mutational analyses of both germline and somatic alterations in the NF2 gene revealed a wide variety of inactivating mutations, most of which are predicted to truncate the protein. Interestingly, no mutations have been reported in either exon 16 or exon 17, implying that both merlin isoforms might be necessary for tumour suppressor function [1]. Structure-function analyses performed with naturally occurring NF2 mutations reveal a significant increase in detergent solubility of mutant merlin in comparison with wild type, indicating a decreased ability of mutant merlin to interact with the cytoskeleton. Localization studies further demonstrated that many of the mutants likely to impair tumour suppressor activity of merlin failed to maintain a stable interaction with actin [29,44]. Merlin isoform 2, owing to its 'open' state, is able to interact more effectively with NHE-RF and the actin cytoskeleton than merlin isoform 1, in which the equivalent sites are masked in the 'closed' state. NHE-RF links merlin isoforms to various ion channels and receptors that provide new possibilities for effects on intracellular signalling. Merlin isoform 2 in its constitutively active state is likely to have more effects on intracellular signalling mediated either through NHE-RF or other partners, and thus might have a role distinct from that of merlin isoform 1.

The key components controlling actin cytoskeletal organization are the Rho-GTPase family members, Rho, Rac and Cdc42. They are believed to act as molecular switches regulating signal-transduction pathways that link membrane receptors to the cytoskeleton [45,46]. The N-terminal halves of the ERM proteins bind to the RhoGDP dissociation inhibitor (RhoGDI), releasing bound GDP-Rho from RhoGDI and initiating the activation of Rho family members [47]. Rho-kinase, a downstream target of active Rho, can phosphorylate ERM proteins *in vitro*, stabilizing the active state of these proteins [38]. Thus ERM proteins are clearly implicated in the Rho-GTPase signalling pathway. On the basis of the N-terminal sequence similarity of merlin and the ERM family members, the ability of merlin's N-terminus to bind RhoGDI was tested and found to bind RhoGDI [28]. Merlin's role in the small Rho-

GTPase signalling cascade is not yet defined. By acting as a linker connecting plasma membrane proteins to the actin cytoskeleton, merlin is likely to be an active player in this signalling cascade leading to actin reorganization, the disruption of which might lead to NF2-related tumours.

We thank Dr K. Barkalow for advice on actin fluorescence measurements, Dr N. Ramesh for generously providing purified N-WIP and for valuable discussions, Denise Pinney-Michalowski for her technical expertise, and members of our laboratory for helpful comments on the manuscript. This work was supported by NIH grant NS24279, U. S. Army grant (V. R.) and NIH HL56949 (J. H. H.). M. F. J. was supported by a NRSA post-doctoral fellowship from NIH, no. F32-NS10699.

REFERENCES

- Gusella, J. F., Ramesh, V., MacCollin, M. and Jacoby, L. B. (1999) Merlin: the neurofibromatosis 2 tumor suppressor. *Biochim. Biophys. Acta* **1423**, M29–M36
- Chishtii, A. H., Kim, A. C., Marfatia, S. M., Lutchman, M., Hanspal, M., Jindal, H., Liu, S. C., Low, P. S., Rouleau, G. A., Mohandas, N. et al. (1998) The FERM domain: a unique module involved in the linkage of cytoplasmic proteins to the membrane. *Trends Biochem. Sci.* **23**, 281–282
- Bretscher, A. (1999) Regulation of cortical structure by the ezrin–radixin–moesin protein family. *Curr. Opin. Cell Biol.* **11**, 109–116
- Tsukita, S. and Yonemura, S. (1999) Cortical actin organization: lessons from ERM (ezrin/radixin/moesin) proteins. *J. Biol. Chem.* **274**, 34507–34510
- Reczek, D., Berryman, M. and Bretcher, A. (1997) Identification of EBP50: a PDZ-containing phosphoprotein that associates with members of the ezrin–radixin–moesin family. *J. Cell Biol.* **139**, 169–179
- Murthy, A., Gonzalez-Agosti, C., Cordero, E., Pinney, D., Candia, C., Solomon, F., Gusella, J. and Ramesh, V. (1998) NHE-RF, a regulatory co-factor for Na⁺/H⁺ exchange is a common interactor for merlin and ERM proteins. *J. Biol. Chem.* **273**, 1273–1276
- Yun, C. H., Lamprecht, G., Foster, D. V. and Sidor, A. (1998) NHE3 kinase a regulatory protein E3KARP binds the epithelial brush border Na⁺/H⁺ exchanger NHE3 and the cytoskeletal protein ezrin. *J. Biol. Chem.* **273**, 25856–25863
- Hall, R. A., Ostedgaard, L. S., Premont, R. T., Blitzer, J. T., Rahman, N., Welsh, M. J. and Lefkowitz, R. J. (1998) A C-terminal motif found in the β_2 -adrenergic receptor, P2Y1 receptor and cystic fibrosis transmembrane conductance regulator determines binding to the Na⁺/H⁺ exchanger regulatory factor family of PDZ proteins. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 8496–8501
- Hall, R. A., Premont, R. T., Chow, C. W., Blitzer, J. T., Pitcher, J. A., Claing, A., Stoffel, R. H., Barak, L. S., Shenolikar, S., Weinman, E. J. et al. (1998) The β_2 -adrenergic receptor interacts with the Na⁺/H⁺-exchanger regulatory factor to control Na⁺/H⁺ exchange. *Nature (London)* **392**, 626–630
- Short, D. B., Trotter, R. W., Reczek, D., Kreda, S. M., Bretscher, A., Boucher, R. C., Stutts, M. J. and Milgram, S. L. (1998) An apical PDZ protein anchors the cystic fibrosis transmembrane conductance regulator to the cytoskeleton. *J. Biol. Chem.* **273**, 19797–19801
- Wang, S., Raab, R. W., Schatz, P. J., Guggino, W. B. and Li, M. (1998) Peptide binding consensus of the NHE-RF-PDZ1 domain matches the C-terminal sequence of cystic fibrosis transmembrane conductance regulator (CFTR). *FEBS Lett.* **427**, 103–108
- Bernardo, A. A., Kear, F. T., Santos, A. V., Ma, J., Steplock, D., Robey, R. B. and Weinman, E. J. (1999) Basolateral Na⁺/HCO₃⁻ cotransport activity is regulated by the dissociable Na⁺/H⁺ exchanger regulatory factor. *J. Clin. Invest.* **104**, 195–201
- Breton, S., Wiederhold, T., Marshansky, V., Nsumu, N. N., Ramesh, V. and Brown, D. (2000) The B1 subunit of the H⁺ATPase is a PDZ-domain binding protein: colocalization with NHE-RF in renal B-intercalated cells. *J. Biol. Chem.* **275**, 18219–18224
- Scoles, D. R., Huynh, D. P., Morcos, P. A., Course, E. R., Robinson, N. G. G., Tamanoi, F. and Pulst, S. M. (1998) Neurofibromatosis 2 tumor suppressor schwannomin interacts with BII-spectrin. *Nat. Genet.* **18**, 354–359
- Gonzalez-Agosti, C., Wiederhold, T., Herndon, M. E., Gusella, J. and Ramesh, V. (1999) Interdomain interaction of merlin isoforms and its influence on intermolecular binding to NHE-RF. *J. Biol. Chem.* **274**, 34438–34442
- Sherman, L., Xu, H. M., Geist, R. T., Saporito-Irwin, S., Howells, N., Ponta, H., Herrlich, P. and Gutmann, D. H. (1997) Interdomain binding mediates tumor growth suppression by the NF2 gene product. *Oncogene* **15**, 2505–2509
- Gronholm, M., Sainio, M., Zhao, F., Heiska, L., Vaheri, A. and Carpen, O. (1999) Homotypic and heterotypic interaction of the neurofibromatosis 2 tumor suppressor protein merlin and the ERM protein ezrin. *J. Cell Sci.* **112**, 895–904
- Huang, L., Ichimaru, E., Pestonjamas, K., Cui, X., Nakamura, H., Lo, G. Y. H., Lin, F. I. K., Luna, E. J. and Furthmayr, H. (1998) Merlin differs from moesin in binding to F-actin and in its intra- and intermolecular interactions. *Biochem. Biophys. Res. Commun.* **248**, 548–553
- Hirao, M., Sato, N., Kondo, T., Yonemura, S., Monden, M., Sasaki, T., Takai, Y., Tsukita, S. and Tsukita, S. (1996) Regulation mechanism of ERM (ezrin/radixin/moesin) protein/plasma membrane association: possible involvement of phosphatidylinositol turnover and Rho-dependent signaling pathway. *J. Cell Biol.* **135**, 37–51
- Turunen, O., Wahlström, T. and Vaheri, A. (1994) Ezrin has a COOH-terminal actin-binding site that is conserved in the ezrin protein family. *J. Cell Biol.* **126**, 1445–1453
- Martin, M., Andreoli, C., Sahuquet, A., Montcourrier, P., Algrain, M. and Mangeat, P. (1995) Ezrin NH₂-terminal domain inhibits the cell extension activity of the COOH-terminal domain. *J. Cell Biol.* **128**, 1081–1093
- Pestonjamas, K., Amieva, M. R., Strassel, C. P., Nauseef, W. M., Furthmayr, H. and Luna, E. J. (1995) Moesin, ezrin, and p205 are actin-binding proteins associated with neutrophil plasma membranes. *Mol. Biol. Cell* **6**, 247–259
- Nakamura, F., Huang, L., Pestonjamas, K., Luna, E. J. and Furthmayr, H. (1999) Regulation of F-actin binding to platelet moesin in vitro by both phosphorylation of threonine 558 and polyphosphatidylinositides. *Mol. Biol. Cell* **10**, 2669–2685
- Gonzalez-Agosti, C., Xu, L., Pinney, D., Beauchamp, R., Hobbs, W., Gusella, J. and Ramesh, V. (1996) The merlin tumor suppressor localizes preferentially in membrane ruffles. *Oncogene* **13**, 1239–1247
- Xu, L., Gonzalez-Agosti, C., Beauchamp, R., Pinney, D., Sterner, C. and Ramesh, V. (1998) Analysis of molecular domains of epitope tagged merlin isoforms in Cos-7 cells and primary rat Schwann cells. *Exp. Cell Res.* **238**, 231–240
- den Bakker, M. A., Riegman, P. H., Hekman, R. A., Boersma, W., Janssen, P. J., van der Kwast, T. H. and Zwarthoff, E. C. (1995) The product of the NF2 tumour suppressor gene localizes near the plasma membrane and is highly expressed in muscle cells. *Oncogene* **10**, 757–763
- Sainio, M., Zhao, F., Heiska, L., Turunen, O., den Bakker, M., Zwarthoff, E., Lutchman, M., Rouleau, G. A., Jaaskelainen, J., Vaheri, A. and Carpen, O. (1997) Neurofibromatosis 2 tumor suppressor protein colocalizes with ezrin and CD44 and associates with actin-containing cytoskeleton. *J. Cell Sci.* **110**, 2249–2260
- Maeda, M., Matsui, T., Imamura, M. and Tsukita, S. (1999) Expression level, subcellular distribution and rho-GDI binding affinity of merlin in comparison with ezrin/radixin/moesin proteins. *Oncogene* **18**, 4788–4797
- Deguen, B., Merel, P., Goutebroze, L., Giovannini, M., Reggio, H., Arpin, M. and Thomas, G. (1998) Impaired interaction of naturally occurring mutant NF2 protein with actin-based cytoskeleton and membrane. *Hum. Mol. Genet.* **7**, 217–226
- Martin, M., Roy, C., Montcourrier, P., Sahuquet, A. and Mangeat, P. (1997) Three determinants in ezrin are responsible for cell extension activity. *Mol. Biol. Cell* **8**, 1543–1557
- Roy, C., Martin, M. and Mangeat, P. (1997) A dual involvement of the amino-terminal domain of ezrin in F- and G-actin binding. *J. Biol. Chem.* **272**, 20088–20095
- Mangeat, P., Roy, C. and Martin, M. (1999) ERM proteins in cell adhesion and membrane dynamics. *Trends Cell Biol.* **9**, 187–192
- Xu, H. and Gutman, D. H. (1998) Merlin differentially associates with the microtubule and actin cytoskeleton. *J. Neurosci. Res.* **51**, 403–415
- Hartwig, J. H. (1992) Mechanisms of actin rearrangements mediating platelet activation. *J. Cell Biol.* **118**, 1421–1442
- Rohatgi, R., Ma, L., Miki, H., Lopez, M., Kirchhausen, T., Takenawa, T. and Kirschner, M. W. (1999) The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. *Cell* **97**, 221–231
- Barkalow, K., Witke, W., Kwiatkowski, D. J. and Hartwig, J. H. (1996) Coordinated regulation of platelet actin filament barbed ends by gelsolin and capping protein. *J. Cell Biol.* **134**, 389–399
- Hartwig, J. H. and DeSisto, M. (1991) The cytoskeleton of the resting human blood platelet: structure of the membrane skeleton and its attachment to actin filaments. *J. Cell Biol.* **112**, 407–425
- Matsui, T., Maeda, M., Doi, Y., Yonemura, S., Amano, M., Kaibuchi, K., Tsukita, S. and Tsukita, S. (1998) Rho-kinase phosphorylates COOH-terminal threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to tail associations. *J. Cell Biol.* **140**, 647–657
- Henry, M. D., Agosti, C. G. and Solomon, F. (1995) Molecular dissection of radixin: distinct and interdependent functions of the amino- and carboxy-terminal domains. *J. Cell Biol.* **129**, 1007–1022
- Hall, A. (1994) Small GTP-binding proteins and the regulation of the actin cytoskeleton. *Annu. Rev. Cell Biol.* **10**, 31–54
- Yao, X., Cheng, L. and Forte, J. G. (1996) Biochemical characterization of ezrin–actin interaction. *J. Biol. Chem.* **271**, 7224–7229

-
- 42 Tsukita, S., Hieda, Y. and Tsukita, S. (1989) A new 82-kD barbed end-capping protein (radixin) localized in the cell-to-cell adherens junction: purification and characterization. *J. Cell Biol.* **108**, 2369–2382
- 43 Shuster, C. B. and Herman, I. M. (1995) Indirect association of ezrin with F-actin: isoform specificity and calcium sensitivity. *J. Cell Biol.* **128**, 837–848
- 44 Stokowski, R. P. and Cox, D. R. (2000) Functional analysis of the neurofibromatosis type 2 protein by means of disease-causing point mutations. *Am. J. Hum. Genet.* **66**, 873–891
- 45 Hall, A. (1998) G proteins and small GTPases: distant relatives keep in touch. *Science* **280**, 2074–2075
- 46 Bishop, A. L. and Hall, A. (2000) Rho GTPases and their effector proteins. *Biochem. J.* **348**, 241–255
- 47 Takahashi, K., Sasaki, T., Mammoto, A., Takaishi, K., Kameyama, T., Tsukita, S., Tsukita, S. and Takai, Y. (1997) Direct interaction of the Rho GDP dissociation inhibitor with ezrin/radixin/moesin initiates the activation of the small G protein. *J. Biol. Chem.* **272**, 23371–23375

Received 28 September 2000/23 February 2001; accepted 12 March 2001