Binding of the merlin-I product of the neurofibromatosis type 2 tumour suppressor gene to a novel site in β-fodrin is regulated by association between merlin domains

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The mechanism underlying the tumour-suppressor activity of the neurofibromatosis type 2 (NF2) gene product, merlin, is largely undefined but there is evidence that the biological function of the protein might be mediated partly through interactions with the cytoskeleton. Merlin is expressed predominantly as two isoforms that differ at their C-termini owing to alternative splicing of exon 16. By expressing merlin isoform I as bait in a yeast two-hybrid screen, we isolated a clone encoding a region of the cytoskeletal protein β-fodrin. Confirmation of the merlin–fodrin interaction was provided by using the mammalian two-hybrid system and binding assays *in vitro*. In addition, these assays and co-immunoprecipitation from mammalian cells revealed that the binding site for fodrin is located in the C-terminal half of merlin at a site that is masked in the native protein. Co-expression of the N-terminus of merlin decreased the interaction of its C-terminus with fodrin, implicating homophilic interactions of merlin isoform I in masking the fodrin-binding site. The effect of three disease-associated mutations on the merlin–fodrin interaction and merlin dimerization was also investigated. The mutation L535P, but not L360P or K413E, significantly decreased the merlin–fodrin interaction but not dimerization, indicating that the tumour suppressor ability of merlin might reside partly in its ability to interact with the cytoskeleton via fodrin.

Key words: ezrin–radixin–moesin, schwannomin, spectrin.

INTRODUCTION

Neurofibromatosis type 2 (NF2) is an autosomally inherited disorder characterized by bilateral vestibular schwannomas of the vestibular branch of the eighth cranial nerve, as well as other tumours of the central nervous system including meningiomas, ependymomas and gliomas. Originally identified as being mutated in schwannomas and meningiomas [1,2], extensive analysis of the *NF2* gene in NF2-associated tumours has revealed many germline and somatic mutations providing evidence that the protein product has tumour-suppressor activity [3]. In addition, mutations have been identified in sporadic tumour types not characteristic of NF2, including mesothelioma [4,5] and melanoma [6]. The *NF2* gene is expressed predominantly as two major isoforms, merlin-I and merlin-II, that differ at their C-termini owing to alternative splicing of exon-16-encoded sequences [1,2,6].

As expected for a tumour-suppressor protein, merlin suppresses cell growth. Overexpression of merlin-I inhibits the growth of NIH 3T3 cells [7] and rat schwannoma cells [8] and suppresses the H-*ras* transformed phenotype of NIH 3T3 cells [9]. Conversely, suppression of merlin expression with anti-sense oligonucleotides increases cell proliferation in Schwann-like cells [10]. Importantly, $NF2^{+/-}$ mice develop a wide range of malignant tumours, although these do not reflect the specific pathology of the human disease, indicating a species-specific difference [11]. Tumours that develop in $NF2^{+/-}$ mice are highly prone to metastasis; functional studies in cultured cells have shown changes in cell motility and adhesion associated with alterations in the expression of merlin [10,12,13]. Recently, conditional inactivation of the *NF2* gene in the Schwann cell lineage has produced a murine model that matches the pathology of human NF2 more closely [14]. The phosphorylation status of merlin in NIH 3T3 cells is affected by changes in cell adhesion and confluence, indicating that merlin might function as part of a signal transduction pathway influencing cell–cell and cell–matrix interactions [15].

The N-terminus of merlin contains the FERM domain [16], first characterized in the erythrocyte protein 4.1R [17]. Merlin displays the highest similarity with the protein 4.1 subfamily members ezrin, radixin and moesin (ERM proteins) and is postulated to have similar properties by acting as a membrane–cytoskeleton linker. Similarly to ERM proteins, merlin binds to the transmembrane glycoprotein CD44 [18–20] and to the regulatory cofactor NHE-RF/EBP50 of the Na^+–H^+ exchanger, which might provide an indirect link to the plasma membrane [21,22]. Despite 40% similarity at the amino acid level with the conserved C-terminal F-actin-binding sequence of the ERM proteins, merlin-I does not bind to F-actin in identical blot overlay assays [23]. In contrast, merlin might interact with the cytoskeleton through its N-terminal region, which binds to both actin and microtubules *in vitro* [24]. However, exogenously expressed merlin-I does not co-localize with microtubules in HeLa cells, nor is its distribution affected by nocodazole, suggesting that merlin-I does not bind to microtubules *in vivo* [25]. Further evidence for an association of merlin with the actin-based cytoskeleton is provided by subcellular fractionation and actin-cytoskeleton-disrupting agents. Merlin-I is detected in the cytoskeletal fraction of detergent-extracted COS and HeLa cells; treatment of these cells with cytochalasins B and D induces a redistribution of merlin-I into actin-rich clusters [19,25]. Exogenous expression of N-terminal and C-terminal deletion

Abbreviations used: 3-AT, 3-aminotriazole; ERM, ezrin–radixin–moesin; FLAG, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; GST, glutathione S-transferase; LB, Luria broth; MAPK, mitogen-activated protein kinase; NF2, neurofibromatosis type 2.

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mutants provides evidence that both domains are required for cytoskeletal binding [25,26]. Evidence for merlin's binding directly to the cytoskeleton through a distinct mechanism has also been demonstrated. A clone encoding a region of β-fodrin (β-II spectrin) was isolated with merlin-II as bait in a yeast two-hybrid screen; merlin and fodrin co-localized in various tissues and cell lines [27].

Although merlin is expressed as two main isoforms, the growth suppressor properties are associated specifically with merlin-I and not merlin-II [8]; transcripts of isoform I are the predominant type detected in the eighth cranial nerve [6]. In an attempt to explain the function of merlin-I in cell growth control, we have employed the yeast two-hybrid system to identify proteins that interact with this isoform. One cDNA clone isolated encoded a region of the cytoskeletal protein β-fodrin distinct from the merlin-binding site described previously [27]. The merlin–fodrin interaction was further verified by using different binding assays; fodrin was found to bind specifically to the C-terminus of merlin-I at a site that is masked by the N-terminus. Finally, of three disease-associated mis-sense mutations assayed, L535P significantly decreased the merlin–fodrin interaction, whereas no mutations inhibited merlin dimerization, indicating that the tumour-suppressor ability of merlin might reside partly in its ability to interact with the cytoskeleton.

MATERIALS AND METHODS

Vector construction

NF2 cDNA encoding human isoform I was obtained as a gift from Dr Vijay Ramesh (Massachusetts General Hospital, Boston, MA, U.S.A.). All constructs were created by subcloning or by cloning PCR-generated sequences digested with the appropriate restriction enzymes. Constructs were verified by restriction digest and DNA sequencing (T7 Sequenase version 2.0; Amersham). Sequences encoding merlin-I (pY9NF2), MerN (residues 1–380, pY9NF2N) and MerC (residues 324–595, pY9NF2C) were cloned into the yeast vector pYTH9 (a gift from Dr Robin Brown, GlaxoWellcome, Stevenage, Herts., U.K.) and expressed as GAL4-binding-domain fusion proteins. Similarly, sequences encoding merlin (pMNF2), MerN (residues 1–378, pMNF2N) and MerC (residues 323–595, pMNF2C) were cloned into the mammalian vector pM (Clontech) and expressed as the same fusion protein type. The fodrin fragment was cloned into the mammalian two-hybrid vector pVP16 (Clontech) and expressed as a fusion protein with the activation domain of herpes simplex virus (pVP4). Sequences for FLAG-tagged merlin (pC3FNF2), FLAG-tagged MerC (residues 323–595, pC3FNF2C), MerN1 (residues 1–378, pC3NF2N) and Myc-tagged fodrin (pC3m4Δ12) were generated by PCR and cloned into pCDNA3 (Invitrogen). The peptide sequences of the Myc and FLAG epitope tags were Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu and Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys respectively and were incorporated at the N-termini of proteins. Glutathione S-transferase (GST) fusion proteins were expressed from sequences cloned into pGEX 4T-3 (Pharmacia). Mutant merlin proteins were generated by site-directed mutagenesis (Quick Change Site-Directed Mutagenesis Kit; Stratagene).

Tissue culture and transfection

HEK-293 cells were grown at 37 °C in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10 % (v/v) newborn calf serum, 2 mM L-glutamine (Sigma), penicillin and streptomycin (respectively 50 i.u. and 50 μg/ml final) (Sigma) and buffered with air/CO_2 (22:3). For transfection purposes, 2×10^5 cells were seeded in each well of a six-well plate and incubated overnight before transfection on the following day. Two methods for the transient transfection of HEK-293 cells were employed with the lipid reagents LIPOFECTAMINE (Gibco BRL) or Fugene (Boehringer Mannheim) in accordance with the manufacturers' instructions.

SDS/PAGE and Western blotting

Protein samples were separated on 10 % (w/v) polyacrylamide gels by the method of Laemmli [28]. For immunodetection, separated proteins were transferred from the gel to nitrocellulose membrane (Hybond-C Extra; Amersham) at a constant current of 400 mA for 2 h in 1 × transfer buffer [0.025 M Tris/HCl (pH 8.3)/0.19 M glycine]. The membrane was stained with a Ponceau Red solution [0.2 % (w/v) Ponceau Red/2 % (v/v) acetic acid] to detect protein transfer and compare protein levels, then washed in PBS before being blocked in PBS containing 0.1 % (v/v) Tween and 5 % (w/v) milk powder (PBS-T-MP) at room temperature (23 °C) for 1 h or at 4 °C overnight. The primary antibody was diluted in PBS-T-MP and incubated with the blot for 1 h at room temperature (23 °C), with rotation. The blot was washed three times for 10 min each in PBS-T-MP before incubation with the secondary antibody (diluted in PBS-T-MP) for 1 h at room temperature (23 °C) with rotation. The blot was finally washed once for 10 min in PBS-T-MP, twice for 10 min each in PBS-T and once for 5 min in PBS before protein detection with Supersignal (Pierce) and light-sensitive film (XLS film; Kodak). Primary antibodies used were sc789 (Santa Cruz Biotechnology), recognizing the Myc epitope tag, and sc807 (Santa Cruz Biotechnology), recognizing the FLAG epitope tag. The secondary antibody used was anti-rabbit NA934 (Amersham).

Two-hybrid systems

The yeast two-hybrid system was essentially performed in accordance with the manufacturer's protocol (Clontech). In brief, Y190-YTH9, Y190-NF2, Y190-NF2N and Y190-NF2C cells were made by transforming Y190 cells with the linearized vectors pYTH9, pY9NF2, pY9NF2N and pY9NF2C respectively. Transformed cells were plated on both –Leu,–Trp and –Leu,–Trp,–His media (Bio 101) to ensure protein expression from both plasmids; all triple dropout media contained 25 mM 3-aminotriazole (3-AT; Sigma) (except when glycerol stocks of 80 isolated colonies were streaked on media containing 25, 50 and 100 mM 3-AT to assess *His3* reporter activity). β-Galactosidase activity was determined by the filter lift assay. Y190-NF2 cells were transformed with 50 μg of a HeLa Matchmaker cDNA library (Clontech) to screen 1.5×10^6 colonies theoretically; library clones were isolated by using the Yeast DNA Isolation System (Stratagene).

Mammalian cells were transfected with 1.0 μg of each expression vector, 0.4 μg of the luciferase reporter vector pT109-5UAS (Dr Graham Goodwin, Institute of Cancer Research, Sutton, Surrey, U.K.) and 0.4 μg of the β-galactosidase reporter vector pCMV-β (Promega) to standardize transfection efficiency. Control expression vectors were pM53, pVP16-T (Mammalian Matchmaker Two-hybrid Assay Kit; Clontech), pEXV-MAPK (Mike Lau, Institute of Cancer Research, Sutton, Surrey, U.K.) and pCDNA3p85α (a cDNA encoding the bovine p85 subunit of phosphoinositide 3-kinase cloned into pCDNA3). Cells were harvested 48 h after transfection and reporter activity was analysed. Luciferase activity was measured with a commercial kit in accordance with the manufacturer's protocol (Luciferase Assay System; Promega). To analyse β-galactosidase activity, lysate was mixed with Chlorophenol-Red-β-D-galactopyranoside (CP-

Table 1 Expression and reporter activity analysis in the yeast two-hybrid screen

Vector	Transformed cell type	Growth on selection medium		
		–Leu,–Trp	–Leu,–Trp,–His *His3* reporter	*lac z* reporter activity
Clones 1–6	Y190-NF2	+	+	+
Clones 1–6	Y190-YTH9	+	−	−
Clones 1–6 + pVA3	Y190	+	−	−
pVA3 + pTD1	Y190	+	+	+
pGAD4	Y190-NF2N	+	−	−
pGAD4	Y190-NF2C	+	+	+

RG) mix [7.97 ml of water, 2 ml of 5 × Z buffer (0.3 M Na_2HPO_4, 0.3 M NaH_2PO_4, 50 mM KCl, 5 mM $MgSO_4$), 14 ml of 2-mercaptoethanol, 10 ml of CPRG (Boehringer Mannheim) in 10 ml of solution] and activity was measured as A_{570} in a plate reader. Basal level control values were taken as the average standardized luciferase activity of cells co-transfected with pMNF2/pVP16 and pM/pVP4; these were given an arbitrary value of 1 to determine the fold increase in reporter activity for each experimental transfection.

Co-immunoprecipitation

Mammalian cells were transfected with pC3m4Δ12 (2.0 μg) and either pC3FNF2 (1.0 μg), pC3FNF2C (1.5 μg) or pC3Fbks [29] (1.0 μg; Dr Philip Mitchell, Institute of Cancer Research, Sutton, Surrey, U.K.). At 48 h after transfection, cells were lysed in 1 ml of ice-cold RIPA buffer [50 mM Tris/HCl (pH 7.5)/150 mM NaCl/1 % (v/v) Nonidet P40/0.5 % sodium deoxycholate/0.1 % SDS] supplemented with 0.5 mM Na_3VO_4 and protease inhibitors (Complete; Boehringer Mannheim). Cell lysates were gently vortex-mixed and resuspended on ice for 15 min, then centrifuged for 10 min at 4 °C. A 10 μl sample of lysate supernatant was kept for protein expression analysis; 0.8 ml of lysate supernatant was mixed with 50 μl of washed anti-FLAG M2 affinity gel slurry (Kodak) and incubated, with rotation, for 45 min at 4 °C. After incubation, beads were washed extensively in RIPA buffer and boiled in 50 μl of 2 × sample buffer. After a brief centrifugation, 10 μl of the supernatant was used for Western blotting.

Expression and purification of GST fusion proteins

BL21(DE3)pLysS cells (Stratagene) were transformed with 0.5 μg of the various pGEX constructs. A 1 ml sample of recovery culture in Luria broth (LB) was subcultured in 9 ml of LB/ampicillin (100 μg/ml) and grown overnight at 37 °C, with shaking (250 rev./min). A 2 ml sample of this culture was further subcultured in 20 ml of LB/ampicillin (100 μg/ml) and grown at 37 °C for 2 h, with shaking (250 rev./min). Expression of fusion proteins was induced by 1 mM isopropyl β-D-thiogalactoside, with incubation for a further 2 h. The cells were centrifuged for 5 min at 1560 ***g*** (3000 rev./min; Denley BR 401) and the cell pellet was resuspended in 20 ml of PBS followed by the same centrifugation step and resuspension of the pellet in 5 ml of PBS containing Complete (Boehringer) protease inhibitors (PBS-PI). A 1 ml sample of the resuspended cells was removed and diluted with 3 ml of PBS-PI and the cell suspension was freeze–thawed twice in ice/solid CO_2. The cell suspension was sonicated five times at 14 MHz for 15 s or until the suspension became clear, then microcentrifuged at 16000 ***g*** (14 000 rev./min) for 5 min at 4 °C in two 2 ml aliquots. The supernatant from each tube was added separately to 200 μl of PBS-washed glutathione–Sepharose 4B beads (Pharmacia) and incubated at 4 °C for 45 min, with rotation. The supernatants were removed and the beads were washed three times in 1 ml of PBS-PI to remove non-specific binding. Finally, the beads were resuspended in 0.6 ml of PBS-PI and 0.2 ml of glycerol, then stored at −80 °C.

Transcription and translation *in vitro*

Translations *in vitro* were performed with the TNT T7 Coupled Reticulocyte Lysate System (Promega). DNA templates were either plasmids or PCR products. Full-length merlin-I was translated from pC3NF2 (NF2 cDNA cloned into pCDNA3). MerN and β-fodrin were translated from cDNA amplified by PCR with the use of the following primers and templates: MerN (amplified from pC3NF2 with the sense primer 5′-CTGCTTACTGGCTTATCG-3′ and the anti-sense primer 5′-CAGGTCAGCTGTCTACTCAGACCGCATCAG-3′) and β-fodrin (amplified from pGAD4 with the sense primer 5′-GGATCCTAATACGACTCACTATAGGAACAGACCACCATGGATGATGAAGATACCCCACCAAAC-3′ and the anti-sense primer 5′-AGTTGAAGTGAACTTGCGGGG-3′).

RESULTS

Merlin isoform I binds to β-fodrin

Yeast (Y190) cells were transformed with linearized merlin-I bait vector (pY9NF2); successful integration and expression of recombinant merlin was determined by Western blot analysis of Y190-NF2 cell lysates (results not shown). Screening recombinant merlin-I against a HeLa cDNA library resulted in 80 colonies on triple-medium selection plates with 25 mM 3-AT. Six unique colonies were ultimately identified that grew on plates containing 100 mM 3-AT, indicative of strong *His3* reporter activity. All six colonies also stained positive for β-galactosidase activity (Table 1). None of the clones isolated induced reporter activity in control (Y190-YTH9) cells (containing integrated empty vector), nor was reporter activity induced in cells co-expressing the six clones and recombinant p53 bait as a control fusion protein (pVA3). As a positive control, co-expression of p53 bait and simian virus 40 large T antigen target (pTD1) fusion proteins induced reporter activity (Table 1).

One of the clones isolated, pGAD4, encoded a region of the cytoskeletal protein β-fodrin (βII-spectrin) encompassing residues 982–1188, which includes the entire spectrin repeat 8 motif (GenBank® accession no. Q01082). In addition, there were 11 non-related residues at the C-terminus of the fodrin sequence preceding an in-frame stop codon (Figure 1). The first two bases of the non-related region were GT, which conforms to the consensus sequence of a 5′ splice donor site, indicating that the clone might represent prespliced mRNA-containing intron sequences; this interpretation was supported by comparison with the human genomic sequence encoding β-fodrin. To determine

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caggacctgggcaatgacctggctggcgtcatggccctgcagcgcaagctgaccggcatg
 Q  D  L  G  N  D  L  A  G  V  M  A  L  Q  R  K  L  T  G  M

gagcgggacttggtggccattgaggcaaagctgagtgacctgcagaaggaggcggagaag
 E  R  D  L  V  A  I  E  A  K  L  S  D  L  Q  K  E  A  E  K

ctggagtccgagcaccccgaccaggcccaggccatcctgtctcggctggccgagatcagc
 L  E  S  E  H  P  D  Q  A  Q  A  I  L  S  R  L  A  E  I  S

gacgtgtgggaggagatgaagaccaccctgaaaaaccgagaggcctccctgggagaggcc
 D  V  W  E  E  M  K  T  T  L  K  N  R  E  A  S  L  G  E  A

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 S  K  L  Q  Q  F  L  R  D  L  D  D  F  Q  S  W  L  S  R  T

cagacagcgatcgcctcggaggacatgccaaacaccctgaccgaggctgagaagctgctc
 Q  T  A  I  A  S  E  D  M  P  N  T  L  T  E  A  E  K  L  L

acgcagcacgagaacatcaagaatgagatcgacaactacgaggaggactaccagaagatg
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 R  D  M  G  E  M  V  T  Q  G  Q  T  D  A  Q  Y  M  F  L  R

cagcggctgcaggccctggacactggatggaacgagctccacaagatgtgggagaacaga
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caaaatctcctatcccagtcacatgcctaccagcagttcctcagagacacgaagcaagcc
 Q  N  L  L  S  Q  S  H  A  Y  Q  Q  F  L  R  D  T  K  Q  A

gaagcctttcttaacaaccaggtaaggtttgttcctgcctttgcttcctttcggtga
 E  A  F  L  N  N  Q  V  R  F  V  P  A  F  A  S  F  R  *
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Figure 1 Sequence of a β-fodrin fragment that interacts with merlin-I

Nucleotide and encoded amino acid sequence of the pGAD4 cDNA insert that contains a region displaying exact identity with residues 982–1188 of β-fodrin. Intron-encoded residues are underlined.

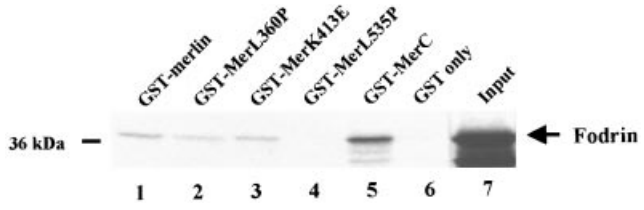

Figure 2 Analysis of the merlin–fodrin interaction *in vitro*

After incubation with radiolabelled fodrin fragment, the fusion proteins GST–merlin (lane 1), GST–MerL360P (lane 2), GST–MerK413E (lane 3), GST–MerL535P (lane 4) and GST–MerC (lane 5), as well as GST only (lane 6), were washed and the precipitates were subjected to SDS/PAGE. Gels were then stained with Coomassie Blue to quantify the loading of fusion proteins (results not shown) and dried at 80 °C for 2 h before autoradiography and densitometry.

whether fodrin binds to the N-terminus (MerN, residues 1–380) or C-terminus (MerC, residues 324–595) of merlin-I, yeast cells expressing each domain (Y190-NF2N and Y190-NF2C) were transformed with pGAD4; expression of recombinant MerN and MerC was determined by Western blot analysis (results not shown). Reporter activity was induced in Y190-NF2C cells but not in Y190-NF2N cells, indicating that fodrin binds to the C-terminus of merlin (Table 1).

Isolated C-terminus of merlin-I binds more efficiently to β-fodrin than does the native protein; the merlin L535P mis-sense mutation selectively abrogates binding

To investigate the merlin–fodrin interaction further, direct binding assays were performed to reveal complex formation. Merlin-I and MerC (residues 324–595) were expressed and purified as GST fusion proteins, then incubated with the fodrin fragment transcribed and translated *in vitro*. Fodrin bound to both GST–merlin (Figure 2, lane 1) and GST–MerC (Figure 2, lane 5) but with a higher efficiency to the latter. No binding was observed to GST only (Figure 2, lane 6). The relative amounts of each fusion protein were comparable (results not shown), suggesting that the binding site for fodrin was masked in the full-length protein and that removal of the N-terminus exposed the binding site.

The effect of three disease-associated mis-sense mutations on the merlin–fodrin interaction was also investigated. Full-length merlin-I was expressed and purified as GST fusion proteins containing each of the three mis-sense mutations L360P, K413E and L535P. Fodrin bound to GST–MerL360P and GST–MerK413E with modestly decreased (68 % and 82 % respectively) efficiencies in comparison with GST–merlin binding (Figure 2, lanes 1–3). However, fodrin did not bind efficiently to GST–MerL535P (Figure 2, lane 4) (27 % in comparison with GST–merlin binding), indicating that this mutation selectively inhibited the merlin–fodrin interaction.

Merlin dimerization is well documented and might be relevant for the control of cell growth [8,23,30–33]. To assess the effect of the mis-sense mutations L360P, K413E and L535P on merlin dimerization, the relevant GST fusion proteins were incubated with merlin and MerN (residues 1–378) transcribed and translated *in vitro*. Merlin and MerN bound with similar efficiencies to each of the fusion proteins (Figure 3, lanes 1–10), demonstrating that merlin dimerization was unaffected by these mis-sense mutations. The decreased capture of radiolabelled merlin on

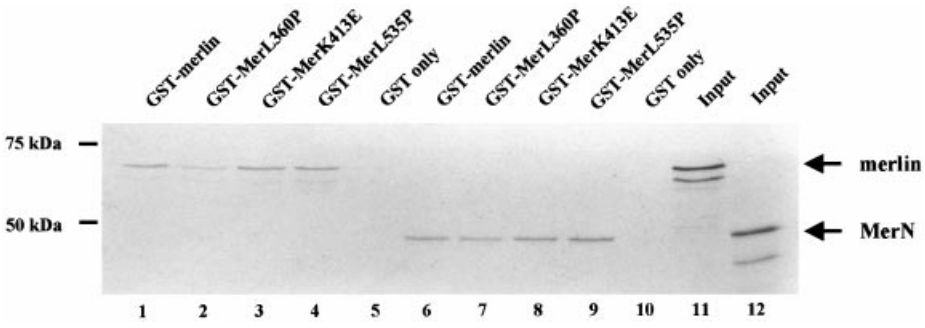

Figure 3 Analysis of the effect of merlin mis-sense mutations on homotypic interactions of merlin

After incubation with radiolabelled merlin and MerN, the fusion proteins GST–merlin (lanes 1 and 6), GST–MerL360P (lanes 2 and 7), GST–MerK413E (lanes 3 and 8) and GST–MerL535P (lanes 4 and 9), as well as GST only (lanes 5 and 10), were washed and the precipitates were subjected to SDS/PAGE. Gels were then stained with Coomassie Blue to confirm equal loading of fusion proteins (results not shown) and dried at 80 °C for 2 h before autoradiography.

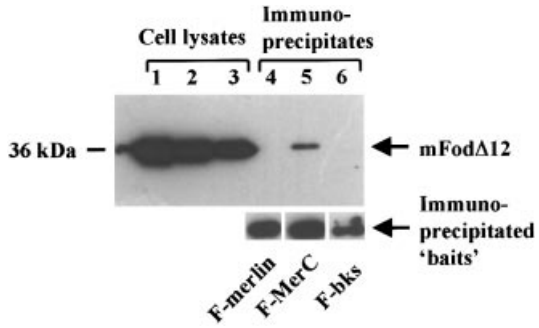

Figure 4 Analysis of the merlin–fodrin interaction *in vivo*

Myc-epitope-tagged fodrinΔ12 fragment (mFodΔ12) was co-expressed with FLAG-epitope-tagged merlin-I (F-merlin) (lanes 1 and 4), tagged merlin-I C-terminus (F-MerC) (lanes 2 and 5) or tagged bks control protein (F-bks) (lanes 3 and 6) in HEK-293 cells. Anti-FLAG immunoprecipitates were resolved by SDS/PAGE, Western blotted and probed with anti-FLAG antibody (lower panels) to detect the respective immunoprecipitated proteins, or probed with anti-Myc antibody (upper panel, lanes 4–6) to detect co-precipitated fodrin fragment. Probing of cell lysates with anti-Myc antibody (lanes 1–3) confirmed comparable expression of the fodrin fragment in each case.

incubation with GST–MerL360P (Figure 3, lane 2) is due to the presence of less fusion protein in the mixture (results not shown). This experiment further verified that the failure of fodrin to bind GST–MerL535P efficiently (Figure 2, lane 4) was specific, because merlin and MerN both bound to this fusion protein (Figure 3, lanes 4 and 9).

To assess the interaction in mammalian cells, FLAG-epitope-tagged merlin-I (F-merlin) and MerC (F-MerC) were co-expressed with Myc-epitope-tagged fodrin (mFodΔ12) in HEK-293 cells. The 12 most C-terminal residues of the fodrin fragment were deleted here to assess the physiological relevance of the intron-encoded amino acids on the merlin–fodrin interaction. mFodΔ12 was detected in anti-FLAG immunoprecipitates from cells co-expressing F-MerC (Figure 4, lane 5) but not F-merlin (Figure 4, lane 4) or a negative control protein F-bks [29] (Figure 4, lane 6). These results confirmed that fodrin interacted with the C-terminus of merlin-I at a site that was masked in the native protein. Furthermore, the intron-encoded residues were not required for the merlin–fodrin interaction.

N-terminus of merlin inhibits binding of the C-terminus to fodrin

To semi-quantify the merlin–fodrin interaction, a mammalian two-hybrid system was employed. All merlin proteins were expressed as GAL4 DNA-binding domain fusion proteins and the fodrin fragment was expressed as a fusion protein with the activation domain of herpes simplex virus VP16. Co-expression of recombinant MerC (323–595) with recombinant fodrin induced a more than 60-fold increase in normalized GAL4-binding-site-dependent luciferase reporter activity compared with the control (Figure 5A, columns 2 and 4). By comparison, co-expression of merlin-I and fodrin fusion proteins induced only a 3-fold increase in reporter activity (Figure 5A, column 1); co-expression of MerN (residues 1–378) and fodrin fusion proteins produced no increase in reporter activity (Figure 5A, column 3). These results again confirmed that fodrin interacted more strongly with the C-terminus of merlin-I than with the native protein. We therefore investigated the effect of the N-terminus of merlin on the MerC–fodrin interaction. Co-expression of the merlin N-terminal domain (residues 1–378) with fodrin and MerC fusion proteins decreased reporter activity by approx. 70% (Figure 5B, columns 1 and 2), whereas co-expression of two

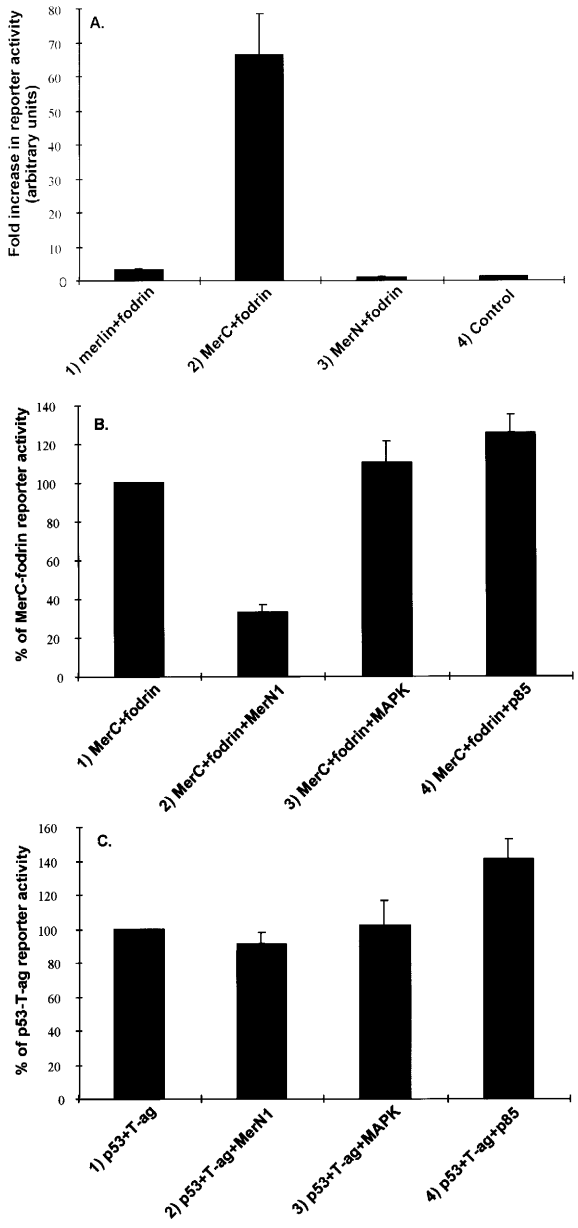

Figure 5 Analysis of the merlin–fodrin interaction and the role of the merlin N-terminal domain in inhibiting binding, with a mammalian two-hybrid system

(**A**) A fusion of the fodrin fragment with the VP16 transcriptional activation domain was co-expressed with fusions of the GAL4 DNA-binding domain with merlin-I (column 1), the C-terminus of merlin-I (MerC) (column 2) or the N-terminal region of merlin (MerN) (column 3) in HEK-293 cells. In each case a GAL4-binding-site-dependent luciferase transcriptional reporter vector was co-transfected. Luciferase levels were measured and normalized against an average of the values obtained by expressing merlin and fodrin fusion proteins separately (column 4, arbitrarily set at 1). (**B**) MerC and fodrin fusion proteins were co-expressed with MerN1 (column 2), MAPK (column 3) or the p85 subunit of phosphoinositide 3-kinase (column 4); standardized reporter activities are compared as a percentage of the control value for the MerC–fodrin interaction (column 1). (**C**) p53 and T-ag fusion proteins were co-expressed with MerN1 (column 2), MAPK (column 3) or p85 (column 4); standardized reporter activities are compared as a percentage of the control value for the p53–T-ag interaction (column 1). Results are means ± S.E.M. for at least four separate transfections.

distinct proteins, mitogen-activated protein kinase (MAPK) or the p85 subunit of phosphoinositide 3-kinase, with MerC and fodrin fusion proteins slightly increased reporter activity (Figure

5C, columns 3 and 4). To control for specificity, the merlin N-terminal domain was co-expressed with p53 and T-ag fusion proteins and found to have no effect on the p53–T-ag interaction (Figure 5C, columns 1 and 2). Similarly, co-expression of MAPK had little effect on reporter activity (Figure 5C, column 3) and co-expression of p85 again increased reporter activity (Figure 5C, column 4). These results indicate that the N-terminus of merlin specifically inhibited complex formation between fodrin and the C-terminus of merlin-I.

DISCUSSION

We have shown that merlin-I binds to β-fodrin in four different binding assays and that the interaction is mediated via the C-terminal domain of merlin-I. The region of fodrin isolated includes residues 982–1188, which encompasses spectrin repeat 8. By employing a similar methodology, Scoles et al. [27] identified a region of β-fodrin that interacts with merlin-II in yeast cells. This region encompasses spectrin repeats 15 and 16 and contains the ankyrin-binding domain. The authors demonstrated that full-length merlin-I does not bind efficiently to this region of fodrin and proposed that this might be due to self-binding of merlin-I. More direct evidence for this is provided in the present study. The C-terminus of merlin-I binds more efficiently to fodrin than does the native protein (Figures 2, 4 and 5A) and expression of the N-terminus of merlin *in trans* specifically inhibits binding of the C-terminus with fodrin (Figures 5B and 5C). Surprisingly, although β-galactosidase activity was not quantified, there seemed to be no noticeable differences in reporter activity between yeast cells co-expressing merlin-I or MerC and fodrin (Table 1), indicating that in this context the full-length protein might not have folded correctly, thus exposing the fodrin-binding site. The possibility that the merlin-II–fodrin interaction is significantly different from the merlin-I–fodrin interaction is suggested by the fact that, in comparison with full-length merlin-II, the C-terminus of merlin-II (residues 256–590) interacts only weakly with fodrin [27]. Another difference between these two studies is the fact that the regions of fodrin isolated were different. Interestingly, a search of the National Centre for Biotechnology Information database with BLAST programs [34] reveals that the region of β-fodrin isolated in the present study displays the strongest similarity to a sequence extending from within spectrin repeat 15 to the N-terminus of repeat 17 of β-fodrin (Figures 6A and 6B), which includes almost 78 % of the sequence studied previously [27]. This suggests that merlin might interact with multiple spectrin repeat motifs in fodrin. However, the C-terminal half of repeat 15 is atypical and predicted to form a β-sheet as opposed to the classical α-helical structure, indicating that this might not be so. Further work will be required to evaluate the specificity of merlin binding to fodrin, especially with regard to spectrin repeats.

Co-localization of merlin and fodrin has been demonstrated in various tissues and cell types; the proteins have been isolated as a complex in Schwann-like cells [27]. Although this provides evidence for a physiologically relevant interaction, the antibody used in these experiments does not distinguish between the two merlin isoforms and, as discussed, the nature of the interaction with fodrin might differ between merlin-I and merlin-II. Furthermore, the growth suppressor properties of merlin are associated with merlin-1 [8] and the transcript for isoform I is the predominant form detected in the eighth cranial nerve [6], indicating that abrogation of merlin-I function is critical in the genesis of at least some NF2-associated tumours.

Conformational differences between the two merlin isoforms have been highlighted recently. Full-length merlin-I binds less efficiently to NHE-RF/EBP50 [32], the Rho GDP dissociation

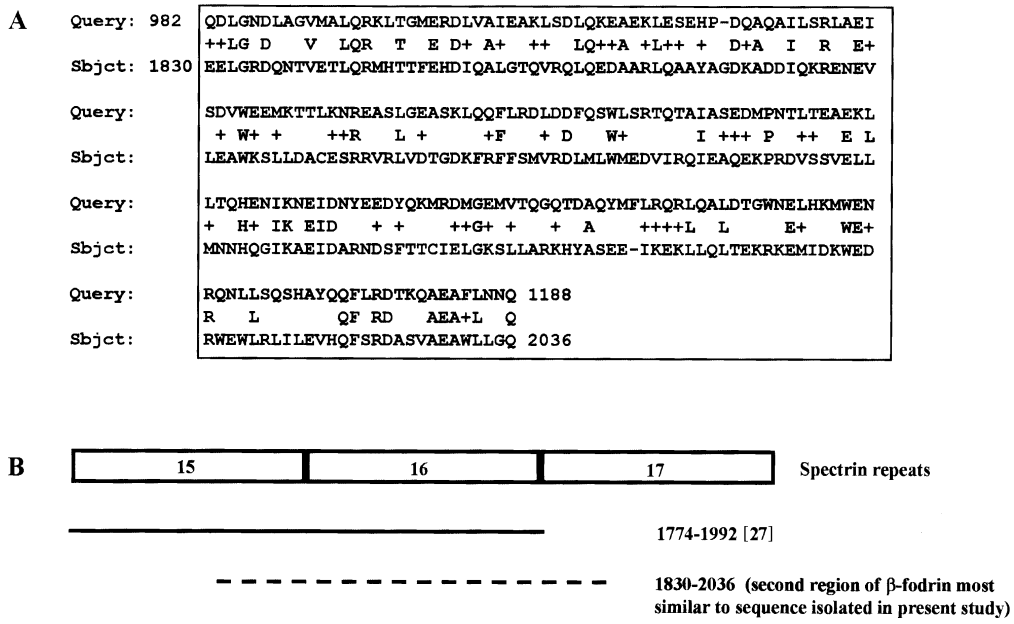

Figure 6 Sequence similarity of β-fodrin spectrin repeats associated with merlin binding

(**A**) The encoded sequence (residues 982–1188) of the β-fodrin cDNA isolated in this study displays 25% identity and 46% similarity to residues 1830–2036 of the same protein. (**B**) Schematic representation indicating the overlap between the region of β-fodrin studied previously (solid line) [27] and the region of β-fodrin showing the strongest similarity to the sequence studied currently (broken line).

inhibitor (Rho GDI) [35] and the hepatocyte-growth-factor-regulated tyrosine kinase substrate [36] than does full length merlin-II; a novel protein, SCHIP-1, also binds with less efficiency to merlin-I than to various mutant merlin proteins in mammalian cells [37]. These differences might be attributable to the ability of merlin to form homophilic interactions. Merlin-II does not form inter-domain interactions [8,32] and thus its binding sites are normally exposed. In contrast, as has been well characterized for ERM proteins [38–40], merlin-I is subject to intramolecular and/or intermolecular homophilic interactions [8,23,30–33,41] which seem to mask the binding sites for certain merlin-interacting proteins including fodrin. We have so far been unable to detect reproducibly an association of 'endogenous' β-fodrin with its binding region on merlin-I *in vivo* by co-immunoprecipitation. Although this might be attributable to technical difficulties, such as disruption of the interaction by the lysis conditions required for solubilizing the cytoskeletal proteins, another explanation is possible. Specific intracellular conditions, associated with the regulation of signalling pathways, might be required for efficient β-fodrin–merlin-I binding. An important goal for future research will be to identify signalling pathways that regulate the association through modifying protein conformations and/or post-translational modifications, because this will give valuable insights into the cell regulatory mechanisms in which merlin's tumour suppressor function participates. By comparison with ERM proteins, exposure of merlin-I's binding sites might be regulated by phosphoinositides or phosphorylation. The binding of full-length ERM proteins to CD44, ICAM-I (intercellular adhesion molecule 1) and ICAM-2 is enhanced in the presence of PtdIns4*P* and PtdIns(4,5)P_2 [42,43], and recent evidence has shown that the binding of merlin-I to NHE-RF/EBP50 is enhanced by PtdIns(4,5)P_2 [32]. Merlin-I binding to fodrin might similarly be regulated by PtdIns(4,5)P_2. β-Fodrin contains a pleckstrin homology domain that binds PtdIns(4,5)P_2 [44]; this might localize or sequester the phospholipid to activate merlin or it might localize merlin to PtdIns(4,5)P_2-rich domains. Phosphorylation of ezrin and moesin on a homologous C-terminal threonine residue by protein kinase C-θ or Rho-kinase exposes the binding site for NHE-RF/EBP50 and F-actin [45,46]. Merlin also contains a homologous threonine residue as well as several other potential phosphorylation sites [2]; protein conformation might therefore be regulated by a combination of phosphorylation and phosphoinositides. Differential phosphorylation of merlin is associated with changes in cell adhesion, supporting a role for this process in regulation of the protein [15]. Other studies have implicated a role for merlin in cell adhesion. Merlin co-localizes with E-cadherin [35]; McCartney and Fehon [47] stated that, in *Drosophila*, Merlin co-localizes with Armadillo in adherens junctions of wing imaginal discs. Merlin localizes to focal adhesions in fibroblasts [48] and to adherens junctions in glioma cells [49]. Suppression of merlin expression with anti-sense oligonucleotides and overexpression of mutant merlin proteins abrogate cell adhesion [10,12,33]; *NF2*$^{+/-}$ mice are highly susceptible to metastatic tumours [11], providing functional evidence that merlin might be involved in cell adhesion. Interestingly, spectrin has been implicated in cell–substrate adhesion in a complex with ankyrin, which additionally interacts with adhesion proteins such as integrins and CD44 [50]; in *Drosophila*, βH-spectrin (a high-molecular-mass spectrin isoform) co-localizes with E-cadherin at adherens junctions in the wing and eye imaginal discs [51].

This study has shown that the disease-associated mutation L535P significantly decreases the merlin–fodrin interaction (Figure 2, lane 4), whereas the other mutations studied, L360P and K413E, do not do so to a comparable extent (Figure 2, lanes 2 and 3). Preliminary studies with the mammalian two-hybrid system have verified that only L535P completely inhibits merlin-I binding to fodrin (G. W. Neill and M. R. Crompton, unpublished work). The effects of mis-sense mutations on merlin function have been investigated in several systems. With regard to cytoskeletal association, L535P decreased the binding of merlin-II to fodrin by 75% and L360P decreased binding by 67% in comparison with wild-type, as determined by β-galactosidase activity in the yeast two-hybrid system [27]. The latter value is in contrast with the finding of this study and might reflect such variables as the different binding assay employed, the merlin isoform used and the region of fodrin isolated. Because both L360P and L535P result in a relaxed association of merlin-I with the actin-based cytoskeleton [25,33], multiple protein–protein interactions probably underlie this localization. L535P might also affect the ability of merlin to interact with the cell membrane because the mutation is one of several that inhibit the binding of merlin to NHE-RF/EBP50 [33]; both L360P and L535P display a decreased interaction with the hepatocyte-growth-factor-regulated tyrosine kinase substrate associated with early endosomes [36]. Functional assays have shown that overexpressed K413E and L535P have no effect upon the growth or motility of rat schwannoma cells, whereas native merlin-I is inhibitory [13,30] and various mis-sense merlin-I mutants including L535P decrease cell adhesion in culture plates [33]. The mutation L535P is associated with a mild clinical phenotype [52] and is predicted to break the α-helix of the C-terminus. This indicates that disruption of the helix with a subsequent abrogation of the merlin–fodrin interaction might not fully inhibit the tumour suppressor ability of merlin. Merlin L535P might still interact with the actin-based cytoskeleton via its N-terminal actin-binding site [24] or through unidentified factors and might thereby retain partial function in signalling pathways that regulate cell growth. The various mutations in *NF2* found in tumours possibly affect binding to several different proteins, resulting in differing outcomes with regard to growth control. Furthermore, merlin is subject to calpain-induced proteolysis [53], and mutant merlin proteins might be more susceptible to this process by virtue of weakened or abrogated protein interaction(s), such as that with fodrin.

The ability of merlin-I to homodimerize is well characterized [8,23,30–33,41] and might be relevant to its ability to function as a negative regulator of cell growth. We have shown that merlin-I is capable of forming homodimers when one molecule contains the mutation L360P, K413E or L535P (Figure 3). This is in agreement with the results of Gutmann et al. [30], who also demonstrated that K413E and L535P do not decrease dimerization efficiency. Recent quantitative analysis has shown that L535P actually increases the affinity almost 3-fold between the mutant and wild-type proteins compared with wild-type merlin-I dimers [33]; several other disease-associated natural mutations affect merlin dimerization [13,33], providing evidence that the function of merlin might be affected by changes in its ability to form intramolecular or intermolecular homophilic interactions. NF2-associated tumours are frequently characterized by loss of the wild-type *NF2* allele, with the remaining allele harbouring the mutation; intermolecular associations might therefore be more seriously affected between molecules containing the same mutation. Merlin also forms heterotypic interactions with ERM proteins [31,32,41] and could therefore influence signalling pathways mediated by these proteins, including those involving the Rho GTPase family [54–56]. Mutations affecting merlin dimerization might similarly affect merlin–ERM heterodimers and subsequently alter signalling pathways involving ERM proteins.

Because merlin interacts with Rho GDI [35], it might influence Rho signalling directly or in combination with ERM proteins.

In conclusion, merlin-I binds to β-fodrin at a site that has not been described previously. This interaction is via the C-terminus of merlin-I and might be subject to regulation by the merlin N-terminal domain. The fact that the disease-associated mutation L535P abrogates merlin–fodrin, but not merlin–merlin, binding dissociates these two interactions with regard to their potential tumour suppressor functions. The recent structural characterization of the FERM–tail interaction of moesin, with the similarities implicated for merlin, should help to unravel the molecular basis of target binding by merlin [57]. None of the protein–protein interactions described for merlin have been unequivocally implicated in the tumour suppressor mechanism; studies in this area have a priority for the future.

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