Defects in neurofibromatosis 2 protein function can arise at multiple levels

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Neurofibromatosis 2 (NF2) is an inherited cancer syndrome resulting from mutations in the NF2 tumor suppressor gene. Analysis of NF2 mutations has revealed some general genotype–phenotype correlations. Severe disease has been associated with mutations that produce a premature termination while more mild disease has been associated with missense mutations. Here, we provide experimental proof for these genotype–phenotype correlations by demonstrating that nonsense mutations fail to produce stable merlin protein while missense mutations result in the generation of merlin proteins defective in negative growth regulation. This inability to suppress cell growth may result from defects in the function of merlin at several levels, including failure to form an intramolecular complex. Based on these findings, we propose a model for merlin growth suppression that provides a framework for analyzing NF2 patient mutations and merlin function.

INTRODUCTION

Neurofibromatosis 2 (NF2) is an autosomal dominant disorder in which affected individuals develop schwannomas, meningiomas and ependymomas (1,2). The clinical hallmark of the disease is the development of bilateral schwannomas involving the eighth cranial nerve (vestibular schwannomas). For this reason, affected individuals develop balance and hearing problems. Positional cloning facilitated the identification of the NF2 tumor suppressor gene, termed merlin or schwannomin (3,4). Database homology searches demonstrated that merlin has structural similarity with a family of proteins that link the actin cytoskeleton to cell membrane glycoproteins. Members of this family include ezrin, radixin and moesin (ERM proteins) (5–8).

ERM proteins are expressed in a wide variety of cell types, where they associate with cell membrane glycoproteins using N-terminal residues and the actin cytoskeleton by virtue of an actin-binding region in the C-terminus. Subcellular localization studies have shown that ERM proteins are associated with membrane ruffles as well as cytoskeletal stress fibers. Although the functions of these ERM proteins are not known, they have been implicated in cellular remodeling and the formation of membrane ruffles.

Alignment of the predicted merlin amino acid sequence demonstrates that the regions of greatest structural similarity to the ERM proteins lie within the N-terminal two-thirds of merlin (glycoprotein association and α-helical domains) (9). The C-terminus of merlin is unique and lacks the conventional actin-binding region found in other ERM proteins.

The NF2 gene is composed of 17 exons and codes for a protein of 595 amino acids (3,4). Alternative splicing of exon 16 generates merlin molecules lacking exon 16 (wild-type NF2.17) or merlin molecules containing exon 16 (NF2.16). Due to the presence of a premature termination codon in exon 16, NF2.16 produces a merlin molecule containing 11 unique residues and no exon 17 sequences.

Merlin is expressed in several different cell types, including neurons, Schwann cells and meningeal cells. In the rat sciatic nerve, merlin co-localizes with RhoA at paranodal incisures (10). Subcellular localization studies have shown that merlin associates with F-actin filaments at ruffling membrane edges (11). However, merlin does not localize to the same subcellular structures as either ezrin or moesin in human meningioma cells, suggesting it may have different function(s) than ERM proteins.

Analysis of the NF2 gene in patients affected with NF2 has demonstrated mutations in the coding region of the gene that are predicted to result in truncated proteins (insertions, deletions and premature terminations) or, less commonly, dysfunctional proteins secondary to missense mutations. Genotype–phenotype correlations have been established based on studies of families with NF2 (12–14). In some families, NF2 appears to be inherited as an early onset, clinically more severe disease with rapid progression of symptoms. In other families, the age of onset is later and the clinical progression is slower. Families with more severe disease tend to harbor NF2 mutations that result in premature protein termination due to frameshift mutations (deletions or insertions) or the presence of a premature termination codon. In families with more mild clinical disease, the mutations are either missense mutations or are...
not found by conventional mutation detection methods. It is predicted that nonsense mutations would result in unstable truncated merlin proteins whereas missense mutations might generate full-length merlin proteins incapable of growth suppression.

Previous studies in our laboratory and others have demonstrated that merlin can suppress the growth of fibroblasts and schwannoma cells in vitro and in vivo (15–17). Overexpression of the NF2 gene in NIH-3T3 cells resulted in a 3-fold decrease in cell proliferation in one study (17) and reversion of Ras-induced transformation in another (16). In RT4 and JS1 rat schwannoma cells, overexpression of the NF2 tumor suppressor gene resulted in reduced cell proliferation in vitro and reduced tumor formation in nude mice in vivo (15). In these experiments, it was demonstrated further that merlin function is dependent upon the formation of an intramolecular complex (15). This intramolecular association requires an interaction between N- and C-terminal residues and is dependent on the presence of exon 17 sequences. The inability of merlin proteins with altered C-termini to form these physical complexes was associated with a failure to negatively regulate schwannoma cell growth. These findings suggested that mutations in the NF2 gene could have differential effects on merlin function based on their ability to disrupt merlin protein interactions.

To test the hypothesis that NF2 gene mutations give rise to defects in merlin function that arise at multiple levels, including protein stability and protein–protein interactions, we analyzed the effect of selected NF2 patient mutations on merlin function. In this report, we demonstrate that merlin forms both intra- and intermolecular complexes using a series of naturally occurring and laboratory-generated merlin cDNA constructs. Moreover, we provide experimental proof for the notion that merlin nonsense mutations do not produce stable merlin proteins and that missense mutations can result in the generation of stable but defective merlin tumor suppressor proteins. A molecular model for merlin function is presented as a framework for the analysis of NF2 patient mutations.

RESULTS

Merlin forms intramolecular complexes

Previously, we demonstrated that merlin forms intramolecular complexes by virtue of an association between its N- and C-termini (15). In these experiments, co-immunoprecipitation and glutathione S-transferase (GST) fusion protein interaction assays were performed to demonstrate this interaction. Moreover, co-expression of the N-terminus (residues 1–302) and the C-terminus (residues 299–595) as separate transgenes in RT4 cells resulted in growth suppression similar to that observed with full-length merlin.

Previously, we demonstrated that exon 17 sequences were necessary for the formation of this merlin intramolecular complex. To determine which N-terminal residues of merlin were required for this self-association, several merlin–GST fusion proteins were generated and purified on glutathione–agarose beads. These GST–N-terminal merlin fusion proteins were then used as bait with a radiolabeled in vitro transcribed and translated C-terminal merlin fragment (residues 299–595). Quantitation by phosphorimage analysis demonstrated that 40–50% of the C-terminal fragment bound to N-terminal 400 (residues 9–400) compared with 1% binding of a C-terminal fragment truncated at residue 547 (15). As can be seen in Figure 1, C-terminal binding to the N-terminus of merlin was dramatically reduced when the N-terminal merlin bait was shortened from 400 residues to 288 residues. Longer exposures demonstrated weak binding of C-terminal merlin to N-terminal 288 (20% of NF2.400 binding), but not to N-terminal 185 (2% of N-terminal 400 binding). These results suggest that at least one region in the amino-terminal domain required for merlin self-association resides between residues 288 and 400 of merlin. Therefore, merlin intramolecular complex formation requires an interaction between exon 17 sequences in the C-terminus and N-terminal residues between amino acids 288 and 400.

Merlin forms intermolecular complexes

Previous studies on other ERM proteins have shown that ezrin and radixin form homo- and heterodimers (18–22). To determine whether merlin forms similar complexes, we used a series of N-terminal merlin–GST fusion proteins as bait in concert with a panel of in vitro transcribed and translated merlin targets. In direct binding experiments, NF2.400– and NF2.185–GST fusion proteins were used as bait. Radioactive N-terminal merlin target fragments were assayed for their ability to bind to these N-terminal merlin fusion protein baits. Both bound and unbound fractions were analyzed by SDS–PAGE and autoradiography. In these experiments, binding of N-terminal (residues 1–302), N-terminal 122–302 (residues 122–302), N-terminal 185–3 (residues 1–185 lacking exon 3) was demonstrated (Fig. 2 A). In addition, N-terminal radixin (residues 1–323) associated with the N-terminal merlin–GST fusion protein bait. These results suggest that merlin forms intermolecular associations with ERM fusion protein bait N-terminal residues in vitro. These intermolecular complexes require residues in the N-terminus between amino acids 122 and 185.

To determine whether this association occurs in the context of the full-length merlin protein, two additional experiments were performed. The first utilized full-length merlin (residues 9–595) as GST fusion protein bait and full-length merlin as target, while the second employed NF2.288 as the GST fusion protein bait. Instead of analyzing the amount of target bound and unbound to the GST–merlin bait, equal amounts of radioactive target were divided into two tubes, one containing GST fusion protein alone and another containing GST–merlin fusion protein, and the
Figure 2. Merlin forms intermolecular complexes. (A) The ability of merlin to form intermolecular complexes was examined using GST–merlin fusion proteins. NF2.400 and NF2.185 were used as GST fusion protein baits with various N-terminal merlin constructs. Both N-terminal merlin constructs (N-terminal, lane 1; N-terminal 185–302, lane 3; N-terminal 122–302, lane 4) and N-terminal radixin (lane 2) formed complexes with the N-terminus of merlin. (B) The ability of merlin to form intermolecular complexes was quantitated using a GST fusion protein binding assay. Unbound radiolabeled merlin was collected and analyzed by SDS–PAGE and autoradiography. Approximately 25–35% of radiolabeled NF2.17 bound to GST–NF2.288 or GST–NF2.17 (residues 9–595) as determined by phosphorimage analysis. M denotes GST–NF2.288 or GST–NF2.17 (residues 9–595) while G denotes GST alone. (C) Discontinuous glycerol gradients were employed to determine whether merlin forms high molecular weight complexes. In these experiments, RT4 cells overexpressing full-length NF2.17 were lysed using non-reducing NP-40 or reducing RIPA detergent extraction and subjected to glycerol gradient ultracentrifugation. Under non-reducing NP-40 lysis conditions, merlin is detected in a bimodal distribution, peaking at 66 kDa (fractions 3–4) and 140 kDa (fraction 6) whereas merlin was maximally detected in the BSA fraction (fraction 3) with RIPA lysis. The positions of BSA (66 kDa) and ADH (150 kDa) are depicted.

amount of unbound target was analyzed after incubation. This allows for a more quantitative estimate of the amount of in vitro transcribed and translated merlin target that binds to the merlin fusion protein bait. In these experiments, full-length merlin associated with both full-length merlin (residues 9–595) and NF2.288 merlin baits as demonstrated by a reduction in merlin target radioactivity in the unbound fraction after incubation with merlin–GST fusion protein (Fig. 2B). Approximately 25–35% of merlin target associated with NF2.17 (residues 9–595) or NF2.288 merlin bait, as determined by phosphorimage analysis. Identical results were obtained with NF2.400– and NF2.185–GST fusion protein baits (not shown).

To determine whether homotypic merlin complexes form in vivo, two approaches were taken. First, glycerol gradient analysis of merlin overexpression in RT4 rat schwannoma cells was performed. In these experiments, RT4 schwannoma cells stably expressing full-length merlin (NF2.17) were lysed using non-reducing NP-40 or reducing RIPA lysis buffer and fractionated on 9–14% discontinuous glycerol gradients by ultracentrifugation. Bovine serum albumin (BSA; 66 kDa) and alcohol dehydrogenase
(ADH; 150 kDa) were used as internal standards for molecular size (Fig. 2C). With RIPA lysis, Merlin was maximally detected by western blot in fraction 3 (∼66 kDa), whereas in the non-reducing NP-40 lysis experiment, Merlin immunoreactivity was detected in two peaks, one at ∼66 kDa (fractions 3–4) and another at ∼140 kDa (fraction 6) by scanning densitometric analysis. These results suggest that Merlin may form higher molecular weight complexes, perhaps with other Merlin molecules. Similar experiments performed with NF2.17 demonstrated identical results (not shown).

To directly demonstrate the formation of Merlin homotypic complexes in vivo, co-immunoprecipitation experiments using epitope-tagged Merlin constructs were designed. COS-1 or NIH-3T3 cells were transiently co-transfected with myc- and FLAG-tagged Merlin cDNAs. Forty eight hours later, the cells were lysed in non-reducing NP-40 lysis buffer and protein was immunoprecipitated with the 9E10 myc monoclonal antibody. Immunoprecipitates were then analyzed by western blotting using FLAG antibodies. In these experiments, both epitope-tagged Merlin transgenes were observed in cell lysates; however, no co-immunoprecipitated FLAG-tagged Merlin was detected reproducibly in the 9E10 immunoprecipitates. These negative results suggest that if homotypic Merlin complexes form in vivo, they do so only under certain conditions.

NF2 nonsense mutations fail to produce stable Merlin proteins

Genotype–phenotype analyses of NF2 families have suggested that NF2 mutations that result in premature truncations are associated with a more severe clinical phenotype. In addition, several studies examining tumor specimens looking for truncated Merlin proteins have failed to identify such protein species (23–25). To determine whether NF2 nonsense mutations result in the production of stable truncated Merlin proteins, two representative NF2 mutations, Q111X and R466X, were analyzed. These mutations were generated by oligonucleotide-directed mutagenesis and sequenced in their entirety. To demonstrate that the mutations were generated correctly, in vitro transcription–translation experiments were performed (Fig. 3A). The presence of a truncated product was observed in both transcription–translation experiments were performed in two peaks, one at ∼66 kDa (fractions 3–4) and another at ∼140 kDa (fraction 6) by scanning densitometric analysis. These results suggest that Merlin may form higher molecular weight complexes, perhaps with other Merlin molecules. Similar experiments performed with NF2.17 demonstrated identical results (not shown).

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Despite the ability to produce these truncated proteins in vitro, multiple attempts to generate stable RT4 or JS-1 rat schwannoma cell lines with these mutations were unsuccessful. In total, >40 individual genetin-resistant clones were screened for each nonsense Merlin construct without evidence of truncated Merlin protein expression. In contrast, screening of RT4 or JS1 rat schwannoma cell clones transfected with full-length NF2.17 or NF2.17 containing nonsense mutations (L64P, K413E or L535P) resulted in a 30–40% success rate for each construct. To demonstrate that JS1 cell lines contained the nonsense NF2 construct, but failed to express truncated Merlin proteins, representative genetin-resistant clones for each construct were selected and analyzed by RT-PCR. Using primers that recognize the T7 site (present in the pcDNA3 NF2 transgene) and sequences within the NF2 gene, expression of the transgene was detected under conditions previously shown to be semi-quantitative in our laboratory. In all cases, amplification of the transgene was detected in the nonsense (Q111X and R466X) and NF2.17, but not the pcDNA3 vector, transfected RT4 cell lines (Fig. 3C). Cyclophilin was included as an internal control for RNA quality and quantity. No product was observed if reverse transcriptase was omitted, arguing that we were not detecting the transfected and integrated pcDNA3 NF2 transgene plasmid DNA (data not shown). The levels of NF2 transgene mRNA expression did not differ substantially from NF2.17 or other NF2 transgenes, suggesting that significant mRNA instability does not account for the reduced Merlin protein expression observed. These results make the case that NF2 nonsense mutations do not produce truncated Merlin proteins in vivo and in effect lead to absent Merlin production from that allele. This result is in keeping with results from our laboratory and others that truncated Merlin proteins are not detected in schwannoma tumor specimens.

NF2 missense mutations result in a defective Merlin protein

To further our analysis of the effect of NF2 patient mutations on Merlin function, we next analyzed three representative missense mutations in spatially distinct regions of the protein. These mutations included L64P (N-terminal domain), K413E (α-helical domain) and L535P (C-terminal domain). For each construct, at least three stable RT4 cell lines were selected with approximately equivalent amounts of Merlin overexpression, as determined by western blot (Fig. 4A; K413E not shown). In contrast to the nonsense mutants, we were able to generate missense Merlin cell lines at an efficiency similar to wild-type NF2 expressing cell lines (30–40% success rate).

To evaluate the stability of Merlin missense mutants, we performed pulse–chase labeling experiments with [35S]methionine on NF2.17 (wild-type Merlin) and two missense mutants, L64P and L535P. In these experiments, the half-life of NF2.17 was calculated to be 20 h, whereas both L64P and L535P had half-lives between 15 and 17 h. Despite these minimal differences in protein turnover, abundant mutant Merlin protein was detected by western blot (Fig. 4A).

To determine the effect of Merlin missense mutants on RT4 schwannoma growth, we performed several growth assays, including direct cell counting (Fig. 4B), thymidine incorporation (Fig. 4C), and the Promega CellTiter nonradioactive proliferation assay (not shown). For each construct, three representative RT4 cell lines with equivalent amounts of Merlin overexpression were used. Analysis of representative RT4 cell lines overexpressing NF2 missense mutations L64P and L535P are shown in Figure 4A (protein expression), Figure 4B (direct cell counting at 60 h) and Figure 4C (thymidine incorporation). Both L64P and L535P Merlin-expressing cell lines grew at the same rate as the untransfected RT4 cells or RT4 cells stably transfected with pcDNA3 vector alone (pcDNA.1 and pcDNA.2 RT4 cell lines). No effect on RT4 schwannoma growth was observed with another NF2 missense mutation (K413E; not shown). In contrast, RT4 cells overexpressing full-length Merlin (NF2.17) had significantly slower growth (40–50% of the vector controls) as assayed by direct cell counting (Fig. 4B), thymidine incorporation (Fig. 4C) and the non-radioactive proliferation assay (not shown). Collectively, these data support the hypothesis that Merlin missense mutations are defective as negative growth regulators.
Figure 3. NF2 nonsense mutations fail to produce stable protein. (A) In vitro transcription and translation in the presence of [35S]methionine demonstrates the production of merlin proteins representing NF2.17 (lane 1; 69 kDa), N-terminal (lane 2; 33 kDa), NF2.17Q111X (lane 3; 14 kDa) and NF2.17R466X (lane 4; 55 kDa). (B) COS-1 cells were transiently transfected with pcDNA3 vector alone (lane 1), NF2.17 (lane 2; 69 kDa), NF2.17Q111X (lane 3; 14 kDa), NF2.17R466X (lane 4; 55 kDa) and NF2.17L535P (lane 5; 69 kDa). Proteins were detected with the FLAG antibody. The bottom of the gel was exposed for 20 min to detect the NF2.17Q111X 14 kDa protein. (C) RT-PCR experiments were performed on geneticin-resistant stable RT4 cell lines transfected with NF2.17 (lane 2), pcDNA3 vector alone (lane 1) and NF2.17Q111X (labeled –1, –2, –3 and –4) using the T7 and M554AS primer set (top panel). Identical RT-PCR analyses were also performed on geneticin-resistant stable RT4 cell lines transfected with NF2.17 (lane 1), pcDNA3 vector (lane 2) and NF2.17R466X (labeled –1, –2 and –3) using the T7 and M554AS primer set (bottom panel). Only the transgene is amplified in this reaction. In all RT4 NF2.17 transfectants, RNA is detected from the transgene.

Another property of transformed cells is their loss of contact inhibition-mediated growth arrest. Both RT4 and JS1 cells continue to grow after reaching confluence in vitro. To determine whether wild-type merlin, but not merlin containing missense mutations, could partially restore this contact inhibition growth arrest phenotype, RT4 cells overexpressing wild-type merlin (NF2.17) and mutant merlin (NF2.17L535P) were grown to confluence and the number of cells counted in quadruplicate as a function of time. One such representative experiment is shown in Figure 4D. Whereas NF2.17-expressing RT4 cells slowed their growth after reaching confluence, RT4 cells expressing mutant (NF2.17L535P) merlin continued to grow. Untransfected RT4 cells and RT4 cells stably transfected with pcDNA3 vector alone had growth patterns similar to stably transfected mutant merlin RT4 cell lines (not shown).

The effect of NF2 missense mutations on merlin protein interactions

The inability of a stably expressed mutant merlin molecule to negatively regulate cell growth might result from altered merlin protein interactions. To determine whether NF2 missense mutations generated merlin proteins with altered protein associations, we analyzed the ability of these mutant merlin proteins to behave as wild-type merlin in a variety of cell extraction and protein interaction paradigms. First, merlin, like other ERM proteins, partitions upon Triton X-100 extraction into both the soluble and insoluble fractions with greater enrichment in the Triton-soluble pellet. As shown in Figure 5A, NF2 mutations L64P and L535P had similar Triton X-100 extraction profiles as NF2.17 (wild-type merlin). Second, merlin is partitioned equally into membrane and cytosol
Figure 4. Analysis of mutant merlin overexpression on RT4 cell growth. (A) Western blotting of equal amounts of protein from RT4 cell lines transfected with pcDNA3 vector alone (labeled ‘pc’), NF2.17, NF2.17L64P (three clones, labeled ‘–1, –2 and –3’), and NF2.17L535P (three clones, labeled ‘–1, –2 and –3’). Merlin was detected using the WA30 antibody. (B) Direct cell counting experiment of representative RT4 cell lines. Ten thousand cells were seeded in quadruplicate in 24-well plates and cells were counted at 60 h. Significantly reduced RT4 cell proliferation (asterisk) was observed with NF2.17 transfectants, but not with either NF2 missense mutant. No changes in cell viability were observed, based on trypan blue exclusion. Standard deviations are depicted for each cell line. (C) Thymidine incorporation of merlin missense mutant RT4 cell lines was performed as described in Materials and Methods. Significantly reduced RT4 proliferation (double asterisk) as measured by thymidine incorporation (c.p.m.) was observed with the wild-type merlin RT4 cell line (NF2.17) but not with the L64P or L535P missense mutant RT4 cell lines. Standard deviations are depicted for each cell line. (D) Direct cell counting of wild-type (NF2.17) and mutant (L535P) merlin RT4 cell lines grown past confluence. Cells were 100% confluent at 96 h after plating (denoted by an asterisk). The growth of RT4 cells expressing wild-type merlin (NF2.17) was significantly reduced compared with cells overexpressing mutant (L535P) merlin. No changes in cell viability were observed, based on trypan blue exclusion. Standard deviations are depicted for each cell line.

fractions upon hypotonic lysis. Similarly, as shown in Figure 5B, NF2 mutations L64P and L535P had similar hypotonic lysis extraction profiles to NF2.17 (wild-type merlin). Third, merlin can form intermolecular complexes with other merlin proteins in vitro. To determine whether the ability of NF2 missense mutants to associate with full-length merlin was impaired, we performed binding experiments using in vitro transcribed and translated NF2 cDNA targets and full-length merlin–GST fusion protein baits as described in Figure 2B. As seen in Figure 5C, both L64P and L535P had similar binding (25–35%) to the full-length merlin–GST fusion protein bait as wild-type merlin (NF2.17). Similar results were observed with the K413E mutant (not shown). Fourth, merlin forms intramolecular associations by virtue of N- and C-terminal interactions. To determine whether the C-terminus of merlin containing the K413E mutation could associate with the N-terminus, GST–merlin fusion protein interaction experiments were performed. As observed with the wild-type C-terminus (C-term), C-term containing the K413E mutation also associated with the N-terminal merlin bait with similar percentage binding (Fig. 5D).

Lastly, we have recently shown that merlin interacts with polymerized microtubules using a region in the N-terminus that is masked in the full-length protein (26). In these experiments,
Figure 5. Analysis of missense mutations on merlin protein interactions. (A) Selective extraction using Triton X-100 was performed on RT4 cells overexpressing wild-type and mutant merlin, as described in Materials and Methods. Equal volumes of supernatant (S) and pellet (P) were analyzed by western blotting. Detection was accomplished using WA30 anti-merlin antibodies. Mutant merlin (L64P and L535P) had similar Triton X-100 extraction profiles to wild-type merlin (NF2.17). (B) Membrane fractions were prepared from RT4 cell lines overexpressing NF2.17, NF2.17L64P and NF2.17L535P as described in Materials and Methods. Equal volumes of the supernatant (S) and membrane (M) fractions were analyzed by western blotting. Detection was accomplished using WA30 anti-merlin antibodies. Mutant merlin (L64P and L535P) had similar membrane extraction profiles to wild-type merlin (NF2.17). (C) The ability of merlin to form intermolecular complexes was quantitated using a GST fusion protein binding assay (described in Fig. 2B). Unbound radiolabeled merlin was collected and analyzed by SDS–PAGE and autoradiography. Equal binding of wild-type (NF2.17) and mutant merlin targets were observed (25–35% as determined by phosphorimage analysis). M denotes GST–NF2.17 while G denotes GST alone. (D) The ability of merlin to form intramolecular complexes was determined using a GST fusion protein binding assay. Bound (B) and unbound (S) wild-type (C-term) or mutant (C-termK413E) C-terminal merlin fragments were collected and analyzed by SDS–PAGE and autoradiography. Binding of both mutant (K413E) and wild-type C-terms to NF2.400, but not GST, was observed. (E) Microtubule assembly assays were performed using in vitro transcribed and translated NF2 cDNA products as described in Materials and Methods. Equal volumes of supernatant (S) and microtubule pellet (M) were analyzed by western blot using Wa30 rabbit polyclonal antibodies. In this experiment, neither the in vitro transcribed and translated wild-type NF2 product (NF2.17) or NF2.17L535P significantly associated with polymerized microtubules, whereas the NF2.17L64P did. The positions of the merlin products are denoted by the arrow. The position of the molecular size markers is shown along the left side of the blot.

N-terminal merlin (residues 1–302), but not full-length merlin (NF2.17), associates with polymerized microtubules. Merlin proteins with alterations in the C-terminus (merlin containing exon 16 and merlin truncated at residue 547) cannot form intramolecular complexes as determined by GST fusion protein binding and co-immunoprecipitation experiments. These C-terminal merlin mutants do not suppress RT4 or JS1 rat schwannoma cell growth in vitro and in vivo. Unlike wild-type full-length merlin, merlin containing exon 16 as well as merlin truncated at residue 547 associated with polymerized microtubules, suggesting that ‘open’ and ‘closed’ conformations of merlin exist. In the ‘open’, but not the ‘closed’, conformation, merlin can bind polymerized microtubules. We used this assay to determine whether any of the merlin missense mutants associated with polymerized microtubules as an indication of an ‘open’ or non-functional conformation. In these experiments, the L64P mutant, but not the L535P or K413E (not shown) mutants, bound polymerized microtubules (Fig. 5E). These results argue that the L64P mutation changes the conformation of merlin in such a
DISCUSSION

The ability of merlin to function as a negative growth regulator probably requires several important protein interactions (Fig. 6). Based on the results presented in this report, we propose that merlin growth regulation is dependent on (i) the production of a stable protein, (ii) the formation of intramolecular complexes and (iii) interactions with critical effector proteins. Defects at any of these levels result in a dysfunctional merlin molecule. It is also possible that additional interactions may be required, including intermolecular homotypic merlin complex formation, actin association and membrane localization, as further analyses are performed.

First, the NF2 mutation must allow for the synthesis of a stable merlin protein. Failure to generate a stable protein would result in no merlin expression from that mutant allele. This has been demonstrated formally herein for two nonsense mutations, Q111X and R466X. Although predicted to produce truncated proteins, no such protein species were observed in RT4 or JS1 cells. This result is in keeping with data from our laboratory and others examining schwannomas, meningiomas and ependymomas from individuals with and without NF2 for the presence of truncated merlin proteins (23–25).

NF2 missense mutations could also result in the production of an unstable merlin protein. This has been demonstrated for several other proteins, including the superoxide dismutase enzyme (SOD-1), in which missense mutations result in reduced protein stability yet retain partial enzyme activity (27). Examination of the L64P and L535P NF2 missense mutations demonstrated a mildly decreased protein half-life compared with wild-type merlin; however, these proteins were easily detectable when overexpressed in RT4 and JS-1 rat schwannoma stable cell lines. In addition, all merlin mutants when stably expressed were still unable to suppress schwannoma cell growth.

Second, merlin forms intramolecular associations that are critical for its ability to regulate cell growth. Previous studies from our laboratory have shown that merlin forms a self-complex joining its N- and C-termini (15). Alterations in the C-terminus (NF2 containing exon 16 or NF2 lacking both exons 16 and 17) result in no merlin self-association and defective merlin growth suppression. Here, we demonstrate that the determinants required for the N-terminal association with the C-terminus lie in the extreme N-terminus, between residues 288 and 400. The region can probably be narrowed to include residues 288 and 302, if one considers that self-association occurs with co-expression of the N- (residues 1–302) and C-terminus (residues 299–595).
Using microtubule association as an indicator for the conformation of the merlin protein, we have identified ‘open’ and ‘closed’ states relevant to merlin growth suppression (26). In these experiments, no association between full-length merlin (NF2.17) and polymerized microtubules could be demonstrated. However, N-terminal merlin constructs (N-terminal residues 1–302) associated with polymerized microtubules. These results suggested that the N-terminal microtubule association region is masked by intramolecular folding of the full-length merlin. In the ‘open’ conformation, the N- and C-termini do not associate and growth suppression is abrogated, whereas in the ‘closed’ conformation, merlin forms a self-complex and can function as a tumor suppressor. Analysis of two truncated merlin molecules that are both defective as growth regulators (NF2.16 and NF2.547) demonstrated association with polymerized microtubules. In this study, we demonstrate that the naturally occurring L64P mutant associates with microtubules and is therefore in the ‘open’ conformation. It is likely that this conformation is not permissive for growth suppression and may explain why this mutant is not active. The substitution of a leucine residue for a proline residue may alter the conformation of the N-terminus sufficiently to eliminate productive self-association. Analysis of the extreme N-terminal domain of merlin will be required to determine whether additional residues beyond those between amino acids 288 and 400 are required for merlin intramolecular complex formation.

The ability of merlin to form intermolecular complexes through homo- or heterotypic associations is supported by the GST fusion protein and glycerol gradient experiments presented herein. However, no direct interaction could be demonstrated by co-transfection and co-immunoprecipitation of merlin in several different cell lines. The inability to demonstrate directly a physical interaction in living cells suggests that if these interactions occur in vivo, they do so only under certain conditions. Further studies will be required to determine whether merlin forms oligomeric complexes in vivo that are important for growth suppression.

Third, merlin must interact with its critical effector proteins in order to effect a growth inhibitory signal. Intense efforts in many laboratories using a variety of different biochemical and genetic techniques have been focused on identifying these proteins. Our results demonstrate that some merlin missense mutations (K413E and L535P) are defective in growth regulation, but are still capable of forming self-complexes and intermolecular associations, and can localize at the plasma membrane. It is likely that these missense mutants are unable to interact productively with the merlin effector protein(s) required to transduce merlin’s tumor suppressor signal.

Several proteins have been identified that may serve as merlin effector proteins. One of the candidates was isolated by yeast two-hybrid interaction cloning and shown to be fodrin, an actin-binding protein (28). Using GST affinity chromatography, several additional proteins have been shown to associate with merlin (29). The identity of these 85, 125 and 165 kDa proteins remains to be determined. Future analysis of the association of merlin and its missense mutants with these effectors should clarify the significance of such interactions. Using the molecular framework outlined in this report, the functional abilities of merlin mutations can be tested efficiently and their relationship to merlin growth suppression determined. With additional analyses, more predictive genotype-phenotype correlations may be possible.

MATERIALS AND METHODS

Antibodies and western blotting

The WA28 and WA30 antibodies are rabbit polyclonal antiserum generated against merlin peptide sequences as previously reported (10,23). Proteins were separated by SDS–PAGE and transferred onto Immobilon-P filters (Millipore) for western blotting as previously described (10). WA28 and WA30 antibodies were used at a dilution of 1:1000. The rabbit FLAG polyclonal and myc monoclonal (clone 9E10) antibodies were purchased from Santa Cruz Biotechnology and used as recommended. Development was accomplished using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL; Amersham).

Co-immunoprecipitation experiments were performed by cotransfecting COS-1, RT4 or NIH-3T3 cells with NF2 cDNA constructs using DOTAP or Lipofectamine cationic lipid reagent (10,15). Proteins were harvested in non-reducing NP-40 lysis buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 0.5% NP-40) containing protease inhibitors (aprotinin, leupeptin and benzamidine) 48 h after transfection and incubated with a 1:100 dilution of the 9E10 antibody for 1 h followed by an overnight incubation with protein G–Sepharose beads at 4 °C on a rotating wheel. Immunoprecipitates were washed 4–5 times in non-reducing NP-40 lysis buffer and eluted in 1× Laemmli buffer for SDS–PAGE and western blot analysis.

Western blot analysis was performed as previously described. Briefly, proteins were separated by SDS–PAGE and transferred onto Immobilon-P filters (Millipore). WA28 and WA30 antibodies were used at a dilution of 1:1000 while the tubulin monoclonal antibodies (Sigma; clone DM-1A) were used at a 1:1000 dilution, as recommended by the manufacturer.

Merlin cDNA constructs

Merlin cDNA constructs were generated by PCR using the full-length NF2 cDNA as template. Merlin containing exon 16 was generated by RT-PCR as previously described. N-terminal radixin (residues 1–323) was generated by RT-PCR from human brain RNA and sequenced in its entirety using the Sequenase reagent (US Biochemicals). All merlin cDNAs generated proteins of the predicted molecular weights.

Patient mutations were generated using oligonucleotides as previously reported (30). Fragments of the NF2 gene were cloned into pSELECT-1 (Promega) and single-stranded DNA generated as template for oligonucleotide-directed mutagenesis. The oligonucleotides used for NF2 mutagenesis included the following: L64P (5′-CTG GTT CTG GGC CCC GGA GAT CAC AAT C-3′), Q111X (5′-GGT GGT TTA GGA GAT CAC-3′), K413E (5′-GCA GGC CAT CGA GGC CAT AGC-3′), R466X (5′-CCA GGC GTA GTG AAC AGC AAA G-3′) and L535P (5′-AGC AAC AAT CCG CAG GAG-3′). The mutant NF2 fragments were cloned back into the full-length NF2 cDNA for expression studies. All mutants were sequenced using the Sequenase reagent to verify the introduction of the specific NF2 mutation.

Epitope-tagged merlin constructs were generated by PCR using the NF2 cDNA as template. Each tagged construct was sequenced (Sequenase) and tested using the appropriate FLAG or myc antibody.
GST–merlin fusion proteins were generated as previously described (15). Briefly, merlin containing residues 9–400 (NF2.400), 9–288 (NF2.288) or 1–185 (NF2.185) was cloned into pGEX.2T (Pharmacia) and transformed into DE3(BL21) competent cells for fusion protein production. Bacteria were induced overnight in 0.4 mM isoprropyl-β-D-thiogalactopyranoside (IPTG) at room temperature and GST–merlin fusion proteins collected on glutathione–agarose beads (Sigma) for the interaction experiments.

GST–merlin binding experiments
Merlin–GST fusion proteins were prepared as above for merlin interaction experiments with in vitro transcribed and translated merlin proteins, as previously described (15). In vitro transcribed and translated merlin proteins were synthesized in the presence of [35S]methionine using the TnT protocol (Promega), according to the instructions supplied by the manufacturer, and detected by autoradiography. Two different experiments were performed. In the first method, radiolabeled merlin proteins were incubated with equal amounts of GST–merlin fusion protein immobilized on glutathione–agarose beads for 2 h at 4°C. The unbound fraction was washed and the agarose beads were then washed four times in TEN buffer (10 mM Tris, pH 7.5; 150 mM NaCl; 5 mM EDTA; 1% Triton X-100) and eluted in 1× Laemmlı buffer. An equal fraction of the supernatant and eluted bound fraction was separated by SDS–PAGE and analyzed by autoradiography. In all experiments, no binding was observed with immobilized GST alone or with irrelevant neurofibromatosis I (NF1) fusion protein baits (not shown). The second method was devised to provide more direct quantitative information regarding merlin binding. In this approach, equal amounts of radiolabeled merlin proteins in TEN buffer were incubated with either GST or GST–merlin bound to glutathione–agarose beads. Equal amounts of GST and GST–merlin protein were used in all experiments. After the 2 h incubation at 4°C, equal amounts of supernatant were removed and separated by SDS–PAGE for autoradiography and phosphorimage analysis. No binding was seen with irrelevant NF1 targets (not shown).

Subcellular fractionation
Detergent extraction cell fractionation was performed as previously described for ezrin (31). Briefly, cells were extracted for 40 s in MES extraction buffer (50 mM MES, pH 6.4; 3 mM EDTA; 5 mM MgCl2; 0.5% Triton X-100) containing protease inhibitors (aprotinin, leupeptin and benzamidine). The soluble and insoluble fractions were collected, and equal volumes of each fraction were separated on SDS–polyacrylamide gels. Membrane fractions were prepared by washing the cells in phosphate-buffered saline (PBS) and then re-suspending them at a concentration of 107 cells per ml in hypotonic lysis buffer (10 mM KCl; 1 mM MgCl2; 10 mM Tris, pH 7.4) with protease inhibitors for 15 min on ice. Cells were then homogenized in a hand-held Dounce homogenizer and then centrifuged at 4000 r.p.m. for 5 min at 4°C. The supernatant was then removed and centrifuged at 100 000 g for 40 min at 4°C in a Beckman ultracentrifuge. The pellet containing the membrane fragments was re-suspended in RIPA buffer (150 mM NaCl; 50 mM Tris, pH 7.5; 1 mM EDTA; 1% Triton X-100; 0.1% SDS; 1% sodium deoxycholate) while the supernatant was saved. Equal volumes of each fraction were separated by SDS–PAGE.

Microtubule polymerization
Assembly of bovine brain microtubules with in vitro transcribed–translated merlin constructs was performed as previously described (26,32,33). Bovine microtubules were homogenized in PEM buffer (100 mM PIPES, pH 6.6; 1 mM EDTA; 1 mM MgSO4) containing protease inhibitors and centrifuged at 18 000 r.p.m. for 1 h at 4°C in an SS-34 Sorvall rotor. Then 1.0 ml of microtubule homogenate per reaction was assembled in vitro in the presence of in vitro translated and transcribed NF2 cDNA constructs, 1 mM GTP and 20 µM taxol for 40 min at 37°C, followed by centrifugation at 30°C for 30 min at 18 000 r.p.m. After centrifugation, the supernatant was removed (1.0 ml) and the polymerized microtubule pellet was resuspended in 1.0 ml of PEM buffer. Equal volumes of supernatant and polymerized microtubule fractions were separated by SDS–PAGE and analyzed by western blotting using WA30 (merlin) and tubulin antibodies.

Glycerol gradients
Glycerol gradients were generated by sequentially layering 400 µl of 14, 13, 12, 11, 10 and 9% glycerol in lysis buffer in micro-ultracentrifuge tubes to form discontinuous gradients. Pre-cleared cell lysates (200 µl) from RT4 cells overexpressing NF2.17 were layered on top and centrifuged for 6 h at 48 000 r.p.m., 4°C. Two hundred µl fractions were collected from top to bottom and analyzed by SDS–PAGE. In separate parallel tubes, BSA (66 kDa) or ADH (150 kDa) was similarly centrifuged and the sedimentation profiles determined by a modified Bradford reaction (BIORAD). RT4-NF2.17 cells were lysed in NP-40 lysis buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 0.5% NP-40) or RIPA buffer containing 1 mM dithiothreitol (DTT) for these analyses.

RT4 cell lines and growth assays
RT4 rat schwannoma cell lines were established as previously described (15). Briefly, transfections of RT4 cells were performed using DOTAP lipofection reagent (Gibco-BRL) according to the manufacturer’s recommendations. Stable cell lines were selected in 500 µg/ml G418 (geneticin) and expanded for western blot analysis using WA30 merlin antibodies. At least three cell lines with equivalent overexpression of merlin (5- to 10-fold overexpression relative to RT4 cells) were selected for analysis.

Two assays were performed to analyze the effect of merlin overexpression on schwannoma cell growth. The first method involved seeding 10 000 RT4 cells per well in quadruplicate in a 24-well plate and direct cell counting using a hemocytometer. Three timepoints were chosen (12, 36 and 60 h) based on previous experiments in our laboratory. Merlin growth suppression was also determined using RT4 cell lines grown to confluence in quadruplicate in 24-well plates. Cells were counted 24 and 48 h later using a hemocytometer.

Results obtained with the direct cell counting method were verified using the Cell Titer 96AQ growth assay (Promega) according to the manufacturer’s recommendations. For these experiments, 5000 cells were seeded in quadruplicate in 96-well plates and cell proliferation was measured by MTS incorporation at 24 and 48 h. Thymidine incorporation assays were performed as previously described (15). Briefly, 5000 cells were plated in triplicate in 24-well plates without serum for 20 h, then pulsed with 1 µCi of [3H]thymidine for 4 h. Labeled cells were washed
in PBS and solubilized in 200 µl of 0.2 M NaOH. Counts were then determined in a scintillation counter.

For all experiments, mean and standard deviations for each cell line were obtained. Each experiment was performed at least three times with identical results.

Pulse–chase experiments

RT4 cell lines grown in methionine-deficient media were radio-labeled with 50 µCi of [35S]methionine for 16 h and the media replaced with fresh Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics. Cells were harvested at 4, 8, 12 and 24 h and methionine immunoprecipitated with WA25 at 1:100 final dilution. Immunoprecipitates were collected on protein A–Sepharose beads, eluted in 1× Laemmli, and separated by SDS–PAGE for autoradiography and phosphorimaging analysis. Half-lives were determined using Canvas software for the Macintosh.

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