Functional analysis of neurofibromatosis 2 (NF2) missense mutations

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Received March 20, 2001; Revised and Accepted May 2, 2001

Neurofibromatosis 2 (NF2) is a tumor predisposition syndrome in which affected individuals develop nervous system tumors at an increased frequency. The most common tumor in individuals with NF2 is the schwannoma, which is composed of neoplastic Schwann cells lacking NF2 gene expression. Moreover, inactivation of the NF2 gene is observed in nearly all sporadic schwannomas, suggesting that the NF2 gene is a critical growth regulator for Schwann cells. In an effort to gain insights into the function of the NF2 gene product, merlin or schwannomin, we performed a detailed functional analysis of eight naturally occurring non-conservative missense mutations in the NF2 gene. Using a regulatable expression system in rat schwannoma cells, we analyzed proliferation, actin cytoskeleton-mediated events and merlin folding. In this report, we demonstrate that mutations clustered in the predicted α-helical region did not impair the function of merlin whereas those in either the N- or C-terminus of the protein rendered merlin inactive as a negative growth regulator. These results suggest that the key functional domains of merlin lie within the highly conserved FERM domain and the unique C-terminus of the protein.

INTRODUCTION

Neurofibromatosis 2 (NF2) is an autosomal dominant inherited cancer predisposition syndrome in which affected individuals develop schwannomas, meningiomas and ependymomas (1). Because of the increased incidence of nervous system tumors in this population, the NF2 gene has been hypothesized to function as a tumor suppressor gene (negative growth regulator). Support for this notion derives from a number of studies involving residues 303–478) and a unique C-terminus (residues 479–595) which lacks the conventional actin-binding domain found in ERM proteins. Like the ERM proteins, merlin contains an N-terminal domain (residues 1–302) which is highly conserved among all members of the Protein 4.1 family and is thought to mediate interactions with the cytoplasmic tail of cell surface glycoproteins such as glycoprotein C and CD44 (29). The second half of the molecule contains a predicted α-helical domain (residues 303–478) and a unique C-terminus (residues 479–595) which lacks the conventional actin-binding domain found in ERM proteins. Based on studies initially performed on ERM proteins (30–32), we and others have demonstrated that merlin forms intramolecular associations that are critical for its ability to function as a negative growth regulator and mediate interactions with potential effector molecules (7,26,33–37). These interactions involve residues in the N- and C-termini of the protein.

In an effort to gain insights into the function of merlin, we analyzed the effect of eight naturally occurring non-conservative missense mutations in three distinct regions of the merlin molecule on merlin intramolecular folding, merlin growth suppression and merlin’s ability to disrupt actin cytoskeleton-mediated events. In this detailed analysis, we demonstrate that...
mutations in the N- and C-termini of the protein impair merlin function, whereas three mutations within the predicted α-helical domain retain structural and functional properties observed with wild-type merlin.

RESULTS

Regulatable overexpression of merlin impairs cell proliferation, growth in soft agar and actin cytoskeleton-mediated events

Previously, we have demonstrated that constitutive overexpression of wild-type merlin in RT4 and JS1 rat schwannoma cells results in reduced cell proliferation, growth in soft agar and tumor formation in immunocompromized mice (7). In addition, both constitutively and zinc-inducible overexpressing wild-type merlin rat schwannoma cell lines exhibited reduced cell motility in vitro as well as dramatic alterations in actin cytoskeleton organization during cell spreading (8). To avoid the potential effects of high concentrations of zinc on normal cell physiology, we elected to generate inducible merlin-expressing rat schwannoma cell lines using the reverse tetracycline transactivator (rtTA) system. In collaboration with Helen Morrison (Karlsruhe, Germany), we developed RT4 rat schwannoma cell lines that inducibly overexpressed wild-type merlin (9). Multiple wild-type merlin regulatable RT4 cell lines were generated, and two were chosen for further study (clones 6 and 8).

Induction of merlin expression in these RT4 cells was observed by 24 h of treatment with 1 µg/ml doxycycline (Fig. 1A). Induction of merlin resulted in reduced cell growth in vitro as measured by thymidine incorporation (Fig. 1B) and reduced colony formation in soft agar (Fig. 1C). As shown previously for the zinc-inducible RT4 cells, regulated overexpression of merlin results in dramatic alterations in the actin cytoskeleton during the initial phase of cell spreading as visualized by phalloidin cytochemistry (Fig. 1D). As reported previously, wild-type merlin expression also impairs cell motility (8), this time measured by a Boyden chamber migration assay (Table 1). Collectively, these results demonstrate that doxycycline-regulated overexpression of merlin impairs cell proliferation, anchorage-independent growth and actin cytoskeleton-mediated events.

Functional analysis of NF2 patient missense mutations

Germline mutations in the schwannoma susceptibility gene, NF2, are responsible for all cases of NF2-associated and nearly all sporadic schwannomas. Whereas all truncating mutations are considered functionally inactive, many of the missense variants identified to date cannot be readily classified as either disease-associated mutations or as benign polymorphisms. Moreover, the predicted structural domains of merlin suggest that critical regions identified by missense mutations might represent important domains required for merlin growth suppressor activity. In an effort to determine which patient mutations in structurally distinct regions of the molecule might be critical for mediating merlin’s functional activities, we generated multiple individual RT4 rat schwannoma cell clones that inducibly overexpressed each of eight NF2 gene missense mutations (M. MacCollin, International NF2 Mutation Database).

<table>
<thead>
<tr>
<th>Mutant</th>
<th>3H-TdR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Soft agar</th>
<th>Spreading</th>
<th>Motility (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MT&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF2.17</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>71.1 (4.1)</td>
<td>NB</td>
</tr>
<tr>
<td>L64P</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>97.9 (5.7)</td>
<td>B</td>
</tr>
<tr>
<td>K79E</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>97.7 (7.6)</td>
<td>NB</td>
</tr>
<tr>
<td>E106G</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>92.8 (6.9)</td>
<td>B</td>
</tr>
<tr>
<td>Q324L</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>79.3 (4)</td>
<td>NB</td>
</tr>
<tr>
<td>T352M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>71.7 (1.2)</td>
<td>NB</td>
</tr>
<tr>
<td>K413E</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>80.9 (6.9)</td>
<td>NB</td>
</tr>
<tr>
<td>L535P</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>89.8 (2.3)</td>
<td>NB</td>
</tr>
<tr>
<td>Q538P</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>89.2 (3.9)</td>
<td>NB</td>
</tr>
</tbody>
</table>

<sup>a</sup>Thymidine incorporation (<sup>3H</sup>TdR), anchorage-independent growth ('soft agar') and actin cytoskeleton disorganization during cell spreading ('spreading') were performed as described in the Materials and Methods. +, clone functioned like wild-type merlin (NF2.17); –, loss of wild-type merlin function.

<sup>b</sup>The mean percentage of induced merlin clone migration compared with uninduced clone migration for three separate Boyden chamber motility experiments. The standard deviation is shown in parentheses.

<sup>c</sup>Results from the in vitro micotubule-binding assay (NB; not bound; B, bound).

Three mutations were clustered in exons 2 and 3 within the highly conserved N-terminal FERM domain. Three missense mutations were localized to the predicted α-helical sequence while two additional mutations localized to the unique C-terminus of the protein. The alignment of these residues with other members of the Protein 4.1 superfamily is shown in Figure 2. For NF2 missense mutations from individuals where clinical information was available, the K413E, E106G and T352M mutations were reported from individuals with a severe clinical phenotype (16,38) while the L64P and L535P mutations were observed in individuals with a milder clinical phenotype (38; M. MacCollin, personal communication). The Q538P mutation was reported in individuals with both mild and severe clinical phenotypes (39).

NF2 missense mutations in the N-terminus impair merlin function

RT4 rat schwannoma cell lines inducibly overexpressing the L64P, K79E and E106G missense mutations were generated. Multiple independently generated cell lines were derived and representative clones are shown for each mutation. In all cases, multiple independently generated cell lines containing the same patient mutation were functionally equivalent.

Previous work from our laboratory demonstrated that the L64P mutation in exon 2 impaired the ability of merlin to form an intramolecular complex by disrupting the formation of an N-term:N-term interaction (34). Using constitutively overexpressing as well as zinc-inducible RT4 cell lines, we demonstrated previously that merlin containing the L64P mutation was defective as a tumor suppressor and did not impair actin cytoskeleton-mediated events (8,26). These results are in good agreement with our present results using the doxycycline regulatable system (Fig. 3). In two independently generated cell lines (clones 7 and 10), the addition of doxycycline results...
in the induction of merlin expression within 4–6 h as detected by western immunoblotting (Fig. 3A). Induction of mutant merlin expression failed to impair cell proliferation (Fig. 3B) or anchorage-independent growth (Fig. 3C). In addition, regulated overexpression of merlin containing the L64P mutation did not impair actin cytoskeleton organization during cell spreading at 60 or 90 min (Fig. 3D). Lastly, there was no effect of mutant merlin overexpression on cell motility (Table 1).

Similarly, regulated overexpression of merlin containing the K79E missense mutation did not impair cell proliferation or anchorage-independent growth in vitro and had no effect on cell motility or actin cytoskeleton organization during cell spreading (Table 1).

Analysis of RT4 cell lines inducibly overexpressing the E106G missense merlin molecule demonstrated induction of merlin expression within 4–6 h after doxycycline addition.
The L535P mutation, when constitutively overexpressed, had no effect on cell proliferation or anchorage-independent growth (Fig. 6A). Similarly, mutant (Q538P) merlin overexpression had no effect on cell proliferation or anchorage-independent growth (Fig. 6B and C). Likewise, there was no effect of mutant merlin overexpression on actin cytoskeleton organization during cell spreading (Fig. 6D) or cell motility (Table 1).

The NF2 missense mutant E106G demonstrates impaired folding in vitro

Previous work from our laboratory demonstrated that wild-type merlin does not associate with polymerized tubulin in an in vitro microtubule assembly assay (40). The inability of wild-type merlin to bind microtubules in vitro was shown to reflect the formation of an intramolecular complex that obscured the microtubule-binding domain within the N-terminus of merlin. The L64P mutation, but not the K413E or L535P mutations, resulted in binding to microtubules in vitro, suggesting an unfolded conformation (40). To determine whether the additional mutants resulted in impaired merlin intramolecular associations, we performed a microtubule-binding assay as described previously on the five remaining mutations not yet reported. Only the E106G mutation had significant microtubule binding, suggestive of an unfolded conformation. None of the other four mutations (K79E, Q324L, T352M and Q538P) significantly associated with polymerized microtubules, and therefore had a pattern of microtubule binding similar to the wild-type ‘folded’ merlin molecule (Fig. 7).

These results suggest that the E106G mutation, which impairs the ability of merlin to function, likely results from impaired merlin intramolecular associations.

DISCUSSION

It is presumed that the NF2 gene mutations identified in cancers are disease-associated and lead to a dysfunctional allele, either by eliminating its expression or by impairing its function. Previous work from our laboratory and others has demonstrated that truncation or frameshift mutations result in the formation of an intramolecular complex that obscured the microtubule-binding domain within the N-terminus of merlin. The NF2 mutation in the C-terminus with the L535P mutation resulted in impaired merlin function, as demonstrated previously on the five remaining mutations not yet reported. Only the E106G mutation had significant microtubule binding, suggestive of an unfolded conformation. None of the other four mutations (K79E, Q324L, T352M and Q538P) significantly associated with polymerized microtubules, and therefore had a pattern of microtubule binding similar to the wild-type ‘folded’ merlin molecule (Fig. 7).

These results suggest that the E106G mutation, which impairs the ability of merlin to function, likely results from impaired merlin intramolecular associations.
mutations is small, conclusions about genotype–phenotype correlations are premature. For missense mutations where clinical information is available, there was significant variability in the disease phenotype. The Q538P mutation was reported in a family in which one individual had a mild disease phenotype while another family member with the identical mutation exhibited a more severe disease course (39). Similar observations have been made for the F62S mutation in which both mild and severe clinical phenotypes have been observed (16,41).

Analysis of the amino acid residues affected by these missense mutants demonstrates a variable amount of sequence conservation amongst members of the Protein 4.1 family (Fig. 2). There does not appear to be a relationship between the degree of sequence conservation at any particular amino acid residue and the functional properties of the mutant. Some residues like L64 and K79 are identical amongst all members

Figure 3. Regulated overexpression of merlin containing the L64P missense mutant does not result in growth suppression or abnormalities in actin cytoskeleton-mediated processes. (A) RT4 rat schwannoma cells containing the rtTA were transfected with pUHD-10.3.NF2.17 (L64P) and several clones were chosen for further study. Two representative clones are shown (clones 7 and 10). Upon the addition of 1 µg/ml doxycycline, an induction of merlin expression was seen by 4–6 h. Merlin was detected using the WA30 polyclonal antibody by western blot. (B) Mutant merlin induction does not affect RT4 schwannoma cell proliferation. Cell proliferation was measured using thymidine incorporation as described in the Materials and Methods. There was no decrease in thymidine incorporation for cells expressing mutant merlin compared with uninduced cells. (+) and (−) denote the addition or omission of doxycycline. The mean and standard deviation for each condition is shown. (D) Induction of mutant merlin did not result in disruption of the actin cytoskeleton during cell spreading within 60–90 min after plating, as assessed using phalloidin-BODIPY immunofluorescence (clone 7). Similar effects were observed for both mutant merlin clones 7 and 10. No effect was observed with cell lines expressing the blank vector alone (data not shown). Photomicrographs were taken at 20×. Bars denote 20 µm.
of the ERM and Protein 4.1 family while L535 demonstrates conservative amino acid changes. In contrast, E106, Q324 and Q538 are highly conserved amongst most members of the ERM family, but not the Protein 4.1 molecules, yet only mutations in E106 and Q538 impair the functional properties of merlin.

In studies reported herein, we generated multiple doxycycline regulatable missense mutant merlin cell lines to carefully characterize the effects of these point mutations on merlin function. We took this labor-intensive approach to eliminate clone-to-clone variability, problems with constitutive over-expression, and the difficulties in interpreting experiments in which massive overexpression results from transient transfection. The use of the doxycycline regulatable cell lines provides built-in internal controls, in that each cell line in the

![Figure 4](image-url)
absence of doxycycline behaves exactly the same as vector transfected cell lines. Our ability to tightly regulate merlin overexpression allows us to address more precisely the effects of merlin mutations on the function of this tumor suppressor.

The three N-terminal domain mutants studied (L64P, K79E and E106G) reside within exons 2 and 3. All of these missense mutant molecules were functionally impaired as growth regulators and had no effect on actin cytoskeleton-mediated events. These mutations occur in two critical exons within the FERM domain, which we have previously shown to be important for the formation of an intramolecular complex within the N-terminus of merlin (34). Removal of exons 2 and 3 dramatically alters merlin’s function and generates a mutant with dominant-negative

Figure 5. Regulated overexpression of merlin containing the K413E missense mutation results in growth suppression and abnormalities in actin cytoskeleton-mediated processes. (A) RT4 rat schwannoma cells containing the rtTA were transfected with pUHD-10.3.NF2.17 (K413E) and several clones were chosen for further study. Two representative clones are shown (clones 4 and 8). Upon the addition of 1 µg/ml of doxycycline, an induction of merlin expression was seen by 4–6 h. Merlin was detected using the WA30 polyclonal antibody by western blot. (B) Mutant merlin induction results in a decrease in RT4 schwannoma cell proliferation. Cell proliferation was measured using thymidine incorporation as described in the Materials and Methods. There was a decrease in thymidine incorporation for cells expressing merlin compared with uninduced cells. (+) and (–) denote the addition or omission of doxycycline. The mean and standard deviation for each condition is shown. Asterisks denote statistical significance using Student’s t-test (P < 0.05). (C) Soft agar growth assays were performed in quadruplicate as described in the Materials and Methods. A decrease in the number of colonies was seen in cells expressing mutant merlin compared with uninduced cells. (+) and (–) denote the addition or omission of doxycycline. The mean and standard deviation for each condition is shown. Asterisks denote statistical significance using Student’s t-test (P < 0.05). (D) Induction of mutant merlin was associated with disorganization of the actin cytoskeleton during cell spreading within 60–90 min after plating, as assessed using phalloloidin-BODIPY immunofluorescence (clone 8). Similar effects were observed for both merlin clones 4 and 8. No effect was observed with cell lines expressing the blank vector alone (data not shown). Photomicrographs were taken at 40×. Bars denote 20 µm.
properties. In this regard, tissue-specific overexpression of a merlin mutant lacking exons 2 and 3 using the Schwann cell-specific P0 promoter results in schwannomas in mice (42). The finding that E106G can associate with polymerized microtubules in the in vitro binding assay also supports our previous work, demonstrating that the L64P mutant is unable to form an intramolecular complex (40). Additional support for the notion that this region of merlin is critical for merlin function derives from studies in which the K79E mutant merlin molecule, when overexpressed in transient transfection assays, had a different subcellular localization and demonstrated decreased binding to one known merlin interactor, EBP-50 or NHE-RF (43). Moreover, merlin’s ability to function as a negative growth regulator is, in part, reflected by its ability to bind to the cytoplasmic tail of CD44. The L64P mutant fails to bind to CD44 and may be functionally impaired as a growth

Figure 6. Regulated overexpression of merlin containing the Q538P missense mutant does not result in growth suppression or abnormalities in actin cytoskeleton-mediated processes. (A) RT4 rat schwannoma cells containing the rTA were transfected with pUHD-10.3.NF2.17 (Q538P) and several clones were chosen for further study. One representative clone is shown (clone 8). Upon the addition of 1 μg/ml of doxycycline, an induction of merlin expression was seen by 24 h. Merlin was detected using the WA30 polyclonal antibody by western blot. (B) Mutant merlin induction does not affect RT4 schwannoma cell proliferation. Cell proliferation was measured using thymidine incorporation as described in the Materials and Methods. There was no decrease in thymidine incorporation for cells expressing mutant merlin compared with uninduced cells. (+) and (−) denote the addition or omission of doxycycline. The mean and standard deviation for each condition is shown. (C) Soft agar growth assays were performed in quadruplicate as described in the Materials and Methods. No decrease in the number of colonies was seen in cells expressing mutant merlin compared with uninduced cells. (+) and (−) denote the addition or omission of doxycycline. The mean and standard deviation for each condition is shown. (D) Induction of mutant merlin did not result in actin cytoskeleton disorganization during cell spreading within 60–90 min after plating, as assessed using phalloidin-BODIPY immunofluorescence. No effect was observed with cell lines expressing the blank vector alone (data not shown). Photomicrographs were taken at 20×. Bars denote 20 μm.
regulator because of its inability to form a meaningful complex with CD44 (9).

The predicted α-helical region of merlin connects the N-terminus with the C-terminus. One model for merlin function envisions that this α-helical region serves as a hinge to allow the N- and C-termini to form the required intramolecular associations necessary for merlin growth suppression and key effector protein interactions. Analysis of the three missense mutations contained within the α-helical region (Q324L, T352M and K413E) demonstrated that all mutants were capable of reducing schwannoma cell proliferation and anchorage-independent growth in vitro. In addition, all three of these mutants were capable of reducing cell motility in a Boyden chamber assay. Our previous study of constitutive RT4 cell lines expressing K413E merlin mutant failed to demonstrate this effect on cell motility, which likely reflected the limitations of studies using constitutively expressing cell lines, as identical effects on cell proliferation and motility were observed with the zinc- and tetracycline-regulatable L64P and L535P cell lines (8). Studies by Stokowski and Cox (43) demonstrated that the T352M mutation had a normal subcellular distribution when overexpressed and demonstrated normal binding to EBP-50 or NHE-RF. These results suggest that these mutations do not impair the known functions of merlin and represent either polymorphisms or non-disease causing mutations. Furthermore, our observations support the notion that the α-helical region might serve as a hinge to bring the FERM and unique C-terminal domains together to mediate merlin’s function.

Mutations in the non-conserved unique C-terminus of merlin had dramatic effects on merlin function. The two mutations studied, L535P and Q538P, were defective as negative growth regulators and did not impair actin cytoskeleton-mediated events. These results argue that sequences contained within the unique C-terminus of the protein are critical for merlin’s function above and beyond its ability to form an intramolecular complex. Whereas both the L535P and Q538P are capable of forming an intramolecular association, they are defective as negative growth regulators. Previous work by Scoles et al. (44,45) demonstrated that the L535P and Q538P mutations dramatically alter the ability of merlin to interact with two merlin-interacting proteins, βIII spectrin and hepatocyte growth factor-regulated tyrosine kinase substrate. In addition, the L535P mutant was unable to bind EBP-50 or NHE-RF in vitro (43).

Although much is known about the ability of merlin to form intra- and intermolecular complexes and associate with a growing number of potential effector proteins, the exact mechanism of action underlying merlin’s growth suppressor activity remains unknown. In an effort to provide insights into the possible mechanism of action of merlin, studies in our laboratory have focused on analyzing NF2 disease-associated missense mutations that might discriminate between critical regions required for growth suppression and the binding of merlin interactors. As missense mutations represent nature’s mutagenesis screen for critical residues, we believe that these studies form the foundations for future experiments that will define regions important for mediating specific functions of merlin and delineate which effector protein interactions are relevant to growth regulation by Protein 4.1 superfamily members.

**MATERIALS AND METHODS**

**Antibodies, cDNA constructs and cell lines**

The rabbit polyclonal antibodies that specifically recognize merlin (WA30) have been described previously (22). The merlin cDNAs used in these experiments were of human origin. The eight missense mutants were generated by oligonucleotide-directed mutagenesis using sense oligonucleotides containing a single nucleotide substitution using the Promega Gene Editor kit according to the instructions provided by the manufacturer. Each construct was sequenced to verify the desired mutation.

Merlin-expressing RT4 cell lines were established by transfecting RT4 cells containing the rtTA transcriptional regulator (developed by Helen Morrison, Karlsruhe, Germany) and puromycin resistance (pBABE.PURO) with either wild-type or mutant NF2.17 cloned into pUHD10.3 and pcDNA3 to confer G418 resistance (9). Several independent clones were selected in 500 µg/ml G418 and 1 µg/ml puromycin. Positive clones were screened for merlin expression using the WA30 or C18 (Santa Cruz Biotechnology) rabbit polyclonal antibodies. At least four to five positive clones per merlin construct were analyzed and two representative clones were used for the analysis. In some cases, data is presented for a single clone, whereas in other cases, data from both clones were shown. RT4 cell lines transfected with pUHD-10.3 vector alone demonstrated no changes in cell proliferation, anchorage-independent growth, cell spreading and cell motility upon the addition of doxycycline.

**Thymidine incorporation and growth in soft agar**

Thymidine incorporation was performed as described previously on subconfluent cultures of RT4 schwannoma cells containing the doxycycline-regulatable wild-type or mutant merlin constructs (7,26). For merlin induction, 1 µg/ml doxycycline was added to the medium for 24 h with 1 µCi/ml tritiated thymidine included for the last 4 h. Twenty-four hours of induction was chosen to ensure that all lines had maximal merlin expression. Each condition (± doxycycline) was
performed in six duplicate wells and cells were harvested in 0.2 M NaOH. Thymidine incorporation was measured on a scintillation counter and the mean and standard deviation determined for each condition. Each experiment was repeated three times with identical results.

Soft agar growth assays were performed four times either in the presence or absence of doxycycline (7). Briefly, 1000 RT4 cells were plated in 24-well plates with medium containing 0.3% Noble agar for 14–21 days. The number of colonies was determined by direct counting on an inverted microscope, and the mean and standard deviation determined for each condition. Each experiment was repeated three times with identical results.

Actin cytoskeleton organization during cell spreading and cell motility

Glass coverslips were coated with 10 µg/ml laminin (Sigma) in PBS overnight at 4°C. Coverslips were then aspirated. Inducible merlin RT4 cells, cultured in DMEM + 10% FBS, were treated with 1 µg/ml doxycycline for 24 h, and then removed from dishes by trypsinization. Cells were washed twice in PBS, resuspended in DMEM + 10% FBS with and without doxycycline, and plated onto the coverslips at approximately 100 000 cells/well. After 60–90 min, cells were fixed in 4% paraformaldehyde for 20 min at room temperature, permeabilized in PBS containing 0.1% Triton X-100, and stained with BODIPY-conjugated phalloidin (Molecular Probes, 0.2 U in 50 µl) for 20 min. Coverslips were then washed in PBS, mounted in one drop of Fluoromount G (EM Sciences), and examined on a Zeiss Axiophot microscope (8). Each experiment was repeated three times with identical results.

Cell motility was determined in Transwell chambers containing 8 µm membranes. Briefly, the bottom surface of the membrane was coated with Matrigel (Collaborative Research) and 10 000 cells grown for 24 h either in the presence or absence of doxycycline were seeded on the outside of the chamber and allowed to attach for 1 h. Cells were gently washed and then the Transwells were inverted for 48 h at 37°C to allow for migration. Cells were then fixed in cold methanol for 30 min prior to staining with a LeukoStat staining kit (Fisher Scientific) and counted visually. The number of migrating cells was counted in quadruplicate and the mean and standard deviation determined for each condition. Each experiment was repeated three times with identical results (Table 1).

Microtubule polymerization assay

Assembly of bovine brain microtubules with in vitro transcribed and translated merlin molecules was performed as described previously (40). Bovine microtubules were homogeneous in PEM buffer containing protease inhibitors and centrifuged at 18 000 r.p.m. in a Sorval SS-34 rotor for 30 min at 30°C. The supernatant (1 ml) was removed and transferred to a fresh tube (labeled ‘supernatant’) and the resulting microtubule pellet was resuspended in 1 ml of PEM buffer (labeled ‘pellet’). From each fraction (total, supernatant and pellet) 50 µl was separated by SDS–PAGE and analyzed by western blotting. The relative merlin binding to polymerized microtubules was determined by scanning densitometry. Less than 2% binding was scored as insignificant binding. Each experiment was repeated at least three times with identical results.

ACKNOWLEDGEMENTS

We thank the members of our laboratory for their expert assistance during the execution of this project. We also thank Dr Mia MacCollin (MGH) for helpful discussions. This work was funded by a grant from the National Institutes of Health (NS35848 to D.H.G.).

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