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Effects of *Nf2* Missense Mutations on Schwannomin Interactions

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Most benign brain tumors are associated with loss of the *Nf2* gene tumor suppressor product schwannomin/merlin. Interactions between schwannomin fragments have given rise to hypotheses of *in vivo* schwannomin folding and dimerization. Previously, we showed that schwannomin with missense mutations L360P, L535P, and Q538P alters interaction with βII-spectrin and Hrs. Using yeast two-hybrid tests of interaction, we now show the effects of 11 *Nf2* missense mutations on schwannomin self-interaction as well as schwannomin interaction with Hrs isoforms 1 and 2, βII-spectrin, and p110. Missense mutations L46R and K364I significantly decreased affinity of schwannomin for binding all interacting proteins. The schwannomin L46R mutation may result in a complex conformational change that alters folding and denies βII-spectrin access to an intact binding site in the C-terminal half of schwannomin. We show that unique inter- and intramolecular interactions occur for schwannomin isoform 2, suggesting that this schwannomin isoform has unique functional properties compared to schwannomin isoform 1.

Key Words: **neurofibromatosis; NF2; schwannomin; merlin; βII-spectrin; Hrs; p110; missense; mutation.**

Neurofibromatosis 2 (NF2) is an autosomal dominant disorder characterized by benign tumors of the neural crest. The *Nf2* gene is commonly mutated in benign tumors of the human nervous system, including nearly all vestibular schwannomas, most sporadic meningiomas and some ependymomas (1–5). Familial NF2 is also characterized by a variety of nontumor features in addition to tumors, including cataracts, retinal hamartomas, and café-au-lait spots (6).

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The *Nf2* gene product schwannomin (SCH), also named merlin, is a tumor suppressor whose biochemical actions are not fully understood. SCH has high homology with ezrin, radixin, and moesin (ERM proteins) of the protein 4.1 superfamily that link the cytoskeleton to the plasma membrane (7, 8). Schwannomin occurs in two predominant isoforms, SCHi1 and SCHi2. Alternative splicing of *Nf2* exon 16, which encodes an alternative termination codon, results in the replacement of the last 16 amino acids (aa) encoded by exon 17 of SCHi1 (595 aa) with 11 aa of SCHi2 (590 aa) (9, 10).

Binding sites in the SCH N-terminal domain can be masked by conformational changes, similar to the binding site masking that occurs in ERM proteins (11, 12). This is interesting in the context that missense or truncating *Nf2* mutations may alter conformation resulting in unmasking or further masking of binding sites on schwannomin. Unfolding of ezrin unmasks an N-terminal domain binding site for the ezrin binding phosphoprotein EBP50 (13), and a C-terminal domain binding site for F-actin (14). In schwannomin, tubulin binding sites in the N-terminal domain are masked in full-length protein (15). In addition, interaction tests using the yeast two-hybrid method showed full-length SCH binds N- and C-terminal SCH fragments but not N- and C-terminal ezrin fragments unless SCH is truncated (16). This suggested that homodimerization sites on SCH are exposed while the binding sites for ezrin on SCH are masked (16). Thus full-length SCH possesses masked tubulin binding sites in the N-terminal domain, as well as masked binding sites for ezrin that are likely unique from the SCH homodimerization sites.

While efforts have been made to demonstrate the effects of schwannomin truncation on schwannomin interactions, little information is available on the effects of natural *Nf2* missense mutations on schwannomin interactions. Previously we identified the HGF-regulated tyrosine kinase substrate (HRS) isoform 1 (HRSi1), HRS isoform 2 (HRSi2) (17), βII-spectrin (18), and the eukaryotic initiation factor 3 (eIF3) p110 sub-

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unit eight (Scoles and Pulst, in preparation) as schwannomin interacting proteins. Each of HRSi1, HRSi2, and βII-spectrin interact with the C-terminal half of schwannomin and bind to SCHi2 more strongly than SCHi1 as measured by the yeast two-hybrid method. p110 binds the N-terminal domain of SCHi1 or SCHi2 with similar affinity. In the present study we tested the ability for eleven different naturally occurring mutant schwannomins to interact with its binding partners HRSi1, HRSi2, βII-spectrin, and p110, and to self-interact with wild-type (wt-) SCHi1, wt-SCHi2, and N- and C-terminal SCH fragments, using the yeast two-hybrid method. We showed that many of the missense mutations altered the SCH binding properties for HRS, βII-spectrin, and p110, and SCH self-binding.

MATERIALS AND METHODS

Plasmid constructs. The cDNA encoding full-length HRS isoform 1 in vector pGAD10 was obtained by yeast two-hybrid screening and was modified to encode the full-length HRS isoform 2 of 110 kDa as previously described (17). The pGAD10–βII-spectrin construct was obtained by two-hybrid screening and this construct encodes amino acids 1716–1998 of βII-spectrin. The pACT–p110 construct was obtained by two-hybrid screening and this construct encodes amino acids 69–635 of the eIF3 p110 subunit (Scoles and Pulst, in preparation). The pGBT9–Nf2i1 and pGBT9–Nf2i2 plasmids were made as previously described (18). The pGAD10–Nf2i2 construct was made by excising the full-length Nf2i2 from pGBT9–Nf2i2 with *Sma*I and *Bam*HI then ligating in pGAD10 prepared by digesting with *Xho*I, followed by blunt filling with T4 DNA polymerase, then digesting with *Bam*HI. The pGAD10–Nf2i1 construct was made just as was pGAD10–Nf2i2, but starting with pGBT9–SCHi1. The pGBT9–Nf2i1(256–595) construct was made by generating the Nf2i1(256–595) fragment by PCR using primers 5′-GATCGAATTCCCGTGGAATGAAATCCGAAAC-3′ and 5′-TTTGGAATTCTCAAATGCAGATAGGTCTTCT-3′ which was then digested with *Eco*RI and ligated in the *Eco*RI site of pGBT9. The pGBT9-Nf2i2(256–590) construct was made by generating the Nf2i1(256–595) fragment by PCR using primers 5′-GATCGAATTCCCGTGGAATGAAATCCGAAAC-3′ and 5′-GCTGGAATTCCTGCTAGAGCTCTTCAAA-3′ which was then digested with *Eco*RI and ligated in the *Eco*RI site of pGBT9. The pGBT9–Nf2i1(469–595) construct was made by generating the Nf2i1(469–595) fragment by PCR using primers 5′-GCGAGAATTCAAGCAGAAGCTCCTGGAGATT-3′ and 5′-AAGAAACTTCTCGAGATCGTCCTTAAGGTCG-3′ which was then digested with *Eco*RI and ligated in the *Eco*RI site of pGBT9. The pGAD10-Nf2i2(469–590) construct was made by generating the Nf2i2(469–590) fragment by PCR using primers 5′-GCGAGAATTCAAGCAGAAGCTCCTGGAGATT-3′ and 5′-TCTTCTGGATAGAGCTAAACTCTTAAGTTTG-3′ which was then digested with *Eco*RI and ligated in the *Eco*RI site of pGBT9. The pGAD10–Nf2i1(519–595) construct was made by generating the Nf2i1(519–595) fragment by PCR using primers 5′-GCGGGAATTCATGGAGATAGAGAAAGAAAAA-3′ and 5′-AAGAAACTTCTCGAGATCGTCCTTAAGGTCG-3′ which was then digested with *Eco*RI and ligated in the *Eco*RI site of pGBT9. The pGAD10–Nf2i2(519–590) construct was made by generating the Nf2i2(519–590) fragment by PCR using primers 5′- GCGGGAATTCATGGAGATAGAGAAAGAAAAA-3′ and 5′-TCTTCTGGATAGAGCTAAACTCTTAAGTTTG-3′ which was then digested with *Eco*RI and ligated in the *Eco*RI site of pGBT9.

PCR-based site directed mutagenesis. Three PCRs were performed in two steps to produce a fragment of the *Nf2* gene with nucleotide base changes to introduce single amino acid substitution mutations as previously described (18). The mutated PCR products were ligated in place of the normal counterpart excised from pGBT9–Nf2i2.

Two-hybrid tests of interaction. Yeast strain Y190 double-transformants were grown on SC media with leucine, and tryptophane dropped out, and 2% glucose as previously described (18). β-Galactosidase production was assayed using the filter binding assay by incubating freeze-fractured colonies on nitrocellulose in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0, 0.03 mM β-mercaptoethanol, and 2.5 μM X-gal) at 37°C for 15 min to 8 h. We determined the relative strengths of interaction using semiquantitative liquid assays for β-galactosidase by incubating yeast extracted in Z buffer and 5% chloroform with 0.6 mg/ml o-nitrophenyl β-D-galactopyranoside for 40 min. β-Galactosidase units = $1000 \times [OD_{420}/(OD_{600} \times \text{time (in min)} \times \text{culture volume (in ml)})]$ (19).

RESULTS

The locations of the studied *Nf2* missense mutations relative to all known *Nf2* missense mutations are provided in Fig. 1. We tested the ability for wt-SCHi1, wt-SCHi2, and SCHi2 missense mutants to bind HRSi1, HRSi2, βII-spectrin, and p110 using the yeast two-hybrid system (20). The p110 protein interacts with the schwannomin N-terminal half, within schwannomin residues 1–305 (Scoles and Pulst, in preparation). SCHi2 interacted considerably stronger with HRSi1, HRSi2, or βII-spectrin, all of which bind the SCH C-terminal half, than did SCHi1 (Fig. 2). The strength of interaction between SCHi2 and p110 was about equal to that of SCHi1 (Fig. 2). Of the eleven tested schwannomin missense mutations, L46R located in the SCH N-terminal half, decreased interaction with each of the schwannomin binding proteins (Fig. 2). The decrease for HRSi2, βII-spectrin, p110 amounted to a complete loss of interaction, while residual binding remained for HRSi1 (Fig. 2).

Among the tested *Nf2* missense mutations, the effect of those in the C-terminal half of the protein was greatest, resulting in loss or near loss of interaction. Schwannomin with the missense mutation L360P did not interact with HRSi1 or HRSi2, but did interact with βII-spectrin and p110 (Fig. 2). Schwannomin with either of the missense mutations K364I, L535P, or Q538P did not significantly bind to HRSi1, HRSi2, βII-spectrin, or p110 (Fig. 2).

Some *Nf2* missense mutations resulted in elevated schwannomin interaction with HRSi1, HRSi2, βII-spectrin, or p110. These mutations are all located between amino acids 219 and 352. The mutations V219M, N220Y, I273F, and T352M elevated interactions with HRSi1 two- to threefold (Fig. 2). Mutations N220Y and I273F elevated interaction with HRSi2 by 155 and 137%, respectively (Fig. 2). The I273F mutation elevated βII-spectrin binding by 241% (Fig. 2).

To show the effects of schwannomin missense mutations on interactions with other schwannomins, we tested the ability for wt-SCHi1, wt-SCHi2, and SCHi2

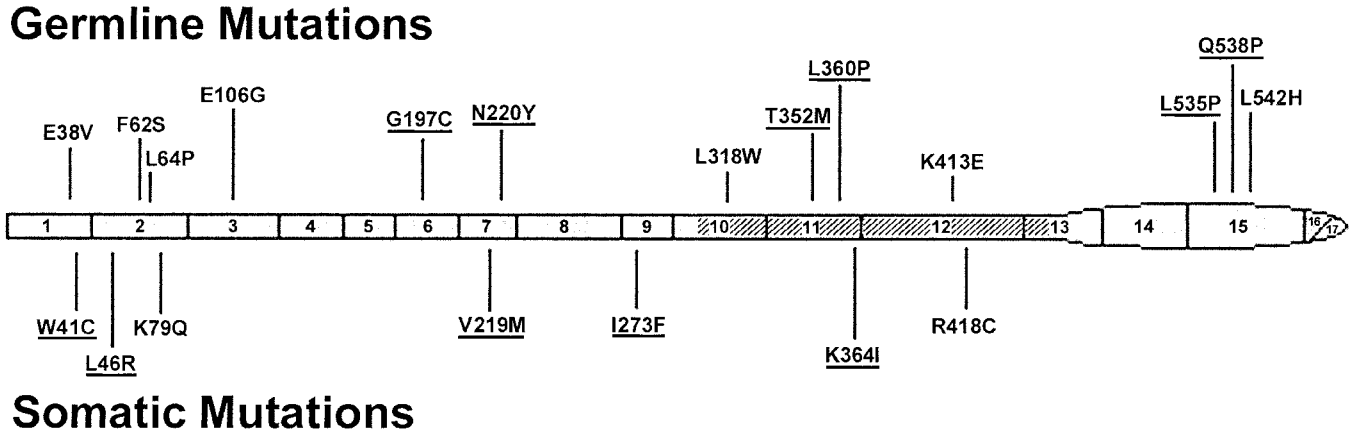


FIG. 1. Schwannomin domains and locations of naturally occurring amino acid substitutions. The N- and C-terminal domains of schwannomin are separated by the α-helical (hatched) central domain. Germline mutations are at the top of the diagram and somatic mutations are at the bottom. Missense mutations that are underlined were included in the study.

missense mutants to bind wt-SCHi1, wt-SCHi2. The interaction of SCHi1 with itself was as strong as with SCHi2, whereas the self-interaction of SCHi2 was stronger that its interaction with SCHi1 (Fig. 3).

Most of the *Nf2* missense mutations had little effect on SCH self-binding, and in general, the mutations decreased SCHi2 binding more than SCHi1 binding. Wt-SCHi1 interaction with wt-SCHi2 was of the approximate same strength as for missense SCHi2 proteins with the W41C, L46R, G197C, V219M, N220Y, I273F, and T352M mutations (Fig. 3). SCHi1 interaction was reduced with SCHi2 proteins mutated at L360P, K364I, L535P, and Q538P (Fig. 3). Most of the tested mutant SCHi2 proteins had decreased interaction with wt-SCHi2 compared to the strength of interaction observed between two wt-SCHi2 proteins (Fig. 3). SCHi2 with the K364I mutation did not bind wt-SCHi2, and SCHi2 with the L46R mutation did not interact with either SCHi1 or SCHi2 (Fig. 3).

We determined that sites for schwannomin self-interaction are different between schwannomin isoform 1 and schwannomin isoform 2 by interacting full-length SCHi1 or SCHi2 with C-terminal fragments of SCHi1 and SCHi2. Strengths of interactions between C-terminal fragments of SCH isoform 1 or isoform 2 (including amino acids 256–595 and 256–590, respectively) and wt-SCHi1 were insignificant (Fig. 4). In contrast, the strengths of interactions between the same C-terminal fragments and full-length SCHi2 was significantly higher than control tests ($P < 0.01$, Student's t test) (Fig. 4).

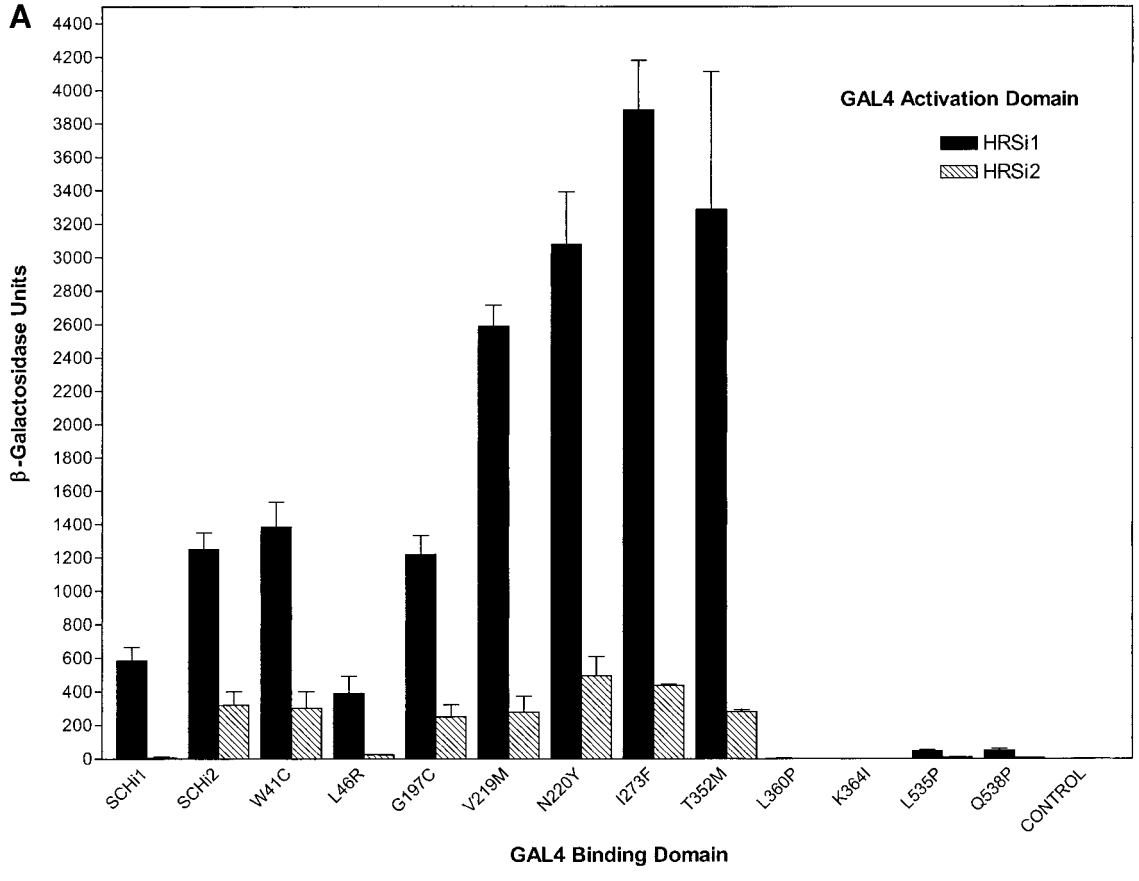
The masking of binding sites may complicate the interaction between schwannomin and other proteins. To demonstrate this, we determined the strengths of interaction between full-length or C-terminal fragments of SCHi2 or SCHi1 with βII-spectrin using the yeast two-hybrid system. βII-Spectrin interacted more strongly with full-length SCHi2 than SCHi1, and successive N-terminal truncation resulted in loss of βII-spectrin binding (Fig. 5). However, the interaction of SCHi1 C-terminal fragments was stronger than full-length SCHi1, suggesting that the N-terminal half of schwannomin isoform 1 masks a site for βII-spectrin binding in the C-terminal domain (Fig. 5).

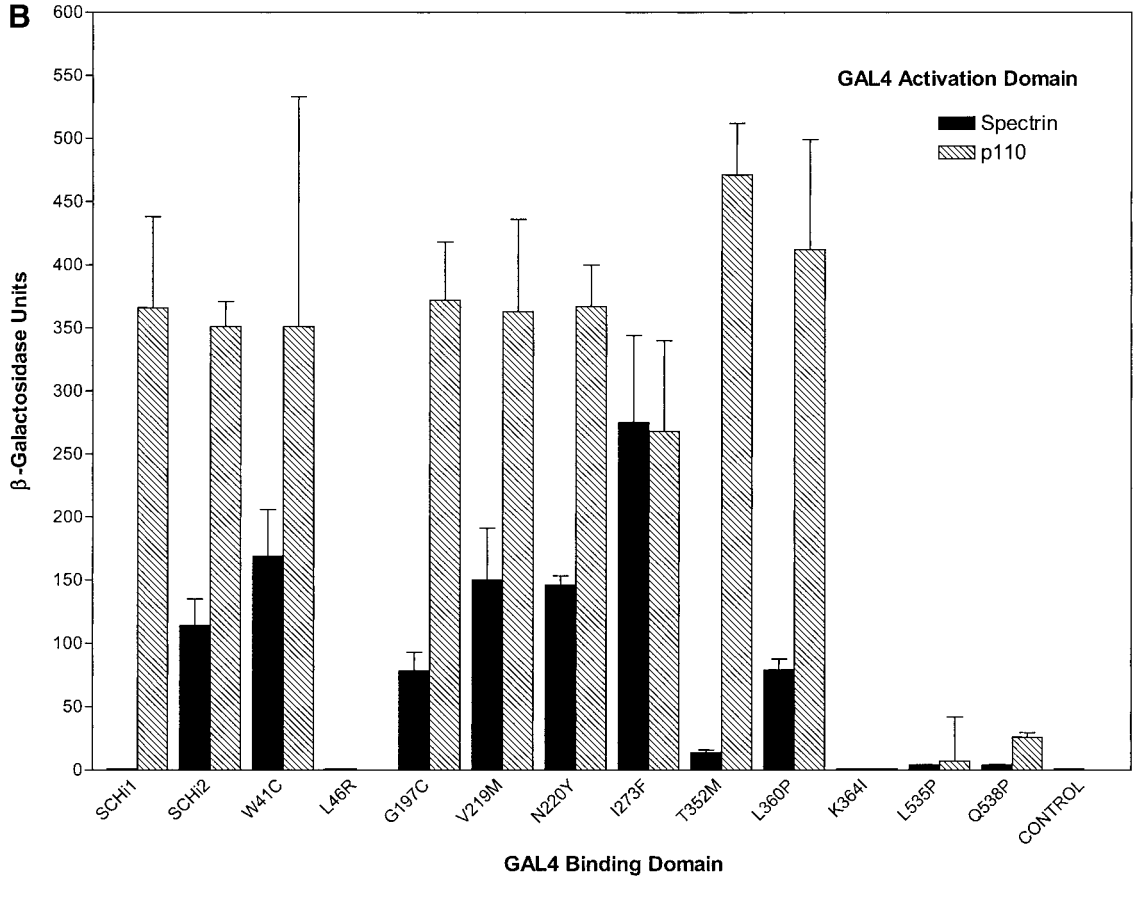
The effect of *Nf2* missense mutations on schwannomin interaction with each of the interacting proteins is summarized in Table 1.

DISCUSSION

A general framework for how SCH intramolecular interactions might influence SCH intermolecular interactions can be obtained by comparing SCH to ERM family proteins. The identities between ezrin and schwannomin N-terminal, α-helical, and C-terminal domains are 61, 30, and 22%, respectively (21). Ezrin exists in at least two conformational states, folded monomers and elongated dimers, both containing masked C-terminal domain binding sites for interaction with N-terminal domains of other ERM proteins that are unmasked in both the monomeric and dimeric states (12, 22). Moesin also folds like ezrin in a manner involving interaction between the N- and C-terminal domains (11).

FIG. 2. Yeast two-hybrid tests of interaction between schwannomin isoform 1, schwannomin isoform 2, or schwannomin isoform 2 with the indicated missense mutations and (A) HRSi1 and HRSi2 or (B) βII-spectrin and p110. Each of the SCHi1, SCHi2, or SCHi2 mutant proteins as indicated on the *X*-axis were expressed as fusions to the GAL4 binding domain and tested in yeast cells for interaction with HRSi1, HRSi2, βII-spectrin, or p110 fused to the GAL4 activation domain. Values are β-galactosidase units and are reported as means ± SD of three replicates.


A
β-Galactosidase Units
GAL4 Activation Domain
HRSi1
HRSi2
SCHi1
SCHi2
W41C
L46R
G197C
V219M
N220Y
I273F
T352M
L360P
K364I
L535P
Q538P
CONTROL
GAL4 Binding Domain


B
β-Galactosidase Units
GAL4 Activation Domain
Spectrin
p110
SCHi1
SCHi2
W41C
L46R
G197C
V219M
N220Y
I273F
T352M
L360P
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Q538P
CONTROL
GAL4 Binding Domain

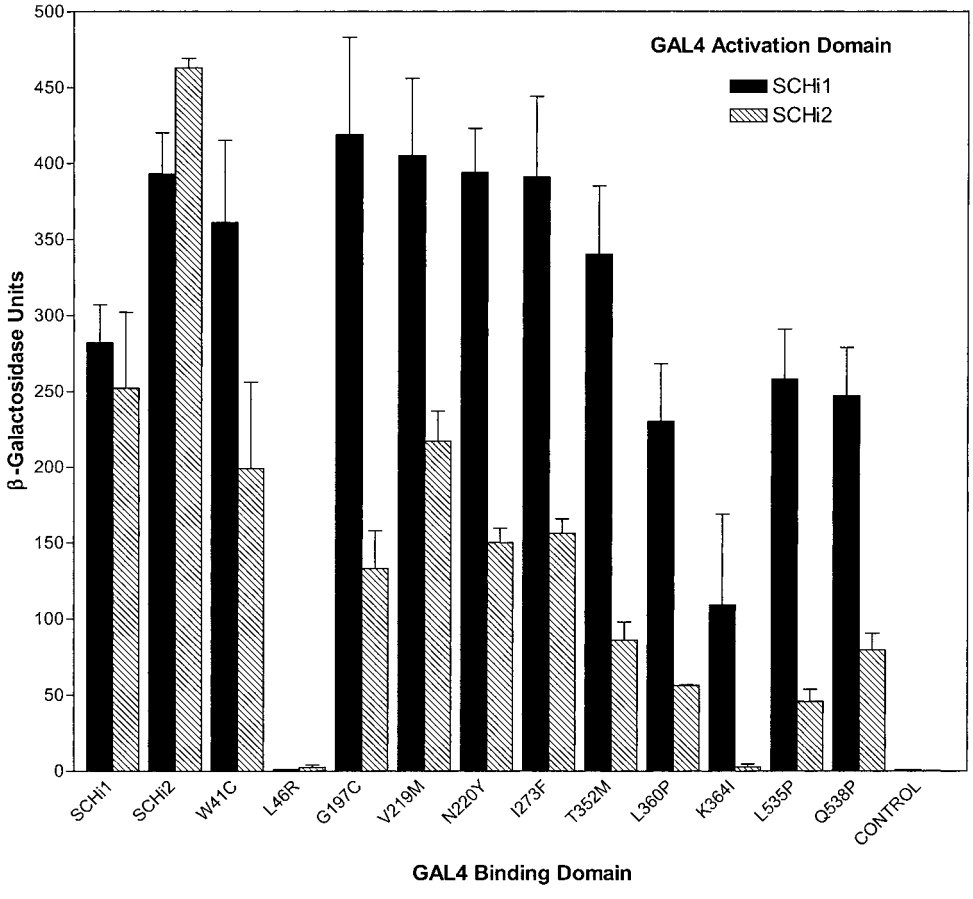

FIG. 3. Yeast two-hybrid tests of interaction between schwannomin isoform 1, schwannomin isoform 2, or schwannomin isoform 2 with the indicated missense mutations and schwannomin isoform 1 or schwannomin isoform 2. Each of the SCHi1, SCHi2, or SCHi2 mutant proteins as indicated on the *X*-axis were expressed as fusions to the GAL4 binding domain and tested in yeast cells for interaction with SCHi1 or SCHi2 fused to the GAL4 activation domain. The control tests show the levels of background obtained in the control interaction between SCHi1 or SCHi2 and the GAL4 binding domain with no fusion. Values are β-galactosidase units and are reported as means $\pm$ SD of three replicates.

Schwannomin Folding

Schwannomin folds in a head-to-tail manner. Gutmann *et al.* (23) demonstrated that two intramolecular interactions in schwannomin mediate the folded conformation. One of these occurs between two regions within the schwannomin N-terminal domain (residues 8–121 and 200–320). The other occurs between N-terminal and C-terminal schwannomin regions (residues 302–308 and residues 580–595). The latter N- to C-terminal domain interaction could not be demonstrated in schwannomin isoform 2 (24) or in a L64P schwannomin isoform 1 mutant protein that had lost the ability for N-terminal domain self-association (23). Gutmann *et al.* (23) hypothesized that the N-terminal domain self-association is required for the N- to C-terminal domain interaction to take place. This hypothesis was strongly supported by Grönholm *et al.* (16) who showed that schwannomin N- and C-terminal domain fragments of residues 1–339 and 252–595 interacted, but schwannomin fragments of residues 1–167 and 252–595 did not. But inconsistently, Grönholm *et al.* (16) also showed that a schwannomin fragment of residues 339-585 interacted with schwannomin fragments of residues 1–167 and 252–595. This result suggested that a masked binding site in the C-terminal domain, present in both schwannomin isoforms, interacted with the unfolded N-terminal domain, and placed into question whether schwannomin isoform 2 could undergo folding. This is of particular interest since it is believed that schwannomin must be folded to function as a negative growth regulator (24).

We tested the effects of mutation on schwannomin isoform 2 interactions using the Gal4-based yeast two-hybrid system in the framework of two hypotheses.

Hypothesis I. Any mutation that disrupts schwannomin self-association alters its ability to bind other proteins.

Hypothesis II. Schwannomin isoform 2 is folded differently than schwannomin isoform 1, perhaps explaining why schwannomin isoform 2 is not a negative growth regulator.

TABLE 1

Nf2 Missense Mutation, Mutation Type (Somatic or Germline), Patient Phenotype (for Germline Mutations Only), and Effect on Schwannomin Interaction

Mutation	Somatic/Germline	Patient phenotype	Effect of the mutation on binding
Trp41Cys (25)	Somatic	—	Interacted with all proteins like the nonmutant SCHi2
Leu46Arg (26, 27)	Somatic	—	Disrupted interactions with all proteins, but interacted strongly with HRSi1 and moderately with HRSi2
Gly197Cys (25)	Somatic	—	Interacted with all proteins like the nonmutant SCHi2, but the mutation decreased binding to βII-spectrin and SCHi2
Val219Met (9, 28)	Somatic	—	Interacted with all proteins like the nonmutant SCHi2, but the mutation elevated HRSi1 binding and decreased SCHi2 binding
Asn220Tyr (2)	Germline	Mild	Interacted with all proteins like the nonmutant SCHi2, but the mutation elevated HRSi1 and HRSi2 binding and decreased SCHi2 binding
Ile273Phe (29)	Somatic	—	Interacted with all proteins like the nonmutant SCHi2, but the mutation elevated HRSi1, HRSi2, and βII-spectrin binding and decreased SCHi2 binding
Thr352Met (30)	Germline	Severe	Interacted with all proteins like the nonmutant SCHi2, but the mutation elevated HRSi1 and βII-spectrin binding and decreased SCHi2 binding
Leu360Pro (7, 26)	Germline	Mild	The mutation did not alter p110 binding, but disrupted interaction with HRSi1 and HRSi2 and weakened binding to βII-spectrin, SCHi1, and SCHi2
Lys364Ile (29)	Somatic	—	The mutation weakened binding to SCHi1 and disrupted binding to HRSi1, HRSi2, βII-spectrin, p110, and SCHi2
Leu535Pro (31)	Germline	Mild	The mutation weakened interaction with HRSi1, HRSi2, βII-spectrin, p110, and SCHi2
Gln538Pro (32)	Germline	Mild/Severe	The mutation weakened interaction with HRSi1, HRSi2, βII-spectrin, p110, and SCHi2

Our approach to test hypothesis I was to assess the ability for full-length schwannomin isoform 1, isoform 2, and isoform 2 mutants (Fig. 1) to interact with HRSi1, HRSi2, βII-spectrin, p110 and with wild-type schwannomin isoform 1 and isoform 2. The second hypothesis was tested by comparing the binding of C-terminal fragments with that of full-length proteins.

In some cases, interactions in yeast may not wholly reflect interactions in mammalian cells. For example, Meng *et al.* (33) showed SCHi1 and ezrin interacted by coimmunoprecipitation of overexpressed proteins from A431 cells, but the authors were unable to show these two proteins interacted using a transcriptionally based yeast two-hybrid method. In the Gal4-based yeast two-hybrid method, the SCHi1-ezrin protein complex may fail to translocate to the nucleus, or perhaps the conformation of the complex does not allow the Gal4 activation domain the correct positioning for transcriptional activation. In addition, while yeast two-hybrid experiments cannot address the stability of missense or nonsense containing transcripts under physiologic conditions, they can make important contributions to structure/function studies. However, comparisons of the β-galactosidase values among Figs. 2 and 3 suggest that all mutant constructs were similarly expressed in yeast.

Effect of Nf2 Mutation on Binding to HRS, βII-Spectrin, and p110

Nf2 mutations significantly altered schwannomin binding to HRS, βII-spectrin, and p110. Schwannomin isoform 2 mutated at L46R had reduced or no binding to HRS isoform 2 and βII-spectrin, which interact with the schwannomin C-terminal half, and p110 which binds the schwannomin N-terminal half (Fig. 2). L46 is within the region involved in N-terminal domain folding (23). Schwannomin binding to each of HRS, βII-spectrin, or p110 was abolished by mutations K364I, L535P, and Q538P. The crystal structure of moesin showed that the surface residues where contact is made between the N- and C-terminal domains share 81% identity to schwannomin, suggesting a similar mechanism in schwannomin folding (11). Because moesin residues V518 and H521, which lie on the interface where the moesin N- and C-terminal domains make contact, correspond to schwannomin residues L535 and Q538 (11), schwannomin mutations at these locations are likely to alter schwannomin folding and binding properties. Missense mutations in the middle domain of SCH (V219M, N220Y, I273F, and T352M) elevated strengths of interactions with HRS and βII-spectrin. The elevated interaction may be of no consequence to SCH loss of function, or may result in loss of schwannomin-mediated signaling due to irreversible binding. Schwannomin isoform 2 mutated at L46R, K364I, L535P, or Q538P is either misfolded or in an otherwise unfavorable conformation that denies sites for binding.

Effect of Nf2 Mutation on Binding of Other SCH Proteins

Only two *Nf2* mutations abolished schwannomin isoform 2 binding to other schwannomin proteins as well

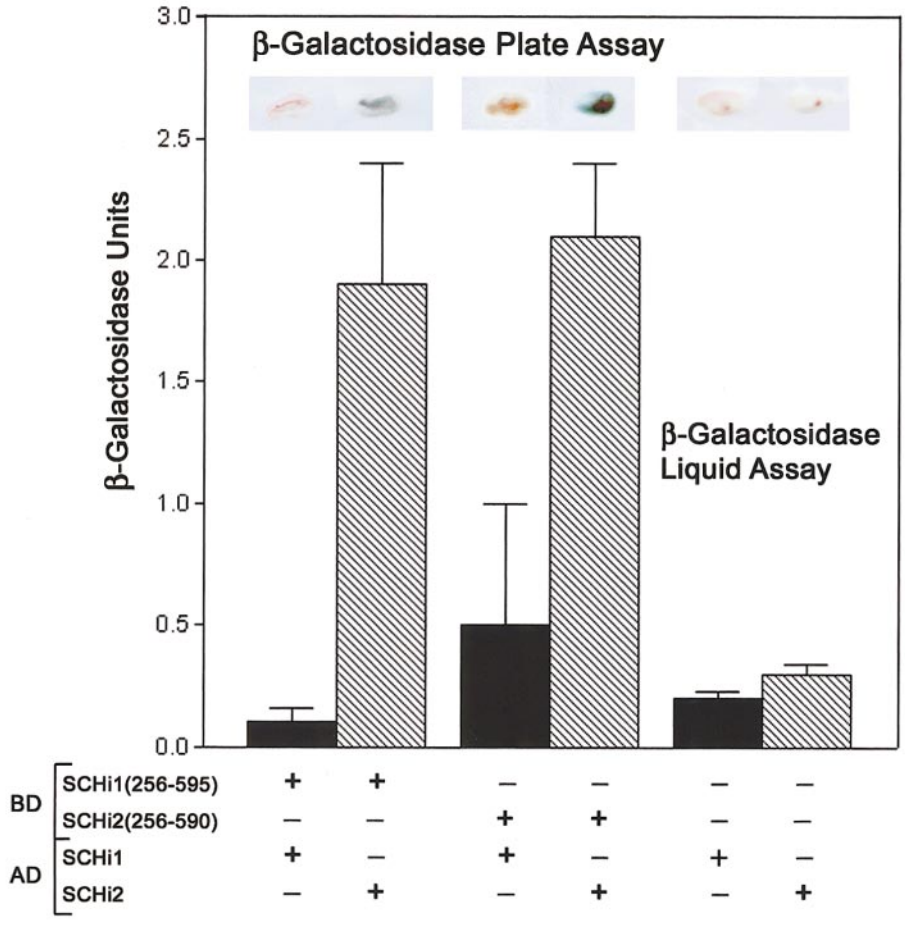

FIG. 4. Yeast two-hybrid tests of interaction between schwannomin isoform 1 or 2 and schwannomin isoform 1 or 2 C-terminal fragments. SCHi1(256–595) and SCHi2(256–590) were expressed as fusions to the GAL4 binding domain (BD) and tested in yeast cells for interaction with SCHi1 or SCHi2 fused to the GAL4 activation domain (AD). The control tests show the levels of background obtained in the control interaction between SCHi1 or SCHi2 and the GAL4 binding domain with no fusion. For the plate assay data, blue indicates the presence of β-galactosidase and a positive interaction. For the liquid assay values given are β-galactosidase units, reported as means ± SD of three replicates.

as to other schwannomin interacting proteins. The schwannomin isoform 2 mutated at L46R failed to interact with either wild-type SCHi1 or SCHi2, demonstrating that this mutant does not self-associate (Fig. 3). Schwannomin isoform 1 and 2 differed remarkably in their affinity for binding SCHi2 mutated at K364I. Schwannomin isoform 1 interacted with the K364I mutant while SCHi2 did not (Fig. 3). The SCHi2 mutated at K364I also had reduced affinity for SCHi1 compared to all other mutant SCHi2 proteins except L46R (Fig. 3). HRS, βII-spectrin or p110 did not interact with SCHi2 mutated at K364I. Our hypothesis I is strongly upheld for L46R and K364I. In addition, the mutations L535P, and Q538P that reduced but did not abolish schwannomin self-interaction had significant effect on schwannomin interaction with HRS, βII-spectrin and p110. Likewise, the mutation L360P that reduced but did not abolish schwannomin self-interaction had significant effect on schwannomin interaction with HRS, but did not disrupt schwannomin binding with βII-spectrin or p110.

Full-Length SCH Isoform 2 Can Bind SCH C-Terminal Domain of Either Isoform

We tested the hypothesis II that schwannomin isoform 2 is unable to fold like schwannomin isoform 1 by assessing the ability for SCHi1 or SCHi2 C-terminal domain fragments (residues 256–595 and 256–590, respectively) to bind full-length SCHi1 or SCHi2. Full-length schwannomin isoform 1 did not interact with the C-terminal fragment of either isoform while full-length schwannomin isoform 2 interacted with the C-terminal fragments of both isoforms (Fig. 4). The schwannomin isoform 1 may be so strongly folded in our assay that a binding site for the C-terminal fragment of schwannomin isoform 1 was masked. Schwannomin isoform 2, which previously was believed to not

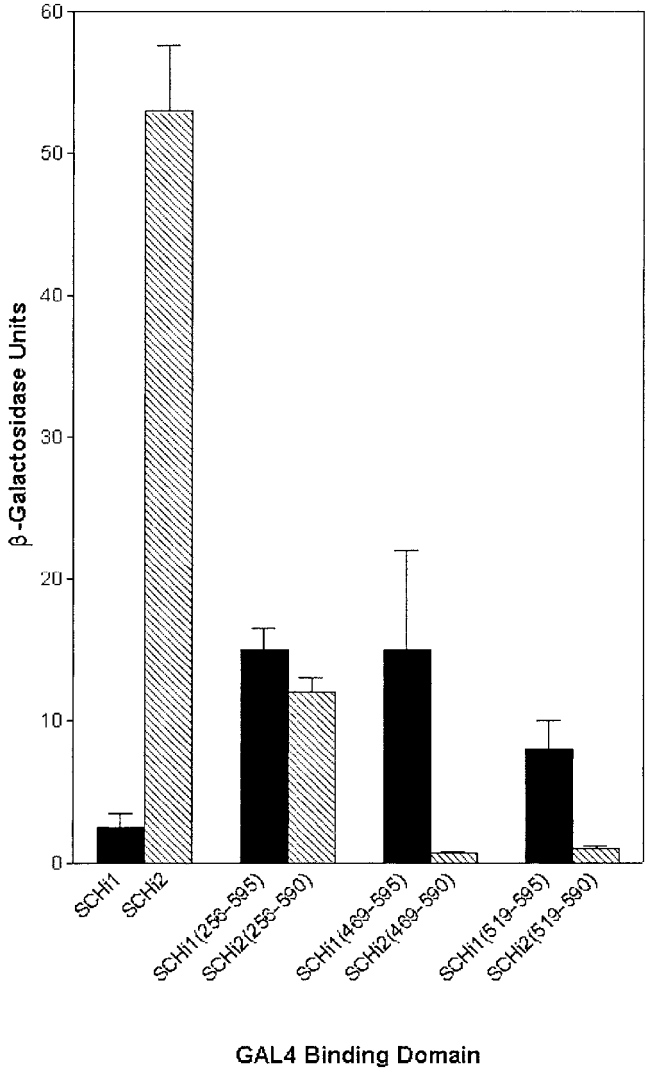

FIG. 5. Yeast two-hybrid tests of interaction between βII-spectrin and full-length or truncated schwannomin isoform 1 (black) or schwannomin isoform 2 (hatched) proteins. Each of the schwannomin proteins indicated on the *X*-axis were expressed as fusions to the GAL4 binding domain and tested in yeast cells for interaction with βII-spectrin residues 1716–1998 fused to the GAL4 activation domain. The control tests show the levels of background obtained in the control interaction between SCHi1 or SCHi2 and the GAL4 binding domain with no fusion. Values are β-galactosidase units and are reported as means ± SD of three replicates.

undergo N- to C-terminal domain folding, interacted with the C-terminal fragments of both isoforms. Thus we conclude that a site that binds the C-terminal half of either isoform of schwannomin is available in the full-length schwannomin isoform 2 that is unavailable in schwannomin isoform 1 in our assay. This is likely due to differences in the folding strengths of the full-length proteins. Schwannomin isoform 2 may undergo N- to C-terminal domain folding in a manner that is entirely different from that in schwannomin isoform 1.

Schwannomin Isoforms Bind βII-Spectrin Uniquely

Because the interactions between schwannomin C-terminal fragments and full-length proteins were all exceedingly weak (Fig. 4), we supported the conclusions of those tests, that schwannomin isoforms 1 and 2 fold uniquely, by testing the ability for truncated schwannomin proteins to bind βII-spectrin. Interactions by SCHi1 and SCHi2 for βII-spectrin and SCHi2 L46R support that the schwannomin isoforms are differently folded and present some unique binding properties. We previously showed that C-terminal fragments of schwannomin interacted with βII-spectrin (18). We have now compared βII-spectrin binding affinities between the schwannomin isoforms using matched sets of C-terminal domain fragments in effort to identify differences in schwannomin isoform conformations. We found that deletion of residues 1–255 markedly decreased SCHi2 affinity for βII-spectrin, but allowed SCHi1 to more strongly bind βII-spectrin (Fig. 5). Further truncation abolished βII-spectrin binding by SCHi2, but not by SCHi1 (Fig. 5). βII-Spectrin binding properties are remarkably unique between the two schwannomin isoforms. The βII-spectrin binding site is masked in full-length schwannomin isoform 1 but not 2, and in truncated schwannomin isoform 2 but not 1.

Our hypothesis II is strongly supported by these tests. The data strongly show that the schwannomin isoform 2 folded conformation is unique from that of schwannomin isoform 1. We further hypothesize that the differences in growth regulatory properties between schwannomin isoform 1 and 2 result from differences in protein binding or folded conformations.

Mutation in the *Nf2* gene severely affects schwannomin normal function by disrupting interactions between schwannomin with itself and its interacting proteins. Schwannomin isoform 1 is a verified growth suppressor while no such function has been proven for schwannomin isoform 2 (24). The growth suppressor function in schwannomin isoform 1 has been tied to schwannomin intramolecular interactions (23). We now show that unique inter- and intramolecular interactions occur for schwannomin isoform 2, suggesting that this schwannomin isoform has unique functional properties.

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