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Alternative transcripts in the mouse neurofibromatosis type 2 (NF2) gene are conserved and code for schwannomins with distinct C-terminal domains

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Mutations in the neurofibromatosis type 2 (NF2) gene predispose individuals to the development of nervous system tumors and ocular abnormalities. The NF2 gene product, schwannomin, is a member of a superfamily of proteins thought to link cytoskeletal elements to cell membrane components. These proteins share significant homologies in the N-terminal and α-helical domains, but diverge in the C-terminus. During our efforts to characterize mouse NF2 transcripts, we identified four different transcripts by cDNA analysis and reverse-transcribed PCR that contained different sequences in the 3′ end of the coding sequences. In human cell lines three isoforms encoding two distinct schwannomins were detected. The mouse and human transcripts containing 61 and 60 bp inserts, respectively, have not been previously described. The isoforms encode schwannomins with significantly altered C-termini and were expressed at different relative levels in adult mouse tissues and during mouse embryogenesis. These results suggest that schwannomin isoforms have distinct functional roles and predict the existence of human mutations involving the C-terminus of schwannomin.

INTRODUCTION

Neurofibromatosis type 2 (NF2) is an autosomal dominant inherited disorder that predisposes to benign tumors of the nervous system as well as cutaneous neurofibromas and schwannomas and a variety of ocular abnormalities (1,2). Bilateral vestibular schwannomas are the hallmark of NF2. The clinical burden and in many cases, debilitating chronic course, result from multiple brain and spinal tumors, hearing loss due to vestibular schwannomas (VSs), and compromised vision due to cataracts. The human NF2 gene encodes a 595 amino acid protein—dubbed schwannomin (3) or merlin (4)—with a predicted molecular weight of 66 kDa. Antibodies raised against different peptides of the human NF2 protein confirmed the presence of a 66 kDa protein in normal human sciatic and eighth cranial nerve (6).

Potential functions for schwannomin were suggested based on its primary and secondary structural similarity with a family of membrane-organizing proteins. The primary amino acid sequence of the NF2 protein is highly similar to meosin (7), ezrin (8), and radixin (9) with highest homology (62%) observed in the amino terminal half of the protein (3,4). It also exhibits significant homology with other cytoskeletal associated proteins such as protein tyrosine phosphatase (10,11), non-erythroid (12) and erythroid protein 4.1 (13), and talin (14). The predicted secondary structure of schwannomin is similar to meosin-ezrin-radixin proteins with a long N-terminal domain (300 aa residues) followed by a large α-helical center (173 aa residues), and a smaller β-turn-helical C-terminal domain (3), suggesting that schwannomin may link cell membrane components to the cytoskeleton (15,16).

The NF2 protein is highly conserved with 97% amino acid identity between mouse and human (17,18). During the isolation of mouse cDNA clones we identified two clones with divergent sequence at the 3′ end of the coding region. Here we report the characterization of these clones and analysis of schwannomin isoforms in mouse and human tissues.

RESULTS

Isolation of mouse cDNA clones containing alternative transcripts

Using probes derived from PCR amplification of mouse RNAs, we isolated two mouse NF2 cDNA clones in addition to those previously described (18). Sequence analysis identified two clones, NF245 and NF261, that contained different nucleotide sequences in the 3′ end (Fig. 1). Clone NF245 contained a 750 bp cDNA fragment with a 16 bp insert (type II transcript, Figure 2). Clone NF261 contained a 4.9 kb insert. This clone was partially sequenced and contained a 61 bp insert (type IV transcript, Fig.2). Both inserts occurred at nucleotide 2057 (18). Clone NF261 contained an 857 bp deletion in the coding region. This likely represents a cloning artifact since PCR amplifications of multiple tissues using primer pairs flanking this region did not detect any PCR product missing the 857 bp segment (unpublished data).

In order to study the expression of the alternative transcripts, we designed primers flanking the alternative splicing site (primer M10A = AACGAGCTCAAGACGGAGATC, corresponding to nucleotides 1935–1956 specific for the mouse NF2 cDNA sequence (18), and primer M15B = GCTGGGTCACCTGCTAGA, complementary to the human NF2 cDNA nucleotides 1787–1804 (3) or 2104–2121 of the mouse NF2 cDNA (18)), and performed RT–PCR of adult mouse tissue mRNAs. The primer pair M10A–M15B amplified four products: a 186 bp

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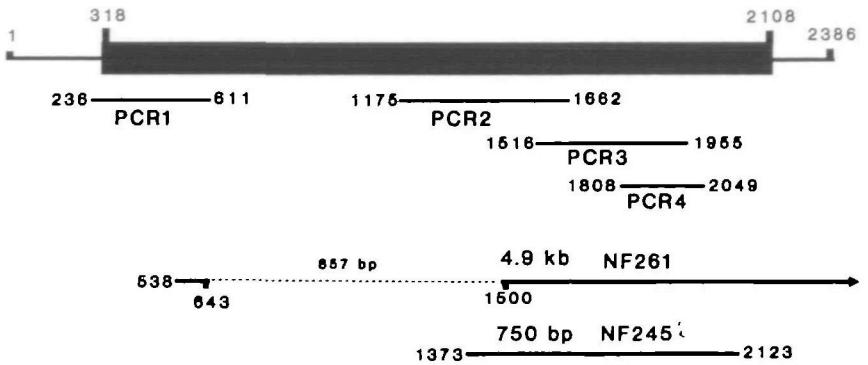

Figure 1. Schematic map of mouse cDNA clones. The top bar shows the coding region (solid bar) and noncoding regions (thin lines). Arrow head in NF261 cDNA clone indicates undetermined sequences. PCR1, 2, 3, and 4 fragments were used to screen a cDNA library for NF2 cDNAs Clone NF261 contained the 61 bp insert (Type IV) and a 857 bp deletion (dashed bar). Clone NF245 contained the 16 bp insert (Type II). Number system is based on the mouse NF2 cDNA sequence (18).

Figure 2. Mouse and human alternative transcripts. Boxed dinucleotides represents alternatively 5′ donor or 3′ acceptor sites. Lowercase letters represent the original NF2 nucleotide sequence in mouse (18) or in human (3), and the uppercase letters indicate nucleotide sequences of the inserts. (A) Partial amino acid sequence and nucleic acid sequence of mouse alternative transcripts. The insertion of alternatively spliced exons occurs between nucleotides 2057 and 2058 (arrowhead). Type IV consists of the 16 bp insert of type II (underlined) and the 45 bp insert of type IIIa. (B) Partial amino acid and nucleotides sequences of human transcripts. Type IIIb consists of the 45 bp insert of type IIIa and a new 15 bp segment in the immediate 3′ untranslated end. The GCG database accession number for the mouse NF2 isoform is L28838 and for the human isoform is L28839.

PCR product of the original NF2 sequence (type I), a 202 bp product containing a 16 bp insert (type II), a 231 bp product containing a 45 bp insert (type IIIa), and a 247 bp product containing a 61 bp insert made up of the 16 bp (type II) and 45 bp inserts (type IV). All four PCR products from adult mouse brain were sequenced, and the inserts were found to occur at the same nucleotide position 2057 (18). The four transcripts encode three schwannomin isoforms with distinct carboxyl ends due to a shift of the reading frame. Type II and type IV transcripts both encode the same truncated schwannomin containing 4 amino acid residues (val-pro-glu-met) due to a premature stop codon at nucleotide 13′ of the alternatively spliced exon. Type IIIa (and type IIIb in human, see below) transcript encodes another schwannomin isoform containing eleven different amino acids (pro-gln-ala-gln-ala-lys-lys-pro-ile-cys-ile) at the carboxyl end.

Relative expression of isoforms differs in adult mouse tissues

In order to study the expression of different NF2 transcripts, we performed RT−PCR of mouse tissue mRNAs using the M10A/M15B primer pair. Figure 3A shows an autoradiograph of transcripts detected in different mouse tissues. The expression of NF2 mRNAs occurred widely as previously reported (3,4,19). Type IV mRNA (61 bp insert) was low in all tissues except the brain and heart. Type IIIa (45 bp insert) predominated in muscle, heart, liver, and lung. Both type I and type IIIa were equally expressed in mouse brain. Type II (16 bp insert) was

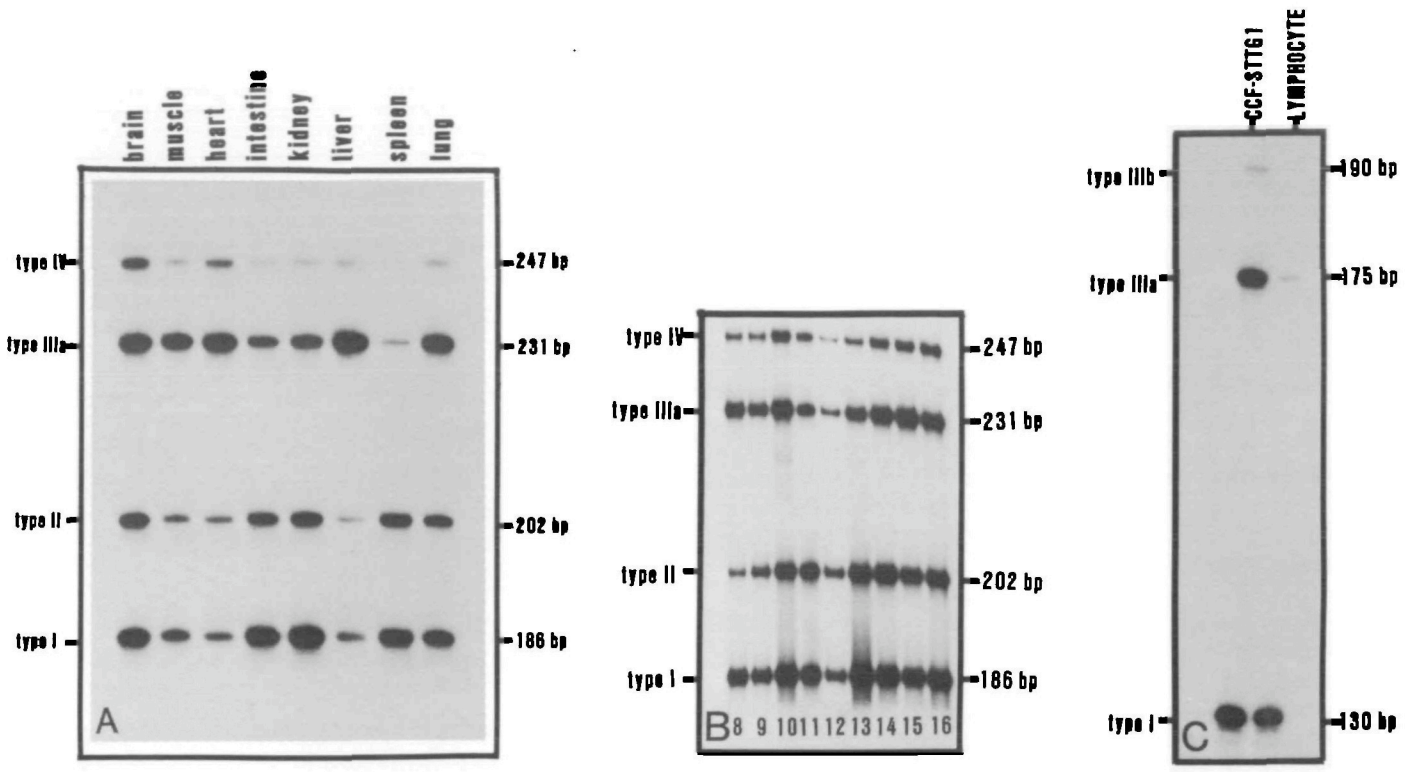

Figure 3. Autoradiographs of PCR products obtained by RT−PCR of mouse and human RNAs. Molecular weights show the actual number of base pairs elucidated by nucleotide sequencing. (**A**) Expression of NF2 in adult mouse tissues. The relative amounts of transcripts vary greatly between different tissues. (**B**) Expression of NF2 transcripts in mouse embryos. Numbers 8−16 correspond to developmental stages from E8 to E16. (**C**) Expression of NF2 transcripts in human cell lines. Only type I, type IIIa, and type IIIb transcripts are seen.

intermediately expressed in brain, intestine, kidney, spleen and lung, and at low levels in muscle, heart, and liver. Type I (no insert) was predominately expressed in brain, intestine, kidney, spleen, and lung. Overall, muscle, heart, and liver expressed the lowest relative amounts of type I and II mRNAs.

Expression of NF2 isoforms during development

To evaluate the relative level of expression of different NF2 transcripts during mouse development, we used the M10A−M15B primer pair to perform RT−PCR of total RNA from mouse embryos at E8 through E16. All isoforms were detected in embryonic tissues as early as E8 (Fig. 3B). Types I and IIIa were the predominant transcripts at E8 and E9. Beginning at E9, type II transcript increased and was as abundant as types I and III at E10.

Alternative transcripts are also found in human cell lines

To test whether human cell lines expressed alternative transcripts, we used a human M9A−M15B primer pair (3), which covered nucleotides 1671 through 1805 of the human NF2 cDNA (3). This primer pair amplified three PCR products: a PCR product of 130 bp (type I), of 175 bp product containing a 45 bp insert (type IIIa), and of 190 bp product (type IIIb) containing 60 bp insert made up of the 45 bp insert of type IIIa and a 15 bp segment in the immediate 3′ untranslated region (Fig. 3C). We detected both type I and IIIa mRNAs in cells from human astrocytoma cell line CCF-STTG1 and a lymphoblastoid cell line from a normal individual (Fig. 3C). Lymphoblastoid cells expressed very low levels of type IIIa mRNA. Neither cell line expressed type II mRNA whereas astrocytoma cells expressed very low levels of the type IIIb transcript. We named the 60 bp insert type IIIb because its first 45 nucleotides were identical to human type IIIa. The nucleotide sequence of type IIIa insert was highly conserved between mouse and human with only one nucleotide substitution (T to C) at position 38′ of the alternatively spliced exon, resulting in a human type IIIa schwannomin having 100% identity with the mouse type IIIa isoform.

DISCUSSION

Using PCR- and hybridization-based methods, we detected three additional NF2 transcripts in mouse tissues and two more transcripts in human cell lines. These transcripts are predicted to encode schwannomins with drastically altered C-terminal domains. All transcripts arise by the insertion of new DNA sequences between nucleotides 1737−1738 of the human NF2 cDNA sequence (3) or between nucleotides 2057−2058 of the mouse NF2 cDNA sequence (18). Recently, an identical transcript containing a 45 bp insert was described in human tissues (5), and the two transcripts containing 16 bp and 45 bp inserts were found in mouse tissues (19,20). In addition to these transcripts (type II and IIIa transcripts in this paper), we also discovered two novel transcripts: the human type IIIb transcript which contained a 60 bp insert consisting of the 45 bp insert of type IIIa plus an additional 15 bp segment in the immediate 3′ untranslated region; and the mouse type IV transcript (Fig. 3A

and 3B) which contained a 61 bp insert consisting of the 16 bp insert of type II and the 45 bp insert of type IIIa transcripts.

The type II and IV (like human type IIIa and IIIb) transcripts, although having different 3′ untranslated flanking sequences, encode identical proteins. It is possible that the different 3′ untranslated regions influence mRNA stability. However, the flanking sequences in type II and IV (like the human type IIIa and IIIb) transcripts have no known consensus sequences (21) to act as either mRNA stabilizing or destabilizing signals under different cell conditions.

Possible mechanisms for the formation of type II and IIIa transcripts were recently described (19). Sequence analysis of mouse genomic DNA fragments at the alternative splicing region revealed that the 16 bp exon was contiguous to the immediate 5′ upstream exon, and that a 1.4 kb intron separated the 16 bp and the 45 bp exons (19). The formation of the human type IIIb transcript probably uses similar mechanism. The newly described type IV transcript is probably formed as a result of the lariat excision of the 1.4 kb intron and ligation of the 5′ end of the 45 bp exon to the 3′ end of the 16 bp exon.

Mouse RNA−PCR analyses confirmed the wide tissue distribution of NF2 transcripts (3,4,19). In brain, type I, II, and IIIa were the predominant transcripts, whereas type IIIa predominated in liver, and to a lesser degree in heart, lung, and skeletal muscles. Significant type IV expression was observed only in brain and heart. These results may suggest tissue specific functions for different isoforms.

Previous studies had shown that the NF2 gene is expressed during mouse development (18). We now demonstrate that the relative amount of NF2 transcripts change from E8 through E16. Beginning at E9 and clearly visible at E10, the relative expression of type II transcript increased. At this stage in mouse development, telencephalic vesicles and different ganglia are markedly differentiated. The trigeminal (V), facial (VII), and acoustic (VIII) ganglia are now identifiable (22). In addition, other organs such as the heart, primitive gut, and urogenital system show a significant degree of differentiation at this stage (22). Thus, different NF2 isoforms may be critical for proper development of the mouse embryo. For the NF1 gene, differential regulation and anatomical distribution of neurofibromin isoforms were recently demonstrated (23). Although major developmental abnormalities are not part of the human NF2 phenotype, it will be important to analyze NF2 expression in human embryos particularly in regard to the presence of a type II transcript.

Little is known about the function of the C-terminal domains in the family of cytoskeletal associated proteins. This domain shows greater sequence divergence between mouse and human schwannomins and between schwannomin and other cytoskeletal associated proteins compared to the N-terminal and α-helical domains (17,18). The analysis of human NF2 mutations supports an important functional role for the C-terminus. In one schwannoma, a frameshift mutation predicts a schwannomin with an altered and extended C-terminus (6). Functional significance of different schwannomin isoforms is further suggested by the fact that the amino acid sequence has remained identical across 20 million years of evolution. A potential phosphorylation site in the C-terminus for a serine/threonine kinase is conserved in mouse and human schwannomin as well as in mouse and human meosin and ezrin (18). Only type I schwannomin isoform contains this site. Thus, differential phosphorylation of schwannomin isoforms may lead to different subcellular compartmentalization similar to the effects of phosphorylation on ezrin (8,24).

MATERIALS AND METHODS

Isolation of total RNA

Total RNA were isolated from adult mouse tissues, whole mouse embryos, and CCF-STTG1 and lymphoblastoid cells using the TriReagent kit (Molecular Research Center, IN) as previously described (18,22). One μg of total RNA was then used for 1st strand cDNA synthesis in a 20 μl reaction volume using a Promega cDNA synthesis kit. Mouse embryos were purchased from Harlam BioProducts for Science (Indiana).

Polymerase chain reaction (PCR) and PCR fragment isolation

PCRs were performed as previously described (18,23). Primers A-B included PCR1 (A = GCCTGTGCAGCAACTCCAGG, 236−255; B = GGCCAAGAA-GAGAAAGGTGA complementary to 592−611); PCR2 (A = CTCCTC AAA-GCTTCGTGTTA, 1175−1194; B = TGGCCCTCCTCTCTGACTCC, complementary to 1643−1662); PCR3 (A = AGAAGGTACTGGAGGCTGA, 1516−1534; B = GATCTACGTCTTGAGC TCTT, complementary to 1936−1955; PCR4 (A = CTTCAACCTCATTGGTGACAG, 1808−1828; B = TGGTATTGTGCTTGCTGCTG, complementary to 2030−2049); M9A (TCTGGATATTCTGCACAATGAGA, 1671−1693; human specific primer [3]); M10A (AACGAGCTCAAGACGGAGATC, 1618−1638 (ref 3) or 1935−1955 (ref. 18); M15B (GCTGGGTCACCTGCTAGA, complementary to 1787−1805 (3) or 2104−2121 (18); specific for both mouse and human).

PCR fragments were separated by 1% LMP agarose (GIBCO-BRL) gel electrophoresis containing 0.5 μg/ml ethidium bromide. Desired bands (300 μl final volume) were cut with a sterile razor blade and incubated at 70°C for 20 minutes to melt the gel. The PCR fragment was then purified from agarose with the Magic PCR PREPs DNA Purification System (Promega). The purified PCR fragment was used for PCR reamplification or double stranded DNA sequencing.

Radiolabelled PCR and PCR fragment purification from polyacrylamide gels

Radiolabelled PCR reactions were performed as previously described (23).The labelled PCR fragments were separated by a 6% denatured polyacrylamide gel, exposed to a Kodak X-OMAT AR film for 1 hour at −70°C, and then developed in an automatic X-ray film developer. The gel was aligned with the developed film, and desired fragments were cut at 2×6 mm^2 gel. Each gel was incubated with 100 μl of sterile ddH_2O overnight at 4°C. Ten μl of the eluted DNA fragment was reamplified in a 100 μl PCR reaction volume at standard PCR conditions for 42 cycles of amplification, separated in a 1% LMP agarose gel, and the reamplified PCR band was purified using the Magic PCR PREPs DNA Purification System (Promega).

To detect alternatively spliced products in mouse tissues, we used the M10A−M15B primer pair. To detect human transcripts, we used primer M9A in place of primer M10A. Both primers M10A and M9A were 5′-end labeled with ^{32}P. The annealing temperature for both primer pairs was 55°C. The PCR reaction mixture was incubated in Biometra cycler with standard reaction conditions with 35 cycles amplification.

Double stranded DNA sequencing was performed using the Circumvent Thermal Cycle Dideoxy DNA Sequencing Kit with Vent$_R$ (exo$^-$) DNA Polymerase (Biolabs).

Hybridization screening of a mouse brain cDNA library

Hybridization screening using different ^{32}P-labeled PCR-generated fragments was described previously (18).

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ABBREVIATIONS

PCR, polymerase chain reaction; cDNA, complementary deoxyribonucleic acid.

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