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A Novel Moesin-, Ezrin-, Radixin-like Gene Is a Candidate for the Neurofibromatosis 2 Tumor Suppressor

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Summary

Neurofibromatosis 2 (NF2) is a dominantly inherited disorder characterized by the occurrence of bilateral vestibular schwannomas and other central nervous system tumors including multiple meningiomas. Genetic linkage studies and investigations of both sporadic and familial tumors suggest that NF2 is caused by inactivation of a tumor suppressor gene in chromosome 22q12. We have identified a candidate gene for the *NF2* tumor suppressor that has suffered nonoverlapping deletions in DNA from two independent NF2 families and alterations in meningiomas from two unrelated NF2 patients. The candidate gene encodes a 587 amino acid protein with striking similarity to several members of a family of proteins proposed to link cytoskeletal components with proteins in the cell membrane. The *NF2* gene may therefore constitute a novel class of tumor suppressor gene.

Introduction

Neurofibromatosis (NF) is a term used to describe two major human genetic disorders both of which display autosomal dominant inheritance and involve tumors of the nervous system, but are distinct clinical entities (Mulvihill et al., 1990). NF1, or von Recklinghausen NF, is more common (incidence of 1 in 4000) and is characterized by the highly variable expression of an array of features that include neurofibromas, cafe-au-lait macules, Lisch nodules of the iris, and a predisposition to certain malignant tumors (Riccardi, 1981; Riccardi and Eichner, 1986). It is caused by defects in a chromosome 17 gene that has recently been isolated and characterized (Viskochil et al., 1990; Cawthon et al., 1990; Wallace et al., 1990). The *NF1* gene product, termed neurofibromin, is a large protein with a GTPase-activating protein-related domain and is presumably involved in modulating a signal transduction pathway whose disruption can lead to tumor formation (Ballester et al., 1990; Buchberg et al., 1990; Xu et al., 1990; DeClue et al., 1992; Basu et al., 1992).

By contrast, NF2, which occurs in about 1 of 40,000 live births (Evans et al., 1992), is characterized by bilateral schwannomas that develop on the vestibular branch of the eighth cranial nerve. Pressure from these tumors often causes hearing loss and vestibular symptoms in the second and third decade. Other tumors of the brain, especially meningiomas, and schwannomas of other cranial nerves and spinal nerve roots (Martuza and Eldridge, 1988) as well as posterior capsular lens opacities (Kaiser-Kupfer et al., 1989) are common and are generally present in the young adult. The *NF2* gene is highly penetrant, and individuals with it have a 95% chance of developing bilateral vestibular schwannomas. NF2 is often more severe than NF1, with teenage or early adulthood onset of multiple slow growing tumors that can gradually cause deafness, balance disorder, paralysis, or increasing neurological problems necessitating repeated surgical procedures.

NF2 was shown to be genetically distinct from NF1 by linkage studies that assigned the *NF2* gene to chromosome 22 (Rouleau et al., 1987; Wertelecki et al., 1988; Rouleau et al., 1990; Narod et al., 1992). The tumor types that occur in NF2 are found more frequently in the general population as solitary, sporadic tumors. Since frequent loss of alleles on chromosome 22 from both sporadic vestibular schwannomas and meningiomas and from their counterparts in NF2 had been noted previously, the localization of the inherited defect to the same chromosome region suggested that the *NF2* locus encodes a recessive tumor suppressor gene (Knudson, 1971) whose inactivation leads to tumor formation (Seizinger et al., 1986, 1987a, 1987b). A number of studies of both sporadically occurring tumors and tumors from NF2 patients have provided consistent support for this hypothesis (Couturier et al., 1990; Rouleau et al., 1990; Fiedler et al., 1991; Fontaine et al., 1991a, 1991b; Bijlsma et al., 1992; Wolff et al., 1992). The combined use of family studies and tumor deletion mapping has progressively narrowed the location of *NF2* within the q12 band of chromosome 22, defining a candidate region in which to search for the genetic defect (Rouleau et al., 1990; Wolff et al., 1992). Here, we report a gene from the *NF2* region that has suffered nonoverlap-

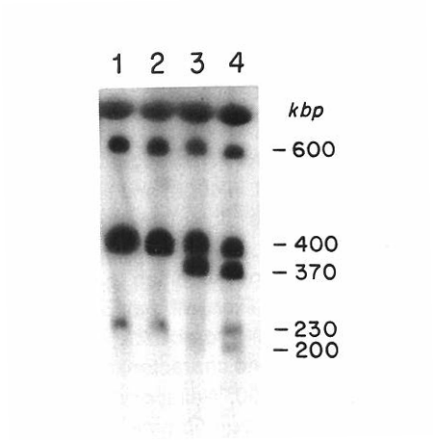

Figure 1. Pulsed-Field Gel Analysis of Lymphoblast DNA from NF2 Patients

DNA in agarose blocks was digested with NotI, subjected to electrophoresis, blotted, and hybridized to a radiolabeled 4 kb *NEFH* probe. Lane 1, GUS6274 (affected NF2 unrelated to lanes 3 and 4); lane 2, GUS7870 (normal human); lane 3, GUS5068 (affected daughter); lane 4, GUS5069 (affected mother).

ping and interstitial deletions in four independent NF2 patients, suggesting that it encodes the *NF2* tumor suppressor. This candidate gene encodes a novel protein related to the moesin (membrane-organizing extension spike protein)–ezrin (cytovillin)–radixin family of cytoskeleton-associated proteins (Gould et al., 1989; Turunen et al., 1989; Funayama et al., 1991; Lankes and Furthmayr, 1991; Sato et al., 1992). This protein, which we have named merlin (for moesin–ezrin–radixin-like protein), may represent a new class of tumor suppressor whose function is mediated by interactions with the cytoskeleton.

Results

Scanning the *NF2* Candidate Region for Rearrangement

The *NF2* candidate region on chromosome 22 lies between *D22S1* and *D22S28* and is estimated to encompass 6 Mb of band q12 (Frazer et al., 1992). Since some germline *NF2* mutations might involve a deletion of the tumor suppressor gene as has been found in Wilms' tumor and retinoblastoma (Riccardi et al., 1978; Francke et al., 1979; Dryja et al., 1986), we set out to scan the candidate region for such alterations. Pulsed-field gel blots containing lymphoblast DNAs from various NF2 patients were probed for several loci in the candidate region, including *D22S1*, *D22S15*, *D22S28*, *D22S32*, *D22S42*, *D22S46*, *D22S56*, the leukemia inhibitory factor gene, and the neurofilament heavy chain gene (*NEFH*). This analysis revealed that in a lymphoblast cell line (GUS5069) derived from a female

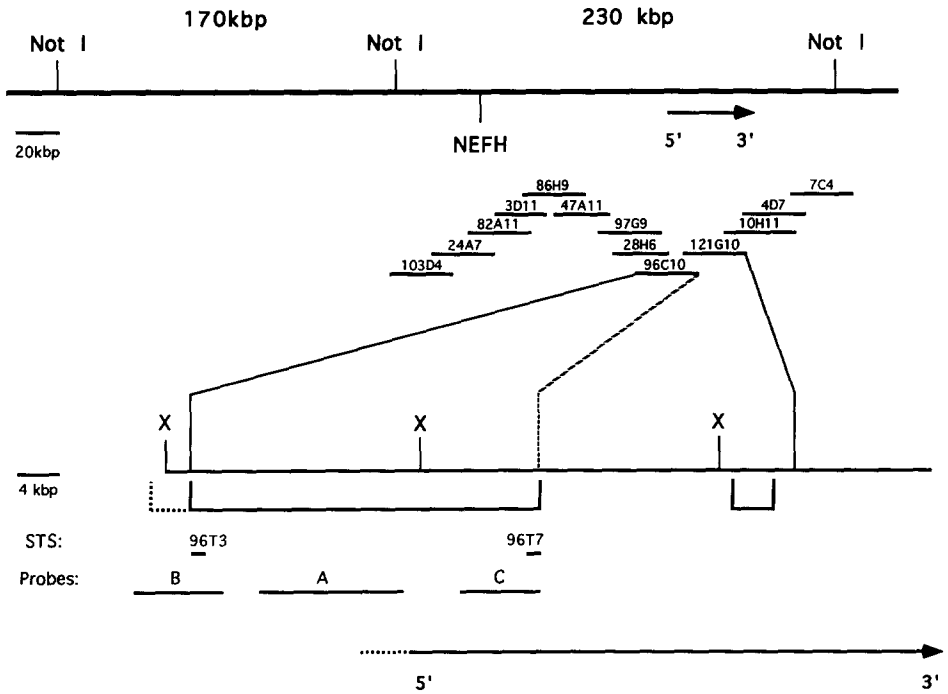

Figure 2. Physical Map of the Chromosome 22 Region Surrounding the NF2 Deletions

NotI sites were determined by pulsed-field gel analysis and confirmed in cosmids. Cosmids were named according to their standardized library coordinates. The enlarged region shows details of the merlin gene vicinity with deleted regions denoted by underlying brackets. Probes used in the deletion analysis: A, 14 kb HindIII fragment from cosmid 96C10; B, 8.5 kb HindIII fragment from 28H6 that spans the T3 end of 96C10; C, 8 kb HindIII end fragment from the T7 end of 96C10; 96T3 (primer pair: 5′-CAGATTGTTCATTCCAAGTGG-3′ and 5′-ACCCTGAGGAATCCACTACC-3′, product size = 124 bp) and 96T7 (5′-TGCACACACATCCTTTTCAC-3′ and 5′-GAGAGAGACTGCTGTCTCAAAAA-3′, product size = 92 bp) are STSs derived from the T3 and T7 ends of 96C10, respectively. X, XhoI recognition site. Arrows indicate the orientation of transcription and approximate genomic coverage of cDNA JJR-1.

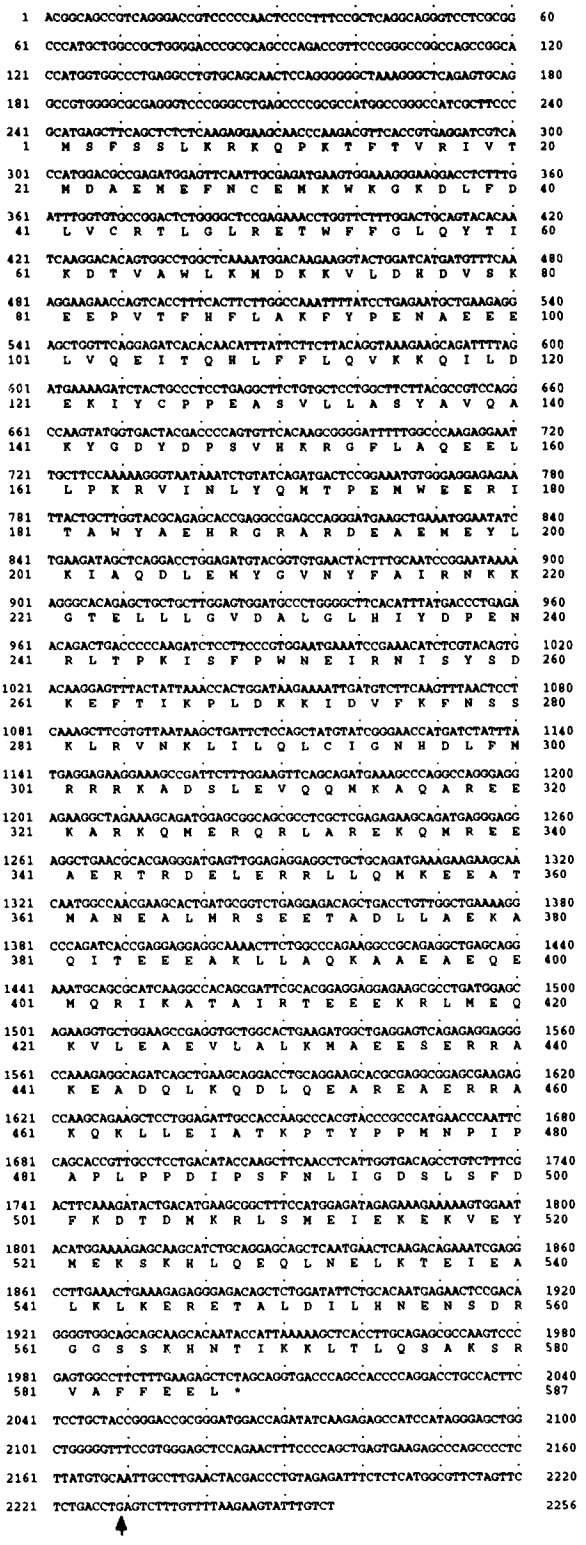

Figure 3. Complete Nucleotide and Predicted Amino Acid Sequences of the Merlin JJR-1 cDNA Clone

Common moesin–ezrin–radixin domain spans amino acid residues 1–350. Arrow indicates where poly(A) addition has occurred in two independent clones (JJR-6 and JJR-9).

NF2 patient, a probe for the *NEFH* locus hybridized to apparently altered fragments of reduced size with both NotI and NruI.

In NotI-digested DNA (Figure 1) the *NEFH* probe detected fragments of approximately 600 kb, 400 kb, and 230 kb in most lymphoblast cell lines. We were unable to confirm that the 600 kb fragment originates from chromosome 22, leaving the possibility that it may derive from hybridization to a related locus (A. G. M. et al., unpublished data). Variable intensity of the 230 kb fragment in many samples suggested that it resulted from partial digestion of the 400 kb fragment. In GUS5069, additional fragments of approximately 370 kb and 200 kb were observed, consistent with the possibility of a deletion within the region common to the 400 kb and 230 kb fragments. The alteration was transmitted along with *NF2* from the patient to her affected daughter (represented by GUS5068) (Figure 1).

Chromosome Walking toward the Putative Deletion

To isolate DNA corresponding to the region of chromosome 22 apparently deleted in GUS5069, a bidirectional cosmid walk was initiated from *NEFH*. At each step, single restriction fragments of the cosmids were used as probes on pulsed-field gels to establish the location of the putative deletion relative to *NEFH*. On the 5′ side of *NEFH*, we soon identified a NotI site that was rarely cleaved in lymphoblast DNA. Probes beyond the NotI site detected the same ~400 kb NotI fragment along with a 170 kb fragment of variable intensity. Thus, infrequent cleavage of this NotI site divides the 400 kb fragment into fragments of 230 kb and 170 kb. Since the putative deletion in GUS5069 affects the 230 kb fragment but not the 170 kb fragment, we continued to walk only 3′ of *NEFH*, again probing pulsed-field gel blots containing DNA from GUS5069.

The NotI pulsed-field gel map, a minimal set of clones representing the cosmid walk, and the extent of the genomic deletion (see Figure 5b) are shown in Figure 2. The deletion was reached when we tested a probe (Figure 2, probe A) from cosmid 96C10 that failed to detect the altered NotI fragment in GUS5069. However, various probes from cosmids 28H6 and 121G10 did detect the altered fragment. To estimate the extent of deletion, probes B and C (Figure 2) were tested. Probe B is an 8 kb HindIII fragment from 28H6 that overlaps with the T3 end of 96C10. Probe C is a 9 kb HindIII fragment from the T7 end of 96C10. Probe B detected both the normal and the altered NotI pulsed-field gel fragments, but probe C detected only the normal fragment.

For more precise analysis of the deletion, the altered chromosome 22 from GUS5069 was segregated from its normal counterpart in human X hamster somatic cell hybrids. We created sequence-tagged site (STS) assays for the T3 and T7 ends of 96C10 and tested hybrids containing the separated chromosomes 22. In contrast with the above hybridization results, the T3 end of 96C10 was absent in hybrid GUSH134A3, which contains the deleted chromosome, but present in GUSH134A10, which contains the

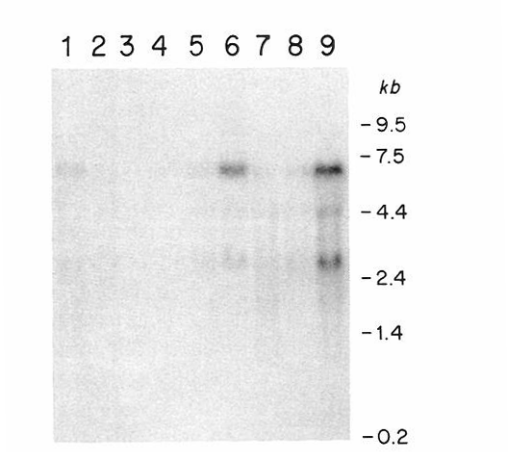

Figure 4. Northern Blot Analysis of RNA Derived from Human Tumor Cell Lines

RNA was size fractionated by electrophoresis in formaldehyde–agarose, blotted, and hybridized as described in Experimental Procedures. The blot was hybridized with a radiolabeled probe corresponding to bases 1253–1942 of the JJR-1 cDNA. Lane 1, SK-N-NB neuroblastoma; lane 2, T98G glioblastoma; lane 3, WERI retinoblastoma; lane 4, MCF-7 breast carcinoma; lane 5, HOS osteosarcoma; lane 6, HEPG2, liver carcinoma; lane 7, CATA4 kidney carcinoma; lane 8, SW480 colon carcinoma; lane 9, EJ bladder carcinoma.

normal chromosome. Moreover, the T7 assay was positive in both hybrids. The locations of probes B and C and of both STS assays were confirmed on the cosmid walk. Thus, the failure of probe C to detect the altered fragment suggests that the deletion spans most but not all of this sequence. Similarly, the other deletion breakpoint must occur within the region spanned by probe B. Therefore, the combination of hybridization and polymerase chain reaction (PCR) results indicates that the deletion must encompass almost all of 96C10 and up to an additional 5 kb of 28H6. This 35–45 kb region is expanded below the cosmid walk in Figure 2.

Identification and Characterization of the Merlin cDNA

Exon amplification (Buckler et al., 1991), which produces cloned "trapped exons," was applied to cosmids 28H6, 96C10, 121G10, 123F5, 10H11, and 7C4 surrounding the site of the NF2 deletion as a rapid method of obtaining exonic probes for cDNA cloning. Each exon clone can represent a single exon or multiple exons spliced together in the trapping procedure. We obtained and sequenced 24 exon clones, 6 of which displayed sequence similarity with the cytoskeleton-associated proteins moesin, ezrin, and radixin (see below). The latter exons were used to screen human frontal cortex and hippocampus cDNA libraries.

Figure 3 shows the complete DNA sequence of JJR-1, the longest clone obtained in cDNA screening. This sequence contains eight of the cloned exon segments as shown in Figure 5a. The cDNA is 2256 bp long and shows no evidence of a poly(A) tail. However, two shorter cDNA clones, JJR-6 and JJR-9, which overlapped the restriction map of JJR-1, had apparent poly(A) tails beginning at base 2230. JJR-1 contains an open reading frame of 1761 bp encoding a predicted protein of 69 kd. There are two in-frame stop codons within 80 bp upstream of the putative initiator methionine. The JJR-1 cDNA spans at least 50 kb of genomic DNA and is transcribed in the same orientation as *NEFH*, as shown by the arrows in Figure 2.

Both the JJR-1 DNA sequence and the predicted protein product were used to search for similarity in nucleic acid and protein data bases using the BLAST network service of the National Center for Biotechnology Information (Altschul et al., 1990). The DNA sequence displayed significant similarity to moesin and ezrin genes from several species, including humans ($P = 9.0e^{-125}$ and $9.0e^{-122}$), to mouse radixin ($1.1e^{-102}$) and to Echinococcus multilocularis tegument protein ($2.4e^{-21}$). Striking similarity was also detected at the amino acid level with these same proteins ($2.5e^{-146}$, $5.0e^{-146}$, $2.7e^{-145}$, and $7.6e^{-73}$, respectively) and to a potential product of a sequence tag from Caenorhabditis elegans ($3.7e^{-43}$). Weaker similarities were detected to the sequences of two protein-tyrosine phosphatases, PTP-MEG and PTP-H1 ($1.3e^{-17}$ and $9.6e^{-16}$, respectively), to erythrocyte protein 4.1 ($9.9e^{-14}$) and to a wide range of myosin, tropomyosin, and paramyosin proteins. Because this novel gene is most closely related to moesin, ezrin, and radixin (45%–47% identity), we have called it merlin.

Northern blot analysis using total RNA from various cultured human tumor cell lines (Figure 4) revealed two major hybridizing species of 2.6 kb and 7 kb, and a less intensely hybridizing RNA of 4.4 kb. A similar pattern was detected in poly(A)$^+$ RNA from various human tissues, including heart, brain, lung, skeletal muscle, kidney, pancreas, and liver (weakly), indicating that the merlin gene is expressed widely. The apparent poly(A) tails detected in JJR-6 and JJR-9 suggest that these clones may have derived from the ~2.6 kb RNA. The JJR-1 clone likely derived from one of the larger RNAs that apparently has a much longer 3′ untranslated region. However, it cannot be excluded that the larger RNAs arise by alternative splicing that alters the length and composition of the coding sequence or by hybridization to related family members.

Nonoverlapping Deletions Interrupt the Candidate *NF2* Gene

To determine whether the deletion detected in GUS5069 interrupts the merlin gene, we prepared exon probes from across the coding sequence (Figure 5a) and analyzed Southern blots containing DNA from GUSH134A3 and from GUSH134B1, two independent hybrid lines containing the deleted chromosome 22. The results for probes I and II, shown in Figure 5b, demonstrate that the probe I sequence was absent from both hybrids, while the probe II sequence was present in both. Thus, the genomic deletion truncates the merlin gene within the coding sequence between probes I and II, removing the 5′ end.

In a search for additional alterations in the merlin gene, we scanned blots of restriction-digested DNA from 33 unrelated NF2 patients using the cDNA as probe. One patient, represented by cell line GUS5722, displayed altered fragments with several restriction enzymes suggestive of a small ~3–4 kb genomic deletion. This patient was analyzed in the Southern blots shown in Figure 5c. Probes III,

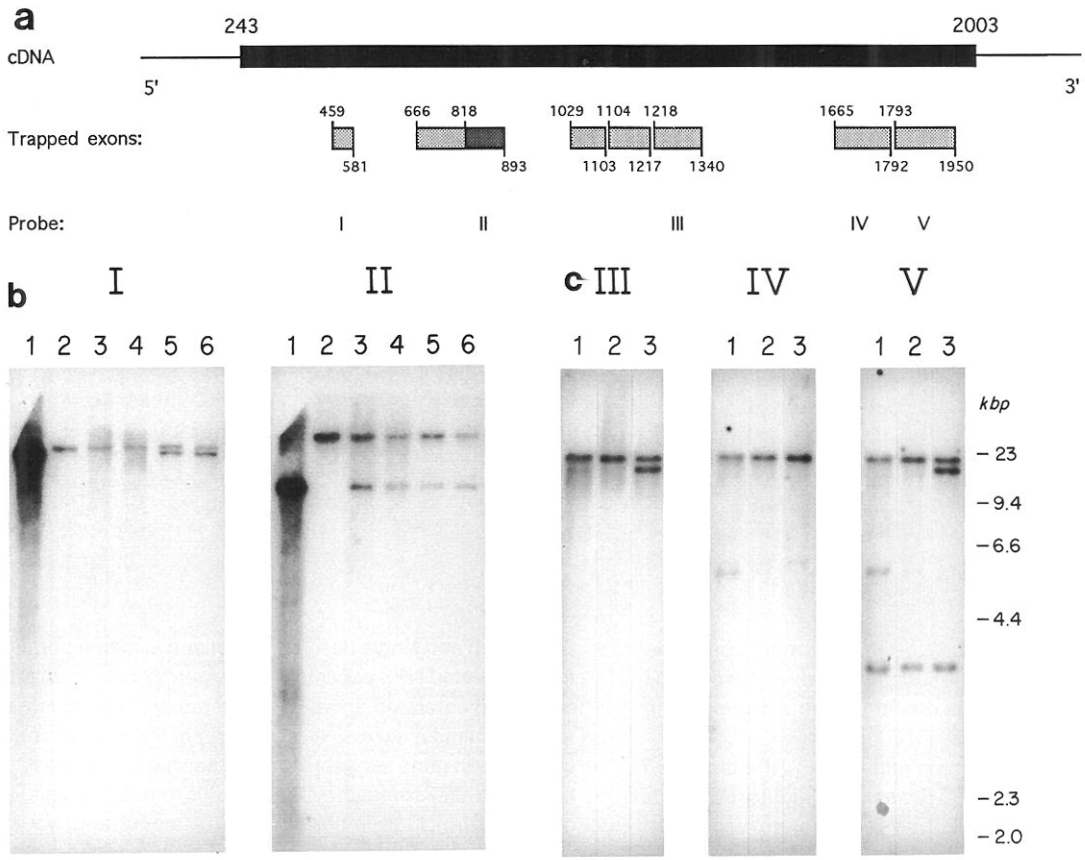

Figure 5. Detection of Genomic Germline Deletions in NF2 Patients

(a) Location of trapped exons in JJR-1 cDNA. The closed box in the cDNA indicates the open reading frame; thin lines indicate both 5′ and 3′ untranslated regions. Probe names correspond to those used in (b) and (c). Probe II represents base pairs 818–893 of JJR-1 and is overlapped by another trapped product. These overlapping products may have arisen from partial digestion of the cosmid or alternative splicing in COS7 cells occurring during exon amplification analysis.

(b) Southern blot analysis of GUS5069 X hamster hybrid cell lines harboring the altered chromosome 22. DNA samples were digested with BamHI, size fractionated in agarose gels, blotted, and hybridized with radiolabeled probes indicated above each panel. Lane 1, GUS1323 (human control); lane 2, CHTG49 (hamster control); lane 3, GUSH134A3 (hybrid with deleted homolog); lane 4, GUSH134B1 (hybrid with deleted homolog); lane 5, GM10888 (chromosome 22-only hybrid); lane 6, Eye3FA6 (hybrid containing chromosome 22 and smaller portions of two to three other chromosomes).

(c) Southern blot analysis of NF2 patient lymphoblast DNA. Samples were digested with EcoRI, size fractionated, blotted, and hybridized with radiolabeled probes indicated above each panel. Lane 1, GUS1323 (human control); lane 2, GUS5069 (NF2 affected); lane 3, GUS5722 (NF2 affected). Probe V comprises multiple exons and recognizes multiple genomic fragments.

IV, and V all reside on the same 21 kb EcoRI fragment. In GUS5722, probes III and V detect both the normal EcoRI fragment and a second fragment reduced in size by the deletion. Probe IV fails to detect the altered fragment in GUS5722 because it lies within the region deleted. We have performed PCR amplification of first strand cDNA from GUS5722 and confirmed the presence of two types of PCR product (Figure 6A). Direct sequencing revealed that the novel PCR product was missing bases 1559 to 1792 of the cDNA, representing deletion of at least two exons. The absence of this segment would remove 78 amino acids from the protein, while leaving the reading frame intact. The GUS5722 cell line was generated from a member of a large NF2 kindred (family 3 in Narod et al., 1992), and the deletion was present in 5 affected members and absent in 11 unaffected members of this pedigree.

The presence of nonoverlapping deletions affecting the merlin gene in two independent families suggests that this gene represents the *NF2* tumor suppressor. We searched for additional alterations by single-strand conformational polymorphism (SSCP) analysis of PCR-amplified first strand cDNA from tumor and lymphoblast samples. We used mRNA from four primary cultures of meningiomas (three from NF2 patients with a family history of the disorder, and one from a probable new mutation to NF2) and analyzed only selective regions of the mRNA. Two of the tumors yielded aberrant patterns.

A meningioma from a female patient likely to have NF2 (see Experimental Procedures) displayed a reduced size for the expected nondenatured PCR product on SSCP gels (Figure 6B). The vastly reduced level of the normal-sized PCR product suggests that this tumor had lost alleles in this region of chromosome 22. However, lymphocyte DNA was not available from this patient to confirm this.

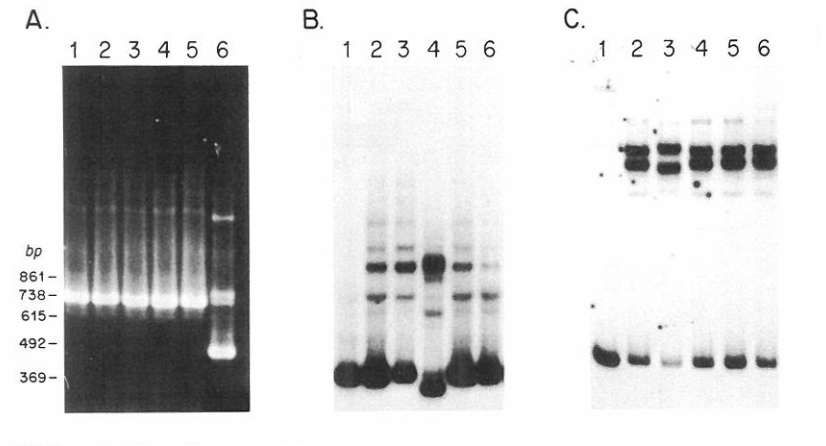

Figure 6. Analysis of Alterations in RNA

(A) PCR analysis of RNA from lymphoblasts of affected NF2 patients. RNA PCR was performed as described in Experimental Procedures. Samples were subjected to electrophoresis in a 1.5% agarose gel and visualized by staining with ethidium bromide and ultraviolet illumination. The products seen correspond to RNA sequence encompassed by bases 1258 to 1947 (primer pair: 5′-AGGAGGCTGAACGCACGAG-3′ and 5′-TGGTATTGTGCTTGCTGCTG-3′). Lanes 1, 2, and 4, lymphoblasts from independent NF2 patients; lane 3, GUS5068; lane 5, normal control lymphoblast; lane 6, GUS5722.

(B and C) SSCP analysis of RNA samples derived from primary cultures of meningioma tumors from NF2 patients. Lane 1, undenatured PCR product from the tumor in lane 2; lanes 2, 3, 4, and 5, denatured PCR product from independent primary NF2 meningiomas; lane 6, denatured PCR product from GUS5068 lymphoblast. The PCRs amplified the following segments: in (B), base pairs 1709–1947 (primer pair: 5′-CTTCAACCTCATTGGTGACAG-3′ and 5′-TGGTATTGTGCTTGCTGCTG-3′); in (C) base pairs 457–730 (primer pair: 5′-AGGTACTGGATCATGATGTTTC-3′ and 5′-TTTGGAAGCAATTCCTCTTGG-3′). All lanes contain a proportion of undenatured product, detected by comparison with lane 1.

Direct sequence analysis of the PCR product confirmed the presence of a 4 bp deletion that removes bases 1781 to 1784 thereby altering the reading frame and generating a shorter protein.

A meningioma from a male patient with NF2 displayed an altered pattern on SSCP analysis (Figure 6C). This meningioma was known to have lost heterozygosity on chromosome 22 based on prior comparison of polymorphic markers in blood and tumor DNA. Thus, the tumor suppressor model would suggest that the normal homolog had been lost and that the remaining copy of the gene represented the altered *NF2* allele. Direct sequence analysis revealed a single base pair deletion at position 488 (Figure 3), which introduced a frameshift that dramatically alters the predicted protein by introducing a stop codon within 100 bases.

Discussion

The delineation of nonoverlapping germline deletions affecting different portions of the same chromosome 22 gene in two independent NF2 families suggests strongly that the *NF2* tumor suppressor has been found. It is conceivable that one or both of these two deletions also affect a second gene in the area. However, for such a gene to supplant our candidate as the *NF2* tumor suppressor, it would have to be affected by both deletions and must therefore be composed of exons interspersed with those of the merlin gene. The larger of the deletions truncates the 5′ end of the merlin gene, removing at least 112 amino acids. In addition, the extent of this deletion suggests that the 5′ regulatory elements may also be missing. The smaller germline deletion removes 78 amino acids from the C-terminal portion of the protein. One would anticipate that such alterations would have drastic consequences for the function of the merlin protein. However, ultimate proof that this locus is *NF2* will require reversal of the tumor phenotype by reintroduction of the merlin gene.

The 4 bp and single base pair deletions in meningiomas from unrelated NF2 patients could arguably be of somatic origin and unrelated to the inherited predisposition. However, the almost exclusive expression of the altered copy of the merlin gene suggests that the normal sequence has been lost as a somatic event in tumor formation. This is consistent with the tumor suppressor model and would suggest that the frameshift alterations actually represent germline mutations in these patients, further implicating the merlin gene.

The merlin protein encoded at the candidate *NF2* locus is a novel member of a growing family of proteins that have been proposed to act as links between the cell membrane and the cytoskeleton (Luna and Hitt, 1992; Sato et al., 1992). All members of the family, which includes moesin, ezrin, radixin, erythrocyte protein 4.1, and talin, contain a homologous domain of ~200 amino acids near the N-terminus, followed by a segment that is predicted to be rich in α helix structure, and a highly charged C-terminal domain. Where they have been characterized from more than one mammalian species, members of this family are remarkably conserved. Moreover, highly related genes have been detected in the nematode, C. elegans (Waterston et al., 1992), and in the parasitic cestode, E. multilocularis (Frosch et al., 1991).

Although most distantly related to merlin, protein 4.1 and talin are the best studied members of this family of proteins and have been most revealing concerning their apparent function. Protein 4.1 plays a critical role in maintaining membrane stability and cell shape in the erythrocyte by connecting the integral membrane proteins glycophorin and protein 3 (the anion channel) to the spectrin–actin lattice of the cytoskeleton (Leto and Marchesi, 1984; Conboy et al., 1986). Genetic defects in protein 4.1 lead to one form of hereditary elliptocytosis (Tchernia et al., 1981; Delaunay et al., 1991). The binding site for glycophorin in protein 4.1 has been mapped to the N-terminal domain, suggesting that the homologous region in other family mem-

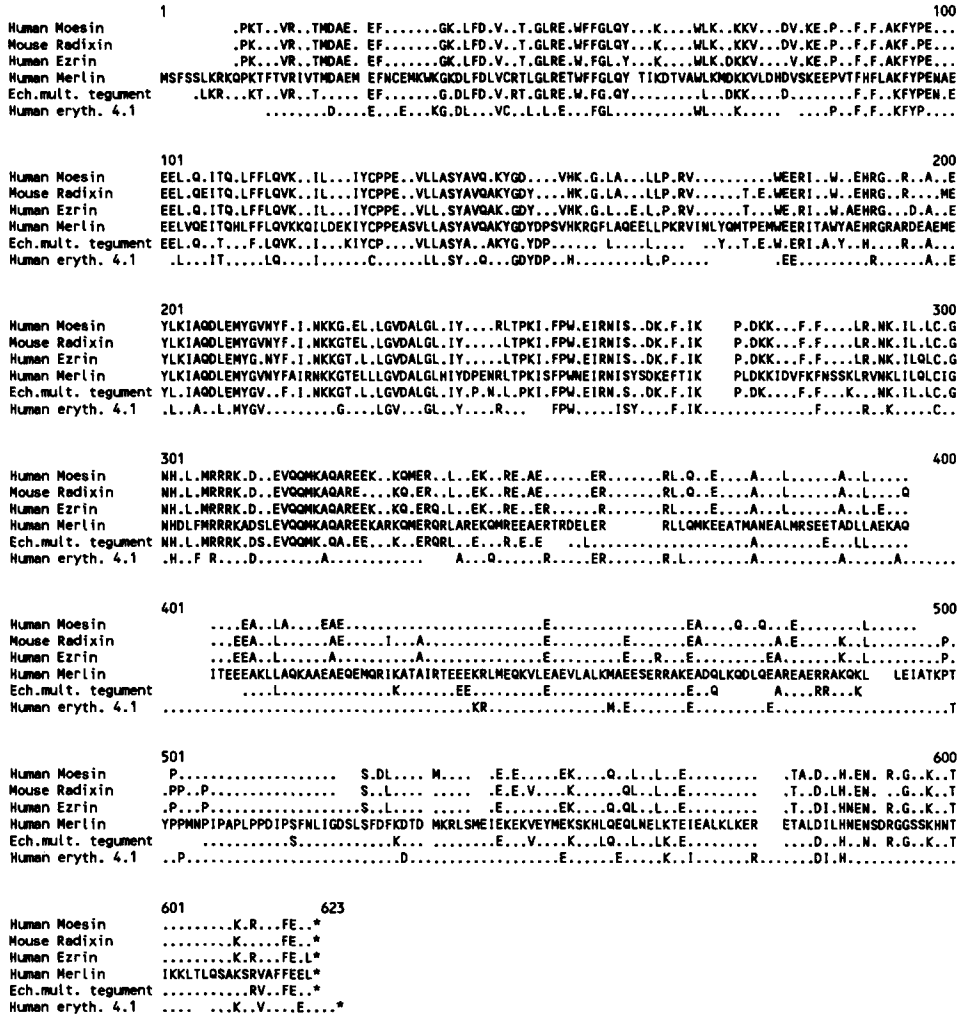

Figure 7. Comparison of Sequence Identities in Merlin-Related Proteins

The program PILEUP of the GCG package (Devereux et al., 1984) was used to generate an optimal alignment of the protein products translated from the following GenBank files: human moesin (M69066); mouse radixin (S66820); human ezrin (X51521); E. multilocularis tegument protein (M61186); human erythrocyte protein 4.1 (M14993). Only amino acid identities are shown; nonidentical residues are indicated by dots, and gaps introduced by the program are represented by empty spaces.

bers might also bind to proteins in the membrane (Leto et al., 1986). Interestingly, a related domain is also found in two protein-tyrosine phosphatases, PTP-MEG and PTP-H1, perhaps allowing these enzymes to associate with the membrane or the cytoskeleton (Gu et al., 1991; Yang and Tonks, 1991). Binding of protein 4.1 to spectrin is mediated by the α-helical region of the protein, suggesting that the analogous segments of the other family members might also bind to cytoskeletal components (Correas et al., 1986). Talin, a large protein found in regions of focal adhesions at cell–cell or cell–substrate contacts, appears to behave similarly, binding to the integrins in the cell membrane and to vinculin, thereby connecting the extracellular adhesion matrix to the cytoskeleton (Rees et al., 1990; Luna and Hitt, 1992).

Moesin, ezrin, and radixin are highly related proteins (~70%–75% amino acid identity) that have each been postulated to provide a link between the cytoskeleton and the cell membrane. Each of these proteins shares 45%–47% amino acid identity with merlin. Moesin, originally proposed as a receptor for heparan sulfate, has been found at or near the membrane in filopodia and other cell surface protrusions (Lankes and Furthmayr, 1991; Furthmayr et al., 1992). Ezrin (cytovillin) has been seen in association with microvilli and cellular protrusions in many cell types (Pakkanen, 1988; Gould et al., 1989; Turunen et al., 1989; Hanzel et al., 1991; Birgbauer et al., 1991). Rapid redistribution of ezrin to regions of membrane remodeling, such as microvillar formation and membrane ruffling in response to growth factor stimulation, may be regulated by phosphorylation of the protein on both tyrosine and serine residues (Bretscher, 1989; Krieg and Hunter, 1992). Radixin was isolated from the cell–cell adherens junction, where it is proposed to cap actin filaments and provide for

their attachment to the cell membrane (Tsukita et al., 1989; Funayama et al., 1991). Interestingly, in mitotic cells, radixin is concentrated at the cleavage furrow (Sato et al., 1991).

Merlin possesses an N-terminal domain that is similar to protein 4.1 (28% identity) and to talin (21% identity), but is much more closely related to moesin, ezrin, and radixin (Figure 7). Amino acid identity between merlin and the three latter proteins is concentrated in the first 342 residues (~63% identity). Like these other family members, the merlin protein is predicted to have a very long α-helical domain spanning 160–170 amino acids, beginning around residue 300. The first third of this domain overlaps with the region of strongest homology to moesin, ezrin, and radixin. The remaining stretch, however, shows limited similarity with these proteins and with a wide variety of myosins and tropomyosins. The C-terminal region of merlin contains a hydrophilic domain analogous to those of the other family members. The similarity in structure of merlin to the other members of this family suggests that it too may normally act as a link between the cytoskeleton and the cell membrane and may thus represent a new class of tumor suppressor gene.

The cytoskeleton of mammalian cells is a complicated latticework of many different kinds of interconnected filaments (Luna and Hitt, 1992). It participates in a wide range of crucial cellular activities, including determining and altering shape, movement, cell division, cell–cell communication, cell anchorage, and organization of the intracellular milieu (Bernal and Stahel, 1985). A defect in a protein that connects some component of this network to the plasma membrane could affect any of these processes and have a consequent effect on growth control. Whether inactivation of the merlin protein disregulates growth by disrupting a signal transduction pathway, by altering anchorage dependence, by upsetting the cell cycle regulation, or by some other mechanism remains to be determined. However, the characteristic structure of the merlin protein suggests that a search for its membrane and cytoskeletal binding targets might provide a logical route to exploring this question.

Experimental Procedures

NF2 Cell Lines

Lymphoblast cell lines were established (Anderson and Gusella, 1984) from affected members of NF2 pedigrees and from their unaffected relatives. Diagnosis of NF2 conformed to the criteria set forward by the National Institutes of Health Consensus Development Conference on neurofibromatosis (Mulvihill et al., 1990), except for the patient whose meningioma displayed a 4 bp deletion. This patient had a right vestibular schwannoma and multiple meningiomas. Although she did not have a history of NF2, she probably represents a new mutation. Primary meningioma cells were cultured as described (Logan et al., 1990) and analyzed after less than five passages.

Somatic cell hybrids were prepared by fusing GUS5069 lymphoblasts with a Chinese hamster cell line deficient in hypoxanthine phosphoribosyltransferase activity (CHTG49; Athwall and McBride, 1977) using GIBCO PEG 4000. Fused cell lines were selected by their ability to grow in media containing hypoxanthine, aminopterin, and thymidine. Hybrids were screened for the chromosome 22 homologs using the polymorphic simple sequence repeat marker, *TOP1P2* (Trofatter et al., 1992). Control hybrids GM10888 and Eye3FA6 (NA10027) are described in the listing of the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository collection (Coriell Institute, Camden NJ).

DNA/RNA Blotting

DNA was prepared from cultured cells, and DNA blots were prepared and hybridized as described (Gusella et al., 1979; Gusella et al., 1983). For pulsed-field gel analysis, agarose DNA plug preparation and electrophoresis were carried out as described (Bucan et al., 1990). RNA was prepared, and Northern blotting was performed as described by Buckler et al. (1991).

Cosmid Walking

The *NEFH* probe used for blot analysis and to initiate cosmid walking was pJL215, representing a 4.4 kb KpnI–XbaI genomic fragment containing exon 4 and 3′ untranslated region (Lees et al., 1988). Cosmid walking was performed in an arrayed cosmid library prepared from DNA of flow-sorted human chromosome 22 (LL22NC03; Lawrence Livermore National Laboratory). Cosmid overlaps were identified either by hybridization of whole cosmid DNA or isolated fragments to filter replicas of the gridded arrays or by PCR screening of row and column DNA pools. STSs were developed by direct cosmid sequencing using the T3 or T7 end primers (McClatchey et al., 1992a).

cDNA Isolation and Characterization

Human frontal cortex and hippocampus cDNA libraries in λZAPII (Stratagene) were screened using exon probes isolated and prepared as described by Buckler et al. (1991). cDNA clones and trapped exon were sequenced as described (Sanger et al., 1977). Direct PCR sequencing was performed as described (McClatchey et al., 1992b). Screening for variations by SSCP analysis followed the procedure described by Ambrose et al. (1992). RNA was reverse transcribed using oligo(dT) primer (BRL reverse transcriptase) to prepare first strand cDNA. Portions of the cDNA were amplified using the following primer sets: 5′-CCAGCCAGCTCCCTATGGATG-3′ and 5′-AGCTGAAATGGAATATCTGAAG-3′ to amplify base pairs 824–2100 and 5′-GCCTTCTCCTCCCTGGCCTG-3′ and 5′-GATGGAGTTCAATTGCGAGATG-3′ to amplify base pairs 314–1207. These cold PCR products were then reamplified with specific regional primers for SSCP as described in Figure 6.

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GenBank Accession Number

The accession number for the sequence reported in this paper is L11353.

Note Added in Proof

The 1 bp deletion detected in the NF2 meningioma is also present in lymphoblasts from this patient, indicating that it is a germline deletion.