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CHARACTERIZATION OF THE NEUROFIBROMATOSIS TYPE 2 GENE

by

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A Thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

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McGill University, Montreal, Quebec

To those who lived with the ill-matched 22q ;
To those who live with the ill-matched 22q.
May hope find its way.

ABSTRACT

Neurofibromatosis type 2 (NF2) is a dominantly inherited genetic disorder predisposing to the development of nervous system tumors such as bilateral vestibular schwannomas, cranial and spinal meningiomas, nerve root schwannomas and ependymomas. The majority of NF2 patients develop juvenile lens opacities often presenting prior to the development of tumors usually in early teens. The *NF2* gene had been identified by positional cloning. It encodes a recessive tumor suppressor protein mutated in both sporadic and familial schwannomas and meningiomas. We have isolated the mouse homologue of the *NF2* gene (*Nf2*) from an 18-day old mouse brain cDNA library. *Nf2* encodes a 596 amino acid-protein, schwannomin (alternatively merlin), with 98% identity to the human *NF2* protein. By characterizing a dinucleotide repeat polymorphism within the 3' untranslated region of *Nf2*, we have mapped the mouse gene to the proximal end of chromosome 11 at a small region of conserved synteny to human chromosome 22. These results indicate that *Nf2* is highly conserved and suggest that analysis of the mouse *Nf2* gene might yield insights into the human gene.

Schwannomin shares homology with members of erythrocyte band 4.1 superfamily which are known to be localized in the membrane-cytoskeleton interface. The predicted secondary structure of schwannomin includes an amino-terminal domain which is highly conserved within the superfamily and is proposed to associate with plasma membrane proteins, and a carboxy-terminal domain of lower homology but hypothesized to associate with the cytoskeleton. By Northern and Western analyses, *Nf2* is expressed in all tissues studied. However, analysis by RNA *in situ* hybridization and immunohistochemistry revealed a widespread but cell-type specific pattern of expression. Furthermore, immunofluorescence technique showed cytoplasmic localization in neuronal and non-neuronal cells. When analysed in lens and Schwann cells, two cell types affected by the NF2 phenotype, schwannomin localized to dynamic structures such as the ruffling

membrane and leading edges. Fractionation of cellular proteins revealed the presence of schwannomin in the detergent-insoluble cytoskeletal fraction as an ~80 kDa protein, consistent with the hypothesis that it functions in association with cytoskeleton proteins. Moreover, schwannomin's expression pattern in the lens indicated that it plays a role in differentiation-specific events. With these observations together with information on related proteins, we propose a working model that defines the role of schwannomin as a membrane organizing protein in the leading edge.

RÉSUMÉ

La neurofibromatose de type 2 (NF2) est une maladie héréditaire dominante prédisposant au développement de tumeurs du système nerveux. Les schwannomes vestibulaires bilatéraux, les méningiomes craniaux et spinaux, les schwannomes des racines nerveuses et les épendymomes sont les tumeurs les plus fréquemment observées chez les personnes atteintes de la NF2. De plus, une majorité des patients présente des opacités du cristallin. Celles-ci apparaissent souvent avant les tumeurs, généralement dès l'adolescence. Le gène responsable de la NF2 a été identifié par clonage positionnel; il code pour une protéine suppresseur de tumeur que l'on retrouve mutée dans les schwannomes et méningiomes, autant familiaux que sporadiques. Nous avons isolé l'homologue murin du gène *NF2* (nommé *Nf2*) à partir d'une banque de cDNA du cerveau d'une souris âgée de 18 jours. Le gène *Nf2* code pour une protéine de 596 acides aminés, nommée schwannomine (ou merline), dont la séquence montre une identité de 98% avec la protéine humaine. La caractérisation d'un marqueur polymorphe dans la région 3' non-codante de *Nf2* a permis de localiser le gène dans une petite région de l'extrémité proximale du chromosome 11 de la souris, une région synthénique au chromosome 22 humain où se trouve le gène *NF2*. Ces résultats suggèrent que les gènes *NF2* et *Nf2* sont hautement conservés et que, par conséquent, l'analyse du gène de souris pourrait permettre d'éclaircir le rôle du gène humain.

La schwannomine présente des homologies de séquence avec plusieurs membres de la superfamille des protéines érythrocytaires Bande 4.1, protéines qui sont localisées à la jonction de la membrane et du cytosquelette. La structure secondaire de la schwannomine, prédite à partir de la séquence des nucléotides, comprend un domaine N-terminal hautement conservé dans l'ensemble de la superfamille et que l'on croit associé aux protéines de la membrane plasmique de même qu'un domaine C-terminal de moindre homologie qui serait associé au cytosquelette. Les analyses de type Northern et Western

montrent que *Nf2* est exprimé dans tous les tissus étudiés. Toutefois, l'immunohistochimie et l'hybridation *in situ* d'ARN, bien que confirmant cette large expression, la restreigne à des types particuliers de cellules. Enfin, la technique d'immunofluorescence montre une localisation cytoplasmique de la schwannomine dans les cellules neuronales et non neuronales. L'analyse des cellules du cristallin et des cellules de Schwann, deux types cellulaires affectés par la NF2, montre que la schwannomine y est localisé avec des structures dynamiques de la cellule comme les ondulations de la membrane et les cônes de croissance. Lors du fractionnement des protéines cellulaires, la schwannomine apparait dans la fraction des protéines insolubles du cytosquelette sous la forme d'une bande de 80 kDa, ce qui cadre bien avec l'hypothèse d'une protéine fonctionnant en association avec le cytosquelette. Pour sa part, le patron d'expression de la schwannomine dans le cristallin indique que cette protéine joue probablement un rôle dans certains événements liés à la différenciation cellulaire. Ces observations, combinées aux donnés sur les protéines de la superfamille Bande 4.1, nous amènent à proposer un modèle où la schwannomine participe à l'organisation membranaire dans les cônes de croissance.

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I value the diversity of Montreal inscribed by the heritage of its people. The richness of its culture was a major incentive in my coming to McGill. Its diversity extended through the people in our laboratory benches, in the the lunch room and in journal club tables.

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To my parents and my brother for their love and understanding and prayers. To them go my undying gratitude.

To HIM, the basis of my faith, the source of my strength, for making it happen.

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LIST OF ABBREVIATIONS

BCR	Breakpoint cluster region
BRCA1	Breast cancer 1
CHRPE	Congenital hypertrophy of the retinal pigment epithelium
DCC	Deleted in colon carcinoma
EGF	Epidermal growth factor
ERM	Ezrin, radixin, moesin
ERMAD	Ezrin, radixin, moesin association domain
EWS	Ewing sarcoma
FAP	Familial adenomatous polyposis
GAP	GTPase-activating protein
GFAP	Glial fibrillary acidic protein
GM-CSF	Granulocyte /macrophage colony stimulating factor
GRD	GAP-related domain
kb	kilobase
kDa	kilodalton
LIF	Leukemia inhibitory factor
LOH	Loss of heterozygosity
NCAM	Neural cell adhesion molecule
NEFH	Neurofilament heavy chain polypeptide
NF1	Neurofibromatosis type 1
NF1	The NF1 gene
NF2	Neurofibromatosis type 2
NF2	The NF2 gene
Nf2	The mouse homologue of the *NF2* gene
OMIM	Online Mendelian Inheritance of Man
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PTP	Protein tyrosine phosphatase
RFLP	Restriction fragment length polymorphism
SCH	Schwannomin
SH3	Src homology domain 3
SSCA	Single strand polymorphism analysis
VHL	Von Hippel-Lindau syndrome
WARG	Wilms' Tumor, aniridia, genitourinary abnormalities, mental retardation
WT	Wilms' Tumor
YAC	Yeast artificial chromosome

PREFACE

Manuscripts of papers which have been published or which have been submitted for publication were incorporated in the thesis. This format is in accordance with the "Guidelines for Thesis Preparation" of the Faculty of Graduate Studies and Research and has been approved by the Division of Experimental Medicine, Department of Medicine. The following paragraphs are quoted directly from the Guidelines:

"Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". The thesis must include: A Table of Contents, an abstract in English and French, an Introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in

the candidate's interest to make perfectly clear the responsibilities of the authors of the co-authored papers."

Each of the manuscripts included in this thesis appears as individual chapters. In Section 1 of Chapter 1, excerpts of short book articles on the Neurofibromatoses were incorporated. Manuscripts in Chapters 2, 3 and 4 have been published; Chapter 5 contains manuscript that has been submitted for publication. The General Discussion which appears in Chapter 6 relates to the combined work presented in Chapter 2, 3, 4 and 5, and manuscripts that appear in the Appendix section. Chapter 6 also contains excerpts of book articles written by the author.

The work described in this thesis was performed by the author under the supervision and guidance of Dr. Guy A. Rouleau at the Center for Research in Neuroscience, Montreal General Hospital. The author received technical assistance from Claude Marineau for the sequencing of the mouse *Nf2* gene; the data appear in Chapter 2. Danielle Malo, Assistant Professor of the Center for Host Resistance, Montreal General Hospital, provided helpful mapping strategies for the localization of the mouse homologue of the *NF2* gene; the results are presented in Chapter 3. Mohini Lutchman, previously a graduate student in the same laboratory where this research was conducted, initiated the development of the antibody against the *NF2* protein, which was used in experiments that appeared in Chapters 4 and 5. Robert Veneziale provided useful data on the expression of the *NF2* protein in chicken lens. His data appeared in Figure 2B of Chapter 5. The author also contributed to the identification and sequencing of germline mutations of NF2 patients. Data obtained from this collaborative effort with Dr. Martin Ruttledge, a postdoctoral fellow in the laboratory where this work was conducted, appeared in the Appendix. Kateri Brisebois provided technical assitance for the breeding and maintenance of ongoing transgenic mice experiment; preliminary data appear in the Appendix.

CHAPTER 1*

TYPES, SITES and FAMILY
(General Introduction)

*Section 1 of this chapter contains excerpts of book articles written on the current understanding of the neurofibromatoses.

Claudio, J.O., Belliveau, M. and Rouleau, G.A. Neurofibromatosis type 2. In: Meyers, R.A., ed. Encyclopedia of Molecular Biology and Molecular Medicine, VCH Verlagsgesellschaft, Weinheim *(in press)*.

Claudio, J. O. and Rouleau, G.A. Neurofibromatosis: Type 1 and Type 2. In: Jameson, J. L. ed. Textbook of Molecular Medicine, Blackwell Scientific Publications, Inc. *(in press)*.

Claudio, J. O. and Rouleau, G.A.. Neurofibromatosis Type 2: Some Answers, More Questions. In: Adelman, G. and Smith B.H., eds. Encyclopedia of Neuroscience, Elsevier Science *(in press)*.

SECTION 1
THE NEUROFIBROMATOSES

Neurofibromatosis is a Mendelian genetic disorder that primarily affects the nervous system. It is characterized by an inherent predisposition to develop tumors of the brain and spinal cord, and a variety of other neoplasms involving tissues derived from neural crest cells or from the neural ectoderm. It consists of two major types which are genetically and phenotypically distinct, neurofibromatosis type 1 (NF1) and neurofibromatosis type 2 (NF2).

The first medical description of NF1 can be traced back in the 16th century to an Italian physician and naturalist, Ullise Aldrovandi (Hecht, 1989; Mulvihill, 1988), but the eponymous credit is ascribed to a German pathologist, Friedrich Daniel von Recklinghausen, who first described the cellular component of the tumors seen in this disease in 1882 (Mulvihill, 1988). von Recklinghausen made no distinction between NF1 and NF2 despite the fact that J.H. Wishart reported 60 years earlier in 1822 the first documented case of NF2 (OMIM 101000; Worster-Drought et al., 1937). The genetic distinction between NF1 and NF2 became clear in 1987 when the genes responsible for these disorders were simultaneously localized to separate chromosomes (Barker et al., 1987, Rouleau et al., 1987). The mapping of the *NF1* locus to the proximal long arm of chromosome 17 and *NF2* to the long arm of chromosome 22 established a precise classification and set the pace for the positional cloning of the *NF1* and *NF2* genes.

1. Comparisons between NF1 and NF2

1.1 Distinguishing Clinical Features

Although NF1 and NF2 are distinct disorders, both show remarkable variable clinical expressivity. Both disorders usually present early; NF1 at about age 10 (Kanter et al., 1980; Eldridge, 1981) and NF2 in teens or early 20's (Kanter et al., 1980; Eldridge, 1981; Evans et al., 1992a; Evans et al., 1992b; Parry et al., 1994). The major defining features of NF1 include café au lait spots (Crowe, 1964), peripheral neurofibromas (Crowe et al., 1956; Knight et al., 1973), and Lisch nodules (Riccardi and Eichner, 1986; Zehavi et al., 1986; Ragge et al., 1993). Additionally, an NF1 patient may suffer from orthopedic problems such as scoliosis and pseudoarthrosis (Riccardi, 1981), may develop plexiform neurofibromas (Knight et al., 1973), or may be intellectually handicapped (Easton et al., 1993). A fraction of NF1 cases develop myeloid leukemia. Tumors such as rhabdomyosarcomas, optic gliomas, phaeochromocytomas, and neurofibrosarcomas may occur (D'Agostino et al., 1963; Sorensen et al., 1986). By contrast, the hallmark of NF2 is the occurrence of bilateral vestibular schwannomas (Eldridge, 1981; Nance et al., 1992); however, schwannomas may occur on any cranial nerve, nerve root, or peripheral nerve. Meningiomas occur in approximately 50% of NF2 patients and ependymomas occur rarely (Evans et al., 1992a; Evans et al., 1992b; Martuza and Eldridge, 1988). Optic gliomas do not occur in NF2 but juvenile subcapsular lenticular opacities are common (Kaiser-Kupfer et al., 1989), often presenting before tumors arise, and so they are useful as a predictive test for at risk individuals. For NF2, café au lait spots and neurofibromas are infrequent, but when present may cause misdiagnosis as NF1. The most useful distinguishing feature for differential diagnosis between the two disorders is the presence of axilliary freckling and Lisch nodules in NF1, and the lower number of café au lait spots in NF2. Furthermore, NF1 individuals never develop bilateral vestibular schwannomas.

Table 1. Clinical Synopsis of the Neurofibromatoses

	Neurofibromatosis Type 1		Neurofibromatosis Type 2
Skin	Neurofibroma Plexiform neuroma Café-au-lait spots Axillary freckling Inguinal freckling	**Skin**	Usually < 6 café-au-lait spots Often no peripheral neurofibroma Spherical subcutaneous tumors on pheripheral nerve
Eyes	Lisch nodules Glaucoma Hypertension	**Eyes**	Visual loss Juvenile posterior subcapsular or nuclear cataract No Lisch nodules Macular hamartoma Lagophthalmos Decreases lacrimal secretion Corneal hypesthesia
Spine	Scoliosis		
Skull	Sphenoid dysplasia		
Limbs	Pseudoarthrosis Thinning of long bone cortex Massive leg overgrowth	**Ears**	Hearing loss Tinnitus
Neuro	Mental retardation Aqueductal stenosis and hydrocephalus	**Neuro**	Bilateral vestibular schwannoma Meningioma Glioma Schwannoma of other nerves Generalized and isolated neuropathy
Oncology	Pheochromocytoma Meningioma Optic glioma Hypothalamic tumor Neurofibrosacrcoma Rhabdomyosarcoma Duodenal carcinoid Somatostatinoma Parathyroid adenoma		
Endocrine	Hypertension Hypophosphatemic osteomalacia		
Metabolic	Hypoglycemia		
Abdomen	Intraperitoneal mesodermal tumor		
Vascular	Arterial occlusive disease		
GU	Clitoral hypertrophy		
Lung	Papillary adenomas Interstitial pulmonary fibrosis Pulmonary hypertension		

Source: Online Mendelian Inheritance in Man, OMIM. Johns Hopkins University. Baltimore, MD MIM 162200 and 101000. WWW URL: http://www.3.ncbi.nlm.nih.gov/omim/.

The clinical synopses of NF1 and NF2 shown in Table 1 present the overall phenotypic characteristics of both disorders.

1.2 Diagnostic Criteria

It had often been thought that Joseph Merrick, a.k.a. Elephant Man, had NF1, until 1987 when Pyeritz (OMIM 162200; Cohen, 1988) suggested that he likely suffered from another disorder known as Proteus syndrome. On the other hand, NF2 had always been associated with a less familiar name, Michael Blair, who was JH Wishart's patient when he was the President of the Royal College of Surgeons of Edinburgh. Michael Blair was blind in the right eye since about 4 months after birth and was suffering from bilateral deafness by age 21. On his death, his autopsy revealed tumors of the dura matter, brain and both "auditory nerves." This classical description of NF2 is embodied by the proposed diagnostic criteria for NF2 (Table 2) set out in 1987 by the National Institutes of Health Consensus Conference on Neurofibromatosis which also recommended the numerical classification of the neurofibromatoses. This original set of criteria has been expanded for NF2 (Table 2) to accommodate diagnosis of individuals with no family history (Evans et al., 1992b).

1.3 Subtypes of NF2

There are two subtypes of NF2, often referred to by eponyms: the mild Gardner subtype and the severe Wishart subtype. The Gardner subtype is generally characterized by late onset (usually >25 years) of bilateral vestibular schwannomas and few associated brain or spinal tumors. The Wishart subtype is more severe with earlier onset (usually <25 years) and accompanied with rapid and progressive growth of bilateral vestibular schwannomas, multiple intracranial and spinal schwannomas, meningiomas, and ependymomas.

Table 2. Diagnostic Criteria for NF1 and NF2

I. Neurofibromatosis Type 1

NF1 may be diagnosed in an individual when two or more of the following are present:

1. Six or more café au lait macules with diameter of more than 5 mm in prepubescent patients and more than 15 mm in postpubescent patients.
2. One plexiform neurofibroma *or* two or more neurofibromas of any type.
3. Freckling in the axillary *or* inguinal region.
4. A distinctive osseous lesion as sphenoid dysplasia *or* thinning of long-bone cortex, with or without pseudoarthrosis.
5. Optic glioma.
6. Two or more Lisch nodules (iris hamartomas).
7. A parent, sibling *or* child with neurofibromatosis 1 on the basis of the above criteria.

II. Neurofibromatosis Type 2

NF2 may be diagnosed when one of the following is present:

1. Bilateral eighth cranial nerve masses seen by magnetic resonance imaging with gadolinium.
2. A parent, sibling, *or* child with NF2 plus:
   a. unilateral eighth cranial nerve mass *or*
   b. any one of the following:
      - neurofibroma
      - meningioma
      - glioma
      - schwannoma
      - posterior subcapsular cataract or opacity at a young age
3. Unilateral vestibular schwannoma plus one or more of the following:
      - neurofibroma
      - meningioma
      - glioma
      - schwannoma of any cranial or peripheral nerve
      - posterior subcapsular cataract or opacity at a young age
4. Multiple meningiomas (two or more) plus unilateral vestibular schwannoma
5. Multiple meningiomas (two or more) plus:
      - neurofibroma
      - glioma
      - posterior subcapsular cataract or opacity at a young age

Source: NIH Development Conference (1987) and Evans, et al. 1992b.

2. Molecular Genetics of the Neurofibromatoses

The predisposition to develop NF1 or NF2 is inherited as an autosomal dominant trait but the diseases manifest as recessive traits at the cellular level. This genetic paradox is analogous to the two-hit mechanism of tumorigenesis seen in retinoblastoma (Knudson, 1971) where an individual inherits a mutant copy of the gene in an autosomal dominant pattern but develops tumors only when an inactivating somatic mutation occurs in the otherwise normal homologue. This results in the recessive expression of the disease phenotype in affected cells leading to tumor formation. Thus, the *NF1* and *NF2* genes are considered as recessive tumor suppressors.

NF1 is a more common disease than NF2 with a birth incidence of 1:3,000 (Crowe et al., 1956; Riccardi, 1981) compared to 1: 37,500 for NF2 (Evans et al., 1992b)(Table 3). However, NF2 is a more serious disease with a mean actuarial survival of 15 years after diagnosis (Evans et al., 1992b), whereas NF1 does not affect life span. Both types share certain genetic features, such as high penetrance and high frequency of new mutations. For example, as many as 50% of cases are reported to have no family history and so represent new mutations (Evans et al., 1992b). The penetrance for both diseases is close to 100% .

2.1 The *NF1* and *NF2* Genes

For diseases with no known biochemical abnormality, positional cloning now offers a way to isolate the primary gene defect. The *NF1* and *NF2* genes were identified using this approach. Thus, the search for the gene responsible for each disorder began by mapping the neurofibromatosis loci to specific chromosomes, narrowing down the region, and identifying genes within the defined critical interval. For NF1, two translocation breakpoints within the critical region helped identify the gene, whereas big

Table 3. Comparisons Between NF1 and NF2

Characteristic	NF1	NF2
Other names	von Recklinghausen's disease Peripheral neurofibromatosis	Bilateral acoustic neurofibromatosis Central neurofibromatosis
Clinical Presentation	Variable	More Uniform
Natural History	Unpredictable	More Predictable
Chromosomal location	17q11.2	22q12.1
Inheritance	Autosomal dominant	Autosomal dominant
Expression	Recessive at cellular level in tumors	Recessive at cellular level in tumors
Age of onset	First decade	Second decade
Birth incidence	1:3,000	1:37,500
Penetrance	Close to 100%	Close to 100%
Mutation rate	$3.0\text{-}5.0 \times 10^{-5}$	$1.0\text{-}8.0 \times 10^{-6}$

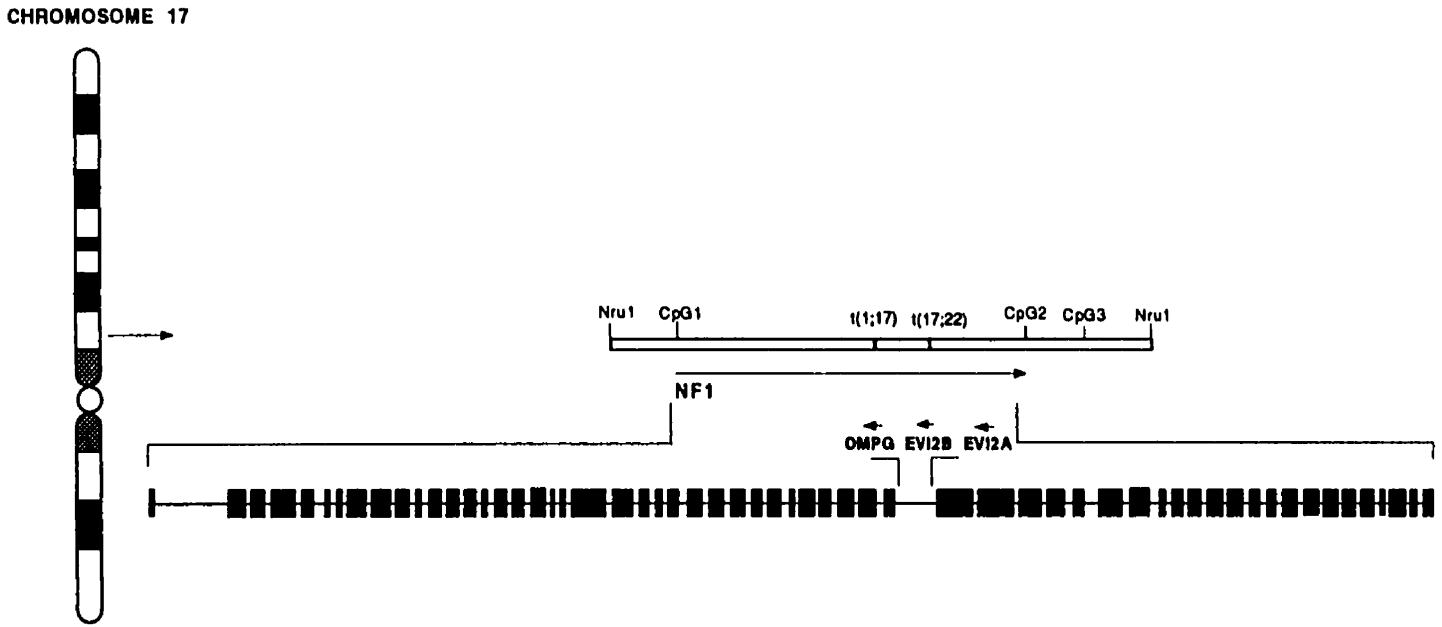
chromosome deletions which segregated with the disease in families provided clues for the positional cloning of the *NF2* gene.

2.1.1 Identification of the *NF1* Gene and Its Protein Neurofibromin

In 1987, two groups reported the localization of the *NF1* locus to the long arm of chromosome 17 (Barker et al., 1987; Seizinger et al., 1987). Using anonymous DNA sequences and cloned genes that detect restriction fragment length polymorphisms (RFLPs), both groups independently identified genetic markers near the centromere that are linked to the *NF1* locus. Fine genetic mapping by an international collaboration established a more precise localization of the *NF1* gene (Goldgar et al., 1989)(Fig. 1). Subsequently, two different chromosomal translocations in two NF1 patients, t(1;17) and t(17;22), were mapped to the *NF1* region (Fountain et al., 1989; Ledbetter et al., 1989; Menon et al., 1989; O'Connell et al., 1989; Schmidt et al., 1987). These chromosomal abnormalities served as key reagents for the identification of the *NF1* gene and provided evidence that NF1 is caused by loss of function mutations. To search for genes within the translocation breakpoint, screening was focused on genes lying within this region. The mouse leukemia gene, *Evi-2*, maps to the distal region of mouse chromosome 11 which is a region of conserved synteny to the *NF1* region on human chromosome 17 (Buchberg et al., 1988; White et al., 1991). Studies on the human homologue of *Evi-2* (EVI2A) indicated that it mapped in between the two translocation breakpoints which are 50 kilobases (kb) apart (O'Connell et al., 1990). Subsequently, two other genes *EVI2B* and oligodendrocyte-myelin glycoprotein (*OMGP*) were discovered within the 50 kb interval (Cawthon et al., 1991; Mikol et al., 1991; Viskochil et al., 1991). All three genes were considered as good candidates for the disease; *Evi-2* is a known proto-oncogene involved in murine myeloid tumors whereas *OMPG* is a cell adhesion molecule in myelin of the nerve sheath

Figure 1

The *NF1* locus in the pericentromeric region of chromosome 17. Fine genetic mapping by pulse field gel electrophoresis identified a 600 kb Nru1 fragment within which two translocation breakpoints from two NF1 patients were identified (4,5). The translocation breakpoints, t(1;17) and t(17;22), were mapped 50 kb apart. Three CpG islands, known marker of the 5' region of a gene, are shown within the NruI fragment. The organization of the 59 exons of the *NF1* gene, shown as black bars, and the three genes originally considered as good candidates, *EVI2A*, *EVI2B* and *OMPG*, embedded in an intron of the *NF1* gene, are indicated. The three genes are transcribed in centromeric direction opposite to the direction of transcription of the *NF1* gene.


CHROMOSOME 17
Nru1
CpG1
t(1;17)
t(17;22)
CpG2
CpG3
Nru1
NF1
OMPG
EVI2B
EVI2A

and expressed only in Schwann cells and oligodendrocytes. However, further analyses indicated that none of the three genes was disrupted by either translocation nor was there any sequence alteration within these genes in NF1 patients. Nevertheless, this strategy allowed the cloning of genomic DNA in the region. By searching for phylogenetically conserved DNA fragments and using these to screen cDNA libraries, a gene was identified which spanned the translocation breakpoint (Viskochil et al., 1990). On further analyses, deletions and point mutations within the cDNAs confirmed the identity of the *NF1* gene (Cawthon et al., 1990). Concomitantly, another group reported the insertion of a 500 base pair (bp) sequence in an NF1 patient which, in subsequent studies, was shown to be an *AluI* repeat that altered the splicing pattern of the *NF1* gene thereby causing exon skipping during RNA processing (Wallace et al., 1991; Wallace et al., 1990).

The *NF1* gene spans at least 350 kb and consists of 59 exons that are processed to yield a transcript of 11-13 kb (Li et al., 1995). Transcription proceeds in telomeric direction opposite to the direction of transcription of the three genes *EVIA2*, *EVI2B*, and *OMGP* that are embedded within an intron of the *NF1* gene (Fig. 1) (White et al., 1991). The *NF1* gene encodes a protein of 2818 amino acids with an estimated molecular weight of 250-320 kDa (DeClue et al., 1991; Gutmann et al., 1991). The *NF1* protein, called neurofibromin, has a central 381-amino acid domain with sequence similarity to p21-*ras* GTPase-activating proteins (GAP) and to IRA1 and IRA2, known yeast inhibitory regulators of the RAS signal transduction pathway (Ballester et al., 1990; Buchberg et al. 1990; Xu et al., 1990b). This domain, the NF1-GAP-related domain (NF1-GRD), may be alternatively spliced generating an additional 21-amino acid residue that retains GAP activity (Takahashi et al., 1994).

2.1.2 Mutation Analysis of the *NF1* Gene

Deletions, insertions, base substitutions, and splice site mutations in the *NF1* gene have been described in a number of NF1 cases (Kayes et al., 1994; Purandare et al., 1994). No hot spot for mutations has yet been identified; however the majority of mutations result in a truncated and presumably non-functional protein. Mutations in the *NF1* gene have also been reported in a variety of malignancies and tumor syndromes from non-NF1 patients including melanomas and neuroblastomas, colon adenocarcinomas, myelodysplastic syndrome and anaplastic astrocytomas (Andersen et al., 1993; Li et al., 1992; The et al., 1993), and in tumors from NF1 patients such as adrenal gland tumor (Gutmann et al., 1994) and malignant myeloid tumors (Shannon et al., 1994). NF1 patients with mosaicism for an *NF1* mutation have been reported and may explain some cases of "new" mutation in a sibship (Lazaro et al., 1994). However, the proportion of somatic and germ cells carrying the *NF1* mutation has not been determined.

2.1.3 Identification of the *NF2* Gene and Its Protein Schwannomin

The identification of the *NF2* gene followed conventional positional cloning techniques. It started with tumor analyses leading to clues for the mapping of the *NF2* gene on chromosome 22. From linkage to the identification of the gene, the following sections describe the strategies initiated to track the gene involved in Neurofibromatosis type 2.

2.1.4 Linkage of *NF2* to Chromosome 22

Cytogenetic and molecular genetic studies in sporadic and NF2-associated meningiomas and schwannomas originally suggested a common pathogenic mechanism for these tumors by the loss of heterozygosity for distinct chromosome 22 loci (Al Saadi et al., 1987; Casalone et al., 1987; Seizinger et al., 1987; Seizinger et al., 1986; Zang, 1982; Zang and Singer, 1967) . Using this information as the starting point for genetic linkage analysis in NF2 pedigrees, Rouleau et al. (1987) identified an anonymous restriction

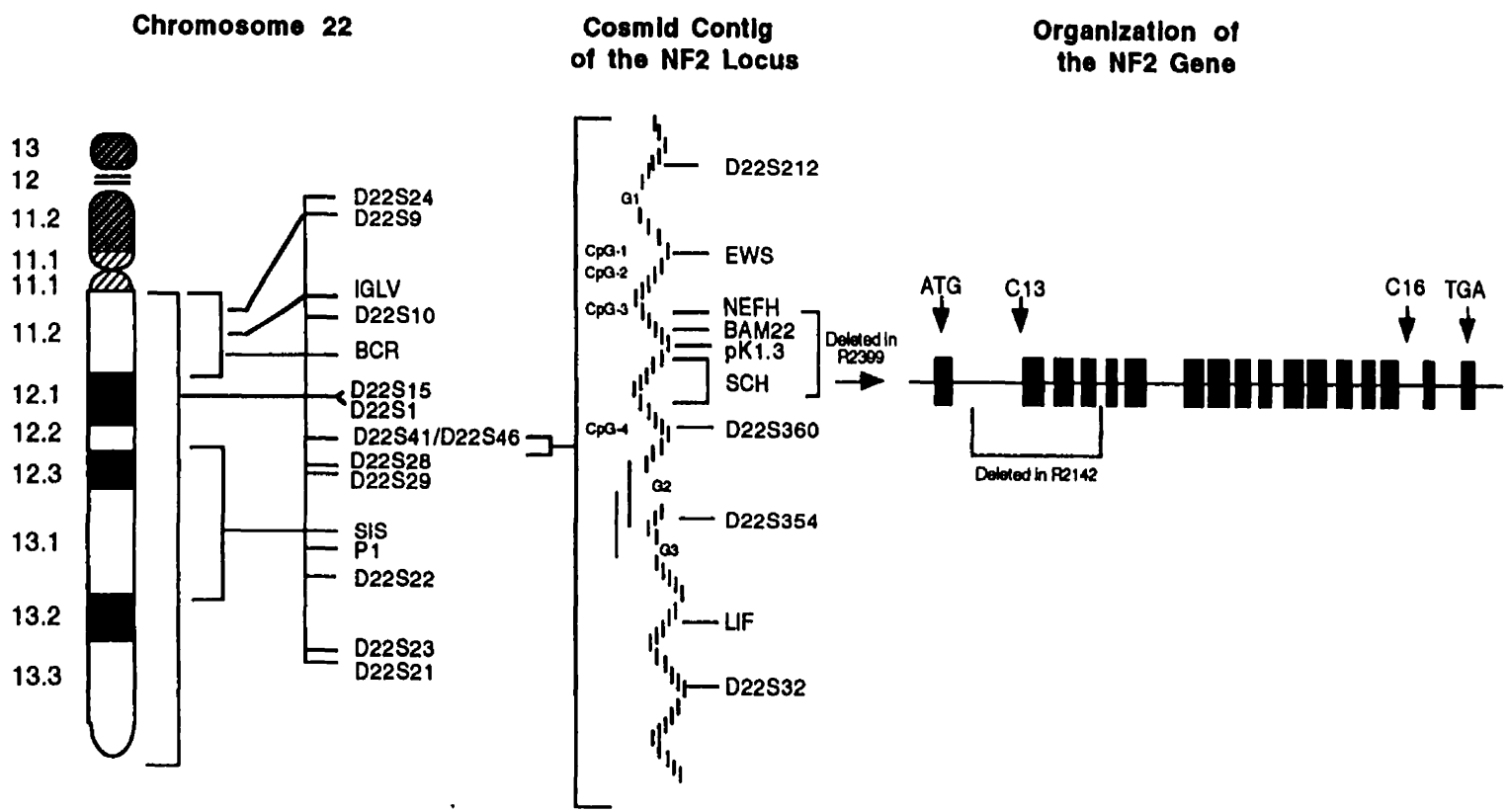
fragment length polymorphic (RFLP) marker, D22S1, which detected the genetic locus on chromosome 22 that was linked to the *NF2* gene (Fig. 2). Significantly, this region was observed to be frequently reduced to hemizygosity in NF2-related tumors (Couturier et al., 1990; Fontaine et al., 1991; Wolff et al., 1992), further providing evidence for the involvement of this locus in the development of the disorder. A candidate gene approach looking for the possible involvement of two closely linked genes in the region soon became a strategy to find the *NF2* gene. Using a large kindred and molecular analysis of NF2 tumors with either interstitial or terminal deletions of chromosome 22 to assess the involvement of nearby genes that include the "breakpoint cluster region" (*BCR*) implicated in the t(9;22) Philadelphia chromosome characteristically seen in chronic myelogenous leukemia (Groffen et al., 1984), and the PDGFß, the homologue of the *sis* oncogene that encodes the platelet derived growth factor (Bolger et al., 1985), both genes were excluded as likely candidates responsible for the disorder (Rouleau et al., 1989; Rouleau et al., 1990).

2.1.5 Positional Cloning of *NF2*

In order to identify the limits of the *NF2* locus, more polymorphic genetic markers were generated for chromosome 22 (Fig. 2) and a genetic map was constructed (Rouleau et al., 1989). Using this map as a tool to analyze NF2 tumors, D22S1 was confirmed as the locus flanking the centromeric limit for the *NF2* gene (Rouleau, 1994; Rouleau et al., 1990). In addition, with the aid of the genetic map, pedigree analysis by multipoint linkage became significantly more informative in delineating the limits of the *NF2* region at its telomeric end. The marker D22S28 was identified as a telomeric flanking marker, thereby establishing the *NF2* locus between D22S1 and D22S28 (Rouleau, 1994). This interval was estimated to be approximately 6 megabases within band q12.1. Subsequently, analysis of other markers identified tumors with deletions occurring between D22S41/D22S46 and D22S56 (Wolff et al., 1992), which map to the D22S1-D22S28

Figure 2

Position of markers on human chromosome 22 used to clone the *NF2* gene. The initial evidence of linkage of the *NF2* gene to chromosome 22 was provided by the marker D22S1. Further characterization of NF2 families by multipoint and haplotype analyses established a critical interval flanked by markers D22S1 and D22S28. This region of about 6 megabases on q12.1 was further narrowed down to a 1 megabase region flanked with D22S212 and D22S32 by segregation analysis and characterization of large germline deletions in affected families. Chromosome walking established a cosmid contig that spans the 1 megabase region and is shown as overlapping vertical lines. The relative position of markers and known genes, including *NF2* (shown as SCH) is indicated. G1, G2 and G3 are gaps within the contig; the G2 and G3 are each spanned by yeast artificial chromosome (YAC). The location of 4 CpG islands are shown in between the markers D22S212 and D22S360. The two patients who showed abnormal fragments as detected by hybridization using C13 and C16 probes are indicated as R2399 and R2142 the extent of the deletion in each patient is shown. The gene mutated in Ewing Sarcoma (*EWS*), the neurofilament heavy chain polypeptide gene (*NEFH*) and SCH are transcribed in telomeric direction; pK1.3 and *BAM22*, two genes with no known definite functions, are transcribed in centromeric direction. The gene encoding for leukemia inhibitory factor (*LIF*) is also shown within the contig. *IGLV*, immunoglobulin light chain variable region gene; *BCR*, breakpoint cluster region gene; *SIS*, represents the platelet-derived growth factor β-chain (formerly known as *sis* oncogene), P1, polymorphic blood marker. The NF2 gene is shown with its 17 coding exons in black bars (Modified from Claudio and Rouleau, 1996c).


Chromosome 22
Cosmid Contig of the NF2 Locus
Organization of the NF2 Gene
13
12
11.2
11.1
11.1
11.2
12.1
12.2
12.3
13.1
13.2
13.3
D22S24
D22S9
IGLV
D22S10
BCR
D22S15
D22S1
D22S41/D22S46
D22S28
D22S29
SIS
P1
D22S22
D22S23
D22S21
D22S212
G1
CpG-1
CpG-2
EWS
CpG-3
NEFH
BAM22
pK1.3
SCH
Deleted in R2399
CpG-4
D22S360
G2
D22S354
G3
LIF
D22S32
ATG
C13
C16
TGA
Deleted in R2142

interval by somatic cell hybrid lines (Fig. 2). This finding narrowed down the *NF2* locus to the interval between D22S41/D22S46 and D22S28 (Wolff et al., 1992; for review see Rouleau, 1994). Segregation studies in affected families and characterization of large germline deletions in NF2 pedigrees further refined the localization of the *NF2* locus to the interval flanked by D22S212 and D22S32 (Rouleau et al., 1993). Contiguous cosmid clones spanning this region established the distance between these two flanking markers to be about one million base pairs (Rouleau et al., 1993).

As part of the cloning strategy, unrelated NF2 individuals were screened for chromosomal rearrangement within this one megabase (Mb) interval using pulse field gel electrophoresis and single copy DNA probes. Yeast artificial chromosome (YAC) and cosmid contiguous sequences, known as contigs, spanning the candidate region were constructed to provide the path for the chromosome walk to identify candidate genes (Fig. 2). This process uncovered two major probes for the identification of the gene. One probe, C16, detected an abnormal fragment in two individuals with NF2 while another probe, C13, located 40 kb centromeric to C16, was deleted in both patients (Fig. 2). Further characterization of the C13 probe showed that it is phylogenetically conserved, suggesting that a gene may be encoded within this DNA fragment. Using C13 to screen a fetal human cDNA library, a 2 kb cDNA clone (N1.1) was isolated that mapped backed by fluorescence *in situ* hybridization to the *NF2* region (Rouleau et al., 1993). When the gene isolated was screened for mutations in NF2 individuals, sequence alterations were found that segregated with the disease. Independently, by chromosome walking and a combination of exon trapping and cDNA library screening, another group identified a candidate cDNA with a sequence identical to N1.1 (Trofatter et al., 1993). Together, these data provided incontrovertible evidence that the cloned cDNA encodes for the protein mutated in NF2.

In order to understand the *NF2* gene better, we studied its mouse homologue. The cloning of the mouse *Nf2* gene provided us with a tool to identify

conserved regions that may potentially provide insights into its function (Claudio et al., 1994a). Additionally, a polymorphism identified within the mouse gene was useful in the mapping of the *Nf2* gene to chromosome 11 (Claudio et al., 1994b). The results of these experiments showed that the mouse homologue of the *NF2* gene is highly conserved. The data are presented in detail in Chapters 2 and 3.

The human *NF2* gene spans about 100 kb of genomic sequence and consists of at least 17 exons that encode a 595 amino acid protein called schwannomin (Rouleau et al., 1993)(alternatively merlin, Trofatter et al., 1993). In human tissue northern blots, transcripts of different sizes, 7.0, 4.4, and 2.6 kb are expressed ubiquitously (Bianchi et al., 1994; Trofatter et al., 1993). It is uncertain whether these transcripts are generated by an alternative polyadenylation signal or by alternative splicing of the *NF2* gene. Indeed, there are reports of different splicing patterns of the *NF2* gene (Arakawa et al., 1994; Bianchi et al., 1994; Pykett et al., 1994) but the biological significance of the mRNA variants has not yet been understood. In mice, only a 4.5 kb mRNA is detected by Northern blot analysis (Claudio et al., 1994a, Haase et al., 1994). Although there is evidence that alternative splicing also occurs in mice (Appendix C, Huynh et al., 1994, Haase et al., 1994), no protein variant has yet been reported. These were explored in studies on the expression of the mouse *Nf2* gene which are presented in detail in Chapter 4.

Schwannomin belongs to a gene superfamily involved in linking plasma membrane proteins to the cytoskeleton. This superfamily includes erythrocyte band 4.1 (Conboy et al., 1986), talin (Rees et al., 1990), a group of proteins known as the ezrin (Gould et al., 1989; Turunen et al., 1989), radixin (Funayama et al., 1991), and moesin (Lankes and Furthmayr, 1991) (ERM) family (Sato et al., 1992) and a rapidly expanding family of protein tyrosine phosphatases (Yang and Tonks, 1991; Gu et al., 1991; Moller et al., 1994; Higashitsuji et al., 1994; Sawada et al., 1994; for review see (Algrain et al., 1993).

2.1.6 The *NF2* gene is a recessive tumor suppressor.

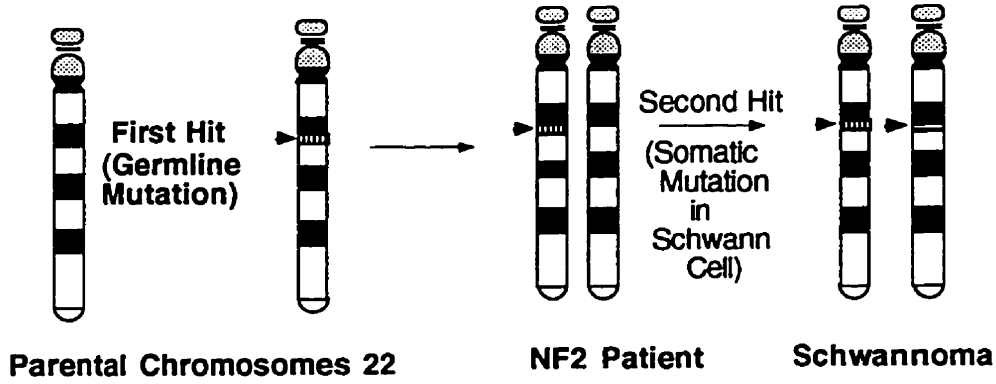
The genetic paradox exemplified by Knudson's hypothesis (Knudson, 1971) for retinoblastoma holds true for NF2. The predisposition to develop NF2 tumors is inherited as autosomal dominant trait in families with the disease, but the characteristic neoplasms occur as a recessive phenotype at the cellular level. For example, when an autosomal dominant mutation in one allele is passed on by a parent to an offspring, tumors develop when a somatic mutation in a cell completely inactivates the tumor suppressor function of *NF2* by knocking out the second normal allele (Fig. 3). The mutated cell, typically a Schwann cell, proliferates by clonal expansion resulting in tumor formation. This hypothesis is supported by data on parallel mutation analyses of blood DNA and schwannomas, (Jacoby et al., 1994; Twist et al., 1994) or meningiomas from NF2 patients (Ruttledge et al., 1994).

2.1.7 Genotype-Phenotype Correlation

With the accumulation of several disease causing mutations, the site of mutations could now be correlated with the disease severity. These mutations could also provide clues on functionally important regions of the *NF2* protein. One large study of this type is presented in the Appendix of which we concluded that the type of mutation in an NF2 family frequently determines the severity of the disease (Ruttledge et al., 1996). A section dealing with the sites of mutations and the potential consequences of the putative functional domains of schwannomin is presented in the General Discussion which appears in Chapter 6.

Figure 3

Illustration of the two-hit mechanism of tumorigenesis in NF2. One allele of the *NF2* gene is passed on to an offspring as a non-functional copy. This event confers to child the predisposition to develop the disease. As another mutation occurs in the otherwise normal homologue in somatic cells (e.g. Schwann cells), the *NF2* gene becomes completely inactivated. Consequently, the tumor suppressor function of *NF2* is lost, the cell proliferates by clonal expansion and eventually forms a tumor.


First Hit
(Germline
Mutation)
Second Hit
(Somatic
Mutation
in
Schwann
Cell)
Parental Chromosomes 22
NF2 Patient
Schwannoma

3. Characterization of the neurofibromatoses gene products

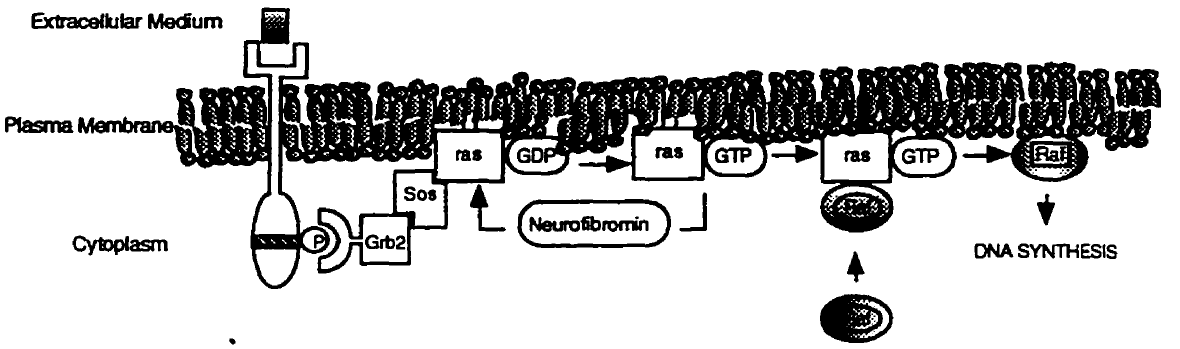
3.1 Neurofibromin

A clue to the function of neurofibromin came from the sequence homology of its central domain to mammalian GAP and to yeast IRA1 and IRA2 (Ballester et al., 1990; Buchberg et al., 1990; Xu et al., 1990b). These proteins down-regulate p21-*ras* by accelerating the hydrolysis of p21-*ras*-GTP to inactive p21-*ras*-GDP (for review see McCormick, 1995; McCormick et al., 1991). Thus, a mutant neurofibromin is thought to be unable to control the continuous mitogenic signal brought about by the unrestricted activity of p21-*ras*. The first evidence supporting this paradigm known as the "upstream model" (Fig. 4A) came from experiments in yeast transfected with the NF1-GRD. Expression of the NF1-GRD in yeast promoted the hydrolysis of GTP by p21-*ras* and was able to complement the heat-shock sensitive phenotype of *ira1* and *ira2* yeast mutants (Xu et al., 1990a). In addition, the GAP-related domain of the neurofibromatosis type 1 gene product had been shown to interact with p21-ras suggesting that it acts as its negative regulator (Martin et al., 1990). However, there are observations that contradict this simple model. For example, active p21-*ras* does not accumulate in mutant cells whose neurofibromin has been lost by mutation, such as neuroblastoma (The et al., 1993) and melanoma cell lines (Johnson et al., 1993); activated p21-*ras* can cause cell cycle arrest in rat Schwann cells (Ridley et al., 1988), and induce differentiation and block proliferation in rat phaeochromocytoma cell line PC12 (Bar-Sagi and Feramisco, 1985); and third, other members of the *ras* superfamily like R-*ras* p23 protein which is regulated by neurofibromin and interacts with Bcl-2, a suppressor of programmed cell death (apoptosis), are expected to be affected by the loss of neurofibromin, thereby altering the apoptotic function of Bcl-2 (McCormick, 1995). The "upstream model" is simple and does not provide explanation to observations described above. Thus, an alternate model known as the "downstream model" postulates that neurofibromin receives a signal from activated p21-*ras* to promote differentiation (Fig. 4B). Therefore, a mutant neurofibromin would be

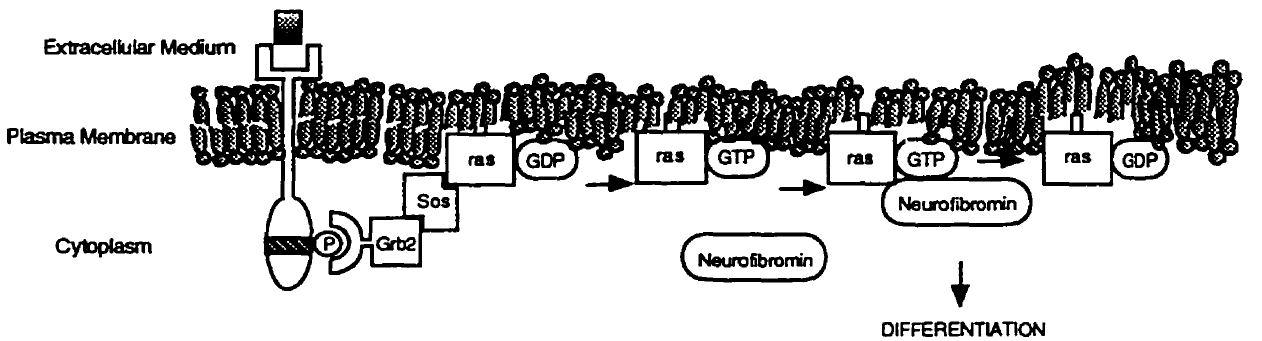
Figure 4

Models of neurofibromin function. **(A)** Upstream model depicting neurofibromin as downregulator of p21-*ras* activity. A growth factor interacting with its receptor initiates the signaling process through p21-*ras* protein. Binding of the growth factor results in phosphorylation of the receptor which, in turn, results in binding of Grb2/Sos to the receptor. This process activates p21-*ras* by promoting exchange of bound GDP for GTP. The level of accumulation of active p21-*ras*-GTP depends on the degree of activation by Sos and the degree of inactivation by neurofibromin which promotes the hydrolysis of GTP. The activation of p21-*ras* leads to recruitment and activation of cytoplasmic Raf thereby initiating a kinase cascade by a pathway that is yet not well defined. This process results in DNA synthesis leading to cell division. **(B)** Downstream model of neurofibromin as an effector of differentiation, either alone or in complex with p21-*ras*. The binding of a growth factor to its receptor promotes phosphorylation of the receptor which, in turn, results in binding of Grb2/Sos to the receptor. The amino-terminal domain of Sos then activates p21-*ras* by exchange of GDP to GTP. The activation of p21-*ras* allows it to interact with neurofibromin; such interaction allows neurofibromin (or in complex with p21-*ras*) to act as an effector of the *ras*-mediated differentiation signal. Loss of function of neurofibromin would cause inhibition of the differentiation signal, and in effect promote cell division.

A. UPSTREAM MODEL



B. DOWNSTREAM MODEL



unable to block cell proliferation because it can no longer effect cell differentiation from a p21-*ras* signal. Support for this model however is not as strong as the former. For example, more recent data had provided additional evidence supporting the model that neurofibromin functions as a negative regulator of p21-*ras* in experiments on primary cell cultures of Schwann cells, and neural crest- and placode-derived sensory neurons from *Nf1* (-/-) embryos (Vogel et al., 1995). Whereas *Nf1* (-/-) neurons extend neurites and survive without neurotrophins *in vitro*, their wild type counterparts die rapidly in the absence of NGF and BDNF. Schwann cells from null mutant embryos develop elaborate processes and, like the neurons from these mutant mice, have elevated levels of *ras*-GTP (Kim et al., 1995). *Nf1* (-/-) neurons survive and acquire mature morphological characteristics in the absence of neurotrophins, just as if Ras signaling pathway were activated constitutively. Similarly, studies on hematopoietic cells from *Nf1* (-/-) mice showed that loss of the *Nf1* gene leads to an increased and prolonged rise in Ras-GTP levels in myeloid cells after granulocyte/macrophage colony stimulating factor (GM-CSF) stimulation, defining a role for neurofibromin in regulating GM-CSF signaling through Ras in hematopoietic cells (Bollag et al., 1996; Largaespada et al., 1996).

Studies on the subcellular distribution of neurofibromin showed that it can associate with cytoplasmic microtubules (Gregory et al., 1993). It is postulated that the interaction of neurofibromin to tubulin reduces its ability to down-regulate p21-*ras*. This observation suggests that neurofibromin may suppress tumor formation in normal cells by an even more complex mechanism.

3.2 Schwannomin

The homology of schwannomin to a group of proteins known to function in maintaining cellular integrity provides evidence of a novel site of action of tumorigenesis. Although there is no current working model for the molecular basis of schwannomin in suppressing tumors of the nervous system, knowledge of its function has been based

primarily on studies of members of the band 4.1 superfamily . For example, schwannomin is postulated to normally act like erythrocyte band 4.1 which links transmembrane glycoproteins to the spectrin-actin complex of the cytoskeleton (for review see Luna and Hitt, 1992); or like talin, which interacts with vinculin and integrins, thereby regulating organization of cell shape. More closely related to schwannomin are ezrin, radixin and moesin which act as structural linkers between cell surface glycoprotein CD44 and actin-based cytoskeleton.

SECTION 2
GENES INVOLVED IN TUMORIGENESIS

There are two classes of genes involved in tumorigenesis: oncogenes and tumor suppressor genes. Oncogenes were first identified as retroviral-encoded genes that produced tumors in rodents and birds which are known to be dominant mutated forms of host genes (proto-oncogenes)(Bishop, 1991; Cantley et al., 1991). Tumor suppressor genes are normal cellular genes whose loss result in deregulated cell proliferation. Thus, oncogenes are often described as dominantly acting oncogenes, where as tumor suppressor genes are generally referred to as recessive oncogenes or sometimes, anti-oncogenes.

1. Oncogenes

There are about a hundred oncogenes described so far (Lewin, 1991); the function of the corresponding proto-oncogenes from which they are derived are classified into 4 classes: growth factors (e.g. v-*sis* or platelet derived growth factor ß chain, PDGFß), growth factor receptors (e.g., v-*erbB*, v-*fms*, v-*kit*), transducers of growth

factor responses (v-*src*, v-*ras*, v-*raf*) and transcription factors that mediate growth factor -induced expression (e.g., v-*jun*, v-*fos*) (Cantley et al.,, 1991; Lewin, 1991). Often, transcription factor type oncogenes cooperate with other classes of oncogenes to accomplish transformation (Hunter, 1991).

Several cellular oncogenes were identified by gene transfer experiments based on the ability of tumor DNA to induce transformation. Some of these oncogenes are homologues of retroviral oncogenes (e.g., *ras* genes). The biologically active cellular oncogenes are mutated forms of normal proto-oncogenes that differ in the regulation of their expression or in the structure and function of their gene products (Cooper, 1990). The deregulation of normal cellular oncogenes by point mutations or DNA rearrangements in human tumors implicates cellular oncogene activation in the pathogenesis of human neoplasms.

2. Tumor suppressor genes

Early evidence suggesting that some neoplastic transformation is a result of loss of function of normal alleles came from somatic cell fusion experiments where hybrid cell lines did not give rise to tumors when injected into suitable hosts (for review see Harris, 1988). The phenomenon of suppression was interpreted as a complementation event of a recessive mutation by the normal alleles from the normal nontumor cell (Stanbridge, 1976; Stanbridge and Ceredig, 1981). These type of genes were often referred to as recessive oncogenes. No mutant recessive oncogene complemented by a normal copy from a nontumor cell has been identified primarily due to the difficulty in determining the chromosomal assignment of the lost alleles. In 1971, Knudson provided a two hit-mechanism of tumorigenesis based on the autosomal dominant form of inheritance of familial cancers and a putative loss-of-function mutations at the cellular level by a recessive mutation. This paradigm, originally established as a working framework for retinoblastoma, has indeed been proven to be true for a number of recessive oncogenes,

now called tumor suppressor genes. The word tumor suppressor genes is more appropriate term, and in fact is the common usage, because there are recent data that argue a dominant negative and dosage effect mechanisms for some of these genes.

The method of pinpointing the location of a tumor suppressor gene in a familial cancer syndrome is technically easier compared to the hybrid system described above due to the common chromosomal alterations such as large deletions seen in tumor cells or the constitutional tissue of an affected individual. Restriction fragment length polymorphisms (RFLP) provide a useful analytical tool in detection of loss of heterozygosity (LOH) brought about by large deletions, aneuploidy, loss/duplication and recombination in tumor suppressor loci. Alternatively, polymerase chain reaction (PCR) provides another useful strategy to identify small deletions.

Except for p53, tumor suppressor genes have mostly been identified by positional cloning, which started from the localization of the tumor suppressor gene to a specific chromosomal segment to the identification of the mutant gene by a combination of chromosome walking, identification of single copy probes within the deleted region or translocation breakpoint, exon trapping/tracking, cDNA library screening and single strand conformation analysis. To date, there are at least 10 tumor suppressor genes cloned by positional cloning strategy. From these positionally cloned human tumor suppressors, insights into the site of action and biochemical functions of this class of genes have been very informative in understanding the molecular mechanism of tumorigenesis.

2.1 *NF1* and *NF2*

NF1 and *NF2*, as described above, are tumor suppressor genes which when inactivated are associated with nervous system neoplasms. Both genes encode cytoplasmic proteins whose exact functional mechanisms have not been fully understood. The *NF1* protein, neurofibromin, is a signaling molecule that is believed to act as downregulator of p21-*ras*, whereas the *NF2* protein, schwannomin, is thought to act as a

membrane organizing protein that may have potential signaling function as well. The mechanism of action of neurofibromin has been described above, and details of a working model for the action of schwannomin action are presented in the Discussion section.

2.2 Retinoblastoma

Retinoblastoma is an early childhood neoplasm of retinal origin. It usually develops bilaterally in children by age 5, with the peak of mortality occurring at ages 2 to 3 (Jensen and Miller, 1971). The risk of osteogenic sarcoma in patients with bilateral retinoblastoma is increased 500 fold particularly in areas exposed to radiation treatment (Abramson et al., 1976) but it also occurs as an accompanying clinical phenotype in retinoblastoma patients (Francois, 1977). The reported incidence rate is 3.5 per million children under age 15 with non-hereditary retinoblastomas occurring 55-65% of all cases (Pendergrass and Davis, 1980). Early cytogenetic studies suggested that deletion on chromosome 13q is mainly responsible for the disease (Francke, 1976; Orye et al., 1974, Riccardi et al., 1979). Later, tight linkage of the retinoblastoma locus to esterase D (ESD) on chromsome 13q confirmed the cytogenetic findings (Connolly et al., 1983).

The gene responsible for the disease was isolated by positional cloning technique by initially characterizing a 1.5 kb DNA sequence that detected deletions on 13q14 in 3 of 37 retinoblastoma (Friend et al., 1986). Using chromosome walking technique, a 30 kb region surrounding this fragment was characterized. This region proved to contain a single copy probe that hybridized with mouse genomic DNA, suggesting that the cloned fragment contained a coding exon. Significantly, the same probe recognized a 4.7 kb transcript in retinal cell line which was not detected in 4 retinoblastomas (Friend et al., 1986). The *RB* gene has an open reading frame of 2,784 bp encoding a 928 amino acid protein called pRB or sometimes p105-RB based on its apparent molecular mass. It is encoded by 27 exons in a span of 180 kb of genomic sequence (Hong et al., 1989; Togochida et al., 1993). Exon

20 encodes a leucine zipper motif, a characteristic domain seen in transcription factors (Hong et al., 1989).

In cells transformed by SV40, adenovirus or human papilloma virus, p105-RB is found in complex with the virus encoded onco-proteins (e.g. E1A transforming protein of adenovirus, large T antigen of SV40, E7 protein of human papilloma viruses) (Hinds and Weinberg, 1994; Marshall, 1991) Thus, it is believed that p105-RB is prevented from doing its function thereby mimicking the loss of function *RB* mutation observed in human tumors.

p105-RB is a nuclear phosphoprotein that exerts its control over cell proliferation by interacting with cellular proteins notably the E2F family of transcription factors (Hinds, 1995). By interacting with an E2F protein, the transcriptional activation of several genes central to the onset of DNA synthesis (S) phase of the cell cycle is prevented (Hinds, 1995; Hollingsworth et al., 1993; Johnson et al., 1994) . The G_1 to S phase model of p105-RB function is regulated by cell cycle dependent phosphorylation. In the G_1 phase of the cell cycle, p105-RB is in hypophosphorylated form which is active in growth suppression and E2F association. It gets hyperphosphorylated in the late G_1 phase by cyclin dependent kinases (Cdks) which are in complex with their regulatory subunits, the cyclins, thereby abrogating p105-RB functions through the S, G_2 and M phases. It reverts back to its hypophosphorylated phase in the late M phase (Hinds, 1995; Hinds and Weinberg, 1994).

2.3 p53

p53 was originally discovered by its reaction to antiserum from animals with SV40 induced tumors (Levine et al., 1991). The large T antigen of SV40 forms a complex with p53 (Lane and Crawford, 1979; Sarnow et al., 1982) and stabilizes it by increasing its half-life from 6-20 min to several hours (Marshall, 1991). It also forms a complex with transforming proteins of other tumor viruses such as E1B of adenovirus

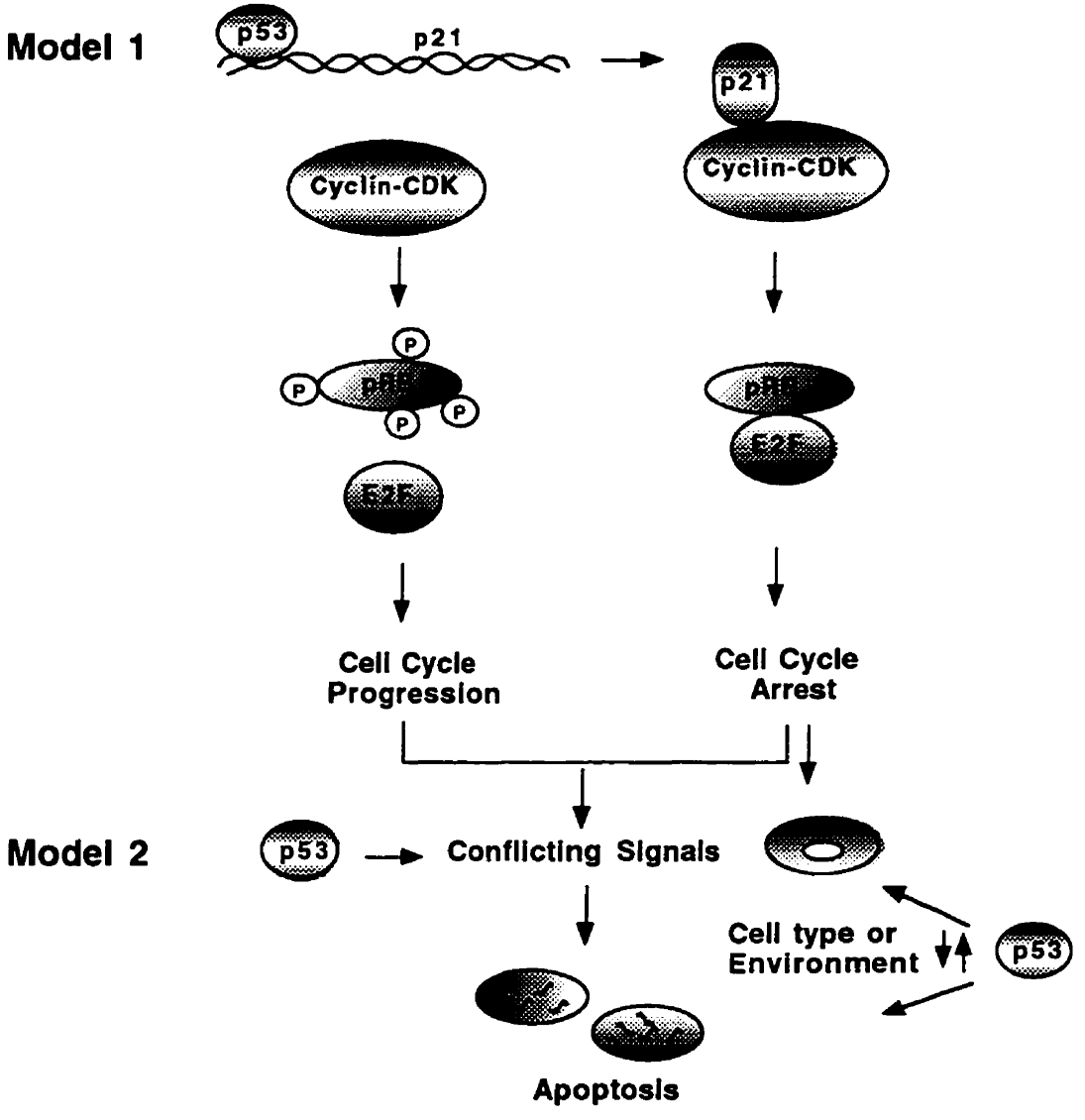
(Sarnow et al., 1982) and E6 of human papillomavirus-16 (HPV-16) and HPV-18 (Werness et al., 1990).

p53 is a 393-amino acid nuclear phosphoprotein implicated in Li-Fraumeni syndrome, an autosomal dominant disease characterized by diverse neoplasms at many sites of the body (Li and Fraumeni, 1969; Malkin et al., 1990). p53 is mutated in about half of all cases of human cancer (Harris, 1993) and has been the most studied of the tumor suppressor genes. Several functions have thus been associated with this molecule including its well known role in cell cycle arrest (Bargonetti et al., 1993; Fields et al., 1990; Raycroft et al., 1990), its involvement in DNA-repair process by binding to ERCC3 (Wang et al., 1994), its binding to single-stranded DNA ends (Bakalkin et al., 1994), its strong DNA-DNA, RNA-RNA strand annealing activity (Bakalkin et al., 1994; Brain and Jenkins, 1994; Oberosler et al., 1993), its function in suppressing signal-mediated transport of proteins into the nucleus (Feldherr et al., 1994) and its property to promote apoptosis (Caelles et al., 1994; Lowe et al., 1993; Morgenbesser et al., 1994; Xu and Levine, 1994). Its roles in signaling checkpoint arrest and apoptosis are favored to account for its tumor suppressor function.

Two models depict the role of p53 in growth suppression (Bates and Vousden, 1996) through G_1 cell cycle arrest or apoptosis, or a combination of both. Model 1 in Fig. 5 illustrates the activation of G_1 cell cycle arrest as a result of transcriptional activation of *CIP1/WAF1*. Several other proteins including Bax, Gadd45, FGF-BP3, mdm2 are also known to be activated by p53. $p21^{CIP1/WAF1}$ is a known inhibitor of cyclin-dependent kinases, one substrate of which includes pRB. Upregulation of p21 results in inhibition of cyclin-CDK activity; pRB remains bound to E2F thereby arresting the cell cycle by preventing E2F-responsive genes expression. Model 2 illustrates the relationship of the function of p53 in cell cycle arrest and apoptosis. The conflicting signal model depicts a cell being driven through the cell cycle simultaneous with a signal for cell cycle arrest. Such signal drives the cell to commit suicide. The alternative pathway

Figure 5

Models of p53 function. The models are not mutually exclusive. Model 1 depicts the activation of transcription of $p21^{CIP1/WAF1}$ by p53. The protein p21 inhibits the activities of cyclin-dependent kinases (CDKs), one substrate of which is pRB. In the hypophosphorylated state, pRB binds to E2F preventing the transcription of E2F-responsive genes, thereby blocking the progression through G_1 of the cell cycle. Conversely, if pRB is hyperphosphorylated, its binding to E2F is inhibited and therefore cell cycle progresses. An alternative model illustrates the relationship between cell-cycle arrest and apoptosis. Conflicting signals of cell cycle arrest and cell cycle progression (Model 2) may activate the apoptotic machinery of the cell. An alternate pathway may occur depending on the cell type or the environment. In this case, the G_1 checkpoint function of p53 is not required for the apoptotic pathway. (Modified from Bates and Vousden, 1996).


Model 1
p53
p21
p21
Cyclin-CDK
Cyclin-CDK
P
P
P
P
E2F
Cell Cycle
Progression
Cell Cycle
Arrest
Model 2
p53
Conflicting Signals
Cell type or
Environment
p53
Apoptosis

hypothesizes that cell type or the environment plays a key role on whether a cell undergoes apoptosis or cell cycle arrest on p53 activation.

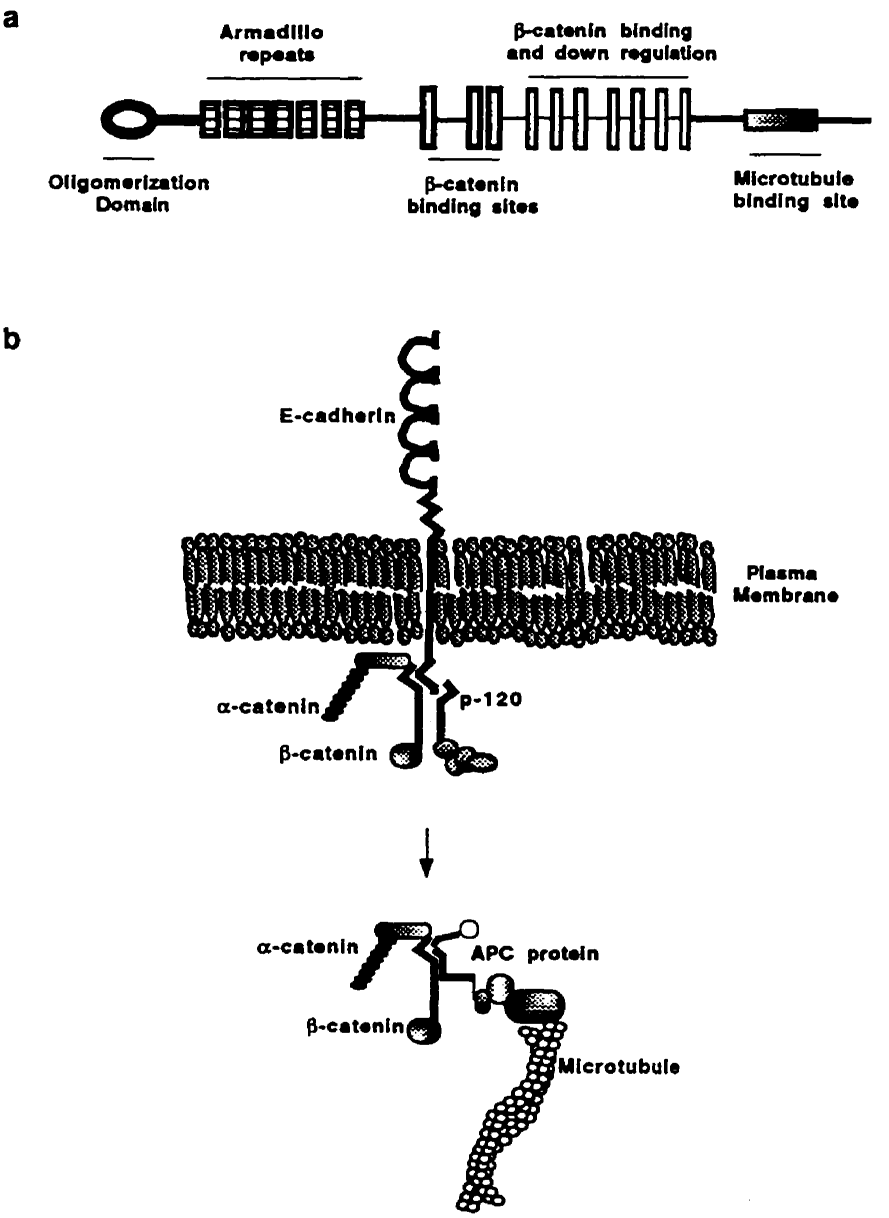
2.4 Adenomatous polyposis coli (APC)

Familial adenomatous polyposis (FAP) is an autosomal dominant disorder characterized by predisposition to develop hundreds to thousands of colorectal adenomas by the third and fourth decade of life, secondary to adenomatous polyps of the colon (Polakis, 1995). Polyps also develop in other sites including the thyroid. A useful sign for presymptomatic diagnosis of the disease is the development of pigmented retinal lesions known as congenital hypertrophy of the retinal pigment epithelium (CHRPE). FAP is a severe disease with polyps progressing to malignancy leaving gene carriers with a life expectancy of about 40 years (Bussey, 1975; Polakis, 1995). Mutations of the gene on chromosome 5q21, *APC*, are responsible for this disorder (Groden et al., 1991; Kinzler et al., 1991; Nishisho et al., 1991). APC mutations have also been implicated in a number of other tumors including colon cancer (Cottrell et al., 1992; Miyoshi et al., 1992; Powell et al., 1992), pancreatic cancer (Hori et al., 1992) and stomach cancer (Nakatsuru et al., 1992). Significantly, the site of APC germline mutation correlates with the manifestation of CHPRE (Olschwang et al., 1993). Retinal lesions are almost always absent if the mutation is 5' to exon 9 and are present if mutations occur 3' to this exon, suggesting the possible involvement of a dominant negative effect of a truncated protein in the development of CHPRE. Indeed, truncated protein products have been shown to interact with the wild-type gene protein through the amino-terminus (Su et al., 1993). A modifier locus Mom-1 (for modifier of *Min*) (Dietrich et al., 1993) was originally identified by analysis of the multiple intestinal neoplasia (*Min*) mutant mouse (Su et al., 1992) which carries a germline mutation in the mouse *APC* homologue.

The APC protein is a 2943 amino acid polypeptide. The putative functional domains of the APC protein (Fig. 6a) provide a useful working model in understanding its

Figure 6

(A) The *APC* protein showing the different functional domains. (B) A cartoon model of the interaction of the *APC* protein with ß-catenin and microtubule. Recently, APC was found to bind to *HDLG*, the human homologue of the *dgl* tumor suppressor gene in *Drosophila.* This interaction involves the carboxy-terminal region of APC and the DLG homology repeat region of DLG (Matsumine et al., 1996). (Modified from Kirkpatrick and Peifer, 1995; Polakis, 1995)



Working model of APC-catenin complex

function. The first 55 amino-acid sequence at the amino-terminus of the protein is responsible for its dimerization/oligomerization (Joslyn et al., 1993; Su et al., 1993). An Arm repeat (imperfect 42 amino acid repeats), seen in Drosophila *armadillo* gene first identified by its role in pattern formation during development (Peifer, 1993), (human homologues are ß-catenin and plakoglobin) is present near the amino-terminal region (Kirkpatrick and Peifer, 1995). This region is followed by ß-catenin-binding sites. A 20 amino-acid repeat interspersed in the middle of the protein is suggested to also bind and down regulate ß-catenin *in vivo*. This domain contains the cluster site for somatic mutation (Polakis, 1995). The microtubule binding site is located near the carboxy-terminus as a domain rich in basic amino acids (Polakis, 1995).

The most recent clue to understanding its function came from the finding that the APC protein interacts with ß-catenin (Rubinfeld et al., 1993; Su et al., 1993). A cartoon model of such an interaction is shown in Fig. 6b. Its interaction with ß-catenin does not occur while ß-catenin is thethered to E-cadherin (Kirkpatrick and Peifer, 1995). It is believed that such interactions provide insights into the role of APC in cell adhesion. It is hypothesized that APC-catenin complex may be linked to the actin cytoskeleton by the ß-catenin/α-catenin complex (Kirkpatrick and Peifer, 1995) and it may interact with microtubules (Munemitsu et al., 1994; Smith et al., 1994). These observations suggest that APC functions as a component of the contact inhibition signal downstream of cadherin-mediated cell adhesion (Kirkpatrick and Peifer, 1995).

2.5 Deleted in Colon Carcinomas (DCC)

The *DCC* gene is another tumor suppressor gene implicated in a large number of tumor types including gastric carcinoma (Uchino et al., 1992), pancreatic carcinoma (Hohne et al., 1992; Seymour et al., 1994), breast carcinoma (Deville et al., 1991; Thompson et al., 1993), prostate carcinoma (Brewster et al., 1994a; Gao et al., 1993; Latil et al., 1994), endometrial carcinoma (Gima et al., 1994), esophageal carcinoma

(Miyake et al., 1994), bladder carcinoma (Brewster et al., 1994b), gliomas (Scheck and Coons, 1993), male germ cell tumors (Murty et al., 1994), and even leukemias (Miyake et al., 1993a; Miyake et al., 1993b; Porfiri et al. 1993). The *DCC* gene spans more than 1.35 megabases of genomic sequence on chromosome 18q21.1 encoding at least 29 exons (Cho and Fearson, 1995; Cho et al., 1994). It was originally identified in the screening of colorectal cancer by the frequent deletion of an anonymous marker p15-65 (Fearson et al., 1990). Analysis of the surrounding sequence of p15-65 revealed a putative gene with sequence similarity to transmembrane proteins of the neural cell adhesion molecule (NCAM) family of cell surface proteins.(Fearson et al., 1990; Hedricket al., 1994).

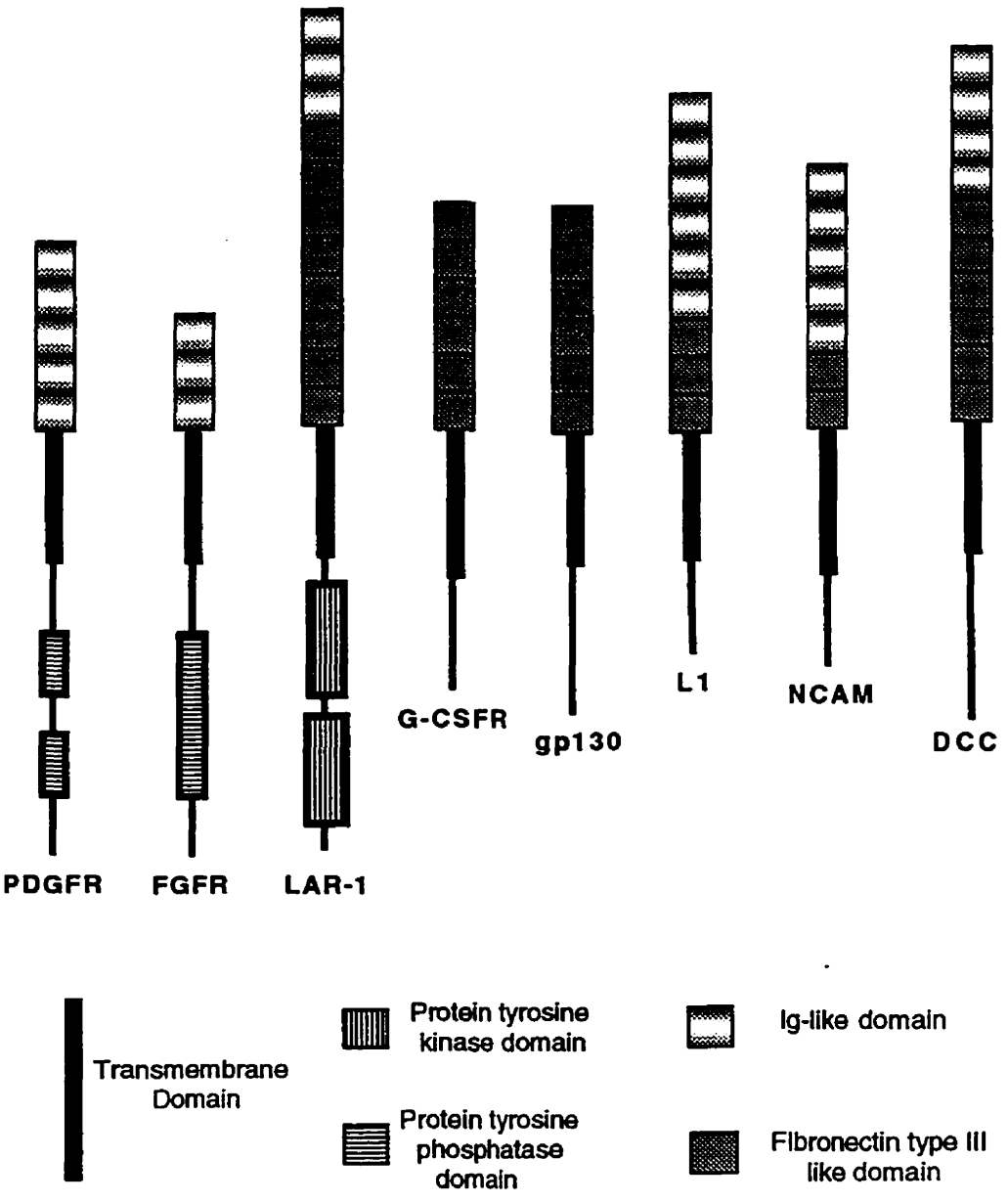
Although the *DCC* protein has sequence similarity to different classes of cell surface proteins like the immunoglobulin superfamily of cell adhesion molecules, not much evidence exitsts to confirm its role in cell-to-cell adhesion. It is proposed that DCC might mediate cell-cell adhesion through Ca^{2+}-independent heterotypic binding (Chuong et al., 1994), but its extracellular domain also has sequence similarity to proteins involved in ligand binding that function as growth surface receptors, rather than cell adhesion molecules (Fig. 7). Thus, two working paradigm might explain the mechanism of DCC function: (1) a cell-cell adhesion function and (2) a signaling molecule function. Nevertheless, the sequence of the *DCC* protein reveals that the plasma membrane-extracellular matrix as one site of action of tumor suppresor genes.

2.6 Wilms' Tumor (WT)

Wilms' tumor occurs as a component of a contiguous gene syndrome that includes aniridia, gonadoblastoma (or ambiguous genitalia/genitourinary abnormalities by some authors) , and mental retardation known as WAGR (Anderson et al., 1978; Miller et al., 1964; Riccardi et al., 1978). The observation of frequent deletion of chromosome 11p in Wilms' tumor suggested the involvement of a locus in the short arm of chromosome 11 in the development of this tumor (Francke et al., 1979; Francke et al., 1978; Riccardi

Figure 7

Schematic representation of the *DCC* protein and selected transmembrane proteins with immunoglobulin (Ig-like and fibronectin like domains. PDGF, platelet-derived growth factor; FGFR, fibroblast growth factor receptor; LAR1, leucocyte antigen related protein; G-CSFR, granulocyte colony stimulating factor receptor; gp130, cytokine receptor dimeric partner protein; NCAM, neural cell adhesion molecule; L1, NCAM-related molecule (Modified from Cho and Fearon, 1995).


PDGFR
FGFR
LAR-1
G-CSFR
gp130
L1
NCAM
DCC
Transmembrane Domain
Protein tyrosine kinase domain
Protein tyrosine phosphatase domain
Ig-like domain
Fibronectin type III like domain

et al., 1978). To identify the *WT* gene, several initial strategies were used to map the Wilms' tumor region including the analysis of somatic cell hybrids with a t(11;14) translocation and Wilms' tumor patient's lymphoblasts with 11p deletion (Lewis and Yeger, 1987), chromosome mediated gene transfer to provide an enriched source of chromosome 11p markers (Porteous et al., 1987) and analysis of deletions in Wilms' tumors which suggested the 11p15.5 locus as likely location of the Wilms' tumor gene instead of 11p13 region as usually reported (Mannens et al., 1988). Using pulsed field gel electrophoresis and irradiation-reduced somatic cell hybrids, a physical map of the 11p14 region was established. A region of 345 kb yielded a transcript encoding a zinc finger protein (Rose et al., 1990; Call et al., 1990). Simultaneously and independently, by chromosome jumping, a gene encoding a Kruppel-like zinc finger protein was identified to be homozygously deleted in Wilms tumors (Gessler et al., 1990). The *WT1* gene spans 50 kb of genomic sequence (Call et al., 1990) and encoded by 10 exons that display a complex pattern of mRNA species (Haber et al., 1990) that are predominantly expressed in condensed mesenchyme, renal vesicle, fetal gonad, mesothelium and a subset of hematopoeitic cells (Call et al., 1990; Pritchard et al., 1990). Mutation of the *WT1* gene is associated with Denys-Drash syndrome (DDS), a rare human condition in which severe urogential aberrations result in renal failure, pseudohermaphroditism and Wilms' tumor (Pelletier et al., 1991). It is believed that the DDS alterations which cluster in the zinc finger encoding exons, operate as dominant negative mutations (Little et al., 1993) presumably incapable of binding DNA but perhaps able to dimerize with *WT1* isoforms disturbing isoform dosage balance (Little et al., 1995; Little et al., 1993). Subnuclear localization of *WT1* protein dramatically differed in cell lines expressing truncated *WT1* and wild type *WT1*. Whereas *WT1* isoform that binds with high affinity to a defined DNA target had a diffuse nuclear staining pattern, *WT1* mutants with disrupted zinc-finger domain had speckled pattern of nuclear expression, suggesting that dominant-negative

WT1 protein physically associates with the wild type protein resulting in its sequestration within subnuclear structures (Englert et al., 1995).

2.7 Von Hippel-Lindau (VHL)

Von Hippel-Lindau syndrome is an autosomal dominant cancer syndrome characterized by the development of renal cell carcinomas, cerebellar hemangioblastomas, retinal angiomata, and pheochromocytomas. The gene responsible for VHL syndrome was mapped to chromosome 3p26-p25 (Seizinger et al., 1988) . It was identified through positional cloning techniques by studying germline deletions from unrelated VHL patients with nested constitutional deletions (Latif et al., 1993). The *VHL* gene encodes a 218 amino acid protein with an apparent molecular weight of 30 kDa (Iliopoulos et al., 1995) that binds with elongation factors elongin B and elongin C (Duan et al., 1995a; Kibel et al., 1995; Duan et al., 1995b). In vitro, pVHL competes with elongin A, for elongin B and C; thus, the normal pVHL is thought to inhibit transcription elongation by binding to elongin B and C (Duan et al., 1995b).

2.8 Breast Cancer 1 (BRCA1)

The first breast cancer susceptibility locus was mapped to chromosome 17q21 using families with early-onset breast cancer (Hall et al, 1990). The same locus was later established, by linkage analysis, to be responsible for the hereditary predisposition to cancer of the breast and ovary (Lynch and Watson, 1992; Narod et al., 1991). Analysis of tumors from breast-ovarian cancer families later demonstrated allele loss from the wildtype chromosome (Kelsell et al., 1993; Smith et al., 1992) suggesting that a tumor suppressor gene is involved in the disorder. The gene for BRCA1 encodes a protein with a zinc finger motif at its amino-terminal region which suggests that it functions as a transcription factor (Miki et al., 1994). The *BRCA1* gene product is thought to encode a 220 kDa nuclear phosphoprotein expressed in normal cells and in breast ductal epithelial cells, but in mutant

form it is often detected in the cytoplasm of breast and ovarian cell lines (Chen et al., 1995). However, recent data indicate that it encodes a 190 kDa protein with sequence homology and biochemical analogy to the granin family of proteins (Jensen et al., 1996). The *BRCA1* protein and granins are localized to secretory vesicles. The granins that include chromogranin A, chromogranin B and secretogranin II (also known as chromogranin C) are postranslationally glycosilated proteins which are responsive to hormones and are secreted by a regulated pathway. They are acidic proteins that bind calcium and aggregate in its presence (Steeg, 1996). This recent finding suggests that *BRCA1* protein acts in a new mechanism never before reported for a tumor suppressor gene. Nevertheless, there is controversy on the significance of the granin homology model, thus more analyses are needed to determine *BRCA1* function.

The mouse *BRCA1* homologue, *Brca1*, shares low sequence homology of only 58% at the amino acid level (Bennett et al., 1995) and maps to chromosome 11 (Bennett et al.,1995; De Gregorio et al., 1996; Schrock et al., 1996).

2.9 Breast Cancer 2 (BRCA2)

The *BRCA2* locus was mapped to chromosme 13q12-q13 by a genome wide search and linkage analysis (Wooster et al., 1994). The locus was also identified to be involved in the susceptibiltiy to male breast cancer (Thorlacius et al., 1995). This region of chromosome 13 often shows loss of heterozygosity in sporadic breast cancer and other neoplasms suggesting that it contains a tumor suppressor gene (Woodster et al., 1995). A transcription unit detected as a 10-12 kb transcript in normal breast epithelial cells, placenta, and breast cancer cell line MCF7 was identified as the *BRCA2* gene because it was disrupted in the germline of breast cancer families (Woodster et al.,1995). The *BRCA2* gene encodes a 3,418 amino acid protein with no obvious membrane spanning region but roughly one-quarter of the residues are highly charged (Tavtigian et al., 1996). It is encoded by 27 exons spanning 70 kb of genomic sequences. Similarities to *BRCA1*

include large exon 11, translational start site that occurs in exon 2 and both have an AT rich coding sequence (Tavtigian et al., 1996). Recently, a sequence homology to the granin family of protein was identified at the carboxy-terminus of *BRCA2* protein (Jensen et al., 1996). This suggests that the both *BRCA1* and *BRCA2* may be involved in similar mechanistic pathway of tumor suppression. Nevertheless, controversies exist on the granin homology model and such interpretation may be premature.

3. Sites of Action of Tumor Suppressor Genes

As more and more human tumor suppressor genes are being identified, the molecular and cellular functions of these genes are revealing several functional mechanisms as well as different sites of action (Table 4).

Tumor suppressors act as negative control elements in a cascade of events that occur from the external millieu or within the cell to the nucleus. Based on studies of tumor suppressor genes, a variety of mechanisms have been identified such as signaling, cell adhesion, transcription, translation and cell cycle control. The sites of action extend from the extracellular matrix to the nucleus. With the identification of the *NF2* protein as a putative membrane-cytoskeleton linker, not only did we identified a novel site of action of tumor suppressor gene, but also gained insights into events leading to tumorigenesis. In addition, the studies on the *NF2* protein is contributing not only to our understanding of tumorigenesis but also on the basic biology of the cell.

In order to understand the basic cell biology of schwannomin in normal cells, we used mouse tissues to look at the cellular expression of the *Nf2* protein at the mRNA and protein levels (Claudio et al., 1995). As well, we looked at its localization in lens and Schwann cells, two cell types affected by the NF2 phenotype (Claudio et al., 1996). In Chapters 4 and 5 we present indirect evidence consistent with the hypothesis that schwannomin acts as a membrane cytoskeleton linker. With our results, together with recent data in the literature, we propose a working model for schwannomin defined by its role in dynamic structures.

Table 4. Functions and sites of action of human tumor suppressor genes.

Gene	Site of Action	Function
p53	Nucleus	Cell cycle regulated nuclear transcription factor
RB-1	Nucleus	Cell cycle regulated nuclear transcription repressor
WT1	Nucleus	Zinc finger transcription factor
VHL	Nucleus	Regulates transcription elongation by binding to elogin B and C.
NF1	Cytoplasm	Downregulates p21-*ras*
NF2	Cell cortex	Membrane-organizing protein.
APC	Beneath the plasma membrane	Component of contact inhibition signal downstream of cadherin-mediated cell adhesion
DCC	Plasma membrane-extracelllular matrix	Cell-cell adhesion molecule with signaling function
BRCA1	Possibly nuclear; Also found in vesicles	With zinc finger motif seen in transcription factors.
BRCA2	Not clear	Possibly transcription factor.

SECTION 3
THE BAND 4.1 SUPERFAMILY

With our limited knowledge on the biology and functional mechanism of schwannomin, insights can be gained by looking at what is known about the band 4.1 superfamily. This section provides an overview of the family members related to band 4.1.

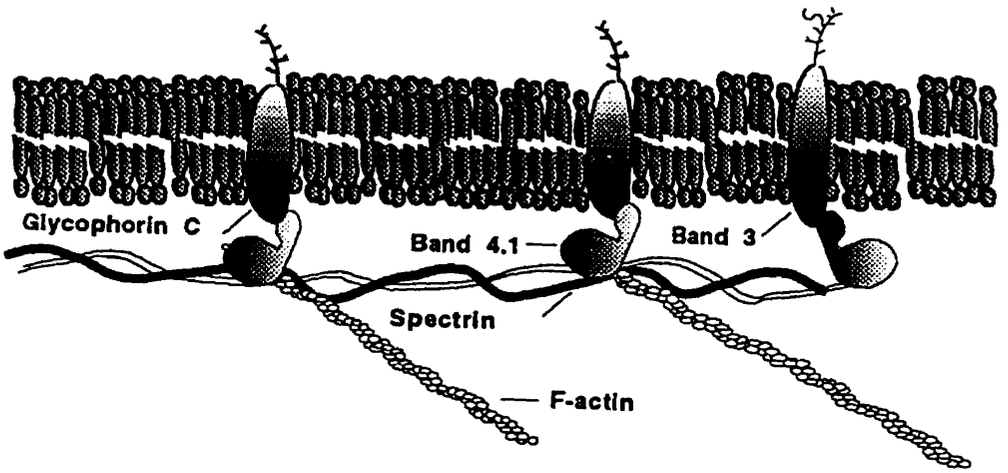
1. Band 4.1

Protein 4.1, or more commonly band 4.1, is a 78 kDa structural protein in erythrocytes and in non-erythroid tissues that regulates the membrane's physical properties of mechanical stability and deformity by stabilizing spectrin-actin interaction (Bennet, 1989; Cohen and Gascard, 1992; Conboy et al., 1986; Tyler et al., 1979). In addition, it binds the cell cytoskeleton to the cell membrane by interacting with transmembrane proteins band 3, an anion exchanger, and glycophorin C, a membrane sialoglycoprotein carrying blood group specifications (Anderson and Lovrien, 1984; Fowler and Taylor, 1980; Pasternack et al., 1985) (Fig. 8). Binding between band 4.1 and band 3 may involve the amino acid sequence LEEDY, near the amino-terminus of band 4.1 and an oppositely charged motif IRRRY or LRRRY in the cytoplasmic domain of band 3, although some other sites had been implicated (Luna and Hitt, 1992). Amino acid sequence YRHKG present in glycophorin C contains the same charge distribution and hydrophobicity as the motif in band 3 (Luna and Hitt, 1992). This sequence motif however has not been reported as the binding site for band 4.1.

Band 4.1 can be phosphorylated by a variety of kinases both *in vitro* and *in vivo*, including PKA, PKC and casein kinase. In general, phosphorylation by each of the kinases results in the reduction in the ability of band 4.1 to bind spectrin and to promote the binding of spectrin to actin (Cohen and Gascard, 1992; Ling et al., 1988). Phosphorylation of band 4.1 on tyrosine 481 by epidermal growth factor (EGF) receptor kinase reduces its ability to promote spectrin/actin binding (Subrahmanyam et al., 1991).

Figure 8

Model of the interaction of band 4.1 to glycophorin C or band 3 and to the actin-spectrin complex in erythrocyte.


Glycophorin C
Band 4.1
Band 3
Spectrin
F-actin

Conversely, dephosphorylation by alkaline phosphatase *in vitro* results in an increased ability of band 4.1 to promote spectrin binding to actin (Cohen and Foley, 1986).

Deficiency of band 4.1 by homozygous mutation has been associated with a hereditary disorder called elliptocytosis (Tchernia et al., 1981). Deficiencies in band 3, band 4.1 and α or ß subunit of spectrin are associated with increased fragility, lysis and ellipsoidal shape of erythrocytes rather than as normal biconcave discs (Luna and Hitt, 1992).

2. Talin

Talin is a 220 kDa protein which has the ability to self associate to form dimers at high protein concentrations. At physiological ionic strength, it forms an elongate flexible rod (Beckerle and Yeh, 1990a). Experiments done *in vitro* show that talin interacts with vinculin (Burridge and Mangeat, 1984) and integrins (Horwitz et al., 1986), two proteins known to be localized at adhesion plaques. Although no direct *in vivo* evidence is available to show the binding of talin and vinculin except for frequent colocalization within cells, several biochemical approaches show that such an association exits (Beckerle and Yeh, 1990a). Binding studies have always failed to show binding of talin with the cytoplasmic domain of ß1 integrin alone. Apparently, binding of talin to integrins of the ß1 class requires the native heterodimeric α and ß integrin complex (Beckerle and Yeh, 1990a).

Talin is phosphorylated on serine, threonine and tyrosine residues *in vivo.* It is hypothesized that phosphorylation might affect the function of talin at sites of cell adhesion by reduction of either developing or existing focal contacts (Beckerle, 1990b; Turner et al., 1989).

3. ERM Family

The ERM family of proteins includes ezrin, radixin and moesin (Sato et al., 1992). These proteins are highly homologous throughout their entire length. Moesin, for

example, is 72% and 80% homologous to ezrin and radixin, respectively. When compared at their amino-terminal halves, their amino acid homology is as high as ~80%, whereas sequence comparison at their carboxy-terminal halves shows homology of ~65%. The ERM proteins are components of actin-rich cellular structures such as membrane ruffles, microvilli, filopodia, lamellipodia, growth cones and cleavage furrows in a variety of cell types (Amieva and Furthmayr, 1995; Berryman et al., 1993; Bretscher, 1989; Franck et al., 1993; Goslin et al., 1989; Henry et al., 1995; Sato et al., 1992; Takeuchi et al., 1994) . These proteins are often seen co-expressed in several cell types, but their pattern of tissue expression is cell-type specific (Amieva and Furthmayr, 1995; Berryman et al., 1993). It has been proposed that redundancy in their function may provide a safety mechanism for the cell (Sato et al., 1992). Indeed, perturbation of cell adhesion and microvilli formation *in vitro* requires the simultaneous suppression of expression of the three proteins (Takeuchi et al., 1994), suggesting a possibly overlapping functions. It is also possible that they have distinct function depending on the cell-type or that they have a synergistic function in several cells.

3.1 Ezrin

Ezrin, named in recognition of the place where it is claimed to have been first purified, Ezra Cornell University (Bretscher, 1983; Bretscher, 1991) is an 81 kDa protein that is a component of microvillar cytoskeleton. In the literature, ezrin was referred to as p81, a protein that becomes phosphorylated on tyrosine residues in human epidermoid carcinoma A431 cell in response to epidermal growth factor (Gould et al., 1986). Cytovillin, a protein that was originally identified by its cross reactivity to an antibody to a synthetic peptide deduced from a cloned human endogenous retroviral *gag*-related sequence, *erv-1* (Suni et al., 1984), turned out to be identical to ezrin after it was cloned (Gould et al., 1989; Turunen et al., 1989). Thus, the general consensus has been to use ezrin for p81 and cytovillin.

Ezrin is the most studied of the ERM protein family. It occurs in monomer form in cell extracts, but dimers and ezrin-moesin heterodimers can also be formed (Berryman et al., 1995; Gary and Bretscher, 1993; Gary and Bretscher, 1995). The ezrin self-association domain has been mapped to the amino- and carboxy-terminus of the protein; dimerization occurs by head-to-tail joining of these domains (Gary and Bretscher, 1995). These domains are also responsible for the homotypic and heterotypic interactions among the ERM proteins, and thus appropriately termed N-ERMAD and C-ERMAD, for ezrin-radixin-moesin association domain. The N-ERMAD was mapped to the first 296 amino acids of ezrin and the C-ERMAD to the last 107 amino acids (Gary and Bretscher, 1995). In ezrin, these domains are masked in native monomers, and can only be unmasked by sodium dodecyl sulfate, suggesting that dimerization *in vivo* requires unmasking of these domains. This may explain the unsuccessful attempts to generate dimeric ezrin species in solution (Gary and Bretscher, 1993). Covalently cross-linked ezrin oligomers which remain associated with the detergent-insoluble cytoskeleton protein extract from microvilli can be detected by western analysis and blot overlay assays (Berryman et al., 1995), suggesting a tight association with the cytoskeleton, presumably actin. Indeed, ezrin has been found to bind actin, and the actin-binding domain has been mapped to the last 34 amino acids at its carboxy-terminus (Turunen et al., 1994). These amino acids are also conserved in radixin and moesin, in CapZ, an actin capping protein, and in a potential actin-binding site of myosin heavy chain (Turunen et al., 1994).

The phosphorylation of ezrin correlates with changes in cell morphology. In gastric parietal cells, stimulation by agents that elevate cAMP induces the phosphorylation of ezrin on serine and threonine residues concomitant with the formation of ezrin-rich microvilli structures (Hanzel et al., 1991; Urushidani et al., 1989). In EGF-stimulated A431 cells, there is a close temporal relationship between tyrosine phosphorylation of ezrin and the rapid formation of microvilli and membrane ruffles (Bretscher, 1989). These observations

suggest that ezrin's phosphorylation might play a role in regulating cell surface cytoskeletal rearrangements.

Although ezrin has not been directly implicated in tumorigenesis, there are observations suggesting a potential role for ezrin in determining the phenotypic characteristics of some tumors. It has been identified that the MTA-I tumor transplantation antigen of methylcholanthrene-induced mouse sarcomas is ezrin (Fazioli et al., 1993). In these cells, upregulation of ezrin was found to correlate with oncogenic transformation and its overexpression subverts its normal physiological localization.

3.2 Moesin

Moesin, for *m*embrane *o*rganizing *e*xtension *s*pike prote*in*, was originally isolated from bovine uterus and characterized as potential receptor for heparan sulfate (Lankes et al., 1988). It is a protein encoded by a 4.2 kb transcript with an open reading frame of 577 amino acids (Lankes and Furthmayr, 1991). It has an estimated molecular weight of 78 kDa on SDS-PAGE (Lankes et al., 1988). Immunofluorescence experiments using a variety of animal cell lines show expression in slender cell processes and frequently observed to co-localize with ezrin (Furthmayr et al., 1992). Other studies show the localization of moesin to be indistinguishable from radixin in mouse fibroblasts (Henry et al., 1995) . Generally, moesin is found to be expressed in blebs, microspikes, filopodia and retraction fibers (Lankes and Furthmayr 1991).

3.3 Radixin

Radixin is a 583 amino acid polypeptide with an estimated molecular weight of 82 kDa (Sato et al., 1992). It is a barbed-end actin capping protein (Tsukita et al., 1989) concentrated at cell-cell adherens junction, cleavage furrows, filopodia, microvilli and ruffling membranes (Henry et al., 1995; Sato, 1991). Epitope tagging experiments reveal that the carboxy-terminal polypeptide is associated with cortical structures whereas parallel

expression of the amino-terminal half showed diffuse cytoplasmic localization (Henry et al., 1995). Molecular dissection of radixin in NIH-3T3 cells shows the localization of radixin in a pattern indistinguishable from moesin staining. Moesin can be displaced by exogenously expressed radixin without any apparent phenotype on cell spreading and cytokinesis (Henry et al., 1995), supporting the notion that they are functionally redundant.

4. Protein Tyrosine Phosphatases (PTPases)

The protein tyrosine phosphatases belong to a rapidly expanding family. Some members of this family have extracellular segment with structural homology to fibronectin type III repeats and immunoglobulin domains, and an intracellular region containing the catalytic protein tyrosine phosphatase domain. These members (transmembrane type) include the receptor protein tyrosine phosphatases. The other class of the PTP family, the intracellular type, does not have extracellular domain, but consists of an amino terminus with sequence homology to the band 4.1 homology domain. The former type is known to localize in cell-cell or cell-matrix contacts which suggests that they could be direct signal transducers of cell contact phenomena (Brady-Kalnay and Tonks, 1995); the latter type may act in the fine tuning of tyrosine phosphorylation of cytoskeletal proteins at focal adhesions.

The intracellular PTPases include PTP 1B from human placenta (Chernoff et al., 1990; Tonks et al., 1988), PTPH1 (Yang and Tonks, 1991), a cytosolic megakaryocyte PTP MEG (Gu et al., 1991), PTPD1 from skeletal muscle (Moller et al., 1994), PTP36 from murine thymus (Sawada et al., 1994), PTP-BAS (Maekawa et al., 1994) and PTP-RL10 from mouse liver (Higashitsuji et al., 1994). These PTPases all have, in their amino terminal region, a sequence that is homologous to the domain in band 4.1, talin and the ERM proteins that is involved in plasma membrane attachment. This suggests that the cytosolic PTPases may be localized underneath the plasma membrane.

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CHAPTER 2*
OF MICE AND MEN

The isolation of the human *NF2* gene provided the initial step in understanding the protein mutated in Neurofibromatosis type 2. This chapter describes the cloning of the mouse homologue of the *NF2* gene and compares its sequence to the human gene at the DNA and protein levels. It concludes that the high conservation of the gene in mice and in vertebrates suggests that studying the mouse *Nf2* gene might yield insights into the functional mechanism of the human gene.

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The Mouse Homologue of the Neurofibromatosis Type 2 Gene is Highly Conserved.

ABSTRACT

Neurofibromatosis type 2 (NF2) is a complex nervous system disorder characterized by the development of schwannomas (especially vestibular), meningiomas, ependymomas and juvenile lens opacities. Mutation in the *NF2* gene, which encodes for the schwannomin protein (SCH), a member of the band 4.1 superfamily of genes, predisposes carriers to these central nervous system tumors. We have isolated a mouse cDNA from a brain library which contains the complete open reading frame of the mouse homologue of the *NF2* gene. This cDNA encodes for a 596 amino acid protein with 98% identity to the human SCH. Cross species hybridization experiments predict that the *NF2* gene is highly conserved in other vertebrates. Northern analysis detects a 4.5 kb transcript in mouse brain, kidney, cardiac muscle, skin and lung suggesting ubiquitous expression. The predicted secondary structure of SCH, which is shared by all members of the band 4.1 superfamily, includes a highly conserved amino-terminal domain which is believed to bind to proteins in the plasma membrane and a large highly charged α–helix domain proposed to associate with the cytoskeleton. The *NF2* gene is the first example of a tumor suppressor gene whose protein product appears to act as a membrane cytoskeleton-linker. These results show that the *NF2* gene is highly conserved and suggest that the analysis of the mouse *Nf2* gene might yield insights into the function of the human gene.

INTRODUCTION

The neurofibromatoses are common autosomal dominant disorders predisposing to various tumors of the nervous system. Two clinically and genetically distinct forms, Neurofibromatosis Type 1 (NF1) or von Recklinghausen's neurofibromatosis and NF2 are known to predispose carriers to tumors derived, primarily, from the neural crest (1-4). The hallmark of NF2 is bilateral vestibular schwannomas, tumors that develop on the vestibular branch of the eighth cranial nerve. Usually NF2 presents in the patient's mid 20's with abnormalities that include multiple cranial and spinal meningiomas, nerve root schwannomas, and brain stem and spinal cord gliomas and ependymomas (1). Additionally, juvenile posterior capsular lenticular opacities are common occuring in up to 80% of NF2 cases (6,7). By contrast, NF1 is characterized by patches of skin pigmentation (cafe-au-lait macules), Lisch nodules of the iris and multiple skin neurofibromas (1,2). Although NF2 is less common (1 in 40,000) than NF1 (1 in 4,000), NF2 is a more serious disease with a mean actuarial survival of 15 years after diagnosis (8).

NF1 maps to chromosome 17 (9) while *NF2* maps to the long arm of chromosome 22 (10) confirming that both disorders are genetically distinct. The genes involved for both forms of neurofibromatosis have been isolated (11-15). The *NF1* protein, neurofibromin, plays a role in the signal transduction pathway and has been postulated to down regulate $p21^{ras}$ (3,16,17), while the *NF2* protein, which we named schwannomin, is a putative membrane organizing protein that belongs to a gene family that is involved in linking the cytoskeleton to the plasma membrane (11). Based on autosomal dominant mutation in one allele that predisposes to the inherited disorder and on the occurrence of frequent somatic inactivation of the normal homologue in tumors, the *NF2* gene is hypothesized to be a recessive tumor suppressor (18,19).

We wished to determine the extent of phylogenetic conservation of the *NF2* gene in order to identify functionally important regions of the protein. In addition, we characterized patterns of expression in mouse tissues. Here we show that the mouse schwannomin is ubiquitously expressed. By secondary structure analysis, the mouse schwannomin represents a highly conserved putative membrane associated protein. Functional conservation, as determined by the high identity of both human and mouse proteins, indicates that the mechanism of action of this novel tumor suppressor can now be studied in mice.

RESULTS

Isolation of the mouse cDNA clones

One million independent recombinants from an 18-day old mouse brain cDNA library were screened using the amino-terminal probe of the human schwannomin gene (Fig. 1). Ten clones were isolated, four of which also hybridized to the carboxy-terminal probe derived from the human gene (Fig. 1); these more complete clones were further studied because they seemed to contain both the amino- and carboxy-terminus. All four clones were found to contain identical 2.6 kb inserts suggesting that they may have been amplified from a single recombinant during the construction of the library. One clone, AB711.1, was further characterized by sequencing both strands. AB711.1 contains 576 bp of 5' untranslated sequence, a 1788 bp open reading frame that codes for 596 amino acids and 233 bp of 3' untranslated sequence. Neither the poly-adenylation tail nor the poly-adenylation signal was detected.

Sequence analysis

Comparison of the coding sequence of clone AB711.1 with the human cDNA revealed overall identity of 90%. Both genes appear to use the same translation start site

and termination codon (11). The mouse cDNA contains three in-frame stop codons located within 57 bases upstream of the putative start codon (Fig. 2a). A 3 bp insertion, which codes for a proline residue, occurs in the mouse SCH at position 571. Thus, the mouse protein is one amino acid longer than the human schwannomin. The amino acid identity is 98.3% suggesting strong functional conservation of the mouse protein. The computed molecular weight is 69.7 kD with two possible N-linked glycosylation sites at amino acid positions 263 and 286, and twenty putative phosphorylation sites.

Sequence alignment of the 5' and 3' untranslated region of mouse SCH showed 80% and 62% identity to the same region in human SCH. The 576 bp 5' untranslated sequence shows 52% sequence identity with the promoter region of mouse breakpoint cluster region (BCR) gene (324 matches, 225 mismatches and 74 gaps)(25).

Predicted secondary structure of the mouse schwannomin

Amino acid sequence analysis of the mouse schwannomin shows no long hydrophobic stretches (Fig. 2b). Thus, it is unlikely that it contains a transmembrane domain. However, the predicted secondary structure (Fig. 2c) consists of domains identical to the human protein (11,12) and to members of the band 4.1 superfamily, particularly radixin, moesin and ezrin (29,30)(Fig. 3). The amino-terminal domain (amino acid 1-302) represents a region of very high homology to human schwannomin (99.67%) with a single conservative and a non-conservative amino acid substitution. This homologous domain is 62% and 59% identical to the same region in mouse moesin and radixin, respectively. By analogy to radixin, this domain might bind to plasma membrane proteins (30). The carboxy-terminal domain is predicted to contain a series of highly charged α-helix structures beginning at amino acid 303 (Fig 2c). Homology of the long α-helix (amino acids 303-478) to the human protein is 100% and 40-42% for mouse moesin and radixin. By analogy to the actin barbed-end capping function of radixin (30) this domain may be involved in cytoskeleton attachment.

Phylogenetic conservation

The strong conservation of the *NF2* gene in mouse prompted us to determine the extent of phylogenetic conservation in other species. We probed EcoR1 digested genomic DNA of different vertebrate species using an XhoI/EcoR1 digest of AB711.1 (Fig. 1). This probe includes the carboxy-terminal half of the protein and the 3' untranslated sequence. Using high stringency hybridization, we determined that the *NF2* gene is conserved in pig, chicken, hamster, cow, turkey and monkey (Fig. 4). Interestingly, the probe hybridized to sequences that include human exons 9 to 15 (11) contained within a ~20 kb EcoR1 fragment (Fig. 4). By contrast, this probe hybridized to at least 5 EcoR1 fragments (Fig. 4) in mouse suggesting that the EcoR1 restriction map of the human and mouse *NF2* gene is different within this region.

Tissue specificity

Northern hybridization using total RNA detected a 4.5 kb transcript in 2-week old mouse and 5-day old rat brain, mouse cardiac muscle and mouse kidney (data not shown). A weakly hybridizing band of approximately the same size was detected in skin and lung. Reverse transcription PCR using primers derived from the carboxy-terminus of the mouse cDNA (Fig. 1) confirmed ubiquitous expression in mouse tissues including liver and spinal cord (data not shown), similar to the widespread expression reported for the human gene (11,12).

DISCUSSION

Animal models are often useful in the study of human diseases (31-33). The striking conservation both at the nucleotide and amino acid level of the human and mouse SCH for its entire length suggests strong functional conservation. Based on these results,

it is now possible to construct a mouse animal model to study the molecular mechanism of SCH function and its role in the disease process.

Moesin, ezrin and radixin belong to a family of proteins which act at the plasma membrane-cytoskeleton interface. Moesin and ezrin are localized in blebs, filopodia and lamellipodia, retraction fibers and microspikes (34). These structures are involved in cell exploration, attachment, movement and events in epithelial-mesenchymal transformations in development. Therefore, it is proposed that SCH might normally lead to stable cell-cell and cell-matrix interactions (11). It has been suggested that a defect in a protein that connects the plasma membrane to a complicated latticework of many different kinds of interconnected filaments that make up the cytoskeleton could consequently contribute to uncontrolled cell growth or transformation (12). A germline in-frame deletion of 3 exons in the highly conserved amino-terminal domain has been identified in a patient with NF2 (11). This suggests that SCH function can be disrupted by mutation that affects its putative association with the plasma membrane. Such a mutation may be used as a basis to study SCH function in mouse models of the disease.

The importance of the *NF2* protein extends to other species. Zoo blot analysis using the carboxy-terminal domain and the 3' untranslated sequence indicates that the *NF2* gene is conserved in vertebrates, highlighting the importance of the protein across species. However, except for its homology to proteins involved in plasma membrane cytoskeleton interaction, no other proteins, that could give further insights to the function of schwannomin in other species, have significant homology. The rat long repetitive elements-1 (LINE-1 or L1Rn) region of human SCH found near the carboxy-terminus is also conserved in mouse (11). L1Rn has 2 overlapping ORFs coding for a DNA/RNA binding protein (ORF1) and reverse transcriptase without RNase H activity (ORF2)(35,36). The homology of 14 of 22 amino acids in the carboxy-terminus observed in ORF1 is unlikely to function as a DNA/RNA binding domain given schwannomin's

putative cytoplasmic subcellular localization. The function of this domain, if any, is yet to be determined.

The amino acid sequence of human and mouse SCH is strongly conserved throughout the whole protein. Only 10 amino acid sequence differences, including 1 insertion, are observed between the mouse and human proteins. Seven amino acid substitutions are located within 108 amino acids from the carboxy-terminus while only 2 are located within 223 residues from the amino-terminus. Of the 9 changes, 6 are conservative amino acid substitutions. The proline insertion occurs near the end of the protein but not within an intron-exon junction site of the human gene suggesting that the insertion is not due to alternative splicing.

Recently, the complete sequence of mouse neurofibromin was reported (37). Both the coding and the 3' non-coding mRNA regions are highly conserved. The coding region of the mouse *NF1* is 98% homologous to the human *NF1* protein, similar to the degree of conservation we have observed between the human and mouse *NF2* protein. Interestingly, the 5' and 3' untranslated region of the mouse *NF2* cDNA contain 80% and 62% homology, respectively, with the same region in the human cDNA. This strong conservation suggests that these regions are also potential sites for mutation in NF2 patients. Although no probable function is known for these regions, search for sequence similarity of the 5' untranslated region revealed 52% similarity to mouse BCR gene promoter region. The function of this region remains speculative whether it is involved in any regulatory mechanism.

By northern analysis, a transcript of 4.5 kb was observed in all tissues examined. Given that NF2 affects mostly cells derived from the neural crest, the tissue expression is unexpectedly diverse. The ubiquitous expression of more than one transcript in human tissues (12) and the widespread expression in mouse suggest that the *NF2* gene might be involved in the development of other tumors as in the case for neurofibromin. The absence of multiple mRNA species in the mouse differs from that reported in humans where four

different sized transcripts are seen (12). Possibly, the 4.5 kb species in mouse, which presumably is the same as the 4.4-4.5 kb transcript in human (11,12) represent homologous messages. The identity of the other different sized human mRNA species needs to be confirmed.

MATERIALS AND METHODS

Probes

The probes used were restriction fragments or PCR product derived from the human NF2 cDNA (11). The amino-terminal probe used for cDNA library screening was generated by PCR amplification of a 668 bp sequence from the human cDNA using (forward) CCTCTTTGATTTGGTGTGCC and (reverse)CGCTGTACGAGATGTTTCGG primers. The PCR reaction contained 250 ng of the human cDNA, 125 ng of each primer, 5 nM dNTP mix, 1x Bio/Can Scientific Taq buffer containing 1.5 mM $MgCl_2$ and 1U Taq polymerase in a total reaction volume of 25 µl. Amplification was done for 35 cycles of 1 min denaturation at 94°C, 45 sec annealing at 55°C and 1 min elongation at 72°C. The PCR product was purified using DNA isolation spin cartridge (Gibco/BRL) and verified by sequencing at both ends. The carboxy-terminal probe for cDNA screening was a 435 bp HindIII/EcoR1 fragment from the human cDNA. An Xho1/EcoR1 fragment of 1003 bp from the mouse cDNA was used for cross species hybridization and northern analysis (Fig. 1).

Cloning and DNA Analysis

The λgt11 cDNA library was prepared from whole brain of 18-day old NIH Swiss mice (ATCC No. 37431) as described (20). Approximately 1 x 10^6 independent recombinants were screened at high stringency and purified by three consecutive hybridizations. Positive clones were subcloned into pBluescript SK(+/-). DNA sequencing

was carried out on double stranded plasmid DNA after denaturation with 2N NaOH. Both strands were sequenced by dideoxynucleotide chain termination procedure (21) using USB Sequenase Kit. The primers used for sequencing were synthetic oligonucleotides derived from human and mouse sequence.

DNA and protein sequence analysis was performed using Intelligenetics Suite and MacVector packages.

Northern and Southern Blotting, Probe Labelling and Hybridization

Total RNA was extracted by the guanidine isothiocyanate method (22). Total RNA (30 μg) from different tissues was run on denaturing formaldehyde gels and blotted onto Hybond-N+ (Amersham) filter as described (23).

Equal amounts of genomic DNA (10 μg) was digested with EcoR1 and run on 1.0% agarose gel and blotted onto nylon filter (Hybond-N+)(Amersham)(23).

Southern and Northern hybridizations were done using standard procedures (23) with slight modifications. Hybridization was performed at 68°C for 18-20 hours using probes labelled by the random priming method (24). Filters were washed with 2x SSC (1x SSC is 150 mM NaCl and 15 mM Na_3 citrate, pH 7.0); 0.3% SDS for 15 min at room temperature followed by a series of washing at 65°C with 1x SSC, 0.3% SDS; 0.5x SSC, 0.3% SDS; 0.2x SSC, 0.3% SDS and a final wash of 0.1x SSC and 0.3% SDS. Filters were exposed to Kodak X-OMAT AR film with intensifying screen at -80°C.

Reverse-Transcription-Polymerase Chain Reaction (RT-PCR)

The antisense primer TATTATGCTTGCTGCTGGG was annealed at 65°C for 10 min with 5 μg of total RNA. Reverse transcription reaction was done with AMV-reverse transcriptase (Pharmacia) using Taq DNA polymerase buffer (BIO/CAN Scientific) at 42°C for 2 hours. Amplification was done together with the sense primer CCACCAAGCCCACCTATCCA using the following conditions for 35 cycles: 94°C denaturation, 55°C annealing and 72°C elongation for 1 min each.

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ABBREVIATIONS

SCH: schwannomin; RNase: ribonuclease, cDNA: complementary deoxyribonucleic acid; ORF: open reading frame; SDS: sodium dodecyl sulfate, SSC: sodium chloride, sodium citrate

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Figure 1

Schematic diagram of human and mouse SCH cDNAs showing probes used for hybridization. (a) Mouse cDNA showing Xho1/EcoR1 probe used for zoo blot and northern hybridization (b) Human cDNA showing the amino- and carboxy-terminal probes used for cDNA library screening. The arrows show the location of primers used for RT-PCR.

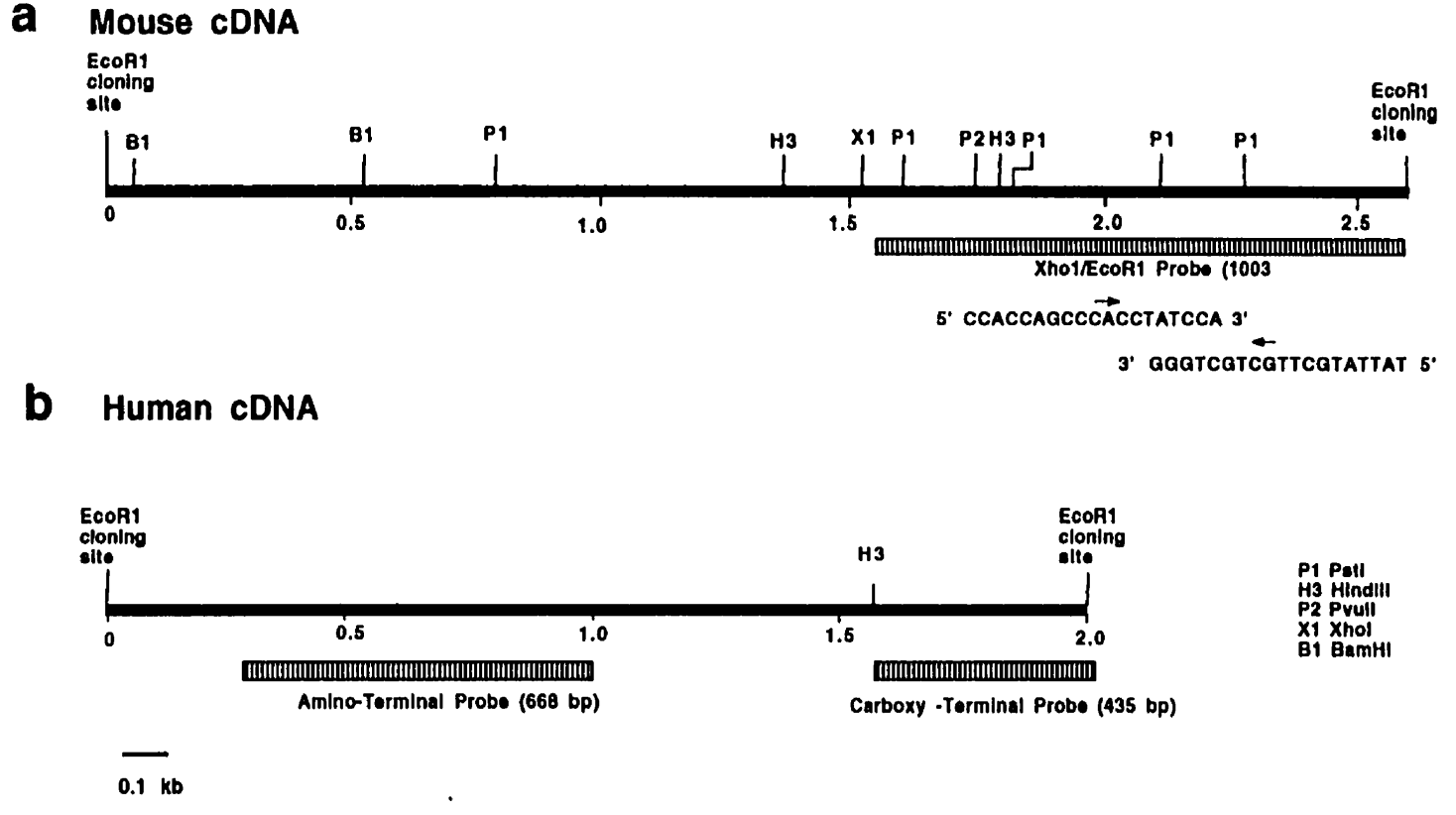
a
Mouse cDNA
EcoR1 cloning site
B1
B1
P1
H3
X1
P1
P2
H3
P1
P1
P1
EcoR1 cloning site
0
0.5
1.0
1.5
2.0
2.5
Xho1/EcoR1 Probe (1003
5' CCACCAGCCCACCTATCCA 3'
3' GGGTCGTCGTTCGTATTAT 5'
b
Human cDNA
EcoR1 cloning site
H3
EcoR1 cloning site
0
0.5
1.0
1.5
2.0
P1 PstI
H3 HindIII
P2 PvuII
X1 XhoI
B1 BamHI
Amino-Terminal Probe (668 bp)
Carboxy -Terminal Probe (435 bp)
0.1 kb

Figure 2

Sequence and predicted structure of mouse schwannomin. (a) Nucleotide sequence of the mouse *NF2* gene and its deduced amino acid sequence. The numbers correspond to the nucleotide (upper line) and amino acid (lower line) sequence. The amino acid sequence with homology to L1Rn retroposon is underlined and the amino acid residues that are different from the human sequence are boxed. The 5' untranslated sequence with homology to the mouse BCR gene promoter region is shown from nucleotide 1-576. (b) Hydrophilicity profile showing no long stretches of hydrophobic residues. Analysis was done using Kyte-Doolittle (26) scale with a window size of seven. (c) Predicted secondary structure based on Chou-Fasmann and Robson-Garnier algorithms (27,28) using MacVector sequence analysis program. Chou-Fasmann (CF), Robson-Garnier (RG), combination of both algorithms (CFRG).

a

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                                                                                CCG CCA ATC ACA GTA TCT TAG AGA AAA AAG TGT TAG     36
GGT GAG TCG GTG AGT AGC GTA GTG GGA GTC CGG AGG CGG GGC GGG GCG GGG CGG GGA TCC GGC AGT CGC TGA GGT GGG GGT TGG TAG AGG    126
ACG CTT CCC GAG GGC GCG CGG ACG AGC TGG TGA CAG ACA CGC ACA CAC GTG TGC GCT CCC AAC TAC TGC GCG GCC CCG GCC CCG GCC CCG    216
GCA CCC TCG CGG GGG TCG CTG AGA GGC CCG GCG TCG GCG CCG CAG CCG TCT AGG ATT TCT GTC CAA AGG CGT CCC TGG CGT CCC CAG CCC    306
GAA GCT CCA AGC TCG GGG CTG CGC GTG CTT TTG CCG GCA TCT CCC CCC GAC CCT CCC GTC GTC AGA GAC TGT CCC TTA ACT CAT CTT TCC    396
GCA CCC ACA GCC GGG TCC TCG CGG CCC ATG CTG GCC GCT GGG GAC CCA GGC AGC CTA GGT CTG TCT CGG GCC GGC CAG CAC GCC ATG GTG    486
GCC CTG AGG CCT GTG CAG CTA CTC CAG GGG GGC TAA GAG ACC CAG AAC GCG GGC TAG GGG TGA GGG GAT CCA AGG CCG GTA CCC CGC GCC    576

ATG GCC GGA GCC ATC GCT TCT CGC ATG AGC TTC AGC TCA CTC AAG AGG AAG CAG CCC AAG ACA TTC ACG GTG CGG ATC GTC ACC ATG GAC    666
MET Ala Gly Ala Ile Ala Ser Arg Met Ser Phe Ser Ser Leu Lys Arg Lys Gln Pro Lys Thr Phe Thr Val Arg Ile Val Thr Met Asp     30

GCC GAG ATG GAG TTC AAC TGC GAG ATG AAA TGG AAG GGG AAG GAC CTG TTT GAT TTG GTG TGC CGG ACA CTG GGG CTT CGG GAA ACC TGG    756
Ala Glu Met Glu Phe Asn Cys Glu Met Lys Trp Lys Gly Lys Asp Leu Phe Asp Leu Val Cys Arg Thr Leu Gly Leu Arg Glu Thr Trp     60

TTC TTT GGA CTG CAG TAT ACA ATC AAG GAC ACG GTG GCC TGG CTC AAA ATG GAC AAG AAG GTG TTG GAT CAT GAT GTT TCG AAG GAA GAA    846
Phe Phe Gly Leu Gln Tyr Thr Ile Lys Asp Thr Val Ala Trp Leu Lys Met Asp Lys Lys Val Leu Asp His Asp Val Ser Lys Glu Glu     90

CCA GTT ACC TTT CAC TTC CTG GCC AAA TTT TAT CCT GAA AAT GCT GAG GAG GAG CTA GTT CAA GAG ATC ACG CAA CAC TTA TTT TTC TTA    936
Pro Val Thr Phe His Phe Leu Ala Lys Phe Tyr Pro Glu Asn Ala Glu Glu Glu Leu Val Gln Glu Ile Thr Gln His Leu Phe Phe Leu    120

CAG GTG AAG AAG CAG ATT TTG GAT GAA AAG[GTC]TAC TGC CCT CCC GAG GCG TCC GTG CTC TTG GCG TCA TAT GCT GTC CAG GCC AAG TAT   1026
Gln Val Lys Lys Gln Ile Leu Asp Glu Lys[VAL]Tyr Cys Pro Pro Glu Ala Ser Val Leu Leu Ala Ser Tyr Ala Val Gln Ala Lys Tyr    150

GGC GAC TAT GAC CCC TCT GTG CAC AAG CGG GGA TTT TTA GCC CAA GAG GAA TTG CTC CCG AAA AGG GTG ATA AAT CTC TAT CAG ATG ACT   1116
Gly Asp Tyr Asp Pro Ser Val His Lys Arg Gly Phe Leu Ala Gln Glu Glu Leu Leu Pro Lys Arg Val Ile Asn Leu Tyr Gln Met Thr    180

CCG GAA ATG TGG GAG GAG AGA ATT ACG GCT TGG TAT GCG GAA CAC CGG GGC AGA GCC AGG GAT GAA GCT GAA ATG GAG TAT TTG AAG ATA   1206
Pro Glu Met Trp Glu Glu Arg Ile Thr Ala Trp Tyr Ala Glu His Arg Gly Arg Ala Arg Asp Glu Ala Glu Met Glu Tyr Leu Lys Ile    210

GCT CAG GAC CTG GAG ATG TAT GGT GTG AAC TAC TTT[ACA]ATC CGG AAT AAA AAG GGC ACA GAG TTG CTG CTT GGA GTG GAT GCT CTT GGG   1296
Ala Gln Asp Leu Glu Met Tyr Gly Val Asn Tyr Phe[THR]Ile Arg Asn Lys Lys Gly Thr Glu Leu Leu Leu Gly Val Asp Ala Leu Gly    240

CTT CAT ATC TAT GAC CCT GAG AAC AGG CTG ACC CCC AAG ATC TCC TTC CCA TGG AAT GAA ATC CGA AAC ATC TCC TAC AGC GAC AAG GAG   1386
Leu His Ile Tyr Asp Pro Glu Asn Arg Leu Thr Pro Lys Ile Ser Phe Pro Trp Asn Glu Ile Arg Asn Ile Ser Tyr Ser Asp Lys Glu    270

TTT ACT ATT AAA CCA CTG GAT AAG AAA ATT GAT GTC TTC AAA TTT AAC TCC TCA AAG CTT CGT GTT AAT AAG CTG ATT CTT CAG CTA TGT   1476
Phe Thr Ile Lys Pro Leu Asp Lys Lys Ile Asp Val Phe Lys Phe Asn Ser Ser Lys Leu Arg Val Asn Lys Leu Ile Leu Gln Leu Cys    300

ATT GGG AAC CAT GAC CTA TTT ATG AGG CGA CGG AAA GCT GAC TCT TTA GAA GTT CAG CAG ATG AAA GCC CAG GCC AGG GAA GAG AAG GCT   1566
Ile Gly Asn His Asp Leu Phe Met Arg Arg Arg Lys Ala Asp Ser Leu Glu Val Gln Gln Met Lys Ala Gln Ala Arg Glu Glu Lys Ala    330

AGA AAG CAG ATG GAA AGG CAG CGG CTG GCT CGA GAG AAG CAG ATG CGG GAG GAG GCC GAG CGT ACA AGA GAT GAG TTA GAG AGG AGG CTC   1656
Arg Lys Gln Met Glu Arg Gln Arg Leu Ala Arg Glu Lys Gln Met Arg Glu Glu Ala Glu Arg Thr Arg Asp Glu Leu Glu Arg Arg Leu    360

CTG CAG ATG AAA GAA GAA GCA ACG ATG GCC AAT GAA GCT CTG ATG CGC TCT GAG GAG ACA GCT GAT CTG TTG GCT GAA AAG GCT CAG ATC   1746
Leu Gln Met Lys Glu Glu Ala Thr Met Ala Asn Glu Ala Leu Met Arg Ser Glu Glu Thr Ala Asp Leu Leu Ala Glu Lys Ala Gln Ile    390

ACA GAG GAG GAG GCC AAG CTT TTG GCA CAA AAG GCT GCA GAG GCT GAG CAA GAG ATG CAG CGA ATC AAG GCC ACG GCC ATT CGG ACA GAG   1836
Thr Glu Glu Glu Ala Lys Leu Leu Ala Gln Lys Ala Ala Glu Ala Glu Gln Glu Met Gln Arg Ile Lys Ala Thr Ala Ile Arg Thr Glu    420

GAG GAG AAG CGC CTG ATG GAG CAG AAG GTG CTG GAG GCT GAA GTG CTG GCA TTG AAG ATG GCT GAG GAG TCA GAG AGG AGG GCC AAG GAG   1926
Glu Glu Lys Arg Leu Met Glu Gln Lys Val Leu Glu Ala Glu Val Leu Ala Leu Lys Met Ala Glu Glu Ser Glu Arg Arg Ala Lys Glu    450

GCT GAT CAG TTA AAG CAA GAC TTG CAA GAA GCC CGA GAA GCA GAG CGA AGA GCC AAG CAG AAG CTC TTA GAA ATC GCC ACC AAG CCC ACC   2016
Ala Asp Gln Leu Lys Gln Asp Leu Gln Glu Ala Arg Glu Ala Glu Arg Arg Ala Lys Gln Lys Leu Leu Glu Ile Ala Thr Lys Pro Thr    480

TAT CCA CCC ATG AAC CCA ATT CCA[CCA]CCA CTG CCT CCT GAC ATA CCG AGC TTC[GAC][ATT]ATT[GCT]GAC AGC TTG TCA TTC GAC TTC AAG   2106
Tyr Pro Pro Met Asn Pro Ile Pro[PRO]Pro Leu Pro Pro Asp Ile Pro Ser Phe[ASP][ILE]Ile[ALA]Asp Ser Leu Ser Phe Asp Phe Lys    510

GAT ACG GAC ATG AAG CGA CTT TCC ATG GAG ATA GAG AAA GAA AAA GTG GAG TAC ATG GAG AAG AGC AAG CAC CTG CAG GAG CAG CTC AAC   2196
Asp Thr Asp Met Lys Arg Leu Ser Met Glu Ile Glu Lys Glu Lys Val Glu Tyr Met Glu Lys Ser Lys His Leu Gln Glu Gln Leu Asn    540

GAG CTC AAG ACT GAG ATC GAG GCC TTG AAA CTC AAA GAG CGG GAG ACG GCC TTG GAC[GTC]CTA CAC[AGC]GAG[AGC]TCA GAC AGA GGC GGC   2286
Glu Leu Lys Thr Glu Ile Glu Ala Leu Lys Leu Lys Glu Arg Glu Thr Ala Leu Asp[VAL]Leu His[SER]Glu[SER]Ser Asp Arg Gly Gly    570

[CCC]AGC AGC AAG CAT AAT ACC ATT AAA AAG CTC ACT CTG CAG AGC GCC AAG TCC CGA GTG GCC TTC TTT GAA GAA CTC TAG CAG GTG ACC   2376
[PRO]Ser Ser Lys His Asn Thr Ile Lys Lys Leu Thr Leu Gln Ser Ala Lys Ser Arg Val Ala Phe Phe Glu Glu Leu  .                 596

CGG CCA CCT CCT GCC AAC ATC TGC TGC TCC TGA CAC CAA CAG GAT GGG CCT GAC CCA AAA GGA ACC ATC AGT AGA GGG CTG GCT TGT TTG   2466
GGA ACT CTT GAG TTG AGG GCC CCG TGC CAC TCT GAC CCT ATA GAG AGG TTT CTC AAT GTG TTC TAG TTC TCC TTG CCT CCT CGA TAC CTG   2556
CAT ATC TCT CTC TCC CTC CCT CTC TCT CTC TCT CCT TCT CG                                                                    2597
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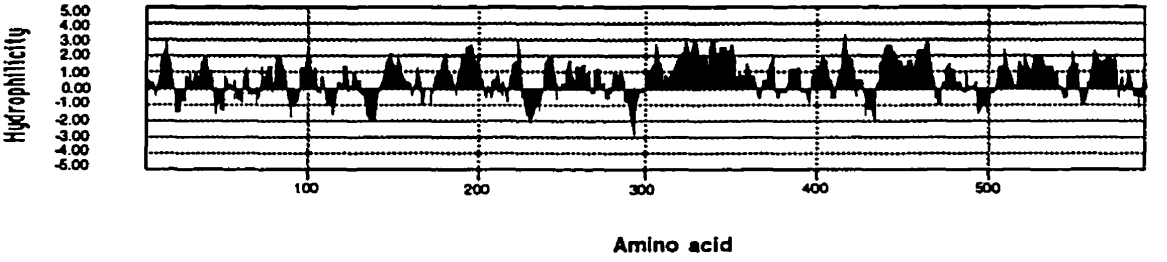
b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5.00
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
-5.00
100
200
300
400
500
Amino acid

c

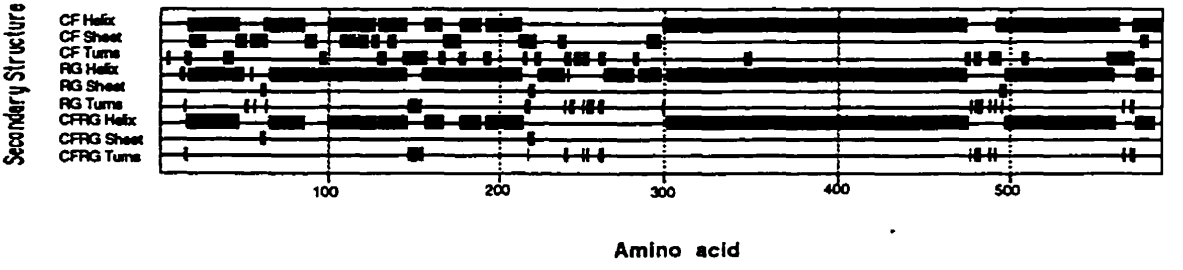

Secondary Structure
CF Helix
CF Sheet
CF Turns
RG Helix
RG Sheet
RG Turns
CFRG Helix
CFRG Sheet
CFRG Turns
100
200
300
400
500
Amino acid

Figure 3

Single letter amino acid sequence alignment of mouse and human schwannomin (11) mouse radixin, moesin and ezrin (29,30). Gaps were introduced to maximize alignment of the sequences. Conserved residues are boxed.

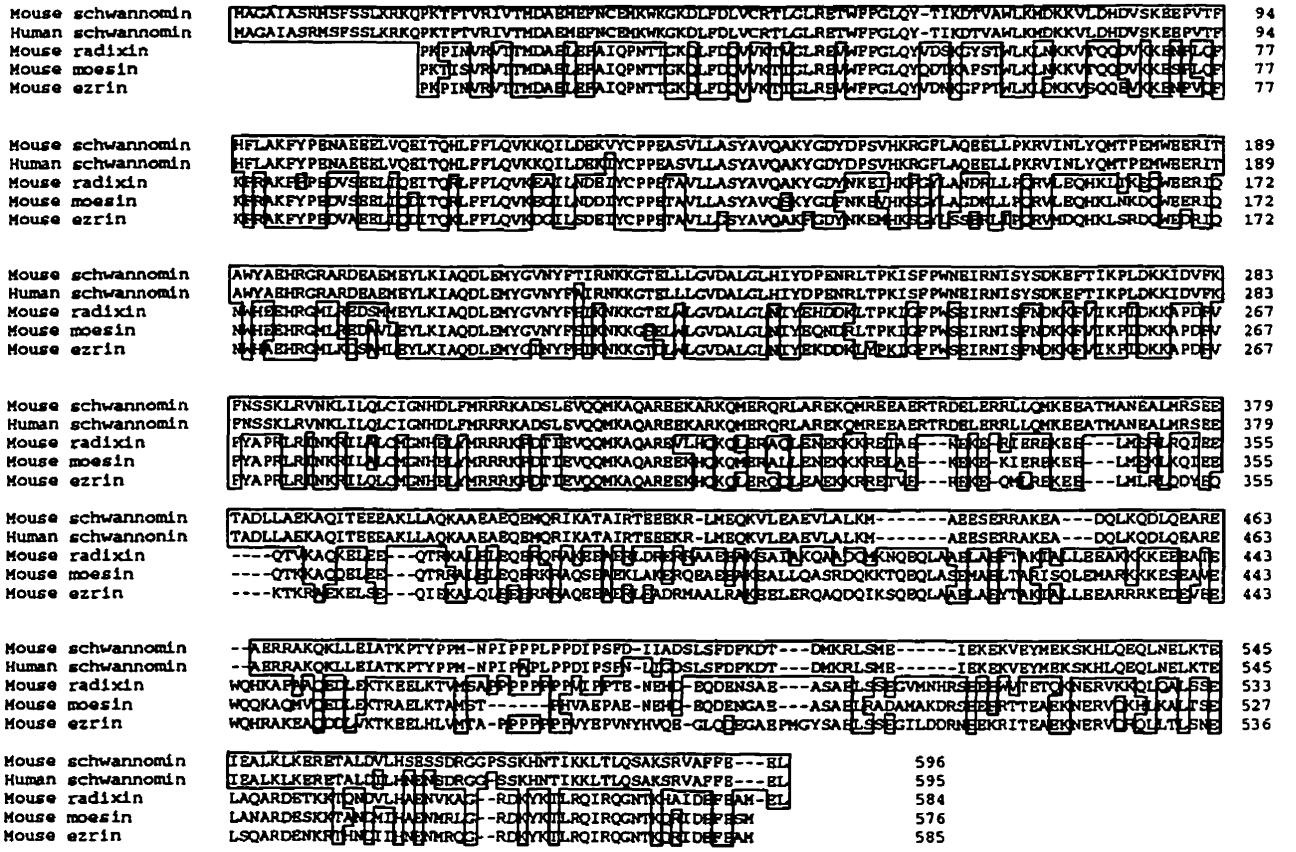
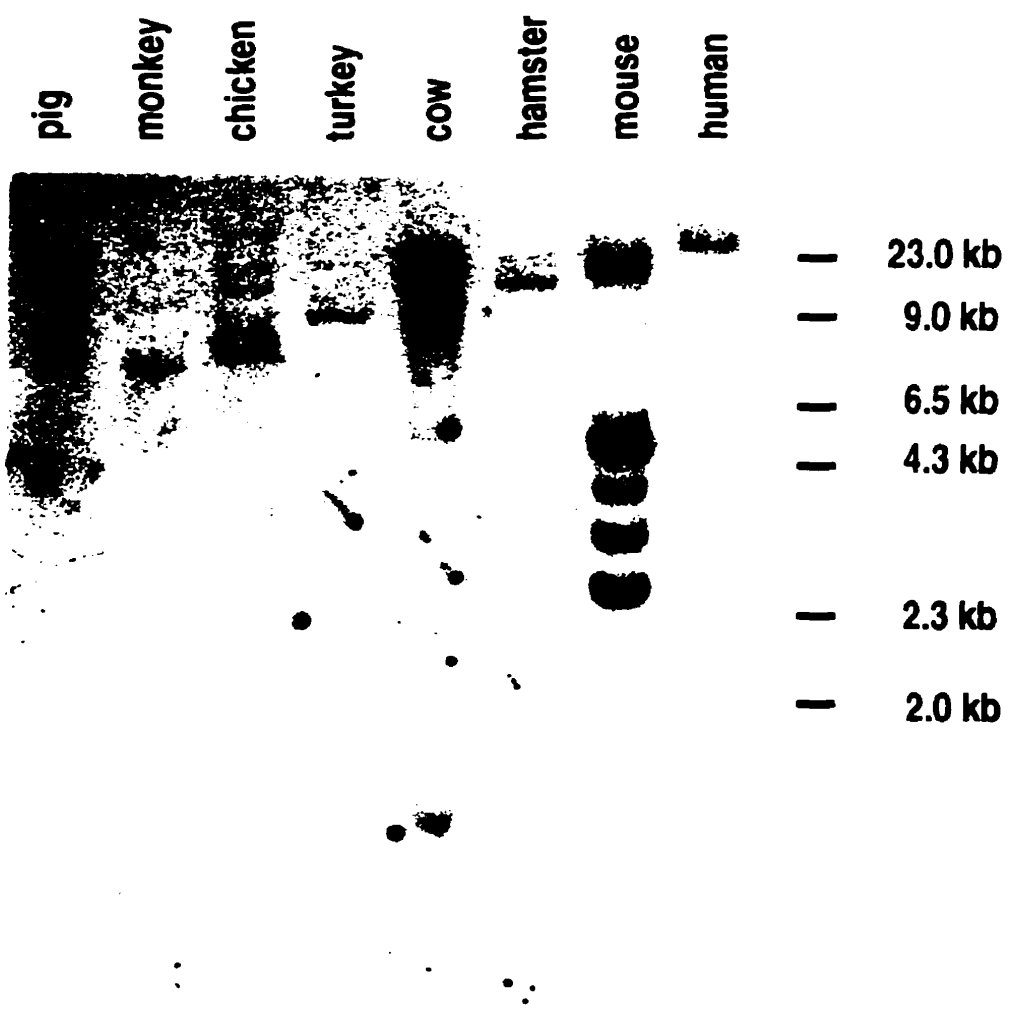

Mouse schwannomin
Human schwannomin
Mouse radixin
Mouse moesin
Mouse ezrin

Figure 4

Phylogenetic conservation of the *NF2* gene in vertebrates. 10 μg of genomic DNA was digested with EcoR1 and run on 1% agarose gel. DNA was transferred to nylon membrane and probed at high stringency with the carboxy-terminal region of the mouse cDNA (See Materials and Methods section).


pig
monkey
chicken
turkey
cow
hamster
mouse
human
23.0 kb
9.0 kb
6.5 kb
4.3 kb
2.3 kb
2.0 kb

Chapter 3*

MOUSE ELEVEN, HUMAN TWENTY TWO

The high conservation of the mouse gene extends further to its chromosome location. By characterizing a dinucleotide repeat polymorphism within the mouse *Nf2* gene, the data presented in this chapter established the location of *Nf2* to the proximal end of mouse chromosome 11, at a small region of conserved synteny to human chromsome 22. The predictive value of comparative mapping using recombinant inbred strain of mice is also discussed.

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The Mouse Neurofibromatosis Type 2 Gene Maps to Chromosome 11

ABSTRACT

Neurofibromatosis type 2 (NF2) is a dominantly inherited disease characterized by the development of bilateral vestibular schwannomas and meningiomas, which together represent 30% of primary brain tumors. The *NF2* gene, which has recently been isolated, maps to the long arm of human chromosome 22. Using recombinant inbred mice, we have determined the chromosomal position of the mouse homologue of the *NF2* gene. Analysis of the allele distribution in AKXD recombinant inbred strains using a simple sequence repeat polymorphism (*D11Mcg1*) in the 3' untranslated region of the mouse cDNA, maps the mouse *NF2* gene to the proximal region of chromosome 11, closely linked to *Pmv-2*. This region also contains the genes for leukemia inhibitory factor and neurofilament heavy chain polypeptide and so represent a region of conserved synteny between human chromosome 22 and mouse chromosome 11. Using additional polymorphic markers, we established the following locus order from the centromere: *D11Mit1/D11Mit72/D11Mcg1-D11Mit74- Pmv-2 - D11Mit2-D11Mit77/D11Mit78/D11Mit63.*

Neurofibromatosis type 2 (NF2) is an autosomal dominant disorder predisposing to the development of central nervous system tumors. The hallmark of NF2 is the frequent occurence of bilateral vestibular schwannomas. Other neoplasms seen in NF2 include cranial and spinal meningiomas, nerve root schwannomas, brainstem and spinal cord gliomas and ependymomas (8,9). Juvenile cataracts may also occur in up to 80% of cases (7,9). In individuals who have inherited an inactive copy of the *NF2* gene, somatic inactivation of the otherwise normal homologue leads to tumor formation. Thus, the *NF2*

gene is recessive at the cellular level, is a tumor suppressor, and displays a dominant pattern of transmission in patients.

The human *NF2* gene maps to the long arm of chromosome 22 (10) within a syntenic chromosomal segment corresponding to the proximal portion of mouse chromosome 11 (1,2). Recently, the human *NF2* gene was positionally cloned between the genes for the leukemia inhibitory factor (*LIF*) and the neurofilament heavy chain polypeptide (*NEFH*) (11,12,14). The predicted *NF2* protein, which we named schwannomin (SCH), based on the characteristic tumors seen on NF2 patients, belongs to a gene family that is thought to be involved in membrane-cytoskeleton organization.

We have recently isolated the mouse homologue of the *NF2* gene and found a high degree of sequence conservation to the human gene (3). Evidence of conservation across many species including monkey, pig, cow, hamster and rat was also detected by zoo blot hybridization. Our mouse brain cDNA clone, AB711.1, which contained the complete 1788 bp open reading frame of the mouse *NF2* gene showed 90% nucleotide sequence identity to the coding region of the human gene (3). AB711.1 also contained 576 bp of 5' untranslated sequence (UTS) and 233 bp of 3' UTS. Interestingly, we observed the presence of a series of dinucleotide $(CT)_n$ repeat within the 3' UTS of this clone. To characterize this dinucleotide repeat further, we sequenced this region in additional clones which hybridized to a probe containing the carboxy-terminal region of the human cDNA (3). One clone, AB211.2, contained the entire dinucleotide repeat and flanking sequences. Further sequencing revealed this repeat to contain the sequence $(CT)_4CCCTCC(CT)_7CCCTT(CT)_{30}$. The mouse *NF2* locus defined by this simple sequence repeat (SSR) was named *D11Mcg1* according to standard nomenclature (5) and the mouse gene was designated *Nf2*. Oligonucleotide primers flanking the repeat were used to amplify by polymerase chain reaction (PCR) the corresponding genomic DNA fragments from inbred mouse strains AKR/J, DBA/2J, C57L/J, C3H/HeJ and C57BL6/J. The length of the PCR-amplified SSR was 292 bp in AKR/J and 288 bp in the 4 other

strains tested.

To determine the chromosomal location of *Nf2* in the mouse, the strain distribution pattern (SDP) of *D11Mcg1* was established in 24 independent recombinant inbred (RI) strains derived from AKR/J and DBA/2J cross (AKXD) (Fig.1a, Table 1). Figure 1a shows the PCR-amplified DNA using *D11Mcg1* in AKXD RI strains. Analyses of the SDP of AKXD RI strains revealed linkage of *Nf2* with mouse chromosome 11 markers. In 24 RI strains, there were two recombinants between *D11Mcg1* and *Pmv-2* (6). These data localize *Nf2* on mouse chromosome 11. The maximun likelihood estimate of the recombination frequencies (13) places *Nf2* at a distance of 2.4 cM (with a 95% confidence limit of 0.3-11.3 cM) from *Pmv-2*. To map more precisely the *Nf2* locus on mouse chromosome 11, we typed 7 additional DNA PCR-based markers (Mouse Map Pairs, Research Genetics) which are informative in AKXD RI strains (Table 1). Minimizing the number of double recombinants in the RI strains analyzed suggests the interlocus distances and order as: (***D11Mit1/D11Mit72/D11Mcg1***)-1.1 cM-***D11Mit74*** -1.1 cM-***Pmv-2***-2.5 cM-***D11Mit2***-2.5 cM-(***D11Mit77/D11Mit78/D11Mit63***). No recombination was detected between *D11Mcg1* and *D11Mit1/D11Mit72* indicating tight linkage of these markers to *D11Mcg1*. More interestingly, the mouse locus carrying *Nf2* is part of a conserved region with human chromosome 22. This syntenic region includes two SCH flanking loci *LIF* and *NEFH* (1,2,11,12).

In order to determine if *D11Mit1/D11Mit72* and *D11Mcg1* polymorphisms are distinct, we hybridized the dinucleotide $(CT)_n$ repeat from clone AB211.1 to the PCR product of markers *D11Mit1*, *D11Mit72* and *D11Mcg1* from AKR/J and DBA/2J genomic DNA. The $(CT)_n$ repeat hybridized to *D11Mcg1* PCR product but not to either *D11Mit1* or *D11Mit72* (data not shown). This suggests that *D11Mcg1* is distinct from *D11Mit1* and *D11Mit72*. Further, comparison of the SSR amplified by *D11Mit1* and *D11Mit72* to *D11Mcg1* obtained from the genetic map of the mouse database confirmed the distict identity of the 3 SSR polymorphic markers.

While typing the alleles for markers *D11Mit2* and *D11Mit63*, we identified two AKXD RI strains (3 and 23) whose alleles differed from either the A (AKR/J) or the D (DBA/2J) allele (see Table 1) by a single repeat unit. Observation of such mutant alleles is not uncommon to SSR loci which have an estimated mutation rate of 10^{-4} to 10^{-3} per locus per gamete per generation for both human and mouse chromosomes (4,15).

Our data demonstrate the predictive value of comparative mapping. The localization of *Nf2* on the most proximal region of mouse chromosome 11 confirms the existence of a small area of synteny with the chromosome segment carrying SCH on human chromosome 22. It is therefore reasonable to assume that other genes mapping to the region such as Ewing sarcoma (11,12) and the novel anonymous gene, pK1.3, which was cloned in the immediate centromeric vicinity of the human *NF2* gene (16) may also map to the same proximal region of mouse chromosome 11. The polymorphic marker *D11Mcg1* should be useful for the mapping of these genes and other closely linked loci in the mouse.

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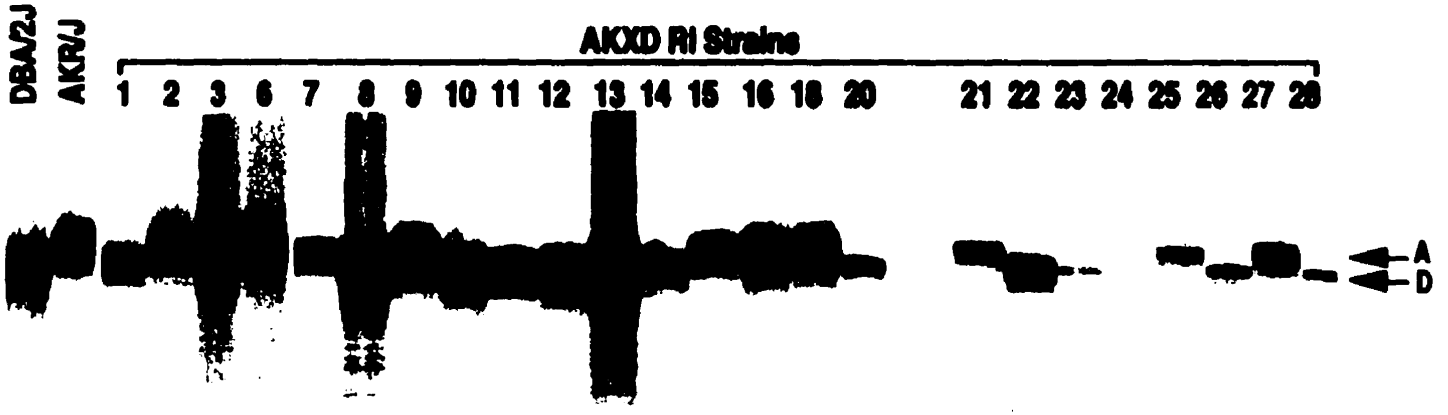
Figure 1. Mapping of the *Nf2* locus to mouse chromosome 11.

(A) *D11Mcg1* strain distribution pattern of AKXD RI strains. Genomic DNA (100ng) was amplified using 100 ng each of forward primer 5'-CTGGCTTGTTTGGGAACTCT-3' and reverse primer 5'-CTCCCACAGCCGATTCTCAAT-3' in a total reaction volume of 12.5 µl containing 100 µM dATP, 200 µM each of dTTP, dGTP, dCTP and 1x Taq buffer (Bio/can Scientific) (1.5 mM $MgCl_2$). The PCR reaction was denatured at 94°C for 10 min before 1U of Taq DNA polymerase (Promega) and 2.5 µCi of ^{35}S-dATP (1000 Ci/mmol) were added at 72°C. Thermal cycling condition was 35, 3-step cycles at 94°C for 1 min, 60°C for 45 sec and 72°C for 1 min. The PCR product was analyzed in denaturing 6% acrylamide gel containing 7M urea. PCR product amplified from AKR/J (A) and DBA/2J (D) DNAs were sized according to their respective migration compared to M13 sequencing reaction.

(B) Position of the *Nf2* locus on chromosome 11 based on data from AKXD RI strains shown in Table 1. Centromere is indicated by a circle. Recombination distances are in centimorgan (cM) and are shown to the left of the chromosome. The percent recombination in a single meiosis between 2 loci was estimated at upper and lower 95% confidence interval (13).

A


DBA/2J
AKR/J
AKXD RI Strains
1 2 3 6 7 8 9 10 11 12 13 14 15 16 18 20 21 22 23 24 25 26 27 28
A
D

B

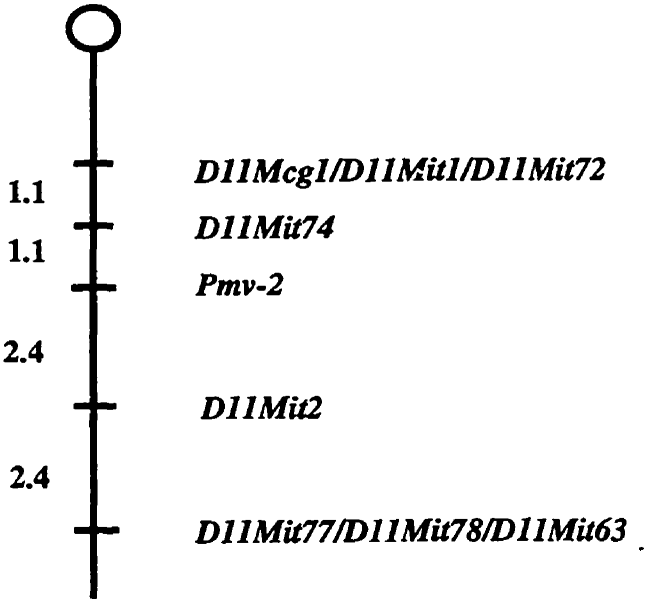

1.1
1.1
2.4
2.4
D11Mcg1/D11Mit1/D11Mit72
D11Mit74
Pmv-2
D11Mit2
D11Mit77/D11Mit78/D11Mit63

Table1. Segregation of D11Mcg1 and chromosome 11 loci in AKXD recombinant inbred mice.

	AKXD																								
Locus	1	2	3	6	7	8	9	10	11	12	13	14	15	16	18	20	21	22	23	24	25	26	27	28
D11Mit1	D	A	A	A	A	D	A	D	D	D	D	D	A	A	A	D	A	D	D	D	A	D	A	D
D11Mit72	D	A	A	A	A	D	A	D	D	D	D	D	A	A	A	D	A	D	D	D	A	D	A	D
D11Mcg1	D	A	A	A	A	D	A	D	D	D	D	D	A	A	A	D	A	D	D	D ×	A	D	A	D
D11Mit74	D	A	A	A	A	D	A	D	D	D	D	D	A	A	A	D	A ×	D	D	A	A	D	A	D
Pmv-2	D	A	A	A	A	D ×	A	D	D	D	D	D	A ×	A	A	D	D	D	D	A	A	D	A	D
D11Mit2	D	A	A	A	A	A	A	D	D	D ×	D	D	D	A	A	D	D	D	*	A ×	A	D	A	D
D11Mit77	D	A	A	A	A	A	A	D	D	A	D	D	D	A	A	D	D	D	D	D	A	D	A	D
D11Mit78	D	A	A	A	A	A	A	D	D	A	D	D	D	A	A	D	D	D	D	D	A	D	A	D
D11Mit63	D	A	*	A	A	A	A	D	D	A	D	D	D	A	A	D	D	D	D	D	A	D	A	D

Strain specific alleles are abbreviated as A (AKR/J) and D (DBA/2J).

Mutant alleles that differed from both A and D are identified with asterisk (*).

Pmv-2 have been typed previously (Frankel et al., 1989).

CHAPTER 4*
SELECTIVE WIZARDRY

By studying the expression of the mouse *Nf2* gene at the RNA and protein level, it should be possible to get insights into the expression of the human gene. This chapter provides this information and presents evidence that there is an apparent selective expression of the *Nf2* protein, schwannomin, despite its widespread expression seen by Northern and Western analysis. Hypotheses as to why tumors occur only in Schwann cells despite the expression of the protein in other cells are presented.

*The manuscript that follows is reproduced with permission. Claudio, J.O., Lutchman, M. and Rouleau, G.A. (1995) Widespread but cell type-specific expression of the mouse neurofibromatosis type 2 gene. *Neuroreport* **6**: 1942-1946.

Widespread but cell type-specific expression of the mouse Neurofibromatosis type 2 gene.

ABSTRACT

Neurofibromatosis type 2 (NF2) is a human autosomal dominant disease in which mutations of the *NF2* gene lead to the development of schwannomas, meningiomas and juvenile cataracts. We studied the mouse *NF2* homologue (*Nf2*) to determine its precise pattern of mRNA and protein expression. *In-situ* hybridization showed that *Nf2* is expressed by neuronal cells in the nervous system as well as in epithelial and fiber cells of the lens. The *Nf2* protein, schwannomin, is expressed as a single protein isoform of ~80 kDa in neuronal and non-neuronal tissues. In Purkinje cells of the cerebellum and motor neurones of the spinal cord, the protein is localized in the cytoplasm. In non-neuronal tissues immunostaining revealed expression in cells of the tunica intima of blood vessels. We conclude that there is a widespread but cell type-specific expression of schwannomin.

Key words: schwannomin, merlin, band 4.1 family, tumour suppressor, ezrin, radixin, moesin, neurofibromatosis

INTRODUCTION

Neurofibromatosis type 2 (NF2) is an autosomal dominant disorder affecting primarily the nervous sytem. It is characterized by the development of bilateral vestibular schwannomas, meningiomas and juvenile posterior lenticular opacities.[1-3] Ependymomas and gliomas may also occur albeit less frequently.[2,4] We and others have previously reported high sequence conservation of the mouse homologue of NF2 [5-7] and have mapped the gene to the proximal region of mouse chromosome 11.[8] The high amino acid sequence homology between the human and mouse gene (*Nf2*) suggests functional conservation of its protein product which we called *schwannomin* (from the characteristic tumour seen in NF2). Hence, we focused our studies on the mouse *Nf2* gene to gain insights into the expression of schwannomin.

Mouse schwannomin is a 596 amino acid protein that belongs to a gene family whose products act as structural linker between plasma membrane protein and the cytoskeleton. This family includes ezrin, radizin and moesin (ERM) which all have ~62% homolgy to the amino-terminal half of schwannomin.[5,9,10] Despite the sequence homologies, the function of schwannomin has yet to be determined.

Nf2 encodes a 4.5 kb transcript that is ubiquitously expressed[5] and could be alternatively spliced.[6,7,11] The functional significance of the alternative splicing is still not clear because no protein variant has yet been reported. We analysed the expression of *Nf2* in the mouse and show that although it is expressed in all tissues, it is present only in a subset of cells. We demonstrated by *in-situ* hybridization that the mRNA for schwannomin is expressed by neuronal cells in the nervous system as well as in epithelial and fiber cells of the lens. By immunoflourescence, distinctly cytoplasmic staining can be seen in Purkinje cells of the cerebellum, in motor neurones of the spinal cord and in goblet cells of the intestine. We also showed that the widespread expression seen by multiple tissue Northern and Western analyses may be due to expression of the gene in blood vessels.

MATERIALS AND METHODS

Preparation of tissue sections and riboprobe: Tissues for *in situ* hybridizations were prepared from paraformaldehyde-perfused C3H mice. Sections (10μm) were cut on a cryostat, mounted on to Probe-On slides (Fisher) and stored at -80°C until used for *in situ* hybridization. For immunohistochemistry, methanol-acetone (50%-50%)-fixed tissues were used immediately after sectioning.

Digoxigenin-labelled RNA probe was derived from the 3'-region of the mouse *Nf2* cDNA (1635-2305, numbering according to ref. 5) and would recognize all the published alternative transcripts of *Nf2*. The probe was prepared from a 670 bp PCR product subcloned into pBluescript KS. The orientations of the subclones were determined by sequencing using USB Sequencing Kit. Digoxigenin-labelled sense and antisense riboprobes were synthesized by *in vitro* transcription using the T7 promoter. The RNA product was digested with DNAse (10 units) for 15 min and precipitated with ethanol. Probes were stored at -80°C until used.

In situ hybridization: Prior to Proteinase-K (5μg/10 ml TE, pH 8.0)(Boehringer Mannheim) treatment for 10-15 min at room temperature, sections were air dried for at least 30 min. Slides were acetylated in 0.25% acetic anhydride for 15 min at room temperature, rinsed with 2x SSC and dehydrated in graded series of alcohol. Hybridization was carried out at 60°C in a box humidified with 2x SSC for at least 16 hours. The hybridization buffer consisted of 50% formamide, 300 mM NaCl, 20 mM Tris pH 8.0, 1 mM EDTA, 1x Denhardt's, 50% dextran sulfate and 100 μg/ml yeast tRNA. After hybridization, the sections were treated with RNAse A (20 μg/ml) for 1 hour at 37°C then washed with 2x

SSC, 1x SSC and 0.5x SSC for 15 minutes each at room temperature. Final washing was done for 1 hour at 60°C using 0.1x SSC.

Sections were preblocked with 2x SSC, 0.05% Triton X-100, 2% normal sheep serum for 1 hour then washed twice with TS buffer (0.1M Tris pH 7.4, 150 mM NaCl) for 3 min each. The alkaline phosphatase conjugated anti-digoxigenin antibody (Boehringer Mannheim)(1:500 in TS buffer plus 1% normal sheep serum, 0.3% Triton X-100) reaction was done by incubation overnight at 4°C. Positively expressing cells were detected using NBT/BCIP substrate (Sigma) for not more than 2 hours in the dark.

Immunofluorescence and immunoblot analysis : The antibody used was developed against a 30-amino acid peptide at the carboxy-terminal end of human schwannomin. The generation, characterization and specificity of the rabbit anti-schwannomin antibody are described elsewhere.[12]

Tissues for immunoblotting were dissected from C3H mice perfused with PBS. Prior to homogenization in buffer containing Nonidet P-40 (100 mM KCl, 1 mM $MgCl_2$, 100 mM Hepes pH 7.5, 0.1% NP40), tissues were washed with at least three changes of PBS. Fifteen micrograms of SDS and heat-denatured supernatants from tissue homogenates were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane using BioRad Blotting apparatus. The membrane was blocked using 10% nonfat dry milk in TBS/Tween 20 (0.2%) at room temperature for 2 hours. The primary antibody (rabbit anti-schwannomin antibody diluted 1:300 in 5% dry milk in TBS) was incubated at 4°C overnight. Thereafter, the membrane was washed with 5% dry milk in TBS 3 times for 15 min each followed by two 10 min washes using TBS. Incubation using the secondary antibody (alkaline phosphatase-congugated goat anti-rabbit IgG diluted 1:5000) was performed at room temperature for 1 hour. The membrane was washed 3 times for 15 min each with TBS and developed using NBT/BCIP (Sigma) for 5-10 min.

For immunofluorescence, sections were permeabilized with 0.2% Triton X-100 in PBS for 15 min then blocked for another 15 minutes with 3% BSA in PBS. The primary antibody, rabbit anti-schwannomin antibody (1:200) in 3 % BSA/PBS was added to the sections and incubated for 30 min at 37°C. Thereafter, fluorescein-conjugated goat anti-rabbit IgG (1:100) was added after three 5 min washes in 3% BSA/PBS. After further incubation at 37°C for 30 min, the sections were washed 5 times for 5 min each and mounted using SlowFade (Molecular Probes, Inc.) mounting medium. Fluorescence was observed under Polyvar photomicroscope and photographs were taken using Kodak Ektachrome P1600.

RESULTS

mRNA expression by in situ hybridization: We looked for schwannomin mRNA expression in cell population of the nervous system and of the lens. Due to the high sequence conservation of the members of the ERM family, we chose a riboprobe derived from a region of the mouse *Nf2* cDNA with no significant sequence homology to either moesin, ezrin or radixin. A PCR product of 670 bp subcloned into pBluescript KS in sense and antisense orientations was transcribed *in vitro* using T7 promoter to generate a digoxigenin-labelled RNA probe. Adult mouse brain and spinal cord sections hybridized with antisense probe showed that schwannomin mRNA is widely distributed in the brain although some regions are more highly expressing than the others (Fig. 1A-C). Within the cerebellum, expression can be seen in the deep cerebellar nuclei (Fig1A). Cellular labelling was evident in the granular layer but not in the molecular layer; the Purkinje cells were distinctly labelled (Fig.1A, 1B, 1D). Cells of the entorrhinal cortex were also labelled (Fig 1B). In the hippocampus, the pyramidal neurones in the CA4 and CA3 regions were strongly labelled as well as the granule cells of the dentate gyrus (Fig. 1B, 1E). In the

thalamus, cells in the medial habenula expressed schwannomin mRNA distinctly (data not shown). The induseum griseum was also labelled (Fig 1C).

mRNA expression was seen throughout the gray matter of the spinal cord but not in the white matter. At higher magnification, the large motor neurone cell bodies were intensely stained (Fig. 1F). Significantly, staining could be observed in the cytoplasmic region confirming that the probe hybridized to mRNA in the cytoplasm and not to genetic materials in the nucleus.

Up to 80% of NF2 patients develop juvenile cataract. Hence, we used mouse lens to gain some insights on the expression of the *Nf2* mRNA in the lens. We chose lens from 16.5 days post coitum embryo because at this time in development the lens fiber cells have terminally differentated but less fragile than adult lens for sectioning. *In-situ* hybridization showed schwannomin mRNA mostly in the single layer of epithelial cells (Fig. 1G). There was no significant expression in the lens fibre cells except in the proliferating cells at the equatorial region. The same labelling pattern was observed in adult lens (data not shown).

Schwannomin expression: In order to determine the subcellular localization of schwannomin, we studied sections of the cerebellum and the spinal cord for immunoflourescence microscopy. Of particular interest were the Purkinje cells of the cerebellum and the large motor neurone cell bodies because both express the mRNA for schwannomin as shown by *in situ* hybridization. An anti-schwannomin antibody, derived from the carboxy-terminus epitope of human schwannomin but cross reacts with mouse *Nf2* protein due to the conservation of the epitope[12] was used. We observed strong immunostaining of Purkinje cells (Fig. 2A and 2B) and motor neuron cell bodies located at the ventral horn of the spinal cord (Fig. 2D) which were not seen using pre-immune controls. Staining was also observed in Schwann cells (not shown). Interestingly, staining can be observed only outside the nucleus suggesting cytoplasmic localization of the protein.

To test the expression of the protein in different tissues we used non-ionic detergent extract for immunoblotting. A predominant band that migrated at approximately 80 kD was detected in all tissues studied (Fig 3) which was absent in blots using pre-immune serum. The 80 kDa protein was expressed in both the brain and the spinal cord as well as in non-neural tissues, including lungs, heart, kidney, spleen and intestine. A lower level of expression was seen in the eye, lens, liver and skeletal muscles. The widespread expression pattern of the protein complements the ubiquitous expression of schwannomin mRNA in the mouse (Fig. 3). Immunohistochemical analysis of sections of heart, kidney and lung showed strong staining in the inner lining of blood vessels, particularly in the tunica intima which contains the endothelial lining (Fig. 2G and 2I). Cytoplasmic staining was also observed in the goblet cells of the intestine (Fig 2G). Weaker staining was detected in the smooth muscles lining the bronchus (Fig 2F), but in no other cells of the tissues tested.

DISCUSSION

In man, schwannomin is expressed in neurones of the cerebral cortex, neural crest derived cells including Schwann cells and endothelial cells.[12] Our data suggest a restricted pattern of expression of schwannomin in the mouse. However, despite this cell type-specific expression of schwannomin, these results contrast with the human disease, which does not involve blood vessels and neurones. It has recently been shown that sporadic and familial schwannomas are caused by loss of function mutations of the *NF2* gene. [9,13-16] Similarly, alteration of the *NF2* gene has been demonstrated as the cause of the majority of sporadic and NF2-associated meningiomas. [9,17] Screening for *NF2* gene mutations in tumours occuring outside the nervous system has not yielded significant evidence to show the involvement of this gene in the genesis of other tumours. Thus, the discrepancy

between expression of schwannomin and the phenotype in patients needs to be explained. There may be redundancy in the tumour suppression provided by schwannomin in neurones and endothelial cells. Alternatively, specific mechanisms may compensate for loss of the *NF2* gene in certain tissues. Finally, perhaps loss of schwannomin function is lethal in certain cells, and so complete loss of function leads to cell death.

The presence of a single 80 kDa band on immunoblotting suggests that only one protein isoform was detected by the antibody. When an antibody derived from the amino-terminal end of schwannomin was used, the same 80 kDa species was detected (data not shown). Similar results were observed in human tissues.[12] These data suggest that the different mRNA splice variants reported [5,6,10] may not be translated into proteins. Alternatively, our technique may not have detected the different isoforms if the splicing events result in expression of proteins with no significant molecular weight differences. Isoforms missing the carboxy-terminus epitope from which the antibody was developed may not also be detected.

In contrast to the tumours seen in NF2, lens opacities seem to represent a true dominant manifestation where a mutation in a single allele leads to cataracts. Our data showing expression of the *Nf2* gene in mouse lens epithelial and fibre cells is consistent with the development of lens opacities in NF2 patients. Immunoblot analysis detected the same 80 kDa protein in adult mouse lens as is seen in other tissues. These results suggest that no special schwannomin protein is produced in the lens which might explain this dominant manifestation. Compared with both the brain and the spinal cord, the level of protein in the lens is significantly lower, probably because it is expressed in only parts of the lens. Immunofluorescence using human lens confirmed the expression pattern in humans (data not shown). Further detailed expression studies of the mouse lens may yield a better understanding of the involvement of the protein in the development of juvenile cataract in NF2 patients.

Our immunofluorescence data show that schwannomin is localized in the cytoplasm of Purkinje cells and motor neurones. In rat primary schwann cell cultures, strong cytoplasmic staining was also observed (data not shown). Expression of the *NF2* protein in NIH 3T3 cells transfected with a human NF2 cDNA also showed cytoplasmic staining.[18] These data taken together support the hypothesis that schwannomin may act as membrane-cytoskeleton linker based on its sequence homology to ezrin, radixin and moesin.

In non-neuronal tissues, we observed strong staining of the walls of blood vessels in all tissues studied. Such staining pattern may explain the widespread expression seen in both RNA and protein analyses.

CONCLUSION

Our results show that although expression of the mouse *Nf2* is ubiquitous, there is an apparent cell-type specific pattern of expression. The expression of schwannomin in blood vessels may account for the widespread expression seen by northern and immunoblot analyses. The cytoplasmic localization of the protein provides indirect evidence supporting the hypothesis that schwannomin links plasma membrane proteins to the cytoskeleton.

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Figure 1

mRNA *in-situ* hybridization of mouse tissues using digoxigenin-labelled probes. A widespread but cell-type specific pattern of staining can be seen. **(A, B, C)** Brightfield photomicrographs of horizontal sections (10 μm) of mouse brain hybridized with antisense probe. In A, labelling is seen in the deep cerebellar nuclei (CN); in B, labelling is seen in the cells at the region of the entorrhinal cortex (EC), the dentate gyrus, and the CA4 and CA3 regions within the hippocampal formation (HF). The cells in the induseum griseum were also labelled (C). **(D, E, F, G)** Sections hybridized with antisense probe; **(H, I, J, K)** corresponding sections hybridized with sense probe (control). D and E show higher magnification of sections seen in A and B. In D, the Purkinje cells (PC, arrowhead) of the cerebellum show distinct labelling as well as the cells in the granule cell layer (GC) but not in the molecular layer (ML). In E, the granule cells of the dentate gyrus (DG) and the CA4 (CA) region were labelled. Only background staining can be seen in control sections H and I. In F, the large motor neuron cell bodies (MN) were labeled but not in control section (J). In G, a section of 16.5 dpc lens cut parallel to the optic nerve shows labeling of the epithelial cells (EC, arrowhead) and the fibre cell (FC) at the equatorial region whereas only background staining can be seen in control section (K). The darkly stained area in K corresponds to the pigment cells of the retina and is not considered positive signal. All sections were not counter stained to preserve the contrast between positive and negative signals. (Scale bars: 455 μm in A, B and C; 220 μm for D-K).

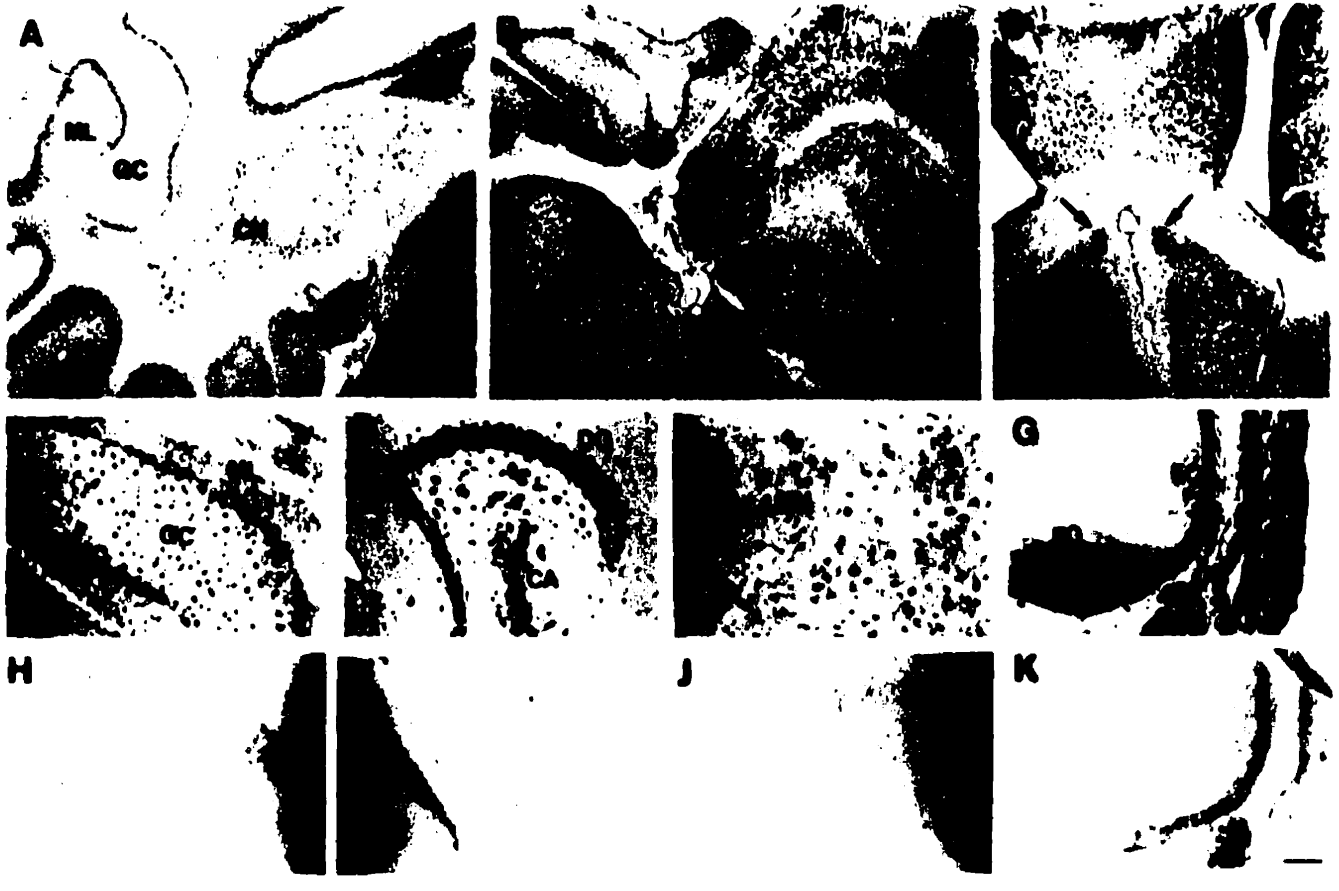
A
ML
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Figure 2

Immunoflourescence detection of schwannomin in sections of mouse tissues. (**A, B**) Strong immunostaining of Purkinje cells (arrow) and the absence of staining in section using pre-immune serum (**C**). [(gc) granule cell layer, (mc) molecular layer]. Notice the cytoplasmic staining of the Purkinje cells in B. (**D**) Cytoplasmic staining of motor neuron cell bodies in the spinal cord; (**E**) shows absence of staining using pre-immune control. (**F**) Section of the bronchus with weak staining of the smooth muscles (sm, arrow). The tunica intima lining the blood vessels in the intestine (**G**, arrowhead) and heart (**I**) shows strong immunostaining compared to the control (**J**). Cytoplasmic labelling can be seen in the goblet cells of the intestine (G, arrows); preimmune control (H). Scale bars: 60 μm in A; 40 μm in B-E; 25 μm in F-I.

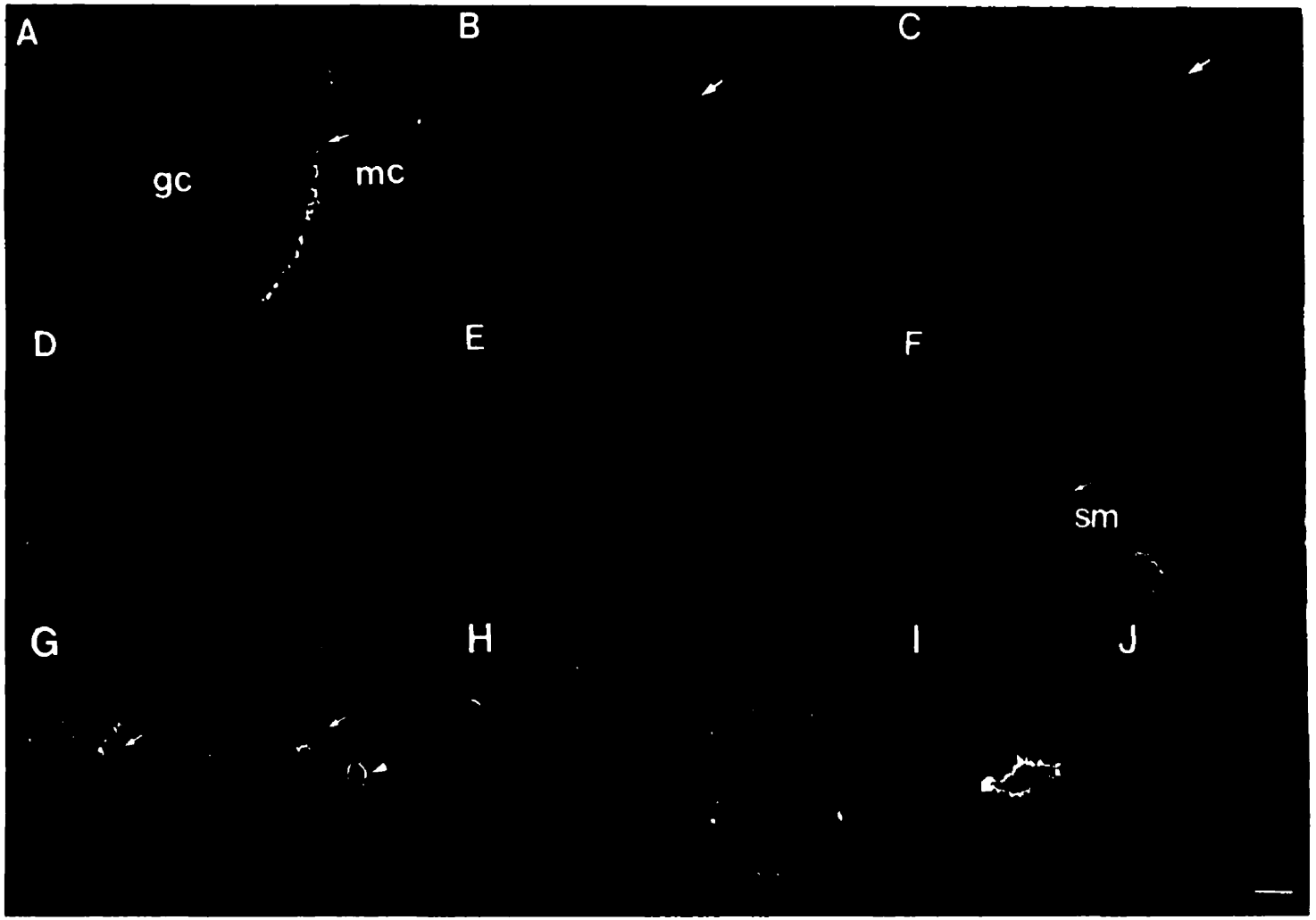
A
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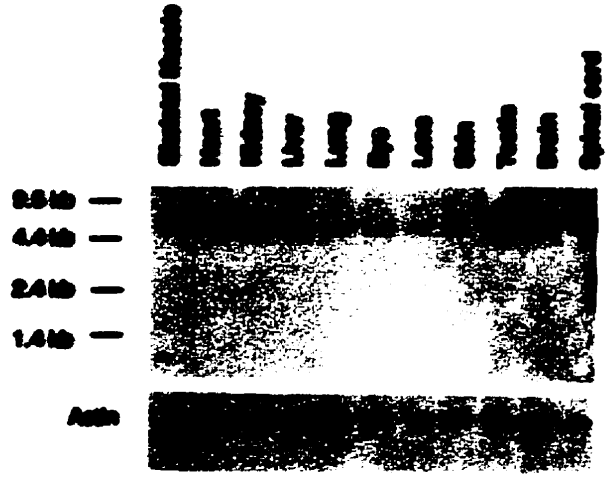
Figure 3

Widespread expression of schwannomin mRNA and protein. (**A**) Multiple tissue northern analysis of schwannomin RNA using a probe with low sequence homology to moesin, ezrin and radixin.[5] A transcript of 4.5 kb can be seen in all tissues albeit with different intensity. Approximately equal amount of total RNA (10 μg) was loaded on each lane and was assessed by a control hybridization using actin probe as shown below each lane. (**B**) Multiple tissue immunoblot analysis. A predominant band of 80 kDa was seen in all tissues studied. A faint band above the protein band in kidney was incosistently observed in other blots. Equal amount of proteins (15 μg) were loaded on each lane. Broad range Biorad prestained protein molecular weight marker was used and the migration pattern is shown on the left.

A



B

CHAPTER 5*

INSIGHTS FROM LENS AND SCHWANN CELLS

In this chapter, the expression of schwannomin in lens and Schwann cells was explored. Its localization in these cell types provides evidence consistent with its putative role as a membrane organizing protein. Moreover, its level of expression in the developing lens inversely correlates with the degree of cell differentiation suggesting that it plays a role in differentiation-specific events.

*This Chapter consists of manuscript which has been submitted for publication.
Claudio, J.O., Veneziale, R.W., Menko, A.S. and Rouleau, G.A. (1996) Schwannomin's pattern of expression in lens and Schwann cells defines its role in dynamic structures and in differentiation. *Manuscript submitted.*

Schwannomin's expression in lens and Schwann cells

Abstract

NF2 is an autosomal dominant genetic disorder characterized by the development of bilateral vestibular schwannomas, meningiomas, ependymomas and juvenile lens opacities. The gene responsible for NF2 encodes a tumor suppressor protein, schwannomin (or merlin), with structural features similar to erythrocyte band 4.1, talin, ezrin, moesin and radixin. Schwannomin is expressed ubiquitously in an apparent cell-type specific pattern. Using an antibody recognizing the carboxy-terminal epitope of isoform 1 of schwannomin, we looked at its expression in lens and Schwann cells, two cell-types affected by the NF2 phenotype. We detected schwannomin in both cell-types as an ~80 kDa protein present in the non-ionic detergent insoluble cytoskeleton fraction. The level of expression of schwannomin in the lens inversely correlates with the degree of lens cell differentiation suggesting a role for schwannomin in differentiation-specific events. On indirect immunofluorescence, schwannomin preferentially localized to leading edges and ruffling membranes, structures which are known to be dynamic cellular regions involved in movement, migration and remodeling. Using these observations, together with information from related proteins, we present a working model that defines the role of schwannomin in these dynamic structures.

Running title: Expression of schwannomin in lens and Schwann cells

Introduction

NF2[3] is a genetic disorder with both recessive and apparently dominant clinical phenotypes. It is characterized by the development of schwannomas and meningiomas which result from the complete inactivation of the *NF2* gene (1-2).The development of these tumors occurs as a manifestation of a recessive phenotype by a two-hit phenomenon (3-11) analogous to the genetic mechanism seen in retinoblastoma (12). An apparently dominant presentation of the disease appears as subcapsular lenticular opacities of juvenile onset which develop in about 80% of patients heterozygous for a mutation in *NF2* (13). These cataracts are used as a predictive test for the disease since they often appear earlier than tumors associated with NF2 (14). It is believed that these patients produce truncated NF2 protein, schwannomin (also called merlin), that may predisposed them more to lens abnormalities compared to patients with *NF2* gene alterations that lead to the absence of the gene product (15).

Schwannomin belongs to a gene superfamily localized to the membrane-cytoskeleton interface which acts as molecular linkers between membrane proteins and components of the cell cortex. Its predicted secondary structure resembles erythrocyte band 4.1, talin and the ezrin, radixin, moesin (ERM) proteins which all have a highly conserved amino-terminal domain followed by a region rich in α-helix and a highly charged carboxy-terminal domain. Schwannomin is thus thought to act as a membrane organizing protein (1) analogous to the function of band 4.1 in linking integral membrane proteins, glycophorin C and band 3, to the actin-spectrin complex in erythrocytes or to talin which links integrins to vinculin [for review see (16)].

The NF2 gene can be alternatively spliced at the carboxy-terminus to generate two isoforms. Isoform 2 introduces a 45-base sequence altering a stretch of 16 amino acids at the carboxy-terminus of isoform 1 into 11 novel amino acids (17-21). Both isoforms are conserved in human, mouse and rat (17-22). We have developed an antibody specific to

the carboxy-terminus of isoform 1 and documented its widespread but apparent cell-type specific expression in the mouse (23). To further understand the expression of schwannomin, we looked at its localization in Schwann cells, schwannomas and lens cell lines by immunoblotting and indirect immunofluorescence. Our results show that schwannomin is a component of the detergent insoluble cytoskeleton fraction as an ~80 kDa protein. Distinct localization of the protein was seen in dynamic structures such as leading edges and ruffling membranes, consistent with the hypothesis that it acts as membrane organizing protein similar to the function of the band 4.1 superfamily of membrane-cytoskeleton linkers that include ezrin, radixin and moesin.

By utilizing embryonic chicken lenses, we looked at the expression of schwannomin during lens differentiation. The epithelial cells of the lens vesicle give rise to all the cell types within the mature lens. The epithelial cells along the anterior face of the lens constitute the central epithelium. The equatorial zone consists of epithelial cells that have divided and migrated towards the equator of the lens. These cells then begin to elongate into the center of the lens and undergo terminal differentiation into secondary fiber cells. The primary fiber cells of the lens are derived from the posterior epithelial cells of the lens vesicle and are in contact with both the anterior epithelial cells and posterior lens capsule. Secondary lens fiber cells are continuously added to the fiber cell mass from the equatorial zone and are therefore not as differentiated as those within the central core. Progressive stages of differentiation can therefore be studied simultaneously within the same embryonic lens. Dissection of the lens and subsequent detergent extraction indicated that schwannomin is expressed at highest levels in epithelial cells and down regulated as lens cells undergo terminal differentiation.

Results

Schwannomin is expressed in rodent Schwann cells and schwannomas.

Schwann cell tumorigenesis in humans is caused primarily by the complete inactivation of the *NF2* gene (4-10). To analyze the expression of the *NF2* protein in Schwann cells, we immunoblotted cell lysates of rodent Schwann cells. The antibody used for immunoblotting recognized a 30-amino acid epitope at the carboxy-terminus of isoform 1 of schwannomin. We detected a protein band with relative mobility of 80 kDa on SDS-PAGE extracts of both sciatic nerve and primary rat Schwann cell cultures (Fig. 1A).

Immunoblotting experiments of rat and mouse schwannomas detected a protein of ~80 kDa which has the same molecular weight as the protein seen in immortalized clonally derived rat Schwann cells and brain lysates (Fig. 1B). A protein band of smaller molecular weight that could possibly represent a truncated protein caused by inframe deletion was not observed. From these observations, it appears that the three schwannomas studied express the rodent schwannomin. We cannot discount the possibility however, that missense mutations or small in-frame deletions, which our method could not discriminate, may have affected the function of schwannomin in these cells.

Schwannomin is a component of the cytoskeleton fraction.

Non-ionic detergents such as Triton X-100 and Non-idet P40 are known to disrupt hydrophobic interactions, but not polar, lipid-protein and protein-protein interactions (16). Organized cytoskeletal and associated proteins are not soluble in 1% Triton. On this basis, we analyzed the expression of the *Nf2* protein in the detergent soluble and insoluble fractions of different cell lines. In all detergent soluble fractions, a distinct band of ~80 kDa was observed when samples were run in a reducing SDS-polyacrylamide gel and a relatively smaller amount of the protein was detected from the detergent insoluble cytoskeleton fractions in all cell lines tested (Fig. 2A). A blot using anti-actin antibody

confirms that actin is enriched in the detergent insoluble fractions of the Schwann cell lines, indicating the effective fractionation of cytoskeleton proteins (Fig. 2A). Similar result was seen using anti-glial fibrillary acidic protein (GFAP) which is an intermediate filament marker for non-myelinating Schwann cells *in vitro* (data not shown).

Role of schwannomin in lens differentiation.

Schwannomin appeared as a band of approximately 80 kDa in western blots of chicken embryonic lens extracts. Both its expression and distribution between the triton soluble and insoluble fractions appears to be developmentally regulated (Fig 2B). The highest level of schwannomin expression was found in the two epithelial cell regions. Schwannomin was found primarily in the soluble fraction where its level of expression increased slightly between the undifferentiated central epithelium and the equatorial zone where lens cell differentiation is initiated. In the peripheral, differentiating region of the fiber cell mass, expression of schwannomin drops significantly and continues to decrease in the differentiated central fiber cells. Schwannomin's association with the triton insoluble or cytoskeletal fraction is differentially regulated with differentiation. Only a very low amount is seen in the triton insoluble fraction of the central epithelium. However, there is a significant increase in its association with the insoluble fraction of the equatorial zone. The level associated with the cytoskeleton decreases in the peripheral fiber cells and is seldom detected in the central fiber cells.

Localization of schwannomin.

The localization of schwannomin in lens cell line and Schwann cells was studied by indirect immunofluorescence. Lens epithelial cells grown in glass slides and labeled with anti-schwannomin antiserum showed expression of the *Nf2* protein in distinct cytoplasmic cellular structures. Most prominent locations are in dynamic structures such as ruffling edges of membranes (Fig.3A, E) and in terminal buttons of cell processes (Fig. 3C, G).

Epithelial lens cells with long processes showed distinct expression in variegated structures periodically located along the length of processes (Fig. 3G). Similar expression in variegated structures has also been observed in insect cells overexpressing an exogenous ezrin (24).

The staining pattern varied from cell to cell depending on the morphology (Fig. 4A, D, G, I). For example, Schwann cells with bipolar shape usually show stronger cytoplasmic staining (Fig. 4A) compared to cells with flattened morphology (Fig. 4D, G). However, the detection of schwannomin in ruffling membranes and leading edges was frequently observed only in cells with flattened morphology (Fig. 4G). Cells with bipolar shape like JS1 labeled no distinct cytoplasmic structures (Fig. 4A). In schwannomas, such as RN22 and JS-1, expression was usually observed as diffused cytoplasmic staining (Fig. 4A, D). When these cells were double labeled with anti-schwannomin antiserum and monoclonal anti-β tubulin antibody, staining of microtubules in both cell lines showed a filamentous network of cytoskeleton protein. This suggests that the fixation technique was not the cause of diffused staining with anti-schwannomin; rather, it is the characteristic pattern of expression in these cells. Unlike moesin and radixin which have been shown to localize in cleavage furrows of dividing cells (25), JS1 cells undergoing mitosis did not show re-localization of schwannomin (Fig 4A, B). In confluent cell cultures, the cell adherens junctions or focal contacts did not show distinct preferential staining (Fig. 4A, D, H) as has been observed for radixin (26). In mouse TR6BC1 schwannoma, strong cytoplasmic staining was observed in bipolar and spindle shaped cells (not shown) while flattened cells frequently exhibited diffused staining (Fig 4J) with some showing a cytoplasmic punctate pattern of staining at low magnification. When viewed at higher magnification, the staining pattern appeared similar to microvilli structures (Fig. 4I) frequently observed in ezrin, radixin and moesin (25-28).

Discussion

Limited role of schwannomin in rodent Schwann cell tumorigenesis

In humans, familial and sporadic schwannomas are known to arise as a result of the complete inactivation of the *NF2* gene (4-10). Analysis of the expression of the *NF2* protein in this tumor type invariably shows the complete absence of expression of schwannomin by immunohistochemistry techniques (10), or when present was detected as a truncated protein[1]. This loss of function mechanism is a frequent phenomenon seen in human schwannomas suggesting that *NF2* is the major gene involved in the genesis of these human tumors. However, based on our immunoblotting results, schwannomin was expressed in the three rodent schwannomas. It is possible that schwannomin may not be the only gene involved in the development of this tumor type in rat and mouse Schwann cells. This hypothesis is supported by the observation that homozygous mutations in the transmembrane region of the *neu* gene had been reported in rat schwannomas derived from ENU-mutagenesis (29), and no such mutation in the human homologue of *neu* in either sporadic or familial schwannomas has been found. These observations suggest that there may be different pathways for Schwann cell tumorigenesis in humans and in rodents. This hypothesis is consistent with the observation that schwannomas are not often seen in mice unless induced by chemical mutagenesis, whereas they occur more frequently in humans due to the high rate of new mutations in the *NF2* gene. It will be interesting to see whether mice with targeted disruption of the *Nf2* gene develop schwannomas.

Role of schwannomin in differentiation.

The NF2 protein in Schwann cells grown in conditions not favorable for myelination to occur, showed a relatively higher level of expression of schwannomin

[1] Lutchman, M.(1995) Cloning and characterization of the human neurofibromatosis type 2 gene. Ph.D. Thesis, Department of Neurology and Neurosurgery, McGill University, Canada.

compared to myelinated Schwann cells from sciatic nerve. Whether this discrepancy is due to different states of myelination is not clear, as we are comparing *in vitro* and *in vivo* conditions, but it will be interesting to study the role of schwannomin in Schwann cell differentiation by determining whether myelination coincides with the changes in the level of expression of schwannomin. It is compelling to suggest that schwannomin may be down regulated after the cells had undergone differentiation. Schwannomin expression in embryonic chicken lenses is consistent with this hypothesis. It appears that lens fiber cells, which are more differentiated, express less schwannomin protein compared to the epithelial regions of the lens which include both the undifferentiated central epithelium and equatorial zone. Cells in both the equatorial zone and peripheral region of fiber cells are undergoing differentiation accompanied by dynamic changes in cell-matrix and cell-cell interactions. In the equatorial zone, the cells migrate along the lens capsule and begin to elongate as differentiation is initiated. Peripheral fiber cells continue to elongate along the epithelial fiber cell interface and the posterior capsule as they begin to establish extensive cell-cell contacts. These events coincide with changes in the level of expression of schwannomin. The less differentiated epithelial cells have higher levels of schwannomin in the triton soluble fractions than the differentiated cells. However, the association of schwannomin with the cytoskeleton is highest in the migratory and elongating regions of the equatorial zone and peripheral fiber cells. Our data suggest that schwannomin associates with the organized cytoskeleton primarily during the initial events of lens differentiation and may play a role in lens epithelial cell migration or elongation.

Similarly, expression of the protein in adult mouse lens by immunoblotting is low compared to its expression during embryonic development or the days following birth (J. Claudio, unpublished observation). This observation together with the data presented provide a working hypothesis on the role of schwannomin in differentiation.

Schwannomin preferentially localizes to dynamic structures.

Schwannomin belongs to a gene superfamily involved in the molecular link between cell membrane proteins and the cytoskeleton. This superfamily includes band 4.1 in erythrocytes which links glycophorin C or band 3 to the actin-spectrin complex (30); talin, which links integrins and vinculins (31); and the ezrin, radixin, moesin (ERM) family (27) which are known to link cell surface glycoprotein CD44 to the actin cytoskeleton in baby hamster kidney cells (32). Indirect immunofluorescence of Schwann cells and lens epithelial cells localized schwannomin in dynamic cellular structures such as membrane rufflings and leading edges. These structures are known to be enriched with actin, α-actinin, caldesmon, ezrin, actin-binding proteins, fimbrin, spectrin, talin and other cytoskeletal proteins [for review see (33)]. The localization of schwannomin in this area suggests it has a role in membrane organization or remodeling. Perhaps, schwannomin may be one of the molecules involved during myelination events providing plasticity to the membrane as the myelin ensheathes the axon.

Fractionation of Schwann cells into non-ionic detergent soluble and insoluble components followed by immunoblotting analysis show that schwannomin is present in both fractions but it is seen at relatively lower amount in the cytoskeleton fraction. It is possible that the lower proportion of schwannomin in the cytoskeleton fraction might represent the active form of the protein present in dynamic structures whereas the protein in the detergent soluble fraction might represent the dormant form of schwannomin. Based on our data on the preferential localization of schwannomin to dynamic structures in cell cultures and on the high sequence homology of schwannomin to ezrin, we hypothesize an analogous role of schwannomin to ezrin in the cell cortex (Fig 5). Ezrin, is known to be activated by a receptor mediated phosphorylation event leading to its polymerization (34). Recruitment into microvilli and membrane ruffles coincides with its phosphorylation on tyrosine residues (35). A similar mechanism may be required for schwannomin leading to its recruitment to dynamic cellular structures. Several putative phosphorylation sites are

predicted from sequence analysis of schwannomin (1, 36). A phosphorylation site that is responsive to growth factor stimulation has not been identified but there is evidence that schwannomin is phosphorylated on serine residues *in vivo* (37). In response to epidermal growth factor (EGF), ezrin becomes phosphorylated on Tyr145 and Tyr353 (38). The tyrosine phosphorylation sites for ezrin are not conserved in schwannomin, but this mechanism suggests that schwannomin may be similarly regulated, perhaps by a different growth factor dependent signaling pathway.

Materials and Methods

Cell lines.

Schwannoma cell line TR6BC1 (ECACC) originated from the trigeminal nerve and brain of a C3H/He mouse intracranialy injected with N-ethyl-N-nitrosourea on the day of birth (39). It was maintained in Dulbeco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 0.12% tryptose phosphate. Rat schwannoma RN22 was derived from a BD-IX rat nerve-root tumor (40) and maintained in DMEM containing 10% fetal bovine serum. JS-1 was described previously as the cell line used for the purification and cloning of schwannoma derived growth factor (41). JS-1 was maintained in DMEM with 10% horse serum. SCL4.1/F7 (ECACC) is a clonally derived rat Schwann cell line (42) and was routinely cultured in DMEM containing 10% fetal bovine serum. Mouse lens epithelial cell line AGO5055 (Coriell Cell Repositories) were grown in minimum essential medium (MEM-Eagle) supplemented with 20% fetal bovine serum and antibiotics. All cell lines were maintained at 37°C with 5% CO_2 except for JS-1 and RN22 which were maintained at 10% CO_2.

Cells used for immunoblotting were grown in plastic flasks or dishes and, when used for immunofluorescence, were seeded on glass chamber slides.

Tissues.

Lenses were obtained by dissection of day 10 white leghorn chicken embryos (Truslow Farms, Inc., Chestertown, MD). Lenses were cleaned of any remaining ciliary epithelium or vitreous body and microdissected into the following regions (listed in order of differentiation): central epithelium, proliferative and equatorial zone, peripheral fiber cell and central fiber cell. The central epithelium was isolated as follows: (1) the lens was placed anterior face up under a dissection microscope; (2) the capsule was cut anterior to the equatorial zone with forceps; and (3) the central epithelium was then peeled away. The remaining epithelium, referred to as the equatorial zones, was collected by removing the remaining lens capsule to which it remains adherent. The central fiber cells which form a tight globular mass are separated from the loosely packed peripheral fiber cells. The central fiber cell fraction is enriched for mature or terminally differentiated primary and secondary fiber cells; whereas, the peripheral fiber cell fraction is enriched for immature or differentiating fiber cells.

Detergent Extraction.

Tissues were extracted at 4°C for 60 min. in a 1% Triton X-100 extraction buffer (5mM EDTA, 10mM imidazole, 100mM KCL, 1mM $MgCl_2$ and 1% Triton-X-100 at pH of 7.2) containing protease and phosphatase inhibitors (50µg/ml aprotinin, 100mM benzamidine, 5µg/ml leupeptin, 0.5mM phenylmethylsulfonyl fluoride, 50mM sodium fluoride, 3mM sodium pyrophosphate and 1mM sodium ortho-vanadate). Extracts were then centrifuged at 16,000g for 10 min. at 4°C. The supernatant was removed and retained as the triton soluble fraction. The triton insoluble pellet was washed in triton extraction buffer and resuspended, in a volume equal to the triton soluble fraction, by homogenation and sonication in a RIPA extraction buffer (1mM EDTA, 150mM NaCl,1% nonidet P-40, 0.25% sodium deoxycholate and 50mM Tris-base at a pH of 7.4) containing the same protease and phosphatase inhibitors as the triton extraction buffer. The resuspended pellet

was then centrifuged at 16,000g for 10 min. at 4°C. The supernatant was removed and retained as the triton insoluble fraction. Triton and RIPA extracts were brought to equal volume before the addition of Laemmli sample buffer.

Detergent insoluble cytoskeleton fractions from cell lines were prepared as described previously for ezrin (34). Briefly, cells grown to confluency in 35 mm dishes were trypsinized, collected, spun down and washed with PBS. The cell pellet was resuspended in 20 µl PBS and extracted for 15 min at room temperature with 20 µl 2x extraction buffer containing 2% Triton X-100, 75 mM KCl, 10 mM Hepes, pH7.4, 1 mM DTT, 1 mM EGTA, 2 mM PMSF, 1 µg/ml leupeptin and 1µg/ml pepstatin. The lysates were centrifuged at 20,000 g for 15 min to pellet the cytoskeletons. The pellets were gently washed with PBS containing 0.1% Triton X-100, recentrifuged and were resuspended in 20 µl PBS and 20 µl of 2x extraction buffer. Equivalent amounts of the soluble and insoluble fractions were loaded in SDS-PAGE and analyzed by immunoblotting. A monoclonal anti-actin antibody (Chemicon International, Inc.) was used to reprobe the same protein blot to confirm that the extraction condition yielded cytoskeleton-enriched fraction. In separate blots, anti-glial fibrilary acidic protein (Sigma) was used to check the enrichment of cytoskeleton in the detergent insoluble fraction.

Immunoblotting.

Proteins used for immunoblotting were extracted using NP-40-containing lysis buffer as described previously (23) or using Triton X-100 as mentioned above. Protein concentration was determined using Bradford reagent (Bio-Rad Laboratories) according to the product specifications. Triton insoluble samples were loaded at a volume equal to that of the associated soluble sample so that the distribution of individual proteins as a percent of the total could be determined. Samples were stored at -80°C until used. Prior to electrophoresis, 7.5 - 15 µg of protein samples were boiled for 5 min. at 80-90°C with or without β-mercaptoethanol immediately prior to gel electrophoresis. Proteins were

separated on 10% sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) and blotted unto 0.2 μm nitrocellulose filters (Schleicher and Schuell) using Biorad's electro blotting apparatus. Protein samples from chicken lens were separated by SDS-PAGE using 4-12% Tris-glycine gels (Novex, San Diego, CA) at 120V and then transferred to Immobilon-P transfer membrane (Millipore Corporation, Bedford, MA) at 290mA for 3 1/2 hours. The protein blots were blocked for 1 hour at room temperature or overnight at 4°C with 10% milk in TBS/ (0.1%) Tween 20, or in the case of chicken lens blots, with 3% nonfat dry milk and 3% bovine serum albumin in TBS/Tween-20 (0.1%) at room temperature for 30 min. The primary antibody, rabbit anti-schwannomin antiserum described previously (23) was diluted 1:300 in 5% milk/TBS. After 1 hour incubation at room temperature, the blots were washed 3 times of 15 min each with TBS containing 0.1% Tween 20. The secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG F(ab')2 (Jackson ImmunoResearch Laboratories) or alkaline phosphatase-conjugated goat anti rabbit IgG (Sigma) was diluted 1:5000 and incubated for 1 hour at room temperature. Washing was done 3 times for 15 min each using TBS containing 0.1% Tween 20. Horseradish peroxidase signal was detected by enhanced chemoluminescence (ECL) reagents (Amersham Life Sciences) and alkaline phosphatase signal by NBT/BCIP substrate (Sigma).

Indirect Immunofluorescence.

Cells grown in glass chamber slides for 48 hours were fixed with ice-cold 50:50 (v/v) methanol:acetone for 2 min. Cells were processed for indirect immunofluorescence by 10 min pretreatment with 0.1% Triton X-100 in PBS, then blocked with 10% goat serum for 1 hour at room temperature. The primary antibody, rabbit anti-schwannomin antiserum (1:300) in 3% BSA was incubated to the cells for 1 hour at room temperature or overnight at 4°C, washed 3 times with PBS, then 1:300 dilution of the secondary antibody, fluorescein-conjugated anti-rabbit IgG (H+L)(Vector Laboratories) was incubated for 1

hour at room temperature. Monoclonal anti-ß-tubulin (Sigma) was used at a dilution of 1:1000 and was detected with Texas red-conjugated anti-mouse IgG (Vector Laboratories). Coverslips were mounted onto glass slides using SlowFade light antifade reagent (MolecularProbes, Inc.) and cells were examined using Polyvar microscope under appropriate filters. Pictures were taken using Kodak P3200 black and white film.

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Figure 1

Schwannomin is an ~80 kDa protein in lens and Schwann cells. (A) Cells were extracted with NP-40 buffer centrifuged and the supernatants used for protein analysis on 10% SDS-PAGE under reducing condition. Equivalent amounts of proteins (7.5 μg) were loaded on each well (see Materials and Methods). Gels were either blotted on nitrocellulose membrane and used for immunoblotting (IB) or were stained with Coomassie blue (CB). The anti-schwannomin antiserum detected an 80 kDa protein in primary rat Schwann cell cultures and in protein extracts from rat sciatic nerve as shown on panel IB. A Coomassie blue (CB) stained gel of the same samples run on separate gel is shown on the right. (B) Expression of schwannomin in rodent schwannoma cell lines. Schwannomin was detected as an 80 kDa band in mouse schwannoma TR6BC1, rat schwannoma JS1 and RN22, and in immortalized rat schwann cell line SCL4.1/F7. A protein band of the same mobility was observed in rat and mouse brain controls. The bands migrating more slowly than the 80 kDa schwannomin signal in mouse brain and TR6BC1 schwannoma was inconsistently observed in other blots.

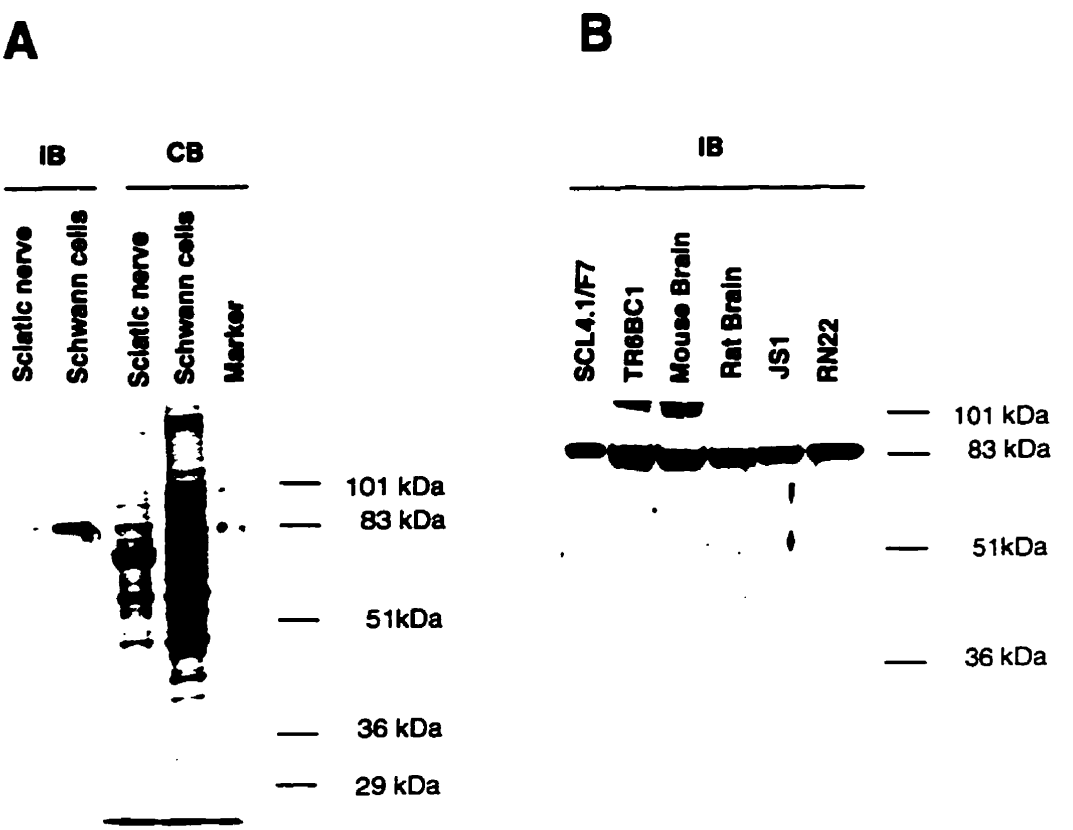
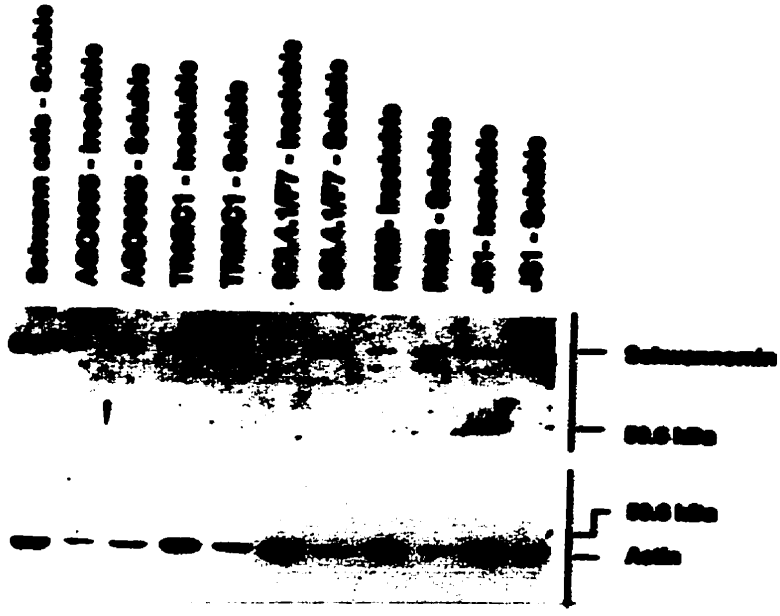

A
IB
CB
Sciatic nerve
Schwann cells
Sciatic nerve
Schwann cells
Marker
101 kDa
83 kDa
51kDa
36 kDa
29 kDa
B
IB
SCL4.1/F7
TR6BC1
Mouse Brain
Rat Brain
JS1
RN22
101 kDa
83 kDa
51kDa
36 kDa

Figure 2

Schwannomin is a component of the non-ionic detergent-insoluble cytoskeleton fraction. (A) Triton X-100 soluble and insoluble fractions were fractionated by centrifugation at 4°C. Detergent-insoluble fractions were run in reducing conditions alongside with detergent soluble fractions of schwannoma and lens cell lines. Equivalent amount of detergent-soluble and insoluble proteins were resolved by SDS-PAGE under reducing conditions, blotted on nitrocellulose membrane and processed for immunodetection using anti-schwannomin antiserum. Schwannomin was present in both detergent-soluble and-insoluble fractions but most of the proteins were detected in the former. When the same blot was stripped and a control antibody against cytoskeleton protein was used as shown on the lower panel, it confirmed that the detergent insoluble fraction was enriched with actin. AGO5055 is a lens epithelial cell line; TRCBC1, RN22 and JS-1 are rodent schwannomas; SCL4.1/F7 is an immortalized rat Schwann cell line. (B) Schwannomin's association with the organized cytoskeleton during lens cell differentiation as determined by 1% Triton X-100 extraction. Triton soluble samples were loaded at 12 μg per sample and Triton insoluble samples were loaded at a volume equal to that of the associated soluble sample (see Methods). Control cytoskeleton proteins such as vinculin, fodrin, and talin were used to check the efficient fractionation of soluble and insoluble fractions (data not shown). The faint cross reacting bands in the PF and CF cytoskeletal lanes are inconsistently observed in other blots. Central epithelium (CE), proliferative and equatorial zone (EZ), peripheral fiber cell (PF) and central fiber cell (CF);

A



B

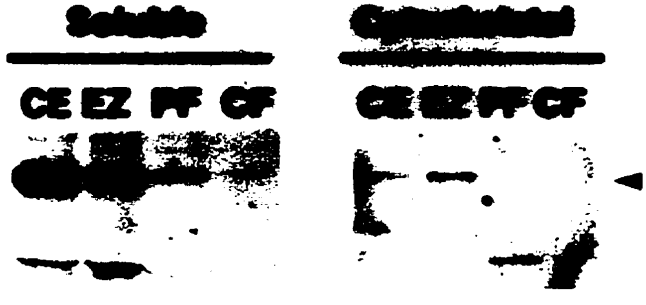


Figure 3

Localization of schwannomin in lens epithelial cells. Cells were processed for indirect immunofluorescence using anti-schwannomin antiserum (A, C, E, G). Their corresponding phase contrast photomicrographs are shown on the right panel (B, D, F, H). Preferential staining was observed in ruffled membranes (arrowheads in A and E), in leading edges of cell processes (big arrows in C and G) and in variegated structures (small arrows in G). Bar, 30 μm.

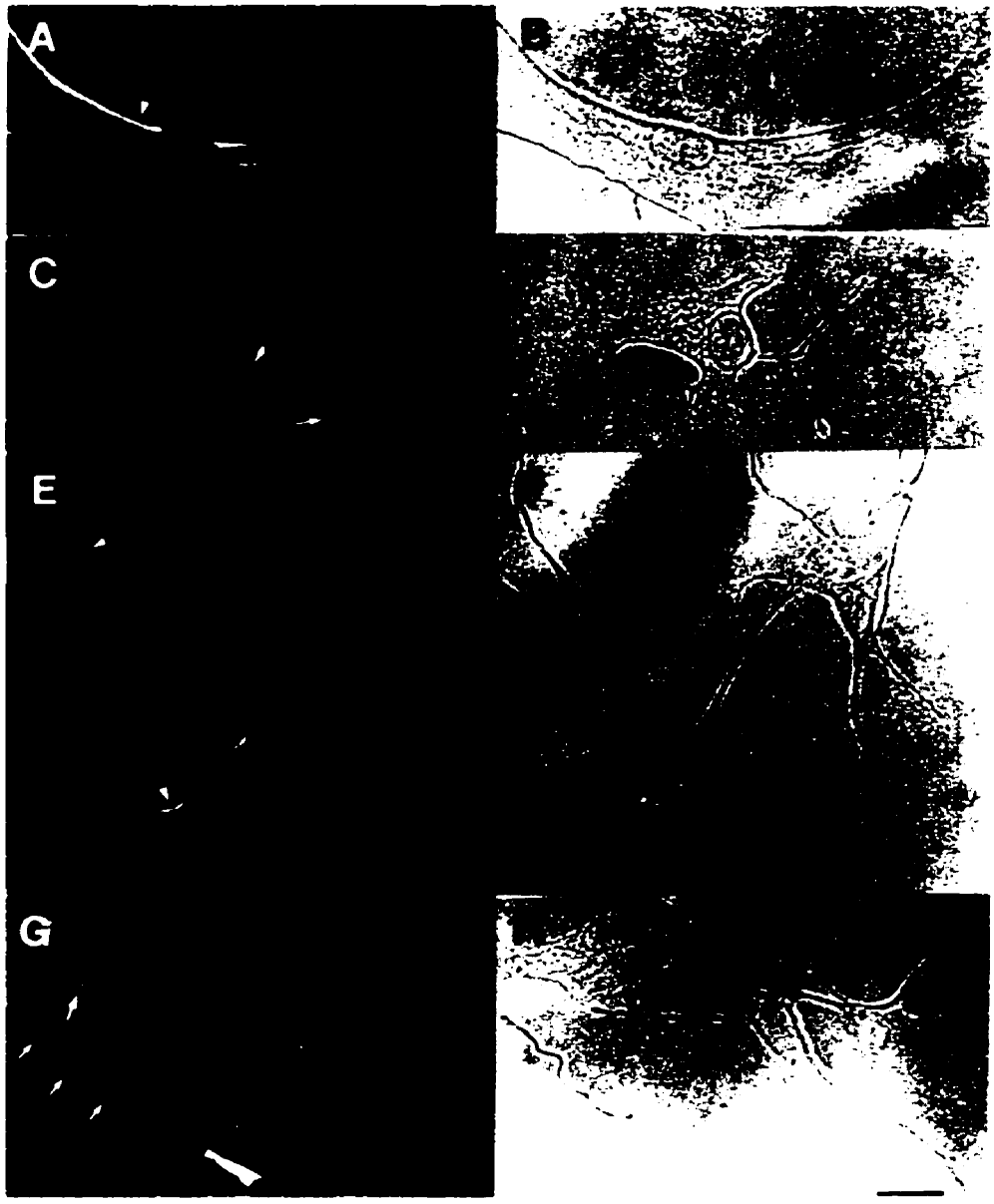
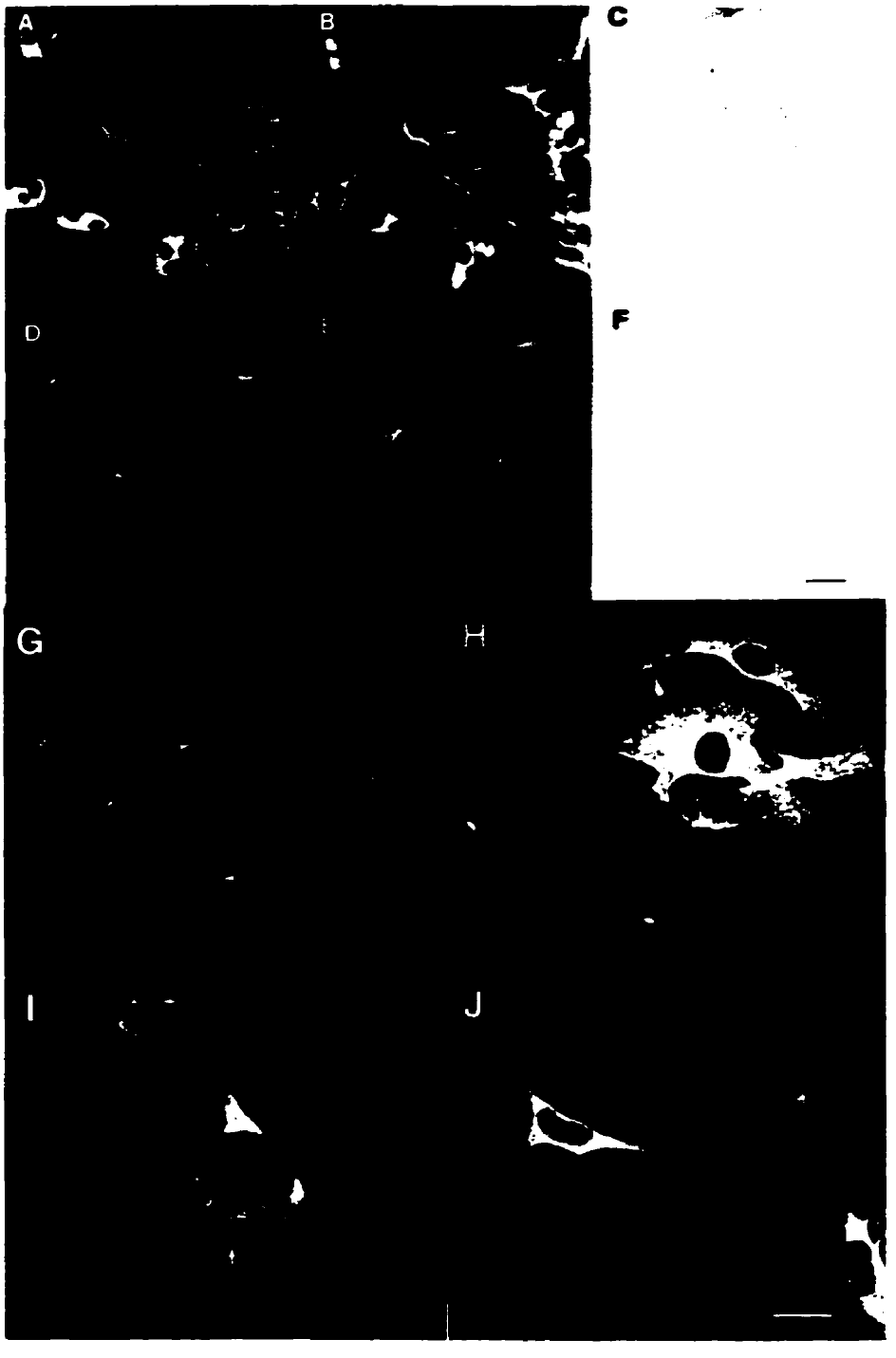

A
B
C
E
G

Figure 4

Localization of schwannomin in Schwann cells. Indirect immunoflourescence showing cytoplasmic localization of schwannomin in rat schwannomas, JSI (A, B, C) and RN22 (D, E, F). JS1 (A) and RN22 (D) show diffused staining pattern with the anti-schwannomin antiserum compared to a fibrous cytoskeletal pattern of staining of the same cells double labeled with monoclonal anti-β tubulin antibody (B and E). Phase contrast photomicrographs are shown in C and F. There is no preferential staining of schwannomin in cleavage furrows of mitotic cells (arrows in A) nor in cell to cell focal contacts. Rat Schwann cell line SCL4.1/F7 shows strong staining in ruffling membranes and leading edges of cell processes (G). In some cells, staining can be observed in the cytoplasm with varying degrees of intensity as in SCL4.1/F7 (H) and in mouse schwannoma TR6BC1 (J). A punctate pattern of staining was observed in some TR6BC1 cells (I, arrows) which appears like microvilli structures at high magnification. Bar, 30 μm.


A
B
C
D
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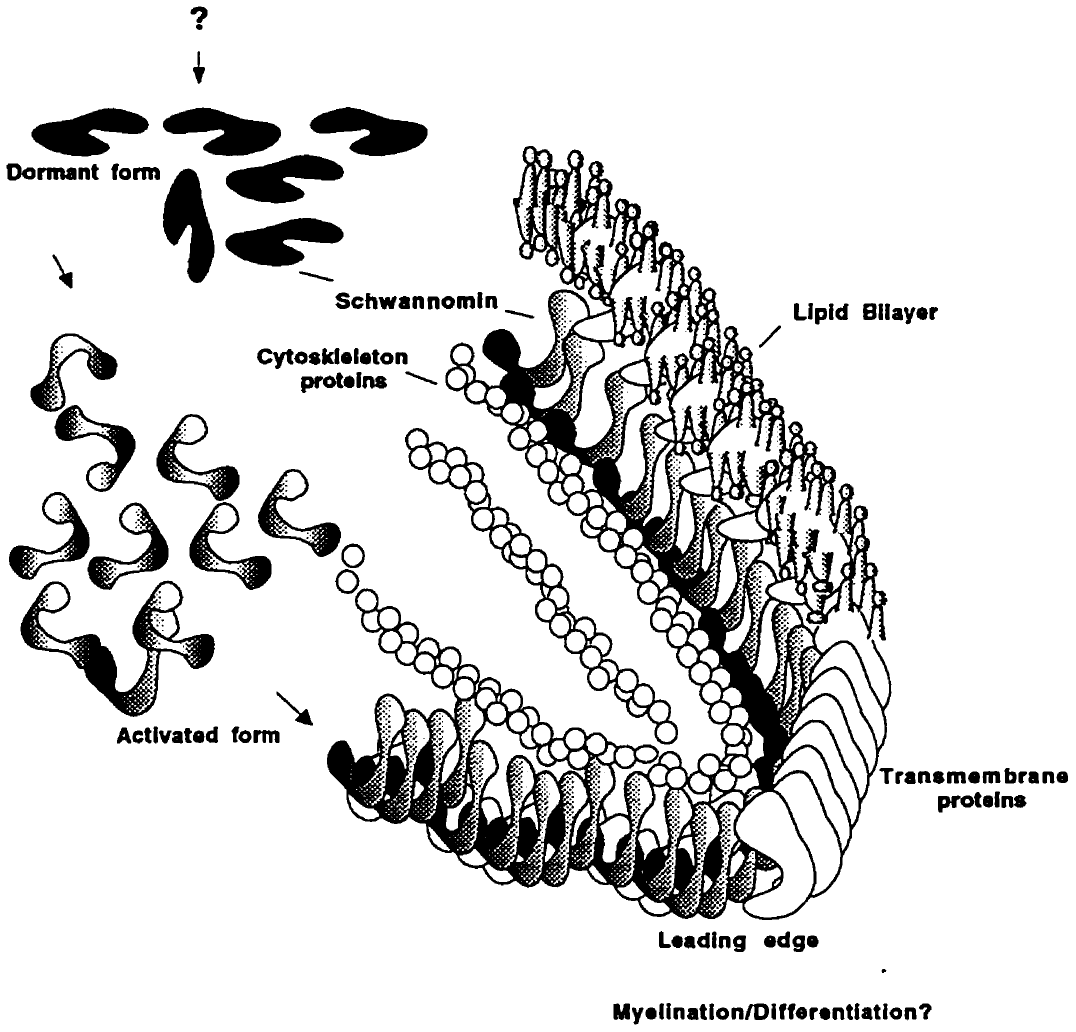
H
I
J

Figure 5

Working model for the proposed function of schwannomin in leading edges. This hypothetical model speculates the recruitment of schwannomin in dynamic structures through an activation step (?) brought about by a membrane-associated factor, possible growth factor. This activation step may arise from receptor specific phosphorylation events leading to changes in conformation of the dormant schwannomin into active monomers. The activated monomers are recruited to the cell surface as they bind to cytoskeleton and plasma membrane protein partners. Such interaction may allow the formation of membrane ruffles in migrating cells or leading edges of cell processes. The model depicts schwannomin arranged at intervals either as monomeric molecules or homo-oligomers bound to membrane and cytoskeleton protein partners.


?
Dormant form
Schwannomin
Lipid Bilayer
Cytoskieleton proteins
Activated form
Transmembrane proteins
Leading edge
Myelination/Differentiation?

CHAPTER 6

IN SEARCH OF ANSWERS
(Overall Discussion)

OVERALL DISCUSSION

1. Insights from across species

When a human gene is cloned, one strategy to characterize the protein is to study its homologues in other species. In the case of the *NF2* gene, we sought this route to gain insights into the gene and its protein product, schwannomin (or merlin). As described in Chapter 2, the *NF2* gene is conserved across species as indicated by zoo blot analysis (Claudio et al, 1994a). This conservation is obvious when we performed a sequence comparison of the *NF2* gene and its homologue *Nf2* (Claudio et al., 1994a). The human and the mouse *NF2* proteins are 98% identical, with only 10 amino acid differences including a proline insertion at position 571 in the mouse protein. Thus, the mouse gene encodes a 596-amino acid protein whereas the human protein is one amino acid shorter. Like most of the amino acid changes, the insertion of the proline residue occurs near the carboxy-terminal end of the protein but not in the proline-rich region (see below). Seven of the amino acid substitutions are located outside of the band 4.1 homology domain.

Using recombinant inbred strains of mice, we mapped *Nf2* to the proximal region of mouse chromosome 11 (Claudio et al., 1994b). This region contains the mouse homologues of the genes for leukemia inhibitory factor (LIF), neurofilament heavy chain polypeptide (NEFH) and the gene disrupted in Ewing sarcoma (EWS) (see Fig. 2 in Chapter 1 for the human gene positions). These mouse homologues, including *Nf2* , are all located on the proximal end of mouse chromosome 11 suggesting that this is a region of conserved synteny to a small segment of human chromosome 22. This region in mouse chromosome 11 has no known natural mouse mutant that could be assessed as a potential model of NF2. Nevertheless, the high sequence conservation of the *NF2* gene in mice suggests that insights into the function of the protein can be gained by studying the mouse *Nf2* gene.

The antibody against schwannomin used in this study recognized the carboxy-terminal epitope of the human, rat, mouse and chicken protein (Claudio et al., 1996). This result is consistent with the predicted conservation of the gene across vertebrate species as shown by zoo blot hybridization (Claudio et al., 1994a). Interestingly, the conservation of the *NF2* gene extends even to invertebrates such as *Drosophila* (McCartney and Fehon, 1996) and *C. elegans* (Gobel et al., 1994). The *Drosophila* homologue of *NF2* is 55% identical to the human protein, but evidence that this gene is indeed the functional homologue of *NF2* and not one of the ERM proteins comes from complementation assays. In *Drosophila* a mutation in the *NF2* homologue could be complemented by the human *NF2* gene. Similarly, conservation of the *NF2* gene in nematode extends further to the genomic organization of its homologue. The intron-exon junctions of the human gene are preserved in the *NF2* gene homologue in *C. elegans*. Although mutations in these homologues do not produce a phenocopy of the human disease, analysis of these genes is useful in understanding the pathways in which they act. Hopefully, studies of the *NF2* protein in other species will yield useful insights into the mechanism of action of this human tumor suppressor gene.

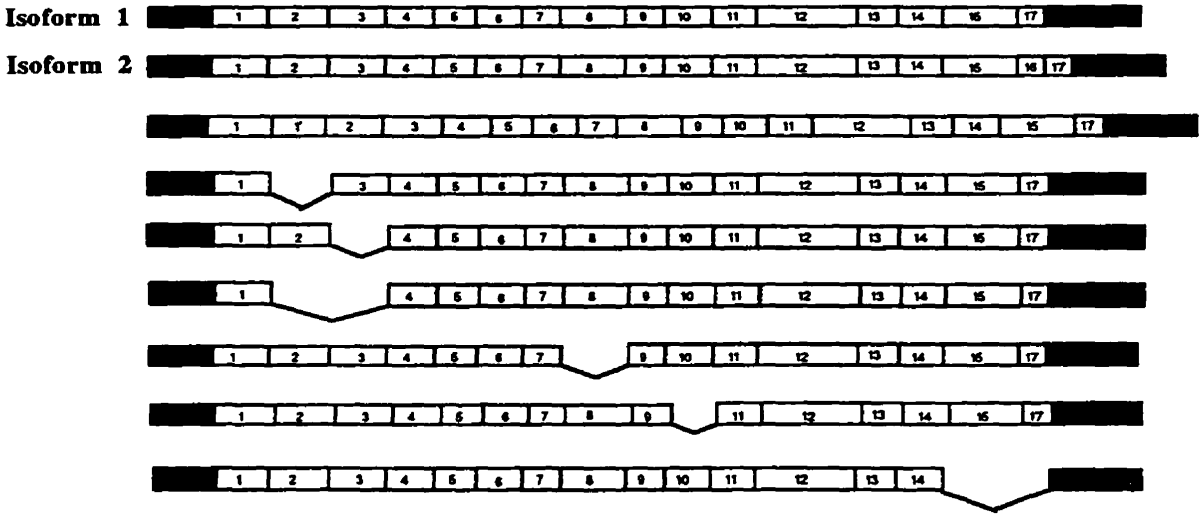
2. Alternative splicing

Nf2 generates one transcript of ~4.5 kb by Northern blot analysis of all mouse tissues (Claudio et al., 1995; Claudio et al., 1994a) which contrasts to at least 3 transcripts, 7.0 kb, 4.4 kb and 2.6 kb, that are observed in human (Bianchi et al., 1994; Trofatter et al., 1993). It is not certain whether the different transcripts in human result from alternative splicing, different polyadenylation sites or uncloned related genes. Although there is evidence to support the alternative splicing hypothesis in both human and mice (Arakawa et al., 1994; Haase et al., 1994; Hara et al., 1994; Huynh et al., 1994; Pykett et al., 1994), the presence of one transcript in mouse Northern blots (Claudio et al., 1995, Claudio et al., 1994a, Haase et al., 1994, Hara et al., 1994), in contrast to the

Figure 1

Schematic representation of a series of alternatively spliced transcripts that are thought to occur within the *NF2* gene. The major transcript is shown as isoform 1. Isoform 2 uses an alternate exon at its 3' end thereby introducing 11 new amino acids and truncating the protein prematurely. The putative exon skipping forms are shown occurring independently; whether skipping occurs as a combination of several exons is not known. The biological significance of this phenomenon is not clear and is hypothesized to be a result of leaky splicing since no protein variants have yet been reported that corresponds to each putative variant (reproduced with permission from Claudio et al., 1996).

Putative Isoforms of the NF2 gene



multiple transcripts in human (Bianchi et al., 1994; Trofatter et al., 1993), is inconsistent with the hypothesis that the different human transcripts are generated by alternative splicing.

Reports that the *NF2* gene encodes multiple alternatively spliced transcripts (Fig. 1) have been puzzling in view of the fact that mutations have been found in every exon of the major isoform except in the alternate exons used by isoforms 1 and 2 (see below). Isoform 1 represents the original sequence reported previously (see Chapter 2 Fig. 2a) (Claudio et al., 1994a; Rouleau et al., 1993; Trofatter et al., 1993) and isoform 2 contains an insertion of 45 bp sequence at the carboxy-terminal end of the protein, introducing 11 novel amino acids and a premature stop codon (Appendix C, Arakawa et al., 1994; Bianchi et al., 1995; Haase et al., 1994; Hara et al., 1994; Huynh et al., 1994; Pykett et al., 1994). Although the biological significance of the alternative splicing is not known, we speculate that most of the alternately spliced transcripts result from leaky splicing. Evidence for this hypothesis comes from protein analysis described in Chapter 4 and mutation analysis which will be described later in this Chapter. There has yet been only one reported protein isoform, presumably from the major transcript known as isoform 1 (Claudio et al., 1995, Claudio et al., 1996, Sainz et al., 1994). Protein that corresponds to isoform 2 has not been reported and no antibody specific to this isoform has been generated to confirm its biological significance or its role in tumorigenesis. More importantly, Western analysis has consistently detected a single protein band suggesting that if the alternately spliced variants are translated into protein at all, the amount may be almost nil and any biological function, if any, would be difficult to evaluate without offsetting the normal ratio of the other isoforms *in vivo*. One way to answer this question would be to analyze splice site mutations that may possibly result in exon skipping and yield transcript analogous to a known splice variant and correlate the severity of the phenotype.

3. Functional homology domains of the Band 4.1 superfamily

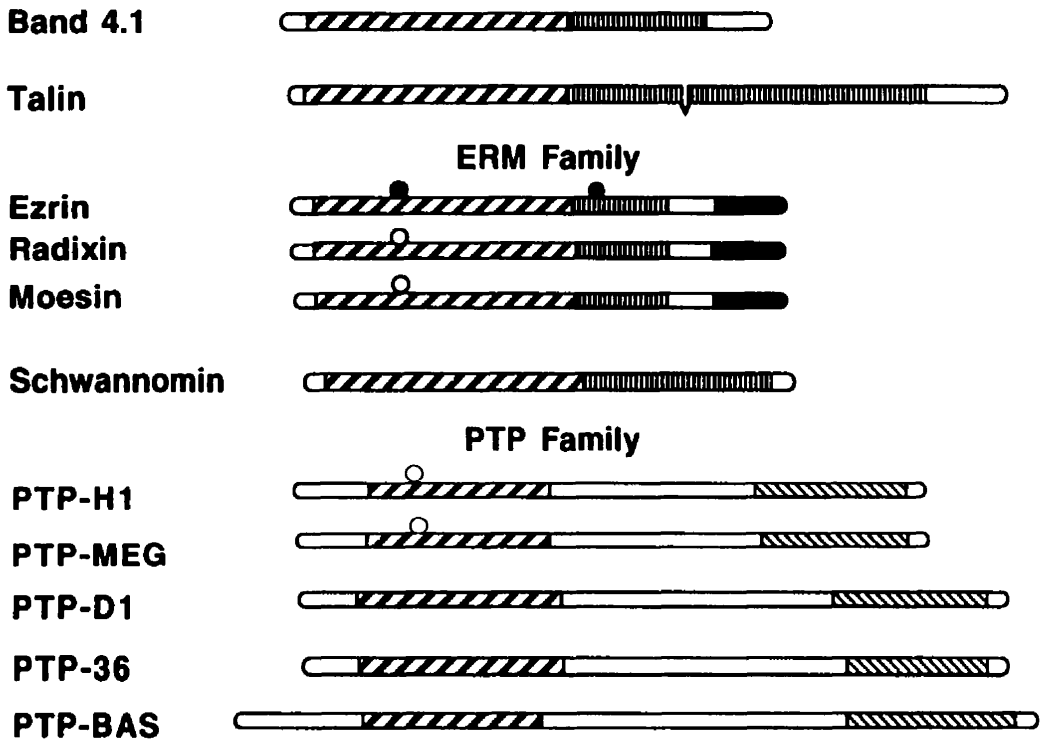
Schwannomin belongs to a superfamily of proteins that includes band 4.1 protein in erythrocytes, talin, the ezrin-radixin-moesin (ERM) family and a rapidly expanding family of protein tyrosine phosphatases (Fig. 2). This superfamily, based on studies of band 4.1 and talin, is thought to be involved in linking membrane protein to the cytoskeleton. These proteins, with the exception of the protein tyrosine phosphatase family, have common structural organization: a globular amino-terminal domain followed by a long α-helical structure and a highly charged carboxy-terminal domain.

Among the members of this superfamily, schwannomin shares highest homology to the ERM proteins. The first 342 residues at the amino-terminus of schwannomin is ~63% identical with ezrin, radixin and moesin. The ERM proteins interact with actin through a 34-amino acid domain at their carboxy-terminus (Turunen et al., 1994) and to CD44, a broadly distributed cell surface glycoprotein, via their amino-terminus (Tsukita et al., 1994). The interaction of the amino-terminus of the ERM proteins to CD44 has only been observed in baby hamster kidney cells. Whether such interaction exists in other cells has yet to be examined. The actin binding domain of the ERM proteins is not conserved in schwannomin suggesting that the analogous region of schwannomin does not bind actin. The possibility that another undefined sequence of schwannomin could interact with actin remains speculative. On the other hand, whether schwannomin interacts with CD44 is not evident, because CD44 monoclonal antibody co-immunoprecipitates ezrin, radixin and moesin but not schwannomin (Tsukita et al., 1994). If it does associate with CD44, such interaction might be weak or indirect. Strategies such as yeast two-hybrid system and immunoprecipitation using monoclonal antibodies against schwannomin or fusion proteins may yield some answers. Indeed, a GST-schwannomin bacterial fusion protein used to assay schwannomin-interacting proteins has identified 5 proteins designated only by their apparent molecular weight as p165, p145, p125, p85 and p70 (Takeshima et al., 1994).

Figure 2

Schematic structural model of schwannomin and related proteins. Schwannomin belongs to a superfamily that includes erythrocyte band 4.1, talin, ERM family and protein tyrosine phosphatase family. The members of this superfamily have a common structural organization: a globular amino-terminal domain followed by a region rich in α-helical structure and a highly charged carboxy-terminal domain. The overall homology of schwannomin to the ERM proteins is 48% but the amino terminal half of schwannomin is 63% identical to the same region in ERM family. Lower amino acid homology is seen between band 4.1, protein tyrosine phosphatases and talin (~43% at the amino terminus). The tyrosine phosphorylation sites for ezrin is conserved in the ERM family but not in schwannomin. The putative tyrosine phosphorylation sites of two of the tyrosine phosphatases are also shown as open circles. The last 34 amino acids at the carboxy-terminus of the ERM proteins are responsible for binding to actin; the amino terminal region binds to CD44. A domain known as ezrin-radixin-moesin association domain (ERMAD) lies within amino acids 1-296 (N-ERMAD) and the last 107 amino acid residues (C-ERMAD) of ezrin. This domain is responsible for head to tail homodimerization of ezrin and heterodimerization with moesin or radixin. Although N-ERMAD is conserved in schwannomin, C-ERMAD is not. The amino-terminal domain of band 4.1 interacts with glycophorin C and its carboxy-terminal domain binds to the actin-spectrin complex in erythrocytes. Similarly, talin links integrins to vinculin through its amino- and carboxy-terminal domains, respectively. The stippled region represents the N-terminal homology domain; regions with a series of diagonal lines represent sequences rich in α-helix; shaded boxes represent the actin-binding domain of ERM proteins; and hatched boxes represents protein tyrosine phosphatase domain of the PTP family. Shaded circle indicates known phosphorylation site and open circle represents putative phosphorylation site. A gap within the α-helical domain of talin indicates that it is shortened to allow comparison (reproduced with permission from Claudio et al., 1996).

BAND 4.1 SUPERFAMILY



The identity of these proteins remains unknown and the validity of the results awaits independent confirmation.

4. Proline-rich region

Proline-rich regions have been implicated as targets for Rho/Rac like GTPases (Symons et al., 1996) or as Src homology 3 (SH3) binding domain (Barfod, et al., 1993). In the case of schwannomin, such a region is evident in the carboxy-terminal half of the protein (Fig. 3). SH3 domains are involved in the interaction of Ras proteins and their regulators, as well as in the association of certain proteins with cytoskeletal elements (see Pawson and Schlessinger, 1993 for review). The Rho family of GTPases have been previously implicated in distinct dynamic processes involving the assembly of focal adhesions and stress fibers (Ridley and Hall, 1992a) and membrane ruffling (Ridley et al, 1992b). Whether schwannomin is involved in these signaling mechanisms remains an interesting supposition that needs to be explored.

5. Protein structure and mutations

In the absence of a lot of information on the cell biology of schwannomin, analysis of different disease-causing mutations have been useful in providing clues to the understanding of the *NF2* protein. The graphical presentation of the distribution of mutations as shown in Fig. 4, identifies no hotspots for mutation. A vast majority of mutations are known to result in truncated proteins. Inframe deletion and missense mutations are uncommon and comprise a very low percentage of the known mutations. Based on these observations, it is apparent that a truncated schwannomin may be non-functional. However, whether some truncating mutations that occur near the 3' end of the gene or missense mutations may have dominant-negative activity or residual activity remains unclear. Furthermore, the relative stability of a mutant *NF2* protein has not been determined.

Figure 3

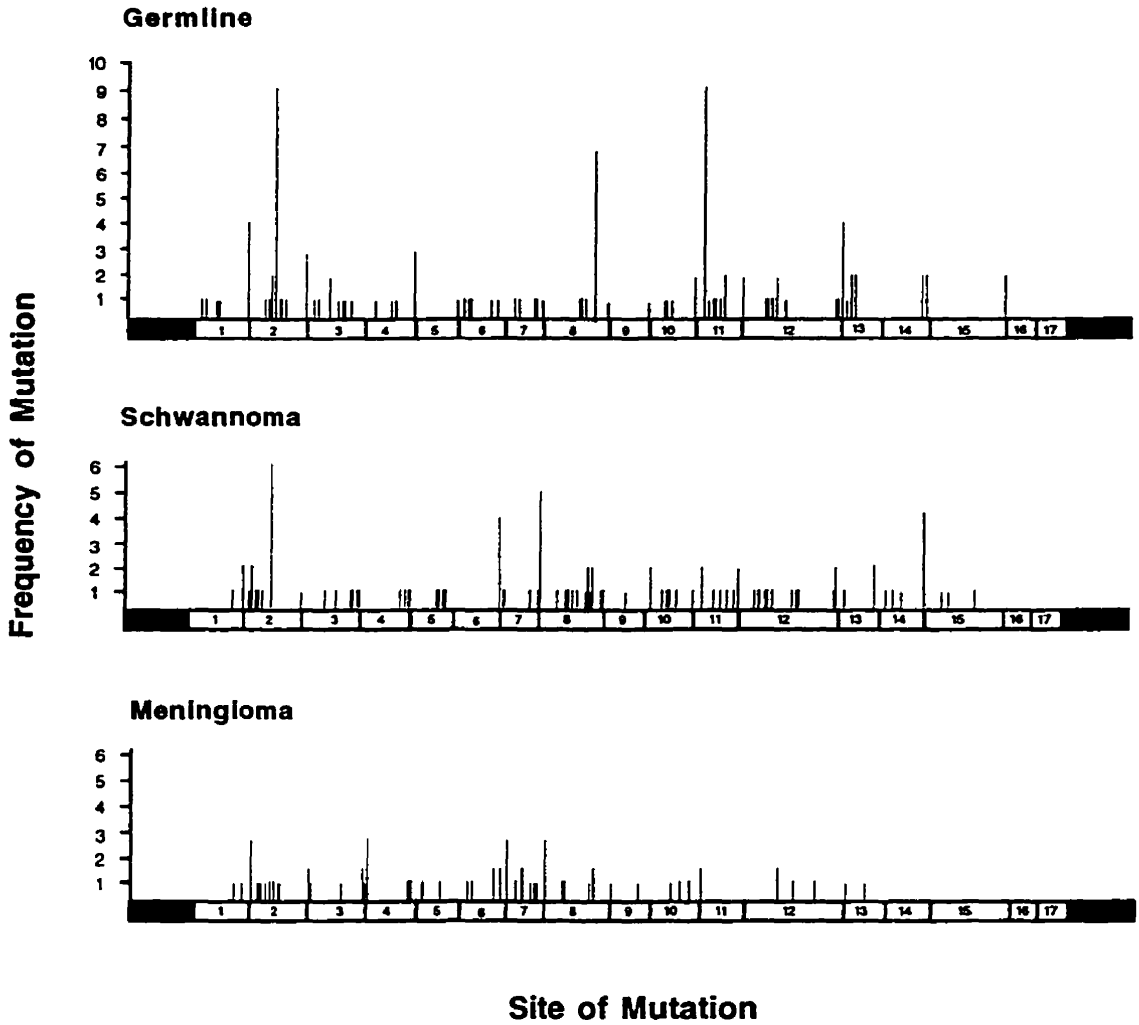
Sequence of the proline rich region of schwannomin, ezrin and radixin. Moesin is lacking a polyproline repeat. Proline rich regions have been implicated as binding site for Rho/Rac like GTPases or as binding site for SH3 domain.

Proline-rich Region

```
Human SCH    TKPTYPPM-NPIPAPLPPDIPSFN-LIGDSL
Mouse SCH    TKPTYPPM-NPIPPPLPPDIPSFD-IIADSL
Ezrin        TKEELHLVMTA-PPPPPPPVYEPVNYHVQE-
Radixin        KEELKTVMSAPPPPPPPPVIPPTENEHD-E
Moesin       RAELKTAMST------PHVAEPAE-NEHD-E
```

Figure 4

Schematic representation of published germline and somatic mutations relative to the 17 exons of the *NF2* gene as detected by polymerase chain reaction. No apparent clustering of mutations exists to date except for the frequent nonsense mutations in codon 57 (exon 2) and codon 262 (exon 8). The vast majority of mutations would result in premature stop codon presumably generating truncated schwannomin. Missense mutations are rare and comprise a small percentage.


Germline
Schwannoma
Meningioma
Frequency of Mutation
Site of Mutation
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

5.1 Germline Mutations

Germline mutation analyses attempting to correlate genotype to phenotype provide preliminary evidence that mild manifestations of the disease may be associated with mutations that preserve the carboxy-terminus of the protein (Ruttledge et al., 1996; refer to Appendix A). For example, missense mutations or small inframe deletions or insertions significantly correlate with the mild-Gardner subtype of NF2 whereas frameshifts and nonsense mutations which are predicted to produce truncated *NF2* proteins are often associated with the severe-Wishart subtype of NF2. This finding suggests that a residual activity may be present in schwannomin with missense mutation. However, until a functional analysis is done on this type of mutations, such a postulate remains speculative.

Notably, germline mutations have been identified in only slightly more than half of NF2 patients screened for alterations in the entire coding region of the *NF2* gene (MacCollin et al., 1994; Merel et al., 1995; Ruttledge et al., 1996). However, because of the genetic homogeneity of NF2 (Narod et al., 1992), it is unlikely that there is another gene involved. The most reasonable explanation for the unaccounted mutations is the possibility that mutations occur within the promoter and other regulatory sequences, and that large deletions affecting both intron and exons are not detected by polymerase chain reaction (PCR) screening method. As the entire genomic sequence of the human *NF2* gene has been determined, search for the other unidentifed mutations will elucidate the nature of the elusive genetic lesions (Thomas, G., personal communication).

5.2 Mutation of the NF2 gene in Schwannomas

The frequent occurrence of schwannomas in NF2 patients has led to the hypothesis that functional loss of the *NF2* protein is a frequent and fundamental event in the genesis of this tumor type. When schwannomas from NF2 patients were analyzed in parallel with blood DNA, alterations corresponding to germline and somatic mutations were identified (Bijlsma et al., 1994; Deprez et al., 1994; Irving et al., 1994; Jacoby et al., 1994; Twist

et al., 1994) (Fig. 3). These data provided evidence at the DNA level that complete inactivation of the *NF2* gene is an event associated with the pathogenesis of schwannoma. At the protein level, at least one study has shown the absence of immunohistochemical staining in schwannomas using anti-schwannomin antibody (Sainz et al, 1994) or when present was detected as truncated protein (Lutchman 1995). Together, these observations provide compelling evidence that the tumorigenesis in NF2 is unmasked by recessive expression of independent mutations on each allele of the *NF2* gene.

Mutation of the *NF2* gene is not restricted to vestibular and spinal schwannomas but has also been reported in intracerebral schwannomas and in schwannomas isolated from tibial nerve and accessory nerve (Bijlsma et al., 1994). A disease presenting with Schwann cell tumors that include multiple cutaneous and spinal neurilemmomas known by the Japanese as neurilemmomatosis, and thought to be distinct from NF2 because of the absence of other features of the disease, has been identified to be caused by mutation of the *NF2* gene (Honda et al, 1995). This finding proves that neurilemmomatosis is a form of NF2. The basis for the clinical differences between neurilemmomatosis and NF2, however, remains unknown. Nevertheless, this implies that human Schwann cell tumorigenesis, in general, may be caused by alteration of the *NF2* gene.

Conversely, the findings we presented in Chapter 5 on the expression of the *NF2* protein in rodent schwannomas suggest that the rodent homologue of *NF2* may not be the sole determinant of Schwann cell tumorigenesis in rodents. We speculate that a different or alternate pathway may be involved in this tumor type in rodents. For example, the *neu* gene had been found to be mutated in rat schwannomas induced by ethylnitrosourea mutagenesis (Perantoni et al., 1987). While this fact has been reported in rodents, no mutation of the *neu* gene in human schwannomas has been identified.

5.3 Mutation of the NF2 gene in Meningiomas

In addition to schwannomas, up to half of NF2 patients develop cranial or spinal meningiomas. Studies on this tumor type indicate loss of constitutional heterozygosity on chromosome 22 in 60% of sporadic meningiomas. When analyzed for mutation of the *NF2* gene (Fig. 3), the same proportion of sporadic meningiomas revealed sequence alterations which would cause the complete inactivation of the *NF2* gene in majority of sporadic meningiomas (Ruttledge et al., 1994). According to one study, mutation of the NF2 gene occurred more frequently in fibroblastic and in transitional meningioma than in meningiothelial meningioma indicating a potentially different molecular pathogenic mechanism for each histological subtype of meningioma (Wellenreuther et al., 1995). The remaining 40% of meningiomas thus seem to harbor mutations from another gene(s) suggesting that the tumorigenesis of meningiomas, unlike human schwannomas, involve multiple, possibly independent genes.

5.4 Analysis of the NF2 gene in other tumors.

Gliomas are tumors that are rarely seen in NF2 patients; however, frequent deletions encompassing the NF2 region in gliomas has implicated the *NF2* gene in the development of a proportion of gliomas. Nevertheless, no somatic mutation within the *NF2* gene have been found in gliomas (Hoang-Xuan et al., 1995), suggesting that *NF2* plays no significant role in the genesis of these tumors even those with known loss of heterozygosity on 22q. Mutations of the *NF2* gene in mesothelioma have been reported but the extent of the involvement of *NF2* gene has not been ascertained (Bianchi et al., 1995; Sekido et al., 1995). Similarly, evidence for the role of *NF2* in multiple tumor types including melanoma and breast carcinoma has not been compelling (Bianchi et al., 1994) and have not been replicated in larger samples of tumors (Arakawa et al., 1994). Studies screening for *NF2* mutations in lung cancer cell lines likewise excluded the involvement of

the *NF2* gene in this malignancy (Sekido et al., 1995). Thus, the *NF2* gene is involved in a limited number of tumor types.

6. Cellular expression of schwannomin

The *NF2* protein schwannomin has an estimated molecular weight of ~80 kDa on SDS-PAGE (Claudio et al., 1995; Claudio et al., 1996). Immunohistochemistry using an anti-schwannomin antibody shows expression of schwannomin in Purkinje cells of the cerebellum, motor and sensory neurons of the spinal cord, cells of the tunica intima of blood vessels and goblet cells of the intestine (Claudio et al., 1995). These results suggest that expression of the *NF2* gene is not limited to neuronal tissues. Furthermore, although expression is widespread there is an apparent cell-type specific expression of schwannomin. Interestingly, the schwannomin expressing cells in non-neuronal tissues are not involved in the tumorigenic phenotype of NF2 suggesting that a cell-specific mechanism may be present in these cells to compensate for inactivating mutation of the *NF2* gene. One can speculate that the discrepancy between expression of schwannomin and the phenotype in patients may be due to redundancy in the tumor suppression provided by schwannomin in other cells. Alternatively, functional loss of schwannomin may be lethal in certain cells, and so complete loss of function may lead to cell death.

Functional studies of schwannomin *in vitro* have demonstrated that it can reverse the v-Ha-*ras*-induced anchorage-independent phenotype of NIH/3T3 cells (Tikoo et al., 1994). Additionally, when NIH/3T3 fibroblasts are transfected with the full length NF2 cDNA, a concomitant one-third reduction of growth rate, as well as phenotypic changes such as extension of varying number of thin processes are observed compared to controls (Lutchman and Rouleau, 1995). These data support the tumor suppressor function of the *NF2* gene.

7. Schwannomin: 'Merlin' at the cell cortex

The ERM proteins are found preferentially in dynamic structures; ezrin is found in actin-containing cell-surface structures; moesin is found in microspikes, blebs, retraction fibers, filopodia and lamellipodia; radixin is found in the cell-to-cell adherens junctions and clevage furrows of mitotic cells. Immunofluorescence analysis of schwannomin in lens and Schwann cells as described in Chapter 5 showed preferential localization of schwannomin in leading edges. Similar localization was observed in a meningioma cell line (Gonzales-Agosti et al., 1996). These observations suggest that schwannomin is a functional, member of the ERM family. However, while there has been an increased understanding on the functional biology of the ERM proteins, namely as substrate for protein kinases and as molecular linkers of plasma membrane proteins and the cytoskeleton, the significance of these information in terms of schwannomin's function remains unclear.

The observations on the transient phosphorylation of ezrin at Tyr145 and Tyr353 in response to epidermal growth factor (EGF) or platelet derived growth factor (PDGF) and the concomitant phosphorylation of a yet unidentified tyrosine residue of radixin in response to PDGF but not EGF (Fazioli et al., 1993) suggest that phosphorylation events regulate the function of this family by a growth factor mediated mechanism. Similar phosphorylation of band 4.1 at Tyr 418 occurs upon EGF stimulation resulting in inhibition of spectrin-actin complex formation (Subrahmanyam et al., 1991). Although these amino acid residues are not conserved in schwannomin, there are 20 identified putative phosphorylation sites in the protein (Claudio et al., 1994a; Rouleau et al., 1993). These data provide insights into possible downstream signaling pathway for schwannomin by a receptor specific mechanism.

Although it is currently unknown how schwannomin functions as tumor suppressor, recent data from members of the family provide clues to our understanding of this protein. For example, the amino-terminal and carboxy-terminal regions within the

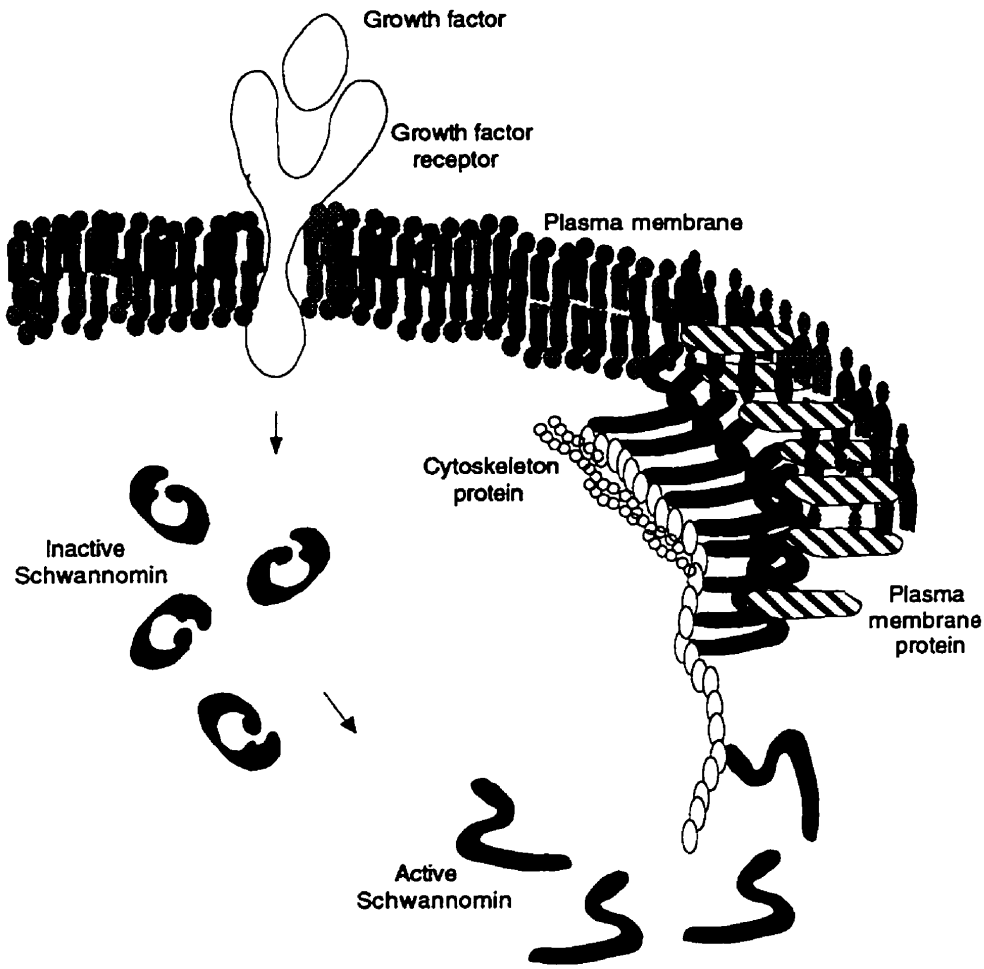
ERM proteins known as ezrin-radixin-moesin association domain (ERMAD), have been identified as being responsible for the self association in head-to-tail joining of N-ERMAD (amino acid 1-296 of ezrin) and C-ERMAD (last 107 amino acid residues of ezrin), and for homotypic and heterotypic associations among the ERM proteins (Gary and Bretscher, 1993; Gary and Bretscher, 1995). Although the presence of active N- and C-ERMAD were identified in ezrin, the conservation of these domains in radixin and moesin suggests similar interactions for both proteins. However, the homology of the C-ERMAD with the carboxy-terminus of schwannomin is low compared to N-ERMAD. This raises the possibility that the C-ERMAD of schwannomin may not be an active ERM association domain. Nevertheless, some insights can be obtained based on studies of ezrin. For example, activation and subsequent oligomerization of ezrin could not occur unless the C-ERMAD is unmasked by conformational change, possibly by phosphorylation (Gary and Bretscher, 1995). Secondly, up-regulation of ezrin has been correlated with oncogenic transformation (Fazioli et al., 1993; Jooss and Muller, 1995). One can hypothesize that schwannomin might heterodimerize with activated ERM proteins that have been activated by mitogenic signals to form heterodimers that do not promote proliferation (Gary and Bretscher, 1995). This concept, however, is speculative and no evidence has been presented to support it.

8. Mechanistic model

While there is lack of information on the cell biology of schwannomin, we consider a working mechanistic model (Fig. 5) based on its relative structural homology to band 4.1, talin and ERM proteins and some recent data in the literature including those presented in Chapters 4 and 5. Schwannomin is thought to sublocalize near the cell membrane with its amino-terminus interacting with plasma membrane protein and its carboxy-terminus to the cytoskeleton. It may normally act like band 4.1 which links transmembrane

Figure 5

A working model of schwannomin's functional mechanism. The model supposes that an active and inactive form of the protein exists. The inactive form can be activated and recruited to dynamic structures by a receptor-specific mechanism. Activation may involve phosphorylation events allowing conformational change in the protein so that its amino-terminus could then interact with membrane proteins and its carboxy-terminus to components of the cytoskeleton. In addition to its role as a putative membrane organizing protein, schwannomin also acts in the complex process of signal transduction. Its proline-rich region may possibly be involved in a pathway involving Rho/Rac or related molecules.


Growth factor
Growth factor receptor
Plasma membrane
Cytoskeleton protein
Inactive Schwannomin
Plasma membrane protein
Active Schwannomin

glycoproteins to the spectrin-actin complex of the cytoskeleton, or like talin which interacts with vinculin and integrins, thereby regulating organization of cell shape, and possibly stable cell-cell and cell-matrix interactions. The conformation of schwannomin may occur in active and inactive state similar to ezrin, because it could be recovered in both the detergent soluble and insoluble cellular fractions (Claudio et al., 1996). Although the radical idea that schwannomins in the soluble fraction are the inactive form of the protein is speculative, it remains an interesting proposition until definite evidence have been obtained to refute it. It may require an 'external factor', (possibly growth factor) to transform the inactive conformation into active conformation that allows it to be recruited beneath the cell membrane. The transformation into active form may be mediated by phosphorylation event brought about by the external factor. Further, our model supposes that schwannomin functions within the complex process of signal transduction. A proline rich region in schwannomin, radixin and ezrin (Fig 3), occurs in the carboxy-terminal region. Proline rich regions have been implicated as targets of Rho/Rac like GTPases in cytoskeletal organization (Symons ety al., 1996). The processes of membrane ruffling and/or formation of stress fibers and focal adhesions are regulated by Rac and Rho (Ridley, 1995). Perhaps, this region in schwannomin may be involved in the ruffling membrane formation observed *in vitro*, by a signaling mechanism involving Rho related proteins.

9. Some answers, more questions

The identification and subsequent findings of high conservation of the mouse homologues of the *NF2* gene made it possible to study the genetic defect in mice. Experimental designs to generate NF2 mutant mice by conventional gene targeting technique have resulted in the understanding that the failure of chorio-allantoic fusion and defect in cardiac development define the embryonic lethality during gastrulation of homozygous mice (McClatchey A and Jacks T, personal communication). However, the exact clinical phenotype of NF2 is not reproducible in heterozygous mice. In view of these

observations, we initiated a transgenic approach to understand the involvement of the *NF2* gene in the genesis of juvenile cataract in NF2 patients (Appendix B). Using a lens specific promoter to express a mutation modeled from an NF2 family that developed cataracts, we have generated mice expressing a lens specific mutant protein. The rationale behind the strategy lies on our hypothesis that a truncated protein presumably acts in a dominant negative fashion to cause lens opacities. As the experiment is ongoing, conclusions are yet to be drawn on the role of truncated schwannomin in the development of lens opacities. Clearly, some questions have been answered but more answers are yet to come.

In the meantime, we summarize some of the answers we now know based on the data presented in this thesis. In chapters 2 and 3, we described the high conservation of the *NF2* gene between human and mouse. This conservation extends from the chromosomal location of nearby genes to the nucleotide sequence of *Nf2*. This findings prompted us to use the mouse to look for the pattern of expression of schwannomin. By looking at the expression of *Nf2* both at the mRNA and protein levels, we observed an apparent widespread but cell type specific expression. Consistent with the putative role of schwannomin as a membrane organizing protein, we observed cytoplasmic localization of schwannomin in Purkinje cells and motor neurons. In lens and Schwann cells, we localized schwannomin in dynamic cellular regions undergoing membrane remodelling such as ruffling membranes and leading edges. When these cells were fractionated and analysed by immunoblotting, schwannomin was found as a component of the detergent insoluble cytoskeleton, albeit at a lower level compared to the detergent soluble fraction. Furthermore, its pattern of expression in lens cells defined a role for schwannomin in differentiation. These data suggest that schwannomin plays a role in the normal biology of a cell other than mere suppression of unrestricted proliferation.

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Appendix A

Typos in the Genetic Script

Type of Mutation in the Neurofibromatosis Type 2 Gene (*NF2*) Frequently Determines Severity of Disease

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Summary

The gene predisposing to neurofibromatosis type 2 (*NF2*) on human chromosome 22 has revealed a wide variety of different mutations in NF2 individuals. These patients display a marked variability in clinical presentation, ranging from very severe disease with numerous tumors at a young age to a relatively mild condition much later in life. To investigate whether this phenotypic heterogeneity is determined by the type of mutation in *NF2*, we have collected clinical information on 111 NF2 cases from 73 different families on whom we have performed mutation screening in this gene. Sixty-seven individuals (56.2%) from 41 of these kindreds revealed 36 different putative disease-causing mutations. These include 26 proposed protein-truncating alterations (frameshift deletions/insertions and nonsense mutations), 6 splice-site mutations, 2 missense mutations, 1 base substitution in the 3′ UTR of the *NF2* cDNA, and a single 3-bp in-frame insertion. Seventeen of these mutations are novel, whereas the remaining 19 have been described previously in other NF2 individuals or sporadic tumors. When individuals harboring protein-truncating mutations are compared with cases with single codon alterations, a significant correlation ($P < .001$) with clinical outcome is observed. Twenty-four of 28 patients with mutations that cause premature truncation of the NF2 protein, schwannomin, present with severe phenotypes. In contrast, all 16 cases from three families with mutations that affect only a single amino acid have mild NF2. These data provide conclusive evidence that a phenotype/genotype correlation exists for certain NF2 mutations.

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Introduction

Neurofibromatosis type 2 (NF2), or central neurofibromatosis, is a severe, often fatal condition in which patients usually present with symptoms from tumors affecting the CNS (Eldridge 1981; Martuza and Eldridge 1988). The most common tumors found in NF2 are vestibular schwannomas, schwannomas at other sites, meningiomas, and ependymomas (Evans et al. 1992*b*). These tumors are often benign and slow growing, but their location predominantly within the CNS may have catastrophic effects on sensitive intracranial and intraspinal structures, thus causing a high rate of morbidity and mortality. Affected individuals typically develop symptoms such as hearing loss (often bilateral), imbalance, tinnitus, facial weakness, and headache (Kanter et al. 1980; Martuza and Eldridge 1988). Approximately half of all NF2 patients also develop posterior capsular lens opacities (Pearson-Webb et al. 1986; Kaiser-Kupfer et al. 1989). The overall incidence of NF2 in Western populations is ~1/40,000 live births, and the average age at onset is in the mid 20s (Kanter et al. 1980; Martuza and Eldridge 1988; Evans et al. 1992*b*; Narod et al. 1992).

It has been proposed that NF2 patients may be clinically subdivided into a severe (Wishart) type and a mild (Gardner) subtype (Eldridge et al. 1991; Evans et al. 1992*a*). This classification is based on the age at onset of symptoms, number and type of tumors developing, and duration of disease. Individuals with the severe-Wishart form of the disease usually present before the age of 25 years, develop numerous tumors (more than three), require repeated surgical intervention, and often do not survive past 50 years of age. Patients with the mild-Gardner subtype usually present with symptoms later in life (classically after 25 years of age), develop a smaller number of more slowly growing tumors (often only bilateral vestibular schwannomas [BVS]), and generally survive beyond the 5th decade. It has been reported that in the majority of familial cases of NF2 there is predominantly only one form of the disease, either

Table 1

Clinical Data for 41 NF2 Families with Mutations in the *NF2* Gene

Family	Patient	Present Age (years)	Age at Onset (years)	Lens Opacity[a]	Tumors Present[b]	Family History[c]	Clinical Subtype[d]
799	782402	17	15	Negative	BVS, mM, mSS	Negative	Severe
240	R1923			Unknown		Unknown	
780	R6307	12	11	Negative	BVS, mM	Negative	Severe
736[e]	R6867 (II-1)	74		Unknown		Positive	Asymptomatic
	R6859 (II-2)	71		Unknown		Positive	Asymptomatic
	R6868 (II-3)	70	22	Unknown	BVS	Positive	Mild
	R6873 (II-5)	57	54	Unknown	BVS	Positive	Mild
	R6865 (II-7)	66		Unknown		Positive	Asymptomatic
	R6872 (II-8)	64	40	Negative	BVS	Positive	Mild
	R6869 (II-10)	59	41	Negative	BVS	Positive	Mild
	R6874 (III-4)	36	33	Negative	UVS	Positive	Mild
	R6862 (III-5)	29		Unknown		Positive	Asymptomatic
	R6861 (III-8)	36		Unknown		Positive	Asymptomatic
794	MR111			Unknown		Unknown	
225	G3060	35	12	Negative	BVS, mM	Negative	Severe
261	R5268	37	18	Positive	BVS, mSS, 1 M	Negative	Severe
202	G5789	29	12	Negative	BVS, 1 M	Negative	Severe
214	G6763			Unknown		Unknown	
223	VMR0195			Positive	BVS	Negative	
254	590402	36	16	Negative	BVS, mSS, 1 M, 1 E, 1 N	Negative	Severe
745	G5145	40	19	Unknown	BVS, mN, 1 M	Negative	Severe
201[f]	G4393	61[g]	42	Negative	BVS, mM	Positive	Mild
220	G9823/0391		17	Unknown	VS, mSS, 2 M	Negative	Severe
758	NNFF103	37	6	Positive	BVS, mS, 1 M	Negative	Severe
781	G2815	27	10	Positive	BVS, 1 M, 1 S	Negative	Severe
249	R3188	32	14	Positive	BVS, 2 M, 1 S	Negative	Severe
234[e]	II-1	34[g]	20	Negative	BVS, 1 M	Positive	Severe
	II-5	33[g]	24	Positive	BVS, 1 M	Positive	Severe
	R3681 (III-1)	32		Negative	UVS, mM	Positive	Asymptomatic
	R3807 (III-2)	31	12	Negative	UVS, mM, 1 N	Positive	Severe
	R3572 (III-4)	30	5	Positive	BVS, mM	Positive	Severe
244	R2848	25	18	Positive	BVS, 1 M, 1 N	Negative	Severe
209	G5095	45	42	Positive	BVS, 1 M	Positive	Mild
535	R0004	37	31	Unknown	BVS, 1 S, 1 M	Negative	Moderate
540	R0005	38[g]	21	Unknown	BVS, mSS	Positive	Severe
783	R5866	37	28	Negative	BVS, 2 M	Negative	Moderate
716	552709	39	18	Unknown	BVS, mS	Positive	Severe
	580802	37		Unknown	BVS, 3 M	Positive	Severe
256[e]	I-2	75	40	Unknown	BVS	Positive	Mild
	I-3	65	20	Positive	BVS	Positive	Mild
	II-2	49	20	Unknown	BVS	Positive	Mild
	II-3	36		Unknown		Positive	Asymptomatic
	II-4	37	35	Negative	UVS, 1 S	Positive	Mild
800	800208	15	13	Negative	BVS, mSS	Negative	Severe
222	G9882/0402		18	Unknown	BVS, SS 1 M	Positive	Severe
785	R7122	30	15	Positive		Negative	Severe
205	G4920	37	24	Unknown		Negative	
757	R4519	20	15	Unknown	BVS, mM, 1 S	Negative	Severe
255	3295D	34	24	Positive	BVS, mM	Negative	Severe
213	G9054	48	28	Unknown	BVS, PN	Positive	Mild
744				Unknown		Unknown	
725	R6364	46	28	Unknown	BVS	Positive	Mild
218	G6274			Unknown		Positive	
779	R6816 (II-4)	29	27	Negative	BVS	Positive	Mild
	I-2	57	40	Unknown	BVS	Positive	Mild
	II-2	36		Unknown	BVS	Positive	Mild
	II-3	33		Unknown	BVS	Positive	Mild

(*continued*)

Table 1 (continued)

Family	Patient	Present Age (years)	Age at Onset (years)	Lens Opacity[a]	Tumors Present[b]	Family History[c]	Clinical Subtype[d]
204	G5434		25	Unknown	BVS, mM, 2 S	Positive	Severe
793	MR112			Unknown		Unknown	
260	R5528 (II-1)	21	20	Positive	BVS, mS, 1 G	Positive	Severe
	II-2	19	19	Unknown	BVS	Positive	Severe
	II-3	16	16	Unknown	BVS	Positive	Severe
	I-2	45	29	Unknown	BVS, 1 M	Positive	Severe
203	G4924	46	41	Unknown	BVS, 2 SS	Positive	Mild
	G5144	50	30	Unknown	BVS	Positive	Mild
	G4913	35	30	Unknown	mSS	Positive	Mild
226	R0622	32	12	Positive	BVS, mS	Negative	Severe

NOTE.—Clinical data are provided only for the families in which sequence variants were identified in NF2.

[a] Eye examination to detect posterior subcapsular lens opacities was performed whenever possible.

[b] Tumors with which each patient has been diagnosed (either by direct histopathological examination of tumor tissue or by radioimaging techniques): M = meningioma (mM = multiple meningiomas); SS = spinal schwannoma (mSS = multiple spinal schwannomas); UVS = unilateral vestibular schwannoma; E = spinal ependymoma; N = neurofibroma (mN = multiple neurofibromas); VS = vestibular schwannoma; S = schwannoma (mS = multiple schwannomas); PN = peripheral neurofibroma; and G = glioma.

[c] Patients are regarded as having a family history if at least one first-degree relative has been diagnosed with NF2.

[d] Patients have been classified according to whether they display a phenotype consistent with the severe or mild form of NF2. This classification is based on age at onset of symptoms, number and type of tumors present, and survival time from diagnosis. Two affected individuals have been classified as having a moderate phenotype, since they do not clearly fall into either of the above two categories.

[e] Pedigrees for three of the largest previously undescribed families (736, 234, and 256) are shown in figure 1.

[f] Clinical data for 22 other affected members of this family may be found in the work of Wertelecki et al. (1988).

[g] Age at death.

the mild-Gardner or severe-Wishart type (Eldridge et al. 1991; Evans et al. 1992*a*). However, this is not exclusively the case, since families with both extremes and with intermediate cases have been observed (Kanter et al. 1980).

We wished to determine whether there is a correlation between the molecular defects in the NF2 gene (*NF2*) and the disease phenotype in affected individuals. That is, does the site or type of mutation play a major role in the severity of NF2, or are there additional factors that play a significant part in the development of this disease? Phenotype/genotype associations have been identified in other disorders, such as colon cancer (for review, see Foulkes, in press), and in some cases this has resulted in a better understanding of the disease and in improvement of the clinical care of patients. Moreover, this type of analysis often results in identification of specific parts of the protein that are critical for normal functioning. To this end, we have screened 73 NF2 families (comprising 111 NF2 individuals) for mutations in the entire coding region of *NF2*, using single-strand conformational analysis (SSCA). For each of the individuals (n = 67) in whom a mutation was identified we have evaluated, wherever possible, the clinical presentation of the disease. For 59 of these cases we have determined age at onset of symptoms, present age, number and type of tumors present, and whether the patient developed posterior capsular lens opacities. Our results demonstrate a statistically significant phenotype/genotype correlation in NF2, for the majority of mutations identified.

Subjects, Material, and Methods

Patient Material

A total of 73 NF2 families containing 111 individuals were screened for mutations in *NF2* (clinical data for the 41 families in which a mutation was identified are given in table 1). These patients were collected as part of our ongoing efforts to elucidate the role of *NF2* and its protein product, schwannomin (SCH), in the disease process. Most cases were referred to us by various clinics in Canada and the United States, but a number were received from Japan, Germany, and Poland. Patients developing at least three tumors (schwannomas, meningiomas, or ependymomas) at an early age (usually before age 25 years) are classified as having severe-Wishart NF2 (n = 49). Individuals presenting later in life (after 30 years of age) and with three tumors or fewer are considered as mild-Gardner cases (n = 38). Four individuals (R0004, R5866, D1956, and D1204) were classified as having moderate NF2, since they did not clearly fall into either of the above two categories (two of these cases are shown in table 1). A further 12 individuals who are presently asymptomatic are known to have NF2 either because a mutation is present or because there is radioimaging evidence of intracranial or intraspinal tumors.

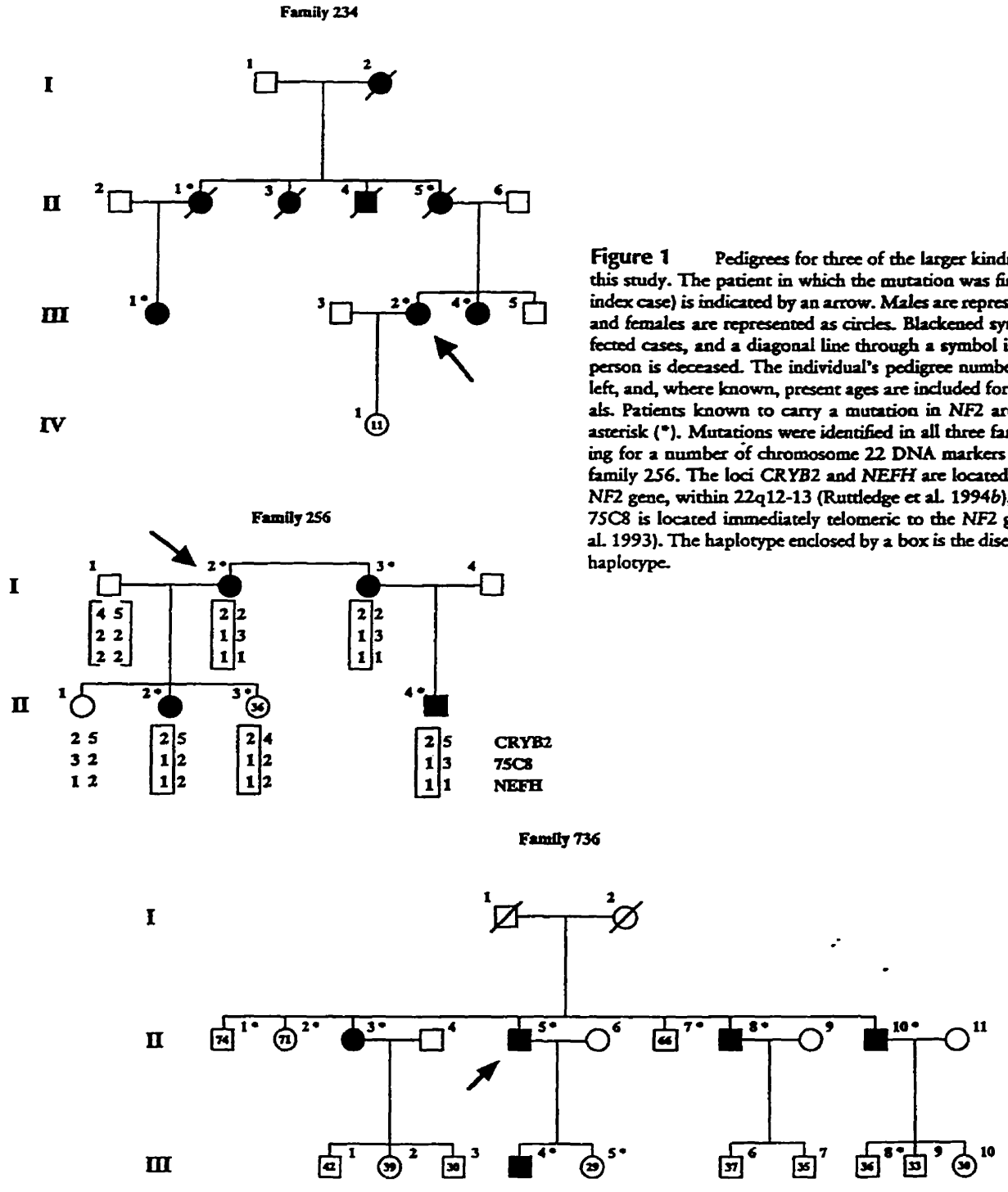

Figure 1 Pedigrees for three of the larger kindreds examined in this study. The patient in which the mutation was first identified (the index case) is indicated by an arrow. Males are represented as squares, and females are represented as circles. Blackened symbols denote affected cases, and a diagonal line through a symbol indicates that the person is deceased. The individual's pedigree number is given to the left, and, where known, present ages are included for at-risk individuals. Patients known to carry a mutation in *NF2* are denoted by an asterisk (*). Mutations were identified in all three families. Allelotyping for a number of chromosome 22 DNA markers are included for family 256. The loci *CRYB2* and *NEFH* are located proximal to the *NF2* gene, within 22q12-13 (Ruttledge et al. 1994*b*), and the marker 75C8 is located immediately telomeric to the *NF2* gene (Rouleau et al. 1993). The haplotype enclosed by a box is the disease-predisposing haplotype.

Of the 73 index cases investigated, 28 (38.4%) had a previous family history of the disease, 37 (50.7%) had no other family members presenting evidence of CNS tumors or peripheral nervous system tumors, and in the 8 (11%) remaining cases no other family information was available. Family 201 is the large NF2 pedigree (BANF 1/family 1) that was originally used to genetically map the *NF2* gene to chromosome 22 (Rouleau et al. 1987, 1990; Wertelecki et al. 1988). We have previously performed presymptomatic diagnosis on many members of this pedigree, using DNA markers flanking the *NF2* gene (Ruttledge et al. 1993). Subsequently, the disease-predisposing mutation has been identified in this family (MacCollin et al. 1993; present report). Families 203 and 218 are two NF2 cohorts (BANF 2 and BANF 9, respectively) that were used for presymptomatic diagno-

Table 2

Oligonucleotide Primers for SSCA and Direct Sequencing Analysis of the *NF2* Gene

Exon	Oligonucleotide Sequences[a]	PCR Product Size (bp)	Nucleotides Screened[b]
1	Forward 5′-AGG CCT GTG CAG CAA CTC-3′ Reverse 5′-GAG AAC CTC TCG AGC TTC CAC-3′	261	−60→114 (174)
3	Forward 5′-GCT TCT TTG AAG GTA GCA CA-3′ Reverse 5′-GGT CAA CTC TGA GGC CAA CT-3′	275	241→363 (123)
4	Forward 5′-CCT CAC TTC CCC TCA CAG AG-3′ Reverse 5′-CCC ATG ACC CAA ATT AAC GC-3′	188	364→447 (84)
6	Forward 5′-CAT GTG TAG GTT TTT TAT TTT GC-3′ Reverse 5′-GCC CAT AAA GGA ATG TAA ACC-3′	161	517→599 (83)
7	Forward 5′-CAG TGT CTT CCG TTC TCC-3′ Reverse 5′-AGC TCA GAG AGG TTT CAA-3′	123	600→675 (76)
9	Forward 5′-GTT CTG CTT CAT TCT TCC-3′ Reverse 5′-GTA ATG AAA ACC AGG ATC-3′	138	811→885 (75)
10	Forward 5′-CCT TTT AGT CTG CTT CTG-3′ Reverse 5′-TCA GTT AAA ACA AGG TTG-3′	166	886→999 (114)
13	Forward 5′-GGT GTC TTT TCC TGC TAC CT-3′ Reverse 5′-GGG AGG AAA GAG AAC ATC AC-3′	227	1341→1446 (106)
14	Forward 5′-TGT GCC ATT GCC TCT GTG-3′ Reverse 5′-AGG GCA CAG GGG GCT ACA-3′	253	1447→1574 (128)
15	Forward 5′-TCT CAC TGT CTG CCC AAG-3′ Reverse 5′-GAT CAG CAA AAT ACA AGA AA-3′	245	1575→1737 (163)
16	Forward 5′-CTC TCA GCT TCT TCT CTG CT-3′ Reverse 5′-CCA GCC AGC TCC TAT GGA TG-3′	178	1738→1876 (149)
17	Forward 5′-GGC ATT GTT GAT ATC ACA GGG-3′ Reverse 5′-GGC AGC ACC ATC ACC ACA TA-3′	148	

[a] Oligonucleotide sequences for exons 2, 5, 8, and 12 have been given by Ruttledge et al. (1994*x*). Sequences for exons 3, 4, 6, 13, 14, 16, and 17 were provided by Dr. Lee Jacoby. The antisense oligonucleotide from each primer set was biotinylated at the 5′ end for sequencing.

[b] From Rouleau et al. (1993). The total number of nucleotides examined is given in parentheses.

sis and genetic homogeneity testing (Narod et al. 1992; Ruttledge et al. 1993). Family 752 has been described by Pastores et al. (1991). Clinical details for family 213 (BANF 14) will be described elsewhere. Pedigrees for families 736, 234, and 256 (which have not been described previously) are given in figure 1. Substantial clinical information was unavailable for other affected family members in the remaining kindreds.

Mutation Screening by SSCA

Isolation of constitutional DNA directly from peripheral blood leukocytes or from transformed cell lines was performed as described elsewhere (Ruttledge et al. 1993). All 17 exons of the *NF2* gene were examined for point mutations by using SSCA with the primers listed in table 2. Approximately 50–100 ng of genomic DNA was used for PCR (with S^{35}-dATP) in a total reaction volume of 12.5 μl. The conditions for PCR were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of alternate steps of annealing, polymerization, and denaturation as outlined elsewhere (Ruttledge et al. 1994*a*). To this reaction 8 μl of stop buffer (95% formamide, 10 mM EDTA pH 8.0, 0.025% each of xylene cyanol FF and bromophenol blue) was added, and 5 μl of this mix was heated to 85–90°C for 2 min and then loaded onto a 6% nondenaturing polyacrylamide gel (with and without 5%–7% glycerol). A nondenatured control was also included, so that the single-stranded fragments could be identified easily. The samples were electrophoresed at a constant current of 20 mA at 4°C for 4–8 h, the time being dependent on the size and pattern of migration of the fragment being

Table 3

Families in Which Constitutional Mutations in the *NF2* Gene Have Been Identified

Family	Patient	Exon[a]	Type of Mutation	Nucleotide(s) Affected	Codon(s) Affected	Predicted Consequence on SCH[b]	Family History	Clinical Subtype[c]
799	780224	1	Nonsense	CAA(Gln)→TAA(Stop) at 52	18	X at 18	Negative	Severe
240	R1923	1	Deletion	TC at 41-42	14	F→X at 47	Unknown	
780	R6307	1	Insertion	A at 27-28	9–10	F→X at 48	Negative	Severe
736	R6873	2	Insertion	GAT TTG→GAT TTG TTG[d]	49	I at 49	Positive	Mild
794	MR111[e]						Unknown	
225	G3060	3	Deletion	A at 270	90	F→X at 122	Unknown	Severe
261	R5268	3	Deletion	T at 287	96	F→X at 122	Unknown	Severe
202	G5789	3	Complex	GAGA[A]TGCTGAA→GAGATTAAATTGCTGAA[f]	103–104	F→X at 122	Negative	Severe
214	G6763	4	Insertion	CTCCTGG at 417	139	F→X at 154	Unknown	
223	VMR0195	6	Nonsense	TAT(Tyr)→TAA(Stop) at 531	177	X at 177	Negative	
254	590402	6	Nonsense	CGA(Arg)→TGA(Stop) at 586	196	X at 196	Unknown	Severe
745	G5145	6	Nonsense	CGA(Arg)→TGA(Stop) at 592	198	X at 198	Negative	Severe
201	G4393	7	Missense	AAC(Asn)→TAC(Tyr) at 658	220	M at 220	Positive	Mild
220	G9823/0391	8	Nonsense	TGG(Trp)→TGA(Stop) at 774	258	X at 258	Negative	Severe
758	NNFF103	8	Nonsense	CGA(Arg)→TGA(Stop) at 784	262	X at 262		Severe
781	G2815	8	Nonsense	CGA(Arg)→TGA(Stop) at 784	262	X at 262	Negative	Severe
249	R3188	8	Deletion	C at 768	256	F→X at 295	Negative	Severe
234	R3807	8	Splice	GAG:gta→GAA:gta		SE	Positive	Severe
244	R2848	10	Deletion	T at 945	315	F→X at 321	Negative	Severe
209	G5095	11	Nonsense	CGA(Arg)→TGA(Stop) at 1021	341	X at 341	Positive	Mild
535	R0004	11	Nonsense	CGA(Arg)→TGA(Stop) at 1021	341	X at 341	Negative	Moderate
540	R0005	11	Nonsense	CGA(Arg)→TGA(Stop) at 1021	341	X at 341	Positive	Severe

783	R5866	11	Nonsense	CGA(Arg)→TGA(Stop) at 1021	341	X at 341	Negative	Moderate
716	552709	11	Nonsense	CGA(Arg)→TGA(Stop) at 1021	341	X at 341	Positive	Severe
256	I-2	11	Missense	CTG(Leu)→CCG(Pro) at 1079	360	M at 360	Positive	Mild
800	800802	11	Insertion	AG at 1032-1033	344–345	F→X at 364	Negative	Severe
222	G9882/0402	11	Splice	CTG:gtg→CTG:ttg		SE	Positive	Severe
785	R7122	12	Nonsense	CAG(Gln)→TAG(Stop) at 1165	389	X at 389	Negative	Severe
205	G4920	12	Deletion	G at 1177	393	F→X at 425	Negative	
757	R4519	12	Deletion	AGAGAGGAG:gtg→AGGAG:gtg	445–446	F→X at 453	Negative	Severe
255	3295D	12	Deletion	AG at 1336-1337	446	F→X at 493	Negative	Severe
213	G9054	13	Splice	cag:GGC→caa:GGC		SE	Positive	Mild
744		14	Deletion	atccgaaatttctcattaacag:CC→atag:CC		SE	Unknown	
725	R6364	14	Insertion	T at 1518–1520	506–507	F→X at 513	Positive	Mild
218	G6274	14	Deletion	T at 1499	500	F→X at 514	Positive	
779	R6816	15	Splice	cag:AGT→cac:AGT		SE	Positive	Mild
204	G5434	15	Nonsense	GAA(Glu)→TAA(Stop) at 1580	527	X at 527	Positive	Severe
793	MR112						Unknown	
260	R5528	14	Deletion	GAGA at 1564–1567	522–523	F→X at 549	Positive	Severe
203	G4924	15	Insertion	200 bp at AG:gtacccaggg[g]		SE	Positive	Mild
226	R0622	16	Substitution	G→A at 1833	3′ UTR	Unknown	Negative	Severe

NOTE.—A total of 73 NF2 families were screened for mutations in all 17 exons of the *NF2* gene by SSCA. The mutations identified in 41 of these families are described here.

[a] Exons of the *NF2* gene that were screened by SSCA; this includes intronic splice-site sequences on both sides of the exon in all cases except for exons 1 and 16, where one of each pair of the PCR primers was situated in the 5′ and 3′ UTR of the *NF2* cDNA, respectively.

[b] According to the amino acid sequence reported by Rouleau et al. (1993): X = stop at the codon indicated; F→X = frameshift to a stop at the codon indicated; I = insertion of a single amino acid at the codon indicated; M = missense at the codon indicated; and SE = putative splicing error.

[c] Patients have been classified as in table 1.

[d] The 3-bp in-frame insertion in exon 2 is predicted to introduce an additional leucine residue into SCH at position 49.

[e] The mutation has been described by M. Sainio (personal communication).

[f] Complex rearrangement involves deletion of an A nucleotide (boxed) at position 311 of the *NF2* cDNA (Rouleau et al. 1993) and concomitant insertion of a novel 6-bp sequence (underlined), the net result being insertion of 5 bp into the open reading frame and a frameshift resulting in the introduction of a premature stop codon at position 122 of the NF2 protein, SCH.

[g] An ~200-bp insertion in the intron between exons 15 and 17, located 10 bp 3′ of the end of exon 15 and presumably causing aberrant splicing of the *NF2* transcript.

tested. The gel was then dried and exposed to X-ray film for 1–3 d. When an abnormal migration pattern was detected, the PCR was repeated and run together with negative controls on a new nondenaturing polyacrylamide gel.

Direct Sequencing of Aberrant PCR Fragments

The exons showing an abnormal SSCA pattern were amplified by PCR from ~300–500 ng of genomic DNA in a total reaction volume of 100 μl by using the same conditions as described above. The amplified fragments were excised from a 1% agarose gel and were purified as described elsewhere (Ruttledge et al. 1994*a*). After precipitation, the DNA was resuspended in 50 μl of TE buffer (10 mM Tris-Cl pH 7.6, 1 mM EDTA pH 8), and the complementary strands were separated by using streptavidin-coated magnetic beads (Dynal) as described elsewhere (Ruttledge et al. 1994*a*). The nucleotide sequence of both single strands was independently determined by using the Sequenase version 2 DNA sequencing kit (United States Biochemical). Mutations were verified by repeating the sequencing from a second PCR, and, in cases where other family members were available, these were also included. The primers that were employed for PCR reactions were also used for sequencing, except that nonbiotinylated oligonucleotides were used in the antisense direction.

Results

Clinical details for the 41 NF2 families (67 individuals) in which mutations were identified in *NF2* are given in table 1. Pedigrees for three of the larger families that have not been described elsewhere are shown in figure 1. A total of 36 different putative disease-causing mutations were identified in these 41 kindreds after SSCA and direct sequencing analysis of constitutional DNA (table 3). Of these 36 variants, 15 are insertions or deletions presumably causing frameshifts, 11 are nonsense mutations, 6 are believed to result in defective splicing, 2 are missense changes, 1 is an in-frame insertion of 3 bp in exon 2, and in a single case a base substitution in the 3′ UTR of *NF2* was identified. The age at onset of symptoms in 83 of the 95 NF2 individuals for whom data were available ranged from 1 to 54 years (mean 24.8 years). In the remaining 12 cases either a mutation in *NF2* has been identified or there is diagnostic imaging evidence of intracranial tumors, but the patient remains asymptomatic at ages ranging from 10 to 74 years (mean 37.3 years). On the basis of these ages at onset, the number of years since diagnosis, and the number and type of tumors presenting, we have classified these patients as either mild-Gardner NF2 or severe-Wishart NF2.

The number of individuals from each clinical group with a specific type of mutation is summarized in table 4. When protein-truncating mutations are compared with single codon changes (missense mutations in families 201 and 256 and a 3-bp in-frame insertion in family 736), a significant correlation with phenotype is found (by χ^2 test; $P < .001$). Two of 28 patients with protein-truncating mutations have mild NF2, whereas all 16 cases with single codon changes are classified as having this phenotype. When all individuals in table 4 are taken into account, a total of nine different alterations (table 3) are detected in 27 individuals (table 1) who have been classified as mild-Gardner NF2 ($n = 20$) or who are asymptomatic ($n = 7$). Twenty-five (92.6%) of these 27 cases are found to have either a splice-site alteration ($n = 9$) or a single amino acid change—that is, two missense mutations ($n = 6$) or a single 3-bp insertion ($n = 10$)—in *NF2*. In contrast, only 5 (15.6%) of the 32 severe-Wishart ($n = 30$) or moderate ($n = 2$) NF2 cases (constituting 18 different mutations) have one of the latter types of alteration. The vast majority (26/32) of severe-Wishart or moderate NF2 cases have nonsense or frameshift-causing deletion/insertion mutations, all of which are predicted to result in the production of a substantially truncated SCH protein.

With the possible exceptions of the two missense changes (families 201 and 256; table 3) and the single base substitution in the 3′ UTR of *NF2*, we did not identify any rare polymorphisms in these 111 NF2 cases. Furthermore, in direct sequencing of all 17 exons in 28 unrelated individuals we did not encounter any sequence changes that were not believed to be associated with the disease process.

The 3-bp in-frame insertion (table 3) that has been identified in kindred 736 (fig. 1) is present in 10 family members, only 5 of whom are affected with NF2 (table 1 and fig. 1). A further seven, unaffected individuals (family 736, cases III-1, III-2, III-3, III-6, III-7, III-9, and III-10 in fig. 1) do not harbor this alteration. The clinical phenotype associated with this mutation is very mild-Gardner NF2. This is represented by the fact that five mutation carriers (average age 55.2 years) still show little or no signs of disease. One of these individuals (III-5) has experienced some balance problems, but magnetic-resonance imaging (MRI) at age 28 years failed to detect intracranial tumors. Furthermore, the five affected cases appear to develop only vestibular schwannomas with no evidence of other intracranial or intraspinal tumors, even though MRI or computed-tomography scans have been performed in all cases.

The two missense mutations that have been identified are present in a total of 23 individuals from two NF2 kindreds, families 201 and 256 (table 3 and fig. 1). The first of these variants was found in family 256 (fig. 1), and it results in the substitution of a proline for a leucine residue at amino acid position 360 of the protein sequence reported by Rouleau et al. (1993). This mutation

Table 4

Number of Individuals in Each Clinical Group, for Different Classes of Mutation in *NF2*

	Protein-Truncating Mutations (n = 26)[a]	Splice-Site Mutations (n = 6)	Single Codon Changes (n = 3)[b]	3′ UTR (n = 1)	Total
Mild	2	9	16	0	27
Moderate	2	0	0	0	2
Severe	24	5	0	1	30
Unknown	7	1	0	0	8
Total	35	15	16	1	67

NOTE.—Sixty-seven individuals from 41 families with 36 different mutations are presented; sufficient clinical data were available for 59 cases to be classified as mild, moderate, or severe NF2.

[a] Includes nonsense changes and frameshift insertions/deletions leading to a premature translation stop.

[b] Two missense mutations in families 201 and 256 and the 3-bp in-frame insertion in family 736 (table 3).

has previously been identified in a presumably unrelated individual displaying a mild-Gardner phenotype (Rouleau et al. 1993; Merel et al. 1995). In family 256, this mutation is present in four individuals (I-2, I-3, II-2, and II-4) with the mild-Gardner subtype of NF2 (fig. 1 and table 1). An additional family member (II-3) also has the missense mutation but was asymptomatic on the basis of normal MRI results of the entire neuraxis at age 36 years. Individual II-3 previously had been predicted to carry the mutated *NF2* gene, on the basis of haplotype analysis using DNA markers flanking the *NF2* gene on chromosome 22 (fig. 1). Two affected members of family 256 (I-3 and II-4) have been examined by an ophthalmologist for lens abnormalities. No evidence of lens opacities was found in case II-4. Individual I-3 presented with a subcapsular cataract of the right lens at age 65 years, the relationship to NF2 being unclear.

The second missense mutation identified in this study (patient G4393 in family 201; table 1) has been reported independently elsewhere (MacCollin et al. 1993). It results in the conversion of an asparagine to a tyrosine residue at amino acid position 220 of SCH. This change is found in 12 other affected members and in 5 presently asymptomatic members of family 256 whom we have tested. We had previously predicted that all 18 of these patients—that is, individuals 37, 16, 20, 18, 49, 53, 52, 60, 57, 46, 67, 69, 79, 81, 82, 85, 75, and 76 in figure 2 of the report by Ruttledge et al. (1993)—would carry the mutated *NF2* gene. Another 20 unaffected members of this pedigree were found not to harbor this missense mutation, results consistent with our previous haplotype analysis (Ruttledge et al. 1993).

An insertion of ~200 bp was identified in the intron between exons 15 and 16 in constitutional DNA from patient G4924 (table 1; family 203), a member of a large Italian/American family that previously has been used in genetic linkage studies to narrow down the *NF2* locus on chromosome 22 (Narod et al. 1992; Ruttledge et al. 1993). In addition to the normal 245-bp PCR product for exon 15 (table 3), a second novel band, of ~450 bp, was seen in this patient by agarose-gel electrophoresis (data not shown). After direct sequencing of both the normal and novel fragments, an ~200-bp insertion was found in the larger fragment. The 200-bp insertion is located 10 bp 3′ of the splice-donor junction of exon 15 (table 1). No significant sequence homology was identified between this 200-bp fragment and sequences in the database. The smaller, 245-bp band contained the normal published sequence for exon 15 and its surrounding introns. The variant identified in patient G4924 is also present in seven other affected family members and in four presently unaffected at-risk individuals. The clinical course of NF2 in three of these affected cases whom we have followed is one of relatively late onset (in the 4th and 5th decades) with predominantly BVS and a limited number of tumors at other sites (table 1). The clinical picture in this kindred is thus most consistent with the mild-Gardner form of NF2.

Discussion

Previous studies have provided evidence of an association between the severe-Wishart form of NF2 and protein-truncation mutations (Bourn et al. 1994*a*, 1994*b*; Merel et al. 1995). We confirm this correlation, since 80% of our 30 severe NF2 cases have nonsense changes or frameshift causing deletion/insertion mutations. Our data, however, also reveal a highly significant ($P < .001$) association between mild-Gardner NF2 and single codon alterations. Furthermore, there is also a predominance of mild NF2 in splice-site-mutation carriers. In total, six mutations are associated with a milder phenotype, and these include two missense changes, three splice-site alterations, and a single 3-bp in-frame insertion (tables 1 and 3). In all but 2 of the 27 mild-phenotype or asymptomatic NF2 patients in whom mutation data were available, one of these three types of mutation was observed.

Probably the most striking example of a phenotype/genotype correlation is in family 736, where a 3-bp in-frame insertion is observed between codons 48 and 49 of *NF2* (tables 1 and 3). The clinical picture observed in this kindred is remarkable because thus far, at a relatively late age (29 to 74 years), five mutation carriers have not developed any detectable tumors. In addition, the five affected mutation carriers have presented only with vestibular schwannomas. Two of these patients (individuals II-8 and II-3 in fig. 1) are in their 7th and 8th decades, respectively. The presence of this 3-bp insertion in the five known affected individuals in family 736 suggests that either this is the pathogenic mutation in this kindred or it is segregating with the disease allele. Support for a causative role of this variant in NF2 is provided by the observations that (1) it has not been identified in >500 unrelated individuals whom we have screened by SSCA, (2) it is segregating with the disease in five affected individuals, and (3) it is found in a highly conserved region of *NF2* (Rouleau et al. 1993; Trofatter et al. 1993). In addition, given that the predicted result of this alteration is the introduction of a single leucine residue, without any other known consequence on SCH, it is conceivable that such a mild phenotype may result. On the basis of both the unusual nature of this insertion and the clinical phenotype, it is possible that some residual protein activity still exists in tumors, and this may account for their generally slow-growing nature. This hypothesis of a partially acting protein differs from the all-or-none concept that generally is proposed for tumorigenesis in NF2 (Seizinger et al. 1987; Rouleau et al. 1993; Trofatter et al. 1993).

The two missense mutations that are found in 23 affected individuals in families 201 and 256 (mutations affecting codons 220 and 360, respectively) are presumably located in regions of SCH that are of functional importance, since both amino acids are highly conserved between SCH and the human band 4.1 proteins (Rouleau et al. 1993; Trofatter et al. 1993). Whatever the function of these codons, it appears that their substitution by other amino acids results in a predominantly mild form of NF2. These missense mutations do allow for the specific formation of vestibular schwannomas and other tumors associated with NF2, but they do so at a lower frequency than is seen in cases that have protein-truncation mutations. Family 201 has been examined in detail elsewhere (Rouleau et al. 1987; Wertelecki et al. 1988; MacCollin et al. 1993; Ruttledge et al. 1993), with affected individuals displaying a generally mild form of NF2 in which no presenile lens opacities (believed to be associated with NF2) have been identified. In the original report of this family (Wertelecki et al. 1988), only 3 (13.0%) of the 23 affected individuals presented with symptoms of NF2 before 20 years of age, and 13 (56.5%) of 23 remained asymptomatic until at least the 4th decade. Therefore, although a small proportion of affected members of this family have developed numerous tumors (some at a relatively young age), the overall clinical picture is one of mild-Gardner NF2, with the majority of carriers displaying one or two neoplasms.

The milder symptoms observed in most of the individuals with missense mutations may be attributable to the fact that these changes only partially disrupt SCH or alter its half-life. Thus, residual SCH activity may lead to slower tumor growth in these cases. In contrast, by deleting a substantial portion of SCH (e.g., in cases with protein-truncation mutations), total loss of SCH function occurs after loss of the wild-type allele in tumor tissue. In this situation the rate at which tumors grow usually increases, resulting in both earlier onset of symptoms and a larger number of tumors presenting (on average) in each individual.

An association between splice-site mutations and mild NF2 is also evident in our data. Six mutations listed in table 3 are likely to affect splicing, and three of these (in families 213, 779, and 203) are associated with a mild phenotype in eight different affected individuals. Such mutations might result in partial splicing, where the mutant site is used less frequently than its normal counterpart, thus allowing a certain amount of normal mRNA and protein to be produced and resulting in a less severe form of the disease. Further support for a relationship between splice-site mutations and a mild-Gardner NF2 phenotype is provided in a recent report by Merel et al. (1995). That report describes a total of four mild NF2 cases, three of which harbor splice-donor mutations; however, a further six splice-site mutations are found in patients with a severe clinical course. Moreover, two of the remaining three splice-site mutations in the present study are associated with a severe phenotype. When the results from both data sets are combined, there is no clear indication as to which type of splice-site alteration (e.g., splice-donor or -acceptor mutations) gives rise to a mild or severe phenotype.

The role of the base substitution in the 3′ UTR of the NF2 cDNA in family 226 is unclear. Conceivably, it may affect the half-life of the *NF2* transcript in this individual, or it may be involved in deregulating the splicing machinery of the cell. On the other hand, it is possible that it is a benign polymorphism that is not the causative mutation of *NF2* in this case. It should be noted that, after the entire coding region of the *NF2* gene was screened by SSCA, only ~60% of affected families were shown to have mutations. This figure may represent genetic heterogeneity in NF2 (Narod et al. 1992). A more likely explanation for the lack of mutation detection in the remaining 40% of affected cases, however, is that either there is a shortfall in sensitivity of SSCA or mutations lie outside the coding exons of the gene (e.g., in the introns or regulatory regions). Thus, it is conceivable that the pathogenic mutation in family

226 has not yet been identified. To aid in the determination of the role of this base substitution in NF2, it would be helpful to examine both parents for the presence or absence of this variant. If this mutation occurred de novo in individual 226, it may be of greater significance, since there is no known family history of the disease. Unfortunately, neither parent was available for study, and thus the role of this base substitution in the development of NF2 remains to be determined

We were interested to determine whether a particular type of mutation in NF2 is associated with the development of presenile lens opacities. Of the 111 individuals included in this study, 57 were examined for lens opacities, with 26 being found positive for them. Fourteen of these 26 cases were also found to have a mutation in *NF2*, with 12 cases harboring a splice-site or protein-truncation mutation. The role of the remaining two mutations (in families 256 and 226, discussed above) in the disease process is uncertain. Thus, our data neither support nor refute the hypothesis of a truncated-protein/presenile-lens-opacity correlation.

We have considered all of the affected individuals in family 234 as having severe-Wishart NF2—except patient III-1, who, at age 32 years, still shows no clinical signs of the disease, even though she has radioimaging evidence of several intracranial tumors (table 1). This severity is evident when the pedigree for this family is examined (fig. 1); all four sibs in the second generation of this kindred died at an early age (34 and 33 years for individuals II-1 and II-5, respectively), because of the serious complications associated with NF2. In contrast, all four affected cases in family 256 (fig. 1) are alive and are coping well. This comparison highlights the disastrous affects of some mutations in *NF2*—and the relatively benign nature of others—on the individuals who harbor them. In conclusion, we believe that most protein-truncation mutations are associated with severe-Wishart NF2, whereas other specific mutations (two missense mutations, some splice-site alterations, and the 3-bp insertion in family 736) are associated with a more mild-Gardner form of the disease. However, other factors certainly play a role in determining the severity of this condition in certain individuals.

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Appendix B

Perspective from the Lens

Introduction

Lens opacities of juvenile onset occurs in up to 80% of NF2 patients (Kaiser-Kupfer et al, 1989; Nance et al, 1992). It is believed that this manifestation of the disease occurs as dominant phenotype, in contrast to the development of tumors which is caused by recessive expression of mutations on both alleles of the *NF2* gene. Presumably, a mutant *NF2* protein acts in a dominant negative mechanism, disrupting the normal function of the native protein. To test this hypothesis, we generated transgenic mice with targeted expression of an inframe mutation in their lens. The mutation, patterned from an NF2 family that developed lens opacities, deletes a 333 bp sequence corresponding to exons 2, 3 and 4 (Rouleau et al, 1993) thereby disrupting the highly conserved amino terminus of schwannomin. This domain is highly conserved in the band 4.1 family, and is hypothesized to interact with plasma membrane proteins.

Materials and Methods

Expression constructs:

To generate the *NF2* construct for specific expression in lens fiber cells, the Xba1/HindIII fragment from an expression construct which contains the regulatory sequences of the γ-crystalline promoter (Goring et al, 1987) was subcloned into the Xba1/HindIII sites of pBluescript SK generating pγXH101. A ligation site for the NF2 cDNA was generated in pγXH101 by ligating an oligoucleotide AGCTGAATTC to the HindIII site, thereby creating an EcoR1 site. This plasmid was named pγNE114. The NF2 cDNA with inframe deletion of 333 bp corresponding to exons 2, 3 and 4 (see below) was ligated into the EcoR1 site of pγNE114, generating pγNF2Δ19. To introduce a polyadenylation signal into the construct, an 800 bp HincII fragment from pMARßgal

which contains an SV40 consensus polyadenylation signal sequence was blunt ligated into the HincII site of pγNF2Δ19, generating pNF2ΔSV156.

The cDNA missing 333 bp sequence was constructed by RT-PCR of RNA extracted from patient R2142 which has an intragenic germline deletion within the *NF2* gene spanning exons 2, 3 and 4 (Rouleau et al., 1993)(Fig. 1a). The primers used for RT-PCR, forward: 5'-TCTCAAGAGGAAGCAACCCA-3' and reverse: 5'-GAACTTCCAAAGAATCGGCT-3', located in exon 1 and 9, respectively amplified a 917 bp fragment that was subcloned into pBluescript-TA vector generating pTA7172. A Mun1/Bgl2 fragment from pTA7172 which contains the region with the 333 bp deletion was ligated into the same site of pNF2 which contains the complete open reading frame of the human NF2 gene. The clone generated was named pNF2Δ234.

DNA used for microinjection was digested with Not 1/Kpn1, run on agarose gel and purified using Gibco/BRL DNA purification kit.

Immunoblotting

Protein used for immunoblotting was extracted using NP-40-containing lysis buffer as described previously (Claudio et al, 1995). Protein concentration was determined by Bradford reagent from Bio-Rad Laboratories. Samples were stored at -80°C until used. Prior to electrophoresis, 7.5 - 15 μg of protein, depending on the size of the wells, were heat denatured in the presence of sample loading buffer containing ß-mercaptoethanol. Proteins were separated on 10% sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) and blotted unto 0.2 μm nitrocellulose filters. The protein blots were blocked for 1 hour at room temperature or overnight at 4°C with 10% milk in TBS. The primary antibody, polyclonal rabbit anti-schwannomin antiserum described previously (Claudio et al., 1995) was diluted 1:300 in 5% milk/TBS. After 1 hour incubation at room

temperature, the blots were washed 3 times of 15 min each with TBS containing 0.2% Tween 20. The secondary antibody, horseraddish peroxidase-conjugated goat anti-rabbit IgG F(ab')2 (Jackson ImmunoResearch Laboratories) or alkaline phosphatase-conjugated goat anti rabbit IgG (Sigma) was diluted 1:5000 and incubated for 1 hour at room temperature. Washing was done 3 times for 15 min each using TBS containing 0.2% Tween 20. Horseradish peroxidase signal was detected by enhanced chemoluminescence with ECL reagents (Amersham Life Sciences) and alkaline phosphatase signal by NBT/BCIP substrate (Sigma).

Results and Discussion

Expression of schwannomin in the lens

Two lines of mice, 4375 and 4359, with targeted expression of truncated human schwannomin were generated by microinjection of the expression construct into fertilized mouse embryos. Immunoblot analysis to detect the expression of the construct show the presence of a truncated protein product with molecular weight of about 69 kDa in addition to the endogenous protein migrating at about 80 Kda (Fig. 2a). The truncated protein product was observed in non-ionic detergent soluble extracts of transgenic mice eyes but not in other tissues (Fig 2b) confirming the γ_F-crystalline promoter-specific expression of the exogenous protein.

Consequence of expression of truncated protein in the lens fiber cells

Comparison of the level of expression of the endogenous wild-type schwannomin compared to the exogenous truncated protein at post natal day 20 shows an equivalent amount of proteins (Fig. 2). Expression of the truncated protein decreases with age similar

to the endogenous protein. A marked difference in the level of expression can be observed even by 3 months. By 12 months of age, the exogenous protein is already hardly visible by immunoblot analysis (data not shown). Thus, if overt clinical manifestations of lens opacity were apparent, such phenotype would be detected early in life. Histological analysis of sections of mouse lens at E12.5 and E13.5 shows no observable morphological changes (not shown). The lens fiber cells reached the anterior epithelium as would be expected by E13.5 suggesting that there is no disruption in the differentiation schedule of the developing lens fiber cells. Thus, if the truncated protein were acting in a dominant negative effect by competing with the wild-type endogenous schwannomin for binding to cytoskeletal proteins, as hypothesized, a marked histological phenotype, however subtle, would have been observed. These results taken together provide evidence that co-expression of a wild type schwannomin with a mutant *NF2* protein containing inframe deletion within the highly conserved amino-terminal domain does not result to differentiation defect in the lens fiber cells.

Ongoing experiments

We have generated transgenic mice expressing mutant *NF2* protein. In parallel experiment a wild-type *NF2* construct has been utilized as control. As the experiments are ongoing, definite conclusions are yet to be drawn. However, based on current available information from the transgenic mice, the mutant protein appears not to affect the differentiation schedule of the lens fiber cells. Studies whether these mice develop mild lens opacities are ongoing.

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Figure 1

Strategy in the generation of transgenic mice expressing a mutant NF2 protein. (a) NF2 genomic structure showing the location of germline deletion in an NF2 family from which the mutation used to generate transgenic mice was modeled. (b) Construct used to generate transgenic mice. (c) Domain structure of the wildtype NF2 protein (d) Domain structure of the mutant NF2 protein. (e) Southern analysis of F1 from founder 4375 showing transmission of the transgene in B303 mouse.

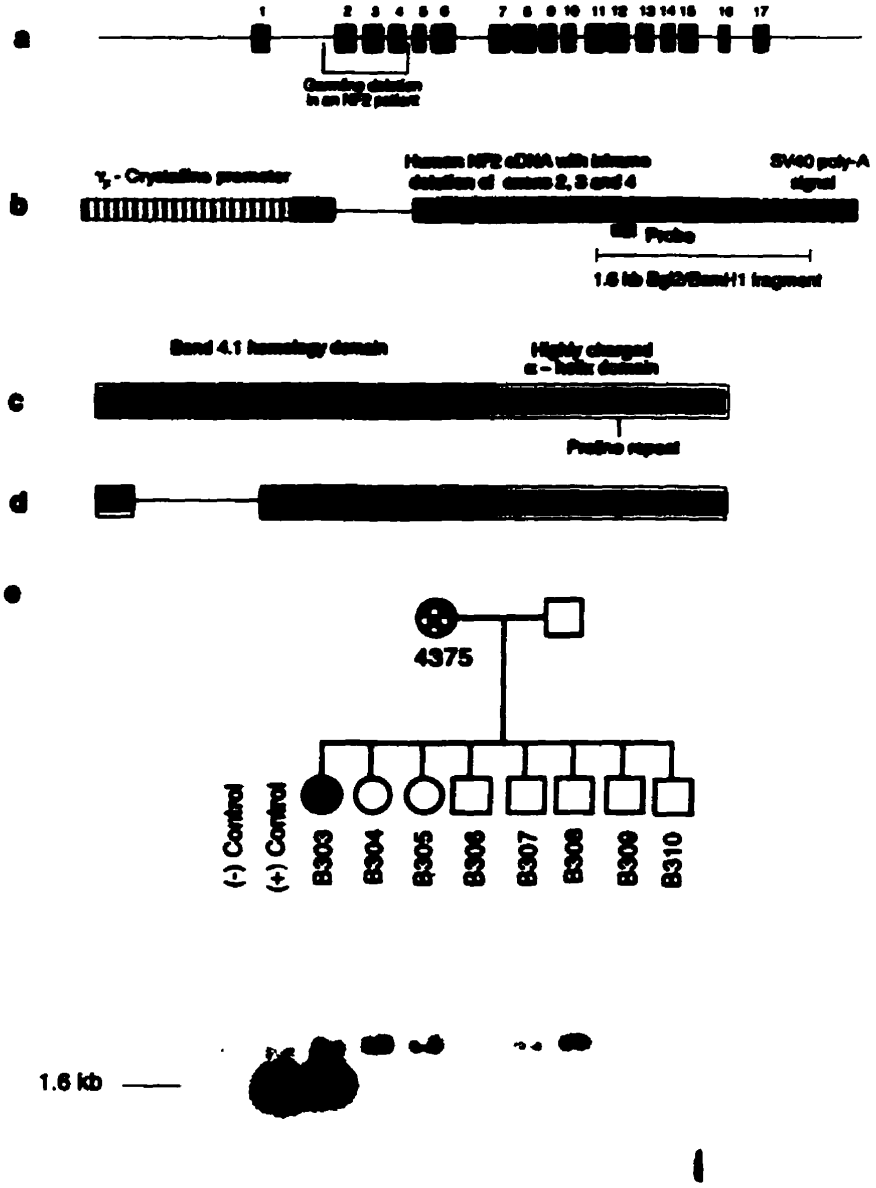
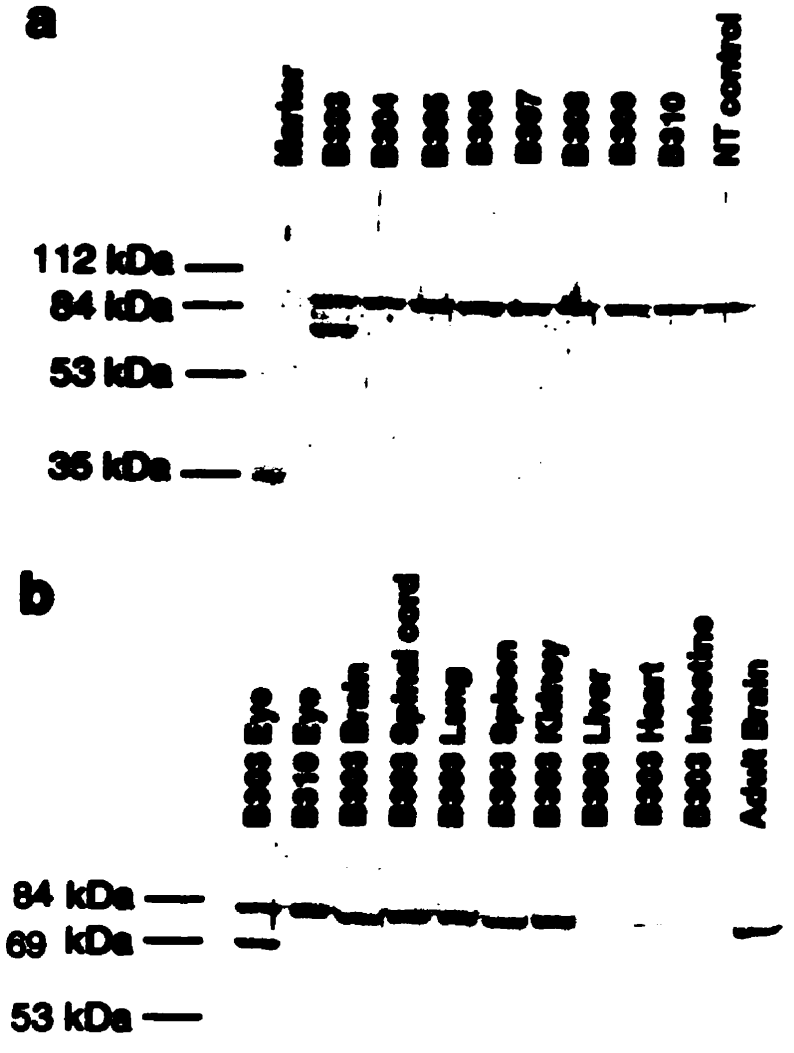

a
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
b
γ - Crystallins promoter
SV40 poly-A signal
Probe
c
Band 4.1 homology domain
Highly charged
Proline repeat
d
4375
(-) Control
(+) Control
B303
B304
B305
B306
B307
B308
B309
B310
1.6 kb

Figure 2

Expression of the transgene by immunoblotting. (a) Expression of the transgene as a truncated protein in mouse B303. (b) Eye-specific expression of the mutant NF2 protein in B303 mouse.


a
Marker
E5.5
E6.5
E7.5
E8.5
E9.5
E10
NT control
112 kDa
84 kDa
53 kDa
35 kDa
b
E5.5 Eye
E10 Eye
E5.5 Brain
E5.5 Spinal cord
E5.5 Lung
E5.5 Spleen
E5.5 Kidney
E5.5 Liver
E5.5 Heart
E5.5 Intestine
Adult Brain
84 kDa
69 kDa
53 kDa

Appendix C

Isoform 2

Figure 1

Sequence of a mouse cDNA clone showing the site of alternative splicing. A 45 bp sequence is introduced between exons 15 and 17. The alternate exon creates 11 novel amino acids but truncates the protein prematurely at the 20th codon upstream of the stop codon of isoform 1. This sequence was deposited in EMBL with accession no. X75759. The arrows indicate the PCR primers used to amplify the dinulceotide repeats described in Chapter 3.

Clone AB211.1 Isoform 2 (EMBL Accession No. 75759)

```
                                   CAA AAG GCT GCA GAG GCT GAG CAA GAG ATG CAG CGA ATC AAG GCC ACG GCC ATT CGG ACA GAG   1836
                                   Gln Lys Ala Ala Glu Ala Glu Gln Glu Met Gln Arg Ile Lys Ala Thr Ala Ile Arg Thr Glu    420

GAG GAG AAG CGC CTG ATG GAG CAG AAG GTG CTG GAG GCT GAA GTG CTG GCA TTG AAG ATG GCT GAG GAG TCA GAG AGG AGG GCC AAG GAG   1926
Glu Glu Lys Arg Leu Met Glu Gln Lys Val Leu Glu Ala Glu Val Leu Ala Leu Lys Met Ala Glu Glu Ser Glu Arg Arg Ala Lys Glu    450

GCT GAT CAG TTA AAG CAA GAC TTG CAA GAA GCC CGA GAA GCA GAG CGA AGA GCC AAG CAG AAG CTC TTA GAA ATC GCC ACC AAG CCC ACC   2016
Ala Asp Gln Leu Lys Gln Asp Leu Gln Glu Ala Arg Glu Ala Glu Arg Arg Ala Lys Gln Lys Leu Leu Glu Ile Ala Thr Lys Pro Thr    480

TAT CCA CCC ATG AAC CCA ATT CCA CCA CCA CTG CCT CCT GAC ATA CCG AGC TTC GAC ATT ATT GCT GAC AGC TTG TCA TTC GAC TTC AAG   2106
Tyr Pro Pro Met Asn Pro Ile Pro PRO Pro Leu Pro Pro Asp Ile Pro Ser Phe Asp Ile Ile Ala Asp Ser Leu Ser Phe Asp Phe Lys    510

GAT ACG GAC ATG AAG CGA CTT TCC ATG GAG ATA GAG AAA GAA AAA GTG GAG TAC ATG GAG AAG AGC AAG CAC CTG CAG GAG CAG CTC AAC   2196
Asp Thr Asp Met Lys Arg Leu Ser Met Glu Ile Glu Lys Glu Lys Val Glu Tyr Met Glu Lys Ser Lys His Leu Gln Glu Gln Leu Asn    540

GAG CTC AAG ACT GAG ATC GAG GCC TTG AAA CTC AAA GAG CGG GAG ACG GCC TTG GAC GTC CTA CAC AGC GAG AGC TCA GAC AGA GGC GGC   2286
Glu Leu Lys Thr Glu Ile Glu Ala Leu Lys Leu Lys Glu Arg Glu Thr Ala Leu Asp Val Leu His Ser Glu Ser Ser Asp Arg Gly Gly    570

CCC AGC AGC AAG CAT AAT ACC ATT AAA AAG CCT CAA GCC CAA GGC AGA AGA CCT ATC TGC ATT TGA GTC CTC AAA CTC ACT CTG CAG AGC   2376
Pro Ser Ser Lys His Asn Thr Ile Lys Lys Pro Gln Ala Gln Gly Arg Arg Pro Ile Cys Ile  *                                    591

GCC AAG TCC CGA GTG GCC TTC TTT GAA GAA CTC TAG CAG GTG ACC CGG CCA CCT CCT GCC AAC ATC TGC TGC TCC TGA CAC CAA CAG GAT   2466
GGG CCT GAC CCA AAA GGA ACC ATC AGT AGA GGG CTG GCT TGT TTG GGA ACT CTT GAG TTG AGG GCC CCG TGC CAC TCT GTC CCT ATA AGA   2556
GAG GTT TGT CAC AAT GTG TTC TAG GTT CTC CCT TGC CTC CTG AAT ACC CTG CAT ATC TCT CTC TCC CTC CCT CTC TCT CTC TCT CCC TTC   2646
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TCC TTC CTG CAC CCA AGC ACT GTG GTG CAC AGG TTG CTG TGC TTG CTA GAC ATT GAG AAT CGG CTG TGG GAG TCC AGT GTG AAA GCT GGC   2826
TGA AAT CTA CCC TAG CAG TGG CTA CCC ACT CCC CAA CTG GCT TCT GGG ATG GGC CTC CCC TCC AAG CAG GGG TAG A                     2902
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Appendix D

Claims to Originality

CLAIMS TO ORIGINALITY

Chapter 2 describes the cloning of the mouse homologue of the *Nf2* gene. It concludes that the *NF2* gene is conserved in mice and across vertebrate species.

(1) The mouse neurofibromatosis type 2 gene is 90% identical to the human gene and 98% homologous to the human protein.

(2) Most of the amino acid changes occur at the carboxy-terminus of the protein, outside of the band 4.1 homology domain.

(3) The mouse protein encodes a 596 amino acid-protein, schwannomin, one amino acid longer than the human protein due to the insertion of a proline residue at position 571.

(4) Zoo blot analysis predicts the conservation of the gene across vertebrate species.

Chapter 3 demonstrates the predictive value of comparative mapping. This method was used to identify the chromosomal location of *Nf2*.

(1) The dinucleotide $(CT)_n$ repeat at the 3' untranslated region of the mouse *Nf2* cDNA is polymorphic in inbred strains of mice. This polymorphic marker was named *D11Mcg1*.

(2) The mouse *Nf2* gene maps to the proximal arm of chromosome 11. This region also contains the homologues of neurofilament heavy chain polypeptide gene (*NEFH*), leukemia inhibitory factor (*LIF*) and the gene disrupted in Ewing sarcoma (*EWS*), confirming this segment of mouse chromosome 11 as a region of conserved synteny to a small segment of human chromosome 22.

(3) The locus order, relative to *Nf2*, from the centromere is : *D11Mit1/D11Mit72/D11Mcg1-D11Mit74-Pmv-2-D11Mit2-D11Mit77/D11Mit78/D11Mit63.*

Chapter 4 provides evidence of the widespread but cell type-specific expression of the mouse *Nf2* gene.

(1) The mouse *Nf2* gene product, schwannomin, is an ~80 kDa protein.

(2) Schwannomin is expressed not only in cells affected by the NF2 phenotype, like lens and Schwann cells, but also in other cells not involved in the tumorigenic property of inactivated schwannomin.

(3) Immunofluorescence using mouse tissues localized the protein in the cytoplasm, providing indirect evidence for the hypothesis that schwannomin acts in the cytoskeleton-membrane interface.

Chapter 5. Demonstrates the localization of schwannomin in lens and Schwann cells and its possible role in differentation-specific events.

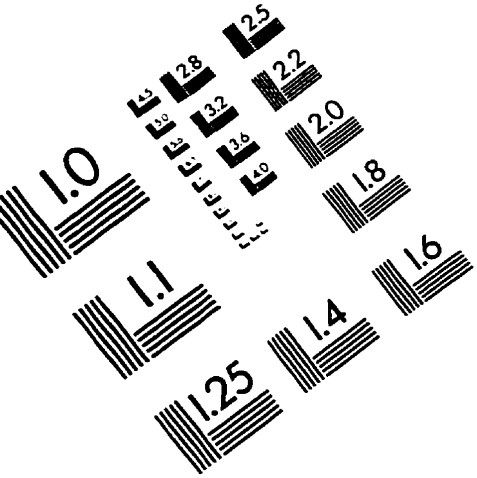
(1) Schwanomin is preferentially localized to dynamic structures of lens and Schwann cells.

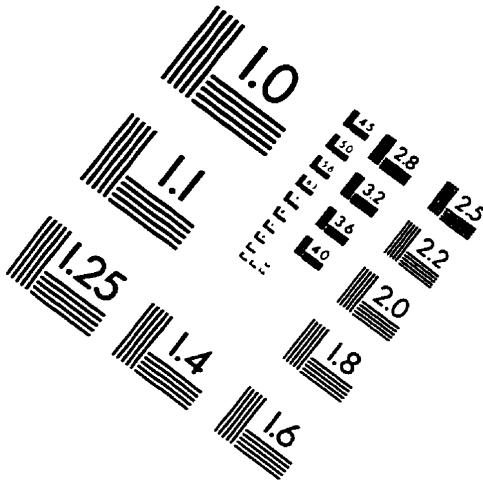
(2) Schwannomin is a component of the detergent insoluble cytoskeleton.

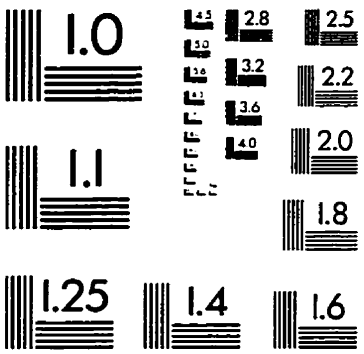
(3) Schwannomin is expressed in rodent schwannomas suggesting a limited role of *Nf2* in this tumor type in rodents.

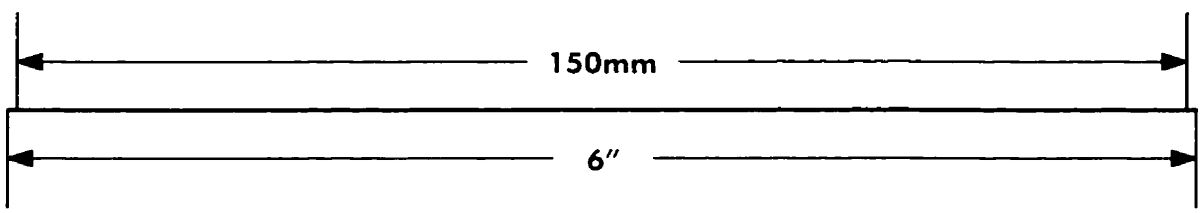
(4) Schwannomin's expression is regulated during lens cell differentiation, suggesting its role in differentiation-specific events.

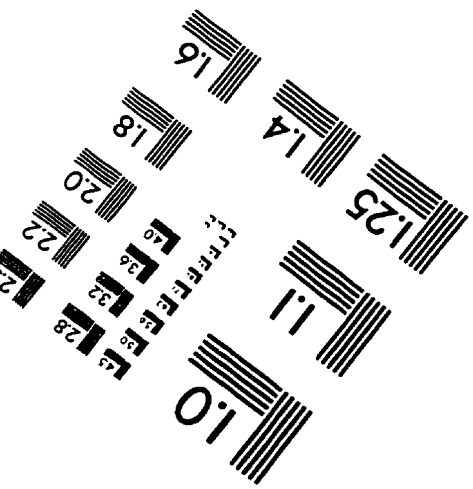
IMAGE EVALUATION TEST TARGET (QA-3)











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