Tissue distribution and functional aspects of the NF2 tumour suppressor gene

Expressie in weefsels en functionele aspecten van het NF2 tumor suppressor gen

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Even the most difficult problems can be solved, and even the most precipitous heights can be scaled, if only a slow, gradual, step-by-step pathway can be found. Mount Improbable cannot be assaulted. Gradually, if not always slowly, it must be climbed.

Richard Dawkins, "Climbing Mount Improbable"

For my mother,
Ter nagedachtenis aan mijn vader,
voor Andrea

CONTENTS

List of abbreviations

ERM	ezrin, radixin, moesin
NF2	neurofibromatosis type 2
NF1	neurofibromatosis type 1
NER	nucleotide excision repair
MMR	mismatch repair
RT-PCR	reverse transcriptase-polymerase chain reaction
CIN	chromosomal instability
XP	xeroderma pigmentosum
HNPCC	hereditary non-polyposis colorectal cancer
VS	vestibular schwannoma

CHAPTER I

General introduction

1. Hereditary cancer syndromes

Neoplastic disease in humans may occur sporadically, that is without a clear familial disposition, or less commonly as an inherited disease. Several distinct familial cancer syndromes are known and the underlying genetic cause has been identified of a number of these [46]. The tumours in these syndromes are usually phenotypically identical to their sporadically occurring counterparts. Both syndromes with benign and with malignant tumours are known. In cancer syndromes tumours generally develop at a younger age than those seen in the non-familial setting and are often multiple [101]. Most inherited cancer syndromes with an autosomal dominant inheritance pattern are caused by mutations in tumour suppressor genes. Far fewer inherited cancer syndromes are caused by mutations in (proto-)oncogenes, notably multiple endocrine neoplasia type 2 and familial medullary thyroid cancer caused by mutations in the RET gene [99, 107], hereditary papillary renal cell carcinoma caused by mutations in the MET gene [124] and some cases of familial melanoma caused by mutations in the CDK4 gene [165]. In addition to the classical tumour suppressor genes and proto-oncogenes, other genes involved in hereditary cancer are DNA repair genes.

1.1 oncogenes

Oncogenes are the mutated counterparts of cellular genes, proto-oncogenes, that physiologically function as regulators of cell proliferation and programmed cell death (apoptosis). The products of oncogenes, also known as oncoproteins, act as growth factors, growth factor receptors, proteins involved in intracellular signal transduction and proteins involved in apoptosis [101]. Mutations in oncogenes are of a dominant nature, resulting in gain of function and loss of susceptibility to control. The oncogene concept was formed after the discovery that in animals retroviral gene integration in host DNA could lead to tumour formation [153]. Two mechanisms may lead to transformation by retroviral oncogenesis. Firstly, a viral oncogene (v-onc) may be inserted in the host DNA. In this case the viral oncogene is derived from a cellular homologue, c-onc. Alternatively, retroviral insertion of DNA may lead to altered expression of cellular proto-oncogenes. In humans retroviral oncogenesis is exceedingly rare, in most cases cellular proto-oncogenes are activated by mutations and translocations. To date more than 100 (cellular) oncogenes have been identified.

1.2 tumour suppressor genes

Tumour suppressor genes are cellular genes both alleles of which must be inactivated for oncogenesis. Evidence for the existence of tumour suppressor genes came from cell fusion experiments in which the tumour-forming capacity of cancer cells was lost after fusion with normal cells or other cancer cells. Loss of chromosomes from these hybrid cells could restore the oncogenic nature of cells. Therefore loss of or, as would be shown later, inactivation of specific genes could lead to malignant transformation of cells [84]. The concept of tumour suppressor genes, also called anti-oncogenes, was further developed by Knudson by observation of the epidemiology of sporadic (unilateral) and familial

(bilateral) cases of retinoblastoma. In this model, called the "two-hit" hypothesis, a germline mutation in a tumour suppressor gene (the first hit) is followed by a somatic mutation in a given target cell (the second hit), thus resulting in a total lack of functional gene product [78]. Although mutations in tumour suppressor genes are of a recessive nature, meaning that both alleles must be inactivated for tumorigenesis, the resulting inherited phenotypic trait is dominant. To date, at least 12 tumour suppressor genes have been identified and partially characterized. However, the frequent observation that tumour cells show loss of chromosomes or parts of chromosomes indicates that many more tumour suppressor genes exist in the human genome [101]. It is most likely that these tumour suppressor genes do not lead to inherited cancer syndromes, possibly owing to embryonic lethality with haplo-insufficiency, but do play a role in multistep carcinogenesis and progression [159].

1.3 genes involved in genetic integrity

The DNA of the human genome is constantly threatened by endogenous and exogenous factors. Over time a sophisticated system has developed through evolutionary pressure to maintain the integrity of the genome. DNA damage leads to mutations. If activating mutations in growth regulating proteins (proto-oncogenes) and inactivating mutations in tumour suppressor genes are not corrected by the DNA repair machinery, uncontrolled growth, cancer, may ensue. Repair genes, alternatively known as mutator genes, encode proteins that function in the proof-reading and correction of replication errors in DNA. Two major forms of DNA repair are nucleotide excision repair (NER) and mismatch repair (MMR). Germline defects in NER genes lead to well-defined clinical syndromes, including xeroderma pigmentosum. In addition to non-neoplastic features, cancer frequently develops in patients with NER defects [155]. Mutations in mismatch repair genes result in a dominantly inherited cancer syndrome in which the most frequently encountered neoplasm is colon cancer (hereditary non-polyposis colorectal cancer, HNPCC). Mutations in several genes engaged in mismatch repair may lead to HNPCC. The characteristic feature of this syndrome is found in microsatellite instability, resulting from faulty repair or replication of di- or trinucleotide repeats [75]. In addition to instability caused by NER and MMR defects, gross chromosomal abnormalities such as complete loss or gain of chromosomes or parts thereof are often seen in cancer. Underlying this phenomenon, named CIN for chromosomal instability, may be faults in genes involved in structural organisation of the chromatin, genes coding for proteins involved in the mechanics of mitoses (including centrosome formation) and checkpoint genes monitoring cell cycle progression [83].

2. Neurofibromatoses

The neurofibromatoses are a hereditary group of neurocutaneous disorders (phakomatoses) of which the 2 major forms are neurofibromatosis type 1 (NF1), also known as von Recklinghausen's disease, and neurofibromatosis type 2 (NF2). Until 1987, NF1 and NF2 were considered variants of a single form of neurofibromatosis. The first extensive description of neurofibromatosis by Friedrich von

Recklinghausen dates from 1882 [110] (the history of the neurofibromatoses is reviewed by Ahn [1]). Features described as characteristic of neurofibromatosis were the numerous skin tumours, often leading to severe malformation. In a minority of neurofibromatosis patients the disease was dominated by intra-cranial tumours with few or no skin manifestations. This "central" form of neurofibromatosis (as distinct from "peripheral" neurofibromatosis) was first described by Wishart in 1822 in a patient with bilateral tumours at the cerebello-pontine angle [160]. In addition to these bilateral cerebello-pontine tumours, Wishart described other intracranial tumours connected to the meninges. These tumours are now known as meningiomas. The familial nature of neurofibromatosis was noted soon after the first description by von Recklinghausen. An extensive survey of patients with bilateral cerebello-pontine angle tumours by Gardner and Frazier in 1930 confirmed the dominant inheritance pattern of central neurofibromatosis [49]. Furthermore, it was found that only approximately 1 in 20 patients with neurofibromatosis presented with the central form. Despite these findings the central from of neurofibromatosis was regarded as a variant and not as a distinct disease. In 1987 linkage analysis assigned the NF1 gene locus to the pericentromeric region on chromosome 17 [10, 129] whilst in the same year the NF2 gene locus was mapped to chromosome 22 [114], providing definitive proof for segregation of these diseases.

2.1 neurofibromatosis type 1

NF1 is a hereditary disease with an autosomal dominant inheritance pattern in which the main symptoms are caused by hamartomatous, dysplastic and benign neoplastic lesions which may occasionally undergo malignant transformation.

2.1.1 epidemiology

The incidence of NF1 is estimated at 1:3000 [66]. A minor reduction in the life span was found in one study. However, the cause of death did not differ significantly from that of the general population. Some rare diseases, including juvenile chronic myeloid leukemia and pheochromocytoma are seen with an increased frequency in individuals affected by NF1 [140]. Genetic fitness is reduced in NF1 patients.

2.1.2 clinical signs and symptoms

The diagnostic criteria for NF1 have been established by the National Institute of Health [104]. Common findings in NF1 are café-au-lait spots, neurofibromas, Lisch nodules and skin-fold freckling [56]. Café-au-lait spots are pigmented skin macules characterized by increased pigmentation of basal keratinocytes. The café-au-lait spots increase in number and size with ageing and are generally the first signs to appear in NF1 or are present at birth [56]. Lisch nodules are hamartomatous lesions of the iris, these do not cause any visual impairment. Neurofibromas are benign, nodular, sometimes pedunculated, non-encapsulated growths composed of elements similar to those in normal nervous

tissue but with a disorganised pattern. Severe malformation and cosmetic problems may be caused by plexiform neurofibromas. These tumours diffusely infiltrate large nerve trunks and are composed of constituents of normal nerves with a disproportionate amount of matrix substance. Plexiform neurofibromas may attain a large size. Additional findings in NF1 patients include intellectual impairment, macrocephaly, dysplastic bone lesions, short stature, hypertelorism and the presence of high-signal-intensity lesions on magnetic resonance images in the brain [56, 67]. Complications resulting from plexiform neurofibromas, skeletal malformation and brain tumours include scoliosis, epilepsy, endocrinological abnormalities and an increased risk of malignant tumours [34].

To date there is no cure for NF1. Surgery may be necessary for neurofibromas, which owing to their disfiguring nature may lead to psychosocial problems. The complete removal of plexiform neurofibromas is often not possible because of their large size and infiltrative growth characteristics. NF1 patients are best managed by a multidisciplinary team, with a single co-ordinator who may involve other specialist team-members when the need arises [67].

2.1.3 the NF1 gene and protein

Cloning of the NF1 gene was accomplished in 1990 [29, 154, 156]. The gene spans 300kb and consists of 59 exons separated by 58 introns. Embedded within an intron in the NF1 gene are 3 other genes which are transcribed in the opposite direction of the NF1 gene. After splicing an 11-13kb NF1 mRNA is formed [85]. The mutation rate of the NF1 gene is higher than would be expected on statistical grounds, even when the large size of the gene is taken into account [34]. The large size of the NF1 gene hampers mutation detection in affected and at risk individuals. However, analysis of mutations in the NF1 gene has confirmed its role as a tumour suppressor gene. In NF1 disease severity varies widely and is unpredictable even within a single affected family. This disease heterogeneity is likely to be caused by modifier genes [41].

The NF1 gene encodes a 2818 aminoacid protein, named neurofibromin. This protein contains a GAP (GTPase activating protein) related domain (GRD) and acts as a negative regulator for the RAS signal transduction pathway [47]. However, the exact mechanism of tumour formation by lack of neurofibromin remains to be resolved.

2.2 neurofibromatosis type 2 (NF2)

NF2 is now recognised as a distinct clinical entity. This is reflected in the names by which NF2 was previously referred to: bilateral acoustic neurofibromatosis (BANF), central neurofibromatosis, hereditary bilateral vestibular-schwannoma syndrome and Wishart-Gardner-Eldridge syndrome [1, 67].

2.2.1 epidemiology

NF2 is about 10 times rarer than NF1 with an incidence of about 1:33.000-1:40.000 live births. Mean survival after diagnosis is 15 years; first signs and symptoms usually present in the second or third decade. Penetrance is close to 100% at 60 years [42, 43].

2.2.2 clinical signs and symptoms

The distinguishing feature of NF2 are bilateral vestibular schwannomas, previously referred to as acoustic neuromas. These occur in over 98% of NF2 patients [105]. These neoplasms are benign proliferations of Schwann cells, with a characteristic histological appearance and originating from the nerve sheath. Although schwannomas are located eccentric to the nerve proper and do not directly involve the nerve fibers they produce symptoms through pressure effects, especially in confined spaces. The schwannomas in NF2 develop on the vestibular branch of the eighth cranial nerve. In a population-based study [43] first symptoms were deafness in 44% of patients, tinnitus in 10% and balance problems in 8%; in the remaining patients presenting symptoms were caused by other central nervous system tumours.

Approximately half of NF2 patients develop meningiomas [43, 105]. Like schwannomas, these are benign tumours which probably arise from arachnoidal cells in the meninges [4, 17]. Symptoms caused by these tumours result from their space-occupying nature and include headaches and seizures. In addition to these neoplastic features NF2 patients have ocular disease manifestations of which only (sub)capsular cataracts cause symptoms [22, 72, 74, 82].

Apart from vestibular schwannomas, schwannomas may develop in other sites in NF2 patients including other cranial nerves, skin and in particular along spinal nerve roots [19, 43, 93, 94].

The diagnostic criteria for neurofibromatosis type 2 (NF2) were established in 1988 [104]. Slight modifications were introduced in 1997 (Table I) [56].

Epidemiological and clinical data indicated that 2 clinical forms of NF2 could be discerned. The more clinically severe form, called the Wishart type, has an early age of onset, rapid disease course, multiple meningiomas and bilateral VS's; the latter less often as presenting sign. A milder form named the Gardner type, has a later onset and presents with VS's and fewer meningiomas. Disease progression is slower [67].

2.2.3 NF2 gene and genetics

Genetic linkage analysis located the NF2 gene on chromosome 22 in 1987 [114]. After narrowing down the region with new markers (reviewed by Bijlsma [18]) the gene was cloned 1993 [113, 147].

2.2.3.1 human, mouse, rat and Drosophila NF2

The human NF2 gene, consisting of 17 exons, is located on the long arm of chromosome 22 and spans approximately 100kb [113, 147]. Two major splice variants are known: in isoform I exon 16 is skipped

and all aminoacids encoded by exon 17 are included, generating a 595 aminoacid protein, in isoform II exon 16 containing an in-frame stop codon is included, producing a 590 aminoacid protein with 11 new C-terminal aminoacids [14, 109]. The isoforms thus differ in their C-terminal composition, whereby the isoform I C-terminus has a predicted helical configuration and is more hydrophobic than isoform II. No functional specificity is known for the isoforms. In northern blots a predominant 4.5kb mRNA was seen in a wide range of tissues, together with weaker messenger signals at 2.6 and 7kb [113, 147]. In addition to these variants, several other less prominent splice variants have been described [63, 109].

Table I. Diagnostic criteria for NF2 [56]

Individuals with the following clinical features have confirmed (definite) NF2:
I. bilateral vestibular schwannoma (VS), or
II. family history of NF2 (first-degree family relative) plus:
1. unilateral VS under 30 years of age, or
2. any 2 of the following: meningioma, glioma, schwannoma, juvenile posterior subcapsular lenticular opacities / juvenile cortical cataract.
Individuals with the following clinical features should be evaluated for NF2 (presumptive or probable NF2):
I. unilateral VS under the age of 30 years plus at least one of the following: meningioma, glioma, schwannoma, juvenile posterior subcapsular lenticular opacities/ juvenile cortical cataract.
II. Multiple meningiomas (≥ 2) plus unilateral VS under 30 years of age or one of the following glioma, schwannoma, juvenile posterior subcapsular lenticular opacities/ juvenile cortical cataract.

The protein encoded by the NF2 gene is homologous to a family of proteins of which the principal members are ezrin, radixin and moesin. This homology is reflected in one of the names suggested for the NF2 protein, merlin, for moesin, ezrin and radixin like protein [147]. Alternatively, the NF2 protein is known as schwannomin [113]. Ezrin, moesin and radixin (collectively the ERM proteins) are part of a superfamily related to the erythrocyte band 4.1 protein.

Phylogenetic conservation has been proven for the NF2 gene, with murine and rat NF2 homologues sharing 90% sequence identity with human NF2 and 98% identity on protein level. Northern blotting and RT-PCR analysis revealed the presence of transcripts in a wide variety of tissues [31, 32, 58-60, 68, 76].

In Drosophila a NF2 homologue has been described with 55% sequence identity with human NF2. Although the homology of Drosophila NF2 is greatest in the N-terminus, the C-terminal similarity is of note as this region of NF2 shows less overlap with other homologous proteins (see below, 4. ERM and related proteins) [81, 95].

2.2.3.2 mutations in NF2 related- and sporadic vestibular schwannomas and meningiomas

Mutation detection in the NF2 gene is hampered by the relatively low sensitivity of the techniques employed, single strand conformational polymorphism (SSCP) analysis and denaturing gradient gel electrophoresis (DGGE). Theoretically DGGE should have a high sensitivity for mutations in the NF2 gene. However, intronic mutations and deletions of complete exons are not uncovered by this technique. In addition, mosaicism will hamper detection by DGGE. Maximal sensitivity of SSCP and DGGE is at best 60% for the NF2 gene. Alternative techniques such as southern blotting or the protein truncation test (PTT), may be more sensitive but are more labour intensive or may not be consistently successful because of technical difficulties ([79]; personal observation). Using SSCP and DGGE mutations have been detected in NF2 gene that segregate with the disease [166](and references herein). Most mutations found in the NF2 gene lead to truncation of the NF2 protein, suggesting inactivation. Truncating mutations have likewise been described in sporadically occurring schwannomas and meningiomas. The frequent loss of the second NF2 allele in sporadic and NF2 related tumours confirms the NF2 gene as a classical tumour suppressor gene. No clear mutation hotspots are present in the NF2 gene, although combining published data and taking the effects of mutations on secondary structure into account indicate that the distribution of mutations is not random [55, 150, 166].

2.2.3.3 genotype-phenotype correlation

Analysis of mutations segregating in NF2 families shows that a clear genotype-phenotype correlation exists in NF2. Mutations resulting in NF2 protein truncation more often lead to severe (Wishart) phenotype, whilst missense mutations are most often associated with a mild (Gardner) phenotype [44, 118, 166]. In theory the association of truncating mutations with the severe NF2 phenotype could be caused by a dominant negative effect. However, several mutations described in the literature lead to severe truncation of the NF2 protein. It is questionable whether the resulting extremely short proteins retain any biological activity [166]. It seems more likely that truncating mutations result in an unstable NF2 protein that is rapidly degraded [57]. Conversely, missense mutations may result in either a less stable NF2 protein or in a NF2 protein that is functionally less effective. In this setting complete absence of NF2 protein resulting from accelerated degradation would lead to a severe phenotype as seen in NF2 patients with truncating mutations and some missense mutations. Most patients with missense mutations presenting with a mild phenotype would harbour a NF2 protein with diminished function. However, identical mutations have been identified in patients with distinct clinical subtypes [21, 127]. In addition, phenotypic variation has been observed in monozygotic twins, although concordance for disease severity (in severe and mild forms) was preserved [11]. Also, in patients with mild phenotypes splice-site mutations theoretically leading to truncated proteins and whole gene deletions leading to an absence of NF2 protein have been described. [27, 98, 120, 158], this thesis, chapter VI). It is possible that mosaicism underlies some cases of phenotypic variation [21, 77]. Alternatively, modifier genes may cause disease variation in selected cases [27], although the phenotypic variation

within families does not support the idea of their existence in NF2. In splice-site mutations some residual wild-type protein is likely to be formed, thus leading to a mild phenotype.

2.2.3.4 NF2 mutations in other tumours

Several different types of human neoplasms that show loss of chromosome 22 markers have been screened for NF2 mutations. Apart from in pleural mesotheliomas and melanoma metastases, NF2 mutations have only sporadically been detected in other tumour types [8, 15, 16, 36, 37, 64, 71, 76, 102, 116, 117, 130, 138, 141, 157].

2.2.3.5 NF2 mouse models

Attempts to generate a mouse (knock-out) model for NF2 have yielded some surprising results. Homozygous NF2 knock-out mice fail at the gastrulation stage, whilst heterozygotes develop metastatic epithelial and mesenchymal tumours [96, 97]. No schwannomas or meningiomas were observed. Recently, NF2 transgenic mice were generated with mutant NF2 genes under control of the Schwann cell specific P0 promoter [52]. Mice carrying a transgene with an internal deletion (aminoacids 39-121) demonstrated generalised Schwann cell hyperplasia (schwannosis) and Schwann cell derived tumours. Conversely, mice transgenic for a NF2 gene with a C-terminal deletion developed comparable to non-transgenic littermates and did not show evidence of Schwann cell hyperplasia or schwannomas. In both transgenic strains the mutant protein was detected along with endogenous wild-type NF2 protein. The latter finding is surprising considering the unstable nature of mutant NF2 protein in humans. Given the fact that both normal and mutant NF2 protein is detected, it is suggested that the mutant NF2 protein may function in a dominant negative way.

2.3 other neurofibromatoses

A further sub-classification of neurofibromatosis was suggested by Riccardi [112] giving 7 distinct neurofibromatosis subgroups based on clinical findings. The identification of the NF1 and NF2 genes and the clinical segregation of NF1 and NF2 using diagnostic criteria set out by the NIH render a further sub-classification of neurofibromatosis of little use. Nevertheless, some forms of neurofibromatosis may present as an overlap syndrome with features of NF1 and NF2, possibly meriting a distinct class.

3. The cytoskeleton

The identification of the NF2 gene and the realisation that its protein product resembled cytoskeleton interacting proteins led to the assuption that the NF2 protein would also function in association with the cytoskeleton.

The cytoskeleton is a filamentous structure involved in virtually all cellular processes and is not, as frequently and erroneously portrayed, simply a rigid scaffold. There are 3 major distinct cytoskeletal

systems: 1) the actin-based cytoskeletal system, also known as microfilaments; 2) intermediate filaments and 3) microtubules. As a general rule the proteins constituting the cytoskeleton are considered resistant to lysis and solubilisation with non-denaturing detergents.

3.1 actin cytoskeleton

The actin-based cytoskeletal system is a highly dynamic structure with a key function in cell shape and motility. The major protein in this system is actin present either as monomers, G- (for globular) actin, or as a polymerised form, denoted F- (filamentous) actin. The polymerisation/de-polymerisation process is the key mechanism underlying the dynamics of the actin system. Specialized cell surface extensions such as lamellipodia, microvilli and microspikes are formed by discrete aggregations of actin molecules. In addition a sub-membranous crosslinked actin network constitutes the cell cortex. F-actin, but not G-actin, resists extraction by non-ionic detergents. The actin cytoskeletal system is supplemented by a great number of binding proteins and interacts with the other elements of the cytoskeleton. The organisation and induction of specialised actin-containing membrane protrusions is under control of a group of ras related proteins (small G-proteins), in particular Rho, Rac and Cdc42 [2, 80, 139].

3.2 intermediate filaments (IF)

So named because of their intermediate size when compared to actin (microfilaments) and microtubules ("macrofilaments"), the IF's are a heterogeneous group of proteins with a common molecular arrangement. The exact function of IF's is unknown, although they do provide resistance to mechanical stress. Intermediate filaments are highly resistant to solubilisation and are formed by polymerisation of (soluble) tetrameric precursors [80].

3.3 microtubules

Microtubules are formed from polymerised heterodimers of α- and β-tubulin and associated proteins (microtubule associated proteins, MAP's). In contrast to the actin and IF cytoskeleton, microtubules are not attached to the cell membrane. Microtubules function in maintaining the overall architecture of the cytoplasm and thus are co-determinants of cell shape. In addition, microtubules form a major component of cilia and flagella and are essential parts of the mitotic spindle. Like other cytoskeletal elements the dynamics of microtubules are governed by polymerisation. In contrast to micro-filaments (actin) and intermediate filaments, microtubules do not resist extraction by non-ionic detergents.

4. ERM and related proteins

The ezrin-radixin-moesin (ERM) proteins, with which the NF2 protein shares considerable homology, are a group of proteins with sequence, structural and functional similarity, acting in close concert with cytoskeletal and membrane proteins. As a group the ERM proteins are related to the erythrocyte

cytoskeletal protein band 4.1 and are part of a large superfamily of interrelated proteins [30, 152]. To extend the family connection it has recently been suggested that the ERM family should be renamed FERM ("F" for 4.1) [30]. Alternatively, the homology of the NF2 protein has led to the suggestion of renaming the family MERM ("M" for merlin)[88].

4.1 ezrin, radixin and moesin

The molecular build of ERM proteins is structurally similar. A globular N-terminal domain of about 300 aminoacids is followed by an extensive alpha-helical segment of 160-170 aminoacids and a charged C-terminal segment of approximately 100 aminoacids with the variable presence of a proline rich stretch (Fig 1). ERM proteins are highly homologous and share many functional similarities resulting to a certain extent in functional redundancy. This is in part reflected by: a) their ability to form homo- and heterotypic interactions, [7, 51, 150], b) the observation that the intracellular concentration of all three ERM proteins ezrin, moesin and radixin needs to be reduced before cell-morphological changes are observed [144] and c) the fact that moesin knockout mice develop normally without obvious cytoskeletal dysfunction [39]. However, several findings indicate that the individual ERM proteins do in part function independently. For instance, radixin, unlike moesin, ezrin and the NF2 protein binds to the barbed end of actin fibers and is located at the cleavage furrow during cytokinesis [48, 122, 148].

Furthermore, the tissue distribution of ERM proteins is partly non-overlapping [5, 6, 12, 91, 126, 135], the ERM proteins exhibit a distinct receptor specific phosphorylation pattern [45] and ERM family members differ in their sensitivity to calpain mediated cleavage [108, 135]. From an evolutionary point of view it appears that there exists considerable pressure to withstand change in the composition of ERM proteins, indicating functional constraint. In addition, the

similarity of ERM proteins to each other, coupled with this evolutionary constraint, indicates that individual ERM proteins have at least some distinct properties [150].

4.1.1 ezrin

The biological characteristics of ezrin are the subject of ongoing investigations and are thought to be representative of other ERM proteins. The 585 aminoacid ezrin protein has been localised to actin-containing structures including membrane ruffles, microvilli, filopodia and possibly cell-to-cell contact points [5, 12, 23, 26, 54]. There is strong evidence that ezrin is a regulator of cell morphology and is itself controlled by homotypic inter- and intramolecular interaction and possibly by heterotypic interactions. Regulation of ezrin and its effect on cell morphology was demonstrated by addition of epidermal growth factor to A-431 cells. This resulted in recruitment of ezrin to microvilli and membrane ruffles with concomitant phosphorylation on tyrosine residues [24]. Initial experiments failed to demonstrate strong ezrin-actin-interaction [23]. However, it was later shown that ezrin contains a C-terminal actin-binding domain that is masked by the N-terminus through intramolecular and possibly

intermolecular interaction [3, 50, 51, 151]. The C-terminal actin-binding domain was also shown to be present in other ERM proteins, but not in the NF2 protein [106]. Actin-association was further substantiated by immunoelectron-microscopy, demonstrating localisation of actin along the length of the microvillus [12]. Apart from the C-terminal actin-binding domains in ezrin an internal F-actin binding domain has been identified with the use of a solid phase assay. This domain is located between aminoacids 1-310. In addition in this assay it was found that aminoacids 13-30 are also important for actin-binding and that low affinity binding of G-actin is also seen in ezrin [115]. Elimination of ERM proteins results in loss of microvilli and cell-cell contacts [144], whilst transfection of C-terminal ezrin domains induces the formation of cell surface extensions in Sf9 cells [89]. Moreover, these morphological effects of C-terminal ezrin domains are negatively regulated by head-to-tail (N-terminal – C-terminal) interaction [35, 89, 90]. The N- and C- terminal association domains involved in this interaction, termed N- and C-ERMAD for ezrin-radixin-moesin association domains, have subsequently been confirmed by other techniques [13]. More recent experiments with deletion constructs show that additional domains in ezrin are involved in morphological changes, indicating that the situation is more complex than had been thought [26, 90].

A working model has been proposed whereby ezrin is thought to be predominantly present in the resting cell in an inactive "dormant" state with masked C- and N-terminal ERMADs and F-actin binding sites. Activation of ezrin, probably involving phosphorylation, on serine and/or threonine residues, results in relaxation of the protein structure with exposure of actin-binding sites [25, 26, 137].

4.2 ERM effectors

Previous observations have identified the Rho protein family as important regulators of cytoskeletal organisation (reviewed by Aspenstrom and Sasaki [9, 121]). Recently it was shown that ERM proteins are phosphorylated in-vitro by Rho-kinase in a Rho dependent way [92]. In addition, it has been shown that redistribution of ERM proteins in actin-containing membrane protrusions is Rho dependent and that this redistribution is preceded by phosphorylation of ERM proteins [132]. Furthermore, the formation of stress fibers, focal adhesions and other actin-containing structures is dependent on an intact Rho pathway and the presence of moesin [87]. Taken together, it seems likely that cytoskeletal organisation involves interplay of ERM- and Rho protein families.

Studies on non-cytoskeletal ERM binding proteins are ongoing. Proteins that may interact with ezrin and other ERM proteins include CD44, ICAM-1, ICAM-2 and ICAM-3, CD43, protein kinase C, protein kinase A, Rho GDP dissociation inhibitor (RhoGDI), a regulatory protein for the NHE3 Na+/H+ exchanger named E3KARP and a homologous phosphoprotein dubbed EBP50 (for ezrin binding phosphoprotein 50) [40, 61, 62, 131, 142, 149, 152, 163, 164].

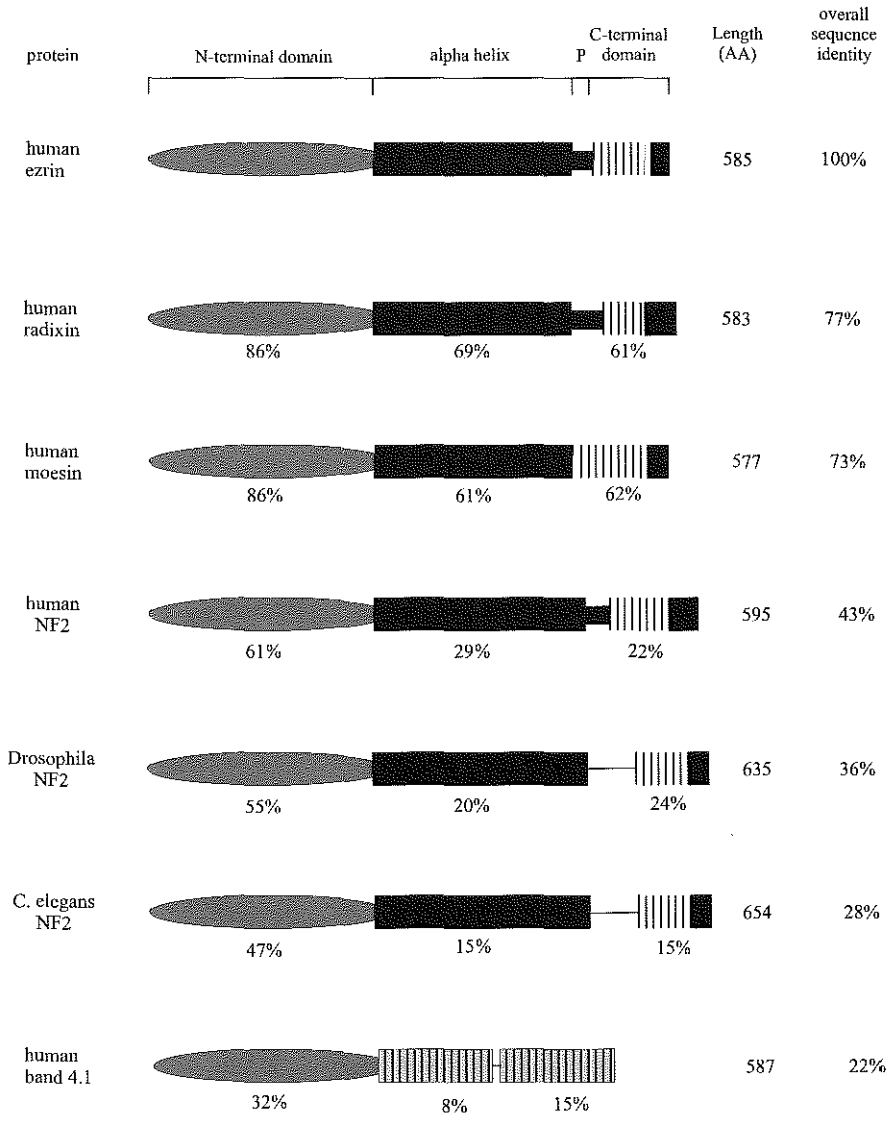

Figure 1.
Schematic representation of ERM proteins
Adapted from Vaheri *et al.* Curr Opin Cell Biol (1997) 9:659-66,
with kind permission from Elsevier Science Ltd. London

Co-localisation and interaction with CD44 has been documented for all three ERM proteins and has also been shown for the NF2 protein [119]. The interaction of ERM proteins with the cytoplasmic tail of CD44 appears to require phosphoinositol biphosphate (PIP_2) [62, 103]. Integrating the experimental data suggests that ERM proteins may have multiple activities including:

1. Regulation of cell surface topography
2. Regulation/relocation/concentration of integral membrane proteins (ICAM, CD43, CD44; NHE via EBP50/E3KARP)
3. Remodelling of the cytoskeleton/stress fiber induction (via Rho signalling)
4. Indirect effects on transcription through the Rho/Rac/Cdc42 pathway

These effects are likely to be governed by homo- and/or heterodimerization / oligomerization, residue specific phosphorylation and phosphoinositides [25, 26, 88, 152].

4.3 ezrin, radixin and moesin in neoplastic disease

Despite the seemingly important role of ERM proteins in the cell, no direct links of ezrin, radixin and moesin with malignant transformation are known. Indirect evidence for involvement of ERM proteins in malignancy is found in cell transformation by overexpression of ezrin in-vitro and the high levels of ezrin found in hemangioblastoma [20, 73]. In addition, moesin upregulation has been found to be associated with the estrogen receptor-negative breast cancer phenotype; it is suggested that upregulation of moesin may be associated with tumour invasiveness and metastasis [28]. Conversely, decreased moesin expression was observed in cutaneous melanoma [70].

4.4 ERM related proteins

Of note is the recent report of a new member of ERM family with characteristics of a tumour suppressor gene. This gene, named DAL-1 for differentially expressed in adenocarcinoma of the lung, is located on chromosome 18 (band p11.3) and is expressed in a variety of tissues including intestine, kidney, pancreas, brain, lung and testis. The gene encodes a 503 aminoacid protein which localises to cell-cell junctions. The homology of DAL-1 aminoacids 132-468 to the N-terminus of band 4.1 is 73%. This region corresponds to the ERM homology (FERM) domain. Re-introduction of DAL-1 in non-expressing non-small cell lung carcinoma suppressed growth, supporting its function as a tumour suppressor gene [146].

5. The NF2 protein, merlin/schwannomin, cytoskeleton and cell biological aspects

The NF2 gene encodes a 595 aminoacid protein which on the basis of its similarity to the ERM protein was thought to function as a membrane-cytoskeleton linking protein [113, 147]. A second less abundant isoform consists of 590 aminoacids. In western blotting the NF2 protein migrates at approximately 69kD while its calculated molecular weight is slightly lower (66kD). The NF2 protein frequently presents as a doublet in western blots, probably due to phosphorylation ([134, 143], our own unpublished

observations). The overall homology of the NF2 protein to ERM proteins is greatest in the N-terminal globular region. The NF2 protein is 43% homologous to ezrin overall and 61% in the N-terminal domain (for details of the secondary structure of ERM proteins see [150]).

5.1 morphogenetic effects of NF2 expression

In a similar way to ezrin, modulation of intracellular NF2 protein content may lead to morphological changes, although this is not found by all investigators [133, 162]. Elongation of cell-processes by overexpression of wild-type NF2 constructs was described by Sainio et al. and Koga et al. [79, 119], but no induction of specialized cell surface projections was observed. Reducing intracellular NF2 protein by introduction of anti-sense oligonucleotides resulted in reversible rounding of cells and loss of adhesion to the substratum [69]. In addition, overexpression of mutated NF2 constructs likewise induced loss of attachment [79].

Although some functional overlap between NF2 and other ERM proteins is likely, localisation studies do not reveal identical spatial localisation for NF2 and other ERM proteins. A relationship between of ezrin and NF2 is indicated by redistribution of ezrin by over-expressed NF2 protein in cells [119], but immunofluorescent staining demonstrates distinct sub-cellular localisation. Moreover, the NF2 protein, in contrast to ezrin is not found in microvilli. Co-localisation with moesin was found in fibroblasts [125], but not in cultured meningioma cells [53].

5.2 sub-cellular localisation, actin binding

Initial studies focused on confirming the proposed role for the NF2 protein as a membrane organizing protein (work described in this thesis, chapter 1 and 2). Subcellular localisation studies have been performed in transfected cells and in cells endogenously expressing the NF2 gene. A general finding is sub-membranous NF2 protein localisation and diffuse cytoplasmic presence. In addition, NF2 protein appears to be concentrated in membrane ruffles and other specialized membrane structures, but not in microvilli where ezrin in particular is found [33, 38, 53, 79, 119, 123, 125, 133, 162].

5.3 NF2 associating proteins

Co-localisation studies in conjunction with GST pull-down experiments support interaction of the NF2 protein with CD44 as has been demonstrated for ezrin [119, 149]. Other candidate NF2-associating proteins have also been identified. Using the yeast 2-hybrid system Scoles et al. identified the actin-binding protein βII-spectrin (fodrin) as a NF2 interacting protein [128]. The interaction was strongest for the type 2 NF2 isoform, possibly owing to masking of binding sequences in the type 1 isoform by intra- or inter-molecular association (see below). In addition, it was shown that naturally occurring missense mutants have a reduced affinity for βII-spectrin. It is possible that the association of NF2 protein with spectrin constitutes the NF2-F-actin link.

In rat Schwann cells NF2 protein was found to co-localise with Rho at paranodes [123]. Rho is an attractive partner for NF2 considering its role in signal transduction and cytoskeletal arrangement and the documented interaction of ezrin with RhoGDI.

Another putative binding partner for the NF2 protein uncovered by the yeast 2-hybrid system is a regulatory cofactor for the Na+/H+ exchanger (NHE-RF) [100]. It was shown that this protein co-localised with the NF2 protein to actin-rich cytoskeletal structures where it also co-localised with moesin. In GST pull-down experiments NHE-RF bound to N-terminus of the NF2 protein. The inter-relatedness of ERM proteins, including NF2, is further substantiated by the independent finding that ezrin similarly binds NHE-RF, called EBP-50 by Reczek et al. [111].

In addition to these cytosolic and membrane proteins, other cytoskeletal proteins may interact with the NF2 protein. Conformation-dependent binding of NF2 protein to microtubules has been described, with localisation of the binding domain in the amino-terminal half [57, 161]. That additional NF2-interacting proteins may exist is indicated by the report of at least 5 associating proteins identified in GST pull down experiments [143].

5.4 intra- and intermolecular association

It has become clear that, similar to ezrin, homotypic and heterotypic interactions govern NF2 functioning. Whilst introduction of NF2 mutants and isoform 2 in tumorigenic cell lines fail to induce growth suppression, this can be achieved by the introduction of wild-type isoform 1 NF2 [57, 69, 86, 145], (our unpublished observations). Furthermore, deletion of C-terminal aminoacids abolished the growth suppressive action. Neither a N-terminal or C-terminal construct alone could induce growth suppression but when combined, growth is suppressed, indicating that N- and C- termini interact [136]. The interaction of NF2 amino-terminal and carboxy-terminal sequences is confirmed by in-vitro GST-binding assays and the yeast 2-hybrid system [57]. In addition, homotypic interaction of C-terminal NF2 sequences is also detected which are dependent on sequences distinct from those required for N-terminal C-terminal interaction [65]. However, convincing demonstration of NF2-NF2 interaction in-vivo has not yet been achieved.

6. Scope of this thesis

The cloning of the NF2 gene in 1993 revealed its similarity to the band 4.1-related proteins ezrin, radixin and moesin. On the basis of the homology to these membrane-organising proteins, it was postulated that the NF2 protein likewise would function as a membrane-cytoskeleton linking protein. The general aim of the work described in this thesis was to gain insight into functioning of the NF2 protein and characterise the role of the NF2 protein in tumorigenesis. In addition, we aimed to determine in which human tissues the NF2 gene is expressed and to compare the expression pattern of the NF2 gene with other ERM proteins. The specific goals were to:

1) Find evidence for and, if present, characterise a NF2-cytoskeletal association.
2) Document the presence of NF2 protein in normal human tissue
3) Detect (mutated) NF2 protein from tumours and determine the effect of mutations on cytoskeletal interaction.

To study the NF2 protein in cells and in tissues we chose to generate polyclonal and monoclonal antibodies. To this end we adopted the use of synthetic peptides to avoid homologous stretches in other ERM proteins. In anticipation of detecting truncated NF2 proteins the synthetic peptides were selected along the length of the protein from the N-terminus to the C-terminus. To determine the sub-cellular localisation of NF2 protein we transiently transfected COS cells with NF2 cDNA isolated from a human fetal brain library. In addition to immunofluorescent staining with anti-NF2 antibodies, the putative cytoskeletal association was studied by detergent extraction and cytochalasin treatment of cells. The generation of antibodies and the sub-cellular localisation studies are described in chapters II and III. The elucidation of a putative cytoskeletal attachment region in the NF2 protein using detergent extraction of mutant NF2 protein, is described in chapter IV. Immunohistochemical- and mRNA-ISH staining techniques were adopted to determine the presence of NF2 protein in human tissues and cultured human cells, this work is discussed in chapters II, III and
V. Mutations in the NF2 gene theoretically lead to protein truncation in a large number of cases. In chapter VI an example of an intronic mutation segregating in a family with a mild phenotype is described. This mutation theoretically results in a truncated NF2 protein. The observed mild phenotype probably results from a degree of residual wild-type splicing in tumour cells. In chapter VII investigations are described aimed at detecting truncated NF2 protein in meningiomas and schwannomas.

7. References

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CHAPTER II

The product of the NF2 tumour suppressor gene localizes near the plasma membrane and is highly expressed in muscle cells

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Abstract

Neurofibromatosis type 2 (NF2) is a disease resulting in the formation of schwannomas of the eighth cranial nerve, and other central nervous system tumours. A tumour suppressor gene has been found to be responsible for this disorder. The 595 amino acid NF2 protein shows a great deal of homology to a superfamily of membrane organizing proteins. To generate antibodies against the NF2 protein four synthetic peptides (SP) were injected in rabbits. COS cells transfected with an NF2 cDNA construct in an expression vector were used for immunocytochemical staining experiments; lysates of transfected COS cells were used for western blotting experiments, as were lysates of E.coli cultures transformed with an NF2 cDNA construct subcloned in a prokaryotic expression vector. In western blots all sera detected a band indicating the appropriate molecular weight in lysates of transfected COS cells and E.coli. Immunocytochemical staining experiments indicate that the NF2 protein localizes in or near the cell membrane. Immunohistochemical staining of human tissue sections demonstrates the presence of the NF2 protein in muscle-, and Schwann cells. These results support the hypothesis that the NF2 protein functions as a membrane organizing element.

Introduction

The neurofibromatoses comprise a group of two clinically and genetically distinct disorders [24, 25, 29, 31]. Neurofibromatosis type 1, otherwise known as Von Recklinghausen's disease, is characterized by areas of localized skin pigmentation (café-au-lait spots), multiple cutaneous neurofibromas, and hamartomas of the iris (Lisch nodules). The gene for Neurofibromatosis type 1 maps to chromosome 17, and codes for a protein named neurofibromin, probably functioning in the RAS signal transduction pathway [10, 11, 20, 39]. The distinguishing feature of Neurofibromatosis type 2, is the bilateral development of schwannomas of the vestibular branch of the eighth cranial nerve, occurring in over 85% of the patients [8]. Other characteristics of this disorder are the development of schwannomas on other (peripheral) nerves and spinal nerve roots, the occurrence of intracranial, or spinal neoplasms such as meningiomas (often multiple), ependymomas, and gliomas [7, 22]. A non-tumour feature are the posterior lens capsule opacities, which occur in up to 80% of NF2 patients [16, 27]. Additional ocular manifestations, in the form of pigment epithelial and retinal hamartomas, and epiretinal membranes, have been described in NF2 [18]. The incidence of NF2 has been estimated at ranging from 1:33.000 to 1:40.000; average age at diagnosis is in the second or third decade [8]. The gene responsible for NF2 has been identified and maps to chromosome 22, band q12 [32, 37]. The autosomal dominant inheritance pattern, loss of heterozygosity studies and analysis of the NF2 gene in tumours have shown that aberrations of both NF2 alleles are common in NF2 related tumours and also in a variety of non-NF2 related tumours. Mutations in the NF2 gene have been found in sporadic meningiomas, sporadic schwannomas, and melanomas. [4, 14, 15, 19, 33, 34, 38]. This data indicates that the NF2 gene acts as a tumour suppressor gene.

The NF2 gene codes for a protein of 595 amino acids, designated "merlin" [37] or "schwannomin" [32]. The coding sequence of the gene is divided over 16 exons. Alternative splicing of the gene has been described [3, 28]. Northern blotting and reverse transcriptase mediated PCR (RT-PCR) experiments, have demonstrated that the NF2 gene is highly conserved, and is expressed in a variety of human tissues [4, 6, 12, 32, 37]. The protein, with a calculated molecular weight of 66kDa, shows a great deal of homology to the erythrocyte band 4.1 related superfamily of membrane organizing proteins. Three proteins in particular, Ezrin, Radixin, and Moesin, referred to as the ERM family [35] share a great deal of homology with the NF2 protein. The homology is greatest in the first 350 amino acids, reaching 63%. Extending the homology to the functional level results in the postulation that the NF2 protein might also act as a membrane organizing protein, functioning as a link between the cell membrane, and the cytoskeleton [32, 37]. However, these postulations await confirmation. As a first step it is essential that the putative localization of the NF2 protein is confirmed. To determine the (sub)cellular localization of the NF2 protein by immunocytochemistry, we have generated antibodies against specific regions of the NF2 protein. Furthermore we demonstrate the localization of the NF2 protein in human tissue sections. To avoid cross-reaction with homologous proteins the synthetic peptide approach was used. Four synthetic peptides were selected for synthesis. A model system for immunocytochemistry, and western blotting was developed using NF2 transfected COS cells.

Results

In order to determine the sub-cellular localisation of the NF2 protein, by immunocytochemistry, we developed polyclonal antibodies against 4 different synthetic NF2 peptides (table 1).

Table 1. Synthetic NF2 peptides

Synthetic peptide	amino acid sequence	position[a] AA (*NT*)	lenght AA	poly-clonal anti-body
SP276	YAEHRGRARDEAEMEYLK-*C*[b]	192-209 (*574-627*)	18	1395NF2 1396NF2
SP277	KMAEESERRAKEADQLKQDLQEAREAE-*C*	439-465 (*1315-1395*)	27	1397NF2 1399NF2
SP278	DFKDTDMKRLSMEIEKEKVEYMEKSK-*C*	508-533 (*1522-1599*)	26	1398NF2 1400NF2
SP279	*C*-LHNENSDRGGSSKHNTIK	561-578 (*1681-1734*)	18	1401NF2 1402NF2

[a] AA are amino acids, NT are nucleotides. Numbers denote the position from the initiation codon ATG (A=1), or first amino acid residue.

[b] *C* is a cysteine residue added for coupling purposes

Eight rabbits immunized with synthetic NF2 peptides demonstrated a specific immune response after the first boost, when tested in a dot-immunobinding assay, with increased titers after the second boost (data not shown). As a control, sera were tested in a dot-immunobinding assay against a non-relevant NF2 synthetic peptide.

To test the sera on the complete NF2 protein, rather than on the synthetic peptides, an assay was set up based on transfected COS cells. An NF2 cDNA clone, containing the complete open reading frame, subcloned in a eukaryotic expression vector, in the sense-, and antisense orientation, was used for transfection experiments in COS cells, and for the generation of a fusion protein in E.coli.

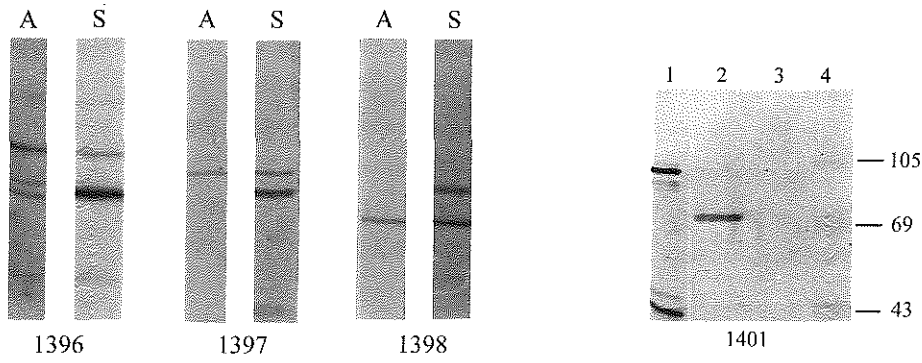


Figure 1

Western blots containing lysates of NF2 transfected COS cells, and E.coli lysates.

a) Results from three antisera, obtained after the second boost are shown: 1396NF2, 1397NF2, and 1398NF2, representing 3 synthetic peptides. Non-specific bands are detected in sense-, and antisense transfected cells. "A"=NF2 antisense transfected COS cell lysate; "S"=NF2 sense transfected COS cell lysate. The position of the 69kDa molecular weight marker is shown. All sera are diluted 1:750.

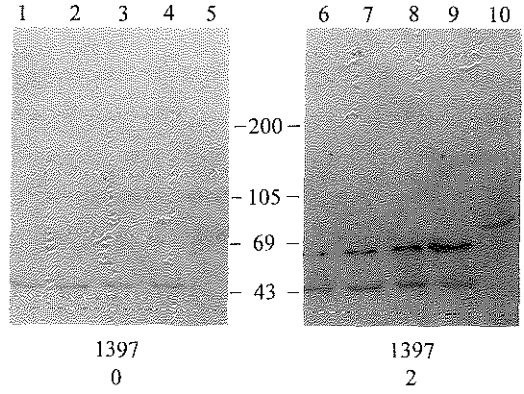
b) Results obtained with polyclonal serum 1401NF2, diluted 1:5000. Lane 1 contains E.coli lysates carrying the NF2-pGEX plasmid. Lanes 2 and 3 contain sense-, and antisense transfected COS cells respectively. In lane 4 a molecular weight marker is shown.

Dilutions of sera from all immunised animals, obtained after the second boost detected an approximately 70kDa band on western blots containing lysates of NF2 sense-transfected COS cells. This band was absent when the blot was incubated with presera from the same animals, nor was a similar band observed on western blots containing lysates of NF2 antisense-, or non-transfected COS cells, when these blots were incubated with serum obtained after the second boost. (fig. 1a,b). On western blots containing lysates of isopropyl ß-D-thiogalactopyranoside (IPTG) induced cultures containing the NF2-pGEX-3X plasmid (see material and methods) a band of approximately 90kDa is observed, representing the NF2 fusion protein (fig. 1b). This band is absent in non-induced cultures, or

when the blot is incubated with presera from the same animal, in the same dilution. To test the specificity of the immune serum, blocking experiments were carried out, by pre-incubating the serum with the synthetic peptide against which antibodies had been raised. As a control another NF2 synthetic peptide was used. On western blots complete absence of the signal was observed when the immune serum pre-incubated with the relevant peptide was used. Pre-incubation with an irrelevant NF2 peptide did not result in loss of signal (results not shown).

To establish the expression of the NF2 protein in human tissues, immunoprecipitation experiments with polyclonal sera were carried out. Polyclonal sera 1396NF2, 1397NF2, 1398NF2, and 1401NF2, representing antibodies against all four synthetic peptides, were used. All sera precipitated an approximately 55kDa protein from human prostate tissue (fig. 2). The 55kDa protein precipitated by any of the four sera could be detected by all four sera on blots. A 55kDa protein was also precipitated in a lysate of human myometrial tissue by sera 1397NF2, 1398NF2, and 1401NF2 (data not shown).

Figure 2
Immunoprecipitation of a 55kDa protein with four antisera. Human prostate lysate was used for the immunoprecipitation. Detection of the precipitated protein is shown here by antiserum 1397NF2. Absence of the 55kDa band is shown in lanes 1-4, incubated with presera. The arrow denotes the position of the immunoglobulins precipitated by the Sepharose-protein A conjugates, and detected by the secondary goat-anti-rabbit antibody. Lane 1, 2, 3, and 4 contain immunoprecipitates, precipitated by respectively 1401NF2, 1398NF2, 1397NF2, and 1396NF2, incubated in presera 1397NF2. Lane 5 portrays a molecular weight marker. Lanes 6, 7, 8, and 9 contain the same precipitates as lanes 1-4, incubated in serum 1397NF2 taken after the second boost. Both presera and immune sera were diluted 1:2500. Lane 10 contains a NF2 sense transfected COS cell lysate.



To determine the staining properties of the polyclonal sera, transfected COS cells were used for immunocytochemical staining experiments. The staining pattern appears to be membrane bound. With immunofluorescent staining of COS cells discrete local punctate areas of staining were observed, in addition to overall staining of the membrane (fig. 3). Peri-nuclear staining was observed in a small subset of cells. Non-transfected cells show slight background staining, comparable to staining produced by presera (not shown). In order to visualize the NF2 protein in human tissues, we selected acetone fixed frozen sections of human colon for immunostaining purposes. In the first experiments human colon was selected for the variety of cell types present in this tissue.

Six of the eight sera, representing all four peptide epitopes, showed an identical staining pattern on human colon sections. Non-specific staining was observed with serum from two animals.

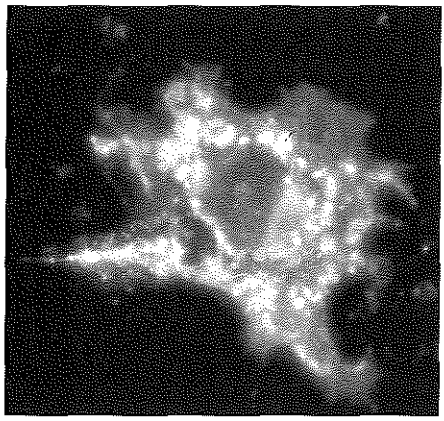


Figure 3
Immunofluorescent staining of NF2 transfected COS cells.
NF2 transfected COS cells were stained using an indirect avidin-biotin method. For the immunofluorescent staining experiments sera 1401NF2 was used. Note punctate, and membranous staining pattern.

Specific staining was observed in the smooth muscle layers of the muscularis mucosae and muscularis externa as well as in the smooth muscle layer surrounding blood vessels (results not shown). No staining was observed in lymphoid tissue. Non-specific staining of mucus in goblet cells in the villi was observed with immune and non-immune sera, and phosphate buffered saline (PBS) controls. Immune sera 1396NF2 and 1398NF2, raised against respectively SP276 and SP278 were used for immunohistochemical staining of human prostate cryostat sections.

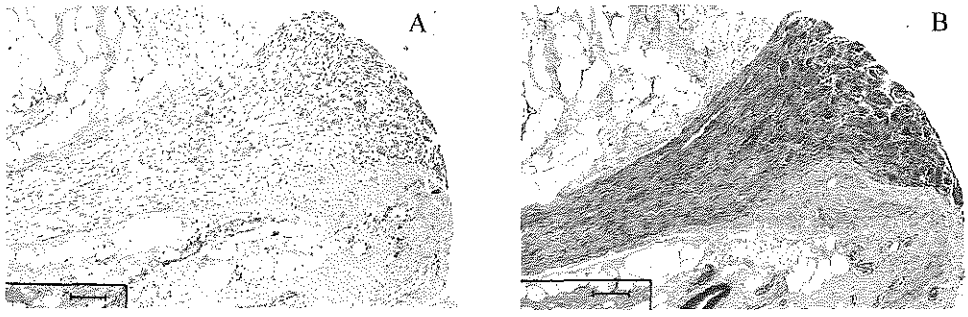


Figure 4

Immunohistochemical staining of formalin fixed paraffin embedded sections of human colon. Sections were not counterstained. Sera were diluted 1:800. The size bar represents 200µm.

a) Negative control, incubation with presera 1398NF2.

b) Incubation with serum 1398NF2 obtained after the second boost. Smooth muscle cells of the tunica media and lamina propria show an intense staining.

Both sera stained smooth muscle fibers in the stroma. Less intense staining of the epithelium was also observed (results not shown).Formalin fixed paraffin embedded sections of cardiac-, and skeletal muscle, prostate and colon were stained with sera 1396NF2, 1397NF2, 1398NF2, and 1402NF2. Staining of smooth muscle cells surrounding blood vessels, smooth muscle cells of the muscularis mucosae and muscularis externa of the colon (fig. 4a,b), and smooth muscle cells present in the prostate was observed.Less intense staining of skeletal and cardiac muscle was also observed (results not shown). No staining of epithelial cells of the prostate or colonic mucosa was observed, nor of the endothelial cells lining the blood vessels, in the paraffin embedded sections. Staining of paraffin embedded sections of human sympathetic trunk was performed with sera 1396NF2, 1397NF2, and 1401NF2. Both sera 1396NF2 and 1397NF2 demonstrate specific staining of Schwann cells, and smooth muscle cells surrounding blood vessels present in the sections (fig. 5a,b). No nuclear staining was observed in either smooth muscle cells or Schwann cells. Ganglion cells present in the sections also appear to stain with immune serum, however due to the presence of Nissl bodies in the cytoplasm of these cells, a clear distinction could not be made between specific cytoplasmatic staining and non-specific colouring of Nissl bodies. Nissl bodies were also coloured with presera, and PBS controls. Staining with serum 1401NF2 resulted in high background staining of the complete section.

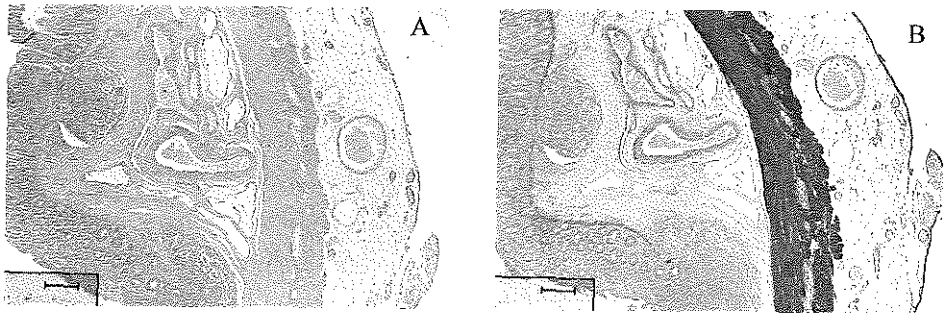


Figure 5
Immunohistochemical staining of paraffin embedded section of human sympathetic trunk. Sections were not counterstained. The size bar represents 100µm.
a) Negative control, incubation with presera 1397NF2, diluted 1:400
b) Incubation with serum 1397NF2 obtained after the second boost, diluted 1:800.

Discussion

The synthetic peptide strategy provides the opportunity to raise antibodies against selected antigenic parts of a particular protein of interest, avoiding stretches of homology to other proteins [40]. To prevent cross-reactions with ERM family members we adopted the synthetic peptide strategy to generate antibodies against the NF2 protein. Experiments with antibodies to one of the ERM family members,

generated by using purified proteins as immunogens, have been hampered by cross-reactions of these antibodies with other ERM family members [9, 35].

A potential problem with antibodies raised against synthetic peptides is the failure of these antibodies to recognize the native protein. This is especially important if antibodies are to be used for immunostaining purposes. For evaluation of the antibodies, we therefore devised an assay using transfected COS cells and immunocytochemical techniques. After acetone fixation the NF2 protein was still detected by all eight sera in COS cells. Six of the eight sera specifically stain smooth muscle cells in acetone fixed frozen sections of human colon. This would indicate that these six polyvalent sera raised against four synthetic NF2 peptides also show immunoreactivity for the native protein.

The polyclonal antibodies detect the NF2 protein on western blots containing lysates of NF2 transfected COS cells. A band indicative for the same molecular weight was observed when the NF2 construct subcloned in the eukaryotic expression vector was used for in vitro translation experiments (results not shown).

In immunoprecipitation experiments, using lysates of human tissue, which in staining experiments had been shown to express the NF2 protein, a shorter band of approximately 55kDa is detected. This shorter protein might represent tissue specific alternative splicing of the NF2 gene resulting in a shorter protein. For instance, alternative splice variants lacking exons 2 (126 nucleotides), or 3 (123 nucleotides) would result in a truncated protein of approximately 61kDa. A protein of approximately 56kDa would be formed as a result of a splice variant lacking both exon 2 and 3 [3, 28].

The subcellular localization of the NF2 protein, as judged by immunocytochemistry on COS cells supports the hypothesis that the NF2 protein is associated with the cell membrane. The homology of the NF2 protein to erythrocyte band 4.1 related proteins and its predicted secondary structure led to the postulation that the NF2 protein might function as a membrane organizing protein [1, 32,37]. Members of the band 4.1 superfamily of proteins show a common arrangement. An α-helical stretch is preceded by a globular N-terminus, and followed by a hydrophillic C-terminus [30]. The association of band 4.1 related proteins with the cell membrane has been demonstrated [2, 5, 23]. Band 4.1 itself has been shown to associate with the membrane proteins glycophorin C and the anion channel band 3 [21]. On the grounds of these homologies the theory has been put forward that the NF2 protein interacts with membrane proteins, possibly integrins, by means of its globular amino-terminal domain, similar to Ezrin. Interaction with cytoskeletal elements could take place through its α-helical domain, or its charged carboxy-terminus [1].

We could not detect the presence of the NF2 protein in non-transfected COS cells by immunocytochemistry, with any of the sera. This would indicate that the NF2 protein is not endogenously expressed in COS cells, or that the homology between the monkey NF2 protein and the human NF2 protein is limited. The latter possibility seems unlikely, considering the phylogenetic conservation of the NF2 gene [6]. In immunofluorescent stained NF2 transfected COS cells, a punctate

staining pattern is observed, possibly representing discrete local concentrations of NF2 protein. These focal staining intensities might be due to overexpression of the NF2 construct from the eukaryotic expression vector. In this vector the NF2 cDNA is under control of the cytomegalovirus (CMV) promoter. Expression of the cDNA under control of this promoter might lead to an aspecific localization of the protein in the cell. Conversely the staining pattern might represent a so far undefined NF2 related subcellular structure, for instance vesicles, or cytoskeleton related structures. Experiments with antibodies to Ezrin, used for immunocytochemical staining of overexpressed Ezrin protein, in transfected CV1 cells, indicate that the sub-cellular distribution of this protein is not modified in these cells [2]. In addition, these authors show a punctate localisation of the Ezrin protein in these cells, similar to our results. Therefore it seems likely that the distribution of the NF2 protein observed in COS cells represents the natural situation.

The mechanism by which mutations in the NF2 gene cause schwannomas and other tumours remains to be elucidated. Loss of cell-cell contact possibly resulting from mutations in the NF2 gene in Schwann cells could result in loss of growth inhibition, favouring tumour formation. Mutations of the NF2 gene that have been described would lead to the formation of a truncated protein, probably unable to fulfil its putative membrane organizing function. Further experiments are needed to confirm the co-localization, and direct association of the NF2 protein with cytoskeletal proteins.

The staining of smooth muscle cells of human intestine, prostate, and blood vessels indicates that these cells expresses the NF2 protein at high levels. The staining pattern of cardiac and skeletal muscle is less intense, possibly indicating a lower level of expression in these tissues. Forces generated in muscle cells are transmitted to the surrounding environment through interactions between the contractile elements, cytoskeleton, cell membrane, lamina basalis and reticular fibres of the surrounding connective tissue. Proteins mediating the interaction between the cell membrane and cytoskeleton thus play an important role in muscle cells. The staining of Schwann cells, in sections taken from the human sympathetic trunk, confirms the presence of the NF2 gene product in cells of the type which give rise to vestibular schwannommas, the hallmark of NF2. Further immunohistochemical staining experiments with these sera will have to demonstrate the absence of the NF2 protein in NF2 related tumours.

NF2 expression has been found to be present in a large number of tissues by northern blot analysis and RT-PCR [4, 6, 12, 32, 37]. Apart from the smooth muscle layers in the human colon, and the muscle fibers in the stroma of the prostate, the smooth muscle layers of blood vessels in the tissue sections also stain with six of the eight sera. Although further immunohistochemical staining experiments on various tissues remain to be done, it is possible that the ubiquitous expression of the NF2 gene, found by other groups, is in fact a result of detecting NF2 mRNA transcripts originating from smooth muscle cells in blood vessel walls; blood vessels being present in all tissues analyzed so far. Expression of the NF2 gene could, in this respect, be restricted to far fewer tissues, or cell types, than as suggested by other workers.

Materials and Methods

Selection and synthesis of peptides.

Selection and synthesis of the peptides were performed essentially as described by Zegers *et al.* [40]. With the aid of a computer prediction program (Genetics computer group, Wisconsin, USA), the NF2 cDNA sequence was screened for stretches of amino acids with a high antigenic index. The selection was based on hydrophilicity, protein surface probability, and expected flexibility. Furthermore, peptides with a high degree of homology to other proteins were rejected as candidates for synthesis. Coordinates and sequence data of the peptides are given in table I. Synthesis of the peptides was performed on a Milligen 9050 pepsynthesizer. An extra cysteine residue was added to the N-terminus of SP276, SP277, and SP278 ; and to the C-terminus of SP279 for coupling purposes. Coupling of the peptides to Keyhole Limpet Hemocyanin (Calbiochem, La Jolla, USA) was achieved with *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide (MBS) ester. Purification of the peptides was by high performance liquid chromatography (HPLC)

Immunisations

250µg of each peptide coupled to Keyhole Limpet Hemocyanin was diluted in 250µl of PBS and mixed with an equal volume of complete Freunds adjuvant (Difco laboratories, Detroit, USA). Each peptide was injected intradermally in 2 rabbits. All animals were boosted twice with three weekly intervals. For the first and second boosts incomplete Freunds adjuvant was substituted for the complete Freunds adjuvant. Blood samples were taken after the first and second boosts. Blood samples were allowed to clot for 1 hour at 37°C. After loosening of the clot, samples were centrifuged at 2500rpm, serum was collected, and stored in aliqouts at -20°C.

Dot-immunobinding assay

The dot-immunobinding assay was performed essentially as described by Hawkes [13]. A 5mm grid was drawn on a sheet of virgin nitrocellulose (Schleicher and Schuell Co., Dassel, Germany), the nitrocellulose was floated on Phosphate buffered saline (PBS) and allowed to hydrate for 20 minutes. After excess moisture had been drained from the sheet, it was incubated in a peptide solution (0.1 ml/cm^2 nitrocellulose; 1µg SP/ml PBS) for 1 hour. After incubation the membrane was air dried. A 3% Bovine Serum Albumin (BSA) solution in PBS (fraction V, Boehringer Mannheim, Germany) was used to block remaining sites on the membrane. Filters were either used directly, or stored at -20°C. Dilutions of immune-sera and sera from pre-bleeds were spotted directly on the membrane, which had been placed on a sheet of damp blotting paper. After 1 hour of incubation the sheet was washed 3 times in PBS. A second incubation was carried out with a peroxidase conjugated goat-anti-rabbit secondary antibody (DAKO, Glostrup, Denmark) for 30 minutes at room temperature. After washing 3

times with PBS, 0.06% (w/v) 3,3'diaminobenzidine (DAB, Sigma, Brussels, Belgium) and 0.01% H_2O_2 was added as a substrate. The reaction was stopped by washing the membrane in water.

Isolation of cDNA clones, and subcloning of the expression constructs.

1 x 10^6 plaque forming units from a Human Fetal Brain cDNA library in lambda Zap (Stratagene, La Jolla, USA) were hybridized with an NF2 PCR probe, generated using primers 5m4 and 3m6 for RT-PCR. (Bianchi *et al.*, 1994). The cDNA was subcloned in the pBluescript plasmid, using the *in-vivo* excision method (Stratagene, La Jolla USA). An approximately 2kb NF2 cDNA clone containing the complete open reading frame together with parts of the 5' and 3' untranslated regions was subcloned in the EcoRI site of the multiple cloning site of the pcDNA-3 eukaryotic expression vector (Invitrogen, San Diego, USA). The vector was transfected to COS cells by electroporation (200V, 500µF). After 48 hours of culture the COS cells were either harvested, lysed in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) buffer [17] and used for western blotting, or if grown on microscope slides, acetone fixed and used for immunocytochemical staining, either directly or after storage at -20°C. A SmaI-EcoRI fragment of the NF2 cDNA was inserted in frame in the pGEX-3X vector (Pharmacia, Uppsala, Sweden). As a result of the cloning procedure 18 nucleotides prior to the ATG codon were incorporated in the insert. A fusion protein of approximately 90kDa was expected, consisting of the NF2 protein (65kDa), glutathion-S-transferase (GST, 26kDa) from the vector, and 6 amino acids incorporated from the NF2 cDNA prior to the ATG initiation codon. Cultures of E.coli strain PC2495 transformed with the pGEX-NF2 construct were grown until the optical density was 0.3-0.5 prior to induction with IPTG. After induction the cultures were grown for another 2 hours. Aliqouts of the culture were lysed in SDS-PAGE buffer, and used for western blotting.

Western blotting

Samples of transfected COS cells, and bacterial cultures were lysed in SDS-PAGE buffer, boiled for 5 min, and sheared through a 26-gauge needle. Prior to loading the samples on the gel, these were boiled for 5 min. Samples were separated on a 7.5% polyacrylamide slab gel. Electrophoresis and electroblotting to nitrocellulose, were carried out according to the manufacturer's recommendations (Biorad mini-protean 2D cell; BioRad, Hercules, CA, USA). Visualisation of transferred protein was performed by incubating the membrane in Ponceau red (Sigma). Blocking the membrane was achieved by incubation for 2 hours in a 3% BSA (Fraction V, Boehringer Mannheim) solution in PBS, pH 7.4. Primary antibodies were diluted in the blocking solution. Incubations were carried out at room temperature for 2 hours, or at 4°C overnight. After washing, detection was accomplished with a secondary alkaline phosphatase conjugated goat-anti-rabbit antibody (1:2000 TAGO, Burlingame, USA). As a substrate 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer Mannheim), or Naphtol AS-MX phosphate and 4-aminodiphenylamine-diazonium sulfate

(Sigma) was used. Blocking experiments were performed by pre-incubating serum dilutions with 20μg of synthetic peptide, overnight at 4°C with continuous agitation. In a separate experiment the serum was also pre-incubated with a non-relevant NF2 synthetic peptide as a control.

Immunoprecipitation

2.5 grams of human prostate obtained from a radical prostatectomy. The tissue sample was taken from an area not involved in the disease process. Myometrial tissue was obtained from a hysterectomy specimen. The tissues were homogenized in a buffer containing 150mM NaCl, 50mM Tris, 1% Nonidet P-40, 0.5% Sodium Deoxycholate, 0.1% Sodium Dodecyl Sulfate. Insoluble debris was removed by centrifugation at 9000g for 5 min. Pefabloc (Boehringer Mannheim) to 1mM was added to the supernatant. The immunoprecipitation experiments were performed essentially as described by Pakkanen for ezrin [26], except that in the wash buffers 1% Nonidet P-40 was substituted for Triton X-114. After the immunoprecipitation the samples were suspended in SDS-PAGE buffer and fractionated on a 7.5% polyacrylamide gel. Western blotting was performed as described above.

Immunohistochemical staining.

Transfected COS cells, frozen tissue sections, and paraffin embedded tissue sections were stained using a standard peroxidase-anti-peroxidase technique [36]. Transfected COS cells, and frozen sections were fixed in acetone. After fixation, sections were rinsed with PBS. Blocking of endogenous peroxidase was not performed. Formalin fixed, paraffine embedded sections were subjected to protease treatment (Pronase E, Sigma) for 10 minutes at 37°C, prior to incubations. Blocking of endogenous peroxidase activity was accomplished by incubation in 3% H_2O_2 in methanol for 15 minutes. Following pre-incubation with 10% normal goat serum (DAKO), incubation with the primary antibodies was carried out, either at room temperature for 2 hours, or at 4°C overnight. After washing, incubation with unconjugated goat-anti-rabbit (DAKO) antibodies was carried out for 30 minutes at room temperature, followed by washing and incubation with the rabbit peroxidase-anti-peroxidase complex (DAKO). DAB supplemented with H_2O_2 was used as chromogen. If applicable, Mayer's haematoxylin was used as a nuclear counterstain.

Immunofluorescence microscopy

Acetone fixed COS-cells or frozen tissue sections were sequentially pre-incubated with 10% normal goat serum (DAKO), followed by incubation with the primary antibodies, diluted in 3% BSA in PBS for 2 hours at room temperature. After washing the slides were incubated in biotinylated goat-anti-rabbit antibodies (1:400, DAKO), after washing, fluorescein isothiocyanate (FITC) conjugated to avidine (1:200) Vector laboratories, Burlingame, USA) was added for 20 minutes. Amplification of the signal was achieved by subsequent incubation for 20 minutes in biotinylated goat-anti-avidine

antibodies (Vector laboratories), followed by a second incubation in FITC conjugated avidine for 20 minutes. Between incubations slides were washed 5 times for 5 minutes in PBS. Slides were mounted in Gelvatol. Fluorescence was observed with a Zeiss epifluorescence microscope, fitted with the 4F fluorescence kit. Detection of FITC fluorescence was achieved with a 450-500nm excitation filter, an LP 515 longpass filter, an FT510 dichroic mirror, and an Osram 50W mercury vapour lightsource. Results were photographed on Kodak Ektachrome 160 (EPT 135-36) film.

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CHAPTER III

Neurofibromatosis type 2 protein co-localizes with elements of the cytoskeleton

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Abstract

The product of the neurofibromatosis type 2 (NF2) tumor suppressor gene is a 595 amino acid protein bearing resemblance to a family of band 4.1 related proteins. These proteins, including ezrin, radixin, and moesin (ERM), probably function as molecular linking proteins, connecting the cytoskeleton to the cell membrane. On the grounds of the homology to the ERM proteins and on the basis of its predicted secondary structure, the NF2 protein is also thought to act as a cytoskeleton-cell membrane linking protein. Using monoclonal antibodies to N- and C-terminal synthetic NF2 peptides we demonstrate the co-localization of the NF2 protein with elements of the cytoskeleton in a COS cell model system and in cultured human cells. Furthermore, the presence of the NF2 protein in tissue sections is shown. The monoclonal antibodies specifically stain smooth muscle cells and the stratum granulosum of the human epidermis. In cultured smooth muscle cells the NF2 protein co-localizes with actin stress fibers. Immuno-electron microscopy demonstrates the presence of the NF2 protein associated with keratohyalin granules and to a lesser extent with intermediate filaments in the human epidermis. We conclude that the NF2 protein is indeed associated with multiple elements of the cytoskeleton.

Introduction

Neurofibromatosis type 2 (NF2) is a disease resulting in the formation of bilateral vestibular schwannomas. These tumors occur in over 98% of NF2 patients [1]. Additional manifestations of the disease are posterior lens capsule opacities, retinal abnormalities and schwannomas of spinal nerve roots [2-8]. In contrast to Neurofibromatosis type 1 patients (NF1; von Recklinghausen's disease), patients suffering from NF2 have few skin disorders [9,10]. Skin manifestations occurring in NF2 include cutaneous schwannomas, neurofibromas and a third pigmented, hairy lesion with a roughened skin surface [2]. Neurofibromatosis type 2 has been subdivided in 2 clinical subtypes, a more severe phenotype designated the Wishart type and a milder variant, the Gardner subtype [1,2,11]. The gene responsible for NF2 has been identified and has been shown to act as a classical tumor suppressor gene [12,13]. The gene, located on chromosome 22 band q12, has also been implicated in sporadic schwannomas and sporadic meningiomas [14-19]. Expression of the NF2 gene has been found in many human tissues by reverse transcriptase mediated PCR (RT-PCR) and northern blotting experiments [12,13,19,20]. The product of the NF2 gene, called merlin [13] or schwannomin [12], is a 595 amino acid protein belonging to a band 4.1 related subset of proteins. These proteins, ezrin, radixin and moesin are referred to as the ERM family [21]. Several other proteins, including talin, share a common design to the ERM proteins, but show a more limited homology. The ERM proteins are thought to function as molecular linkers, connecting the cytoskeleton to the plasma membrane [22]. A similar role has been postulated for the NF2 protein, based on the high degree of homology with the ERM proteins [12,13]. The ERM and related proteins, including the NF2 protein, all share a central α-helical part that is preceded by a globular N-terminus, and followed by a charged C-terminus. Apart from moesin the ERM and NF2 proteins also contain a proline rich stretch at the C-terminus. It has been postulated that the NF2 protein is an F-actin associated protein, based on its homology to ERM proteins. Recently, it has

been shown that ERM family members contain a C-terminal actin-binding site. However, this actin-binding site is not present in the NF2 protein [23,24]. We have recently shown that the NF2 protein is highly expressed in smooth muscle cells, and to a lesser extent in other muscle cell types and in Schwann cells [25]. In Ras transformed NIH/3T3 cells it has been shown that the NF2 protein can reverse the malignant phenotype [26]. The same properties have been observed for other actin-binding proteins [27-29]. The NF2 protein is unique in the sense that it is the first of the ERM related plasma membrane cytoskeletal linking proteins implicated in human disease. We here demonstrate, with the use of monoclonal antibodies to synthetic NF2 peptides, the co-localization of the NF2 protein with F-actin. In addition we provide evidence for the association of the NF2 protein with other components of the cytoskeleton. Furthermore, the association of the NF2 protein with the cytoskeleton was confirmed by detergent extraction of cellular proteins.

Material and methods

Cell culture, detergent extraction and cytochalasin D treatment.

COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum and antibiotics. Human intestinal smooth muscle (HISM) cells (ATCC CRL-1692, American type culture collection, Rockville, MD, USA) were maintained in DMEM supplemented with 10% fetal calf serum and antibiotics. The X63Ag8.653 myeloma cells [30] and hybridomas were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium supplemented with 10% fetal calf serum and antibiotics. All cell cultures were maintained in a humidified 5% CO_2 atmosphere. Cytochalasin D treatment of HISM cells was performed by culturing cells on slides in medium containing 2.5µM cytochalasin D (Sigma, St.Louis, USA) for 20 minutes at 37°C. The slides were fixed immediately in acetone, and used for immunostaining. The detergent extraction of COS and HISM cells was carried out essentially as described by Kreis [31] and Algrain [23]. The extraction buffer contained 80mM Pipes-KOH pH 6.4, 5mM EGTA, 1mM $MgCl_2$, and 0.5% Triton X-100.

Peptide synthesis, generation and characterization of monoclonal antibodies.

The N-terminal SP-0 (RAIASRMSFSSLKRKQPKTF-C) peptide was synthesized essentially as described [32], the terminal cysteine residue was added for coupling purposes. Peptide SP279 has been described in detail elsewhere [25]. Female BALB/c mice were immunized intraperitoneally with 50µg of synthetic peptide coupled to keyhole limpet hemocyanin suspended in 250µl of phosphate buffered saline (PBS) and mixed with 250µl of complete Freunds adjuvant. The mice were boosted twice at 2 weekly intervals; for the boosts incomplete Freunds adjuvant was substituted for the complete Freunds adjuvant. To determine if an immune response had developed, test bleeds were obtained after the second boost. The sera were tested in an immunocytochemical assay on NF2 transfected COS cells. The mouse displaying the highest antibody titer was used for the production of hybridomas. Three days prior to the fusion the

mouse was boosted a third time with 50µg of synthetic peptide suspended in 500µl of PBS. On the day of the fusion the mouse was sacrificed and the spleen was aseptically removed. Subsequent fusion with the X63Ag8.653 myeloma cell line, and cell culture was essentially carried out as described [33]. After 10 to 14 days of culture in selective medium clones were screened for antibody production using the dot-immunobinding assay for SP279 essentially as described by Hawkes [34]. A total of 70 positive clones were identified for SP279. These 70 clones were subjected to a second round of screening using an immunocytochemical assay on NF2 transfected COS cells. Three clones were identified (UC2, MH3 and KF10) which specifically stained transfected COS cells. These clones were subjected to limiting dilution cloning, followed by expansion and cryopreservation. The KF10 clone was subsequently cultured in the Tecnomouse (Tecnomara, Integra Biosciences, Switzerland) under serum-free conditions, generating a high titer antiserum.

Culture supernatants of clones identified in the SP-0 fusion were pooled in groups of 5 and tested directly on transfected COS cells. One clone, 15H3, was identified which exclusively stained transfected COS cells. The isotype of the monoclonal antibodies was determined with the Isostrip isotyping kit (Boehringer Mannheim, Germany). ELISA was performed by coating 96 well PVC plates with synthetic peptide. The wells were blocked with a 3% bovine serum albumin (BSA; fraction V, Boehringer Mannheim) solution in PBS for 2 hours at room temperature. After incubation with culture supernatants and washing with PBS, incubation with a secondary peroxidase conjugated rabbit anti-mouse antibody (DAKO, Glostrup, Denmark) was performed. After washing, visualisation of positive wells was achieved by incubation with o-phenylenediamine (Eastman Kodak Co., Rochester, USA) and H_2O_2.

Construction of expression vector, transfection and western blotting.

The cloning of the NF2 cDNA, subcloning of the cDNA in the eukaryotic expression vector pCDNA3, and transfection of the vector to COS-1 cells were performed as described previously [25]. Lysates of COS cells and protein precipitates of detergent extractions were fractionated under reducing conditions, and electroblotted to nitrocellulose according to the manufacturers recommendations (Bio-Rad mini-protean 2D cell; BioRad, Hercules, CA, USA). Protein blots were blocked with a 3%BSA solution in PBS for 2 hours at room temperature or overnight at 4°C. The blots were incubated with the KF10 antibody at a 1:1000 dilution in PBS for 1 hour at room temperature. After washing with PBS, bands were visualised with a secondary alkaline phosphatase conjugated goat anti-rabbit antibody (TAGO, Burlingame, USA). As a substrate 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer Mannheim), or Naphtol AS-MX phosphate and 4-aminodiphenylamine-diazonium sulfate (Sigma, St.Louis, USA) was used.

Immunolabelling studies and competition assays.

Transfected COS cells and HISM cells were grown on microscope slides. After 48 hours of growth after transfection (COS cells), or at 50% confluency (HISM cells) the slides were washed once in PBS and

fixed in acetone for 5 minutes at room temperature. The slides were air dried and used for staining purposes immediately or stored at room temperature. Cryostat sections mounted on 3-aminopropyl-triethoxy-silane (Sigma) coated slides, were air dried for 20 minutes after sectioning and subsequently fixed in acetone for 10 minutes at room temperature. Human tissues were fixed in phosphate buffered formaldehyde 4%, pH7.2, embedded and following sectioning mounted, on coated slides. Immunostaining of COS cells, cryostat sections, and paraffin-embedded sections was accomplished with the KF10 antibody at a 1:50 dilution. For immunostaining with monoclonal antibodies UC2, MH3 and 15H3 undiluted culture supernatant was used. Human tissues were stained with a standard peroxidase-anti-peroxidase (PAP) method [35]. A protease pre-treatment (Pronase E, Sigma) was required for the paraffin-embedded tissue sections. Endogenous peroxidase activity was quenched with 3% H_2O_2 in methanol for 20 minutes at room temperature. Mayer's hematoxylin was used as a nuclear counterstain if applicable. Immunofluorescent staining was accomplished with the KF10 antibody at a 1:300 dilution and a secondary fluorescein isothiocyanate(FITC)-conjugated goat anti-mouse or tetramethyl rhodamine isothiocyanate(TRITC)-conjugated rabbit anti-mouse antibody (DAKO). Phalloidin-FITC staining was accomplished by incubating slides in a 5mg/ml solution of phalloidin-FITC (Fluka, Buchs, Switzerland) for 10 minutes at room temperature. If applicable, propidium iodide was used as a nuclear counterstain. Slides were mounted in Mowiol (Hoechst, Germany) containing 2.5% 1,4-diazobicyclo-[2.2.2]-octane (DABCO; Fluka) to reduce fading, and were examined with a Zeiss epifluorescence microscope fitted with the 4F fluorescence kit. Results were photographed on Kodak Ektachrome 160 (EPT 135-36) film. Confocal laser scanning microscopy was performed on a Bio-Rad MRC1000 employing the advanced XZ-scanning option. Immuno-electron microscopy was performed with the KF10 antibody as described [36]. The competition studies were performed by diluting KF10 in a 40mM SP279 solution in PBS, and mixing overnight at 4°C. As a control a 40mM solution of another synthetic NF2 peptide was used (SP277 or SP278 [25]). After the pre-incubation the solutions were used for immunostaining and immuno-electron microscopy.

Results

Three monoclonal antibodies were raised against a C-terminal NF2 synthetic peptide SP279 (C-LHNENSDRGGSSKHNTIK). Two of the antibodies, KF10 and UC2, reacted similar with regard to staining properties while the third antibody, MH3, displayed a more restricted staining pattern. One monoclonal antibody, 15H3, was raised against a N-terminal peptide SP-0. The immunostaining properties of this antibody were identical to the KF10 and UC2 antibodies. All four monoclonal antibodies were of the IgG1 isotype with a κ-light chain. The specificity of the antibodies was checked by ELISA. The KF10 antibody was only reactive to SP279 and not to any of the other NF2 synthetic peptides (SP-0, SP276, SP277 and SP278). Likewise the 15H3 antibody exclusively reacted to SP-0.

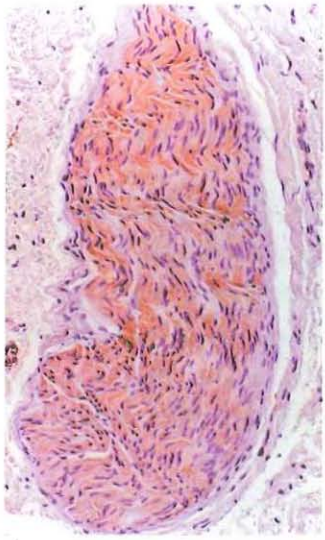
The presence of the NF2 protein in normal human tissues was investigated by immunostaining of formalin-fixed, paraffin-embedded tissue sections, and of frozen sections. A panel of human tissues was

selected including all three types of human muscle, which have been shown to express the NF2 protein. Exclusive staining of smooth muscle cells was observed with the KF10, UC2 and 15H3 antibodies, but not with the MH3 antibody. No staining of skeletal or cardiac muscle was observed (not shown). Vascular and visceral smooth muscle showed an intense cytoplasmic staining pattern (figure 1b). In addition, myoepithelial cells in sections of breast tissue, salivary gland tissue and around merocrine sweat glands in the skin, also stained with the monoclonal antibodies. No difference in staining was observed between frozen sections and paraffin embedded sections.

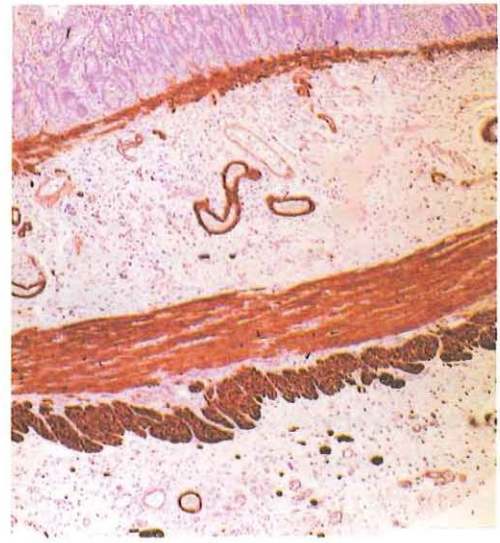
Staining of the epidermis was also observed with all four antibodies (figure 1c). An intense granular staining pattern was observed in the stratum granulosum, decreasing in intensity towards the more basal cell layers. Very weak staining was observed in the stratum corneum. The granular staining pattern of the stratum granulosum was further investigated by immuno-electron microscopy, using the KF10 antibody and secondary antibodies conjugated to 10nm gold particles. Specific staining was found of the keratohyalin granules in the stratum granulosum of the epidermis (figure 1d). Less intense staining was found associated with intermediate filaments in the more basal layers (stratum spinosum and stratum basale epidermidis). To rule out non-specific interactions of the KF10 antibody and components of the keratohyalin granules incubations were performed in the presence of 0.15M NaCl and 0.5M NaCl, or in PBS containing 0.5% Triton X-100. No loss of signal strength was observed by light microscopy of the stratum granulosum and musculus arrector pilli. In addition, competition experiments were performed. The staining pattern of the monoclonal antibodies as observed by light microscopy, and the labelling of the keratohyalin granules in immuno-electron microscopy, could be completely abolished by pre-incubation of a working solution of the antibody with the synthetic peptide against which it had been raised (not shown). Pre-incubation with different NF2 peptides did not result in loss of staining.

Figure 1
Immunohistochemical staining with the KF10 monoclonal antibody.

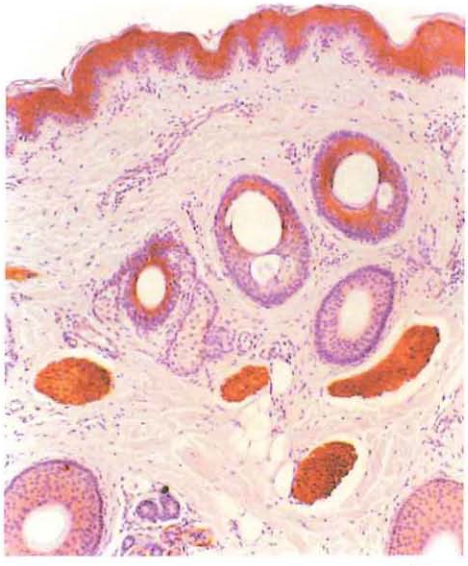
a. Counterstained section of the human parotid salivary gland containing branches of the 7th cranial nerve. Note cytoplasmic staining of Schwann cells. Magnification 400x.
b. Counterstained section of human ileum. Staining of the muscularis externa, muscularis mucosae and tunica media of the blood vessels is observed. Structures staining in the serosal tissue are tangentially sectioned vessel walls. Magnification 50x.
c. Counterstained section of the human skin, stained with the KF10 antibody. Note the staining of the suprabasal layers of the epidermis, musculus arrector pilli and tunica media around the blood vessels. Magnification 100x
d. Immuno-electron micrograph of the human epidermis stained with the KF10 antibody. Specific labeling of a keratohyalin granule is observed. Magnification 30,000x.



A



B



C

D

To determine if the monoclonal antibodies could detect the NF2 protein in Schwann cells, the cells giving rise to the vestibular schwannomas, several tissue sections were included containing peripheral or cranial nerves. In peripheral nerves no staining of Schwann cells was observed.

Branches of the facial nerve (7th cranial nerve) present in a section of a parotid salivary gland were seen to stain with the KF10 antibody. Distinct cytoplasmic staining of the Schwann cells was observed in several branches (figure 1a).

To further evaluate the NF2 expression in smooth muscle we used a smooth muscle cell line derived from human intestine [37]. Immunofluorescent staining of these cells with the KF10 antibody revealed a pattern resembling that of actin stress fibers. Double-staining of HISM cells with the KF10 antibody and FITC-conjugated phalloidin resulted in complete overlap of the staining patterns (figure 2a,b). Treatment of HISM cells with cytochalasin D resulted in disruption of the stress fibers as evidenced by phalloidin staining. The staining patterns produced by KF10 and phalloidin after cytochalasin D treatment did not overlap. Both staining patterns were of a punctate nature, with occasional stress fibers still present (figure 2c,d). Detergent extraction of HISM cells did not interfere with the staining pattern produced by phalloidin or KF10 (not shown).

To study the role of the NF2 protein with respect to its putative membrane-cytoskeletal organising function we used a model system based on NF2 transfected COS cells. Immunocytochemical staining of the transfected COS cells confirmed the punctate staining pattern previously observed with the polyclonal sera. Immunofluorescent staining of the transfected COS cells was used in conjunction with confocal laser scanning microscopy (CLSM) to determine a more precise localization of the NF2 protein in the COS cells (figure 3a,b). Optical sections perpendicular to the culture slide demonstrated a specific membrane bound localization. Furthermore staining was only detected on the dorsal cell membrane, the ventral cell membrane did not stain. A non-continuous distribution of the antigen was observed. Focal staining intensities and non-staining patches were observed. The cytoskeletal association of the NF2 protein was further studied by performing detergent extraction of NF2 transfected COS cells. COS cells extracted with the non-ionic detergent Triton X-100 were analyzed by western blotting and immunocytochemical staining.

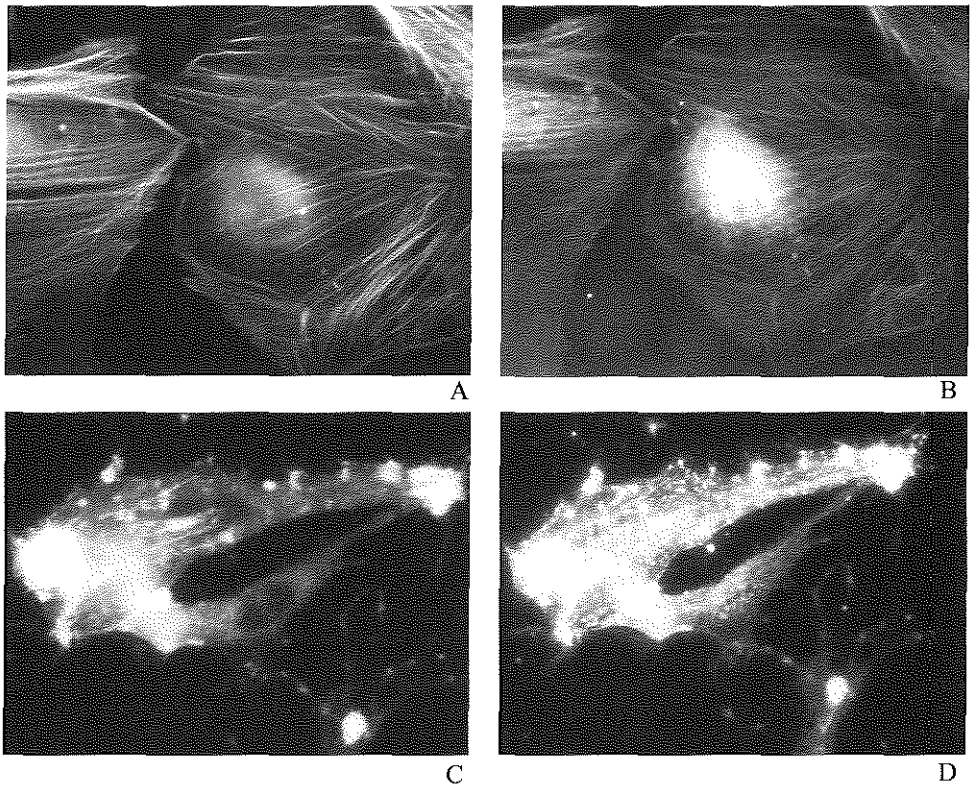
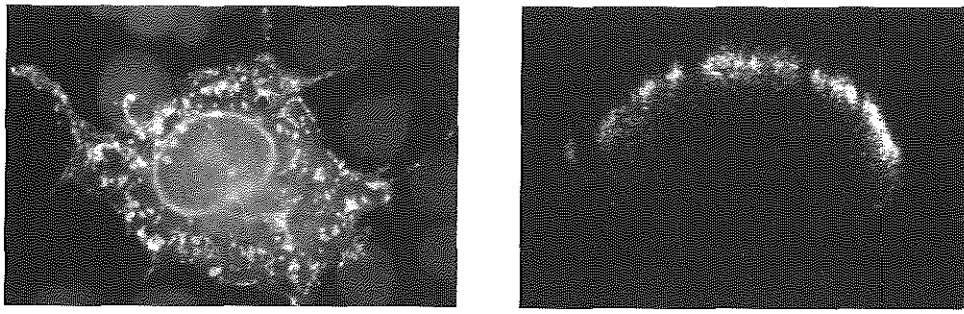


Figure 2
Double-immunofluorescent staining of HISM cells, using the KF10 antibody and TRITC-conjugated secondary antibodies (a,c) and FITC-conjugated phalloidin (b,d); the sections were not counterstained.

a. Phalloidin staining.
b. KF10 staining. Note complete overlap of the staining patterns in a and b, Magnification 1000x.
c. Phalloidin staining of cytochalasin D treated HISM cells.
d. KF10 staining pattern after cytochalasin D treatment of HISM cells. The staining patterns in c and d no longer overlap. Magnification 630x.

After detergent extraction of the COS cells, the staining pattern was virtually unimpaired, and staining intensity was completely retained (not shown). Immunoblotting analysis of the extracted protein and non-extracted protein indicated that the NF2 protein is associated with the non-extractable portion of the cellular proteins (figure 4).

Figure 3



A B

Immunofluorescent staining of NF2 transfected COS cells with the KF10 antibody.

a. Conventional fluoresence microscopy; note the punctate staining pattern. Propidium iodide counterstained section.

b. XZ-scanning CLSM micrograph of a stained COS cell expressing NF2, stained with the KF10 antibody, revealing a membrane bound punctate staining pattern. No signal is detected on the ventral cell membrane.

Discussion

To study the NF2 protein in normal human tissues and cultured cells we produced monoclonal antibodies to synthetic NF2 peptides. The N-terminal part of the NF2 protein is highly homologous to ERM proteins, the homology reaching 63% in amino acids 25-342, and 48% for the whole protein [12,13]. To avoid possible cross-reactivity of antibodies we therefore adopted the synthetic peptide approach [32] to immunize mice for the production of monoclonal antibodies. The N-terminal peptide SP-0 does not bear homology to any of the ERM proteins, ruling out the possibility of cross-reactivity with ERM proteins. Peptide SP279 has 7 of its 18 amino acids in common with ezrin, 4 of which occur at a single stretch. Generally a stretch of 6 amino acids is considered the shortest stretch constituting an epitope [38], making cross-reactivity with the ERM proteins unlikely. The identical staining patterns produced by 15H3 and KF10 indeed indicate that the KF10 antibody is specific for the NF2 protein only.

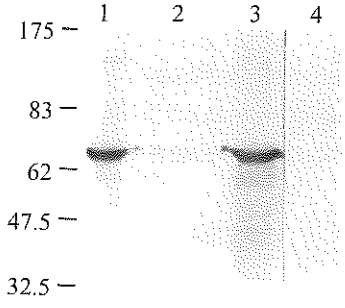


Figure 4
Western blot analysis of detergent soluble, and insoluble proteins from NF2 transfected COS cells. Detected with the KF10 antibody. The postion of the molecular weight markers is indicated. Lane **1**, insoluble protein fraction; **2**, acetone precipitate of extracted protein fraction; **3**, total COS cell lysate; **4**, lysate of mock-transfected COS cells.

The hallmark of NF2 is the development of bilateral vestibular schwannomas. Schwannomas of other cranial-, and peripheral nerves have also been reported [2]. A subset of the dermal abnormalities found in NF2 patients are also schwannomas. The NF2 gene product thus most likely plays an important role in Schwann cells. With the monoclonal antibody KF10 we could only detect the NF2 protein in Schwann cells in a section containing branches of the facial nerve (7th cranial nerve). It is possible that the SP279 epitope is shielded by other proteins binding to the NF2 protein. Proteins interacting with the NF2 protein have been identified [39]. Conversely, alternative splicing of the NF2 gene, or post-translational modification of the NF2 protein could result in abrogation of the SP279 epitope. Various alternatively spliced transcripts of the NF2 gene have been described [20,40,41]. This does however imply that this is not a universal event in Schwann cells, as indicated by the staining of the Schwann cells of the facial nerve. It seems likely that Schwann cells surrounding specific nerves either produce specific isoforms of the NF2 protein, or that a distinct subset of NF2 binding proteins is synthesized in different Schwann cells.

The immunostaining experiments performed on sections of human tissues confirm the presence of the NF2 protein in smooth muscle cells. With the monoclonal antibodies we could not detect the NF2 protein in either skeletal, or cardiac muscle. The immunostaining experiments further indicate that the tissue distribution of the NF2 protein is distinct from that of ezrin and moesin, and possibly partially overlaps that of radixin [22,42].

Having noted the intense staining pattern of smooth muscle cells in tissue sections we studied a human smooth muscle cell line. This cell line has been isolated from a section of human jejunum [37]. The cell line was characterized by its ability to contract in response to cholecystokinin-octapeptide, and by the presence of abundant stress fibers. Staining of HISM cells with the KF10 antibody and phalloidin revealed co-localization of the NF2 protein and actin stress fibers. Detergent extraction of the HISM cells did not result in changes of either the KF10 or phalloidin staining pattern. It thus appears that the NF2 protein is bound to actin stress fibers in smooth muscle cells. This is supported by the finding that the staining patterns produced by KF10 and phalloidin no longer coincide after cytochalasin D treatment. Phalloidin staining after cytochalasin D treatment of the HISM cells shows disruption of the stress fiber arrays. As a result KF10 staining adopts a disorganized punctate configuration, not overlapping with the stress fiber distribution pattern, suggesting aggregation of the NF2 protein in the cytoplasm.

A surprising finding was the specific staining pattern of the monoclonal antibodies of the human epidermis, although several band 4.1 related proteins have been identified in the epidermis by cross-reactivity with antibodies to band 4.1 protein [43]. Keratohyalin granules are notorious for their false positive staining reactions with various monoclonal antibodies. However, antibody incubations performed in high salt conditions, eliminating non-specific electrostatic interactions, or in the presence of non-ionic detergents, preventing hydrophobic interactions, did not impair immunostaining. The specificity was further substantiated by blocking experiments with synthetic peptides. Immuno-electron microscopy analysis demonstrates the association of NF2 protein with keratohyalin granules and intermediate filaments in the human epidermis. The contents of the keratohyalin granules include the protein fillagrin

which is probably involved in bundling cytokeratin filaments in terminally differentiating keratinocytes [44]. The presence of the NF2 protein associated with intermediate filaments in the more basal layers of the skin, and with keratohyalin granules in the stratum granulosum suggests that the NF2 protein might be involved in the terminal differentiation of the human epidermis. Although most of the cutaneous manifestations of the NF2 patients do not constitute disorders of keratinization, demonstration of the presence of the NF2 protein in the epidermis supports its involvement in the NF2-related skin disorders. The localization of the NF2 protein associated with intermediate filaments is unique in the sense that none of the ERM proteins has been found associated with intermediate filaments.

To study the sub-cellular localization of the NF2 protein we used NF2 transfected COS cells. A similar system has been used to investigate the cytoskeletal association of ezrin [23], which is endogenously expressed in COS cells. The specific localization of the NF2 protein in COS cells at the dorsal cell membrane observed with confocal laser scanning microscopy indicates that the NF2 protein is not randomly distributed in the COS cells. A staining pattern suggesting discrete accumulations of the NF2 protein at the dorsal cell membrane, and complete absence of the protein at the ventral cell membrane was observed. It does remain possible however that artifacts are created by the overexpression of the NF2 cDNA under control of the cytomegalovirus (CMV) promoter. It is interesting to note that the NF2 protein is not localized on the ventral cell membrane where focal contacts occur. In this respect the NF2 protein resembles ezrin and moesin as neither of these proteins is associated with focal adhesions [22]. Talin, another band 4.1 related protein is specifically located at focal adhesions [45]. Apparently the NF2 protein does not participate in the formation of focal contacts in COS cells. The NF2 protein also does not seem to co-localize with specialized cell surface structures such as microvilli. Ezrin specifically localizes at these structures in tissues and cultured cells [22,23]. To investigate the association of the NF2 protein with the cytoskeleton we performed extraction experiments. The majority of cytoskeletal proteins are not solubilized by detergent extraction. Immunoblotting analysis of NF2 transfected COS cell extracts with the monoclonal antibodies confirmed the cytoskeletal association of the NF2 protein. A faint band was also observed in the extracted protein probably representing non-cytoskeletal associated NF2 protein. The overproduction of the NF2 protein in COS cells might lead to spill-over of the protein after saturation of NF2 binding sites. Staining of COS cells after detergent extraction did not result in changes of the staining pattern, the punctate membrane-bound staining pattern was unaffected. These results indicate that the NF2 protein is tightly bound to elements of the cytoskeleton.

We have shown that the NF2 protein is associated with 2 components of the cytoskeleton, the microfilament system in smooth muscle cells and the intermediate filament system in the human epidermis. Further support for the cytoskeletal association of the NF2 protein is provided by the COS model system. The function of the NF2 protein is probably modified and controlled by binding proteins. If the NF2 protein acts as membrane-cytoskeletal linking protein, it is likely that amongst these binding proteins are integral membrane proteins.

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CHAPTER IV

Evidence for a cytoskeletal attachment domain at the N-terminus of the NF2 protein.

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Abstract

Neurofibromatosis type 2 is a hereditary cancer syndrome characterized by the development of bilateral vestibular schwannomas. Underlying the disease are inactivating mutations of the NF2 tumour suppressor gene, located on chromosome 22, encoding a 595 aminoacid protein. The NF2 protein, also known as merlin or schwannomin, reported to act as a membrane-cytoskeleton linking protein. This assumption is based on the homology of the NF2 protein to a group of band 4.1 related proteins, ezrin radixin and moesin. The cytoskeletal association of the NF2 protein has in part been confirmed by its ability to resist extraction from cells by non-ionic detergents. We performed detergent extraction on COS cells transfected with NF2 cDNA constructs. The extracts were analyzed by western blotting and immunofluorescent staining with monoclonal anti-NF2 antibodies. The results provide evidence for a major cytoskeleton attachment domain at aminoacids 27-131 and a putative second attachment domain between aminoacids 315 and 475.

Introduction

Introduction

Inactivating mutations of the neurofibromatosis type 2 (NF2) tumour suppressor gene, located on chromosome 22, cause the NF2 syndrome, characterized by bilateral vestibular schwannomas [22, 32]. Other features of NF2 include development of meningiomas, spinal nerve root schwannomas, ependymomas and non-neoplastic ocular manifestations [11]. In addition to the germline mutations found in familial NF2 cases, somatic mutations are found in sporadic schwannomas and meningiomas [37]. Two major isoforms of the NF2 gene are known. Isoform-1 lacks exon 16 and contains all aminoacids encoded by exon 17. Isoform-2 includes exon 16, which contains an in frame stop codon, resulting in a C-terminus with 11 different aminoacids [1, 13]. The 595-aminoacid NF2 protein, alternatively known as merlin or schwannomin, is related to the ezrin-radixin-moesin (ERM)-band 4.1 family of proteins. These proteins are involved in attachment of integral membrane proteins to elements of the cytoskeleton [33, 35]. Most mutations in the NF2 gene theoretically result in truncated NF2 protein [37]. The homology of the NF2 protein to ERM proteins is greatest in the first 300 aminoacids and includes an extensive alpha helical segment common to ERM proteins [10]. The similarity of the NF2 protein to ERM family members suggests that it likewise functions as a membrane-cytoskeleton linking protein. Evidence has accumulated that the integral membrane protein CD44 functions as the membrane bound NF2 associating protein partner [24, 26]. An alternative membrane protein, the regulatory cofactor of the Na+-H+ exchanger (NHE-RF) has been described as a potential NF2 membrane associating protein [21]. Several cytoskeletal proteins have been found to associate or co-localize with NF2 protein including actin, both as cortical actin fibers and as stress fibers, spectrin, tubulin and intermediate filaments [6, 9, 28, 36]. Ezrin, an ERM protein specifically localizing to actin filaments in specialized membrane structures, has been found to contain an actin binding site at the C-terminus, regulated by sequences in the N-terminus [35]. Recently an internal F-

and G-actin binding sequence has been described in ezrin located between aminoacids 281-333 [23]. Although the NF2 protein shares considerable homology with ezrin this is located in the first 300 aminoacids, the C-terminal actin binding sequence of ezrin is not present in the NF2 protein.

Evidence for the cytoskeletal association of proteins may be obtained by their ability to resist extraction from cells by treatment with detergents. We, and others, have previously shown that the NF2 protein resists extraction by non-ionic detergents in keeping with its postulated cytoskeletal association [4-6, 17, 24, 26, 27]. Moreover, immunofluorescent and immuno-electron microscopy localization studies have provided further evidence for the function of the NF2 protein as a membrane cytoskeleton linker. To determine which domain of the NF2 protein might determine its attachment to the cytoskeleton we tested 13 NF2 expression constructs in detergent extraction experiments. Of these 13 constructs 4 harboured mutations found in NF2 patients, including NF2-220N→Y, a construct based on a aac→tac mutation at nucleotide position 657 found in a NF2 family and resulting in substitution of tyrosine for asparagine [20]; constructs NF2-del262-C and NF2-360L→P are both based on mutations described in NF2 patients by Rouleau *et al.* (NF2-del262-C = EB G and NF2-360L→P = RF10) [22]. The mutation in NF2-del262-C is a t→c mutation in exon 8 at position 784 and introduces a stop codon, generating a 261 aminoacid protein. In NF2-360L→P a t→c mutation in exon 11 at position 1079 was described causing a proline for leucine substitution. The NF2-del173-C construct is based on a NF2 gene mutation observed in a NF2 family, in which a g→a transition in intron 5 results in activation of a cryptic exon. [3, 19]. Detergent soluble and insoluble fractions from transiently transfected COS cells were analyzed by western blotting using monoclonal anti-NF2 antibodies. In addition, visualization of NF2 protein from 6 constructs before and after detergent extraction was performed by immunofluorescent staining of cells grown on slides. Our results provide evidence for a major cytoskeleton attachment domain between aminoacids 29-131. Furthermore, all mutant NF2 proteins based on natural mutations in families remained attached to the cytoskeleton in our assays.

Materials and Methods

Construction of expression constructs (Fig. 2)

Cloning and expression of wild-type NF2 isoform 1 (wtNF2iso1) has been described previously (den Bakker *et al.*, 1995a). NF2 isoform 2 (wtNF2iso2) was kindly provided by Dr. Ramesh (Massachusetts General Hospital, Charlestown, MA, USA). All other constructs are based on isoform-1.

Construction of NF2-220N→Y was achieved by PCR using forward primer 5NFA2-121: 5'-gcccaagaggaattgcttc-3' and a reverse primer incorporating the changed nucleotides (capitals) and a BspEI restriction site (bold): 5'-ttat**tccgga**ttgcaaagtagATAacaccgt-3'. The PCR product thus produced spanned 2 internal BspEI sites. After digestion of wtNF2iso1 cDNA and the PCR product with BspEI (New England Biolabs, Beverly, CA, USA) and agarose gel electrophoresis, the appropriate fragments were isolated and ligated. Construction NF2-del262-C and NF2-360L→P was achieved with the aid of

an in-vitro mutagenesis kit (Altered Sites, Promega, Madison, USA). The cloning procedures were performed according to the manufacturers recommendations. For NF2-del262-C forward primer: 5'-ccccaagatctccttcccgtggaatgaaatcTgaaaca-3' in conjunction with the reverse primer from the kit was used. For NF2-360L→P a reverse primer: 5'-aggcCgctgcagatgaaagaagaagcaacaatggccaacga-3' was used in combination with a forward primer from the kit. The sequence of mutants NF2-220N→Y, NF2-del262-C and NF2-360L→P was checked by sequencing using a sequencing kit (Pharmacia, Uppsala, Sweden). NF2-del341-C was constructed by XhoI digestion (New England Biolabs) of pcDNA-3 vector containing the wtNF2iso1 cDNA, preceding preparative agarose gel electrophoresis, fragment isolation and re-ligation of the isolated fragment. Cloning and characterization of construct NF2-del173-C has been described previously.[3] Construction of NF2-del131-254 and NF2-del131-254;262-C was achieved by BglII digestion of respectively wtNF2iso1 cDNA and NF2-del262-C cDNA following subcloning of the cDNA's from the pcDNA-3 vector to pTZ19 (Pharmacia, Uppsala, Sweden) to avoid BglII sites in the pcDNA-3 vector. After digestion and fragment isolation, the inserts were re-inserted in the pcDNA-3 vector. Starting from wtNF2iso1 cloned in the pBluescript plasmid, construction of NF2-delN-28 was achieved by first subcloning the cDNA insert in reverse orientation by digestion with EcoRI followed by ligation and selection for the appropriate orientation by control digestions. After XhoI digestion, the resulting larger XhoI fragment derived from the plasmid was circularly ligated and digested with NcoI. Following isolation of this fragment from agarose gel and circular ligation the product was digested with XhoI and treated with calf intestinal phosphatase (Boehringer Mannheim, Germany). The second fragment from the initial XhoI digestion was isolated from gel and ligated into the modified vector. The resulting insert, now lacking sequences coding for the initial 28 aminoacids of the NF2 protein, was isolated by EcoRI digestion and inserted in the pcDNA-3 vector. The NF2-delN-28;262-C construct was made using NF2-delN-28 as a starting point and the using the same method as described above for NF2-del262-C. Constructs NF2-delN-320 and NF2-delN-204;471-C were constructed by PCR using wtNF2iso1 cDNA as a template. For NF2-delN-320 forward primer FW1168 5'-**ggaatt**CCACCATGaaagcccaggccagggag-3') and reverse primer RV2004 (5'-gc**tctaga**CTAgagctcttcaaagaaggccac-3') were used. For NF2-delN-204;471-C forward primer FW832 (5'-**ggaatt**CCACCATGgaatatctgaagatagctcaggacc-3') and reverse primer RV1633 (5'-gc**tctaga**CTActgcttggctcttcgctccg-3') were used. The forward primers incorporated an EcoRI restriction endonuclease site (bold) facilitating directional cloning and a Kozak consensus sequence flanking an ATG initiation codon (capitals). The reverse primers contained a XbaI restriction endonuclease site (bold) and a CTA termination codon (capitals). Amplification was performed under standard conditions (Perkin Elmer Gene Amp 9600, The Perkin-Elmer Corporation, Norwalk, USA); 30 cycles, 95°C 45 sec, 55°C 45 sec, 72°C 2 min) in the presence of 200ng each of the primers and 1U of Taq polymerase (Promega).Ten µl of the 100µl reaction volume was analyzed by agarose gel

electrophoresis, after which the product was digested by EcoRI and XbaI according to the manufacturers recommendations (Boehringer Mannheim, Germany). After agarose gel electrophoresis the product was isolated and inserted in the pcDNA-3 eukaryotic expression vector (Invitrogen, San Diego, USA). All constructs were tested for expression by in-vitro-transcription-translation experiments using T7 RNA polymerase and 35S-methionine in the TnT coupled reticulocyte lysate system according to the manufacturers recommendations (Promega). Of a 50µl reaction 2 µl was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; mini-protean 2D cell, BioRad, Hercules, CA, USA). After electrophoresis, the gels were dried and analysed by autoradiography.

Cell culture, transfection of constructs, western blotting and immunofluorescent staining of cultured cells were performed essentially as described previously (den Bakker *et al.*, 1995a) Monoclonal antibodies used for immunoblotting and staining included FE9, HB7, AH10 and KF10. Monoclonal antibody FE9 was raised against NF2 synthetic peptide SP-0 [6]. Monoclonal antibodies HB7 and AH10 were raised against NF2 synthetic peptides SP276 and SP277 respectively (manuscript submitted); monoclonal antibody KF10 has been described previously [5]. In these experiments detection in western blotting was performed by incubation of blots in a cocktail of antibodies (AH10 1:1000, FE9 1:1000, HB7 1:1000 and KF10 1:3000) followed by incubation with peroxidase conjugated goat-anti-mouse antibodies. Enhanced chemoluminescence (Pierce, Rockford, USA) with exposure to film was used for visualization of bands. For immunofluorescent staining single monoclonal antibodies were used, nuclei were counterstained with propidium iodide (Sigma, Brussels, Belgium).

Detergent extraction was performed according to the method outlined by Sainio *et al.*. [24] based on the method of Bretscher *et al.* [2]. The results were analyzed by western blotting; no densitometric analysis was performed. Detergent extracted cells grown on slides were immediately fixed in ice-cold acetone and stained. Control experiments included immunofluorescent staining and western blotting of COS cells with an anti-cytokeratin antibody (NCL5D3; DAKO Corporation, Glostrup, Denmark).

Results

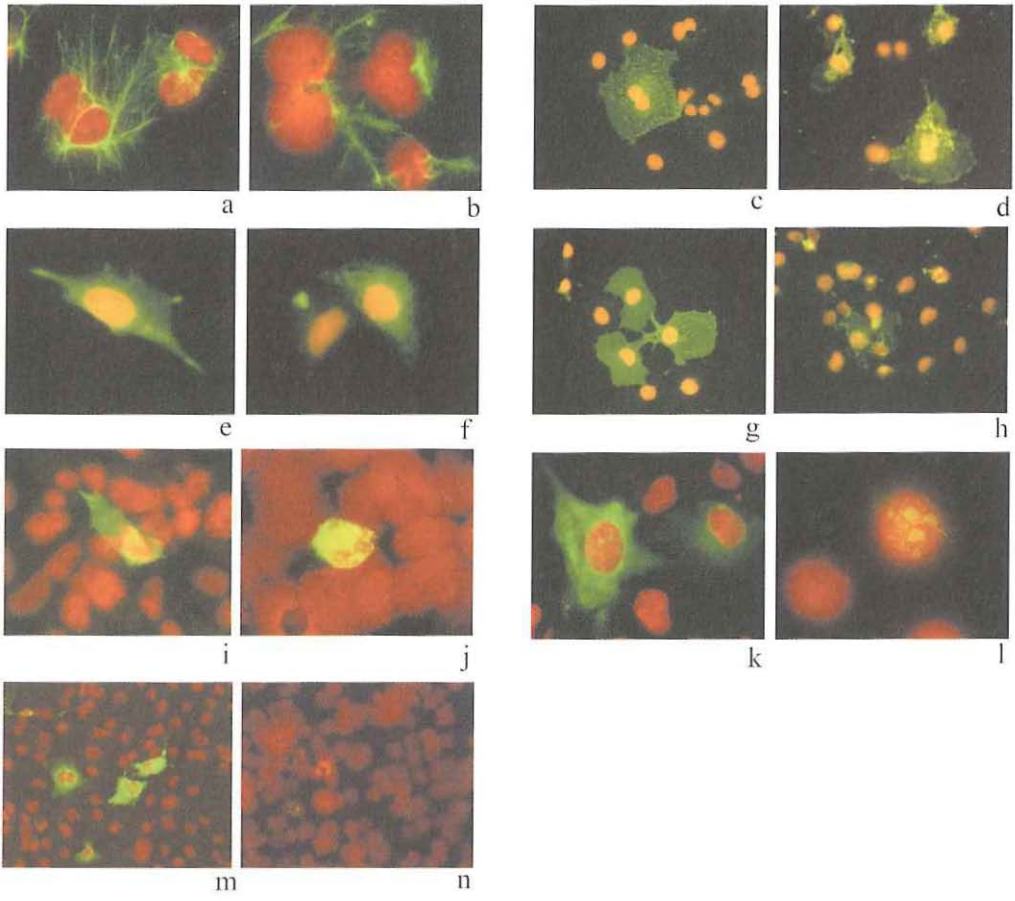
In-vitro-transcription-translation experiments confirmed synthesis of mutant NF2 proteins with expected calculated molecular weights (not shown). All constructs could be detected after transfection to COS cells, both by immunofluorescent staining and by western blotting. However, transfection efficiency of constructs varied considerably, ranging from approximately 1% to 15% of cells. Although some differences in staining patterns were noted for some constructs, these were not found to be readily reproducible. In addition, overexpression of cDNA in the pcDNA-3 vector imparts a considerable degree of difficulty when interpreting qualitative staining results. Therefore we drew no conclusions from the staining patterns. Detergent extraction of cells grown on slides resulted in degeneration of morphology with fuzzy nuclear outlines and loss of cell shape. However, cell ghosts could still be discerned and elements of retained cytoplasm and cytoskeleton were easily recognised

with slight loss of fluorescence intensity as compared to non-extracted cells. In control experiments with wild-type NF2 (wtNF2iso1), and non-transfected COS cells in which intermediate filaments were detected with anti-cytokeratin antibodies, a typical filamentous network was observed (Figure 1a). This network, although somewhat distorted, was still present after detergent extraction with minor loss of staining intensity (Figure 1b). Immunoblotting with the anti-cytokeratin antibody yielded a 60kD band in non-extracted COS cells and only in the insoluble fraction of detergent extracted COS cells (Figure 2). Of note is the observation that the proportion of soluble versus insoluble NF2 protein from one and the same construct varied from one extraction experiment to another. Some NF2 protein was usually found in the soluble fraction. Most likely this results from the overexpression of the cDNA construct and subsequent spill-over into the cytoplasm following saturation of cytoskeleton attachment sites. Therefore, mutant NF2 protein was only deemed soluble if in repeated experiments the bulk of protein was recovered in the soluble fraction.

NF2 protein resulting from transfection of wild type NF2 cDNA to COS cells, was predominantly present in the insoluble fraction. Likewise, isoform-2 NF2 protein was recovered from the insoluble fraction. Apart from constructs, NF2-delN-204;471-C and NF2-delN-320 only minor amounts of mutant NF2 protein from the other constructs was detected in the soluble fraction (Figure 2). Conversely, the mutant NF2 protein from these constructs was easily detected in the insoluble fraction. The molecular weight of the mutant proteins in the extraction experiments was similar to the determination in in-vitro-transcription-translation experiments (not shown). Immunofluorescent staining of COS cells transfected with constructs other than NF2-delN-204;471-C and NF2-delN-320, (wtNF2iso1; NF2-220N→Y; NF2-360L→P; NF2-del262-C) after detergent extraction showed slightly diminished staining intensity compared to non-extracted cells, nevertheless, the fluorescent signal remained associated with cell ghosts (Figure 1 c,d,e,f,g,h,i,j).

Figure 1
Immunofluorescent staining of transfected COS cells with and without detergent extraction.

a. Staining with anti-cytokeratin antibody, non-extracted COS cells, 1000X magnification
b. Staining with anti-cytokeratin antibody, after detergent extraction of COS cells, 1000X magnification
c. $wtNF2^{iso1}$ transfected COS cells stained with KF10, not-extracted, 200X magnification
d. $wtNF2^{iso1}$ transfected COS cells stained with KF10, after detergent extraction, 200X magnification
e. Staining of NF2-220N→Y transfected COS cells with KF10, not-extracted, 400X magnification
f. Staining of NF2-220N→Y transfected COS cells with KF10, after detergent extraction, 400X magnification
g. NF2-360L→P transfected COS cells stained with KF10, not-extracted, 200X magnification
h. NF2-360L→P transfected COS cells stained with KF10, detergent extracted, 200X magnification
i. NF2-del262-C transfected COS cells stained with HB7, not extracted, 400X magnification
j. NF2-del262-C transfected COS cells stained with HB7, after extraction, 400X magnification
k. NF2-delN-320 transfected COS cells stained with AH10, not extracted, 400X magnification
l. NF2-delN-320 transfected COS cells stained with AH10, after extraction, 400X magnification; note minor retention of stainable NF2 protein
m. NF2-delN-204;471-C transfected COS cells stained with AH10, not extracted, 100X magnification
n. NF2-delN-204;471-C transfected COS cells stained with AH10, after extraction, 200X magnification


a
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The mutant NF2 protein of constructs NF2-delN-320 and NF2-delN-204;471-C was predominantly present in the detergent soluble fraction as determined by western blotting (Figure 2). No immunofluorescence signal was observed in COS cells transfected with these 2 constructs after detergent extraction (Figure 1 k,l,m,n).

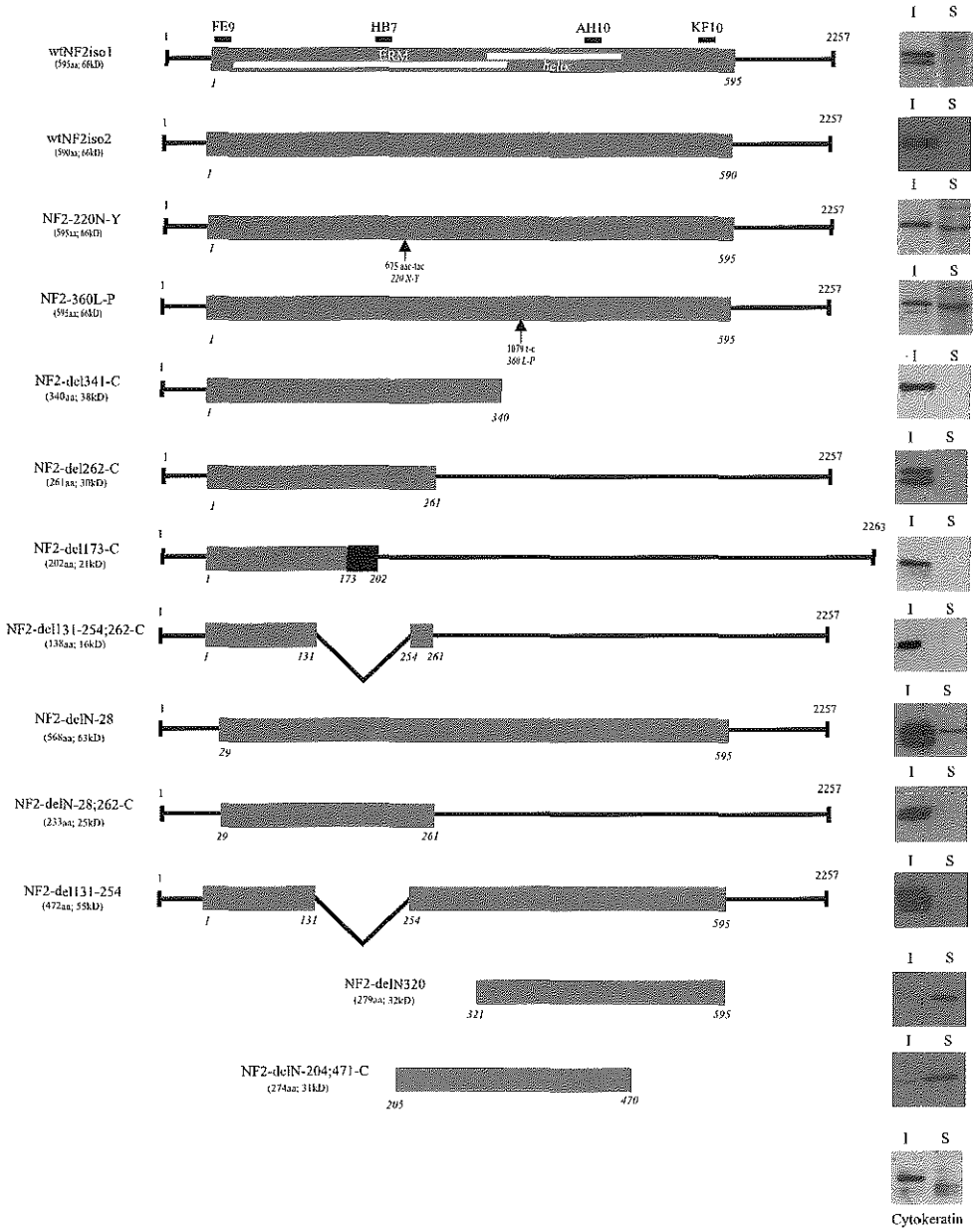
Discussion

The assumption that the NF2 protein is a membrane-cytoskeleton linking protein is based on the homology of the NF2 protein to members of the ezrin-radixin-moesin (ERM) family of proteins, in turn part of a superfamily of proteins based on the erythrocyte cytoskeleton protein band 4.1. A common structure is shared by ERM proteins. A globular N-terminal region is followed by an alpha helical stretch and charged C-terminus [33, 35]. The cytoskeleton binding capacity of ERM proteins has been convincingly demonstrated for ezrin, radixin and moesin. This binding capacity has been shown to reside in a 34 aminoacid F-actin binding sequence in the C-terminus, first described for ezrin. [34]. Although this domain is present in radixin and moesin, it is lacking in the NF2 protein. Using a solid phase assay an additional internal actin binding domain has been described By Roy *et al.* [23] Interestingly, this domain located between aminoacid residues 281-333, falls within the ERM homology region also shared by the NF2 protein. Intermolecular head-to-tail interaction has been demonstrated in ezrin in which the C-terminal F-actin binding site is obscured [7]. Heterotypic interaction has also been demonstrated between moesin and ezrin, further uniting the ERM family as a closely related functional group of proteins [8]. Recently, the membership of the NF2 protein to this family has been further substantiated by the finding that ezrin-NF2 interaction can occur [14] and that NF2 isoform1 protein also may form inter- and intramolecular association. [14, 24, 30]. Sequences facilitating this interaction are located in C-terminal aminoacids encoded by exon 17.

The functional implications of these homotypic interactions have been strengthened by the observation that interaction of N-and C-terminal sequences is essential for growth inhibition in Schwann cells and that overexpression of the C-terminal sequences solely cause cell death in NIH3T3 cells [29, 30]. It would be of interest to determine the effect of mutations on NF2 homotypic and heterotypic interactions in relation to growth inhibition.

The majority of mutations in the NF2 gene theoretically lead to truncated NF2 protein [37]. Truncated NF2 protein however, has not yet been conclusively demonstrated in tumours from NF2 patients [12, 18, 31]; our unpublished observations), although immunohistochemical staining experiments have provided some evidence for their existence [15, 25].

Figure 2
Schematic overview of cDNA constructs and accompanying western blots of soluble (s) and insoluble (I) protein extracts. Numbers above diagrams indicate positions of nucleotides, numbers in italics below diagrams represent aminoacids. Calculated molecular weights are given with construct names. The relative position of peptides to which monoclonal antibodies were raised are given with reference to wtNF2^{iso1}


FE9
HB7
AH10
KF10
wtNF2iso1
(595aa; 66kD)
FERM
Helix
2257
595
wtNF2iso2
(590aa; 66kD)
590
NF2-220N-Y
(595aa; 66kD)
625 aac-tac
220N-Y
NF2-360L-P
(595aa; 66kD)
1079 t-c
360L-P
NF2-del341-C
(340aa; 38kD)
340
NF2-del262-C
(261aa; 30kD)
261
NF2-del173-C
(202aa; 21kD)
2263
173
202
NF2-del131-254;262-C
(138aa; 16kD)
131
254
261
NF2-delN-28
(568aa; 63kD)
29
NF2-delN-28;262-C
(233aa; 25kD)
NF2-del131-254
(472aa; 55kD)
NF2-delN320
(279aa; 32kD)
321
NF2-delN-204;471-C
(274aa; 31kD)
205
470
I
S
Cytokeratin

The lack of detection of truncated NF2 protein may be caused by increased degradation of mutated protein, possibly mediated by calpain dependent proteolysis [16]. As the NF2 protein is thought to act as a linking protein, connecting integral membrane proteins to elements of the cytoskeleton, it would naturally follow that truncated protein would lose the capability to bind to either membrane or cytoskeletal proteins. Therefore determination of the functional domains of the NF2 protein may indicate which mechanism underlies tumour formation in NF2. We therefore have undertaken experiments to establish which domain of the NF2 protein mediates binding to the cytoskeleton. To this end we adopted detergent extraction of COS cells transfected with NF2 mutant cDNA constructs, 4 of which are based on mutations found in NF2 patients. Of note is the finding that the 4 constructs harbouring mutations found in NF2 patients do not seem to be impaired in their ability to resist detergent extraction and thus attachment to the cytoskeleton. Therefore it may be concluded that loss of cytoskeletal interaction by itself is not sufficient to cause NF2.

The data provided here show that the N-terminal sequences of the NF2 protein harbour a high affinity cytoskeleton attachment domain. Constructs NF2-delN-204;471-C and NF2-delN-320 almost totally lose their ability to interact with the cytoskeleton, whilst constructs NF2-del173-C, NF2-del131-254;262-C, NF2-delN-28 and NF2-del262-C retain resistance to detergent extraction. Therefore, we conclude that aminoacids 29-131 harbour a major cytoskeleton attachment domain. At present we do not know whether this domain directly interacts with cytoskeletal proteins, such as actin, or whether this interaction is indirect. There is considerable data from other groups supporting the existence of a cytoskeletal attachment domain in the N terminal region of the protein. In concordance with our results, Koga *et al.* show that several constructs in which C-terminal amino acids were deleted are retained in the insoluble fraction upon extraction with Triton X-100, with the smallest construct representing amino acids 1-173 [17]. In addition, Scherer *et al.* show the same for a construct deleted following aminoacid 303 [26]. Shaw *et al.* show that wild-type NF2 protein and a construct containing the N-terminal sequences localise to cortical actin and in particular areas such as membrane ruffles where dynamic actin rearrangements take place [29].

In addition to this N-terminally located high affinity domain, there is evidence for a second domain more C-terminal in the NF2 protein. From our extraction experiments it appeared that although the majority of protein from deletion constructs NF2-delN-204;471-C and NF2-delN-320 is extracted and recovered in the soluble fraction, some protein remained present in the insoluble fraction. We hypothesize that this is possibly due to a second lower affinity domain located between aminoacids 321-470, being the overlap between these two constructs. Evidence for a C-terminal binding domain has also been presented by Deguen *et al.* In their experiments a deletion construct harbouring amino acids 251-595 is even exclusively found in the insoluble fraction [4]. Further support for a C-terminal binding domain is provided by Scherer *et al.* who show that a protein fragment spanning amino acids 300-595 is recovered in both detergent soluble- and insoluble fractions [26]. Moreover, from other experiments it appears that NF2 protein sequences from 178-367 co-sediment with actin [36].

Combining these data the results would be compatible with a putative actin binding domain between amino acids 321-366 in the NF2 protein. Interestingly, an internal actin binding site was demonstrated for the ERM protein ezrin between ezrin amino acids 281-333 [23]. There is a 30 amino acid overlap between the actin binding region in ezrin and the putative NF2 domain. From these 30 amino acids 20 are identical between both proteins and an additional 5 are similar.

Taken together, it appears that cytoskeletal association of the NF2 protein is complex and most likely involves N- and C-terminal sequences. Furthermore, homotypic and heterotypic interactions probably influence this interaction. The available data seems to point to a major cytoskeleton association domain in the N-terminus of the protein probably regulated by C-terminal sequences. We here provide evidence for a major cytoskeleton attachment domain at the N-terminus of the NF2 protein, located between aminoacids 29-131 and a putative weaker attachment domain at aminoacids 321-470 possibly shared with other ERM proteins.

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CHAPTER V

Expression of the Neurofibromatosis type 2 gene in human tissues

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Abstract

The Neurofibromatosis type 2 tumor suppressor gene is implicated in the hereditary tumor syndrome NF2, hallmarked by bilateral vestibular schwannomas, meningiomas and ocular non-neoplastic features. The gene product has characteristics of a membrane-cytoskeleton linking protein, but the mechanism of tumor suppression by the NF2 protein remains to be elucidated. The NF2 gene is widely expressed in mouse and rat tissues. In humans most of the expression data has accumulated through northern blot analysis, RT-PCR and more recently western blot analysis, providing information on whole tissues and organs rather than on specific cell types. We here report an extensive survey of NF2 gene expression in human tissues using a combination of mRNA in-situ-hybridization (mRNA-ISH) and immunohistochemistry (IH) with a panel of monoclonal antibodies, supplemented by tissue immunoprecipitation experiments with affinity purified polyclonal antibodies. Expression was observed in many different cell types, most of which appear functionally normal in individuals affected by NF2. Surprisingly, expression could not be consistently documented in Schwann cells and arachnoidal cells by immunohistochemistry nor by mRNA-ISH in formalin fixed tissue. However, consistent immunostaining of Schwann cells was seen in frozen sections.

Introduction

Cloning of the neurofibromatosis type 2 (NF2) gene in 1993 [26, 38] led to the decisive segregation of the neurofibromatosis type 1 and neurofibromatosis type 2 syndromes. NF2 is characterized by the development of bilateral vestibular schwannomas. In addition, other central nervous system tumors, in particular meningiomas and to a lesser extent gliomas and ependymomas, occur in NF2 patients [12]. Spinal tumors observed in a high percentage of cases by neuroimaging studies less commonly lead to functional impairment [24]. Non-neoplastic features of NF2 include juvenile subcapsular lenticular opacities, retinal hamartomas and epiretinal membranes. Mutations in the NF2 gene are detected in NF2-related and sporadic schwannomas and meningiomas, supporting its role as a classical tumor suppressor gene [43]. In addition mutations have been detected in pleural mesotheliomas, a tumor that does not occur in NF2 patients [5, 22, 33]. The 595 aminoacid NF2 protein is homologous to a subgroup of band 4.1 proteins, ezrin, radixin and moesin, collectively known as the ERM protein family. ERM proteins function as membrane organizing proteins, linking the plasma membrane to the cytoskeleton [3, 40]. Based on the homology of the NF2 protein to the ERM proteins it has been postulated that the NF2 protein likewise functions as a membrane-cytoskeleton linking protein. The CD44 glycoprotein and a regulatory cofactor of the Na^+-H^+ exchanger are candidate NF2 associating membrane proteins [25, 27]. It has been shown that the NF2 protein co-localizes with elements of the actin cytoskeleton, possibly actin, with reports supporting colocalization with F-actin in stress fibers and subcortical actin fibers [10, 11]. Other reported putative NF2 associating cytoskeletal proteins include spectrin [32], RhoA [30] and tubulin [42]. To date, localization studies and biochemical approaches thus support the postulated

role of the NF2 protein, although conclusive evidence is still lacking and the method of tumor suppression is unclear.

The expression of the NF2 gene has been extensively studied in mouse and rat tissues using various techniques, including mRNA-in-situ hybridization (mRNA-ISH), RT-PCR, immunohistochemistry and western blotting [6-8, 13, 14, 19, 21]. Expression studies of the NF2 gene in human tissue are limited and are mainly based on RT-PCR and northern blotting [2, 4, 17, 18, 26, 28, 34-36, 38]. These approaches provide a general overview of gene expression. Detailed expression studies with visualization at the cellular level may be performed by immunohistochemistry or mRNA-ISH. Immunohistochemical detection of the NF2 protein relies on the availability of suitable antibodies and the accessibility of the epitope in the tissue. Previously, we have performed immunohistochemical detection of the NF2 protein in selected human tissue samples[9, 10]. We here present a detailed expression study of the NF2 gene in human tissue combining mRNA-ISH and immunohistochemical staining with a panel of monoclonal anti-NF2 antibodies. This approach was adopted in favor of immunohistochemistry solely, to avoid epitope masking problems which, with respect to ERM proteins, appear to prohibit an adequate inventory [29].

Materials and methods

Tissue samples and sections.

For mRNA-ISH and immunohistochemistry experiments formalin fixed paraffin embedded archival tissue blocks from surgical specimens were used. All brain sections were obtained from an autopsy in which there was no neurological disease. Following fixation in neutral buffered 10% formalin and paraffin wax embedding, 5μm sections were cut and mounted on 3-aminopropyltriethoxysilane coated slides (Sigma, Chemical Co., St.Louis, MO, USA). Glassware and solutions were autoclaved to inhibit RNase activity; non-autoclavable material was soaked in 0.2M HCl. All manipulations were performed under RNase free conditions.

In-situ-hybridization probes

Digoxigenin (DIG) labelled riboprobes BAK3AS (antisense) and BAK3S (sense) were transcribed from a subcloned 291 bp PstI fragment of the NF2 cDNA (positions 1085-1376 from the ATG initiation codon; Fig. 1). The fragment was subcloned in the pTZ-19 vector (Pharmacia, Uppsala, Sweden) in 2 orientations. After linearization of the plasmid, transcription and digoxigenin-labelling was performed according to the manufacturers recommendations (Boehringer Mannheim, Mannheim, Germany) using T7 RNA-polymerase. The BAK2S and BAK2AS probes (235bp, positions 1376-1611 from the ATG initiation codon; Fig. 1) were transcribed from PCR generated DNA templates. A consensus T7 RNA polymerase binding sequence was incorporated in the primer sets. The antisense BAK2 probe was transcribed from a DNA template generated with primers P1 (5'-

aggaagcacgcgaggcgga-3', forward) and P2T7 (5'-'tgcttgctcttttccatggcatcctaatacgactcactatagg-3', reverse).

For the sense BAK2 probe primers T7P1 (5'-gcatcctaatacgactcactataggaggaagcacgcgaggcgga-3', forward) and P2 (5-'tgcttgctcttttccatg-3', reverse) were used. The PCR products were purified (Qiaquick spin column, Qiagen GmbH, Hilden, Germany) and used for transcription. Transcribed riboprobes were checked by agarose gel electrophoresis and ethidium bromide staining and spot-blotting. Initial hybridization experiments performed on parallel tissue sections with BAK3AS/S and BAK2AS/S indicated that the results with these 2 probe sets were comparable with the BAK3AS/S probe set generally generating a stronger signal, all further hybridizations were carried out with probe set BAK3AS/S.

In-situ hybridization

mRNA-in-situ hybridization was performed essentially as described by Lindenbergh *et al.* [23]. Hybridization was performed at 53°C (2xSSC, 50% formamide) in a humid chamber overnight with a final probe concentration of 100ng/ml. Posthybridization washes were carried out at 45°C. Visualization of RNA hybrids was achieved by incubation with alkaline phosphatase conjugated anti-digoxigenin antibodies (Fab fragments, Boehringer Mannheim); 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-chloro-indolyl-phosphate (BCIP) were used as substrate. The colour development was performed in the dark with periodic visual inspection. The reaction was terminated when a sufficiently strong signal was observed. The sections were counterstained with nuclear fast red, dehydrated through ethanol gradients and mounted with Euparal (Chroma-Gesellschaft, Stuttgart, Germany). Control hybridizations were performed in selected cases with the DIG-labelled insulin-like-growth-factor-binding-protein-5 (IGF-BP5) probe [31], kindly provided by D.Lindenbergh-Kortleve. Control slides were treated with DNase or RNase prior to hybridization as controls. NF2 transfected COS cells were used as hybridization controls; transfection and cell culture were performed as described previously [10].

Antibodies and immunohistochemistry

The production of the monoclonal anti-NF2 antibody KF10 has been described previously [10]. Production of monoclonal antibodies FE9, HB7 and AH10, immunohistochemistry and ELISA were performed as described previously [10]. In ELISA and immunohistochemistry control experiments culture supernatant from the IgG1 secreting myeloma parent cell line (P3-X63Ag8) was used. Monoclonal antibody FE9 was raised against synthetic peptide SPNF2-0 [10] and monoclonal antibodies HB7 and AH10 were raised against synthetic peptides SP276 and SP277 respectively (Fig. 1) [9]. The isotype of the monoclonal antibodies was determined with the use of the Isostrip isotyping kit (Boehringer Mannheim, Germany). Primary monoclonal antibodies AH10 and HB7 were used as undiluted culture supernatants FE9 and KF10 were used at 1:50 to 1:100 dilutions from culture

supernatants from a Tecnomouse incubator (Tecnomara, Integra Biosciences, Wallisellen, Switzerland); all antibody incubations were performed overnight at 4°C. For immunoperoxidase staining the peroxidase-anti-peroxidase (PAP) method was used. Staining intensity for FE9 was improved by wet heat (microwave antigen retrieval) pre-treatment. As a chromogen diaminobenzidine (DAB) was used. Sections were incubated in the chromogen solution with hydrogen peroxide as a substrate for no longer than 7 minutes in the dark with gentle agitation. After rinsing, sections were counterstained with hematoxylin. No enhancement techniques were used. Negative controls included omission of the primary antibody, staining of parallel sections with undiluted culture supernatant from the myeloma parent cell line which secretes an IgG1 antibody, and staining with a commercially available anti prostate specific antigen (PSA) antibody (clone 8, IgG1, Sanbio, Uden, The Netherlands). The polyclonal antiserum 1399NF2 [9] was affinity purified using synthetic peptide SP277 coupled to an immobilized matrix (Sulfolink, Pierce, Rockford, IL, USA) through its terminal cysteine residue according to the manufacturers specifications. The antibody containing fractions were pooled and concentrated by ultrafiltration (Diaflow ultrafiltration membrane PM10, Amicon Corp. Beverly, MA, USA).

Immunoprecipitation

Normal human tissue was obtained from surgical specimens submitted for pathological diagnosis. The samples were analyzed histologically to avoid abnormal tissue. Tissue samples were lysed by mincing in 5 volumes immunoprecipitation buffer (1% NP40; 50mM TRIS-HCl, pH8.0; 150mM NaCl; 5mM EGTA; 5mM EDTA; 15mM $MgCl_2$; 60mM β-glycerophosphate; 1mM DTT; 0.1mM $NaVO_4$; 0.1mM NaF) supplemented with protease inhibitors. The samples were cleared by centrifugation and filtration (Super Acrodisc, Gelman Sciences, Ann Arbor, MI, USA). The obtained supernatant was precleared by adding 5µg of normal rabbit IgG and incubating for 1 hour at 4°C after which 25µl of a 50% slurry of protein-A sepharose (Pharmacia, Uppsala, Sweden) was added to the supernatant and mixed for an additional 30 minutes. The beads were spun down and the precleared supernatant was transferred to a fresh tube. Ten µg of polyclonal antibody 1399A or of the commercially available NF2 antibody A19 (sc331, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to the lysate and incubated for 1 hour at 4°C, after which 20µl of protein-A sepharose beads were added. The immunoprecipitate was allowed to form for 2 hours at 4°C or overnight. Following four washes of the immunoprecipitate in immunoprecipitation buffer and centrifugation, the pellet was resuspended in 50µl of reducing sample buffer (Laemlli). Ten µl samples were analyzed by western blotting as described previously [10].

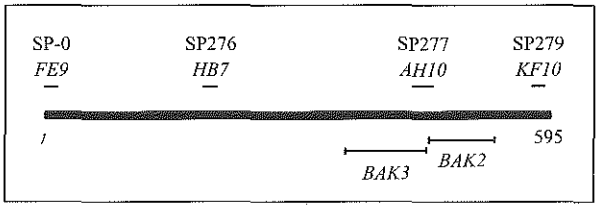


Figure 1.

Schematic diagram of the NF2 protein with relative positions of synthetic peptides and mRNA-ISH probe sets.

Results

mRNA-in-situ-hybridization and antibody controls

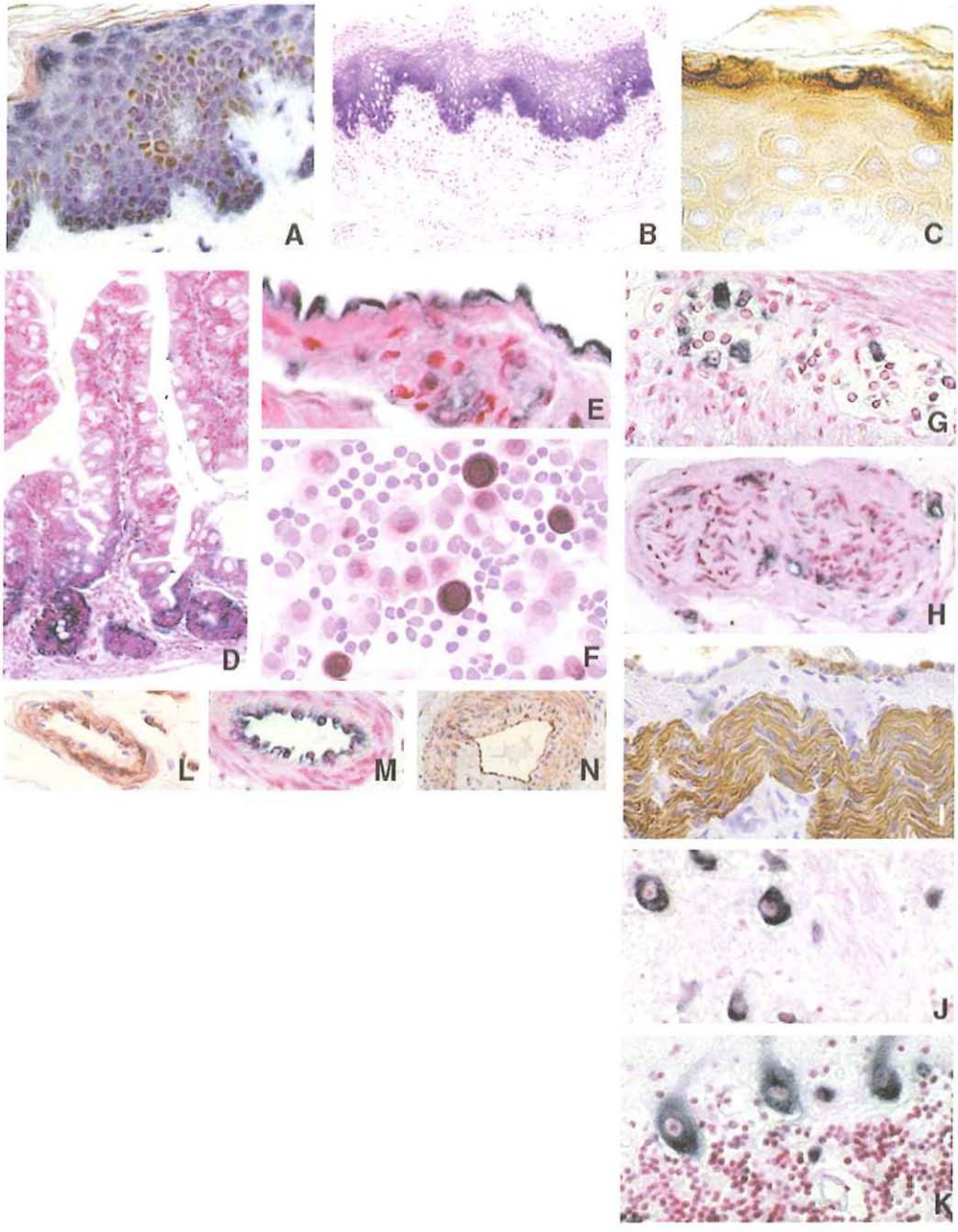
The specificity of the probes was checked by control hybridizations on NF2- and mock transfected COS cells with and without prior DNase or RNase digestion. A strong cytoplasmic hybridization signal was observed in approximately 10% of the cells (not shown), no signal was observed after RNase digestion, but DNase digestion did not alter staining, indicating that the signal was not due to hybridization of the riboprobe to transfected NF2 cDNA. Weak background staining in all COS cells was observed with the anti-sense and sense probes in most experiments.

Antibodies FE9 and AH10 were determined as IgG1(κ) antibodies, while HB7 presented as an IgM(κ) antibody. In solid phase assays (ELISA) using synthetic NF2 peptides and non-relevant protein (bovine serum albumin) the monoclonal antibodies reacted exclusively to the NF2 peptides to which these had been raised. No reactivity in ELISA was seen when culture supernatant from the myeloma parent cell line was used. All mono- and affinity purified polyclonal antibodies detected the 69kD band in western blots of NF2 transfected COS cells. In western blotting experiments and immunocytochemical staining experiments using cultured epithelial cell lines it was found that anti SP-0 antibody FE9 crossreacts with a low molecular weight cytokeratin (not shown), therefore staining of epithelia with this antibody was not interpreted. Staining of nucleoli in some tissue sections was seen with the monoclonal antibody AH10 and to a lesser extent a-cellular stroma was stained with this antibody.

Figure 2.

Immunohistochemical- and mRNA-ISH staining of human tissue.

a. mRNA-ISH of epidermis, BAK3-AS probe, original magnification 200X
b. mRNA-ISH of oesophagus, BAK3-AS probe, original magnification 100X
c. Immunostaining of epidermis, KF10 antibody, original magnification 400X
d. mRNA-ISH of small intestine, BAK3-AS probe, original magnification 100X
e. mRNA-ISH of mesothelial lining of intestine, BAK3-AS probe, original magnification 200X
f. Immunostaining of pleural aspirate, FE9 antibody, original magnification 400X. Note strong staining of mesothelial cells and weaker staining of histiocytes, lymphocytes do not stain.
g. mRNA-ISH of intestinal ganglion, BAK3-AS probe, original magnification 200X. A clear signal is seen in neurons, satellite cells do not stain.
h. mRNA-ISH of peripheral nerve, BAK3-AS probe, original magnification 200X. ISH staining is seen in endothelial cells, Schwann cells are negative.
i. Immunostaining of cryostat section of peripheral nerve in sub-epicardial connective tissue, FE9 antibody, original magnification 200X. Note strong cytoplasmic staining of Schwann cells and weaker staining of mesothelial (epicardial) lining.
j. mRNA-ISH of CNS neurons, BAK3-AS probe, original magnification 400X. Very weak staining of glial cells is observed in addition to strong cytoplasmic staining of neurons.
k. mRNA-ISH of Purkinje cells in the cerebellum, BAK3-AS probe, original magnification 400X.
l. Immunostaining of bloodvessel, KF10 antibody, original magnification 200X. The tunica media is stained, the endothelial lining does not stain with this antibody.
m. mRNA-ISH of bloodvessels in loose subserosal tissue in the intestinal tract endothelial, BAK3-AS probe, original magnification 200X. Note absence of staining of smooth muscle cells of the tunica media, a strong hybridization signal is in endothelial cells.
n. Immunostaining of bloodvessel, FE9 antibody, original magnification 200X. Both smooth muscle cells in the tunica media and endothelial cells are stained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The sections stained with culture supernatant from the myeloma parent cell line demonstrated weak non-specific staining that did not overlap with staining patterns achieved by the NF2 monoclonal antibodies; no signal was observed in sections stained with anti-PSA antibodies.

NF2 expression in tissues by mRNA-ISH and immunohistochemistry

Consistent NF2 gene expression was observed in a number of tissues by mRNA-ISH, immunohistochemistry and immunoprecipitation. An overlapping but distinct staining pattern of the various anti-NF2 antibodies was found.

epithelium and mesothelium

High levels of NF2 gene expression were observed in all layers of the epidermis excluding the a-cellular cornified layer by in-situ-hybridization (Fig 2a). Non-cornifying stratified epithelium of the oesophagus also stained. However, here the ISH signal was limited to the lower layers (Fig 2b). A strong ISH-signal was observed in urothelium and in the epithelium of the small intestine. In the small intestine the signal was confined to the crypts and diminished towards the tips of the villi (Fig 2d). Weak ISH-staining was observed of colonic epithelium, generally located at the basal parts of the crypt. Other epithelia in which a weaker mRNA-ISH signal was observed included pseudostratified epithelium of the trachea, bronchial epithelium, epithelium of eccrine sweatducts in the skin, the acinar cells in the pancreas, cells of the collecting ducts in the kidney and follicular epithelium in the thyroid and parathyroid gland. No ISH signal was observed in fat, cardiac muscle, cartilage, liver, prostate, adrenal gland, spleen, thymus and peripheral nerve. Immunostaining closely mimicked mRNA-ISH staining in stratified epithelia with strong staining by AH10, HB7 and KF10 (Fig 2c). In non-stratified epithelia weak immunostaining was observed with AH10 only.

Hybridization- and immunostaining of peritoneal mesothelial cells was observed, but of variable intensity (Fig 2e). Mesothelial cells in other locations, including pleura and epicardium were also stained with NF2 antibodies, a stronger cytoplasmic immunohistochemical signal was observed when cytological smears from pleural aspirates were used (immunohistochemistry only, Fig. 2f).

neural tissue

Strong and consistent immunohistochemical and mRNA-ISH staining was observed of neurons in the autonomic ganglia throughout the intestinal tract (Fig 2g). However, Schwann cells and satellite cells were negative. Likewise, Schwann cells in other locations, for instance in peripheral nerves or in peripheral sections of cranial nerves, did not show any hybridization signal (Fig 2h) and only weak and non-consistent immunostaining was seen in paraffin sections. However, immunostaining of cryostat sections of peripheral nerve with FE9 was consistently positive (Fig 2i). In the central nervous system neurons were consistently positive with mRNA-ISH but no regional differences were noted. Cortical neurons, neurons of cranial nerve ganglia and neurons of extra-pyramidal locomotory systems

stained with equal intensity (Fig 2j). We did observe diminishing staining intensity towards the deeper layers, most likely due to impaired penetration of fixative in the brains after autopsy. Weak and inconsistent ISH-staining of glial cells was noted. A weak hybridization signal was observed in ependymal cells but arachnoidal cells were negative. Staining of neurons with the monoclonal NF2 antibodies was not observed and no immunostaining of arachnoidal cells was seen. In the cerebellum the Purkinje cells hybridized strongly (Fig 2k) but no ISH-staining of the molecular or granular layer was observed. Immunostaining of cells in the cerebellum was not seen. In the human eye a hybridization signal was observed in amacrine cells in the retina. Very weak ISH-staining was observed in the cornea and in lens epithelium while immunostaining was negative.

blood vessels

Variable NF2 gene expression was found in endothelium in bloodvessels. In bloodvessels located in the loose subserosal tissue in the intestinal tract and in umbilical cord vessels strong specific endothelial ISH- and immunostaining with the FE9 monoclonal antibody was observed but not with any of the other antibodies (Fig 2 l,m,n). In other tissues staining of endothelial cells was weak or absent. Of note is the very weak or complete absence of a ISH-signal in the smooth muscle cells of the tunica media of blood vessels, despite consistent strong positive immunostaining with several antibodies (Fig 2 l,m,n). Skeletal and cardiac muscle demonstrated low expression levels with immunostaining with 1 antibody (AH10) but ISH was completely negative.

other tissues

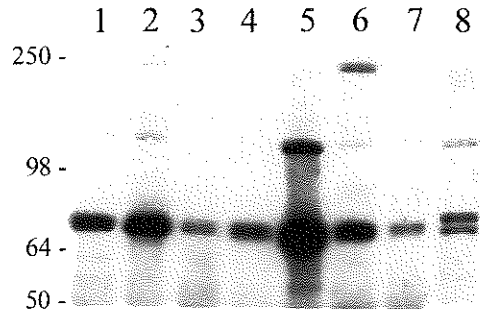
No staining was seen of liver including bile duct epithelium, spleen, prostatic epithelium, thymus (only staining of Hassal's corpuscles), adrenal gland, pneumocytes, circulating blood cells, fat, cartilage, mucus glands in oesophagus and trachea, islet cells and ductular epithelium in the pancreas, glomeruli and renal tubules. Immuno- and ISH staining was seen of tissue macrophages in various tissues; in lymph nodes immunostaining was seen of high endothelial venules.

Immunoprecipitation.

To confirm the presence of NF2 protein in various tissues we performed immunoprecipitation experiments with affinity purified anti-NF2 antibodies. Frozen tissue samples were available for immunoprecipitation experiments for a selection of tissues. From all tissue samples NF2 could be immunoprecipitated by either of the affinity purified polyclonal antibodies A19 or 1399A (Fig 3). Immunoprecipitated protein could be detected with any one of the monoclonal antibodies and with either of the affinity purified polyclonal antibodies. In addition to the expected 69kD band, a slower migrating band of approximately 130 kD was observed in most samples and in lysates of oesophagus and adrenal gland a band of 250 kD was also seen. These additional bands were detected by several NF2 antibodies and therefore possibly represent NF2 dimers. From most lysates the 69kD NF2 band presented as a doublet, probably due to phosphorylation.

Two human smooth muscle cell lines, one an established smooth muscle cell line derived from the muscularis externa of the intestine (human intestinal smooth muscle, HISM (Graham *et al.* 1984)) and a second primary smooth muscle culture from the human airway (kindly supplied by Miss S. McKay, Dept. of Pharmacology, Erasmus University Rotterdam), were used for immunoprecipitation. Both cell lines yielded a 69kD band which was detected with monoclonal antibodies raised against distinct NF2-peptide
epitopes (not shown). In some experiments the 69kD band presented as a doublet.

Figure 3
Immunoprecipitation of NF2 protein from human tissue. Precipitating antibody A19, detection with FE9. Lane 1 to 8: small intestine, adrenal gland, myometrium, myocardium, kidney, oesophageal mucosa, epidermis and NF2 transfected COS cells. Note the slower migrating bands at approximately 130 kD band and 250 kD. The position of the molecular weight markers is indicated on the left.



Discussion

Neurofibromatosis type 2 is characterized by the development of schwannomas of the eighth cranial nerves, and meningiomas. Schwannomas are benign tumors arising from neural-crest derived Schwann cells, in which the inactivation of the NF2 gene is thought to be paramount in the pathogenesis. Therefore expression of the NF2 gene is expected in Schwann and arachnoidal cells.
To further document NF2 gene expression in human tissue, we performed immunohistochemical and mRNA-ISH staining experiments on an extensive panel of human tissues. Although frozen sections may provide increased sensitivity we used formalin fixed paraffin embedded sections for all experiments to ensure a comprehensive tissue panel and optimal morphology after hybridization. An exception was made to demonstrate the presence of NF2 protein in Schwann cells in peripheral nerve. In this case both formalin fixed and cryostat sections were used for immunohistochemical staining. The discrepancy of non-staining tissue in paraffin sections versus clear immunoreactivity in cryostat sections is well known. In a number of tissues in the study presented here, including liver, spleen, adrenal gland, thymus, fat, prostatic epithelium, cartilage, pneumocytes and circulating blood cells we could not document NF2 expression by either mRNA-ISH or IH. It is possible that the sensitivity of the employed methods is insufficient to detect the presence of NF2 protein in these tissues. Therefore, we interpreted positive immuno- or ISH staining as evidence of NF2 gene expression whilst acknowledging that non-staining does not rule out NF2 gene expression below the detection threshold levels.

Expression studies of the NF2 gene have been performed by a number of techniques including northern blotting, RT-PCR, western blotting, mRNA-ISH and immunohistochemistry. Furthermore, the majority of these investigations have been performed on non-human tissues, in particular rodent tissues and on cell lines [2, 4, 6-8, 13, 14, 17-19, 21, 26, 28, 34-36, 38]. A drawback of a number of techniques utilising tissues homogenates such as northern blotting, western blotting and RT-PCR, is the inherent lack of detail. No information on specific cell types can be inferred from these experiments. Furthermore, until evidence exists that the NF2 protein functions similarly in rodent cells and human cells comparison of expression studies should be interpreted with care.

Strong and consistent immuno- and ISH-staining of epithelia, in particular stratified epithelia, was observed. Expression was observed both in keratinizing and non-keratinizing stratified epithelium. Previously we have documented co-localization of NF2 protein with keratohyalin granules and intermediate filaments in keratinocytes [10]. Apart from cutaneous schwannomas NF2 patients do not develop specific skin disorders. Our findings coincide with immunohistochemical and mRNA-ISH data from a developmental study in mice in which expression in keratinocytes in the epidermis was described [21].

NF2 gene mutations have been documented in malignant mesotheliomas [5, 22, 33], a tumour that is not seen with an increased frequency in NF2 patients. In support of the involvement of the NF2 gene in the development of malignant mesotheliomas are our findings of NF2 gene expression in pleural, epicardial and abdominal mesothelium. It remains to be elucidated if, comparable to epithelium, the NF2 protein co-localizes and associates with intermediate filaments in mesothelial cells.

Schwann cells are derived form the neural crest and form myelin sheaths around axons in the peripheral nervous system. Sporadic and NF2 related Schwannomas arise from Schwann cells. Therefore, the presence of NF2 protein is expected in Schwann cells. Surprisingly, no signal was observed in Schwann cells with either of the hybridization probe sets, while immunostaining was weak and variable in paraffin sections. However, strong and consistent positive immunostaining of Schwann cells in frozen sections was seen. Approximately half of NF2 patients develop meningiomas. These tumors, with variable histological appearances, are generally believed to originate from arachnoidal cells in the leptomeninges. Therefore, it is assumed that the NF2 gene is expressed in these cells. We were not able to demonstrate NF2 gene expression in arachnoidal cells with mRNA-ISH. Neither did we detect NF2 protein by immunohistochemical methods with the monoclonal antibodies. The lack of reactivity in mRNA-ISH and immunostaining experiments of Schwann- and arachnoidal cells may be caused by insufficient sensitivity of the employed methods to detect low levels of NF2 mRNA and protein. In previous reports the presence of the NF2 protein in Schwann and arachnoidal cells has been demonstrated by immunohistochemical methods [7, 18, 28, 30, 34, 35]. However, the results of these experiments are not consistent and staining in some reports has only been achieved by very sensitive methods. Investigators using more conventional staining techniques, describe cytoplasmic perinuclear staining in some Schwann cells and variable expression in arachnoidal cells in

frozen sections of human tissue [34]. In addition, fixation time appears critical for immunohistological detection of NF2 protein [30] and it appears that detection of NF2 protein in tissues is dependent on the epitope to which antibodies are reactive. Several investigators have used cultured Schwann cells to detect NF2 protein. It must be borne in mind however, that ERM gene expression may be upregulated in cell culture while corresponding endogenous tissue expression has not been documented for the particular ERM protein [1, 16, 39]. Previously it has been demonstrated that NF2 gene expression in the mouse and possibly in humans is developmentally regulated [21, 26]. Failure of demonstration of NF2 expression in the study in adult human tissue could thus be due to physiological down-regulation in Schwann and arachnoidal cells.

As we and others have noted, the NF2 gene is expressed in smooth muscle cells and endothelial cells [7, 10, 34]. Therefore, expression studies utilizing RT-PCR, northern blotting and to a certain extent western blotting, may erroneously detect NF2 expression originating from blood vessel wall elements in tissues not truly expressing the NF2 gene.

Comparable to rodents, we found a strong ISH signal for the NF2 gene in neurons of the central nervous system, including the Purkinje cells in the cerebellum. In contrast, we were not able to detect NF2 protein in these cells using antibodies to various epitopes. It is possible that the antibodies we employed were directed to masked epitopes. Indeed, a recent report by Stemmer-Rachamimmov describes extensive expression of the NF2 gene in the human CNS by immunohistochemical methods using a monoclonal antibody directed to C-terminal NF2 sequences [35]. In addition to neurons, these authors detect NF2 protein in glial and ependymal cells. Although we did occasionally observe a ISH signal in glial cells this was not readily reproducible and weak ISH staining was also observed after hybridization with sense probes.

Taken together, it appears that the NF2 gene is expressed in many human tissues, most of which are not involved by disease in individuals affected by NF2. NF2 gene mutations in particular affect Schwann cells and arachnoidal cells and do not affect other tissues that apparently express the NF2 gene. It therefore seems plausible that NF2 expression in these tissues may be redundant. Experiments providing support for this hypothesis have been performed by Takeuchi, Huyng and Henry [15, 20, 37]. By introducing anti-sense NF2 oligonucleotides in the Schwann-cell like cell line STS-26 Huyng *et al.* observed morphological and stimulatory proliferation effects, while Takeuchi showed that inhibition of all 3 ERM proteins was required in thymoma and epithelial cells for maximal inhibition of cell adhesion and for morphological changes. Why this putative redundancy might fail in Schwann cells and arachnoidal cells is not clear. Although cell type specific expression of a single ERM protein seems an attractive explanation, Schwann cells have been shown to express moesin [34] as well as NF2 protein thus excluding this hypothesis. An alternative explanation for the apparent discrepancy between NF2 disease symptoms and tissue expression patterns of the NF2 gene may be that, in analogy to loss of the retinoblastoma gene in several epithelia, loss of function of NF2 function in epithelial cells induces hyperproliferation masked by an increased rate of apoptosis [41].

We have provided an inventory of human NF2 gene expression. From this inventory and the available literature data, it can be concluded that NF2 and ERM genes are indeed expressed in an partly overlapping set of tissues. Additional studies are required to focus on common and distinct NF2 and ERM protein functions.

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CHAPTER VI

A G→A transition creates a branch point sequence and activation of a cryptic exon, resulting in the hereditary disorder neurofibromatosis 2

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Abstract

We describe a G→A transition within intron 5 of the NF2 gene. This mutation creates a consensus splice branch point sequence. To our knowledge, this is the first report of a mutation that creates a functional branch point sequence in a human hereditary disorder. The new branch point sequence is located 18 bp upstream of a consensus splice acceptor site. A consensus splice donor site is found 106 bp 3' of the acceptor site. As a consequence the G→A transition results in an alternatively spliced mRNA containing an additional exon 5a of 106 base pairs derived from intron sequences. We cloned the mutant cDNA and show that due to an in frame stop codon the cDNA codes for a truncated NF2 protein. The mutation was observed in 3 affected members of a NF2 family. In a tumour of one of the family members, both alternatively spliced and wild type mRNA were found, although the wild type allele of the gene is absent due to an interstitial deletion on chromosome 22. We also show that immunoprecipitations reveal the presence of full length wild type NF2 protein in the tumour lysate. These data support the hypothesis that some degree of normal splicing of the mutant precursor RNA is taking place. It is therefore likely that this residual activity of the mutant allele explains the relatively mild phenotype in the family. These data also implicate that complete inactivation of the gene is not required for tumour formation.

Introduction

Branch point sequences are located 18-40nt upstream of a splice acceptor site which usually is the first AG downstream of the branch point sequence. In the branch point consensus sequence, Y,N,Y,U,R,A,C (Y=C/T; R=G/A; N=any) the A at position 6 is critical for the formation of a mammalian splice intermediate: the lariat structure. In addition, it has been shown that mutation of this A residue strongly reduces the splicing efficiency of the downstream exon [13]. In human genetic diseases, splicing errors are common, however most of these alterations are due to a mutation of the splice donor or acceptor sites. Only two cases have been described so far in which the splicing error was the result of a mutation of the invariant A residue of the branch point resulting in reduced usage of the downstream exon [12, 14].

Neurofibromatosis type 2 (NF2) predisposes patients to the development of central nervous system tumours such as Schwannomas, meningiomas and ependymomas [5, 10]. Additional manifestations of the disease include peripheral Schwannomas and cataract. NF2 is an autosomal dominant disorder with a birth incidence of 1:35,000. The NF2 gene, located on chromosome 22q12, consists of 17 exons of which exons 16 and 17 are alternative splice forms leading to different COOH-termini of the NF2 protein [1, 15, 19]. Mutations in the NF2 gene have been found in NF2 family members and in sporadic Schwannomas and meningiomas [20]. In addition, mutations were found in malignant mesotheliomas, a tumour that does not develop in NF2 patients [2, 18].

Most of the mutations that have so far been described in the NF2 gene predict that gene function is negatively affected. In general, mutations that lead to a truncation of the protein are found more

frequently in patients with the severe form of the disease. In patients with a mild phenotype missense mutations are more frequent. This suggests that there is a correlation between phenotype and genotype [11, 16, 20]. It has been speculated that the mutations leading to a truncated protein could perhaps function as dominant negative mutations in which the truncated protein interferes with the function of the wild type gene. The reduced activity of the NF2 protein could then trigger some degree of hyperplasia which would increase the chances on a second hit (i.e. loss of the other allele), after which tumour formation would ensue. Such a scenario would explain the larger number of tumours in severely affected patients. However, this hypothesis remains to be proven. Mutations that affect splice sites are encountered in both forms of NF2 and it has been argued that in cases with the mild form of NF2 these mutations do not completely prevent normal splicing. However, the effect of the mutation on mRNA and protein is usually not investigated.

In the current report we describe a G→A transition which causes NF2 in a Dutch family. The mutation creates a consensus splice branch point sequence in intron 5 of the NF2 gene. Due to the presence of downstream splice acceptor and donor sites this mutation results in the incorporation of a additional exon in the NF2 mRNA. We show that the alternative mRNA codes for a truncated protein. In addition, we detected large amounts of wild type mRNA and protein in tumour tissue. This suggests that the mutation still allows normal splicing of the mutant allele. This finding might explain the relatively mild phenotype in the family and that tumourigenesis does not require a complete absence of wild type NF2 protein.

Results

Detection of a point mutation in the NF2 gene

Earlier we reported that in a meningioma from case 2, MN121, the NF2 cDNA contained an extra insert of 106 base pairs [8]. The insertion of 106 bp located between exon 5 and exon 6, suggested a mutation either in the exon 5 splice donor site or in the exon 6 splice acceptor site. In order to find the exact position of the mutation in genomic DNA, we have amplified these exons including the exon/intron borders with exon 5 and 6 specific primers [7]. No altered migration pattern was observed on agarose gels or by SCCA analysis (not shown) nor did the sequence of the 106 bp insertion correspond to the reported exon border sequences. These results indicate that mutations in the exon 5 splice donor or exon 6 splice acceptor sites were not the cause of the insertion.

To determine the origin of the extra 106 bp at the exon 5 and 6 border in MN121 cDNA, we have cloned a 1,0kb PCR fragment encompassing exon 5 and 6 and the intron 5 sequences of the NF2 gene from case 2. To locate the 106nt cDNA insertion in the intronic sequences we used the restriction enzyme *Bpm*I which has one recognition site in the 106nt insertion. *Bpm*I cuts also once in the 1.0kb genomic exon 5-6 fragment. By determining the sizes of the restriction fragments, we were able to locate the 106nt insertion 249 bp downstream of exon 5 (Fig. 1A). PCR, using primers from exon 5 or exon 6 in combination with primers derived from the 106nt sequence confirmed the position of the

106 bp sequence. These primers were also used for DNA sequencing and the sequence of a 430 bp genomic region of MN121 DNA was determined and compared with normal DNA (Fig. 1). It appears that both in control DNA and in DNA of tumour MN121 the sequence of the 106nt insertion is present. However, in MN121 there is a G→A point mutation at position 301 (start of exon 5 is 1), 18 bp upstream of the 106 bp sequence. This is the only difference we could detect in MN121 tumour DNA when compared to the control. The point mutation results in the creation of a putative branch point site just in front of a putative splice acceptor site (Fig. 1).

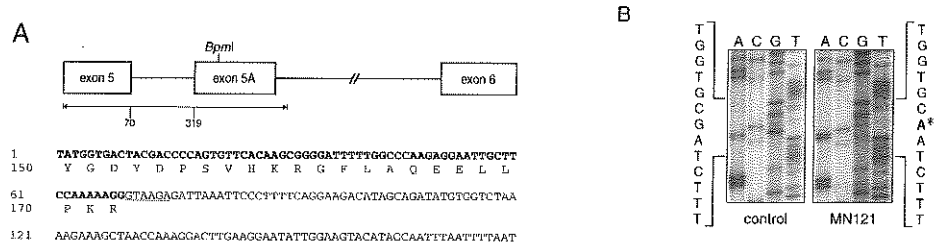

Figure 1.

Identification and position of the G→A mutation and exon 5a relative to exons 5 and 6 of the NF2 gene. **A.** Upper drawing: location of exon 5a within the intron between exons 5 and 6. The *Bpm*I site is indicated. The sequenced area is indicated in the drawing. In the sequence, exon 5 and 5a are in bold letters. Underlined are splice acceptor and donor sites. Bold underlined is the newly created branch point sequence, in which the mutant A is in bold and italic. The *Bpm*I site is doubly underlined. **B.** DNA sequence analysis of the area spanning the mutation in control DNA (left) and DNA from MN121 (right). The altered nucleotide is indicated with an asterisk

The splice acceptor site precedes the genomic copy of the 106 bp insertion. At the 3' end, the 106 bp genomic sequence is flanked by a putative splice donor site. We therefore conclude that the point mutation creates a new splice branch point sequence, which in combination with already existing consensus splice acceptor and donor sites, results in the formation of an extra exon 5a in the NF2 gene.

Effect of the mutation on the NF2 protein

To analyse the NF2 protein encoded by cDNA carrying the insertion, we generated an expression plasmid (pR121) containing a cDNA derived from the mutant NF2 transcript from meningioma

MN121. This mutant cDNA encodes a putative protein of 23.5 kD consisting of 172 amino acids of the normal NF2 protein and 31 amino acids derived from exon 5a. A nonsense codon is present at position 412 in exon 5a (see Fig. 1). When clone pR121 was used for *in-vitro* transcription-translation a truncated protein with the expected molecular weight of about 25 kD was found (Fig. 2A). In addition, a western blot of a lysate of COS cells transiently transfected with the pR121 construct, also showed a protein of about 25 kD (results not shown).

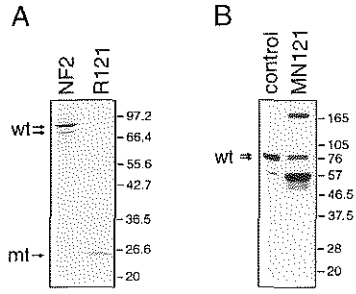

Figure 2.
Analysis of the NF2 protein.
A. *In-vitro*-transcription-translation of wild type and mutant (R121) cDNA. The positions of the wild type (wt) NF2 protein, which migrates at approximately 70 kD, and the mutant (mt) 23 kD protein are indicated.
B. Immunoprecipitation of the NF2 protein from a lysate of meningioma MN121. The wild type NF2 protein produced by transfected COS cells is used as a control for the position of the full length NF2 protein. The bands below the full length NF2 band in MN121 are from the precipitating antibody. The identity of the slower migrating band, which is sometimes observed in these analyses (Den Bakker, unpublished results), is not known at present. The 23 kD mutant NF2 protein cannot be seen. The length of the protein markers is shown at the right side of the panels.

To see whether the truncated protein could be detected in MN121, we used a frozen sample of this tumour for immunoprecipitation. Tumour cells were lysed and used for immunoprecipitation with antibody A19. Detection of the NF2 proteins was with the same antibody. The results of this analysis are shown in figure 2B. As a control, figure 2B shows the position of the wild type NF2 protein, transiently expressed in monkey COS cells. In the lane with the immunoprecipitated MN121 sample a band migrating at the position of full length NF2 is visible, in addition to the bands from the heavy and light chains of the precipitating antibody and a slower migrating band of unknown identity. However, no truncated mutant protein of the expected size can be detected in tumour MN121.

Presence of the G→A mutation in other members of the NF2 family

The G→A transition that was detected in PBL DNA from case 2, results in the loss of a *Bfa*I site and creates a new *Mae*II site. Both these restriction enzymes were used to detect the mutation in other

members of the family and the results are shown in figure 4. Two fragments resulting from a *Mae*II digestion were found in the DNA extracted from peripheral blood lymphocytes of cases 2 and 3, together with the undigested band from the wild type allele. Similarly, two *Mae*II fragments were also found in DNA extracted from meningiomas from case 1 (MN60) and 2 (MN121).

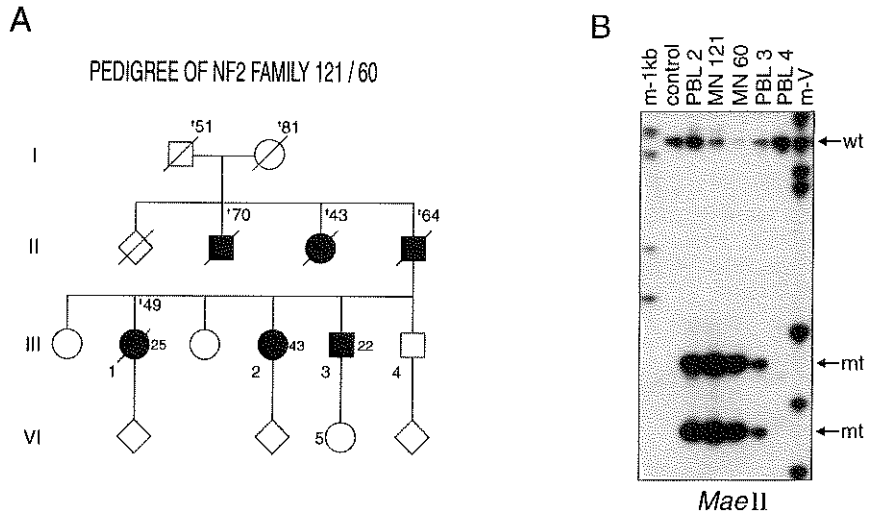

Figure 3.
Diagnostic analysis of the mutation in the family members. A. Pedigree of the family. Individuals with filled symbols are affected. Further information: number in the upper right quadrant depicts age of decease; figure in the lower left quadrant indicates case number; figure on the right is age of onset of clinical symptoms. B. *Mae*II digestion of amplified DNA from MN60, MN121 and PBLs from cases 2-4. DNA from case 5 was analysed separately and is not included in this figure. She did not carry the mutation.

In the DNA of case 4, no new *Mae*II sites or loss of the *Bfa*I site could be detected (Fig. 3). This individual was born in 1938 and has so far not complained of any symptoms reminiscent of NF2, although he has not been tested in a clinical setting. In addition, the G→A transition could not be found in control DNAs extracted from peripheral blood lymphocytes of a series of 50 independent individuals. These results suggest that the G→A transition is indeed the causative mutation in this family. Case 5 was seen for genetic counselling because she wanted to be informed about her carrier status. She was tested according to a presymptomatic counselling protocol. Her DNA was screened using the RFLP analysis. She had a wild type genotype (results not shown).

Discussion

In this paper we describe a mutation which creates a splice branch point sequence in an intron of the NF2 gene. Due to the presence of consensus splice acceptor and donor sites downstream of this

mutation, intron sequences are spliced into the NF2 mRNA. Mutations affecting a branch point sequence are not frequently encountered and only two cases have been described so far. Putnam *et al.* [12] describe a case of congenital contractural arachnodactyly (CCA) in which an A→G mutation destroyed the invariant A residue of the branch point preceding exon 29 of the *FBN2* gene, resulting in a reduced usage of the exon. In a family with X-linked hydrocephalus (HSAS) a A→C change destroys the branch point of exon R [14]. This results in omission of the exon from the mRNA or usage of an alternative splice acceptor site 69 nucleotides upstream of the exon. In addition to this normal splicing of the mutant allele was also observed. In the affected members of the NF2 family described here, the reverse is found: a genuine branch point sequence, -TTCTAAC- is created and 106 bp of intron sequence are spliced in the mRNA between exons 5 and 6. To our knowledge this is the first time such a mutation has been found in a human hereditary disorder.

Mutations that lead to a putative truncated protein are predominantly found in families with the severe form of NF2. In contrast, families with the mild form more often have a missense mutation [20], and references therein, [11, 16]. A special class is formed by the patients in whom a splice site mutation is detected, these may be either of the mild or severe phenotype. A possible explanation of the latter finding is that the mutation may not always exclusively lead to a mutant transcript but that some degree of normal splicing may still be possible. However, the result on mRNA and protein of the splice site mutations is usually not investigated. MN121 contains both mutant and considerable amounts of wild type transcript as judged from the relative intensities of bands obtained in RT-PCR experiments (results not shown). In the tumour, LOH of the wild type allele was observed [9] and densitometry of the RFLP data suggests that the tumour sample contained 80-90% tumour cells (results not shown; non-tumour cells are presumably derived from blood vessels). Thus, we presume that a considerable fraction of the wild type transcript originates from the tumour cells and was produced by normal splicing of the mutant transcript. The detection of full length NF2 protein in the tumour lysate also supports this hypothesis. Therefore, in this family, the occurrence of some degree of normal splicing may explain the relatively mild phenotype.

The nucleotide sequence of the mutant cDNA predicts that translation of this alternative mRNA stops within the insertion at a stop codon located after 31 amino acids within the 106nt fragment. These predictions were confirmed by Western blotting of COS cells transfected with a NF2 expression vector containing the MN121 mutation and *in-vitro* transcription-translation of this construct. With both analyses we demonstrated the formation of a smaller truncated NF2 protein. In whole cell lysates of tumour MN121 the 23 kD mutant NF2 protein could not be detected in contrast to full-length wild type protein. This suggests that the truncated 23 kD protein is unstable. It has been suggested by several investigators that a mutation leading to a truncated NF2 protein may display a dominant negative effect on the product of the wild type allele [11, 16, 20]. However, so far there is no proof for this hypothesis. The data presented in this paper show that we were unable to detect the truncated protein,

even when considerable amounts of mutant transcript were present. Therefore it seems that, at least in this case, a dominant negative effect of the mutation is unlikely.

Approximately half of all NF2 patients are severely affected [5]. However, 80% of the identified mutations are in patients with the severe phenotype. This implicates that there is a bias towards the detection of mutations that lead to the severe form of the disease [11, 16, 20] In the group of patients described by Parry, germ-line mutations were detected in only 36% of families with a mild phenotype. Therefore it is likely that many of the mutations in mildly affected families, are within introns and affect splicing of the transcript to some extent. In the family discussed in this paper, a mutation affecting splicing was revealed that cannot easily be discovered using SSCA on genomic DNA with exon flanking primers. This will also hold for other similar mutations, because it is practically impossible to screen each intron of the entire gene. The most optimal approach to detect mutations like these is by SSCA on cDNA. A problem with this approach is that RNA isolated from lymphoblasts cannot be used for such assays, probably because of the low or absent expression of the gene in these cells [11].The NF2 gene is, however, strongly expressed in the skin [4]. Thus, an alternative and perhaps better approach for mutation detection in NF2 families, would be to use RNA isolated from skin biopsies.

Materials and methods

Patients

The patients mentioned here are members of a family that was described by Delleman *et al.* [3] as having the central form of Von Recklinghausen disease. Figure 3A depicts the updated pedigree. This family is now diagnosed as having NF2, using the current NIH criteria for diagnosis of NF1 and NF2 [10]. The first generation consisted of three affected and four unaffected sibs. The two affected brothers from this generation developed multiple meningiomas and died at ages 70 and 64. The affected sister died at age 43. Upon autopsy, multiple meningiomas and bilateral acoustic neurinomas (vestibular Schwannomas) were discovered. The second generation members are descendants of one of the brothers and comprise 3 affected and (as far as is known) 3 unaffected sibs. Update of the clinical record of this generation:

Case 1. A female patient 1 (FIII, 4 in Delleman *et al.*), born in 1942. From 1967 multiple intracranial and intraspinal meningiomas were discovered. Several of these were removed by surgery during the following years. The patient died in 1991.

Case 2. Sister of case 1 (FIII, 2), was born in 1936. In 1972 a bilateral focal cord paresis and a right-sided radiculopathy of L4 and L5 were diagnosed, for which, at that time, no explanation could be found. In 1979 she presented with hearing loss. A CT-scan showed a right cerebellopontine angle tumour besides multiple supra tentorial high density lesions suggestive of meningiomas. The cerebellopontine angle tumour was partially removed. Histology: fibrous meningioma. When the

tumour recurred in 1992, it was totally removed. The other tumours have not shown progressive growth and the clinical condition of the patient has remained stable.

Case 3. Brother of case 1 (FIII,5), was born in 1944. At age 22 a meningioma was removed from the optic nerve sheath. Presently, he has a harsh voice due to unilateral cord paralysis. Because of an intrathoracal tumour detected in 1993, he is slightly dyspneic. Since 1994 his visual acuity is deteriorating due to cataract. In 1997 he is still able to work.

Case 4: Brother of case 1, born in 1938, is without symptoms of NF2.

Case 5. Daughter of case 3. Was healthy at age 19 although she complained of dizziness.

NF2 mutation detection

For the detection of NF2 mutations in genomic DNA, DNA was extracted using routine procedures [17] from MN60 (case 1) and MN121 (case 2) and peripheral blood lymphocytes of the family members 2 - 5 and control individuals. Primers flanking the exons of the NF2 gene were kindly supplied by Dr JF Gusella [7]. These primers were used to amplify exons 5 and 6 of the NF2 gene. PCR conditions: non-radioactive (50µl containing 100ng genomic DNA; 200 µM dNTP; 7.5 pMol of each primer; 0.1U SuperTaq DNA polymerase (HT Biotechnology) and 1x PCR buffer (PCR optimizer kit, Invitrogen Leek, The Netherlands) or radioactive (15µl : identical with 20 µM dATP and 0.15 µl of ^{32}P-α-dATP(3000 Ci/mmol, Amersham, Little Chalfont, UK)). The non-radioactive PCR products were analysed on 4% agarose gels and radioactive fragments were used for SSCA on 0.5x MDE gels (JT Baker) containing 0%, 5% or 10% glycerol and electrophoresed at 8 Watt at 4 C or RT for 14-19 hours. SSCA gels were transferred to blotting paper, dried and exposed to Fuji Medical X-ray film. A combination of exon 5 and exon 6 and exon flanking primers [7] was used to amplify the region containing the intron sequence. The PCR fragment was subcloned into pCM^{tm}II (TA-cloning kit, Invitrogen) and the 5' part of this fragment was sequenced using a T7 DNA sequencing kit (Pharmacia, Sweden). For the detection of the G→A transition in the family members and control DNAs the primers 5aII (AATCTCCCAGTAAACAGTGT, nt 192-211 in Fig. 1) and 121B (CACTGCAAACAGCTCAATTCC, nt 399-379 in Fig. 1) were used to amplify a 206 bp DNA fragment. After digestion of the radioactive PCR fragment with the restriction enzymes *Bfa*I or a *Mae*II the samples were analysed on 12.5% polyacrylamide gels and exposed to Kodak XAR-5 films.

Construction of a mutant cDNA expression construct

The forward primers A1/5' (CATGGCCGGGCCATCGCTTCC, nt 219-240, relative to the start codon) in combination with the reverse primers A3/3' (CATAAATAGATCATGGTTCCCGAT, nt 1142-1119) [8], flanking the insertion in the NF2 transcript of MN121 were used to amplify the cDNA of MN121. The 1.0 kb PCR product of the primer combination was cloned into plasmid vector pCM^{TM}II, using the TA-cloning kit (Invitrogen). A 0.475 kb *Bgl*II fragment was isolated and

subcloned into a full length NF2 cDNA construct replacing the original *Bgl*II fragment. Clones with a properly oriented *Bgl*II fragment were selected by restriction analysis and recloned into a pCDNA3 mammalian expression vector. The insert of the thus obtained pR121 clone was sequenced using a sequence kit (Pharmacia, Sweden).

Detection of mutant and wild type NF2 protein

In-vitro transcription-translation was performed using the TnT Coupled Reticulocyte Lysate System (Promega, Madison, WI, USA), with the T7 primer, 1 µg of plasmid DNA and 2 µl of ^{35}S labeled methionine (10 mCi/ml, Amersham, Little Chalfont, UK), under conditions described by the manufacturer. Samples were analysed using SDS-PAGE and autoradiography. The expression constructs were transfected to COS cells by electroporation (250V, 500µF). After 48 hours the transfected cells were washed with PBS, harvested by scraping with a rubber policeman, lysed in reducing sample buffer and used for Western blotting. Immunoprecipitations were performed essentially as described by Fornerod *et al.* [6]. Briefly, tumour tissue of MN121 was homogenized by mincing in immunoprecipitation buffer (50 mM Tris-HCl, pH8.0, 150 mM NaCl, 1% Nonidet P40, 5mM EGTA, 5mM EDTA, 15 mM $MgCl_2$, 1 mM Dithiotreitol, 0.1 mM $NaVO_4$, 0.1 mM NaF) supplemented with protease inhibitors. The crude lysate was centrifuged and filtered trough a 45µm filter. After preclearing, specific antibody (A19; sc331, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and 40 µl of a 50% slurry of sepharose-protein A were added. After O/N incubation at 4^0C, the beads were spun down and washed four times in immunoprecipitation buffer. The beads were resuspended in reducing sample buffer and the sample was subjected to electrophoresis and blotting as described previously [4]. After blocking, the NF2 protein was detected by incubating the blots with antibody A19 , followed by a secondary biotinylated goat-anti-rabbit and peroxidase conjugated streptavidin. Bands were visualised by enhanced chemiluminescence and exposure to film.

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CHAPTER VII

Truncated NF2 proteins are not detected in meningiomas and schwannomas.

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Abstract

Neurofibromatosis type 2 is caused by mutations in the NF2 tumour suppressor gene. The NF2 gene encodes a 595 aminoacid protein, presumably functioning as a membrane organising element. Theoretically, the majority of mutations found in the NF2 gene should lead to a truncated protein product. Using immunoprecipitation with an antibody raised to N-terminal sequences of the NF2 protein, we sought to demonstrate the presence of truncated NF2 proteins in tumours. From 17 of 19 tumours (14 meningiomas and 5 schwannomas), 12 of were shown to harbour truncating NF2 mutations, wild-type NF2 protein was immunoprecipitated. From 2 tumours no protein was precipitated. Truncated NF2 proteins were not observed. We conclude that mutant NF2 proteins are unstable and undergo accelerated degradation.

Introduction

Neurofibromatosis type 2 (NF2) is a rare inherited cancer syndrome characterized by the occurrence of bilateral vestibular schwannomas. These tumours, also known as neurilemmemomas, are benign neoplasms originating from the Schwann cells in the nerve sheath. Other NF2 disease manifestations include meningiomas, which are tumours arising from the membranes covering the brain and non-neoplastic lenticular opacities developing at an early age. The disease is caused by mutations in the NF2 tumour suppressor gene, located on chromosome 22. The NF2 gene was cloned in 1993 and encodes a 595 aminoacid protein that is believed to interact with integral membrane proteins and the cytoskeleton [12, 18]. This assumption is based on the homology of the NF2 protein to ezrin, radixin and moesin (collectively ERM proteins) which in turn form part of yet a larger superfamily of proteins related to the erythrocyte band 4.1 protein.

Two subforms of NF2 can be discerned, a clinically severe form, called the Wishart type, with an early onset of symptoms, multiple meningiomas and accelerated disease course. A second milder form is known as the Gardner subtype and presents with a delayed disease onset and fewer tumours [4, 11]. Mutation analysis has shown that a clear NF2 genotype-phenotype correlation can be deduced. Nonsense mutations resulting in a truncated protein are usually associated with a severe phenotype and miss-sense mutations resulting in aminoacid substitutions are predominantly associated with the mild phenotype [13, 20]. Two possibilities exist for tumorigenesis by NF2 gene mutations. Either the mutant protein product acts in a dominant negative way over the wild-type protein or alternatively true loss of function occurs in which case loss of the remaining wild-type allele is expected in tumours. In either case it may be expected that mutant (truncated) NF2 protein is present in tumours. Successful immunological detection of truncated protein may simplify the search for NF2 mutations and aid diagnosis.

To detect truncated NF2 proteins we performed immunoprecipitation with a N-terminal anti-NF2 antibody on fresh tumour samples. Nineteen tumours, 5 schwannomas and 14 meningiomas were analysed (Table 1). Eight of the 14 meningiomas and 4 of the 5 schwannomas have previously been

investigated for NF2 mutations and chromosome 22 copy number [9]. Loss of heterozygosity (LOH) for chromosome 22 was determined for the remaining 7 tumours using polymorphic microsatellite markers. In addition, these 7 tumours not previously investigated were immunohistochemically stained for the endothelial cell marker CD34 and smooth muscle actin (HHF35) to determine residual normal tissue in the form of endothelium and smooth muscle cells of the tunica media.

Materials and Methods

Fresh tissue samples from operative specimens were snap frozen and stored at -80°C until use. For LOH analysis formalin-fixed, paraffin-embedded archival tissue was used. Blocks were selected with a minimal amount of residual normal tissue on the basis of H&E-stained sections.

To evaluate the normal tissues content, sections were stained for the endothelial cell marker CD34 (Qbend 10, Biogenex, San Ramon, CA, USA) and smooth muscle actin (HHF-35, Biogenex). A standard streptavidine-biotin-peroxidase method was used (UltraVision detection system, Lab vision, Fremont, CA, USA) with diaminobenzidine as a chromogen. Normal tissue content was scored visually.

DNA extraction from paraffin blocks was performed by proteinase K (2 mg/ml) digestion of de-paraffinized 10 µm sections, followed by purification using the Qiagen DNeasy kit (Qiagen, Hilden, Germany). Microsatellite primer sequences were obtained form the Cooperative Human Linkage Center (http://www.chlc.org/). Six primer pairs were used: D22S444, D22S445, D22S683, D22S684, D22S685 and D22S686. Amplification and quantification were performed as described previously [17].

Frozen tissue samples were thawed and minced in immunoprecipitation buffer. After centrifugation, immunoprecipitation and western blotting were performed as previously described [2]. An affinity purified polyclonal NF2 antibody was used for immunoprecipitation (A19, sc331, Santa Cruz Biotechnology, Santa Cruz, CA, USA). This antibody was raised to N-terminal NF2 aminoacids and therefore should precipitate all truncated proteins. The ability to immunoprecipitate truncated NF2 proteins was confirmed using mutant NF2 proteins produced *in-vitro*. For these experiments cDNA constructs NF2-del-341-C and NF2-del-N204;471C were used together with an affinity purified polyclonal antibody 1399A raised to synthetic peptide SP277 ([2], den Bakker submitted; Fig. 1a,b). *In-vitro-transcription-translation* (ITT) reactions were performed as described with ^{35}S-methionine as a radiolabel. For immunological detection on western blots monoclonal anti-NF2 antibodies FE9 and KF10 were used [2, 3]. FE9 was raised to a synthetic peptide spanning aminoacids 1-20 of the NF2 protein and therefore should detect all truncated NF2 proteins resolvable by sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The KF10 monoclonal antibody was raised to a C-terminal synthetic peptide and thus only detects full-length NF2 protein. Samples were run on 7.5 and 12 or 15% slab gels to enhance detection of truncated low molecular weight proteins. In addition, multiple exposures of probed western blots were analysed to detect faint bands. Mock

immunoprecipitations were performed on tumour tissues samples by omitting the precipitating antibody.

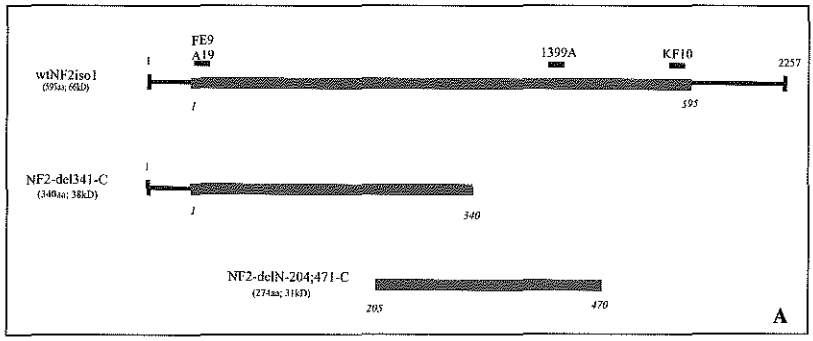
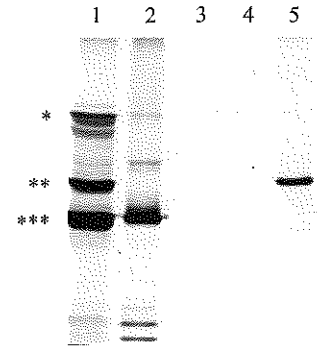


Figure 1

A. Schematic representation of wt-NF2 protein and cDNA constructs with positions of antibody epitopes used for control immunoprecipitation experiments. Construct NF2-del-341-C lacks the C-terminal 255 aminoacids of the NF2 protein. Construct NF2-del N-204;471-C is a NF2 construct spanning aminoacids 205-470. Calculated molecular weights are given with constructs.

B. Autoradiogram of a protein blot of immunoprecipitated protein produced *in-vitro*.
Lane 1: non-immunoprecipitated ITT products, wtNF2 (*), NF2-del-341-C (**) and NF2-del N-204;471-C (***)
Lane 2: construct NF2-del N-204;471-C immunoprecipitated by 1399A
Lane 3: construct NF2-del N-204;471-C immunoprecipitated by A19
Lane 4: construct NF2-del-341-C immunoprecipitated by 1399A
Lane 5: construct NF2-del-341-C immunoprecipitated by A19



Results

Immunohistochemical staining for the endothelial cell marker CD34 and the anti-smooth muscle antibody HHF35 demonstrated a rich vascular network in tumour samples (not shown). However, estimated amounts of normal cells (predominantly endothelium and smooth muscle cells) was not more than 10% in any sample. Five of the 6 meningiomas and the schwannoma demonstrated allelic imbalance for chromosome 22 by PCR with polymorphic microsatellite markers (not shown). From 17 of the 19 tumours full-length NF2 protein was immunoprecipitated, detectable by both FE9 and KF10. From 2 tumours, both schwannomas, no detectable NF2 protein was precipitated (Table I; Fig 2). However, only minor tissue was available from the two tumours from which no NF2 protein was precipitated. The immunoprecipitated protein presented as a doublet in virtually all cases. Truncated NF2 proteins were not identified. On very long exposures additional bands were observed but these

additional bands did not conform to calculated molecular weights resulting from specific mutations in selected tumours and were either detected with FE9 or KF10 but never by both antibodies. In addition, faster migrating bands were also observed in mock immunoprecipitations in very long exposures. Two additional bands migrating at approximately 130 and 200kD were consistently observed in several samples. We have previously observed these bands in immunoprecipitates from normal tissue.

Table 1. Details of tumours and outcome of immunoprecipitations

Meningiomas	chrom. #22	NF2	% tumour	IP
99*	-22	del 448-467; FS	>90	WT
108*	-22	53 CAA-CT; FS	>90	WT
111*	-22	del 76; FS	>90	WT
121*#	-22	ins 106bp 517; FS	>90	WT
127*	-22	1009 CAG-TAG stop	>90	WT
128*	diploid	del 36-37; FS del 82bp + 18bp ins 496-577	>90	WT
140*	-22	del 1223-1236; FS	>90	WT
145*	-22	ins 119bp 364; FS	>90	WT
328	LOH		90	WT
329	LOH		>90	WT
331	no loss		90	WT
334	LOH		>90	WT
337	LOH		>90	WT
399	LOH		>90	WT
Schwannomas				
340	LOH		>90	WT
106*	-22	del 38-58	>90	-
110*	diploid	del 440-492	>90	WT
117*	diploid	del 1023-1044	>90	-
175			>90	WT

* Lekanne-Deprez (1994). [9]
\# meningioma in a NF2 patient

Discussion

Mutation analysis in NF2 families and in sporadic schwannomas and meningiomas has confirmed the role of the NF2 gene as a classical tumour suppressor gene. Inactivating mutations of both alleles of tumour suppressor genes are required for tumorigenesis. In the familial setting a germline mutation inactivates the first allele. The second allele is often inactivated by loss of (parts of) the chromosome on which the gene is located in target cells. Indeed, in tumours with NF2 mutations the second chromosome 22 is usually lost. From these observations it may be expected that loss of functional NF2 protein results in disease. However, a dominant negative effect before loss of the second allele cannot be ruled out. Theoretically, many of the mutations identified in the NF2 gene should result in the production of a truncated protein [20]. Truncating NF2 mutations are associated with a severe phenotype, whilst miss-sense mutations lead to a mild phenotype [13, 20]. We sought to demonstrate the

existence of truncated NF2 proteins from tumours with known NF2 mutations. In addition, meningiomas evaluated for loss of chromosome 22 were included. It has been shown that most if not all meningiomas with chromosome 22 loss, also harbour NF2 mutations [14, 19].

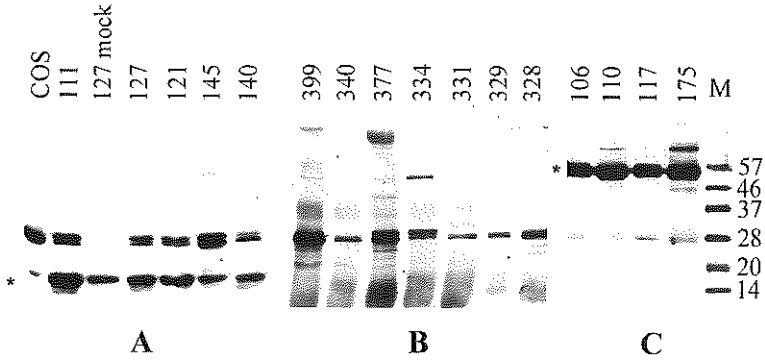


Figure 2
Western blots of protein immunoprecipitated from meningiomas and schwannomas by antibody A19. Tumour numbers are indicated above the lanes (see Table 1), not shown are MN99, 108 and 128.

A. Western blot of 7.5% slab gel, NF2 protein detection by monoclonal antibody FE9. These samples were prepared from tumours with characterised NF2 mutations. COS= direct (non-immunoprecipitated) lysate of NF2 transfected COS cells, serving as positive control. The negative control is 127mock, the precipitating antibody was omitted in this sample. The bands indicated by * are immunoglobulins cross-reacting with the secondary antibody.

B. Western blot of 12% slab gel, loaded with precipitated protein from tumours not evaluated for chromosome 22 loss. Detection by FE9.

C. Western blot of 15% slab gel loaded with precipitated NF2 protein from 4 schwannomas with characterised NF2 mutations. No truncated NF2 protein is observed. Lane M is a biotinylated molecular weight marker.

Truncated NF2 proteins were not detected in any of the tumour samples investigated. From 2 schwannoma samples no protein at all was immunoprecipitated. However, wild-type NF2 protein was found in all but these 2 schwannomas. Although residual normal tissues was less than 10% in all cases it must be assumed that the precipitated wild-type NF2 protein originates from intimal endothelial cells and smooth muscle cells of the tunica media of blood vessels. We have previously shown that NF2 gene expression is demonstrable in both of these cells types [2]. The failure to immunoprecipitate NF2 protein from 2 tumours is likely to be the result of the minimal amount of fresh tissue available from these tumours for investigation.

The failure to detect truncated NF2 protein in tumours may result from defective transcription and/or translation or from decreased stability of the mutant protein. A failure of translation as a result of increased RNA degradation is highly unlikely as RT-PCR analysis is usually performed successfully. In addition, cDNA constructs based on mutations found in patients do yield truncated protein *in-vitro*

(den Bakker *et al.*, submitted). It therefore appears that truncated NF2 proteins are unstable and are rapidly degraded. There is considerable evidence supporting this mechanism. It has recently been shown by Gutmann *et al.* that truncated NF2 proteins are not produced *in-vivo*. Efforts to generate rat-schwannoma cell lines producing truncated NF2 proteins were unsuccessful despite incorporation of the transgene with detection of transcribed NF2 mRNA. In addition, it was shown that NF2 proteins with miss-sense mutations were produced in transgenic cell-lines but these too had a reduced half-life compared to wild-type protein [5]. NF2 protein has been shown to be sensitive to calpain dependent proteolysis and that this proteolytic pathway be excessively activated in meningiomas. In up to 30% of meningiomas loss of chromosome 22 is not observed [10]. It is conceivable that in these meningiomas aberrations of the proteolytic pathway may result in reduced NF2 protein.

The failure to detect truncated proteins in schwannomas and meningiomas is corroborated by data from other investigations [1, 6-8, 15, 16]. Quantitative western blot analyses have shown reduced NF2 protein levels or complete absence of NF2 protein in meningiomas and schwannomas. However, quantitative determination of NF2 protein by western blotting should take into account the vascular density of the tumour to compensate for NF2 expression in endothelial and smooth muscle cells. In the published reports this has not been the case. In none of the reports published to date are truncated NF2 proteins detected by western blotting. Immunohistochemical staining with antibodies to N- and C-terminal NF2 epitopes indirectly supports the presence of truncated NF2 proteins in a single report [7] but these findings are not completely consistent with the molecular data. Our own efforts to detect NF2 protein in normal arachnoidal cells have not met with success [2].

In conclusion, truncated NF2 proteins are not detected in meningiomas and schwannomas. Failure of detection probably results from accelerated degradation of mutant proteins, possibly mediated by calpain dependent proteolysis. These findings strongly indicate that the severe form of NF2 (Wishart type) results from complete loss of NF2 protein, rather than a dominant negative effect of mutant protein over wild-type. The mild form may be explained by some remaining activity of the mutant allele, for instance by residual activity of a mutant protein or alternatively by residual wild-type splicing of an allele with a splice-site mutation.

Acknowledgements

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CHAPTER VIII

Summary and discussion

Samenvatting en discussie

Neurofibromatosis type 2 is a hereditary disease caused by mutations in the NF2 gene located on chromosome 22. The disease is characterised by the occurrence of bilateral vestibular schwannomas. These are benign tumours developing from cells of the nerve sheath of the eighth cranial nerve. Vestibular schwannomas lead to deafness and balance disorders. Schwannomas may also develop in other sites in NF2 patients, in particular on the nerve sheaths of spinal nerve roots. Approximately half of the NF2 patients develop meningiomas. These are benign intracranial tumours originating from the membranes covering the brain. In addition to schwannomas and meningiomas, NF2 patients develop lens opacities at an early age, leading to impaired vision.

The NF2 gene was cloned in 1993. Mutation analysis of the NF2 gene in tumours and NF2 families confirmed its role as a tumour suppressor gene. In the majority of cases the identified mutations theoretically should lead to a truncated NF2 protein product. Two major splice variants of the NF2 gene are known, resulting in either a 595 aminoacid (isoform I) or a 590 aminoacid protein (isoform II). The NF2 protein, also known as *merlin* or *schwannomin*, is highly homologous to three proteins in turn belonging to a superfamily of proteins related to the erythrocyte cytoskeletal protein band 4.1. These three proteins, ezrin, radixin and moesin, link integral membrane proteins to the cytoskeleton. The similarity of the NF2 protein to these proteins, collectively known as ERM proteins, led to the assumption that it likewise would function as a membrane-cytoskeleton linking protein.

Although initial studies documented NF2 gene expression in many tissues by northern blotting and RT-PCR, a detailed analysis of its expression in specific cell types within the various human tissues was not yet performed.

The aim of the work described in this thesis was to gain insight into the mechanisms underlying the pathological phenomena observed in NF2. To this end we sought to investigate and characterise the association of the NF2 protein with the cytoskeleton. Furthermore, by providing a detailed inventory of cells and tissues in which the NF2 gene is expressed the postulated presence of NF2 protein in specific tissues is supported. Finally, immunological detection of mutated NF2 protein in tumours may speed-up and simplify diagnosis. Therefore, using immunological methods, we sought to detect mutated NF2 proteins from meningiomas and schwannomas.

We generated polyclonal antibodies to synthetic NF2 peptides (**Chapter II**). With these antibodies we investigated the subcellular localisation of the NF2 protein and putative cytoskeletal-NF2 interaction. In COS cells transfected with the NF2 cDNA, the spatial arrangement of NF2 protein as determined by immunofluorescent staining was compatible with a cytoskeletal interaction. Immunohistochemical staining experiments with the polyclonal antibodies revealed expression of the NF2 gene in muscle cells, in particular in smooth muscle cells. Immunohistochemical evidence for NF2 protein in Schwann cells was also found although staining was weaker than in smooth muscle cells.

Monoclonal antibodies were raised to the same synthetic NF2 peptides and an additional N-terminal synthetic peptide (**Chapter III**). Immunofluorescent staining of NF2 transfected COS cells with these antibodies, in conjunction with confocal scanning laser microscopy, demonstrated the localisation of NF2 protein at the dorsal cell membrane. This localisation is in accordance with an association with the cortical actin cytoskeleton. Furthermore, the transfected NF2 protein was resistant to extraction with non-ionic detergents, substantiating the postulated cytoskeletal interaction. Immunostaining with the monoclonal anti-NF2 antibodies confirmed the high level of NF2 expression in smooth muscle cells. However, staining of other muscle cells (skeletal and cardiac) was unsuccessful. The cytoskeletal association of the NF2 protein was further studied by immunofluorescent staining of cultured smooth muscle cells. In these cells co-localisation of NF2 protein with actin stress fibers was found. Staining of the human epidermis with the monoclonal anti-NF2 antibodies was also seen. Immuno-electron microscopy of the epidermis with a monoclonal antibody showed association of NF2 protein with keratohyalin granules and intermediate filaments. It is concluded that the NF2 protein shares some characteristics with other ERM proteins, in particular as determined by biochemical assays. But unlike ERM proteins, the NF2 protein also associates with intermediate filaments. Therefore, in comparison with ERM proteins the NF2 protein harbours unique functions.

The gathered data supports an interaction of NF2 protein with the actin cytoskeleton. It is possible that NF2 protein truncation results in impaired cytoskeletal interaction and in this way leads to disease. Therefore determination of NF2 protein domains governing cytoskeletal interaction would further our understanding of NF2 pathogenesis. To determine the domain of the NF2 protein governing cytoskeletal interaction NF2 cDNA constructs with specific mutations were made and transfected to COS cells (**Chapter IV**). The ability of the mutant NF2 protein to resist extraction by non-ionic detergents reflected cytoskeletal association. It was found that a cytoskeletal attachment domain was present in the N-terminus of the NF2 protein between aminoacids 29-131. A second weaker attachment domain was found between aminoacids 321-470. In addition, NF2 mutations found in patients did not result in a NF2 protein with impaired cytoskeletal attachment. In conclusion, The NF2 protein harbours at least 2 cytoskeleton attachment domains that may be common to other ERM proteins. However, impairment of cytoskeletal attachment of NF2 protein by itself does not appear sufficient to cause disease.

Early studies of the expression of the human NF2 gene in tissues were largely based on northern blotting and RT-PCR analysis. Since these techniques are performed on tissue homogenates they do not permit detailed expression studies at the cellular level. In **Chapter V** application of mRNA-ISH and immunohistochemistry with a panel of monoclonal antibodies is described, with generation of detailed NF2 expression data in human tissues. Expression of the NF2 gene was found in many human tissues, partly overlapping with the other members of the ERM family. The expression of the NF2

gene could be confirmed in Schwann cells by immunohistochemistry on frozen sections but not in paraffin sections. Detection of NF2 mRNA in Schwann cells, arachnoidal cells and smooth muscle cells was not successful. In the epidermis and other epithelia an overlapping mRNA-ISH and immunostaining signal was found.

NF2 mutations theoretically lead to a truncated protein in the majority of cases. Truncating mutations are particularly found in patients with a more severe form of NF2. In **Chapter VI** the detection and characterisation of an intronic NF2 mutation in a family with NF2 is described. The mutation results in the introduction of a cryptic exon and theoretically in the formation of a 172 aminoacid protein. Although detection of mutant protein produced *in-vitro* from a cDNA construct with the mutation was possible, no mutant protein was detected by immunoprecipitation from tumour tissue. The results indicate that this truncated protein is unstable and is rapidly degraded.

To determine the presence of truncated NF2 proteins in tumour tissue, meningiomas and schwannomas were subjected to immunoprecipitation. A subset of these tumours harboured previously defined NF2 mutations, theoretically leading to truncated proteins with expected molecular weights. From 17 of 19 tumours wild-type NF2 protein was precipitated. From 2 schwannomas no product was precipitated. No smaller products were seen. The results indicate that truncated NF2 proteins are unstable. The detected wild-type protein most likely originated from blood vessel walls. (**Chapter VII**).

In conclusion, in this thesis evidence for the association of the NF2 protein with the cytoskeleton is provided. Moreover, the NF2 protein interacts with the actin cytoskeleton and also with intermediate filaments. The conclusions are supported by data from other investigators. However, several points remain to be clarified. Exactly which specialized membrane structures is the NF2 protein associated with and does it perform an organising role herein? Are there (additional) functional domains present in the NF2 protein? To what extent are the functions of ezrin and NF2 protein interchangeable? How does the absence of NF2 protein lead to tumorigenesis in Schwann cells? If indeed the NF2 protein is an essential part of specialised membrane structures or even induces them, possible effects pertaining to growth control may be regulation, relocation or concentration of integral membrane proteins, including receptors or regulatory sub-units thereof. However, comparison with other ERM proteins fails to reveal a common theme. No neoplastic disease is clearly associated with ERM proteins as yet. Therefore tumorigenesis induced by the absence of NF2 protein is likely to be the result of loss of interaction with a specific NF2 interacting protein. Conversely, similar to mechanisms proposed for ezrin, indirect effects on transcription through cytoskeleton associated signalling proteins (Rho/Rac/Cdc42?) may occur. It is conceivable that these signalling cascades may be secondary to morphological aberrations induced by loss of NF2 protein involved.

Future investigations should focus on the interaction of the NF2 protein with other proteins. This may be achieved by co-immunoprecipitation or other biochemical techniques. However, it must be appreciated that the NF2 protein resists solubilisation by non-ionic detergents. Therefore techniques relying on solubilisation may fail to identify those proteins that physiologically interact with the NF2 protein. The downstream effects of loss of NF2 may be more efficiently investigated by focusing on the NF2 interacting proteins already identified by other means.

A second conclusion from the work presented here is that the NF2 gene is expressed in a wide variety of human tissues but that gene transcription and the presence of NF2 protein do not always correlate. Of note is the finding that in continually proliferating tissue such as epithelia, both protein and mRNA is detected, whilst in non-proliferating tissues the protein is detected but detection of transcription fails. In general, expression of the NF2 gene in human tissues described here compares with the published expression data in rodents.

Finally, it is concluded that it is not possible to detect truncated NF2 proteins from tumours known to harbour NF2 mutations, probably due to accelerated degradation of mutated NF2 protein. This conclusion is in accordance with published experimental data documenting the instability of mutated NF2 proteins.

Samenvatting en Discussie

Neurofibromatose type 2 is een erfelijke aandoening die veroorzaakt wordt door mutaties in het op chromosoom 22 gelegen NF2 gen. De ziekte wordt gekenmerkt door bilaterale vestibulaire schwannomen. Dit zijn goedaardige gezwellen die ontstaan vanuit cellen van de zenuwschede van de achtste hersenzenuw. Vestibulaire schwannomen veroorzaken doofheid en evenwichtsstoornissen. Ook op andere plaatsen in het lichaam kunnen schwannomen ontstaan bij NF2 patiënten, met name in de zenuwscheden van de spinale zenuwwortels. Bij ongeveer de helft van alle NF2 patiënten ontstaan ook meningiomen. Dit zijn goedaardige tumoren die ontstaan in de hersenvliezen. Behalve deze tumoren krijgen NF2 patiënten ook op relatief jonge leeftijd vertroebelingen van de ooglens die tot een verminderd gezichtsvermogen leiden.

Het NF2 gen werd in 1993 geïsoleerd. Mutatie analyse in tumoren en in NF2 families bevestigde de rol van het NF2 gen als tumour-suppressor-gen. In de meerderheid van de gevallen zouden de gevonden mutaties theoretisch tot een getrunceerd eiwitprodukt leiden. De twee meest voorkomende splice-varianten van het NF2 gen genereren eiwitten van 595 aminozuren (isoform I) en 590 aminozuren (isoform II). Het NF2 eiwit, ook wel *merlin* of *schwannomin* genoemd, toont sterke homologie met drie eiwitten die op hun beurt behoren tot een superfamilie van eiwitten gerelateerd aan het erytrocyten-cytoskelet eiwit band 4.1. Deze drie eiwitten, ezrin, radixin en moesin verbinden celmembraaneiwitten met het cytoskelet. De gelijkenis van het NF2 eiwit met deze eiwitten, tezamen ERM-eiwitten genoemd, leidde tot de veronderstelling dat het NF2 eiwit ook functioneert als brug tussen celmembraan en cytoskelet.

Aanvankelijk werd NF2 gen expressie in veel verschillende humane weefsels beschreven op grond van northern-blotting en RT-PCR experimenten. Een gedetailleerde analyse van NF2 expressie in specifieke humaan weefsels was nog niet verricht.

Doel van het werk dat wordt beschreven in dit proefschrift was inzicht verkrijgen in de mechanismen die de pathologische verschijnselen veroorzaken in NF2. Om dit te onderzoeken hebben wij getracht de samenhang van het NF2 eiwit met het cytoskelet nader te karakteriseren. Verder geven wij een gedetailleerd overzicht van humane cellen en weefsels waarin het NF2 gen tot expressie komt. Tenslotte zou immunologische detectie van gemuteerde NF2 eiwitten de diagnostiek van NF2 kunnen vereenvoudigen en versnellen. Daartoe hebben wij getracht om met immunologische technieken gemuteerde NF2 eiwitten in meningiomen en schwannomen aan te tonen.

Wij hebben polyclonale eiwitten opgewekt tegen synthetische NF2 peptiden (**Hoofdstuk II**). Met deze antilichamen onderzochten wij de intracellulaire lokalisatie van het NF2 eiwit en de mogelijke interactie van het NF2 eiwit met het cytoskelet. In COS-cellen getransfecteerd met het NF2 cDNA werd met behulp van immunofluoresentie gevonden dat de ruimtelijke verdeling van het NF2 eiwit verenigbaar was met een associatie met het cytoskelet. Immunohistochemische kleuringen met de polyclonale eiwitten toonde expressie van het NF2 gen in spiercellen, met name in glad spierweefsel.

Er werd tevens immunohistochemisch bewijs gevonden voor de aanwezigheid van het NF2 eiwit in Schwann-cellen met immunohistochemie, hoewel de aankleuring zwakker was dan in glad spierweefsel.

Er werden monoklonale antilichamen opgewekt tegen dezelfde synthetische NF2 peptiden en tegen een nieuw N-terminaal gelegen synthetisch peptide (**Hoofdstuk III**). Confocale laser-scanning-microscopische analyse van NF2 getransfecteerde, en met immunofluorescentie gekleurde COS-cellen toonde de aanwezigheid van het NF2 eiwit aan de dorsale celmembraan. Deze lokalisatie is in overeenstemming met een associatie met het corticale actine-cytoskelet. Het getransfecteerde NF2 eiwit bleek bovendien resistent te zijn voor extractie met niet-ionogene detergentia. Deze bevinding bevestigde de veronderstelde interactie met het cytoskelet. Immunohistochemische kleuringen met de monoklonale antilichamen bevestigden de sterke NF2 expressie in gladde spiercellen. Echter, immunohistochemisch aankleuren van andere typen spiercellen (skeletspier en hartspier) bleek niet mogelijk. De associatie van het NF2 eiwit met het cytoskelet werd nader onderzocht door immunofluorescentie onderzoek van gekweekte gladde spiercellen. In deze cellen bleek het NF2 eiwit te co-lokaliseren met actine "stress-fibers". Bovendien werd aankleuring van de humane epidermis gezien met monoklonale anti-NF2 antilichamen. In de epidermis werd met behulp van immuno-elektronenmicroscopie associatie van het NF2 eiwit met keratohyaliene korrels en intermediare filamenten gevonden. Geconcludeerd wordt dat het NF2 eiwit gemeenschappelijke biochemische eigenschappen toont met andere ERM-eiwitten. Anders dan ERM-eiwitten kan het NF2 eiwit ook associeren met intermediare filamenten. Derhalve bezit het NF2 eiwit in vergelijking met andere ERM-eiwitten specifieke eigenschappen.

De verzamelde gegevens ondersteunen een interactie van het NF2 eiwit met het cytoskelet. Het is mogelijk dat verkorting van het NF2 eiwit deze interactie verstoort en op deze wijze tot ziekte leidt. Identificatie van domeinen van het NF2 eiwit die van belang zijn voor de interactie met het cytoskelet zou derhalve het inzicht in de pathogenese van NF2 kunnen vergroten. Ten einde domeinen in het NF2 eiwit te identificeren die de interactie met het cytoskelet bewerkstelligen, werden NF2 cDNA constructen met specifieke mutaties vervaardigd en in COS-cellen getransfecteerd (**Hoofdstuk IV**). Het vermogen om extractie door niet-ionogene detergentia te weerstaan geeft een indruk van cytoskelet interactie. Er werd in het N-terminale deel van het NF2 eiwit een cytoskelet bindend domein gevonden tussen aminozuren 29-131. Een tweede zwakker cytoskelet bindend domein werd gevonden tussen aminozuren 321-470. Bovendien bleken specifieke NF2 mutaties, die werden gevonden in patiënten, niet tot NF2 eiwitten te leiden met gestoorde cytoskelet binding. In conclusie blijkt het NF2 eiwit tenminste twee cytoskelet-bindende domeinen te bezitten die mogelijk ook aanwezig zijn in andere ERM-eiwitten. Bovendien lijkt het verloren gaan van cytoskelet interactie van het NF2 eiwit op zich niet te leiden tot ziekte.

Onderzoeken naar de expressie van het humane NF2 gen waren aanvankelijk gebaseerd op northern blotting en RT-PCR analyse. Omdat deze technieken gebruik maken van gehomogeniseerd weefsel laten zij geen gedetailleerde analyse van de expressie van het gen op celniveau toe. In **Hoofdstuk V** wordt immunohistochemie met een panel specifieke monoklonale antilichamen en mRNA-ISH beschreven waarbij gedetailleerde data over NF2 expressie in humane weefsels worden verkregen. NF2 gen-expressie werd gevonden in vele humane weefsels, deels overlappend met andere ERM-eiwitten. De expressie van het NF2 gen in Schwann-cellen kon worden bevestigd in vriescoupes maar niet in paraffine coupes. Detectie van NF2 mRNA in Schwann-cellen, arachnoidale cellen en gladde spiercellen bleef zonder succes. In de epidermis en andere epithelia werd een overlappend in-situ-hybridisatie en immunohistochemisch patroon gevonden.

In een meerderheid van de gevallen resulteren NF2 mutaties theoretisch in een getrunceerd eiwit. Mutaties leidend tot een getrunceerd eiwit worden met name gevonden in patiënten met een ernstige vorm van NF2. In **Hoofdstuk VI** wordt een mutatie in een intron van het NF2 gen beschreven die gevonden werd in een familie met NF2. Het gevolg van deze mutatie is de incorporatie van een cryptisch exon waardoor theoretisch een mutant NF2 eiwit van 172 aminozuren gevormd zou worden. Hoewel detectie van het *in-vitro* geproduceerde mutante eiwit goed mogelijk bleek, kon met behulp van immunoprecipitatie geen mutant eiwit uit tumorweefsel aangetoond worden. De bevindingen wijzen erop dat het mutante eiwit niet stabiel is en versneld wordt afgebroken.

Om de te bepalen of getrunceerde NF2 eiwitten in tumoren aangetoond konden worden, werd immunoprecipitatie uitgevoerd op meningiomen en schwannomen. Een deel van deze tumoren had een NF2 mutatie, waarbij theoretisch gemuteerde NF2 eiwitten met bekende lengte gevormd zouden worden. Uit 17 van de 19 onderzochte tumoren werd uitsluitend wild-type NF2 eiwitten gevonden. Uit twee schwannomen werd geen produkt verkregen. Getrunceerde NF2 eiwitten werden niet gevonden. Deze resultaten wijzen erop dat getrunceerde NF2 eiwitten niet stabiel zijn. (**Hoofdstuk VII**)
Concluderend, in dit proefschrift wordt bewijs geleverd voor de interactie van het NF2 eiwit met het cytoskelet. Het NF2 eiwit associeert met het actine cytoskelet en met intermediare filamenten. Deze conclusies worden gesteund door bevindingen van andere onderzoekers. Echter, een aantal punten behoeven verder onderzoek. Met welke gespecialiseerde celmembraan strukturen is het NF2 eiwit geassocieerd en vervult het hierin een organiserende rol? Zijn er nog andere functionele domeinen aanwezig in het NF2 eiwit? Tot op welke hoogte zijn de functies van het NF2 eiwit en ezrin uitwisselbaar? Hoe leidt de afwezigheid van NF2 eiwit tot tumorvorming in Schwann-cellen? Mogelijk effecten op groei regulatie zouden, indien het NF2 eiwit inderdaad een essentieel onderdeel van gespecialiseerde membraanstrukturen uitmaakt of deze zelfs induceert, een gevolg kunnen zijn van regulatie, relocatie en concentratie van integrale membraaneiwitten, inclusief receptoren en de regulerende onderdelen hiervan. Doch vergelijking met andere ERM-eiwitten toont geen

gemeenschappelijk mechanisme. Er is, voor zover bekend, geen neoplastische afwijking geassocieerd met andere ERM-eiwitten. Tumorgenese geïnduceerd door afwezigheid van NF2 eiwit lijkt derhalve een gevolg van een verlies van interactie van NF2 eiwit en een specifiek geassocieerd eiwit. Anderzijds, overeenkomstig met mechanismen waarvan gedacht worden dat deze een rol spelen bij ezrin, zouden indirekte effecten op transcriptie door tussenkomst van cytoskelet-geassocieerde signaaleiwitten (Rho/Rac/Cdc42?) een rol kunnen spelen. Het is niet ondenkbaar dat deze signaalcascaden worden geactiveerd als gevolg van morfologische veranderingen die het gevolg zijn van NF2 verlies.

Toekomstig onderzoek zal zich moeten toespitsen op de interactie van het NF2 eiwit met andere eiwitten. Dit zou kunnen plaatsvinden door co-immunoprecipitatie of andere biochemische technieken. Er moet echter rekening mee worden gehouden dat het NF2 eiwit niet geëxtraheerd wordt door niet-ionogene detergentia. Met technieken die berusten op het extractie met deze stoffen zouden derhalve potentiële NF2 bindende eiwitten kunnen worden gemist. Het lijkt daarom zinvol om effecten van NF2 verlies te bestuderen door het onderzoeken van reeds met andere methoden geïdentificeerde NF2 geassocieerde eiwitten.

Een tweede conclusie van het hier gepresenteerde werk is dat het NF2 gen in veel verschillende weefsels tot expressie komt doch dat transcriptie en de aanwezigheid van NF2 eiwit niet altijd overeenkomen. Opmerkelijk is de bevinding dat in continu vernieuwend weefsel zoals epitheel, zowel NF2 mRNA en NF2 eiwit aantoonbaar zijn terwijl in niet delend weefsel NF2 eiwit wel aantoonbaar is maar dat transcriptie van het NF2 gen niet is aan te tonen. Over het algemeen is er overeenkomst tussen de hier beschreven expressie van het NF2 gen in humaan weefsel en de gepubliceerde gegevens in knaagdieren.

Tenslotte wordt geconcludeerd dat het niet mogelijk is getrunceerde NF2 eiwitten aan te tonen in tumoren hoewel dat deze een NF2 mutatie hebben. Vermoedelijk is dit een gevolg van versnelde afbraak van het gemuteerde NF2 eiwit. Deze conclusie stemt overeen met gepubliceerde experimentele gegevens waaruit de instabiliteit blijkt van mutant NF2 eiwit.

Overige publicaties

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Neurofibromatosis 2 tumor suppressor protein colocalizes with ezrin and CD44 and associates with actin-containing cytoskeleton
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Pathology Research and Practice (1998) 194; 189-194

Curriculum vitae

De schrijver van dit proefschrift werd op 21 nov 1963 te Vlaardingen geboren. Na het doorlopen van de middelbare school (VWO diploma 1994, Scholengemeenschap "Westland-Zuid" te Vlaardingen) werd in hetzelfde jaar, na uitloting voor de studie geneeskunde, begonnen met de studie psychologie aan de Rijksuniversiteit Leiden. Deze studie werd afgebroken om van mei 1985 tot september 1986 de militaire dienstplicht te vervullen. Thans is de schrijver reserve officier in de rang van ritmeester. Eveneens vond in deze periode inloting plaats voor de studie geneeskunde in Rotterdam, waarmee werd begonnen in september 1986. Het doctoraal examen werd behaald in 1990, het artsexamen in november 1992. Gedurende de doctoraal fase werd onderzoek verricht op de afdeling celbiologie en genetica, onder leiding van dr. G.C. Grosveld en dr. M. Von Lindern (afdelingshoofd prof. dr. D. Bootsma). Dit onderzoek leidde tot de doctoraal afstudeer scriptie "*Constructie van een chromosoom 22 specifieke NotI jumping library*". Na het artsexamen werkte de schrijver als arts-assistent cardiologie in het Schieland ziekenhuis te Schiedam. In april 1993 volgde aanstelling als AIO bij de afdeling pathologie van de Erasmus Universiteit en werd het onderzoek aangevangen dat heeft geleid tot dit proefschrift. Na 2½ jaar als AIO onderzoek te hebben verricht werd begonnen met de opleiding tot patholoog waarbij de schrijver in dienst kwam van de Reinier de Graaf Groep in Delft (opleider Rotterdam: prof. dr. W.J. Mooi, opleider Delft: prof. dr. F. Eulderink). De opleiding werd van 1 oktober 1996 tot 1 oktober 1997 onderbroken voor het verrichten van verder onderzoek in het kader van een door de Nederlandse Kankerbestrijding gefinancieerd "onderzoeksjaar voor arts-assistenten". Na dit onderzoeksjaar werd de opleiding tot patholoog voortgezet.

De schrijver is gehuwd met Andrea de Zeeuw

Dankwoord

Voor u ligt een proefschrift dat tot stand is gekomen mede door de inspanning van veel personen. Zoals gebruikelijk in een dankwoord zullen, ten onrechte, de meesten niet met naam genoemd worden. Mijn waardering voor de bijdrage van deze mensen is echter niet minder groot; *they know who they are*.... Een aantal wil ik toch apart noemen:

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Andrea, een heleboel van jouw tijd zit in dit boekje....., ik beloof dat je het terug krijgt!