Novel alternatively spliced isoforms of the neurofibromatosis type 2 tumor suppressor are targeted to the nucleus and cytoplasmic granules

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INTRODUCTION

The neurofibromatosis type 2 (NF2) gene encodes for a protein of 595 amino acids, named merlin (moesin-ezrin-radixin-like protein), which belongs to the protein 4.1 superfamily due to its striking homology in the N-terminal half (1,2). So far, merlin and the recently cloned DAL-1 (3) are the only members of the protein 4.1 superfamily to act as tumor suppressors. NF2 is an autosomal inherited disorder of the nervous system characterized by multiple schwannomas, typically bilateral of the eighth cranial nerve. About half of the patients also manifest meningiomas and there is an increased risk of ependymomas. The NF2 gene is also involved in the pathogenesis of sporadic schwannomas, as well as in the majority of sporadic mesotheliomas (4). All members of the protein 4.1 superfamily share a function in linking the cytoskeleton to the plasma membrane. The prototypical erythrocyte protein 4.1 has been shown to bind to integral membrane proteins such as glycoporphin C, p55 and band 3 (5–8), to associate with spectrin and actin (9,10), as well as tubulin (11) and myosin (12), and to rearrange to the mitotic spindle during cell division (13). The functional diversity is due to post-translational modifications (14), usage of two translation initiation sites and above all an extraordinarily complex pattern of alternative splicing (10,15–20). In contrast, only a few splicing variants have been identified for the NF2 gene. Splicing out of NF2 exons 2, 3, 8, 10, 15 and 16 (21–24) and insertion of exon 1a (21,22) have been described. These reports, however, are restricted to the splice junctions and their flanking exons, thus characterizing only a fragment of the alternative transcripts. The complete exon composition and consequently the complete encoded protein sequences of the splicing products are lacking. This is true also for the well-known isoform II including exon 16, which has been cloned from three independent groups only in the C-terminal region from exon 11 to 17 (22), exon 13 to 17 (21) or 14 to 17 (23), respectively. It is a commonly held view that its N-terminus is identical to wild-type NF2 (wt-NF2), which, however, has never been confirmed by experimental evidence. Data gained by expressing artificial NF2 constructs are of limited value concerning physiological functions, unless it can be demonstrated that they are identical to naturally occurring isoforms.

We cloned novel splice variants Mer150, Mer151 and Mer162 of the neurofibromatosis 2 (NF2) tumor suppressor, which demonstrate a tissue-specific and development-specific expression pattern. Isoform Mer150 is created by cryptic splicing from exon 8 to 14 and represents an N-terminal truncation of 259 residues. Mer151 is characterized by in-frame splicing out of several exons and a modified C-terminus due to a frameshift in exons 13+14 and premature termination. Mer162 represents a head-to-tail isoform resulting from in-frame skipping of exons 5–16. As a common feature, the α-helical domain and a variable proportion of the ERM homology domain are spliced out in these isoforms. To investigate differences in subcellular localization, we expressed epitope-tagged cDNA constructs of the wild-type NF2 as well as of the three alternatively spliced transcripts in NIH 3T3 cells by nuclear microinjection or lipid-mediated transfection. Subcellular localization of Mer151 in filopodia and ruffling membranes was similar to the wild-type NF2. Mer151, however, was targeted to the nucleus, which was not observed for wild-type NF2, Mer150 or Mer162. A putative nuclear localization signal created by alternative splicing was identified in Mer151. In contrast to Mer151, Mer150 and Mer162 were not found in regions of the plasma membrane, but localized to a granular intracellular compartment. The results suggest that the recently described actin-binding domain in exon 10, but not the presence or absence of exons 2+3, is relevant for subcellular targeting. Although the NF2 protein is known as a cytoskeletal linker, additional functions in a cytoplasmic compartment and in the nucleus may exist.
Isoform Mer150 is created by cryptic splice sites in exons 8 and 14 and represents an N-terminal truncation of wt-NF2 spanning residues 1–258 plus Arg.

Sequencing of the RT–PCR product of 840 bp identified a novel NF2 transcript consisting of exons 1/2/3/4/5/6/7/partial 8/partial 14/17, named Mer150 (Fig. 1). Splicing occurred at a cryptic 5’ splice site of exon 8 (nt 984–986) and a cryptic 3’ splice site of exon 14 (nt 1768–1770). Thus, the terminal third of exon 8 is lacking, as well as the region from exon 9 to 13 and most of exon 14, with the exception of the last eight codons. Both splice sites are characterized by the identical triplet CTT, as indicated in Figure 2B. Interestingly, the cryptic splice sites do not fit the conventional consensus sequence of splice donor and splice acceptor, respectively. The splice junction of exon 8/p14 is shown in Figure 2B. The complete cDNA sequence of Mer150 is available under GenBank accession no. AF123570. The alternative splicing results in a frameshift of exon 14, encoding the amino acids FPWR and a stop codon. Therefore, the Mer150 protein sequence was terminated at a length of 259 amino acids. Surprisingly, unspliced exon 8 would continue encoding for the residues FPWN . . . (Fig. 2B). Taken together, the Mer150 protein sequence is identical to wt-NF2 amino acids 1–258. Only the last amino acid of Mer150 at position 259 is different from wt-NF2, with Asn replaced by Arg. Thus, Mer150 represents an N-terminal truncated wt-NF2 protein of 259 residues.

Mer162 is a head-to-tail isoform resulting from in-frame skipping of exons 5–16.

In addition to Mer150 and Mer151, a very small RT–PCR product of 500 bp was obtained by screening for alternative NF2 transcripts amplifying from exon 1 to exon 17. Sequencing identified a novel splice variant consisting of exons 1/2/3/4/17, named Mer162. The schematic diagram of splicing of Mer162 is shown in Figure 1. The region from exon 5 to 16, which spans 1290 bp and represents 72% of the coding sequence of full-length NF2 is spliced out. The cDNA of the splice junction exons 4/17 is shown in Figure 2C. Alternative splicing, however, does not alter the reading frame and generates Mer162 protein of 165 amino acids (Fig. 2C). The complete cDNA sequence of Mer162 has been submitted to GenBank under accession no. AF122827.

Expression pattern of Mer150, Mer151 and Mer162

To evaluate the expression patterns of the novel isoforms, we performed RT–PCR in human fetal and human adult multiple tissue cDNA panels (Clontech, Heidelberg, Germany). In addition, total RNAs from tumor cell lines, such as A431 (human epidermoid carcinoma) and SKHeP1 (human liver adenocarcinoma), as well as non-tumor cell lines (foreskin fibroblasts and an immortalized lymphoblastoid cell line) were analyzed (Fig. 3). RT–PCR analyses revealed a band of ~1800 bp, representing full-length NF2 as confirmed by sequencing, and a variable expression of the novel isoforms as well as as yet uncharacterized bands. Adult human tissues (lanes 1–4, 6–8, 10 and 12) uniformly showed a strong band of full-length NF2 transcript. With the exception of adult retina expressing Mer151 (data not shown), the three isoforms Mer150, Mer151 and Mer162 were not found in adult tissues (lanes 1–4, 6–8, 10 and 12). In con-
trast, cell lines expressed different levels of the isoforms compared with full-length NF2. In the lymphoblastoid cell line (lane 5) full-length NF2 and both isoforms Mer150 and Mer151 were found. SKHep1 (lane 9) expressed full-length NF2 and Mer151 as a fainter band. In the fibroblast cell line (lane 11), Mer150 and Mer162 were present in addition to full-length NF2. The intensity of the Mer162 allele, however, exceeded all bands, including the full-length NF2 band. In contrast, the A431 cell line (lane 13) expressed full-length NF2 and Mer162 in about equal intensity. In fetal tissues, the intensity of the band of full-length NF2 was variable. Mer162 was found as a faint band in all fetal tissues tested (lanes 14–18).

Fetal thymus (lane 19) predominantly expressed Mer150 and, in about equal intensity, full-length NF2, Mer151 and Mer162.

The expression patterns of the isoforms at the protein level were studied by western blot analysis of the fibroblast cell line, A431 and lymphoblastoid cells (Fig. 5). The predicted molecular weights of Mer150, Mer151 and Mer162 are 30, 24 and 19 kDa, respectively. The sc332 antibody raised against residues 578–595 encoded by exon 17 detected full-length NF2 as a doublet of ~80 kDa, as well as higher and lower molecular weight bands. The epitope detected by the sc332 antibody is not present in isoforms Mer150 and Mer151 due to a premature translation stop. In Mer162, however, the epitope of the sc332 antibody is present. Of

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**Figure 2.** Partial cDNA sequences (top) and corresponding amino acid sequences (bottom) of Mer151, Mer150 and Mer162. (Top) (A) Splice junction of isoform Mer151. Splicing from exon 1 to exon 4 and from exon 4 to exon 8 are both in-frame, whereas the splicing from exon 10 to exon 13 causes a frameshift and premature termination in exon 14. (B) Isoform Mer150. Splicing from exon 10p to exon 14p due to usage of a cryptic splice donor in exon 8 and a cryptic splice acceptor in exon 14 results in a frameshift of the next four codons and truncation. (C) Isoform Mer162. Splicing of exon 4 to exon 17 is in-frame. (Bottom) Nucleotide and predicted amino acid sequences of (A) Mer151, (B) Mer150 and (C) Mer162. The stop codon is indicated by an asterisk. (B) The splicing event of Mer150 is shown. On the right are wild-type nucleotide and amino acid sequences of exons 8 and 14. The nucleotides involved in splicing of both the cryptic splice donor of exon 8 and the cryptic splice acceptor of exon 14 are boxed. Both splice sites are characterized by the identical triplet CTT. On the left, the amino acid sequence of Mer150 created by cryptic splicing is shown. The splicing causes a frameshift of the wild-type exon 14-encoded sequence. Comparison of the amino acid sequence on the left with wild-type exon 14-encoded amino acids (right) demonstrates the frameshift. The residues created by the cryptic splicing PKISFPW, however, are identical to the wild-type exon 14-encoded amino acids (right).
the lower molecular weight bands detected by sc332, a band migrating below 26 kDa correlates to the predicted size of Mer162. This band was not found in lymphoblastoid cells (Fig. 5A, lane 3), which were negative for Mer162 in RT–PCR, but was observed in fibroblast (Fig. 5A, lane 1) and A431 cells (Fig. 5A, lane 2). In order to detect Mer150 and Mer151 we used sc331 antibody raised against residues 2–21 encoded by exon 1 (Fig. 5B). Similar to sc332, an expression pattern consisting of full-length NF2 and higher and lower molecular weight bands was detected. Lymphoblastoid cells (Fig. 5B, lane 1) expressed protein bands of ~30 and 26 kDa, which correspond to the predicted sizes of Mer150 and Mer151, respectively. Fibroblast cells (Fig. 5B, lane 2) demonstrated a prominent band below 26 kDa correlating with the size of Mer162, which was also detected by sc332 (Fig. 5A, lane 1).

Subcellular localization of Mer151 in filopodia and ruffling membranes is similar to full-length NF2 protein

To generate the epitope-tagged constructs, the 6×His epitope sequence was engineered at the 3’ site of the novel merlin alternative splice variants Mer150, Mer151 and Mer162. A His-tagged merlin full-length construct was included in all experiments for comparison. The epitope-tagged isoforms were cloned into a mammalian expression vector and transiently transfected into NIH 3T3 cells (for details see Materials and Methods). Subcellular localization was studied by indirect immunofluorescence using an epitope-specific antibody and confocal laser scanning microscopy. No differences in subcellular localization of each epitope-tagged merlin construct were seen using either intranuclear microinjection or lipid-mediated transfection. Full-length NF2 was observed as a homogeneously distributed labelling over the entire cell. In addition, immunofluorescence was found in ruffling membranes and in filopodia (Fig. 4a). The cells extended numerous thin and long filopodia, which were intensely labelled and gave the cells a characteristically spiny or hairy appearance (Fig. 4b). No staining of the cell nucleus was found. An identical staining pattern was detected using a polyclonal antiserum (sc332) raised against the C-terminus of the full-length NF2 protein (amino acids 578–595) instead of the monoclonal anti-His antibody. The subcellular localization of Mer151 was comparable with wild-type NF2, since intense staining was detected in ruffling membranes and in numerous thin filopodia (Fig. 4c). Similar to wt-NF2, staining of the entire cell area was observed. In addition, an intense perinuclear staining was present. Cross-section analysis of transfected NIH 3T3 cells double labelled for both the epitope tag and the actin cytoske-
Figure 4. Subcellular localization of epitope-tagged NF2 isoforms expressed in NIH 3T3 cells and examined by confocal laser scanning microscopy. (a) This image shows a cell transfected with the epitope-tagged full-length NF2 plasmid. A diffuse staining homogeneously distributed over the entire cell can be observed, as well as prominent staining of ruffling membranes (arrowhead), of a long filopodium (arrow) and numerous shorter filopodia along the right cell margin. (b) Three cells expressing the full-length NF2 protein can be seen similar to (a). To demonstrate the numerous thin filopodial extensions of the cells the confocal data have been processed with the shadow projection algorithms of the Imaris software package. (c) NIH 3T3 fibroblast cell expressing the Mer151 isoform displayed by a shadow projection as in (b). Similar to the full-length protein, a diffuse staining over the entire cell surface can be seen in addition to staining of ruffling membranes (arrows) and long filopodia (arrowhead). (d) Confocal image of a Mer151-expressing cell with prominent labelling of the cell nucleus and intranuclear structures resembling nucleoli. Note also the positive staining of filopodia extending from the cell margin. In the perinuclear region a thread- or net-like staining in an intracellular compartment can be observed. (e) In a subset of Mer151-expressing cells immunofluorescence can be detected only in a perinuclear region of the cell without staining of membrane ruffles or filopodia. This staining was localized in an intracellular compartment. (f) A Mer150-expressing cell, which extends a long cell process to the upper left of the image. Labelling for Mer150 is restricted to cytoplasmic granules in a perinuclear region but spares the cell nucleus. Single positive granules can also be seen in the long cell extension (arrow). (g and h) Double labelling for Mer151 (g) and Mer162 (h) in red in conjunction with Alexa–phalloidin for visualization of the actin cytoskeleton (green). Additionally, cross-sectional images of the cell in a vertical plane along the x–z and y–z axes are displayed along the lower and right side of the picture, respectively. The location of the vertical planes of the cross-sections are indicated by arrowheads. Both Mer150 and Mer162 can be observed in granules distributed predominantly in the perinuclear region of the cells. The cross-sectional images clearly show that staining for these NF2 isoforms is concentrated beneath the actin-rich cell cortex and hence localized in an intracellular compartment. The scale bar in (h) applies to all images and represents: (a) and (d), 32 µm; (b) 39 µm; (c) and (f–h), 34 µm; (e), 47 µm.
weights of Mer150 and Mer151, respectively (arrows in (B) lane 1). At ~30 and 26 kDa were observed corresponding to the predicted molecular size of Mer162 in the fibroblast and A431 cell lines [(A) lane 3 and (B) lane 1]. In the lymphoblastoid cell line two bands of ~80 kDa, as well as higher and lower molecular weight bands. Using either the sc332 or the sc331 antibody, a band below 26 kDa corresponding to the pre-mature termination from 6 to 22 h after transfection. A small subset of transfected cells was found (Fig. 4d). Inside the nucleus, round bodies resembling nucleoli were delineated by increased immunofluorescence. Labelling of the cell nucleus was variable and ranged from no labelling at all to a faint staining to a very prominent and intense immunoreactivity. Nuclei displaying immunoreactivity were observed throughout the observation time from 6 to 22 h after transfection. A small subset of Mer151-transfected cells displayed only a thread or net-like labelling of the cell nucleus and no staining of ruffling membranes or filopodia (Fig. 4e).

The nuclear localization of Mer151 is unique and in contrast to the subcellular distribution of full-length NF2 protein. Furthermore, labelling of the NF2 isoforms Mer150 and Mer162 was never observed in cell nuclei.

Mer150 and Mer162 are localized in cytoplasmic granules

In transfection experiments using epitope-tagged Mer150 or Mer162 constructs, labelling was confined to granules concentrated in a perinuclear localization, which is different from the localization of full-length NF2 protein. In contrast to the latter and Mer151, no labelling of Mer150 or Mer162 was seen in ruffling membranes and filopodial extensions. Furthermore, a diffuse staining within the boundary of the cell was missing. The granules observed by transfection with either Mer150 or Mer162 were transported over long distances into peripheral cell processes (Fig. 4f). Cross-sectional views of these cells in combination with double labelling of actin filaments with Alexa-phallloidin revealed that the fluorescence of Mer150 and Mer162 was below the outer rim of the actin-rich cell cortex and therefore concentrated in an intracellular localization (Fig. 4g and h). The pool of cytoplasmic granules, where Mer150 or Mer162 were found, looked similar, and a distinction between the localization of these two isoforms on the basis of morphological criteria was not possible.

DISCUSSION

We report three novel alternatively spliced isoforms of the human NF2 gene. To our knowledge, this is the first characterization of NF2 splice variants in their complete exon structure. The necessity of cloning the full-length coding sequences has been recognized for the complexly alternatively spliced protein 4.1 to ensure that encoded products correspond to natural isoforms (26). Although artificial deletion constructs are useful to correlate a specific function to a protein domain, they do not represent the whole biological function of the isoform in the in vivo situation. The three novel NF2 isoforms are created by different mechanisms. Mer162 results from en bloc splicing out of exons 5–16 conserving the open reading frame. Thus, the N-terminus spanning amino acids 1–149 is joined to amino acids 580–595 arising from the C-terminus. Mer151 is characterized by splicing out of exons 2+3 as described previously (21,22) and splicing out of exons 5–7, as well as exons 11+12. Skipping of exons 11+12 causes a frameshift in exons 13 and 14 and premature termination. Therefore, a novel C-terminus of 46 residues is created. Mer150, however, arises from cryptic splicing at nt 984–986 of exon 8 to nt 1768–1770 of exon 14. This results in a frameshift encoding four additional amino acids and a stop codon. Paradoxically, the total amino acid sequence of Mer150 is identical to the N-terminal wt-NF2 protein (amino acids 1–259), with the exception of the last amino acid Asn at position 259, replaced by Arg. Interestingly, the splicing events of all three isoforms completely delete the predicted α-helical domain of the full-length NF2 protein and a variable proportion of the ERM homology domain. Analysis of the expression of these isoforms by RT–PCR in human adult and fetal tissues and different cell lines revealed a tissue-specific and development-specific expression pattern. This is not surprising, since regulation of full-length NF2 protein expression during mouse development has been reported (27,28). Furthermore, evidence for the expression of these isoforms as endogenous stable proteins in different cell cultures was found by western blot analyses. In agreement with previous reports, a complex pattern of protein bands was detected (29–32). Full-length NF2 was described as migrating as a doublet at 70 kDa, the species with decreased mobility being phosphorylated (31). The higher and lower molecular weight bands additionally detected in our study have also been described by other groups and the specificity of the bands was demonstrated using a panel of different antibodies (30). Since merlin is known to form intra- and intermolecular self-associations as well as to form heterodimers with members of the ERM family (33), some of the detected bands might be explained by oligomerization.

To characterize biological significance, transfection experiments of epitope-tagged Mer151, Mer150 and Mer162, as well as full-length NF2, were performed. We found the epitope-
tagged full-length NF2 protein in filopodia and ruffling membranes, which is concordant with the previous immunofluorescence experiments of endogenous (29,34–35) or transfected merlin. Apart from staining of ruffling membranes and filopodia, a homogeneous staining of the entire cell body was seen. This staining seemed to be close to the plasma membrane, although the flatness of the transfected cells precluded an unequivocal identification due to the limited resolution of the light microscope. The subcellular localization of the three novel isoforms, however, is only in partial agreement to the hypotheses of previous expression studies using artificial deletion constructs. These studies reported that full-length NF2 as well as the N-terminal half is targeted to the plasma membrane, filopodia and ruffling membranes (36,38). Deletion of exons 2+3 of either the full-length or the N-terminal half, however, led to localization in cytoplasmic granules (37,39). Furthermore, a single mutation delF118 in exon 3 of the full-length NF2 protein caused an aberrant wide distribution in the cytoplasm (37). Based on these findings, it was concluded that deletion of exons 2+3-encoded amino acids as well as delF118 may be responsible for a relocation from the plasma membrane to the cytoplasm. Since these deletions have been identified in NF2 patients, the cytoplasmic localization was regarded as pathological (37). In contrast, we found Mer150, which represents the N-terminal half (amino acids 1–259), including exons 2+3, exclusively in cytoplasmic granules. In addition, Mer162, also containing exons 2+3, was detected in the same cytoplasmic localization as Mer150. On the other hand, Mer151, lacking exons 2+3, was found in membrane ruffles or filopodia in the majority of transfected cells. In summary, we suggest that the presence or absence of exons 2+3 does not correlate with a localization at the plasma membrane, ruffling membranes or filopodia.

The subcellular localization of the novel isoforms to actin-rich compartments might be explained by the recently described putative actin-binding domain of merlin. N-terminal fragments of merlin comprising residues 178–367 bind to actin filaments in vitro (40). The actin-binding domain is believed to occur in a region spanning amino acids 298–318 encoded by exon 10 (40). This motif is present in Mer151, but not Mer162 and Mer150. Thus, it can be postulated that of the three isoforms, only Mer151 can bind to actin filaments. Indeed, we found Mer151 in ruffling membranes and filopodia, but not Mer162 nor Mer150.

Furthermore, in addition to the actin-binding domain a calpain protease cleavage site has recently been identified within exon 10 (41). This cleavage site mapping to residues 295–299 is used to regulate merlin expression and thereby its tumor suppressive potential. Absence of the calpain cleavage domain in Mer151 and Mer162, but its presence in Mer150, suggests that the cellular pathways regulating the expression of these NF2 isoforms are different.

Mer151 was the only isoform visible in the cell nucleus. A nuclear localization of wt-NF2 is not known, although there has been one report about staining of nuclear structures with an antiserum raised against the C-terminus of merlin (42). A subset of isoforms of the 4.1 protein, however, have been shown to be targeted to the cell nucleus by a nuclear localization signal (NLS), which is created by alternative splicing (26). Classical NLSs are generally characterized by one or more clusters of basic amino acids K(K/R)X(K/R) (43,44). Inspection of the Mer151 sequence revealed that a putative NLS (KNKK), generated by alternative splicing from exon 4 to exon 8, is present in this isoform, but not in full-length NF2 nor in Mer150 and Mer162. The first lysine within the cluster corresponds to the last amino acid encoded by exon 4, whereas the other basic residues are the first amino acids encoded by exon 8. This cluster resembles the prototypical NLS of the SV40 large T antigen. Interestingly, a casein kinase II site SDKE (consensus sequence SXSE; reviewed in ref. 45) downstream from the NLS at the end of the exon 8-encoded amino acids was also detected in Mer151. It has been demonstrated that proteins that harbor an NLS very often also contain casein kinase II sites at a distance of ~10–30 amino acids from the NLS (46). While the NLS determines the specificity of nuclear transport, the casein kinase II site determines the rate of transport. An identical mechanism for the generation of an NLS by the joining of two exon sequences regulated by alternative splicing has been described for the nuclear isoform of protein 4.1, named isoform 4.1H (26). Recently, it has been demonstrated that phosphorylation at the NLS blocks nuclear targeting of the protein (47,48). This mechanism might also explain why Mer151 does not localize to the nucleus in all cells and points towards a regulation of nuclear targeting of this isoform.

Additional experiments will be necessary to determine whether the potential NLS and casein kinase II site in Mer151 are essential for translocation of the protein into the nucleus. The cloning of the Mer151 isoform and its identification as a nuclear component opens up future investigations into the role that merlin plays in this cellular compartment.

**MATERIALS AND METHODS**

**Cell cultures**

NIH 3T3 (DSM ACC 59, Swiss mouse embryo), SKHeP1 (DSM ACC 141, human liver adenocarcinoma) and A431 (DSM ACC 91, human epidermoid carcinoma) cells, obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (PAA-Biologics GmbH, Marburg, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin. Human foreskin fibroblasts of healthy donors were cultured in RPMI-1640 with 10% heat-inactivated fetal bovine serum. EBV-transformed leukocytes from healthy donors were cultured according to standard techniques. All cell lines were incubated at 37°C in an atmosphere of 5% CO₂. Before reaching confluence, cells were detached by treatment with 0.05% trypsin, 0.02% EDTA for 3–4 min at room temperature and transferred to a new tissue culture flask (Corning, New York, NY).

**Merlin full-length cDNA**

Total RNA was isolated from human EBV-immortalized lymphoblastoid cell lines and human foreskin fibroblast cells of healthy donors usingpeqGOLD TriFast kit (Peqlab, Erlangen, Germany). Reverse transcription was performed using the First Strand cDNA Synthesis Kit for RT–PCR (AMV) (Boehringer Mannheim, Mannheim, Germany) and the NF2 gene-specific reverse primer 1819 mapping to the 3’-UTR of exon 17, nt 2215–2238 (GenBank accession no. L11353). Full-length NF2 cDNA
was generated by nested RT–PCR. First round primers 1818/1819 mapped to the 5'-UTR (nt 160–184) and 3'-UTR (nt 2215–2238), respectively. Second round primers 1643/1652 mapped to exons 1 (nt 204–228) and 17 (nt 1970–1990), respectively. Amplification was for 25 cycles each, 1 min at 94°C (nt 204–228) and 17 (nt 1970–1990), respectively. The RT–PCR product of the expected length was sequenced using an automated sequencer (LICOR 4000; MWG Biotech, Ebersberg, Germany).

Identification of alternatively spliced isoforms

Screening for alternative splicing products was performed by nested PCR as described above. RT–PCR products of altered size were excised and eluted from the gel (Qiagen Gel Extraction Kit; Qiagen, Hilden, Germany) and sequenced in their entire length.

Analysis of the expression pattern

Multiple tissue cDNA panel I (Clontech no. K1420-1) derived from eight different adult tissues (brain, heart, kidney, liver, lung, placenta, pancreas and skeletal muscle) was used according to the manufacturer’s instructions. In addition, a human fetal multiple cDNA panel (Clontech no. K1425-1) derived from fetal tissues (brain, heart, kidney, liver, lung, skeletal muscle, spleen and thymus) and Marathon-Ready Human Thyroid Gland cDNA (pooled from 41 male/female Caucasians, ages 17–61; Clontech no. 7490-1) were screened for expression of the novel NF2 isoforms. Since the disease neurofibromatosis type 2 also causes retinal hamartomas in a minor proportion of patients, we included in the study Marathon-Ready Retina cDNA (pooled from 76 male/female Caucasians, ages 16–75 years; Clontech no. 7449-1). The above-listed cDNAs have been normalized to the mRNA expression levels of six different housekeeping genes to ensure an accurate assessment of tissue-specific abundance of target mRNAs. Furthermore, total RNA was isolated from cell lines (SKHep1 and A431) and screened by RT–PCR as described above.

Western blot analyses

Western blotting of SDS extracts of cellular proteins of fibroblast cells, A431 cells and lymphoblastoid cells was performed as described previously in detail (29). For detection of the NF2 protein, affinity-purified polyclonal antibodies sc331 and sc332 were used raised against synthetic peptides corresponding to the N-terminus (amino acids 2–21) and the C-terminus (amino acids 578–595) of the NF2 protein, respectively (Santa Cruz Biotechnology, Heidelberg, Germany). The specificity of the antibodies has been shown in previous studies (29,30).

Clones and plasmids

Epitope tagging of full-length NF2 cDNA, as well as of the identified splice variants (Mer151, Mer150 and Mer162) was performed using modified isoform-specific reverse primers including the 6xHis-epitope at their 3' end. The His-tagged cDNAs were cloned into the eukaryotic expression vector TOPO TA cloning Kit (Invitrogen, Groningen, The Netherlands) according to the manufacturer’s instructions. Positive clones were sequenced to confirm correct cDNA sequence and in-frame insertion of the C-terminal His tag. The plasmids were extracted using a Plasmid Midi Preparation Kit (Qiagen) and resequenced.

Transfection experiments and microinjection

NIH 3T3 cells were seeded on Nunc 2-well chamber slides (Nunc, Naperville, IL) 24 h prior to the transfection experiment. Epitope-tagged plasmids were expressed either by lipid-mediated transfection or intranuclear microinjection. For the lipid-mediated transfection FuGene reagent (Boehringer Mannheim) was used according to the manufacturer’s instructions. An aliquot of 1 µg of purified plasmid was added per well. Alternatively, purified plasmids were microinjected into the cell nuclei at a concentration of 100 ng/µl using the Micromanipulator 5171/Transjector 5426 system (Eppendorf, Germany). After transfection and incubation for a time period of 6–36 h cells were fixed and processed for immunofluorescense.

Immunofluorescence and confocal laser scanning microscopy

NIH 3T3 fibroblast cultures were fixed by adding an equal amount of freshly prepared 3% paraformaldehyde in 80 mM K-PIPES pH 7.5 (Sigma-Aldrich, Deisenhofen, Germany) supplemented with 5 mM EGTA and 2 mM MgCl2 to the culture medium for 30 min. Cells were quenched for 15 min in freshly prepared NaBH4 (0.5 mg/ml) (Fuka, Buchs, Switzerland) in phosphate-buffered saline (PBS) pH 7.4 and, after washing with PBS, they were permeabilized for 5 min in 0.1% Triton X-100 in PBS. After incubation in PBS plus 5% normal donkey serum, the slides were washed and incubated with the primary antibody in PBS including 0.2% bovine serum albumin (PBS-BSA). For detection of the 6x-His epitope a mouse monoclonal antibody (Boehringer Mannheim) was used at a 1:100 dilution. After a washing step in PBS the slides were incubated with biotinylated donkey anti-mouse antiseraum at a 1:200 dilution in PBS-BSA (Jackson Laboratories, Dianova, Hamburg, Germany). The biotin was subsequently visualized with streptavidin–Alexa 488 conjugate (Molecular Probes, Leiden, The Netherlands) diluted 1:400 in PBS-BSA in a third step. For visualization of the actin cytoskeleton, Alexa 568–phalloidin was included in the last incubation step at a 1:50 dilution. In an alternative procedure, transfected full-length NF2 protein was visualized with the sc332 rabbit antiseraum raised against the C-terminus (amino acids 578–595) of the NF2 protein (Santa Cruz Biotechnology) diluted 1:100 in conjunction with mouse anti-6x-His monoclonal antibody. The sc332 antiseraum was detected by donkey anti-rabbit Cy3 secondary antiseraum. Slides were mounted in Pro-long Antifade Reagent (Molecular Probes) and examined with a Bio-Rad (Munich, Germany) MRC1000 confocal scanning microscope connected to a Nikon 300 inverted microscope. Serial optical sections were taken and digitally superimposed in the extended depth of focus mode, to obtain an ‘all in focus’ image of all the fluorescence of a cell. The Ikaris software package (Bitplane, Zürich, Switzerland) was used for 3-dimensional analysis of the confocal data, including cross-sections.

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