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Neurofibromatosis Type 2 Protein Co-Localizes with Elements of the Cytoskeleton

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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, probably function as molecular linking proteins, connecting the cytoskeleton to the cell membrane. On the grounds of the homology to the ezrin, radixin, and moesin 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 amino- and carboxyl-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. Immunoelectron 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. (Am J Pathol 1995, 147:1339–1349)

Neurofibromatosis type 2 (NF2) is a disease resulting in the formation of bilateral vestibular schwannomas. These tumors occur in more than 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] NF2 has been subdivided into two 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 polymerase chain reaction (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 with 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 amino terminus and followed by a charged carboxy terminus. Apart from

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moesin, the ERM and NF2 proteins also contain a proline-rich stretch at the carboxy 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 carboxyl-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.

Materials 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) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. The X63Ag8.653 myeloma cells[30] and hybridomas were cultured in 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 2.5 μmol/L cytochalasin D (Sigma Chemical Co., St. Louis, MO) 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 80 mmol/L Pipes-KOH, pH 6.4, 5 mmol/L EGTA, 1 mmol/L $MgCl_2$, and 0.5% Triton X-100.

Peptide Synthesis and Generation and Characterization of Monoclonal Antibodies

The amino-terminal SP-0 (GAIASRMSFSSLKRKQP-KTF-C) peptide was synthesized essentially as described,[32] and 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 50 μg of synthetic peptide coupled to keyhole limpet hemocyanin suspended in 250 μl of phosphate-buffered saline (PBS) and mixed with 250 μl of complete Freund's adjuvant. The mice were boosted twice at 2-week intervals; for the boosts, incomplete Freund's adjuvant was substituted for the complete Freund's adjuvant. To determine whether 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 before the fusion the mouse was boosted a third time with 50 μg of synthetic peptide suspended in 500 μl 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 by 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 by an immunocytochemical assay on NF2-transfected COS cells. Three clones were identified (UC2, MH3, and KF10) that 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, Wallisellen, 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 five and tested directly on transfected COS cells. One clone, 15H3, was identified that exclusively stained transfected COS cells. The isotype of the monoclonal antibodies was determined with the Isostrip isotyping kit (Boehringer Mannheim, Mannheim, Germany). An enzyme-linked immunosorbent assay was performed by coating 96-well PVC plates with synthetic peptide. The wells were blocked with a 3% bovine serum albumin (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, visualization of positive wells was achieved by incubation with *o*-phenylenediamine (Eastman Kodak Co., Rochester, NY) 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 manufacturer's recommendations (Bio-Rad mini-protean 2D cell, Bio-Rad, Richmond, CA). Protein blots were blocked with a 3% bovine serum albumin 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 visualized with a secondary alkaline phosphatase-conjugated goat anti-rabbit antibody (TAGO, Burlingame, CA). As a substrate, 4-nitroblue 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.

Immunolabeling 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-triethoxysilane-coated (Sigma) 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%, pH 7.2, embedded, and after 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 method.[35] A protease pretreatment (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-conjugated rabbit anti-mouse antibody (Dako). Phalloidin-FITC staining was accomplished by incubating slides in a 5-mg/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, Frankfurt, Germany) containing 2.5% 1,4-diazobicyclo-[2,2,2]-octane (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. Immunoelectron microscopy was performed with the KF10 antibody as described.[36] The competition studies were performed by diluting KF10 in a 40-mmol/L SP279 solution in PBS and mixing overnight at 4°C. As a control, a 40-mmol/L solution of another synthetic NF2 peptide was used (SP277 or SP278[25]). After the preincubation, the solutions were used for immunostaining and immunoelectron microscopy.

Results

Three monoclonal antibodies were raised against the carboxyl-terminal NF2 synthetic peptide SP279 (C-LHNENSDRGGSSKHNTIK). Two of the antibodies, KF10 and UC2, reacted similarly with regard to staining properties whereas the third antibody, MH3, displayed a more restricted staining pattern. One monoclonal antibody, 15H3, was raised against the amino-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 an enzyme-linked immunosorbent assay. The KF10 antibody was reactive only 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 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 and 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 toward 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 immunoelectron microscopy with the KF10 antibody and secondary antibodies conjugated to 10-nm 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 nonspecific interactions of the KF10 antibody and components of the keratohyalin granules, incubations were performed in the presence of 0.15 mol/L NaCl and 0.5 mol/L 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 labeling of the keratohyalin granules in immunoelectron microscopy could be completely abolished by preincubation of a working solution of the antibody with the synthetic peptide against which it had been raised (not shown). Preincubation with different NF2 peptides did not result in loss of staining.

To determine whether 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 2, A and 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 2, C and 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 organizing 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 to determine a more precise localization of the NF2 protein in the COS cells (Figure 3, A and B). Optical sections perpendicular to the culture slide demonstrated a specific membrane-bound localization. Furthermore, staining was detected only on the dorsal cell mem-

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,* ×*400.* 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,* × *50.* 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,* × *100.* D: *Immunoelectron micrograph of the human epidermis stained with the KF10 antibody. Specific labeling of a keratohyalin granule is observed. Magnification,* ×*30,000.*