The Neurofibromatosis Type 2 Gene Product, Schwannomin, Suppresses Growth of NIH 3T3 Cells

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Abstract

Cancer is a multistep process that involves the activation of oncogenes and the inactivation of antioncogenes. Recently, a new putative tumor suppressor, the neurofibromatosis type 2 (NF2) gene, was mapped to chromosome 22, cloned, and found to encode for a new protein, merlin/schwannomin, a member of the band 4.1 family of proteins. Members of this family have not been implicated previously in tumorigenesis. They possess significant homology in their NH2-terminal domain, which is thought to be important in the binding of the plasma membrane to the underlying actin cytoskeleton. To determine whether schwannomin may affect cell growth, we transfected NIH 3T3 cells with the wild type and an NF2 cDNA lacking 111 amino acids at the NH2 terminus. We observed slowing of growth and changes in cellular morphology only in cells expressing the wild-type NF2 cDNA. This finding suggests that schwannomin can suppress growth directly and confirms its role in tumor suppression. This system will provide a useful assay to identify important functional domains of the protein.

Introduction

NF2 is an autosomal dominant disease that predisposes patients to the development of vestibular schwannomas, meningiomas, and other tumors of the neuroectoderm (1). The gene was mapped to chromosome 22 (2), and more recently it was cloned (3, 4) and found to encode a protein, SCH, which is a new member of the band 4.1 family of proteins. This family includes numerous structural proteins such as ezrin, radixin, and moesin, as well as enzymes such as the protein tyrosine phosphatases PTPH1 and PTPMEG (5). Protein band 4.1 is an important structural protein that plays a key role in regulating membrane physical properties, including mechanical stability and deformability, by stabilizing spectrin-ankyrin interaction (6). Ezrin, radixin, and moesin appear to be expressed widely but have been found to localize to various subcellular compartments (7-9). Ezrin (or cytovillin) is a microvillar cytoplasmic peripheral membrane protein, with prominent expression in placental syncytiotrophoblasts and certain human tumors (10). Radixin is an actin-barbed end-capping protein that is highly concentrated in the undercoat of the cell-cell adherens junction and the cleavage furrow of cells in the interphase and mitotic phases (8, 11). Moesin is a membrane-organizing extension spike protein that is localized in cultured cells near the plasma membrane especially in microspikes, retraction fibers, blebs, filopodia, and lamellodopa (7, 12). These proteins are all involved in maintaining the focal contact points between the plasma membrane and cytoskeleton. It has been postulated that certain proteins in this class may act as tumor suppressor genes (13).

Many lines of evidence support the hypothesis that SCH is a tumor suppressor. NF2 shares many features with retinoblastoma, the paradigm for a genetic disease caused by mutation of a tumor suppressor (14). These include autosomal dominant mode of inheritance, inactivation of germline mutations, and the occurrence of multiple tumors at a young age. In sporadic and NF2-associated meningiomas and schwannomas, loss of heterozygosity for chromosome 22 and a high frequency of inactivating mutations of the NF2 gene strongly support the hypothesis that SCH is a tumor suppressor (3, 4, 15-25).

To determine whether SCH can suppress growth, we transfected the gene into NIH 3T3 cells in culture. SCH expression significantly slowed growth and caused morphological changes. Transfection with a mutant NF2 gene isolated from an NF2 patient did not cause any phenotypic changes. These results confirm that SCH can act as a tumor suppressor. This system will allow the dissection of key SCH domains.

Materials and Methods

RNA Extraction and Reverse Transcription-PCR. The following cell lines were analyzed for NF2 mRNA: P19 embryonic carcinoma cells; NG108 and SHSY5Y human neuroblastoma cells; and NIH 3T3 Swiss mouse fibroblasts. Total RNA was isolated with the use of the GIBCO-BRL (Gaithersburg, MD) TRizol reagent from 2 × 10⁶ cells and was reverse transcribed for 1 h at 37°C with the use of primer 906 (GCGAGATCCAGAGTCGCTTC). Primers 840 (TGCTGGCTGAGTGATG) and 906 were then used for PCR amplification [94°C for 2 min (hot start) and the addition of Taq polymerase, followed by 94°C for 1 min, 55°C for 30 s, and 72°C for 2 min, for 30 cycles]. The product was visualized on a 1% agarose gel.

Transfection of Mouse Fibroblasts (NIH 3T3 Cells). The full-length 2046-bp wild-type cDNA was subcloned into the EcoRI site of the pcDNAII expression vector (Invitrogen, San Diego, CA). The construct was cotransfected with the β-galactosidase gene into NIH 3T3 Swiss mouse fibroblasts. Total RNA was isolated with the use of the GIBCO-BRL (Gaithersburg, MD) TRizol reagent from 2 × 10⁶ cells and was reverse transcribed for 1 h at 37°C with the use of primer 906 (GCGAGATCCAGAGTCGCTTC). Primers 840 (TGCTGGCTGAGTGATG) and 906 were then used for PCR amplification [94°C for 2 min (hot start) and the addition of Taq polymerase, followed by 94°C for 1 min, 55°C for 30 s, and 72°C for 2 min, for 30 cycles]. The product was visualized on a 1% agarose gel.

Effect of Antisense Oligonucleotides. An antisense oligonucleotide (GGAACGCGATGGCCCGCCAG) from the 5'-end of the gene and crossing the ATG was tested in cell lines transfected stably with the wild-type NF2 gene and then observed for reversal of the growth changes. The same experiment was carried out using 80 μM of the sense oligonucleotide (CCTCCCTACCGGGCGGCT) in 3-h pulses every day for 6 days. Growth rate and thymidine uptake were assessed in treated cells.

Effect of Epidermal Growth Factor on NF2 Stable Transfectants. Approximately 2 × 10⁶ cells were plated in 10-cm tissue culture plates and grown in serum-free medium for fibroblasts (GIBCO-BRL) in the presence and absence of 100 ng EGFR. EGFR at this concentration is known to stimulate growth rate (26). The cells undergoing the different treatment were plated in triplicate. Morphological changes in the cells were assessed, and growth rate, thymidine uptake, and protein content (27) were measured. Characteristics of the SCH protein were investigated by Western blotting.
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Fig. 1. Reverse transcription-PCR of different cell lines tested for NF2 expression. The 1.4-kb product was detected in all cell lines except NIH 3T3 Swiss mouse contact-inhibited fibroblasts. This observation was also confirmed by Northern blotting analysis (data not shown).

Western Blots of Cell Lysates. The polyclonal antiserum used in this assay has been characterized previously. Cell lysates were collected from cultured cells by pelleting by centrifugation at 2500 rpm in a clinical centrifuge and then resuspending the pellet in 200 ml of NP40 buffer as described previously. Total protein extracted was quantified against a BSA standard. Equal volumes of protein (10 µg) were loaded in duplicate on 8 and 10% denaturing polyacrylamide gels electrophoresed at 100 mV for 1 h. After this time one gel was stained with Coomassie and the other was blotted onto nitrocellulose membrane for 1 h at 4°C. The gel was checked to ensure that the protein had transferred with the use of Ponceau-S staining (Sigma Chemical Co., St. Louis, MO). The membrane was preblocked for 1 h at room temperature in TBS-10% milk-0.5% Tween, and then the anti-SCH polyclonal antiserum was bound to the membrane for 1 h at room temperature (28). The membrane was washed 15 min in TBS-5% milk-0.05% Tween, 3 times 5 min in the same solution, followed finally by 2 5-min washes in TBS. The signal was visualized with the use of the Amersham Enhanced Chemiluminescence (ECL; Amersham, United Kingdom) technique (see manufacturer's protocol).

Immunofluorescence of Cultured Cells. Cells in log phase were trypsinized and plated onto 2-mm Corning glass coverslips. The cells were incubated overnight and then fixed with cold acetone (BDH, Germany) for 1 min. They were rinsed twice for 5 min at room temperature in 1× TBS (25 mM TBS (8g NaCl, 0.2 g KCl, 3 g Tris + 800 ml H2O), pH 7.4)-0.05% Tween. Fifty µl of a solution containing DME-10% normal goat serum-1:100 primary SCH antiserum were added to each coverslip and incubated for 1 h at room temperature. The coverslips were rinsed twice for 5 min in 1× TBS at room temperature, and then the secondary antibody, goat anti-rabbit rhodami-

Measurements of Growth Rate and Thymidine Uptake. Six different clones from each of the untransfected, mock transfected, and stably transfected cells (the wild-type NF2 gene and a deletion mutant) were grown to confluence (2 × 10⁶) in 96-well plates, and during the last 16 h of growth they were subjected to 50 mCi of [³H]thymidine. The cells were counted in triplicate for each, put into a 3-ml volume of scintillation fluid (Beckman Instruments, Fullerton, CA) in vials, and then counted with the use of a Beckman counter. In separate experiments, untransfected, mock transfected, stably NF2-transfected, and mutant NF2-transfected NIH 3T3 cells were plated in triplicate with 2 × 10⁶ cells/well in a 96-well plate. The cells were counted every 24 h for 5 days with the use of a haemocytometer, and the results were plotted (Cricket graph).

Image Analysis and Statistics. Tissue culture dishes containing cultures of the untransfected, mock transfected, and stably transfected NIH 3T3 cells were used for image analysis on an inverted light microscope attached to an IBAS computer (IBM) network. Cells (approximately 50 each) were selected, and the length of the cell process and the cell body diameters were measured. The ratio was taken and then compared with the use of the Student’s t test (2-tailed), and significance was determined.

Results

Reverse Transcription-PCR. Reverse transcription-PCR (Fig. 1) was used to test several cell lines for the expression of the NF2 gene product. Confirmation of expression was carried out by Northern blot analysis (data not shown). The anti-SCH antibody was also used to


Fig. 2. Western blot analysis of untransfected (wt), wild-type, and mutant (Mut) NF2 stably transfected NIH 3T3 cells. In the NF2 stably transfected line the Mr 81UKH)SCH protein was present. The protein was shown to be absent in the untransfected and altered by at least one-third of its size in the 111-amino acid deletion mutant. In this case there also appeared to be breakdown products, which are indicative of an unstable protein. Ordinary molecular weight in thousands.
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Fig. 3. Immunofluorescence of transfected NIH 3T3 cells. NIH 3T3 cells were co-transfected with the β-galactosidase gene and stained with X-galactosidase, and the image was seen through an inverted microscope to ensure the transfection had worked (a). The cells were then tested by light microscopy for expression, b, cells with a preabsorbed control of the anti-SCH antibody. The faint appearance of a cell can be seen as a white shadow; c, cells that were labeled throughout the nucleus and the cytoplasm with anti-SCH. X 500.

Table 1 Statistical analysis of the phenotypic changes seen in NIH 3T3 cells overexpressing the NF2 gene

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Significance</th>
<th>Effect on NF2-transfected cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF2 transfected vs. untransfected cells</td>
<td>P &lt; 0.001</td>
<td>Decreased growth</td>
</tr>
<tr>
<td>NF2 transfected vs. vector transfected cells</td>
<td>P &lt; 0.001</td>
<td>Decreased growth</td>
</tr>
<tr>
<td>growth rate</td>
<td>P &lt; 0.0001</td>
<td>Increased ratio*</td>
</tr>
<tr>
<td>diameter:process length ratio*</td>
<td>P &lt; 0.0001</td>
<td>Reversal of decreased growth</td>
</tr>
<tr>
<td>NF2 transfected vs. NF2-transfected cells treated with antisense oligonucleotide</td>
<td>P &lt; 0.0001</td>
<td>Reversal of decreased growth</td>
</tr>
<tr>
<td>NF2 transfected vs. EGF-treated transfected cells</td>
<td>Not significant</td>
<td>No change</td>
</tr>
</tbody>
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* Ratio of cell body diameter versus length of extending processes.

test for the presence of SCH protein. P19 (rat embryonic carcinoma cells), PC12 (mouse pheochromocytoma), and NG108 and S5HY (both are human neuroblastoma cells) were found to express the NF2 gene at about the same relatively high levels. NIH 3T3 Swiss mouse contact-inhibited cells were found to express the gene at extremely low levels. Therefore, these cells were used for the subsequent experiments.

**Transfection Experiments.** Confluent NIH 3T3 cells were transfected with the vector only, the full length wild-type NF2 cDNA, or the mutant cDNA as described in “Materials and Methods.” The transfection efficiency was determined to be more than 50% by cotransfection with the β-galactosidase gene. Western analysis using the SCH polyclonal antiserum of the untransfected, wild-type NF2-transfected, and mutant-transfected cells indicated that the cells expressed no, normal, or mutant protein, respectively (Fig. 2). Overexpression of SCH in the wild-type NF2-transfected cell lines was confirmed by immunofluorescence (Fig. 3).

No distinct phenotypic differences were seen among untransfected cells, mock transfected cells, or cells transfected with the mutant NF2 gene. However, phenotypic changes were seen in cells transfected with the wild-type NF2 gene (Table 1; Fig. 4). There was a 3-fold decrease in the growth rate (Fig. 5) and morphological changes in the cells. Control cells are flattened and spindle shaped with short filopo-
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Fig. 5. Growth curve plots of the untransfected (Untrans), mock transfected (Mocks), and NF2 stably transfected cells. The graph illustrates that there is little difference among the growth rates of the untransfected, mock transfected, and mutant-transfected (Mut) cells. However, the growth rate was slowed almost 3-fold in the wild-type NF2-transfected cells.

Results show that SCH may play a role in this process, which is frequently seen overexpression of the NF2 gene results in decreased growth rate and may result from interactions between SCH and extracellular matrix, cell function, allowing the identification of key functional domains.

These results indicate that this mutant protein is inactive. The same of M, 50,000, the expected size given the 111-amino acid deletion.

Changes. Western analysis showed the presence of a truncated protein or morphology (data not shown). Western analysis showed no change in the size of the protein (data not shown).

Discussion

The NF2 tumor suppressor gene was cloned recently (3, 4) and found to be highly homologous to the band 4.1 family (29), which encodes proteins involved in interaction of the plasma membrane with the underlying actin cytoskeleton (6, 13). We have now shown that overexpression of the NF2 gene results in decreased growth rate and altered morphology of NIH 3T3 cells. These phenotypic changes were not seen in mock transfected cells or cells transfected with an inactivated derivative of the NF2 gene. The phenotypic changes in stably transfected cells could be reversed by exposure to an antisense oligonucleotide designed to span the ATG of the NF2 gene. The sense oligonucleotide had no effect.

Effect of EGF. Incubation of the stably transfected cell lines with medium containing 100 μM resulted in no changes in cell growth rate or morphology (data not shown). Western analysis showed no change in the size of the protein (data not shown).

Acknowledgments

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References

12. Futhymay, H., Lankes, W., and Amiev, M. Moesin, a new cytoskeletal protein and in terminally differentiating cells. Perhaps SCH is important in the organization and maintenance of myelin sheaths surrounding axons, as well as the dendrites of neurons and the extensions of melanocytes, cell types where SCH is expressed highly. Alternatively, SCH may be involved in the formation of growth cones. Moesin is important in the development of filopodial extensions, which are thought to be important in exploration, attachment, and the movement of cells in vitro, as well as events in epithelial-mesenchymal interactions during oncogenic transformation (5, 9).

Transfected cells were incubated in the presence of EGF to see whether there was any change in growth rate or size of the NF2 protein. No significant changes were observed. We conclude that SCH possibly differs from Ezrin where phosphorylation of Tyr 353 permits its translocation from the microvillar core to the membrane in the process of “ruffling of the membrane,” as described by experiments in overexpressing A431 cells treated with EGF (26). Further experiments need to be done to determine whether SCH is phosphorylated.

We conclude that overexpressing SCH results in a decreased growth rate in NIH 3T3 cells. However, how this novel protein interacts, directly or indirectly, with the cell cycle needs to be determined. The observed morphological changes suggest that SCH may be important in exploration and organization of membrane-cytoskeletal elements. In addition, this cell culture assay provides a means for studying the effects of mutations affecting different functional domains of SCH. Results of these studies will provide clues to possible interacting molecules such as growth factors, adhesion molecules, cytoskeletal elements, and signaling molecules, all of which may be important to SCH function and tumorigenesis.
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