Communication

An Anti-Ras Function of Neurofibromatosis Type 2 Gene Product (NF2/Merlin)*

(Received for publication, May 25, 1994, and in revised form, July 13, 1994)

Anjali Tikoo, Mikael Varga, Vijaya Ramesh, James Gusella, and Hiroshi Maruta‡

From the Ludwig Institute for Cancer Research, Royal Melbourne Hospital, Victoria 3050, Australia, and the Molecular Neurogenetics Unit, Neuroscience Center, Massachusetts General Hospital East, Charlestown, Massachusetts 02129-9142

Previously, we have cloned a candidate for the 805-amino-acid neurofibromatosis type 2 tumor suppressor called NF2 or Merlin, with striking sequence similarity to the human chromosome 22q12 chromosome (7, 8). This gene encodes a protein of 595 amino acids called NF2 or Merlin (7, 8). Point mutations or truncation of NF2 have also been found to be closely associated with development of schwannomas and other central nervous system tumors including multiple meningiomas (7-11). Interestingly, NF2 shares around 50% sequence identity in its N-terminal half (residues 1-359) with the corresponding domains of several members of an F-actin-binding protein family called TER, which includes talin, ezrin, radixin, and goesin (7, 8).

Very recently, at least four distinct actin-cytoskeleton-associated proteins were shown to act as tumor suppressors (12-15); overexpression of vinculin and α-actinin can reverse SV40-induced malignant transformation (12, 13), whereas tropomyosin-1 and gelsolin can reverse malignant transformation caused by Ras mutants such as v-Ha-Ras and v-Ki-Ras (14, 15). Interestingly, expression of the genes encoding these four actin-binding proteins is strongly suppressed when normal cells are transformed by SV-40 virus or Ras oncogenes (14). It is now clear that constitutive expression of these genes in SV40- or Ras-transformed cells results in reversion of the malignant state (12-15). Although NF2 is expressed widely in various human tissues (7), it still remains to be clarified whether NF2 expression is down-regulated by Ras or other oncogenes.

In this paper we demonstrate for the first time that (i) overexpression of NF2 can reverse the v-Ha-Ras-induced anchorage-independent growth of NIH/3T3 cells. Like neurofibromatosis type 1 (NF1) fragments (Nur-E-Kamal, M. S. A., Varga, M., and Maruta, H. (1993) J. Biol. Chem. 268, 22331-22337), full-length NF2 can reverse the Ras-induced malignant phenotype, i.e., anchorage-independent growth in a soft agar, and restore contact inhibition of cell growth, indicating that NF2 is indeed a tumor suppressor. Furthermore, NF2-N also suppresses the Ras-induced malignant phenotype, although it appears to be less effective than the full-length NF2. These observations indicate that the anti-Ras function of NF2 resides in part in its N-terminal half. Thus, NF2 appears to be a new member of the tumor suppressor family of actin-cytoskeleton-associated proteins, which includes vinculin, α-actinin, tropomyosin-1, gelsolin, and tensin.

Oncogenic mutants of Ha-Ras, Ki-Ras, or N-Ras are found in around 30% of total human carcinomas, notably in 50% and 90% of colon and pancreatic carcinomas, respectively (1). Oncogenic mutants of Ras are locked in the active GTP-bound form (2). Their intrinsic GTPase activity is low and no longer stimulated by GTPase-activating proteins (GAPs)3 such as GAP1, which is 1044 amino acids, and NF1, which is 2818 amino acids (3, 4).

We have shown previously that neurofibromatosis type 1 (NF1) fragments of 338 and 91 amino acids (NF338 and NF91) can reverse the malignant phenotype, i.e. anchorage-independent growth in a soft agar, caused by Ras mutants such as v-Ha-Ras (5). This was the first demonstration that a target of the tumor suppressor NF1 is the Ras-GTP complex as these NF1 fragments bind the Ras-GTP complex directly (6). Dysfunction or deletion of NF1 develops schwannomas and other tumors (neurofibromatosis 1) in central nervous system of human (6). We and another group recently cloned a candidate for the neurofibromatosis type 2 tumor suppressor gene located on human chromosome 22q12 (7, 8). This gene encodes a protein of 595 amino acids called NF2 or Merlin (7, 8). Point mutations or truncation of NF2 have also been found to be closely associated with development of schwannomas and other central nervous system tumors including multiple meningiomas (7-11).

1 The abbreviations used are: GAP, GTPase-activating protein; PCR, polymerase chain reaction; RT, reverse transcriptase; ACAP, actin-cytoskeleton-associated protein; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase kinase.

* This work was supported in part by grants from the National Health and Medical Research Council of Australia and the Anti-Cancer Council of Victoria (to H. M.) and National Institutes of Health Grant NS24279 (to V. R. and J. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

To whom correspondence should be addressed. Fax: 613-347-3155.

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**Effect of NF2 and NF2-N Transfection on the Colony-forming Ability of v-Ha-Ras-transformed Cells in Soft Agar—**v-Ha-Ras-transformed NIH/3T3 cells were transfected with either NF2/pMV7 or NF2-N/pMV7 as complexes with liposomes as described previously (17). A parallel transfection with vector pMV7 alone was also carried out to serve as a negative control. The resultant G418-resistant transfectants were cloned in the presence of 400 pg/ml G418 (a neomycin analogue) as described previously (5). The colony formation in a soft agar (anchorage-independent growth) of NF2/pMV7 and NF2-N/pMV7 clones, in comparison with pMV7 clones, was examined by incubating 1000 cells/plate at 37°C for 3 weeks under standard culture conditions (5). The colonies were stained with crystal violet and counted.

Reverse Transcriptase (RT) PCR Analysis of NF2, NF2-N, and v-Ha-Ras Expression—Total RNAs from both the parental v-Ha-Ras transformants and NF2/NF2-N transfectants were isolated by the method of Chomczynski and Sacchi (18). 5 μg of each total RNA preparation was then used as a template to synthesize v-Ha-Ras and NF2/NF2-N cDNAs by avian myeloblastosis virus reverse transcriptase (Promega) and a Riboclone cDNA synthesis kit (Promega), using the corresponding antisense PCR primers including the last several codons (17). To compare the amount of these cDNAs between the Ras transformants and NF2/NF2-N transfectants, the same aliquots of all cDNA preparations were then used as PCR templates for Taq DNA polymerase to amplify exogenous (human) or endogenous (mouse) NF2, NF2-N, or v-Ha-Ras cDNA sequences using the corresponding pairs of sense and antisense primers under essentially the same conditions as those described previously (17, 19). Under these PCR conditions, the amount of PCR products is linearly proportional to the initial amount of cDNAs used as templates and the saturation point is achieved when a rise in PCR products plateaus. The titration curves were drawn based on incorporation of [α-<sup>32</sup>P]dTTP into the PCR bands, using both cloned v-Ha-Ras and NF2 DNAs at various concentrations (0.1–10 pg) as the standard PCR templates (data not shown).

The human and mouse NF2 amino acid sequences are 98% identical (20, 21), therefore, some pairs of the sense/antisense PCR primers corresponding to the human NF2 base sequences are able to amplify the corresponding base sequences of mouse NF2 cDNA as well. For instance, sense primer S and antisense primer B, corresponding to codons 9–14 and 354–359 of human NF2 (8), respectively, can be used to amplify both mouse and human sequences, whereas antisense primers A and C, corresponding to codons 335/336–340 (AGCGAGCGCTGCGCC) and codons 590–595 (8), respectively, can be used for amplifying only human, and not mouse, sequences. Thus, PCR primers C and A were used to quantitate the levels of exogenous human NF2 and NF2-N mRNAs, respectively, whereas PCR primer B was used to compare levels of endogenous mouse NF2 mRNAs between normal and Ras-transformed NIH/3T3 cells or compare levels of endogenous (mouse) NF2 mRNAs in parental Ras transformants and exogenous (human) NF2/NF2-N mRNAs in NF2/NF2-N transfectants.

**RESULTS**

**Anti-Ras Action of NF2—**Using a retroviral vector pMV7, we have overexpressed full-length NF2 in v-Ha-Ras-transformed NIH/3T3 cells under the control of long terminal repeats, and examined the effects of NF2 on their growth, in particular the anchorage-independent growth in a soft agar, which correlates with their malignancy. As shown in Fig. 1 (top panel), very few NF2-overexpressing cells (N) formed large colonies in a soft agar. Under the same conditions, parental transformed cells (T) formed many large colonies. Each of these large colonies contains more than 100 cells. Furthermore, although these NF2 transfectants still formed small colonies, the number of small colonies derived from NF2 transfectants was only about 10% of that derived from parental transformed cells (see Table I, experiment 1). A quantitative RT PCR analysis of both v-Ha-Ras and NF2 mRNAs levels (see Fig. 1, bottom panel) has confirmed that this dramatic reduction in both size and number of colonies was due to expression of NF2, and not due to any reduction or loss of v-Ha-Ras expression. Thus, these data clearly indicate that, like NF1 fragments (5), full-length NF2 can reverse a malignant phenotype caused by v-Ha-Ras.

NF2 Restores Contact Inhibition of Cell Growth—Normal fibroblasts such as NIH/3T3 cells in a liquid culture cease to grow when they form a confluent monolayer on a glass or plastic substratum. This phenomenon is called contact (or density-dependent) inhibition of cell growth (or division). Interestingly, when the cells are transformed by v-Ha-Ras or many other oncogenes, they lose this contact inhibition and continue to grow, piling up on one another after they have formed the confluent monolayer. Interestingly, NF2 transfectants derived from v-Ha-Ras-transformed cells are contact-inhibited (see Fig. 2). In a liquid culture, both the control v-Ha-Ras transformants and their NF2 transfectants grow at the almost same rate until they form a confluent monolayer. However, at confluence, NF2 transfectants cease to grow (as do normal NIH/3T3 fibroblasts), whereas control transformed cells continue to proliferate (Fig. 2).

**The Anti-Ras Function of NF2 Resides in Part in Its N-terminal Half—**NF2 shares a high sequence identity (around 50%), mainly in the N-terminal domain (NF2-N, residues 1–358), with several other members of an F-actin-binding protein family (TERM) including talin, ezrin, radixin, and moesin (7). The remaining domain of NF2 has no sequence homology to any domains of the TERM proteins. Thus, if its actin binding is sufficient for anti-Ras action of NF2, NF2-N is expected to...
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Fig. 2. NF2 restores contact inhibition of cell growth that is lost in v-Ha-Ras transformants. In a liquid culture, the v-Ha-Ras transformants (Control, cross) and their NF2 transfectants (NF2, solid square) grow at the almost same rate until they form a confluent monolayer (day 5–6). Then only NF2 transfectants cease to grow (as normal transformants in a soft agar, but its anti-Ras action (average 77% suppression) appears to be less efficient than that of full-length NF2 (average 87% suppression). Quantitative RT PCR analysis has revealed again that NF2-N transfectants express both NF2-N (Fig. 3) and v-Ha-Ras (data not known), clearly indicating that reduction of Ras-induced malignancy is due to NF2-N expression and not due to a loss of v-Ha-Ras expression. These observations confirmed our prediction that the anti-Ras function of NF2 resides in part in its highly conserved N-terminal half, which most likely binds actin filament (F-actin).

NF2 mRNA Level Is Not Affected by v-Ha-Ras—Expression of the four distinct tumor suppressor genes encoding actin cytoskeleton-associated proteins (vinculin, α-actinin, gelsolin, and tropomyosin-1), which can suppress SV40- or Ras-induced malignancies, are down-regulated by either SV40 virus or oncogenic Ras mutants (14). Thus, we have compared the steady-state levels of NF2 mRNA between normal and v-Ha-Ras-transformed mouse NIH/3T3 cells by a quantitative RT PCR technique using a pair of oligonucleotide primers, B (antisense) and S (sense), that can amplify both mouse and human NF2 cDNAs (for detail, see "Experimental Procedures"). As shown in Fig. 3 (right panel), no significant difference in the NF2 message level could be detected between normal (N) and Ras-transformed (T) cells, and the endogenous (mouse) NF2 mRNA level in both cases is far lower than the exogenous (human) NF2-N mRNA level in NF2-N transfectants (R), clearly indicating that v-Ha-Ras does not affect the steady-state level of NF2 mRNAs.

Using another pair of primers, A (antisense) and S, which could amplify the exogenous (human) NF2 message of 1.0 kb in NF2-N transfectants (R, 1–R4) but not the endogenous (mouse) NF2 cDNAs in either normal cells (N) or Ras transformants (T) (Fig. 3, left panel), we have confirmed that the 1.1-kb PCR products generated by primers B and S from both normal and Ras-transformed cells (Fig. 3, right panel) are indeed derived from mouse NF2 mRNAs, and not from a human NF2 cDNA contaminant (this conclusion was verified further by subsequent sequencing). The deduced human and mouse NF2 amino acid sequences are 98% identical (20, 21).

DISCUSSION

We have shown here that both full-length NF2 and its N-terminal half (NF2-N) can reverse the malignant phenotype induced by an oncogenic Ras mutant, but full anti-Ras action of NF2 requires its C-terminal half (NF2-C) as well. The partial requirement of NF2-C for full anti-oncogenicity appears to be compatible with previous observations that majority of mutations associated with neurofibromatosis 2 cause C-terminal truncations (7, 8), suggesting that the C-terminal domain is required for a potential tumor suppressor activity of NF2. In fact, our preliminary results indicate that an NF2-C fragment (residues 354–595) is anti-oncogenic as NF2-N.2 Interestingly, one NF2 patient, represented by cell line GUS5722 (7), was found to carry an NF2 mutant that lacks 78 amino acids (residues 447–524) in the C-terminal domain (7). This missing domain contains two slightly overlapping Pro-rich decapeptide motifs (residues 478–495), which potentially could bind SH3 domains of some intracellular signal transducers such as the N-terminal domain of GAP1 (a Ras GTPase-activating protein of 120 kDa) and the C termini of myosin Is (single-headed myosin isoenzymes).

The domain NF2-N is highly homologous to several other members of the TERC family of F-actin-binding proteins (7, 8). In fact, our preliminary results indicate that NF2-N at least co-sediments with purified F-actin in vitro.3 Thus, it appears that NF2 belongs to a tumor suppressor family of actin-cytoskeleton-associated proteins (ACAPs). This family includes at least four other ACAPs, i.e. vinculin, α-actinin, tropomyosin-1, and gelsolin (12–15). However, it still remains to be clarified whether their F-actin binding is absolutely required for their anti-oncogenicity. How do these actin-binding proteins block the oncogenic Ras signal transduction pathway? It was conceivable that expression of NF2 gene, like other members of this tumor suppressor gene family, could be suppressed by v-

\[ \text{\textsuperscript{2}} A. Tikoo and H. Maruta, unpublished observations. \]
\[ \text{\textsuperscript{3}} A. Tikoo and H. Maruta, unpublished observation. \]
Ha-Ras, and the constitutive expression of NF2 gene might be necessary to modulate the action of the Ras mutant. However, we have demonstrated here that v-Ha-Ras does not affect steady-state levels of NF2 mRNAs in NIH/3T3 cells. Alternatively, NF2 overexpression could lead to overproduction of actin or other ACAPs, which in turn suppress Ras-induced malignant transformation. Interestingly, it was recently reported that a key role of Ras-GTP complex was demonstrated recently that a key role of Ras-GTP complex interacts with the action of p190 by forming a complex with tyrosine-phosphorylated p190. NF2 and other actin-binding tumor suppressors might serve as the downstream targets of p190 or other ACAPs, which in turn suppress Ras-induced malignancy or other forms of malignant transformation.

An interaction of Ras with F-actin itself or an actin-containing complex has been observed previously (25). Interestingly, it was demonstrated recently that a key role of Ras-GTP complex is to recruit cytoplasmic Sed and Rho (30). Perhaps p190 acts as a downstream target of Rho or its C-terminal domain (23, 31); however, it is possible that its N-terminal domain (29), and (ii) p190, a GAP specific for Ras, directly interacts with GAP1 (22, 30, 31), GAP1 binds p190 only when p190 is phosphorylated at specific tyrosine residues (22). p190 activates Ras GTPase through its C-terminal domain (23, 31); however, it is possible that its N-terminal domain, which contains a G protein or GTPase motif, might act as a GTP-activated signal transducer as Ras and Rho (30). Perhaps p190 acts as a downstream target of Ras for organizing actin stress fibers and GAP1 somehow interferes with the action of p190 by forming a complex with tyrosine-phosphorylated p190. NF2 and other actin-binding tumor suppressors might serve as the downstream targets of p190 or form a complex with GAP1, thereby interfering with GAP1-p190 interaction to antagonize the oncogenic action of Ras mutants.

Acknowledgments—We are grateful to Masha Fridman for skillful assistance during preparation of mRNAs and Northern blot analysis, Giovanna D’Aboaco for technical advice, and Dr. Tony Burgess for consistent encouragement and support, as well as invaluable advice and comment throughout this work. We also thank the JBC reviewers for their comments, which were helpful in improving the manuscript.

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