Vol. 187, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1992 Pages 332-339

EXPRESSION OF TWO TYPES OF NEUROFIBROMATOSIS TYPE 1 GENE TRANSCRIPTS IN GASTRIC CANCERS AND COMPARISON OF GAP ACTIVITIES

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Received July 8, 1992

Summary: To understand the molecular mechanism of gastric tumorigenesis, the status of neurofibromatosis type 1 (NF1) gene was analyzed in human gastric cancer cell lines. Although the sequencing of the GTPase activating protein (GAP)-related region of NF1 (NF1-GRD) revealed no apparent mutation, the NF1-GRD transcript (type I) and that containing an additional 63 bp insert in the center of NF1-GRD (type II) were equally expressed in most gastric cancer cells. By contrast, type II was predominantly expressed in normal stomach mucosa. When these two types of NF1-GRD were bacterially expressed and their GAP activities were tested, both types of NF1-GRD similarly stimulated ras GTPase activity. However, arachidonic acid inhibited GAP activities of two types of NF1-GRD to different extents. These results suggest that the increased expression of type I NF1 protein may modulate ras-related signal transduction and it may be related to the control of the gastric cellular proliferation.

Human gastric cancers are most common tumors worldwide, particularly in Japan (1). The molecular basis of the pathogenesis of gastric tumorigenesis has been extensively explored and multiple oncogenes may be involved in the malignant transformation of stomach cells (1). Furthermore, not only activated oncogenes but also inactivation of tumor suppressor genes such as p53 gene (2,3,4) may play an important role in the development of gastric cancers.

In addition to several tumor suppressor genes known so far, the neurofibromatosis type 1 (NF1) gene has recently been isolated as a

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Abbreviations used are: PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; NF1, neurofibromatosis type 1; GAP, GTPase-activating protein; NF1-GRD, GAP-related domain of the NF1 gene.

0006-291X/92 $4.00

gene responsible for the pathogenesis of NF1 (5,6). Of particular interest is the fact that a 360 residue region encoded by the NF-1 cDNA shares structural similarity with the mammalian GTPase activating protein (GAP)(7,8). Furthermore, the GAP-related domain of the NF1 gene (NF1-GRD) product stimulates ras GTPase activity (8). These results strongly suggest that the NF1 gene plays an important role in the ras-related signal transduction pathway and that structural alterations of NF1 gene may be involved in abnormal cellular growth and differentiation (8). NF1 patients have an increased risk of malignant tumors including gastric cancers (9) as well as tumors in the nervous system (9). In the present study, therefore, to better understand the molecular pathogenesis of gastric tumorigenesis we have determined the status of the NF1 gene in gastric cancer cell lines. Although we did not detect any mutations of NF1-GRD, two types of the NF1 gene transcript discovered most recently (12) were differentially expressed in normal stomach tissues and gastric cancer cells.

MATERIALS AND METHODS

Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) and Sequencing

A Complimentary DNA (cDNA) was synthesized by incubating ~2 μg of total RNA with a random hexamer as a primer and 60 units of avian myeloblastosis virus reverse transcriptase.(Boehringer Mannheim) at 42 °C as described (4). PCR amplification in a total volume of 0.1 ml was performed with one-tenth of the cDNA, 100 pmoles each of oligonucleotide primers, 1.25 mM dNTPs and 2.5 units Taq polymerase in PCR buffer (Perkin-Elmer-Cetus) for 30 cycles each of which included denaturation at 94 °C for 1 min, annealing at 55 °C for 1.5 min, and primer extension at 72 °C for 2 min. Sense and antisense PCR primers used for amplification of NF1-GRD were 5'-TATAAGCTTAGGTTACCACAAGGATCTCCAG (nucleotide 2496-2520)(Primer-1) and 5'-ACTTCTAGAGGACCAGTGTGTATCTGCCACAGG (nucleotide 3591-3615)(Primer-2), respectively. The numbers of nucleotides correspond to the numbering used by Xu et al. (7) The PCR products were digested with Hind III and Xba I, ligated to pUC 19 (Takara) and sequenced by dideoxy termination methods (11) using the Sequenase kit (USB).

Detection of Two Types of NF1-GRD Transcripts by RT-PCR

The expression of two different types of NF1-GRD transcripts in cancer cells was analyzed essentially as described previously (10). A cDNA was synthesized by using total RNA extracted from human stomach tissue or various cultured cells. PCR amplification (30 cycles) was performed using cDNA as a template as described above to generate 312 and 375 bp DNA fragments, the latter of which contains an additional 63 bp (10). The primers used were 5'-CAGAATTCCCCCCTCAACTTCGAAG (nucleotide 3062-3083)(Primer-3) and 5'-GAAGGATCCTGCGTGCTGCATCAAAGTTGC (nucleotide 3344-3366)(Primer 4).

Ten microliters of the PCR products were separated on 1.8 % agarose gels and analyzed by ethidium bromide staining. The PCR product was also radiolabeled by the addition of 10 μCi of [α-^{32}P]dCTP (3000 Ci/mmol)(New England Nuclear) to the PCR reaction mixture immediately before the final PCR cycle. Ten microliters of the radiolabeled PCR product was electrophoresed on a 5 % polyacrylamide gel followed by autoradiography. The amount of radioactivity in each band was determined by Fujix BAS 2000 Bio-imaging Analyzer.

Expression and Purification of Glutathione-S Transferase(GST)-NF1 GRD fusion proteins

To determine GAP activities of NF1-GRD gene products, Hind III-Sac I fragments were excised from pUC 19-NF1-GRD constructs, blunt-ended with Klenow fragment of DNA polymerase, ligated to a Bgl II linker and digested with Bgl II and Sma I. The digested DNA fragments were then cloned into the Bam Hl-Sma I restriction site of the pGEX-1 (kindly provided by Dr. Aoyama, 2nd Dept. of Int. Med., Kobe Univ.). Expression and purification of GST-NF1-GRD fusion proteins were essentially performed as described previously (12) and the detail of the procedure was described elsewhere (13).

Assay for GAP Activities of Two Types of NF1-GRD

GAP activities of bacterially expressed NF1-GRD proteins were assayed essentially as described previously (14). Briefly, Ki-ras p21 or H-ras p21 was prebound with [γ-^{32}P]GTP (6000 Ci/mmol)(New England Nuclear) in 100 mM sodium phosphate (pH 6.8) containing 0.5 mM EDTA, 0.05 mg/ml BSA, 0.04 mM dithiothreitol, 0.005 % sodium cholate and 0.5 μM GTP at 30 °C for 15 min. Ki-ras p21 and H-ras p21, both of which were expressed by use of a baculovirus/insect system (15), were kindly provided by Dr. Y. Takai (Dept. of Biochemistry, Kobe Univ.). It was estimated that approximately 1 pmol of each ras p21 was GTP bound form. GTPase activities of two types of NF1-GRD were measured in a solution containing 20 mM Hepes (pH 7.5), 1 mM $MgCl_2$, 0.1 mg/ml BSA for 10 min at 24 °C. Reaction mixtures were then filtered through a nitrocellulose membrane to remove unbound nucleotides and hydrolyzed phosphate, then the radioactivity retained on the membrane was counted.

RESULTS AND DISCUSSION

It is most likely that mutations of NF1-GRD region affect the function of the NF1 gene product in cancer cells. Therefore, we have sequenced the NF1-GRD region (nucleotide 2521-3590)(7) in two gastric cancer cell lines, KATO-III and MKN45. However, sequencing of NF1-GRD region amplified by RT-PCR showed no apparent mutation in these cancer cell lines (data not shown). On the other hand, we have detected two different types of NF1-GRD cDNAs in these cell lines. As recently described by Nishi et al.(10), one (type I) is identical to the originally reported sequence (7) and the other (type II) contained a 63 bp insertion that encodes for a region of 21 amino acids in the center of the NF1-GRD (10). Thus, to compare the expression levels of two types of NF1-GRD transcripts the

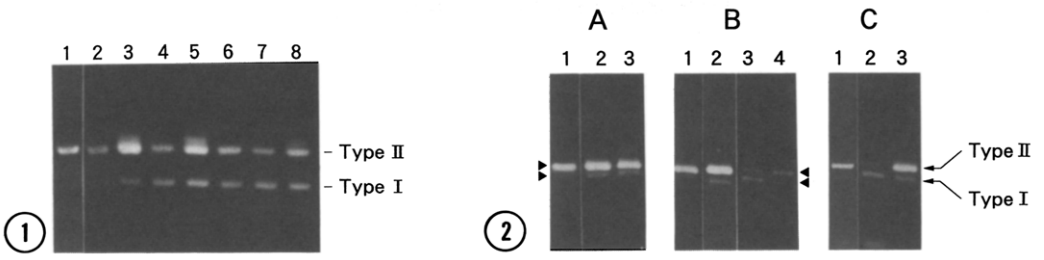

Fig. 1. Expression levels of two different types of NF1-GRD transcripts in human gastric cancer cell lines.
RT-PCR was performed with cDNAs of various cancer cells by using primers 3 and 4 as described in Methods. Representative ethidium bromide staining patterns of PCR fragments derived from type I (312 bp) and type II (375 bp) NF1-GRD transcripts are shown. Lanes 1 and 2, normal stomach mucosa; lane 3, MKN1; lane 4, MKN28; lane 5, MKN45; lane 6, MKN74; lane 7, KATO-III, lane 8, TMK-1.

Fig. 2 Expression levels of two different types of NF1-GRD transcripts in various gastrointestinal cancer cells.
(A) lane 1, normal pancreas; lane 2, Panc-1, lane 3, MIA PaCa-2. (B) lane 1, normal colon mucosa; lane 2, SW 480; lane 3, SW837; lane 4, WiDr. (C) lane 1, normal liver; lane 2, HuH-6; lane 3, HuH-7.

middle portion of NF1-GRD with or without a 63 bp insertion was amplified by RT-PCR using primers 3 and 4 (Fig. 1). The small (312 bp) and the large (375 bp) fragments were amplified and separated on 1.8 % agarose gel. Sequencing of these two PCR fragments revealed that they were derived from type I and type II NF1-GRD transcripts, respectively. In the normal stomach mucosa, the type II transcript was predominantly expressed (Fig. 1, lane 1,2). When compared with normal stomach mucosa, the expression of the type I transcript was increased and both type I and type II transcripts were equally expressed in most of gastric cancer cell lines (Fig. 1). The relative expression levels of type I and type II transcripts in gastric cancer cell lines were further evaluated by radiolabeling RT-PCR analysis. Values of type I/type II ratio were the following: stomach mucosa, 0.149±0.083; MKN1; 0.825±0.062; MKN28, 1.019±0.135; MKN45, 1.103±0.257; MKN74, 0.800±0.104; KATO-III, 1.226±0.340; TMK-1, 0.834±0.079 (mean±SE, n=3). Thus, radioactivities in type I and type II bands were almost equal in gastric cancer cell lines, a finding corresponding to that of ethidium bromide staining. In addition to gastric cancer cells, we also examined the relative expression levels of two types of NF1-GRD transcripts in other gastrointestinal cancers. In the normal pancreas, type II was predominantly expressed (Fig. 2A). Similarly, the type II was predominantly expressed in two

individual pancreatic cancer cell lines (Panc-1 and MIA PaCa-2 cells)(Fig. 2A). In the normal colon mucosa and in two different colon cancer cell lines (SW480, WiDr), the type II transcript of NF1-GRD was preferably expressed and both type I and type II transcripts were equally expressed in SW837 (Fig. 2B). In the normal liver and a differentiated cancer cell, HuH-7, type II NF1-GRD was predominantly observed (Fig. 2C). By contrast, the type I transcript was predominant in an undifferentiated hepatoblastoma cell lines, HuH-6 (Fig. 2C). These data indicate that type II NF1-GRD transcript is predominantly expressed in the normal gastrointestinal tract. It has been shown that the type I NF1-GRD transcript is predominant in the fetal brain and in undifferentiated primitive neuroectodermal tumors (10). In contrast, the type II transcript has been found to predominate in the adult brain and differentiated brain tumor cell lines (10), suggesting that the differential expression of type I and type II NF1-GRD transcripts is related to neuronal cell differentiation. MKN1, MKN28 and MKN74 cells are derived from differentiated gastric cancers, whereas MKN45, KATO-III and TMK-1 are from undifferentiated gastric cancers (4). Thus, the increased expression of the type I transcript as compared with the normal stomach mucosa seems to be not related to differential grades of gastric cancer cells. In the liver, by analogy with the brain (10), present data suggest that differential expression of two types of NF1-GRD might function the differentiation of hepatic cells, although present data is preliminary and further studies should be required.

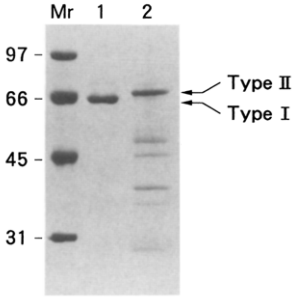

Fig. 3. Expression and purification of two types of NF1-GRD proteins.
Bacterially expressed GST-NF1-GRD fusion proteins were purified on glutathione Sepharose beads, electrophoresed on 10 % polyacrylamide gel and visualized by Coomassie blue staining. Molecular sizes are indicated in kd. lane 1, GST-NF1-GRD type 1 (62.7 kd); lane 2, GST-NF1-GRD type II (66.8 kd).

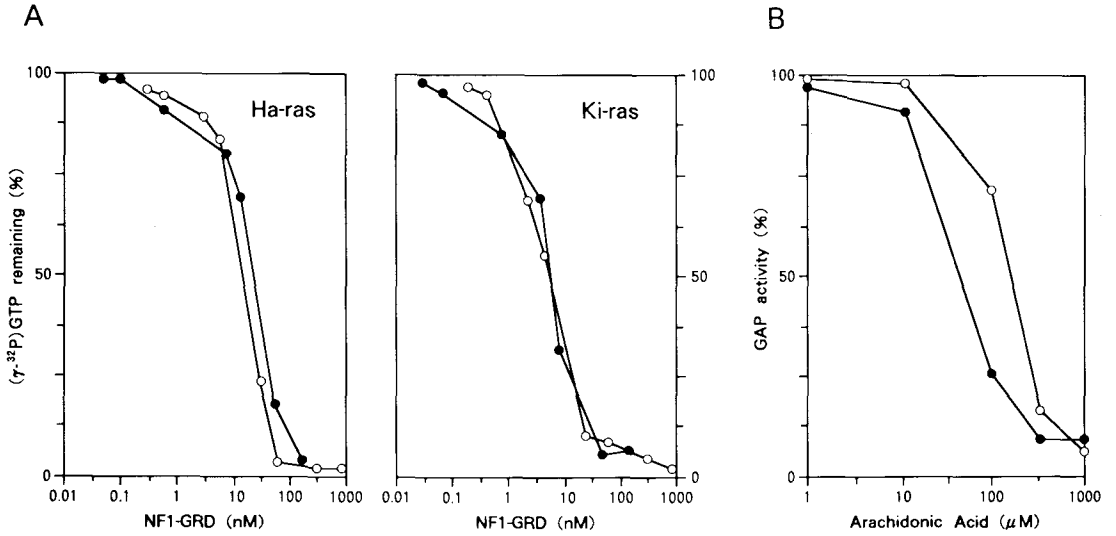

Fig. 4. GAP activities of two types of NF1-GRD on Ki-ras and H-ras (A) and the inhibition of GAP activity by arachidonic acid (B).
GTPase activities of each ras p21 proteins at the indicated concentrations of type I (open circle) or type II (closed circle) NF1-GRD protein were measured for 10 min at 24 °C. Prebinding of Ki-ras p21 (A) or H-ras p21 (A,B) and [γ-^{32}P]GTP was carried out as described in Methods. GTPase activity was expressed as the percentage of the radioactivity of [γ-^{32}P]GTP-bound ras p21 without NF1-GRD (A) or the percentage of GAP activity of NF1-GRD in the absence of arachidonic acid (B). Results are the means of duplicate determinations of a representative of 3-4 separate experiments.

To investigate whether a 63 bp insertion would modulate the GAP activity of the NF-1 gene product, type I and type II NF1-GRD were bacterially expressed by using a GST-fusion protein expression system, then their GAP activities were examined. Two types of GST-NF1 fusion proteins were purified rapidly by affinity binding to glutathione-Sepharose beads as shown in Fig. 3. A small but a significant difference in the apparent molecular size of two GST NF1-GRD fusion proteins reflects a 63 base insertion of the type II NF1-GRD cDNA. As shown in Fig. 4, both types of GST-NF1-GRD fusion proteins stimulated the GTPase activity of H-ras p21 in a concentration dependent manner. However, an apparent difference in GAP activities of both types of NF1-GRD was not observed (Fig. 4A). Furthermore, when GAP activities of NF1-GRD fusion proteins on Ki-ras p21 were examined, type I and type II NF1-GRD similarly stimulated the GTPase activity of Ki-ras p21 (Fig. 4A). In either H-ras or Ki-ras, GST protein alone did not show any significant GAP activity (data not shown). It has been shown that arachidonic acid

(AA) inhibits the GAP activity of NF1-GRD product (16), while p120-GAP activity is only weakly affected by AA (16). When the GAP activities of two GST-NF1-GRD fusion proteins for H-ras p21 were examined in the presence of increasing concentrations of AA, AA inhibited GAP activities of both types of NF1-GRD in a concentration-dependent manner (Fig. 4B). However, the IC_{50} of AA for type I (156±34 μM, mean±SE, n=3) was significantly higher than that for type II (34±8 μM, mean±SE, n=3). Thus, these results indicate that type I and type II NF1 GRD protein possess the same ability to stimulate ras p21 GTPase activity in vitro. In addition, GAP activities of two types of NF1 proteins may be differentially regulated by lipids such as AA. It has been demonstrated that the activation of the ras oncogene product by point mutation is a rare event in gastric cancers (1). However, since ras p21 has been shown to play a crucial role in cellular proliferation and malignant transformation in a number of other cell types (17), an alteration in the ras-related signalling pathway might be involved in gastric tumorigenesis. The present results, therefore, suggest that differential expression of two types of NF1-GRD in cancer cells might affect the ras-related signal transduction through different regulation of both types of NF1-GRD by AA. Alternatively, either an insertion (type II) or a deletion (type I) of 21 amino acids may change the whole NF1 protein structure then altering its GAP activity for ras p21, but it may not affect the GAP activity of bacterially expressed NF1-GRD in vitro. Thus, if type I NF1 protein could antagonize the GAP activity of type II NF1 protein predominantly expressed in normal stomach cells, the increased level of type I NF1 protein might result in increasing the amount of ras-GTP bound form, leading to the cellular proliferation of stomach cells. To further understand the functional difference between type I and type II NF-1 gene products, transfection study using the whole cDNA of both types of the NF1 gene will be required.

ACKNOWLEDGMENTS

We thank Drs. Y. Takai, K. Kaibuchi and A. Kikuchi, Dept. of Biochemistry, Kobe Univ., for providing H-ras and Ki-ras and for helpful discussions. We are grateful to Dr. H. Saya for communicating observations as to two types of NF1-GRD transcripts prior to publication.

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