Neurofibromatosis Type I Tumor Suppressor Neurofibromin Regulates Neuronal Differentiation via Its GTPase-activating Protein Function toward Ras*

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Neurofibromin, the neurofibromatosis type 1 (NF1) gene product, contains a central domain homologous to a family of proteins known as Ras-GTPase-activating proteins (Ras-GAPs), which function as negative regulators of Ras. The loss of neurofibromin function has been thought to be implicated in the abnormal regulation of Ras in NF1-related pathogenesis. In this study, we found a novel role of neurofibromin in neuronal differentiation in conjunction with the regulation of Ras activity via its GAP-related domain (GRD) in neuronal cells. In PC12 cells, time-dependent increases in the GAP activity of cellular neurofibromin (NF1-GAP) were detected after NGF stimulation, which were correlated with the down-regulation of Ras activity during neurite elongation. Interestingly, the NF1-GAP increase was due to the induction of alternative splicing of NF1-GRD type I triggered by the NGF-induced Ras activation. Dominant-negative (DN) forms of NF1-GRD type I significantly inhibited the neurite extension of PC12 cells via regulation of the Ras state. NF1-GRD-DN also reduced axonal and dendritic branching/extension of rat embryonic hippocampal neurons. These results demonstrate that the mutual regulation of Ras and NF1-GAP is essential for normal neuronal differentiation and that abnormal regulation in neuronal cells may be implicated in NF1-related learning and memory disturbance.

Neurofibromatosis type 1 (NF1)† is one of the most common autosomal dominantly inherited disorders, with an incidence of about 1 in 3500 individuals (1). The NF1 hallmark is the development of benign tumors of the peripheral nervous system and the increased risk of developing malignancies. The NF1 phenotype is highly variable; it affects several organ systems, including bones, skin, irises, and central nervous system manifested as gliomas and learning disabilities. The NF1 gene lies on chromosome 17q11.2 and encodes neurofibromin, a large 2818-amino acid protein (2). Since the majority of NF1 gene mutations frequently found in NF1 patients prevent intact neurofibromin expression, functional disruption of neurofibromin is potentially relevant to the expression of some or all of the multiple abnormalities that occur in NF1 patients (3).

A region centered around the 360 amino acids encoded by the NF1 gene shows significant homology to the known catalytic domains of mammalian Ras GAP-activating protein (p120GAP) and is also similar to yeast IRA1/2 proteins, which interact with Ras and mediate hydrolysis of Ras-bound GTP to GDP, resulting in Ras protein inactivation. The GAP-related domain of the NF1 gene product (NF1-GRD) also stimulates Ras GTPase and consequently inactivates Ras protein (4, 5).

Two different isoforms, type I and type II, which are formed by alternative splicing, have been identified in the NF1-GRD region. Type II contains an additional 63-bp insertion (exon 23a) that encodes 21 amino acids in the center of NF1-GRD type I (6). The specific expression patterns of the two isoforms have been studied in several organs and cells (7, 8) and provide a basis for implicating differential expression of NF1 type I and type II transcripts in the regulation of neuronal differentiation and development.

Recent studies using NF1 gene targeting animals reported that Drosophila homozygotes with NF1 null mutation showed significantly decreased olfactory learning performance (9), NF1 heterozygous mice displayed spatial learning disability (10, 11), and mice lacking alternatively spliced NF1 exon 23a exhibited specific learning impairment (12). Furthermore, abnormal Ras activity in NF1 knockout mice disrupted learning and memory, indicating that functional modulation of Ras by neurofibromin is essential for learning and memory (11). These observations prompted us to postulate that neurofibromin plays a key role in the Ras signal-dependent pathway as a GAP in neuronal cells and that functional regulation of neurofibromin, Rap; GAP, GTPase-activating protein; PI3K, phosphatidylinositol 3-kinase.

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† The abbreviations used are: NF1, neurofibromatosis type 1; NGF, neural growth factor; EGF, epidermal growth factor; RT, reverse transcriptase; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; Ab, antibody; GRD, GAP-related domain; DN, dominant negative; GFP, green fluorescent protein; CA-Raf, constitutively active Ras; GTPase-activating protein; PI3K, phosphatidylinositol 3-kinase.

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such as alternative splicing, could be involved in the neuronal development that may be implicated in the learning disability of NF1 patients.

Here, we analyzed alterations in cellular neurofibromin GAP activity (NF1-GAP) using a newly developed NF1-GAP assay system during the neuronal differentiation of PC12 cells. PC12 cells are well known to respond to NGF, followed by activation of Ras-dependent signal pathway, leading to differentiation into a synaptoid neuron-like phenotype (13, 14). Our results demonstrate that the mutation rate of Ras and NF1-GAP is implicated in neuronal differentiation. For further confirmation, rat embryonic hippocampal neurons and PC12 cells were analyzed for morphological alterations after overexpression of NF1-GRD type I dominant negative mutants. Finally, we discuss the cellular function of neurofibromin in the normal development of neuronal cells in conjunction with the Ras-related signal modulation via NF1-GAP and implications for the NF1-related neuronal pathogenesis such as learning disability and memory disturbance.

**EXPERIMENTAL PROCEDURES**

**Preparation of Recombinant Proteins**—Glutathione S-transferase (GST) fusion proteins of human GRD type I (residues 1175–1552), GRD type II (21 amino acids inserted between residues 1345 and 1346 of GRD type I), Ha-Ras, and p120GAP were produced in Escherichia coli under the isopropyl-1-thio-galactopyranoside induction system, and affinity-purified as described (15).

**Construction of Plasmids**—pGEX-2TH plasmids harboring NF1-GRD type I and type II cDNAs, which produce GST-GRD type I and GST-GRD type II fusion proteins, respectively, were constructed as described (16). pGEX-2TH-Ha-Ras and pGEX-2T p120GAP were prepared as described (17, 18). pEGFPc1-GRD type I and type II vectors were constructed by insertion of a HindIII fragment from pGEX-2TH-GRD type I or type II, respectively, into a HindIII site in the pEGFP-c1 (Clontech) vector. pEGFPc1-GRD type I and type II vectors were constructed by insertion of a HindIII fragment from pGEX-2TH-GRD type I or type II, respectively, into a HindIII site in the pEGFP-c1 (Clontech) vector.

**Preparation of Cell Lysates**—COS7 cells were cultured in Dulbecco modified Eagle's medium supplemented with 10% horse serum, 5% cow serum, and 400 μg/ml G418 (Invitrogen). PC12 cells were cultured with 10 ng/ml of 2.5 S NGF (WAKO) or EGF (WAKO) for up to 48 h. For preparation of cellular lysates, cells were solubilized with NF1 lysis buffer (20 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 1 mM 4-(aminomethyl)-benzenesulfonyl fluoride hydrochloride, 1 μg/ml each aprotinin, leupeptin A, and leupeptin), and passed through a 25-gauge syringe 15 times. Lysates were centrifuged at 20,000 × g for 20 min at 4 °C, and the protein concentrations of the supernatants were determined using the BCA protein assay (Pierce).

**GAP Activity Assay**—To prepare the [γ-32P]GTP-bound form of Ras (Ras-γ-32P[GTP]), 40 μl of GST-Ha-Ras (5 μm) was preincubated with 6 μm of 0.5 μCi/ml [γ-32P]GTP diluted with 0.2 μg/ml GST (0.2 μCi/ml) for 2.5 min at 37 °C in 42 μl of Ras loading buffer A (20 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 20 mM EDTA, 100 mM NaCl, 10% glycerol, 2.5 mg of bovine serum albumin). After the incubation, 8 μl of 0.5 M MgCl2 and 240 μl of ice-cold Ras loading buffer B (Ras loading buffer A without EDTA) were added. The indicated amounts of GST fusion proteins or cell lysates in 40 μl of GAP assay buffer (50 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 2.5 μg of bovine serum albumin, 5% glycerol, 0.5 mM GTP) in the presence or absence of 3 μg of anti-NF1-GAP IgG were incubated with 10 μl of Ras-γ-32P[GTP] for 10 min at 30 °C. After a second incubation, the reactions were quenched by keeping the sample on ice and adding 0.8 ml of ice-cold stop buffer (25 mM Tris-HCl, pH 7.6, 100 mM NaCl, 5 mM MgCl2, 1% Triton X-100, 0.5 mM EDTA, 10% glycerol)). The sample was passed through a nitrocellulose filter (0.45 μm), and the filter was washed three times with 0.8 ml of ice-cold stop buffer. After drying the filter, the radioactivity was counted. Total GAP activities were obtained from the differences between the total Ras-γ-32P[GTP] radioactivity and the sample radioactivity hydrolyzed after the GAP reaction. Specific NF1-GAP activity was obtained from the difference between the sample radioactivities after the reaction with and without anti-NF1-GAP IgG, and the activity of other GAPs was obtained by subtraction of NF1-GAP activity from the total GAP activity.

**Ras Activity Assay**—The Ras activity assay was performed using the Ras-binding domain of Raf-1 bound to agarose in a Ras activation assay kit (Cell Signal Technology) according to the manufacturer's instructions and as described (21).

**RT-PCR Analysis of NF1-GRD**—For NF1-GRD amplification by RT-PCR, primers 5′-CAGAGTTTCCCCCTGCAGCCTGG-3′ (NF1 GRD-sense) and 5′-TTCCGGTCCCCATCGACTTG-3′ (NF1 GRD-antisense) were used. First-strand cDNA, which served as the PCR template, was synthesized from 2 μg of total RNA purified using an RNeasy minikit (Qiagen) from PC12 cells or M-M17–26 cells. Reverse transcription was performed using oligo(dT) primer and Superscript II reverse transcriptase (Invitrogen), and the PCR conditions were as described (8). After PCR, each sample was separated in a 7.5% polyacrylamide gel and stained with ethidium bromide. To quantify the PCR products using a BigDye Terminator chemistry, the bands were scanned and analyzed with MacBas2000 (Fuji Film).

**Antibodies**—An anti-NF1-GAP antibody was generated in female Japanese white rabbits immunized against GST-NF1-GRD (amino acids 1175–1552) and affinity-purified on a formylcellulofine column (Seikagaku Co.) covalently coupled with GST-p120GAP protein, followed by a protein G-Sepharose column. A monoclonal anti-FLAG antibody (M5) was purchased from Sigma, and anti-phospho-p44/42 MAPK and anti-p44/42 MAPK antibodies were purchased from Cell Signaling Technology.

**Neuronal Culture, Transfection, and Immunocytochemistry**—Rat hippocampal cell cultures were prepared as described (22). Neurons were cultured from rat embryonic hippocampal cells by plating at high density on coverslips coated with polyethyleneimine (IWAKI). FLAG-R1276A/R1391K (R1276A/R1391K) was transfections into the neurons with Lipo-fectamine 2000 at 12 h after plating. As a control for the morphological analysis, FLAG and GFP vectors were co-transfected and expressed. For morphological studies, neurons were fixed in 4% paraformaldehyde for 15 min at room temperature and permeabilized with 50 μM ammonium chloride for 5 min, and cells were permeabilized with 0.1% Triton X-100. To identify transfected neurons, cells were immunostained with the monoclonal anti-FLAG antibody M5 (Sigma), followed by a fluorescent dye-conjugated secondary antibody, and analyzed using confocal microscopy (Fluoview, FV500; Olympus). To determine the axons and dendrites of neuronal cells, axonal immunostaining was performed with a rabbit polyclonal anti-tau antibody (Sigma), and anti-mitogen-activated protein antibody (Sigma), respectively, and the morphological criteria of axons (thin, uniform caliber, nonbranching processes) or dendrites (thick, tapering with distant processes)
RESULTS

Measurement of the Specific Ras GAP Activity of Neurofibromin—To analyze the specific GAP function of neurofibromin in neuronal cells, we first established a method for measuring NF1-GAP activity using the anti-NF1-GAP IgG (NF1-GAP Ab), prepared from a rabbit antiserum against GST-NF1-GRD type I depleted of fractions reactive to p120GAP. Immunoblotting using NF1-GAP Ab indicated no cross-reactivity with any cellular proteins other than neurofibromin (Fig. 1F), suggesting...
its specificity for NF1-GAP. The dose-dependent GAP activities of both GST-NF1-GRD and p120GAP (Fig. 1, A and B) detected by the original filtration assay (23) were completely blocked by NF1-GAP Ab, whereas GST-p120GAP was not inhibited (Fig. 1, C and D). We also analyzed the specific effects of NF1-GAP Ab on cellular lysates of Nf1−/− mouse embryo fibroblasts, which had GST-NF1-GRD, p120GAP, or GST proteins added. The GAP activity of GST-NF1-GRD in the Nf1−/− mouse embryo fibroblast extracts was completely inhibited by NF1-GAP Ab to the basal level, but that of p120GAP was not inhibited (Fig. 1E). These results indicate that NF1-GAP Ab specifically inhibits the GAP activity of neurofibromin in cellular extracts, but not p120GAP, and is therefore useful for measurement of cellular specific NF1-GAP. The percentages for the hydrolyzed Ras-[γ-32P]GTP with the reaction of GST-NF1-GRD or the cellular lysates without NF1-GAP Ab were subtracted by those with NF1-GAP Ab and plotted as the specific NF1-GAP activity (Fig. 2, A and B).

GAP Activities of NF1 Type I and Type II in Mammalian Cells—The GAP activities of the two alternatively spliced NF1 isoforms, type I and type II, have previously been studied, and it was reported that NF1-GRD type II resulted in a 50% reduction of the GAP activity compared with that of NF1-GRD type I (24). However, when we analyzed the GST-NF1-GRD type I and type II GAP activities using our system, GRD type I GAP activity was more than 10-fold higher than that of GRD type II at their lower protein concentrations (ng level of protein) (Fig. 2A). This difference between NF1-GRD type I and type II was reproduced in the cellular GAP analysis of FLAG-tagged NF1-GRD type I (FLAG-NF1-GRD type I) and FLAG-NF1-GRD-type II overexpressed in COS7 cells (Fig. 2B). The expression levels of each FLAG-GRD in 25 μg of cell lysate were almost equal in intensity (Fig. 2C). The activity of GRD type I expressed in COS7 cells in 5 μg of cell lysate was more than 10-fold higher than that of GRD type II. These results suggest that the alternative splicing of NF1 type I and type II regulates cellular NF1-GAP activity and may control the Ras-related signals, such as those for cellular proliferation and differentiation.

Relationship of Neurite Outgrowth, Ras Activity, and Endogenous NF1-GAP Activity in PC12 Cells—To study the GAP function of neurofibromin in neuronal cell differentiation, we examined NF1-GAP in PC12 cells after stimulation by several different growth factors. After NGF stimulation, neurite outgrowth occurred in PC12 cells within 24 h, followed by significant neurite extension (Fig. 3B). Neurite outgrowth-bearing PC12 cells that carried processes longer than the cell body diameter represented up to 50% of the total cells after 48 h of NGF stimulation (Fig. 3A). In contrast, PC12 cells treated with EGF showed little morphological change, scored less than 2% of neurite outgrowth-bearing cells, and were similar to non-treated cells (Fig. 3, A and B). In the same experiment, the specific NF1-GAP activities of PC12 cells were shown to increase significantly during 12–48 h after NGF stimulation (Fig. 3C). After 48 h, the NF1-GAP activity in NGF-stimulated cells reached 2.5-fold that before stimulation, whereas that of nonstimulated or EGF-stimulated cells only increased 1.25-fold. The activity of other GAPs obtained after subtraction of the specific NF1-GAP activity from the total GAP activity showed a small increase after NGF stimulation but stayed at a lower level compared with the NF1-GAP activity (50% increase in other GAPs versus 180% increase in NF1-GAP compared with each GAP in the nonstimulated state). The effect of EGF stimulation on the activity of other GAPs was also insignificant and as low as the effect on NF1-GAP (Fig. 3D). Cellular NF1-GAP activity also increased in a dose-dependent manner with NGF stimulation (data not shown), indicating that NF1-GAP activity is responsive to NGF stimulation.

Cellular Ras activity was analyzed using an affinity tech-
niue with Raf-1RBD (Ras-binding domain)-agarose. After NGF stimulation, Ras activity quickly reached the maximum level within 5 min and was down-regulated during 15–60 min (Fig. 4, A and B), as reported previously (25). Surprisingly, upon further stimulation with NGF, Ras activity gradually increased up to more than 10-fold the original level, reached a plateau at around 24 h, and was then down-regulated over 24–72-h stimulation (Fig. 4, A and C). EGF stimulation increased Ras activity quickly and down-regulated it during an early phase in the same manner as NGF stimulation. However, later than 60 min after EGF stimulation, Ras activity regulated at the basal level did not change over 72-h stimulation (Fig. 4, A–C). The Ras activity regulation in PC12 cells in the later phase of NGF stimulation was well correlated with the time-dependent increase in cellular NF1-GAP activity as well as with the increase in neurite-bearing PC12 cells (Fig. 3, A and C). Thus, these results prompted us to speculate that the increase in endogenous NF1-GAP activity in PC12 cells may regulate cellular Ras activity and control neuronal outgrowth of PC12 cells during longer NGF stimulation.

Effects of NGF and EGF on NF1 Type I and Type II Alternative Splicing in PC12 Cells—To elucidate how the NF1-GAP activity was increased by NGF stimulation in PC12 cells, we examined the alternative splicing pattern of NF1 type I and type II in PC12 cells by RT-PCR at the later phase of NGF treatment, compared with the pattern with EGF treatment. Interestingly, the transcriptional levels of both the type I and type II isoforms increased after NGF exposure in a time-dependent fashion, but the rate of increase of type I mRNA was significantly higher than that of type II (Figs. 5A and 6, A and B). Before exposure to NGF, the type I/type II transcript ratio was 22%, but after NGF stimulation, it gradually increased in a time-dependent manner, and after 48 h, the ratio was up to 110% (Figs. 5B and 6C). In contrast, EGF stimulation did not increase the type I/type II ratio, even after 48-h stimulation (Fig. 5, A and B), suggesting that the specific splicing of NF1 type I/type II mRNA could be induced by cellular signals via NGF stimulation in particular. The pattern of the time-depend-
ent increase for type I/type II transcription was very similar to that of the NF1-GAP activity in PC12 cells after NGF stimulation (Fig. 3C). These results indicate that NGF stimulation up-regulates the cellular expression of NF1 type I, and since it possesses a much higher GAP activity than type II, this causes the increase in cellular NF1-GAP activity in PC12 cells.

**NF1-GRD Alternative Splicing Requires Ras Activity**—It has been shown that the initial activation of Ras by NGF stimulation acts as a trigger for differentiation of PC12 cells via the transcriptional up-regulation of several genes. To investigate the mechanism for the transcriptional increase of NF1 type I after NGF stimulation, we analyzed the regulation of NF1-GRD alternative splicing in a subline of PC12 cells (M-M17–26) that stably overexpress a dominant inhibitory Ras mutant, Ha-Ras (S17N). Previous analysis of this subline has shown that the absence of Ras activity completely blocks neurite outgrowth after NGF stimulation (26). As shown in Fig. 6, NF1 type I and type II mRNA levels in untreated M-M17–26 cells were similar to those detected in wild-type PC12 cells when the cells were maintained in the absence of NGF. However, the specific time-dependent increase in NF1 type I mRNA normally observed in response to NGF stimulation was significantly inhibited in M-M17–26 cells (Fig. 6, D–F). These results suggest that Ras activity regulates the alternative splicing of NF1 mRNA after NGF stimulation in PC12 cells, resulting in increasing cellular NF1-GAP activity.

**Mechanism of NGF-Ras-induced NF1-GRD Alternative Splicing**—To understand which Ras-mediated signals induced by NGF stimulation in PC12 cells are involved in NF1 type I/type II alternative splicing, we tested the effects of several kinds of pharmacological inhibitors against signals upstream or downstream of Ras, such as MEK1 inhibitor (PD98059), PI3K inhibitor (LY294002), MEK1/2 inhibitor (U0126), p38 MAPK inhibitor (SB203580), and an inhibitor of the NGF re-
ceptor tyrosine kinase (K252a), on the PC12 cells after NGF stimulation. As shown in Fig. 7, A and B, the inhibitors for both the NGF receptor and PI3K effectively inhibited NGF-Ras-induced NF1-GRD alternative splicing, whereas the other inhibitors had no effects. These results suggest that the NGF-induced Ras-PI3K signal plays an important role in NF1-type I/type II alternative splicing in PC12 cells.

NF1-GRD Mutants Function as Dominant Negative Inhibitors of Endogenous NF1-GAP—Two arginine residues of neurofibromin (Arg1276 and Arg1391) in NF1-GRD, whose alterations have been found in NF1 patients with severe phenotypes, are known to be important for catalysis of NF1-GAP activity (27). Mutational and crystallographic analysis of NF1-GRD revealed that alteration of Arg1276 to lysine (R1276K) increased the binding affinity for Ras 1.85-fold but compromised GAP-stimulated GTP hydrolysis to 1/225. It was also reported that the affinity of the double mutant R1276A/R1391K for the Ras-GTP form was slightly increased by 1.08-fold, but the GAP activity was decreased by 1/1080 compared with wild-type NF1-GRD (27, 28). Based on these results with data from the previous mutational and crystallographic analysis, these mutants are thought to act as dominant negative (DN) inhibitors for endogenous NF1-GRD. To confirm this, we prepared expression plasmids for FLAG-tagged NF1-GRD type I with the R1276K (FLAG-R1276K) and R1276A/R1391K (FLAG-R1276A/R1391K) mutations and analyzed their effects in PC12 cells by overexpression of these mutants. Cellular NF1-GAP activities in transfectants of FLAG-R1276K or FLAG-R1276A/R1391K were extremely low compared with those of NF1-GRD type I (Fig. 8A). FLAG-R1276A/R1391K, in particular, showed the lowest GAP activity in all cells tested, and it was lower than that of the mock vector controls, although the levels of those were extremely small and near the detection limit, suggesting the inhibitory effects of the mutants on endogenous NF1-GAP (Fig. 8A).

Next, we examined whether the mutants could affect the Ras activity and subsequent Raf-MAPK signals in PC12 cells via the suppression of NF1-GAP after NGF stimulation. Twenty-four h after the transfection of FLAG-R1276K, FLAG-R1276A/R1391K, or mock vector into PC12 cells, NGF was added to the cells, and their time-dependent alterations in Ras activities and MAPK phosphorylation levels were analyzed (Fig. 8, B and C). The levels of active Ras bound to the RBD in both the mutant cells overexpressing FLAG-R1276K and FLAG-R1276A/R1391K were apparently increased by NGF treatment in a time-dependent manner, and their levels were significantly higher than those in the mock transfectants, especially during the later phase of NGF stimulation. The phosphorylation levels were also increased in both the mutant cells during NGF stimulation, and their levels were kept higher and longer than those in the mock vector transfectants (Fig. 8, B and C). These results indicate that both the R1276K and R1276A/R1391K mutants inhibit endogenous NF1-GAP function in PC12 cells, which up-regulates Ras activity at the later phase of NGF stimulation, followed by successive sustained activation of MAPK signals, and thus should be useful for studies of the cellular GAP function of neurofibromin as dominant negative inhibitors.
NF1-GAP DN Mutants Block NGF-induced Neurite Extension in PC12 Cells—Neurite outgrowth in PC12 cells is basically divided into two steps. Initiation of neurite outgrowth is the first event, which occurs within 12 h after NGF stimulation (early phase), followed by regulated elongation of the neurite (later phase) (29). NF1 type I transcription and activation were observed after 24 h of NGF stimulation. Therefore, neurofibromin may be classified as a later phase protein in PC12 cells. We speculated that NF1-GAP DN mutants R1276K and R1276A/R1391K would affect NGF-induced neurite elongation in PC12 cells. To test this, PC12 cells were transfected with the NF1-GAP DN mutants, and their morphological changes were analyzed by confocal microscopy at 48 h after NGF stimulation. The induction of neurite elongation was significantly blocked in PC12 cells overexpressing FLAG-R1276K and R1276A/R1391K, whereas cells co-transfected with FLAG mock and GFP vectors induced normal neurite elongation (Fig. 9A). The ratios of neurite-bearing cells to total transfectants were 18.15 ± 4.03% for FLAG-R1276K, 17.65 ± 4.31% for R1276A/R1391K, and 44.45 ± 7.03% for mock vector-transfected cells (Fig. 9B). The transfected mutants with GFP-R1276K and R1276A/R1391K also showed similar effects on neurite elongation to those of the FLAG-tagged mutants (data not shown).

To confirm that these NF1-GAP DN-mutants do not directly interfere downstream of the Raf signals during NGF-induced neurite extension of PC12 cells, we tested the effects of the DN mutants on neurite processes induced by constitutively active Raf (CA-Raf) that can alone promote neurite outgrowth in PC12 cells without NGF stimulation. After co-transfection of CA-Raf (pcDNA3-Myc-cRaf-1CAAX) with NF1-GAP DN mutants (FLAG-R1276K or R1276A/R1391K) or FLAG-Mock into PC12 cells, the morphological changes of these transfected cells were analyzed (Fig. 9C). CA-Raf expressing cells showed aggressive neurite outgrowth within 48 h after transfection without NGF stimulation, regardless of its expression with or without the NF1 DN mutants (Fig. 9C), suggesting that the NF1-GAP DN mutants at least have no effect on the neurite outgrowth via CA-Raf-mediated signal activation. Taken to-
FIG. 9. Effects of NF1-GAP-DNs on neurite outgrowth induced by NGF or cRaf-1CAAX in PC12 cells. A, 24 h after the transfection of FLAG-R1276K (R1276K) and FLAG-R1276A/R1391K (R1276A/R1391K) plasmids or co-transfection of FLAG mock and GFP vectors (control) into PC12 cells, cells were treated with 50 ng/ml NGF for 48 h, reacted with the anti-FLAG monoclonal antibody followed by a fluorescein isothiocyanate-conjugated secondary antibody, and analyzed by confocal microscopy to detect cells overexpressing the proteins (left panels). Phase-contrast micrographs are shown in the right panels, and the arrows indicate the fluorescence-positive cells in the corresponding left panel. B, quantification of the neurite outgrowth of PC12 cells transfected with GRD-DN mutants or a mock vector. The percentages of cells whose processes are longer than their cell bodies in the total transfectants (200 cells each) are shown. Data represent means ± S.D. from three experiments. C, PC12 cells were co-transfected with Myc-cRaf-1CAAX and FLAG vector alone (top), Myc-cRaf-1CAAX and FLAG-R1276K (middle), or Myc-cRaf-1CAAX and FLAG-R1276A/R1391K (bottom), as indicated. After 48 h, cells were fixed and stained with a mouse monoclonal anti-Myc antibody and a rabbit polyclonal anti-FLAG antibody. Samples were double-stained with a Texas Red-conjugated secondary antibody for mouse IgG (red) and a fluorescein isothiocyanate-conjugated secondary antibody for rabbit IgG (green) and analyzed with confocal fluorescence microscopy.
Neurofemin Regulates Neuronal Differentiation

To elucidate this, we examined whether an NF1-GAP DN mutant could affect the function of rat hippocampal embryonic neurons, especially with respect to neuronal differentiation. Rat embryonic day 18 hippocampal primary neurons were transfected with FLAG-R1276A/R1391K, and their morphological changes were analyzed during differentiation compared with those of neurons co-transfected with the FLAG mock and GFP vectors. At 72 h after transfection, the mock vector-transfected neurons showed normal polarized differentiation of axons and dendrites at stage 4 (30). In contrast, in the neurons overexpressing FLAG-R1276A/R1391K, dendritic elongation and branch formation were significantly decreased (Fig. 10, A and B). Neurons overexpressing FLAG-R1276A/R1391K were shortened in axon length by an average of 36% of that of control axons (Fig. 10G) and also shortened in dendrite length by 47% of that of control dendrites (Fig. 10H). Furthermore, in the neurons of the transfected mutant, the numbers of secondary axonal branches were significantly decreased compared with those of the control neurons (Fig. 10F). On days 6 and 7 after transfection, the mutant appeared to retract the dendrite formation and axonal elongation compared with the control (Fig. 10, C–F). These results obviously indicate that NF1-GAP plays an important role in the regulation of hippocampal neuronal differentiation, especially in the elongation and extension of both axons and dendrites that coordinate the development of the neuronal cellular structure and network.

In this study, we demonstrated a novel GAP function of neurofemin on the neurite outgrowth of neuronal cells, such as PC12 cells and rat embryonic hippocampal neurons. We showed that NGF-induced Ras activation up-regulates NF1-GAP activity via induction of the NF1-GrD type I splicing variant, and it controls the active state of Ras by feedback regulation, leading to normal neurite extension in neuronal cells at the later phase of the neuronal differentiation process.

Cellular Ras regulation by NF1-GAP has been speculated to be one of the most important events in neuronal development. However, it had not been practically elucidated either how to evaluate the cellular NF1-GAP activity or how neurofemin functions in neurons. Therefore, we first developed a concise procedure for semiquantification of cellular NF1-GAP activity. Originally, neurofemin GAP activity in bovine cerebral lysates was analyzed after immunoprecipitation with an anti-C-terminal NF1 antibody (23) that needed large amounts of cellular neurofemin. In previous reports, including our study, the C-terminal and other regions, such as the N-terminal Cys-Ser-rich and GrD domains, have been suggested to be structurally critical for targeting by cellular interacting proteins and functionally important for regulating the cellular signals (15, 16, 31). Thus, antibodies against not only the neurofemin C-terminal but also the other domains may alter the neurofemin activity during immunoprecipitation and cause difficulties in obtaining quantitative values for cellular NF1-GAP. Although measurement of total cellular GAP activities by the original filtration assay (23) and Ras activity by TLC analysis (32) have been widely used, these assays do not give specific information on NF1-GAP activity. Our procedure is a simple, sensitive direct method for cellular NF1-GAP analysis and can be applied to measure NF1-GAP activity in clinical samples.

Using our method, we found that NF1-GAP activity in PC12 cells increased in a time-dependent manner after NGF stimulation. After the up-regulation of NF1-GAP activity, Ras activity was gradually decreased during the later phase of NGF stimulation (24–72 h). For Ras activity regulation in the early phase of NGF stimulation, it has been reported that a quick increase in Ras activity was subsequently down-regulated during 15–60 min in PC12 cells (25). However, the Ras regulation, especially in the later phase of NGF stimulation, has not been reported. Our precise time course analysis of PC12 cells revealed the surprising result that the Ras activity was dynamically regulated during the early to later phase of NGF stimulation. After a quick up- and down-regulation of Ras activity in the early phase, it gradually increased, reached a plateau, and was then down-regulated over 24–72 h of stimulation, in contrast to the Ras activity after EGF stimulation (Fig. 4). These specific Ras regulations during the later phase of NGF stimulation in PC12 cells were confirmed by the analysis using cells expressing NF1-GAP DN mutants that block endogenous NF1-GAP and could thus inhibit the down-regulation of cellular Ras activity during the later phase of NGF stimulation (Fig. 8). These inhibitions of Ras regulation by the NF1-GAP DN mutants were followed by inhibition of neurite extension in the mutant cells during the later phase of NGF stimulation (Fig. 9, A and B). These results suggest that the dynamic balancing of activities between guanine nucleotide-exchanging factor and GAP may regulate Ras activity during the initiation and maintenance of neurite outgrowth. Previously, in human SK-N-SH neuroblastoma cells, it was shown that differential induction by retinoic acid decreased the cellular Ras GTP level with a correlation to increases in the expression of NF1 mRNA (33). Our findings that the NF1-GAP activity increased concomitantly with neurite outgrowth and Ras down-regulation in PC12 cells in the later phase of NGF stimulation suggest that the Ras activity regulation by NF1-GAP in the later phase may be critical for neuronal cell differentiation.

Interestingly, the up-regulation of NF1-GAP activity at the later phase was correlated with transcriptional elevation of the NF1 type I isoform. The presence of NF1-GAP regulation by alternative splicing of NF1 type I and type II and their implication for neuronal cellular differentiation were originally demonstrated in our previous report (6). The relative expression of these two isoforms in neuronal cell cultures and developing animal brains were widely analyzed in different ways and under various conditions. It was recently reported that NGF leads to an increase in the NF1 type I/II mRNA ratio in PC12 cells and that this was correlated with a decrease in cellular proliferation (8), supporting our hypothesis that up-regulation of the type I isoform plays a role in the differentiation of PC12 cells; in other words, the transcriptional switching from NF1 type II to type I induced by NGF stimulation could be one of the crucial functions in PC12 cells for promotion toward neuronal differentiation through cellular growth inhibition.

(left panel). Neurite outgrowth of PC12 cells co-transfected with Myc-cRaf-1CAAX and GrD-DN mutants was quantified (right panel). The percentage of cells whose processes were longer than their cell bodies in the total transfecants or co-transfecants (200 cells each) are shown. Data are means ± S.D. from three experiments.
The NF1 type II isoform contains a lysine-rich 21-amino acid insertion near the catalytic hydrophobic region of NF1-GRD (6), and several experiments using purified recombinant proteins and a yeast system have shown that the alternative splicing of NF1-GRD affects the GAP activity (34). However, alteration in GAP activity by the alternative splicing has not been demonstrated in living mammalian cells. Here, we showed differences in GAP activity between the FLAG-tagged fragments of NF1-GRD type I and type II in a mammalian expression system. Endogenous NF1-GAP activity in COS7 cells was detected as only a trace level. The expression levels of both the GRD proteins in COS7 cells were almost equal, but the GAP activity of NF1-GRD type I in cells was remarkably higher than that of type II (Fig. 2). Thus, even a small expression of NF1 type I by alternative splicing may produce significant effects on Ras/Ras-related signals.

Expression of a dominant inhibitory form of Ras abolished the NF1 type I mRNA induction as well as the neurite outgrowth in NGF-treated PC12 cells. Conversely, the expression of NF1-GAP DN mutants maintained the Ras activity at a higher level in the later phase of NGF stimulation (Fig. 8), and this was correlated with neurite outgrowth inhibition of PC12 cells (Fig. 9, A and B). These results all prove our proposal that the induction of NF1 alternative splicing triggered by Ras activation may be a negative feedback function in the later phase for the regulation of the Ras activity itself toward the promotion of neurite outgrowth of PC12 cells.

Several transcriptional inductions and alternative splicings triggered by NGF stimulation followed by Ras activation in PC12 cells have been demonstrated (35). However, the specific mechanisms of these alternative splicings linked to the neurite outgrowth have not been studied precisely. Here, we have shown the first evidence that NF1 alternative splicing triggered by the specific activation of the Ras-PI3K pathway induced by NGF, but not EGF, and it was linked to process formation in PC12 cells. These results are supported by previous reports that elevation of PI3K activity was necessary for neurite outgrowth of PC12 cells (36). Further investigation is required to explore the underlying mechanisms of how Ras induces the NF1 type I alternative splicing as well as the

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specific process formation of PC12 cells.

It is well known that overexpression of constitutively active Ras or specific activated forms downstream of the Ras signal, such as cRaf-1CAAX, can induce neurite outgrowth of PC12 cells without NGF stimulation (37–39). However, from our observations, it seems that the morphological patterns of neurite outgrowth induced without NGF stimulation are different from those derived with NGF stimulation. For example, as shown in Fig. 9C, constitutively active cRaf-1CAAX-transfected cells could induce aggressive neurites without NGF stimulation, but the majority of these possessed many processes with thinner and shorter spikes, and some of them expressed advanced corns with filopodia- and lamellipodia-like processes, which were not found in NGF-treated normal cells. In addition, we also noticed that Ras12V transfectants expressed abnormal processes with many aggressive filopodia-like neurites directly from the cell body and many short branches compared with the normal processing induced by NGF using our PC12 cells.7 Based on these observations, we propose that the mechanisms of neurite outgrowth induced by dominant active Ras or Ras-related signals without NGF stimulation could be different from those induced by NGF. In other words, it is suggested that the dynamic balancing of Ras and Ras-related signals by NF1-GAP during long NGF stimulation could be important for the maintenance of the normal neurite extension processes.

In NF1 patients, a cognitive deficit has frequently been found as one of the typical NF1-related phenotypes. For example, 4–8% of NF1 children display mental retardation, and 30–60% show specific learning disability (40). Consistent with the observations in human NF1 patients, Drosophila homozygotes with NF1 null mutation showed significantly decreased olfactory learning performance compared with the wild type (9), and NF1 heterozygous (NF1+/−) mice displayed spatial learning disability by the Morris water maze paradigm, a test for hippocampus-dependent learning (10, 11). Furthermore, immunohistochemical analysis revealed that neurofibromin is expressed in cell bodies and axons but is highly enriched in dendrites (41). These reports are highly relevant to our observation that phenotypic disorders in axonal elongation and dendritic expression were found in NF1-GAP DN mutant-transfected hippocampal neurons at differentiation days 4–7 (Fig. 10). It is a well known fact that loss of the NF1 gene leads to aberrant activation of Ras signals (42). Interestingly, a recent report showed that the learning deficits of NF1+/− mice can be rescued by a genetic cross with a heterozygous K-Ras mutant that also shows a genetic cross with a heterozygous K-Ras mutant which rescues abnormal processes with many aggressive filopodia- and lamellipodia-like processes, which were not found in NGF-treated normal cells.
Neurofibromatosis Type I Tumor Suppressor Neurofibromin Regulates Neuronal Differentiation via Its GTPase-activating Protein Function toward Ras
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