

Identification of a Dominant Negative Mutant of Sprouty That Potentiates Fibroblast Growth Factor- but Not Epidermal Growth Factor-induced ERK Activation*

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Atsuo Sasaki^{‡§}, Takaharu Taketomi^{‡¶}, Toru Wakioka^{‡§}, Reiko Kato^{‡§}, and Akihiko Yoshimura^{‡§||}

From the [‡]Division of Molecular and Cellular Immunology, Medical Institute of Bioregulation, and [¶]Division of Oral and Maxillofacial Oncology, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan, and the [§]Institute of Life Science, Kurume University, Aikawa-machi 2432-3, Kurume 839-0861, Japan

Various mitogenic stimuli such as epidermal growth factor (EGF), fibroblast growth factor (FGF), and phorbol 12,13-dibutyrate (PDBu) activate the Ras-Raf-MEK-ERK pathway, but the regulatory mechanism of this pathway remains to be investigated. Here we found that in 293 cells, mammalian Sprouty2 and Sprouty4 were rapidly induced by EGF, FGF, and PDBu in an ERK pathway-dependent manner. Forced expression of Sprouty2 and Sprouty4 inhibited FGF-induced ERK activation but did not affect EGF- or PDBu-induced ERK activation. To examine whether endogenous Sproutys were also selective inhibitors, we generated a dominant negative form of Sprouty2 (Y55A) and Sprouty4 (Y53A) in which conserved tyrosine residues were mutated. These mutants reverted the suppressive effect of both Sprouty2 and Sprouty4 but not that of RasGAP or SPRED (Sprouty-related EVH1 domain-containing protein), another Sprouty-related Ras suppressor. Expression of dominant negative Sprouty2 and Sprouty4 enhanced and prolonged FGF- but not EGF-induced ERK activation in 293 cells. In PC12 cells, endogenous Sprouty4 was also induced by FGF. Overexpression of wild-type Sprouty4 blocked FGF-induced differentiation, whereas Y53A-Sprouty4 enhanced it. These observations suggest that endogenous Sprouty2 and Sprouty4 are physiological negative feedback regulators of growth factor-mediated ERK pathway and that there are Sprouty-sensitive and -insensitive ERK activation pathways. Finding a dominant negative form of Sproutys will facilitate the study of the molecular mechanism and physiological function of Sproutys.

Cellular growth and differentiation are controlled by multiple extracellular signals, many of which activate the Ras/mitogen-activated protein (MAP)¹ kinase cascade (1–4). Ras directly in-

teracts with and activates Raf. Raf phosphorylates and activates MEK, which in turn phosphorylates and activates MAP kinases including ERK1 and ERK2 (5–7).

Growth factor-induced ERK activity is regulated in multiple ways (8–10). Recently, a novel negative regulator of the growth factor Ras signaling pathway, the *Drosophila* Sprouty (*dSprouty*) gene, was identified as an inhibitor of FGF receptor signaling using genetic screens (11). *dSprouty* inhibits not only FGF receptor signaling but also other receptor tyrosine kinases (RTKs) such as EGF receptor, Torso, and Sevenless (12–14). Subsequently, four mammalian Sprouty genes have been identified based on sequence similarities to *dSprouty* (11, 15, 16). A conserved function between *dSprouty* and vertebrate Sproutys was implicated in organogenesis (16, 17) and angiogenesis (18). However, Sprouty1 and Sprouty2 inhibit FGF- and VEGF-induced ERK activation, but they do not affect EGF-induced or phorbol 12-myristate 13-acetate-induced ERK activation (18, 19). These findings suggest the possibility that mammalian Sprouty proteins are not general inhibitors of RTK-induced ERK signaling but are rather selective inhibitors of RTK signaling. However, most of these studies used an overexpression system, and biochemical analysis of a negative feedback function of endogenous Sproutys remains to be investigated.

We recently identified novel family members of a Sprouty-related EVH1 domain-containing protein, SPRED (20). SPRED proteins contain an N-terminal EVH-1 (Ena/VASP homology-1) domain and a conserved cysteine-rich domain that is related to Sprouty. Like Sprouty, SPREDS also down-regulate Ras/ERK signaling (20). However, the molecular mechanism of Ras/MAP kinase suppression and the functional relationship between SPRED and Sprouty at endogenous protein levels have not been clarified.

In the present study, we have demonstrated that Sprouty2 and Sprouty4 are downstream targets of ERK and selective inhibitors of growth factor-mediated ERK activation. The results strongly suggest the presence of a Sprouty-sensitive and -insensitive ERK activation pathway.

EXPERIMENTAL PROCEDURES

Materials and Cells—Human EGF and bFGF were purchased from PeproTech EC, Ltd. Polyclonal anti-ERK2 and anti-Myc antibodies were from Santa Cruz Biotechnology. Anti-Flag (M2) antibody and PDBu were from SIGMA. Anti-phospho-ERK1/2 antibody was from Cell Signaling. Rabbit serum against mouse Sprouty4 was generated by injecting glutathione S-transferase-fused polypeptide corresponding to amino acids 1–160 of Sprouty4.

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|| To whom correspondence should be addressed: Division of Molecular and Cellular Immunology, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Tel.: 81-92-642-6822; Fax: 81-92-642-6825; E-mail: yakihiko@bioreg.kyushu-u.ac.jp.

¹ The abbreviations used are: MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAP/ERK kinase; RTK, receptor tyrosine kinase; EGF, epidermal growth factor; FGF, fibroblast growth factor; bFGF, basic FGF; NGF, nerve growth

factor; SPRED, Sprouty-related EVH1 domain-containing protein; PDBu, phorbol 12,13-dibutyrate; MOPS, 4-morpholinepropanesulfonic acid; WT, wild type; *dSprouty*, *Drosophila* Sprouty gene; FITC, fluorescein isothiocyanate.

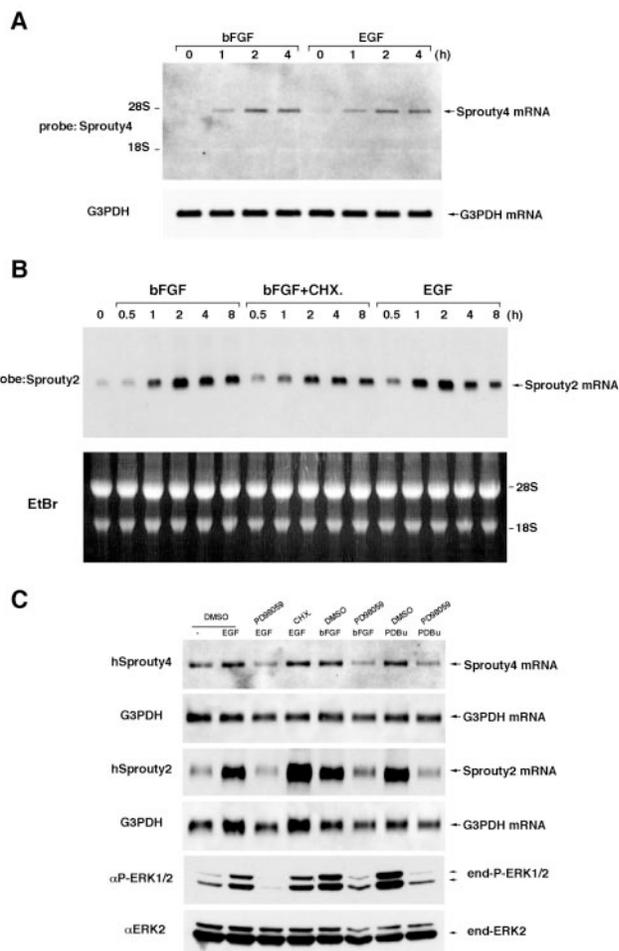


FIG. 1. Sprouty2 and Sprouty4 are induced by mitogenic stimuli via the ERK pathway. *A* and *B*, 293 cells were treated with 25 ng/ml EGF or bFGF for indicated periods in the presence or absence of 50 μ g/ml cycloheximide (*CHX*). Cycloheximide was added 10 min before FGF stimulation. For the detection of Sprouty4 mRNA, poly(A)-RNA was isolated from 40 μ g of total RNA and subjected to Northern blot analysis (*upper panel*). Blots were reprobed with glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) riboprobes (*lower panel*). *B*, for Sprouty2 detection, 20 μ g of total RNA was electrophoresed, stained with ethidium bromide (*EtBr*, *lower panel*), and blotted with a human Sprouty2 riboprobe (*upper panel*). *C*, 293 cells were pretreated with or without 40 μ M PD98059 in Me₂SO (*DMSO*) for 1 h or 50 μ g/ml cycloheximide for 10 min. Cells were then stimulated with 25 ng/ml EGF or bFGF or 1 μ g/ml PDBu for 2 h in the presence of reagents. Cells were analyzed by Northern blot analysis (*upper four panels*) as described in *A* and Western blot with indicated antibodies (*lower two panels*).

Luciferase Assay—Elk-1 activation was measured as described (21). In all reporter assays, 2×10^5 293 cells were plated on 6-well dishes and transfected by the calcium phosphate method.

Plasmid Construction—Full-length cDNA of human Sprouty2, human p120-RasGAP, mouse Sprouty4, and dSPRED was cloned by polymerase chain reaction with primers designed against the published sequence from human placenta, 11.5 day mouse embryo, or 0–4-h *Drosophila* embryo cDNA libraries. Wild-type Sprouty2, dSPRED cDNA, and mutants were subcloned into *EcoRI/XhoI* sites of pcDNA3 with 6xMyc epitope tag at the N terminus or into *EcoRI/SalI* sites of Flag-pCMV2 (Kodak).

Northern Hybridization—cDNAs of mouse Sprouty4 corresponding to codon 1–196, human Sprouty2 corresponding to codon 1–204, and human Sprouty4 corresponding to nucleotides 8–768 of published sequence AF227516 (GenBank accession number) were cloned in T-vector (Promega) and used for riboprobe production. Northern blot analysis was performed as described (21).

In Vitro ERK2 Kinase Assay—Transiently transfected 293 cells were lysed in lysis buffer A (20 mM Hepes, pH 7.3, 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM dithiothreitol, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride,

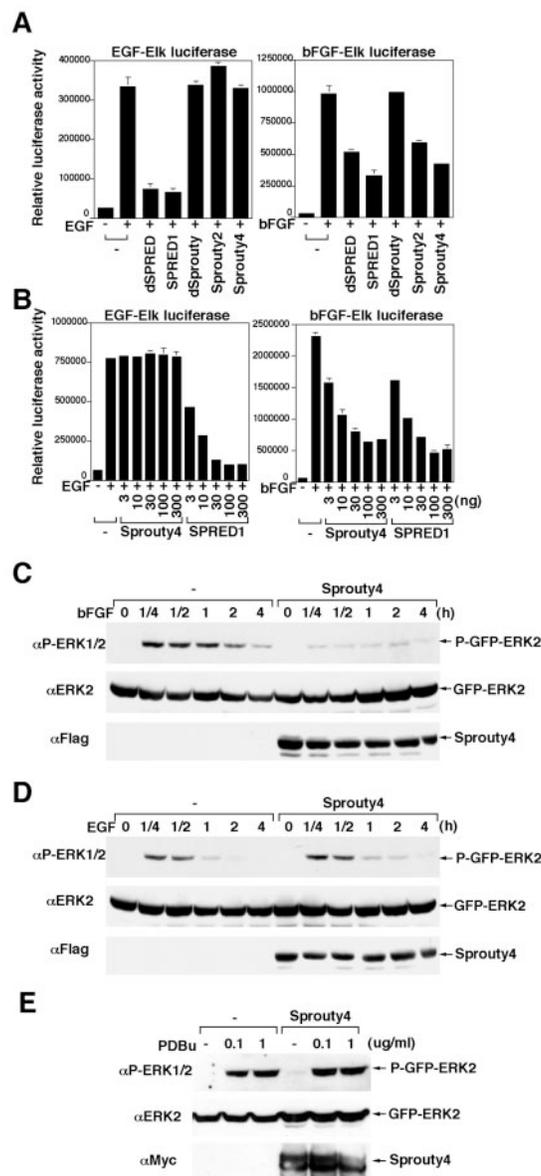


FIG. 2. Sprouty2 and Sprouty4 inhibit FGF- but not EGF-induced ERK activation. *A*, Flag-tagged SPRED or Sprouty plasmids were co-transfected into 293 cells with Elk-1 reporter plasmids. Cells were treated with (+) or without (–) 25 ng/ml EGF or bFGF for 6 h and then analyzed with the luciferase assay. The results presented are for one experiment assayed in duplicate. Similar results were obtained in three independent experiments. *B*, indicated amounts of Flag-Sprouty4 and Flag-SPRED1 plasmids were transfected and assayed as in *A*. *C–E*, GFP-ERK2 (1 μ g) was transfected with a control or a Sprouty4 (1 μ g) vector into 293 cells. After 24 h, cells were treated with 25 ng/ml bFGF (*C*) or EGF (*D*) for indicated times or indicated amounts of PDBu (*E*) for 1 h. Then, cell extracts were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted with indicated antibodies. Similar results were obtained in three independent experiments.

and 1% aprotinin) and immunoprecipitated with anti-Flag antibody. Immunoprecipitates were washed three times with buffer A and once with buffer B (20 mM MOPS (pH 7.2), 25 mM β -glycerol phosphate, 5 mM EGTA, 1 mM sodium vanadate, and 1 mM dithiothreitol). ERK2 kinase activity was assayed in buffer B containing 10 μ Ci of [γ -³²P]ATP, 100 μ M cold ATP, 25 mM magnesium chloride, and 5 mg/ml myelin basic protein (MBP; Upstate Biotechnology, Co.) for 10 min at 30 °C. Reaction products were resolved by SDS-polyacrylamide gel electrophoresis.

RESULTS AND DISCUSSION

Sprouty2 and Sprouty4 Are Induced by Mitogenic Stimuli—After stimulation of human embryonic kidney fibroblast 293 cells with bFGF and EGF, Sprouty2 and Sprouty4 expression

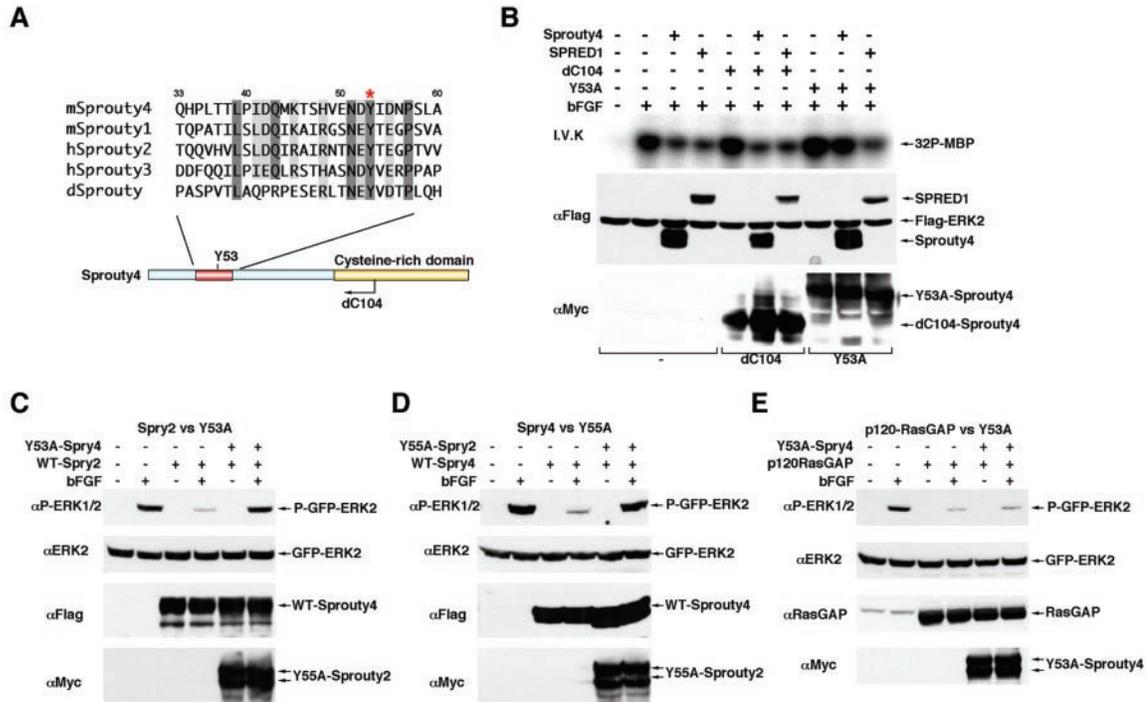


FIG. 3. Mutation of conserved tyrosine residue of Sprouty confers dominant negative activity against WT Sproutys but not SPRED or RasGAP. *A*, amino acid alignment of the five Sprouty proteins with mSprouty4. Conserved tyrosine residues are indicated with a red asterisk above the amino acid sequences in the N-terminal region. *dC104* indicates the deletion of 104 amino acids from the C terminus. *B*, Flag-ERK2 was transfected into 293 cells with (+) or without (-) Flag-Sprouty4 or Flag-SPRED1 in the presence of the Myc-dC104 mutant or the Myc-Y53A mutant. Cells were treated with 25 ng/ml bFGF for 1 h, and Flag-ERK2 proteins were immunoprecipitated with the anti-Flag monoclonal antibody for *in vitro* kinase assay (*I.V.K.*). Cell extracts were also analyzed by Western blot analysis with the indicated antibodies. *C–E*, GFP-ERK2 was transfected into 293 cells with the indicated plasmids. Cells were treated with 25 ng/ml bFGF for 20 min, and then cell extracts were analyzed by Western blots with the indicated antibodies.

was induced rapidly (Fig. 1, *A* and *B*). The mRNA accumulation was seen 1 h after stimulation and was not inhibited by the treatment with cycloheximide (Fig. 1, *B* and *C*), a general translation inhibitor, indicating that *Sprouty2* and *Sprouty4* are early-response genes. To investigate the regulation of Sprouty gene expression, we used extracellular stimulation that activates the ERK pathway. PDBu activated ERK1/2 and induced a strong accumulation of both *Sprouty2* and *Sprouty4* mRNA (Fig. 1*C*). We confirmed PDBu-induced *Sprouty4* protein accumulation by immunoblotting (see Fig. 4*C*). On the other hand, in the presence of PD98059, a specific inhibitor of MEK1, the ability of EGF, FGF, and PDBu to induce *Sprouty2* and *Sprouty4* mRNA was significantly attenuated (Fig. 1*C*). These data suggest that activation of the ERK pathway by mitogenic stimuli induces *Sprouty2* and *Sprouty4* in cultured cells. It has been reported that during embryogenesis the expression of each *Sprouty1–4* gene is associated with FGF signaling in both mouse and chick embryos, even though there are significant differences in the expression pattern among them (16, 22). Recently, Impagnatiello *et al.* (19) reported that murine *Sprouty1* mRNA was transiently down-regulated by FGF2 treatment in endothelial cells (19). Therefore, in addition to spatial and temporal regulation, each Sprouty gene might be differentially regulated at the transcriptional or post-transcriptional levels.

***Sprouty2* and *Sprouty4* Blocked FGF- but not EGF- or PDBu-mediated ERK2 Activation**—To investigate the functional difference in the inhibitory activity of Sproutys for several growth factors, we expressed the dSprouty, human *Sprouty2*, and murine *Sprouty4* proteins under a constitutive heterologous promoter in 293 cells. SPREDs that contain the Sprouty-related domain were also examined (20). As one of the nuclear targets of ERK is Elk-1, a transcription factor of the Ets family, EGF-

induced activation of ERK can be monitored by measuring the rate of Elk-1-dependent transcription. The results show that expression of fly (dSPRED) and mouse SPRED (SPRED1) completely blocked the EGF-mediated activation of Elk-1, whereas expression of fly and mammalian Sproutys could not inhibit EGF-induced Elk-1 activation at all (Fig. 2*A*, left panel). By contrast, under the same experimental conditions, *Sprouty2* and *Sprouty4* as well as SPREDs suppressed bFGF-induced Elk-1 activation (Fig. 2*A*, right panel). Interestingly, although dSprouty has been shown to be a common RTK-induced ERK inhibitor, it did not function in the 293 human cell system (Fig. 2*A*).

To confirm the selective effect of *Sprouty4* on FGF- and EGF-induced ERK activation, we titrated an amount of *Sprouty4* and SPRED1 plasmids and found that *Sprouty4* could not inhibit EGF-induced Elk-1 activation (Fig. 2*B*, left panel) but suppressed FGF signaling in a dose-dependent manner (Fig. 2*B*, right panel). The inhibitory effect of *Sprouty4* on Elk-1 activity was about 70% at most, and *Sprouty4* could not completely inhibit Elk1 activity even when the amount of plasmid was increased. *Sprouty4* inhibited, but not completely, FGF-induced phosphorylation of the conserved TEY motif within the activation loop of ERK (Fig. 2*C*). Suppression of FGF-induced ERK kinase activity was confirmed by using an *in vitro* kinase assay (see Fig. 3*B*). Again, *Sprouty4* did not completely inhibit ERK activation. On the contrary, *Sprouty4* could not reduce EGF-induced ERK phosphorylation but rather slightly enhanced it (Fig. 2*D*). Similar results were obtained with *Sprouty2* (data not shown). We also found that *Sprouty2* and *Sprouty4* suppressed platelet-derived growth factor receptor (PDGFR) and c-Kit (data not shown) but not PDBu-triggered ERK activation (Fig. 2*E*). These data suggest that EGF and PDBu activate ERK mainly through a Sprouty-resistant

pathway, whereas FGF and some other RTKs utilize both a Sprouty-sensitive and -resistant pathway.

Dominant Negative Mutation in the Conserved Tyrosine Residue of Sprouty2 and Sprouty4—To investigate the roles of endogenous Sproutys on ERK activation, we searched the mutations of Sprouty4 that have a dominant negative effect against wild-type (WT) Sprouty4. Among the mutants we created, we identified a dominant negative effect by Y53A-Sprouty4, which carries the substitution of alanine for tyrosine 53. This Tyr⁵³ was conserved among Sprouty proteins (Fig. 3A) and was essential for the function of other Sproutys.² In contrast, C-terminal truncation resulted in just a loss-of-function mutant. As shown in Fig. 3B, the dC104 mutant of Sprouty4 did not affect the inhibitory effect of WT-Sprouty4 and SPRED1. Although the overexpression of the Y53A-Sprouty4 mutant overcame the inhibitory effect of WT-Sprouty4 but not SPRED1 (Fig. 3B). Y53A-Sprouty4 could also overcome the inhibitory effect of WT-Sprouty2 (Fig. 3C). Y55A-Sprouty2, which carries the same mutation as Y53A-Sprouty4, inhibited WT-Sprouty2 and WT-Sprouty4 activity (Fig. 3D and data not shown). These data suggest that the inhibitory mechanisms or targets are common between Sprouty2 and Sprouty4. Because dSprouty has been shown to interact with RasGAP *in vitro* (12), we examined the effect of Y53A-Sprouty4 on RasGAP. As shown in Fig. 3E, Y53A-Sprouty4 did not affect the RasGAP-induced suppression of ERK activation. This suggests that Y53A substitution is a dominant negative-type mutation that is specific to Sproutys. The molecular mechanisms of the dominant negative action of the Y53A mutant are currently not clear. However, our preliminary data suggest that Sprouty2 and Sprouty4 form homo- and hetero-oligomers through their cysteine-rich regions and that these dominant negative Sproutys still bind to WT-Sprouty2 and Sprouty4.² Thus, we speculate that Sproutys function as oligomers and that dominant negative action may be achieved by replacing the WT-Sproutys with a nonfunctional mutant.

Sprouty2 and Sprouty4 Are Negative Feedback Regulators of the FGF-induced ERK Pathway—To determine whether endogenous Sproutys are involved in regulating the FGF-induced ERK pathway, we transfected the dominant negative mutant Sproutys, and then ERK activation was measured with an anti-phospho ERK antibody (Fig. 4, A and B). Transfection of Y53A-Sprouty4 and Y55A-Sprouty2 enhanced and sustained FGF-induced ERK activation (Fig. 4A). By contrast, under the same conditions, it did not enhance EGF-induced ERK activation (Fig. 4B). Because WT-Sproutys affect FGF- but not EGF-induced ERK activation (Fig. 2), the selective effect of Y53A-Sprouty4 and Y55A-Sprouty2 on FGF and EGF supports the dominant negative effect of these mutants against the endogenous Sproutys.

Next we examined whether prior-expression of endogenous Sproutys suppresses FGF-induced ERK activation. Although PDBu induced Sprouty2 and Sprouty4 (Fig. 1C), they did not affect PDBu-induced ERK activity (ref.18 and Fig. 2E). Thus, we treated 293 cells with PDBu for 8–12 h to accumulate endogenous Sproutys, and then cells were stimulated with EGF or FGF (Fig. 4C). ERK activity returned to basal levels, but Sprouty4 protein remained at high levels after PDBu treatment (Fig. 4C, lower panel). EGF-induced ERK activation was slightly enhanced by PDBu treatment, whereas FGF-induced ERK activation was partially suppressed (Fig. 4C). This PDBu-mediated down-regulation of FGF-induced ERK activation was not observed when Y53A-Sprouty4 was overexpressed (Fig.

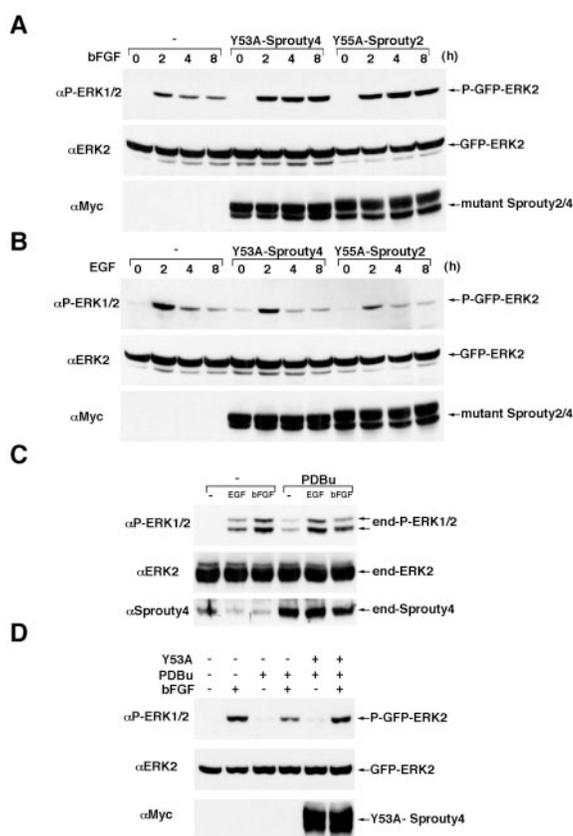


FIG. 4. Dominant negative Sproutys enhanced bFGF- but not EGF-induced ERK2 activation. A and B, GFP-ERK2 was transfected with or without a Myc-Y53A Sprouty4 or a Myc-Y55A Sprouty2 vector into 293 cells. After 24 h, cells were treated with 25 ng/ml bFGF (A) or EGF (B) for the indicated times, and then cell extracts were analyzed by Western blots with the indicated antibodies. Similar results were obtained in three independent experiments. C, cells were prestimulated with or without 500 ng/ml PDBu for 8 h and then stimulated with 25 ng/ml EGF or bFGF for 15 min. Total cell extracts and immunoprecipitates with anti-Sprouty4 antibody were analyzed with the indicated antibodies. Similar results were obtained in two independent experiments. D, GFP-ERK2 was transfected with or without Myc-Y53A Sprouty4 vector into 293 cells. After 24 h, cells were pretreated with 500 ng/ml PDBu for 12 h and then stimulated with 25 ng/ml bFGF for 20 min. Cell extracts were analyzed by Western blots with the indicated antibodies.

4D). These data suggest that the antagonistic effect of PDBu on FGF-induced ERK activation is, at least in part, attributed to the Sprouty proteins induced by PDBu. Therefore, we propose that Sproutys are a negative feedback regulator of ERK but that they exhibit different suppressive effect on ERK activity induced by different stimuli.

Finally, we examined the physiological function of Sproutys on growth factor-induced neurite outgrowth of PC12 pheochromocytoma cells, which is dependent on the ERK pathway (23, 24). Sprouty4 mRNA was strongly induced in response to bFGF and NGF in rat PC12 cells (Fig. 5A). Then, we examined the effect of WT-Sprouty4 or the Y53A dominant negative mutant on an FGF-mediated neurite outgrowth in PC12 cells. As shown in Fig. 5B, WT-Sprouty4 blocked the neurite outgrowth induced by bFGF. In contrast, Y53A increased the number of differentiated cells at low bFGF concentration (Fig. 5C). Similar results were obtained for NGF-induced differentiation (data not shown). Taken together, these results indicate that Sprouty4 and probably other Sproutys are among the negative feedback regulators of FGF and NGF signaling in PC12 cells. As shown in these experiments, the dominant negative mutant

² A. Sasaki, T. Taketomi, T. Wakioka, R. Kato, and A. Yoshimura, manuscript in preparation.

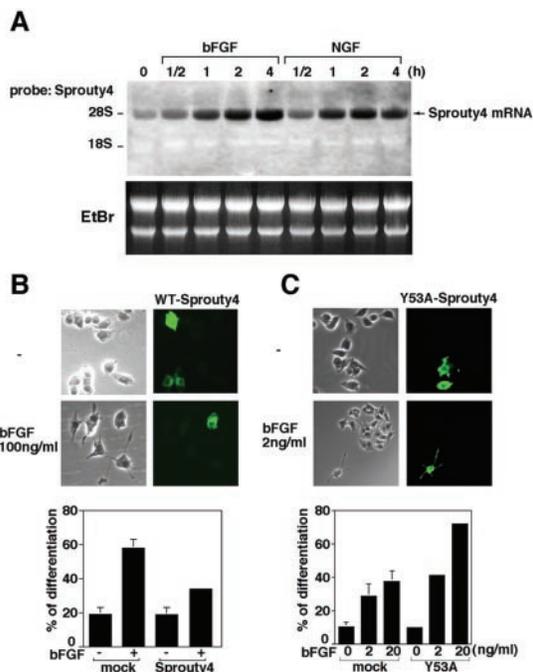


FIG. 5. Sprouty4 is induced by FGF and negatively modulates FGF-induced differentiation of PC12 cells. *A*, PC12 cells were treated with 100 ng/ml bFGF or NGF for the indicated periods, and total RNA was then isolated from them. RNA samples (20 μ g) were stained with ethidium bromide (*EtBr*, lower panel) and blotted with a mouse Sprouty4 riboprobe (upper panel). *B*, Myc-WT-Sprouty4 was transiently transfected into PC12 cells. After 24 h, cells were treated with 100 ng/ml bFGF for 3 days, and then cells were fixed and stained with anti-Myc polyclonal antibody and FITC-labeled secondary antibody (upper panel). Neurite outgrowth was quantified by scoring the number of cells with neurites that were longer than the one-cell bodies among FITC-positive cells (graph, lower panel). Similar results were obtained in three independent experiments under blind testing. *C*, Myc-Y53A-Sprouty4 was transfected into PC12 cells, and cells were treated with 2 ng/ml (upper panel) or indicated concentrations (graph, lower panel) of bFGF for 3 days. Neurite outgrowth was visualized by immunofluorescence microscopy (upper panel) or quantified by scoring the number of cells among FITC-positive cells (lower panels).

Sprouty may be a useful tool for the study of physiological function of Sproutys.

Previous studies suggested the presence of Sprouty-sensitive and -resistant ERK activation pathways in response to different stimuli (18, 19, 25). However, these studies used overexpression of WT-Sproutys, and the effect of endogenous Sproutys on ERK activity remained to be investigated. Because multiple Sprouty genes are induced by a single stimulus, and these genes have similar effect on ERK, the effect of antisense oligonucleotides or disruption of the single gene may be compensated by others. In the present study, we have developed dominant negative Sproutys and shown that endogenous Sprouty2 and Sprouty4 are physiological negative feedback regulators of FGF but not EGF-induced ERK activation. Interestingly, it has been shown that Sproutys regulate other signaling pathways in addition to ERK (19, 25). The effect of

endogenous Sproutys on other signaling pathways will be verified by using the dominant negative form of Sproutys.

It has been reported that MAP kinase phosphatases including MKP-1 and MKP-2 are negative feedback regulators of the ERK pathway (26, 27). However, MAP kinase phosphatases inhibit ERK by dephosphorylation, and therefore they can down-regulate ERK activation in response to any stimuli (28–32). In contrast, although Sproutys are also induced by ERK, they selectively inhibit ERK activity induced by various stimuli. Thus, Sproutys may play a role not only in the negative feedback loop of ERK but also in cross-talk among different stimuli. Further study of the mechanism of selective inhibition of ERK by Sproutys will uncover a novel signaling pathways toward ERK.

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