The Rho family of small GTPases has been implicated in the reorganization of the actin cytoskeleton and subsequent morphological changes in various cells. Rnd1, a member of this family, has a low intrinsic GTPase activity and exerts antagonistic effects on RhoA signaling. However, how the activity of Rnd1 is regulated has not yet been elucidated. Here we have demonstrated that Rnd1 directly associates with FRS2α and FRS2β, which are docking proteins of fibroblast growth factor (FGF) receptors and play important roles in the intracellular signals induced by FGFs. The interaction of FRS2β with Rnd1 suppresses the inhibitory effect of Rnd1 on RhoA. Rnd1 binds to the COOH-terminal region of FRS2β including tyrosine residues essential for the interaction with Shp2. When FGF receptor 1 is activated, it phosphorylates FRS2β, recruits Shp2, and releases Rnd1 from FRS2β. The liberated Rnd1 then inhibits RhoA activity. Furthermore, knockdown of Rnd1 by Rnd1-specific short interfering RNAs suppress the FGF-induced neurite outgrowth in PC12 cells. These results suggest that the activity of Rnd1 is regulated by FGF receptor through FRS2β and that Rnd1 plays an important role in the FGF signaling during neurite outgrowth.

The actin cytoskeleton mediates a variety of essential cellular functions, including motility, cytokinesis, and morphogenesis. Much progress has been made in elucidating the molecular mechanisms that control the organization of the actin cytoskeleton, and it is already well known that members of the Rho family of small GTPases are key regulators of the actin cytoskeleton in various cell types (1). Like other GTPases of the Ras superfamily, they serve as molecular switches by cycling between an inactive GDP-bound state and an active GTP-bound state, and once activated, they can interact with their specific effectors, leading to a variety of biological functions. Activation of the Rho family proteins requires GDP-GTP exchange catalyzed by various guanine nucleotide exchange factors, whereas the activation of the GTPases is down-regulated by GTPase-activating proteins, which stimulate the intrinsic GTPase activities. Presently, at least 20 mammalian Rho family proteins have been identified, Rho (A, B, and C), Rac (1, 2, and 3), Cdc42, TC10, TLR, Wrc1, Cbp/Wrch2, RhoG, RhoD, Rif, RhoH/TF, RhoBTB (1 and 2), and Rnd (1, 2, and 3). Among them, the functions of Rho, Rac, and Cdc42 have been extensively characterized. In neuronal cells, activation of Rac and Cdc42 induces the formation of lamellipodia and filopodia of the growth cone and stimulation of neurite outgrowth. On the other hand, Rho activation induces the inhibition of neuritogenes and neurite retraction. Recent studies have revealed involvement of Rho family GTPases in the downstream signaling pathways of a variety of neurotrophins and axon guidance molecules to regulate the actin cytoskeleton (2).

Fibroblast growth factors (FGF(s)) constitute a large family of growth factors that influence a wide variety of biological processes such as angiogenesis, embryogenesis, differentiation, and proliferation depending on the cell type (3, 4). In the nervous system, they have been shown to stimulate both the differentiation and survival of postmitotic cells as well as being proliferative factors for non-differentiated cells (5). FGFs mediate their pleiotropic responses by binding to and activating a family of receptor tyrosine kinases designated FGF receptors (FGFRs) 1–4 (6). Many of the cellular responses of FGFs are mediated by the membrane-linked docking proteins, FRS2α and FRS2β (7–11). FRS2α and FRS2β contain myristyl anchors and phosphotyrosine-binding domains in their NH2 termini and multiple tyrosine phosphorylation sites in their COOH termini that serve as binding sites for the adaptor protein Grb2 and the protein phosphatase Shp2, transducing signals to mitogen-activated protein kinase cascades (8, 9, 11). Concerning signaling to Rho family GTPases, recent studies indicate that basic FGF induces phosphorylation of the p85 βPIX, a guanine nucleotide exchange factor for Rac1/Cdc42 and that phosphorylated p85 βPIX mediates Rac1 activation and regulates cytoskeletal reorganization at growth cones (12, 13). However, precise mechanisms of FGF signal transduction pathway to Rho family GTPases remain unclear.

The Rnd proteins, Rnd1, Rnd2, and Rnd3/RhoE, comprise a unique branch of Rho family GTPases that lack intrinsic GTPase activity and consequently remain constitutively active (14, 15). Prior studies have suggested that Rnd1 has antagonistic effects on RhoA-regulated signaling pathways, and several downstream effectors have been identified, such as Sos1 and p190RhoGAP (14, 16–18). However, it has been unclear how the activity of Rnd1 is regulated. Here we show that Rnd1 directly interacts with FRS2α and FRS2β. The Rnd1-induced inactivation of RhoA is regulated by the FGF receptor through FRS2β, and Rnd1 is involved in the FGF-

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The abbreviations used are: FGF, fibroblast growth factor; FGFβ, fibroblast growth factor receptor; HA, hemagglutinin; GST, glutathione S-transferase; GFP, green fluorescent protein; nFGF, acidic FGF; siRNA, short interfering RNA; WT, wild type; GTPγS, guanosine 5′-3′-O-thio-triphosphate; SH, Src homology.
induced neurite outgrowth. We propose a new role of Rnd1 in the FGF signaling pathway leading to RhoA inactivation and cytoskeletal rearrangements.

MATERIALS AND METHODS

**Plasmid Constructions and Antibodies**—The coding sequences for rat FRS2α, FRS2β, and FGF-R1 were obtained from rat pheochromocytoma PC12 cells by reverse transcription-PCR. The PCR products were cloned into pCR2.1 vector (Invitrogen) and sequenced completely. For expression in mammalian cells, the cDNAs for wild-type FRS2α and FRS2β were subcloned into pcDNA3 (Invitrogen) encoding the Myc tag sequence at the COOH terminus followed by a stop codon. The cDNA for wild-type FGF-R1 was subcloned into pcDNA3. FRS2α/Y418F/Y456F, FRS2β/Y418F, and FGF-R1/G903N/S946F were generated by PCR-mediated mutagenesis (19). Hemagglutinin A (HA)-tagged Rnd1, Rnd2, Rnd3, RhoA(1-16) Rac1(V12), Cdc42(V12), and green fluorescent protein (GFP)-tagged Rnd1 were obtained as described previously (17). For purification of recombinant proteins, cDNAs of Rnd1, Rac1, FRS2α-WT (amino acids 2–508), FRS2α-CT (amino acids 411–508), FRS2β-WT (amino acids 2–493), FRS2β-CT+ (amino acids 247–493), FRS2β-CT (amino acids 393–493), and FRS2β-ΔCT (amino acids 2–392) were subcloned into pGEX-4T-2 (Amersham Biosciences). The short interfering RNA (siRNA) for rat Rnd1 was designed to target 19 nucleotides of the rat Rnd1 transcript (Rnd1 siRNA-A, nucleotide 74–92, 5′-ggagagggagagggtttgac-3′; Rnd1 siRNA-B, nucleotide 145–163, 5′-ttacacagctgtttggag-3′; Rnd1 siRNA-C, nucleotide 396–414, 5′-ctggagacccactgttga-gaa-3′), and they were expressed by using a siRNA expression vector pSilencer (Ambion). We used a pSilencer plasmid encoding a hairpin siRNA whose sequence is not found in the mouse, human, or rat genome database supplied with the kit as a control.

Antibodies used were as follows: a mouse monoclonal anti-Myc antibody 9E10 (Roche Applied Science); a mouse monoclonal anti-HA antibody 12CA5 (Roche Applied Science); a mouse monoclonal anti-Rac1 antibody (BD Biosciences); a mouse monoclonal anti-α-tubulin antibody (Sigma); a mouse monoclonal anti-GFP antibody, a mouse monoclonal anti-RhoA antibody, a rabbit polyclonal anti-YFRG1 antibody, and anti-Shp2 antibody (Santa Cruz Biotechnology); and horseradish peroxidase-conjugated secondary antibodies (DAKO). A rabbit polyclonal antibody against Rnd1 has been described previously (20). Yeast Two-hybrid Screening—A rat brain cDNA library fused to the GAL4 activation domain of the pACT2 vector (Clontech) was screened using pGBKKT7/Rnd1(y282) as bait in the yeast strain AH109 according to the manufacturer’s instructions. Interaction between the bait and library proteins activates transcription of the reporter genes HIS3, Ade2, and lacZ. From 1.3 × 10⁶ transformants, 647 colonies grew on selective medium lacking histidine and adenine and were also positive for β-galactosidase activity. One of these, clone 2-107 was found to encode the cDNA containing 5′ amino acids of FRS2α (amino acids 1–325) and 3′ amino acids of human FGFR1.

For the β-galactosidase filter assay, colonies of yeast transformants were transferred onto Hybond-N filter papers (Amersham Biosciences) and permeabilized in liquid nitrogen. Each filter was placed on a Whatman No. 2 filter paper that had been presoaked in Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, and 37.5 mM β-mercaptoethanol) containing 0.33 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and was incubated at 30 °C for 8 h.

Cell Culture and Transfection—293T cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 4 mM glutamine, 100 units/ml penicillin, and 0.2 mg/ml streptomycin under humidified conditions in 95% air and 5% CO2 at 37 °C. PC12 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% horse serum and 5% fetal bovine serum. Transient transfections were carried out with Lipofectamine PLUS (Invitrogen) for 293T cells or Lipofectamine 2000 (Invitrogen) for PC12 cells, according to the manufacturer’s instructions.

Immunoblotting—Proteins were separated by 10 or 12.5% SDS-PAGE and were electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore Corp.). The membrane was blocked with 3% low fat milk in Tris-buffered saline (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) and then incubated with primary antibodies. The primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies and the ECL detection kit (Amersham Biosciences).

**In Vitro Binding Assays**—All glutathione S-transferase (GST)-fused proteins were purified from Escherichia coli as described previously (17). Protein concentration was determined by comparing with bovine serum albumin standards after SDS-PAGE and by staining with Coomassie Brilliant Blue.

For pull-down assays, 293T cells transfected with HA-tagged Rho GTPases were rinsed once with phosphate-buffered saline and lysed with the ice-cold cell lysis buffer (20 mM Tris-HCl, pH 7.5, 2 mM MgCl2, 1 mM dithiothreitol, 0.2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Cell lysates were then centrifuged for 10 min at 16,000 × g at 4 °C. The supernatants were incubated for 10 min at 4 °C with 10 μg of GST fusion proteins and subsequently incubated with glutathione-Sepharose beads for 2 h at 4 °C. After the beads were washed with the ice-cold cell lysis buffer, the bound proteins were eluted in Laemmli sample buffer and analyzed by SDS-PAGE and immunoblotting with anti-HA antibody.

To examine direct interaction between the FRS2β and Rnd1, dot blot assay was performed as described previously (21). Five μg of GST and GST-fused FRS2β (amino acids 247–493) were spotted onto a nitrocellulose membrane and allowed to dry for 1 h at room temperature. The membrane was blocked with buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl2, and 1 mM dithiothreitol) containing 5% low fat milk for 1 h at 4 °C. The membrane was then incubated for 1 h at 4 °C in buffer A containing 20 μg of GST-fused Rnd1 or Rac1 preloaded with GTPγS. The membrane was washed with buffer A and then incubated
with 3% low fat milk in Tris-buffered saline containing anti-Rnd1 or anti-Rac1 antibody. These antibodies were detected by using horseradish peroxidase-conjugated secondary antibody and the ECL detection kit.

**Immunoprecipitation**—293T cells cotransfected with Myc-tagged FRS2α or FRS2β and HA-tagged Rnd1 together with or without FGF1 were lysed with ice-cold cell lysis buffer (20 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 1 mM dithiothreitol, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 25 mM NaF, and 1 mM Na₃VO₄). After centrifugation, the supernatants were incubated with anti-Myc polyclonal antibody for 1 h and then with protein A-Sepharose (Amersham Biosciences) for 1 h. The beads were washed with the lysis buffer, and bound proteins were analyzed by SDS-PAGE and immunoblotting.

**Immunofluorescence Microscopy**—PC12 cells were seeded onto round 13-mm glass coverslips coated with poly-β-lysine, and then they were transfected with expression vectors encoding GFP and HA-RhoAV14. At 5 h after transfection, the medium was changed with Dulbecco’s modified Eagle’s medium containing 100 ng/ml acidic FGF (aFGF) (Sigma) and 5 μg/ml heparin. At 48 h after transfection, they were immunostained with 100 ng/ml aFGF and 5 μg/ml heparin. PC12 cells possessing one or more neurites longer than the diameter of the cell body were scored as positive. We scored more than 50 cells in each experiment.

**Measurement of RhoA Activity**—Measurement of RhoA activity was performed as described previously (22). Briefly, 293T cells were transfected with an expression vector encoding HA-tagged wild-type RhoA together with Myc-tagged FRS2β and GFP-tagged Rnd1. At 36 h after transfection, the cells were washed with ice-cold Tris-buffered saline, and lysed with the cell lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 30 mM MgCl₂, 0.5% Triton X-100, 0.5% sodium deoxycholate, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) containing 20 μg of GST-fused Rho-binding domain of mouse Rhotekin. The cell lysates were centrifuged for 5 min at 16,000 × g at 4 °C, and the supernatants were incubated with glutathione-Sepharose beads for 1 h at 4 °C. The beads were washed with the lysis buffer, and bound proteins were analyzed by SDS-PAGE and immunoblotting.

**RESULTS**

**Rnd1 Directly Interacts with FRS2α and FRS2β**—In an attempt to identify binding proteins of Rnd1, we employed a two-hybrid screening of a rat brain cDNA library with Rnd1S229, a mutant of Rnd1 lacking the carboxyl-terminal CAAX motif, as bait. Approximately 1.3 × 10⁷ clones were screened, and several positive clones were isolated. Sequence
The Interaction of Rnd1 with FRS2

Analyses revealed that one of them encoded the COOH-terminal region of rat FRS2β. FRS2α and FRS2β function as mediators of signaling by FGFRs (7–11). To determine whether Rnd1 interacts with FRS2α and FRS2β in vitro, HA-tagged Rnd1 was expressed in 293T cells and pull-down assays were performed by using the purified GST-fused full-length of Rnd1. Myc-tagged FRS2β in the absence or presence of wild-type FGFR1 (FGFR1-WT), or kinase-inactive FGFR1 (FGFR1-KD). Alternatively, they were transiently coexpressed with expression vectors encoding HA-tagged Rnd1, Myc-tagged FRS2β containing Y418F (FRS2β-F418) or Y456F (FRS2β-F456) substitution or both (FRS2β-2F) in the absence or presence of FGFR1-WT. The cell lysates were subjected to immunoprecipitation (IP) with anti-Myc antibody. The immunoprecipitates and the total lysates were analyzed by immunoblotting with anti-HA, anti-Myc, anti-Shp2, anti-FGFR1, or anti-phosphotyrosine antibody (p-FGFR1, p-FRS2β).

To determine whether Rnd1 interacts with FRS2α and FRS2β in mammalian cells, lysates from 293T cells expressing HA-tagged Rnd1 and either Myc-tagged full-length FRS2α or FRS2β were used in a coimmunoprecipitation assay. Rnd1 was coimmunoprecipitated with FRS2α and FRS2β, but RhoAV14 and Cdc42V12 did not, indicating that FRS2 proteins specifically interact with Rnd1 and Rnd2. A dot blot assay with GTPγS-loaded purified GST-Rnd1 as a probe showed that the GTP-bound Rnd1 directly interacted with FRS2α (Fig. 1A).

To determine whether Rnd1 interacts with FRS2α and FRS2β required for the interaction with Rnd1, various truncated forms of FRS2α and FRS2β were purified as GST fusion proteins, and their bindings to HA-tagged Rnd1 expressed in 293T cells were examined by a pull-down assay (Fig. 2). HA-tagged Rnd1 was precipitated by the GST-fused COOH-terminal region of FRS2α (FRS2α-CT, amino acids 411–508) or FRS2β (FRS2β-CT, amino acids 393–493) and the wild-type proteins (FRS2α-WT and FRS2β-WT) or the fragment corresponding to the original yeast clone (FRS2β-CT*, amino acids 247–493), whereas it was not precipitated by the GST-fused FRS2β lacking CT region (FRS2βΔCT, amino acids 2–392) or GST alone (Fig. 2, B and C). These results indicate that the binding site of Rnd1 is located at the COOH-terminal region of FRS2α and FRS2β.

Tyrosine Phosphorylation of FRS2β Releases Rnd1 from FRS2β and Recruits Shp2—Upon FGF stimulation, FRS2α and FRS2β are phosphorylated by activated FGFRs and recruit Grb2 and Shp2. FRS2β-CT contains two tyrosine phosphorylation sites, Tyr-418 and Tyr-456, and their phosphorylations are essential for the interaction with Shp2 (9, 11). Therefore, we next examined whether phosphorylation of FRS2 affects its interaction with Rnd1. 293T cells were cotransfected with Myc-tagged FRS2β, HA-tagged Rnd1, and FGFR1, and the lysates were then immunoprecipitated with anti-Myc antibody. FRS2β was tyrosine phosphorylated when it was coexpressed with FGFR1 and bound to Shp2. In contrast, Rnd1 was dissociated from the phosphorylated FRS2β (Fig. 3A). The dissociation of Rnd1 from FRS2β was not observed when they were coexpressed with kinase-inactive FGFR1 (FGFR1-KD). We found that FRS2α also had Rnd1-releasing activity, although it was weak compared with that of FRS2β (data not shown). We generated FRS2β containing Y418F and Y456F substitutions in the Shp2-binding region (FRS2β-2F) to examine whether tyrosine phosphorylation in this region is required for the dissociation of Rnd1 from FRS2β. As observed in previous studies (9, 11), FRS2β-2F was phosphorylated by FGFR1, probably at

Fig. 3. Interaction of Rnd1 with FRS2β is regulated by phosphorylation of FRS2β. A, 293T cells were transiently transfected with expression vectors encoding HA-tagged Rnd1, Myc-tagged FRS2β in the absence or presence of wild-type FGFR1 (FGFR1-WT), or kinase-inactive FGFR1 (FGFR1-KD). B, alternatively, they were transiently coexpressed with expression vectors encoding HA-tagged Rnd1, Myc-tagged FRS2β containing Y418F (FRS2β-F418) or Y456F (FRS2β-F456) substitution or both (FRS2β-2F) in the absence or presence of FGFR1-WT. The cell lysates were subjected to immunoprecipitation (IP) with anti-Myc antibody. The immunoprecipitates and the total lysates were analyzed by immunoblotting with anti-HA, anti-Myc, anti-Shp2, anti-FGFR1, or anti-phosphotyrosine antibody (p-FGFR1, p-FRS2β).
tyrosine residues in the Grb2-binding region. However, FRS2β-2F did not interact with Shp2 in the presence of FGFR1. On the other hand, Rnd1 interacted with FRS2β-2F in the absence and presence of FGFR1 (Fig. 3A). We further examined whether one or both phosphorylation sites of FRS2β are essential for the loss of Rnd1 binding by using each of single point mutants of FRS2β (FRS2β-Phe-418 and FRS2β-Phe-456). Both FRS2β-Phe-418 and FRS2β-Phe-456 interacted with Rnd1 in the absence of FGFR1 and partially released Rnd1 in the presence of FGFR1 (Fig. 3B). Taken together, these results suggest that the interaction of Rnd1 with FRS2β is regulated by the FGFR-induced phosphorylation of FRS2β and that both Tyr-418 and Tyr-456 are important for the regulation of Rnd1 binding.

The Activity of Rnd1 Is Regulated by Its Interaction with FRS2β—Rnd1 has much lower affinity to GDP than GTP and no detectable intrinsic GTPase activity (14), and no GTPase-activating protein or inhibitory binding protein specific for Rnd1 has been identified so far. Thus, it remains unknown how the cellular activities of Rnd1 are regulated. We next examined the possibility that FRS2β functions as an inhibitory binding protein for Rnd1 and that the activity of Rnd1 is regulated by FGFR-induced phosphorylation of FRS2β. Several lines of evidence indicate that Rnd1 counteracts the biological functions of RhoA (14, 16–18). Therefore, we measured the level of active GTP-bound RhoA in cells by using GST-fused Rho-binding domain of Rhotekin. The amounts of GTP-bound RhoA were determined by immunoblotting with anti-HA antibody (GST-Rho-binding domain pull-down). Expressions of HA-tagged RhoA, GFP-tagged Rnd1, Myc-tagged FRS2β, and FGFR1 were examined whether one or both phosphorylation sites of FRS2β are essential for the loss of Rnd1 binding by using each of single point mutants of FRS2β (FRS2β-Phe-418 and FRS2β-Phe-456). Both FRS2β-Phe-418 and FRS2β-Phe-456 interacted with Rnd1 in the absence of FGFR1 and partially released Rnd1 in the presence of FGFR1 (Fig. 3B). Taken together, these results suggest that the interaction of Rnd1 with FRS2β is regulated by the FGFR-induced phosphorylation of FRS2β and that both Tyr-418 and Tyr-456 are important for the regulation of Rnd1 binding.

The Interaction of Rnd1 with FRS2β (Fig. 4). On the other hand, expression of FGFR1 restored the Rnd1-induced inactivation of RhoA, whereas the expression of FGFR1-KD did not. Furthermore, coexpression of Rnd1 with FRS2β-KD suppressed the Rnd1-induced down-regulation of RhoA activity in the cells coexpressing Rnd1 and FRS2β. As shown in Fig. 4A, expression of FGFR1 induced the down-regulation of RhoA activity in the cells expressing FRS2β, whereas expression of FGFR1 did not in the cells expressing FRS2β-KD. Furthermore, coexpression of Rnd1 with FRS2β-KD did not restore the Rnd1-induced inactivation of RhoA. Therefore, the expression of FGFR1-KD did not. Furthermore, coexpression of Rnd1 with FRS2β-KD suppressed the Rnd1-induced down-regulation of RhoA activity in the cells coexpressing Rnd1 and FRS2β. As shown in Fig. 4A, expression of FGFR1 induced the down-regulation of RhoA activity in the cells expressing FRS2β, whereas expression of FGFR1 did not in the cells expressing FRS2β-KD. Furthermore, coexpression of Rnd1 with FRS2β-KD did not restore the Rnd1-induced inactivation of RhoA.
ulation of RhoA activity in the absence and presence of FGFR1. These results indicate that the Rnd1-induced down-regulation of RhoA activity is regulated by the interaction of Rnd1 with FRS2β.

Knock-down of Rnd1 Suppresses the FGF-induced Neurite Outgrowth in PC12 Cells—FGFs play important roles in neurogenesis, axon growth, and differentiation (24), and previous reports showed that FGFs stimulate neurite extension in PC12 cells (8, 25). Rho family GTPases regulate neuronal morphology through reorganizing the actin cytoskeleton (2, 26, 27). Among them, activation of RhoA has been known to stabilize cortical actin and prevents neurite outgrowth, whereas inactivation of RhoA promotes neurite outgrowth in PC12 cells (2, 26). In addition, ectopic expression of Rnd1 in PC12 cells stimulates neurite extension (28). These observations led us to examine whether Rnd1 is involved in the FGF-induced neurite outgrowth in PC12 cells by inhibiting RhoA activity. Indeed, stimulation of PC12 cells with aFGF decreased GTP-bound RhoA in the cells (Fig. 5A). To determine whether the down-regulation of RhoA activity is required for the FGF-induced neurite outgrowth, we transfected HA-tagged RhoA and examined cell morphology after stimulation of the cells with aFGF. PC12 cells expressing GFP alone extend their neurites after stimulation with aFGF. In contrast, expression of HA-tagged RhoA completely blocked the aFGF-induced neurite extension (Fig. 5B). We next examined the involvement of Rnd1 in the FGF-induced neurite extension by expressing vectors encoding Rnd1-specific siRNAs. Three siRNAs targeting different coding regions of the rat Rnd1 cDNA were designed (Rnd1 siRNA-A, -B, and -C), and two of them (Rnd1 siRNA-A and -B) significantly reduced exogenously expressed Myc-tagged rat Rnd1 (Fig. 1C). However, they had no effect on the Rnd2 expression (data not shown). Similar to untransfected cells, PC12 cells coexpressing GFP and control siRNA or Rnd1 siRNA-C that had little effect on Rnd1 expression caused neurite outgrowth in response to aFGF. However, coexpression of GFP and Rnd1 siRNA-A or -B significantly blocked the aFGF-induced neurite outgrowth (Fig. 5D). We also examined whether expression of FRS2β-2F has the same effect as the Rnd1 siRNA in decreasing neurite outgrowth in PC12 cells, because Rnd1 was not dissociated from FRS2β-2F (Fig. 3A). However, we could not detect PC12 cells expressing the FRS2β-2F mutant, although wild-type FRS2β was expressed in the same transfection condition. This may be because overexpression of FRS2β-2F mutant was toxic to PC12 cells (data not shown). These results indicate that Rnd1 is involved in the FGF-induced neurite outgrowth in PC12 cells.

**DISCUSSION**

Most of Rho family GTPases cycle between inactive GDP-bound and active GTP-bound forms. However, Rnd1 has been shown to be constitutively active, because it has substitutions at amino acid residues known to be important for GTP hydrolysis (14). Although several downstream proteins that interact with Rnd1 have been identified, it has not yet to be elucidated how its function is regulated. Our findings demonstrate an FRS2β phosphorylation-dependent mechanism for regulating Rnd1 function. We showed that FRS2β directly associates with Rnd1 and suppresses the activity of Rnd1. The binding region of Rnd1 is located at the COOH-terminal region of FRS2β including Tyr-418 and Tyr-456, which are essential for the interaction with the SH2 domain of Shp2. Phosphorylation of FRS2β by FGFR1 at Tyr-418 and Tyr-456 recruits Shp2 and instead releases Rnd1 from FRS2β, and then dissociated Rnd1 inhibits RhoA. These results suggest that the activity of Rnd1 is regulated by FGFR through FRS2β. A previous report showed that expression of Rnd3, another Rnd subfamily member that is also known to be a constitutively active GTPase, can be induced by expressing activated Raf in Madin-Darby canine kidney cells, but the expression of Rnd1 is not affected (29). Similarly, stimulation of fibroblasts with platelet-derived growth factor promotes the synthesis of Rnd3 protein (30). Our results showed that Rnd3 does not interact with FRS2α and FRS2β. In this respect, these constitutively active GTPases may be differentially regulated; the activity of Rnd1 is controlled by the interaction with inhibitory binding proteins such as FRS2β, whereas Rnd3 is regulated at the level of transcription or translation.

Rho family GTPases are critical regulators of neurite outgrowth (2, 26, 27). Among them, Rac and Cdc42 activation are involved in promoting neurite outgrowth, whereas RhoA inhibits the outgrowth, and down-regulation of RhoA activity is required for the neurite outgrowth (2, 26, 27). It seems likely that Rho GTPases are also involved in FGF-induced neurite outgrowth. Indeed, a recent study showed that basic FGF induces Rac1 activation through p85/PIX, which in turn regulates cytoskeletal reorganization at growth cones (12, 13). We show here that FGF-induced phosphorylation of FRS2β triggers the Rnd1-induced down-regulation of RhoA activity. The down-regulation of endogenous RhoA activity was observed in PC12 cells when they were stimulated with FGF. In addition, overexpression of constitutively active RhoA, or knock-down of endogenous Rnd1 by Rnd1-specific siRNA inhibited FGF-induced neurite outgrowth in PC12 cells. These observations suggest that down-regulation of RhoA is one of important signaling pathways in FGF-induced neurite outgrowth, and that Rnd1 is involved in this effect.

It has been reported that FGF-2 (basic FGF) increases functional excitatory synapses on cultured hippocampal neurons (31). Recently, we have shown that Rnd1 is highly expressed in hippocampus during the stage of synapse formation and plays a role in spine formation (20). In contrast, active Rho has been shown to strongly induce a loss of mature-shaped spines, whereas inhibition of RhoA activity resulted in elongated spine necks (32, 33). Considering our present results and these observations, FGFs may enhance synapse formation by Rnd1-induced inactivation of RhoA.

In conclusion, we identified FRS2β as an upstream regulator of Rnd1 and demonstrated that Rnd1 plays an important role for FGF-induced neurite outgrowth in PC12 cells. This study not only links FRS2β to Rnd1 but also contributes to the understanding of control mechanism for constitutively active GTPases.

**REFERENCES**

1. Etienne-Manneville, S., and Hall, A. (2002) *Nature* **420**, 629–635
2. Negishi, M., and Rat Hoffman, H. (2002) *J. Biochem.* **132**, 157–166
3. Basilico, C., and Moscatelli, D. (1992) *Adv. Cancer Res.* **59**, 115–165
4. Galzie, Z., Kinsella, A. R., and Smith, J. A. (1997) *J. Biochem. Cell Biol.* **75**, 669–683
5. Gritti, A., Parati, E. A., Cova, L., Frolichsthal, P., Galli, R., Wanke, E., Faravelli, L., Morassutti, D. J., Riesen, F., Nickel, D. D., and Vescovi, A. L. (1996) *J. Neurosci.* **16**, 1091–1100
6. Schlessinger, J. (2000) *Cell* **103**, 211–225
7. Gotth, N., Laks, S., Nakashima, M., Lax, I., and Schlessinger, J. (2004) *FEBS Lett.* **564**, 14–18
8. Kouraha, H., Hadari, Y. R., Spivak-Kroizman, T., Shilling, J., Bar-Sagi, D., Lax, I., and Schlessinger, J. (1997) *Cell* **89**, 693–702
9. Hadari, Y. R., Kouraha, H., Lax, I., and Schlessinger, J. (1998) *J. Mol. Biol.* **28**, 3966–3973
10. Hadari, Y. R., Gotth, N., Kouraha, H., Lax, I., and Schlessinger, J. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 8578–8583
11. Xu, H., and Goldfarb, M. (1998) *J. Biol. Chem.* **20**, 13049–13056
12. Shin, E. Y., Shin, K. S., Lee, C. S., Woo, K. N., Quan, S. H., Seong, N. K., Kim, Y. G., Cha, C. I., Kim, S. R., Park, D., Bokoch, G. M., and Kim, E. G. (2002) *J. Biol. Chem.* **277**, 44417–44430
13. Shin, E. Y., Woo, K. N., Lee, C. S., Koo, S. H., Kim, Y. G., Kim, W. J., Bae, C. D., Chang, S. I., and Kim, E. G. (2004) *J. Biol. Chem.* **279**, 2974–2984
14. Nosè, K., Lauritzen, I., Mattei, M. G., Parisi, S., Hall, A., and Chardin, P. (1999) *J. Cell Biol.* **141**, 187–197
15. Guasch, R. M., Scambler, P., Jones, G. E., and Ridley, A. J. (1998) *Mol. Cell. Biol.* **18**, 4761–4771
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