Pragmin, a Novel Effector of Rnd2 GTPase, Stimulates RhoA Activity*

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The Rho family small GTPases have been implicated in the reorganization of actin cytoskeleton and subsequent morphological changes in various cells. Rnd2 is a member of the Rnd subfamily, comprising Rnd1, Rnd2, and Rnd3. In contrast to Rnd1 and Rnd3, displaying an antagonistic action for RhoA signaling, signaling pathways of Rnd2 are not well known. Here we have performed a yeast two-hybrid screen using Rnd2 as bait and identified a novel Rnd2 effector protein, predominantly expressed in neurons, including cortical and hippocampal neurons. We named it Pragmin (pragma of Rnd2). In in vivo and in vitro binding assays, Pragmin specifically binds to Rnd2 among the Rho family GTPases in a GTP-dependent manner. Rnd2-bound Pragmin significantly stimulates RhoA activity and induces cell contraction through RhoA and the Rho-kinase pathway in HeLa cells. In PC12 cells, expressing Pragmin inhibits nerve growth factor-induced neurite outgrowth in response to Rnd2, and knock-down of Pragmin by Pragmin-specific small interfering RNA enhances neurite elongation. Therefore, Rnd2 regulates neurite outgrowth by functioning as the RhoA activator through Pragmin, in contrast to Rnd1 and Rnd3 inhibiting RhoA signaling.

Rho family small GTPases have been implicated in the regulation of the cytoskeleton and subsequent morphological changes in various cell types (1–3). 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. Activation of the Rho family proteins requires GDP-GTP exchange catalyzed by various guanine nucleotide exchange factors (GEFs), whereas the activation of the GTPases is down-regulated by GTPase-activating proteins (GAPs), 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, TCL, Wrc1, Chp/Wrc2, RhoG, RhoD, RhoH/TTF, RhoBTB (1 and 2), and Rnd (1, 2, and 3). Among them, the functions of Rho, Rac, and Cdc42 have been extensively characterized, and they induce formations of actin stress fibers, lamellipodia, and filopodia, respectively, in fibroblasts (4–7). 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 neuritogenesis and neurite retraction. These actions of Rho family GTPases are mediated by a variety of downstream effectors (8–11). RhoA induces stress fiber formation, cell contraction, and neurite retraction through Rho-associated kinase/ROK/ROCK (referred to as Rho-kinase) (12–15). Activated Rho-kinase increases phosphorylation of myosin light chain and promotes actomyosin-based cytoskeletal contraction (16). Rac and Cdc42 regulate reorganization of actin cytoskeleton through WAVE and N-WASP (17, 18).

The Rnd proteins, Rnd1, Rnd2, and Rnd3/RhoE, comprise a unique branch of the Rho family GTPases that lack intrinsic GTPase activity and consequently remain constitutively active (19). Previous studies have suggested that Rnd1 and Rnd3 have antagonistic effects on RhoA-regulated signaling pathways, and several downstream effectors have been identified, such as Socius and p190RhoGAP (20, 21). In contrast to Rnd1 and Rnd3, little is known about Rnd2, although Rnd2 is expressed specifically in neurons in brain (22, 23), suggesting that Rnd2 plays an important role in neuronal functions. We have recently demonstrated that Vps4-A is a Rnd2-binding protein, involved in endosomal vesicle trafficking (24). In addition, we identified Rapostlin as an effector of Rnd2, inducing neurite branching (25, 26). During screening putative Rnd2-binding proteins by a yeast two-hybrid system, we found a novel effector protein of Rnd2, Pragmin. Pragmin specifically binds to Rnd2 among the Rho family GTPases in a GTP-dependent manner. Pragmin with Rnd2 stimulates RhoA activity and inhibits NGF-induced neurite outgrowth in PC12 cells.

EXPERIMENTAL PROCEDURES

Plasmid Constructions and Antibodies—Wild-type and mutant forms of Rnd2, wild-type Rnd1 and Rnd3, mutant forms of RhoA, Rac1, and Cdc42 were obtained as described previously (14, 20, 27, 28). For a yeast two-hybrid screening, Rnd2V16S224 (Rnd2V16 lacking a farnesylation site motif by a substitution of Cys to Ser) was fused to the GAL4 DNA-binding domain in the yeast expression vector pGBK7T7 (Clontech). For purification of recombinant proteins, cDNAs of Rnd1, Rnd2, Rnd3, and Pragmin-Ct (amino acids 947–1368) were subcloned into pGEX-4T-2 (Amersham Biosciences). For expression in mammalian cells, cDNAs of RhoAV14, Rac1V12, Cdc42V12, Rnd1, Rnd2, Rnd2V16, Rnd2V221, and Rnd3 were subcloned into pcDNA3 encoding an initiating Met followed by the hemagglutinin (HA) epitope tag sequence at the HA terminus. The small interfering RNAs (siRNAs) for Pragmin were designed to target 19 nucleotides of rat Pragmin (Pragmin siRNA-A, nucleotides 224–242, 5′-CCACCATGATGACTTCTGTA-3′; Pragmin siRNA-B, nucleotides 1339–1357, 5′-GAAACCTACTACTGGTA-3′), and the siRNA for Rapostlin was designed to target 19 nucleotides of rat Rapostlin (nucleotides 132–150, 5′-ACAAGCTCAGGAATTTTCA-3′). They were subcloned into an siRNA expression vector pSilencer (Ambion) to express a 21-nucleotides hairpin siRNA
according to the manufacturer’s instructions. A pSilencer plasmid encoding a validated nontargeting siRNA supplied by the manufacturer was used as a negative control.

Antibodies used were as follows: mouse monoclonal anti-Myc antibody 9E10 (Upstate Biotechnology, Inc., Lake Placid, NY); mouse monoclonal anti-HA antibody 12CA5 (Roche Applied Science); mouse monoclonal anti-RhoA antibody 26C4, goat polyclonal anti-Rho8 (Rnd3) antibody C-19, and mouse monoclonal anti-green fluorescent protein (GFP) antibody B-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); mouse monoclonal anti-β-tubulin antibody (Sigma); Alexa Fluor 488/594-conjugated secondary antibodies and Alexa Fluor 488-conjugated phalloidin (Molecular Probes, Inc., Eugene, OR); and horseradish peroxidase-conjugated secondary antibodies (DAKO). Rabbit polyclonal antibodies against Rnd1 and Rnd2 have been described previously (25, 29).

Antibody for Pragmin was generated against bacterially expressed glutathione S-transferase (GST)-fused peptide corresponding to the amino acids 15–297 of Pragmin. The specific antibody was purified by passage over HiTrap NHS-activated HP columns (Amersham Biosciences).

Yeast Two-hybrid Screening—A rat brain cDNA library fused to the GAL4 activation domain of the pACT2 vector (Clontech) was screened using Rnd2Y10356/pGBKKT7 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 gene HIS3, Ade, and lacZ. From 1.2 × 10⁷ transformants, 318 colonies grew on selective medium, containing 2 mM 3-amino-1,2,4-triazole but lacking histidine and adenine, and 139 colonies were also positive for β-galactosidase activity. Positive clones were sequenced using an ABI PRISM 310 DNA sequencer. One of these, a clone F-53 was found to encode the COOH-terminal 422 amino acids of Pragmin.

BLAST search revealed that F-53 is identical to the sequence corresponding to the COOH-terminal region of NCBI reference sequence number XM_224908. To obtain a full-length cDNA, two primers were synthesized: 5'-ATAGAATTCCCATGTCTGCGTGCAGTGACTTTG-3' (5′ end of a putative coding sequence of XM_224908 with EcoRI) and 5'-TAAGCGGCCGCTCAGAGAAGTTGC-3' (3′ end of F-53 with NotI). These two primers were used in reverse transcription-PCR with rat brain RNA as a template, and a 4.1-kb PCR fragment was obtained. The PCR product was subcloned into pCR2.1 vector (Invitrogen) and sequenced completely. For expression in mammalian cells, cDNAs of full-length Pragmin (amino acids 1–1368), the NH2-terminal half region of Pragmin (Pragmin-NT; amino acids 1–829), and the COOH-terminal half region of Pragmin (Pragmin-CT; amino acids 830–1368) were subcloned into pcDNA3 encoding an initiating Met followed by the Myc epitope tag sequence at the NH2 terminus. The cDNA of full-length Pragmin was also subcloned into pEGFP-C3 (Clontech).

**FIGURE 1.** A novel Rnd2 effector, Pragmin, is expressed in neurons in brain. A, schematic of the Pragmin domain structures and various deletion mutants of Pragmin described in this study are shown. Ser, serine-rich region; shaded box, kinase-like domain. B, tissue distribution and developmental expression of Pragmin mRNA in brain were analyzed by reverse transcription-PCR. Total RNAs were isolated from various tissues and various stages of rat brain, followed by amplification with primers specific for Pragmin, as described under “Experimental Procedures.” Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a control. En, embryonic day; Pn, postnatal day. C, in situ hybridization was performed with antisense Pragmin riboprobe (a, c, d, and e) and corresponding sense riboprobe (b) for postnatal day 27 rat of coronally sectioned cerebral cortex (a and b), hippocampus (c), piriform cortex (d), and cerebellar cortex (e). Scale bar, 200 μm.
Reverse Transcription-PCR—Total RNAs were isolated using Isogen (Nippongene, Japan) according to the manufacturer’s instructions, and 30 μg of RNA was used to generate cDNA using Moloney murine leukemia virus reverse transcriptase (Roche Applied Science). Reverse transcription was performed with the primers of Pragmin (5'-ATAGAATTCCCAT-GTCTGGTGCAGTGACTTTG-3' and 5'-TTCTGTGGTCTTGTA-CATG-3') using the generated cDNA as a template.

In Situ Hybridization—Antisense and sense riboprobes were prepared by in vitro transcription of the plasmids encoding bp 3741–4107 of rat Pragmin cDNA sequence with T7 and T3 RNA polymerases and digoxigenin (DIG) RNA labeling mix (Roche Applied Science). In situ hybridization was performed as described previously (29). Briefly, 40-μm-thick coronal sections of postnatal day 27 of rat brains were treated with 1.0 μg/ml protease K (Roche Applied Science) for 7 min at room temperature and then acetylated before hybridization. After prehybridization, the sections were incubated with 500 ng/ml DIG-labeled antisense or sense probe overnight at 55 °C and washed with SSC several times. DIG-labeled probes were immunodetected using alkaline phosphatase-conjugated anti-DIG antibody (1:2000 dilution; Roche Applied Science) and then reacted with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Roche Applied Science).

Cell Culture and Transfection—HeLa and 293T cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum.
serum, 4 mM glutamine, 100 units/ml penicillin, and 0.2 mg/ml streptomycin under humidified air containing 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 Superfect (Qiagen) for HeLa cells, 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 GST-fused proteins were purified from *Escherichia coli* as described previously (14, 30). Recombinant GTPases were loaded with guanine nucleotide as described previously (24). 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 Myc-tagged Pragmin or HA-tagged Rho family GTPases were rinsed once with phosphate-buffered saline (PBS) and lysed with the ice-cold cell lysis buffer A (20 mM Tris- HCl, pH 7.5, 150 mM NaCl, 2 mM MgCl2, 1 mM dithiothreitol (DTT), 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, and 10 μg/ml leupeptin) for Pragmin or the buffer B (20 mM Tris-HCl, pH 7.5, 20 mM MgCl2, 1 mM DTT, 0.5% Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) for Rho family GTPases. Cell lysates were then centrifuged for 10 min at 16,000 x g at 4 °C. The supernatants were incubated for 2 h at 4 °C with 2 μg of GST fusion proteins immobilized on glutathione-Sepharose beads. 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 or anti-Myc antibody.

**FIGURE 3.** Rnd2 and Pragmin induce cell contraction in HeLa cells. A. HeLa cells were transiently transfected with HA-tagged Rnd2V16 (a), Myc-tagged Pragmin (b), HA-Rnd2V16 + Myc-Pragmin (c), or HA-Rnd2N21 + Myc-Pragmin (d), and cells were fixed and stained with Alexa 488-phalloidin (right) and either anti-Rnd2 (a, left) or anti-Myc antibody (b–d, left). Scale bar, 10 μm. B, HeLa cells were transiently transfected with HA-Rnd2V16 + Myc-Pragmin + HA-RhoAN19, and cells were fixed and stained with anti-Myc antibody (left) and Alexa 488-phalloidin (right). Scale bar, 10 μm. C, average cell area of HeLa cells untransfected or transfected with the indicated plasmids. HeLa cells were transiently transfected with the indicated plasmids together with GFP. Cells were fixed, and the morphology of transfected cells was visualized by the fluorescence of GFP. For untransfected cells, the morphology was visualized by F-actin staining with Alexa 488-phalloidin. At least 50 cells were assessed in one experiment, and data are the means ± S.E. of triplicate experiments. *, p < 0.01; **, p < 0.005; Student’s t test, in comparison with untransfected cells. D and E, quantitative analyses of HeLa cell contraction induced by Rnd2 and Pragmin. HeLa cells were transiently transfected with the indicated plasmids. Cell contraction was scored as a percentage of the round and shrunken cells expressing the indicated plasmid of the total number of transfected cells. At least 150 cells were assessed in one experiment, and data are the means ± S.E. of triplicate experiments. *, p < 0.01; **, p < 0.005; ***, p < 0.001; ****, p < 0.0001; Student’s t test, in comparison with GFP (C) or GFP + Pragmin (D).
cells were treated with 10 μM Y-27632 for 14 h. Cells were fixed and incubated with anti-HA monoclonal antibody for 1 h and then with protein A-Sepharose for 3 h at 4°C. The beads were washed with the homogenization buffer, and bound proteins were analyzed by SDS-PAGE and immunoblotting.

**Immunoprecipitation**—293T cells cotransfected with Myc-tagged Pragmin and HA-tagged Rnd2 N21 were washed once with PBS and lysed with the ice-cold cell lysis buffer A. After centrifugation, the supernatants were incubated with anti-HA monoclonal antibody in 10% fetal bovine serum in PBS for 1 h, followed by incubation with Alexa 488/594-conjugated goat anti-rabbit IgG antibody in PBS for 30 min. Cells on coverslips were rinsed with PBS and fixed with 3.7% formaldehyde in PBS for 15 min. After residual formaldehyde had been quenched with 50 mM NH₄Cl in PBS for 10 min, the cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min and incubated with 10% fetal bovine serum in PBS for 1 h. For detection of Myc epitope, cells were incubated with anti-Myc monoclonal antibody in 10% fetal bovine serum in PBS for 1 h followed by incubation with Alexa 488-conjugated phallolidin in PBS (1:500 dilution) for 30 min. The membranes were blocked with dot-blot buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 1 mM DTT) containing 5% low fat milk for 1 h at 4°C. The membrane was then washed with the dot-blot buffer and then incubated with 3% low fat milk in Tris-buffered saline containing anti-Rnd1, Rnd2, or Rnd3 antibody. These antibodies were detected by using horseradish peroxidase-conjugated secondary antibodies and the ECL detection kit.

**Immunofluorescence Microscopy**—HeLa cells were seeded onto round 13-mm glass coverslips in 24-well plates at a density of 1.5 × 10⁴ cells/well, and cultured for 16 h after transfection. PC12 cells were seeded onto round 13-mm glass coverslips coated with poly-D-lysine at a density of 1 × 10⁴ cells/well. After 5 h at transfection, PC12 cells were differentiated with 50 ng/ml nerve growth factor (NGF; Promega Corp.) in serum-free Dulbecco’s modified Eagle’s medium and further cultured for 43 h. For siRNA-mediated knockdown experiments, PC12 cells were differentiated with 50 ng/ml NGF for 24 h and then transfected in the presence of 50 ng/ml NGF followed by further incubation for 36 h. Cells on coverslips were rinsed with PBS and fixed with 3.7% formaldehyde in PBS for 15 min. After residual formaldehyde had been quenched with 50 mM NH₄Cl in PBS for 10 min, the cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min and incubated with 10% fetal bovine serum in PBS for 1 h. For detection of Myc epitope, cells were incubated with anti-Myc monoclonal antibody in 10% fetal bovine serum in PBS (1:1000 dilution) for 1 h, followed by incubation with Alexa 488/594-conjugated goat anti-mouse IgG antibody in PBS (1:2000 dilution) for 1 h. For detection of Rnd2, cells were incubated with anti-Rnd2 polyclonal antibody in 10% fetal bovine serum in PBS (1 μg/ml) for 1 h, followed by incubation with Alexa 594-conjugated goat anti-rabbit IgG antibody in PBS (1:2000 dilution) for 1 h. Filamentous actin (F-actin) was stained with Alexa 488-conjugated phallolidin in PBS (1:500 dilution) for 1 h. Cells on coverslips were mounted in 90% glycerol containing 0.1% p-phenylendiamine dihydrochloride in PBS and photo-

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A dot-blot assay was performed as described previously (25). Briefly, 8 μg of GST, GST-fused Pragmin–CT⁴, Rho-binding domain of Rho-kinase (amino acids 932–1065) (31) was spotted onto a nitrocellulose membrane and allowed to dry for 2 h at room temperature. The membrane was blocked with dot-blot buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 1 mM DTT) containing 5% low fat milk for 1 h at 4°C. Cell lysis was scored as a percentage of the round and shrunken cells expressing the indicated plasmids of the total number of transfected cells. At least 150 cells were assessed in one experiment, and data are the means ± S.E. of triplicate experiments. *, p < 0.01; ***, p < 0.001; Student’s t test, in comparison with each counterpart.
FIGURE 6. **Rnd2 and Pragmin inhibit the NGF-induced neurite outgrowth.**

**A.** PC12 cells were transiently transfected with HA-tagged Rnd2^{V16} and Myc-tagged Pragmin and then treated with 50 ng/ml NGF together with 10 μM Y-27632 for 44 h. Cells were fixed and costained with anti-HA (left, red in the merged image) and anti-Myc (middle, green in the merged image) antibodies. Note that red/green overlap leads to yellow at the tip of a neurite (arrowhead) as well as in the cell body in the merged image (right). Scale bar, 10 μm. **B.** PC12 cells were transiently transfected with GFP (a), HA-tagged Rnd2^{V16} (b), Myc-tagged Pragmin (c), or HA-Rnd2^{V16} + Myc-Pragmin (d) and then treated with 50 ng/ml NGF for 44 h. Cells were fixed and immunostained with anti-Rnd2 (b, left) or anti-Myc antibody (c and d, left) to identify transfected cells. The morphology of the cells was visualized by F-actin staining with Alexa 488-phalloidin (right). Scale bar, 10 μm. **C.** PC12 cells were transiently transfected with Myc-tagged Pragmin-NT (a) or Myc-tagged Pragmin-CT (b) and then treated with 50 ng/ml NGF for 44 h. Cells were fixed and immunostained with anti-Myc antibody (left) to identify transfected cells. The morphology of the cells was visualized by F-actin staining with Alexa 488-phalloidin (right). Scale bar, 10 μm. **D.** Length distribution of NGF-induced neurites in PC12 cells expressing Rnd2 and Pragmin. PC12 cells were transiently transfected with the indicated plasmids and then treated with 50 ng/ml NGF for 44 h. Cells were costained with Alexa 488-phalloidin and either anti-HA or anti-Myc antibody, and positively stained cells were assessed. Cells with neurites (<10 μm, 10–40 μm, and >40 μm) were scored as a percentage of the total number of transfected cells. At least 50 cells were assessed in one experiment, and data are the means ± S.E. of triplicate experiments. *, p < 0.01; ***, p < 0.001; Student’s t test, in comparison with GFP.
graphed with a Leica DC350F digital camera system equipped with a Nikon Eclipse E800 microscope. The images were analyzed with Image-Pro Plus image analysis software.

Measurement of RhoA Activity—Measurement of RhoA activity was performed as described previously (32, 33). Briefly, 16 h after transfection, HeLa cells were washed with ice-cold Tris-buffered saline and lysed for 10 min with cell lysis buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 30 mM MgCl₂, 1% Triton X-100, 10% glycerol, 1 mM DTT, 1 mM PMSF, 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 3 min at 16,000 × g at 4 °C, and the supernatants were incubated with glutathione-Sepharose beads for 50 min at 4 °C. The beads were washed with wash buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 30 mM MgCl₂, 0.5% Triton X-100, 1 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin), and bound proteins were analyzed by SDS-PAGE and immunoblotting. Densitometric analyses were performed with NIH Image software, and the amounts of GTP-bound RhoA were normalized to the total amounts of RhoA in cell lysates.

RESULTS

Cloning of Pragmin, a Novel Rnd2 Effector, and Its Expression—In an attempt to identify effector proteins of Rnd2, we employed a two-hybrid screening of a rat brain cDNA library with Rnd2V16S224, equivalent to a constitutively active Val-12 mutation in Ras (25), lacking the COOH-terminal CAAX motif, as bait. Approximately 1.2 × 10⁷ clones were screened, and several positive clones were isolated. Sequence analyses revealed that one of them encoded the COOH-terminal region of a novel protein. A BLAST search to match the isolated fragment revealed that it shows 98% identity to rat NCBI reference sequence clone XM_224908, its function being unknown. We cloned cDNA encoding full-length of this protein (1368 amino acids) and then named it Pragmin (pragma of Rnd2). Pragmin has two serine-rich regions and a kinase-like domain at the COOH terminus (Fig. 1A). The kinase-like domain has substitutions in the highly conserved DFG triplet within the activation segment critical for kinase activity. The other region of Pragmin showed no significant sequence homology to any other known proteins. To examine the tissue distribution of Pragmin, we carried out reverse transcription-PCR. Pragmin mRNA was detected in rat brain, kidney, spleen, small intestine, and colon (Fig. 1B). The expression level of Pragmin mRNA did not change during the development of brain from embryonic day 14 to adult. To reveal the distribution of Pragmin mRNA in the postnatal day 27 of rat brain, we performed in situ hybridization using a DIG-labeled Pragmin antisense riboprobe (Fig. 1C). Expression of Pragmin mRNA was observed in cortical neurons and hippocampal pyramidal neurons. Pragmin mRNA-expressing neurons were also found in the piriform cortex and cerebellum. This expression pattern was similar to that of Rnd2 mRNA (22).

To determine whether the interaction of Rnd2 with Pragmin is dependent on GTP, full-length Pragmin tagged with Myc was expressed in 293T cells and used in a pull-down assay with GST-fused Rnd2 preloaded with GTPγS or nucleotide-free GST-fused Rnd2. Pragmin interacted with GTPγS-bound Rnd2 but not with nucleotide-free Rnd2 or GST alone (Fig. 2A). To examine whether Pragmin interacts specifically with Rnd2 among the Rho family GTPases, HA-tagged Rnd subfamily GTPases and well-characterized Rho family GTPases were expressed and used in a pull-down assay with the GST-fused COOH-terminal region of Pragmin (Pragma-CT), a fragment originally isolated from the yeast two-hybrid screening. Both wild-type Rnd2 and Rnd2V16 strongly bound to Pragmin-CT, whereas Rnd1, Rnd3, constitutively active RhoA (RhoA V14), Rac1 (Rac1 V12), and Cdc42 (Cdc42 V12) did not, indicating that Rnd2 specifically binds to Pragmin among the Rho family GTPTases (Fig. 2B). To examine whether Rnd2 directly binds to Pragmin, we carried out a dot-blot assay. GST-fused Pragmin-CT was spotted onto a nitrocellulose membrane, and the membrane was overlayed with GTPγS-preloaded Rnd1, Rnd2, or Rnd3. Pragmin interacted with Rnd2 but not with Rnd1 or Rnd3, although the RhoA-binding domain of Rho-kinase, a RhoA-specific effector, failed to interact with three GTPases (Fig. 2C). On the other hand, the COOH-terminal tail of Plexin-B1, which is known to associate with three Rnd GTPTases (31), interacted with Rnd1, Rnd2, and Rnd3. The interaction between Rnd2 and Pragmin also occurred in mammalian cells, as observed by an immunoprecipitation study of 293T cells cotransfected with Myc-tagged Pragmin and HA-tagged Rnd2 V16 (Fig. 2D). In contrast, Rnd2V21, equivalent to a dominant negative Asn-17 mutation in Ras, did not bind to Pragmin in the cells. Furthermore, we examined the endogenous interaction between Rnd2 and Pragmin in the embryonic day 16 of rat brain, since they were highly expressed in the brain (22). Antibody for Pragmin was generated against bacterially expressed the GST-fused NH₂-terminal region of Pragmin and was confirmed to recognize Myc-tagged Pragmin specifically (Fig. 2E). Endogenous Pragmin was coimmunoprecipitated with Rnd2 by anti-Rnd2 polyclonal antibody but not by anti-HA antibody used as a negative control (Fig. 2F).

Pragmin Induces Cell Contraction through RhoA Activation—To investigate the role of the interaction of Rnd2 with Pragmin, HeLa cells were transfected with Pragmin, Rnd2V16, or both, and morphological changes in transfected cells were analyzed by the fluorescence of F-actin. In the Pragmin-expressing cells, Pragmin was diffusely distributed throughout the cell body with some punctate structures (Fig. 3A, b, left). When cells were cotransfected with Pragmin and Rnd2 V16, Pragmin was
accumulated at dense punctate structures in the cell periphery (Fig. 3, a, left). Rnd2V16 expression alone showed slight cell contraction, resulting in 70% of the cell area of untransfected or GFP-transfected control HeLa cells, and cells were fixed and stained with anti-RhoA antibody (right). The transfected cells were shown by the fluorescence of GFP (left). Arrowheads and arrows indicate the transfected and untransfected cells, respectively. Scale bar, 10 μm. C, after NGF-induced differentiation for 24 h, PC12 cells were transiently transfected with GFP together with control siRNA (a), Pragmin siRNA-A (b), or Pragmin siRNA-B (c) for 36 h. Cells were fixed, and transfected cells were shown by the fluorescence of GFP. Scale bar, 10 μm. D, length distribution of NGF-induced neurites in PC12 cells expressing Pragmin siRNAs together with GFP. GFP-positive cells with neurites (<10 μm, 10–40 μm, and >40 μm) were scored as a percentage of the total number of transfected cells. At least 50 cells were assessed in one experiment, and data are the means ± S.E. of triplicate experiments. *, p < 0.01; Student’s t test, in comparison with control. 

**FIGURE 8. Knockdown of Pragmin expression enhances neurite elongation.** A, 293T cells were transiently transfected with GFP-fused Pragmin together with either Pragmin siRNA-A, Pragmin siRNA-B, or control siRNA, and the cell lysates were immunoblotted with anti-GFP or anti-α-tubulin antibodies. B, after NGF-induced differentiation for 24 h, PC12 cells were transiently transfected with GFP together with Pragmin siRNA-B for 36 h, and cells were fixed and stained with anti-RhoA antibody (right). The transfected cells were shown by the fluorescence of GFP (left). Arrowheads and arrows indicate the transfected and untransfected cells, respectively. Scale bar, 10 μm. C, after NGF-induced differentiation for 24 h, PC12 cells were transiently transfected with GFP together with control siRNA (a), Pragmin siRNA-A (b), or Pragmin siRNA-B (c) for 36 h. Cells were fixed, and transfected cells were shown by the fluorescence of GFP. Scale bar, 10 μm. D, length distribution of NGF-induced neurites in PC12 cells expressing Pragmin siRNAs together with GFP. GFP-positive cells with neurites (<10 μm, 10–40 μm, and >40 μm) were scored as a percentage of the total number of transfected cells. At least 50 cells were assessed in one experiment, and data are the means ± S.E. of triplicate experiments. *, p < 0.01; Student’s t test, in comparison with control. E, average length of NGF-induced neurites in PC12 cells expressing Pragmin siRNAs. After NGF-induced differentiation for 24 h, PC12 cells were transiently transfected with Pragmin siRNAs together with GFP or GFP + HA-tagged Rnd2V16 for 36 h. Cells were fixed, and GFP-positive cells were assessed. At least 50 cells were assessed in one experiment, and data are the means ± S.E. of triplicate experiments. **, p < 0.005; ***, p < 0.001; Student’s t test, in comparison with each control siRNA.
ulate RhoA activity. These results indicate that Pragmin stimulates RhoA activity in response to Rnd2.

**Pragmin Inhibits Neurite Outgrowth through the NH₂-terminal Region**—Activation of RhoA has been known to stabilize cortical actin and prevents NGF-induced neurite outgrowth in PC12 cells (34, 35). We then examined the localization of Rnd2 and Pragmin in Y-27632-treated PC12 cells differentiated with NGF because cells overexpressing both Rnd2 and Pragmin were estimated to induce cell contraction and inhibit neurite outgrowth. Rnd2V16 and Pragmin colocalized at the neurite tip as well as in the cell body (Fig. 6A). We next examined the effect of Pragmin on NGF-induced neurite outgrowth. After transfection with Rnd2V16, Pragmin, or both, PC12 cells were treated with NGF, and 48 h later, the morphologies of the transfected cells were examined. Expression of Rnd2V16 slightly inhibited the NGF-induced neurite outgrowth, and expression of Pragmin significantly inhibited the outgrowth (Fig. 6B). On the other hand, coexpression of Rnd2V16 and Pragmin markedly suppressed the NGF-induced neurite outgrowth and induced cell rounding (Fig. 6, B and D). We next examined the region within Pragmin responsible for RhoA activation and inhibition of neurite outgrowth. Expression of Pragmin-NT by itself strongly inhibited the NGF-induced neurite outgrowth (Fig. 6C). On the other hand, the inhibitory activity was not observed with Pragmin-CT (Fig. 6, C and D). Expression of Pragmin-NT, but not Pragmin-CT, markedly stimulated RhoA activity to the same extent as that induced by Rnd2V16 and Pragmin (Fig. 7). Therefore, the Pragmin-induced inhibition of neurite outgrowth is mediated by its NH₂-terminal region.

**Knockdown of Pragmin Expression Promotes Neurite Elongation**—Since overexpression of Pragmin inhibited neurite outgrowth in PC12 cells, we further examined if knockdown of endogenous Pragmin with siRNA induces neurite extension. Between two Pragmin-specific siRNAs designed to target different coding regions of rat Pragmin (Pragmin siRNA-A and -B), Pragmin siRNA-B significantly reduced exogenously expressed Pragmin in 293T cells, whereas Pragmin siRNA-A had no effect, exhibiting the same expression level of Pragmin as control siRNA (Fig. 8A). Next, we confirmed the reduction of endogenous Pragmin in Pragmin siRNA-B–expressing PC12 cells by immunostaining with anti-Pragmin antibody (Fig. 8B, arrowhead compared with arrow). The neurite morphology in the NGF–differentiated PC12 cells expressing Pragmin siRNA-A with GFP was similar to that of control siRNA-expressing cells (Fig. 8C). In contrast, significantly longer neurites were shown in cells transfected with Pragmin siRNA-B (Fig. 8, C–E). Furthermore, Rnd2V16–reduced neurite outgrowth was prevented by Pragmin siRNA-B but not by control siRNA or Pragmin siRNA-A (Fig. 8E), suggesting that endogenous Pragmin is involved in the Rnd2 signal pathway. These results indicate that Pragmin is the effector of Rnd2 for negative regulation of neurite outgrowth in PC12 cells.

**Endogenous Rapostlin Has No Effect on Pragmin-induced Inhibition of Neurite Outgrowth**—We previously reported another Rnd2 effector, Rapostlin, inducing neurite branching in PC12 cells (25, 26). To examine whether endogenous Rapostlin modulates the effect of Pragmin on neurite outgrowth, we examined the effect of knockdown of endogenous Rapostlin by siRNA for Rapostlin on the inhibition of neurite outgrowth by overexpression of Pragmin or Pragmin-NT in PC12 cells. Rapostlin siRNA significantly reduced exogenously expressed Rapostlin in 293T cells (Fig. 9A). Pragmin and Pragmin-NT significantly reduced the NGF-induced neurite outgrowth (Fig. 9B). Rapostlin siRNA did not affect the inhibitory effect of Pragmin and Pragmin-NT (Fig. 9C), suggesting that Rapostlin is not involved in the Pragmin-induced inhibition of neurite outgrowth.

**DISCUSSION**

Rnd2 is a new member of Rho family GTPases, which is expressed in hippocampal neurons in the brain. To identify downstream effectors of Rnd2, we performed a yeast two-hybrid screen with Rnd2 as bait and isolated a novel effector of Rnd2, Pragmin, which stimulates RhoA activity and inhibits NGF-induced neurite outgrowth.

Pragmin has a kinase-like domain at the COOH terminus. The kinase-like domain has substitutions in the highly conserved DFG triplet within the activation segment critical for kinase activity. Therefore, Pragmin is unlikely to have catalytic kinase activity. Rnd2 binds to the COOH-terminal region containing the kinase-like domain, whereas the NH₂-terminal half region is responsible for stimulation of RhoA activity.
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by Pragmin. RhoA is usually activated by a variety of GEFs, and DH domains within GEFs are catalytic domain for RhoA activation (36). However, the NH2-terminal half region of Pragmin shows no significant sequence homology to any other known proteins including Rho family GEFs with DH domain. Thus, Pragmin has no DH domain and does not appear to directly function as a GEF for RhoA. Pragmin may stimulate RhoA activity through association with unidentified RhoA-activating molecule(s) at the NH2-terminal region.

Among Rnd subfamily GTPases, downstream effectors of Rnd1 and Rnd3 have been extensively investigated. Rnd1 and Rnd3 associate with and translocate Socius to plasma membranes, leading to disappearance of stress fibers, a well known action of RhoA activation (20). Rnd3 directly binds to the kinase domain of Rho-kinase, suppressing the kinase activity (37). In addition, Rnd1 and Rnd3 interact with p190RhoGAP, a Rho-specific GAP, and stimulate the GAP activity of p190RhoGAP, causing down-regulation of RhoA activity (21). Although Rnd2 can interact with both Socius and p190RhoGAP in vitro, Rnd2 cannot exhibit inhibition of Rho signaling through the interaction. At any rate, Rnd1 and Rnd3 act as negative regulators for RhoA signaling through their downstream effectors mentioned above. This time we revealed that Pragmin is a specific downstream effector of Rnd2 and stimulates RhoA activity in response to Rnd2. Rnd2-bound Pragmin induces cell contraction in HeLa cells and inhibition of NGF-induced neurite outgrowth in PC12 cells through RhoA activation. These results taken together, G proteins of the Rnd subfamily are thought to be upstream regulators of RhoA activity; Rnd1 and Rnd3 inhibit RhoA activity, whereas Rnd2 stimulates it.

We also identified another Rnd2 effector, Rapostlin, in the previous report (25). Rapostlin interacts with N-WASP, an effector of Cdc42, releases it in response to Rnd2, and induces neurite branching in PC12 cells (26). In this paper, we confirmed that Rapostlin does not affect the Pragmin-induced inhibitory effect of neurite outgrowth by means of knock-down of endogenous Rapostlin. Thus, Rnd2 may be involved in several signal pathways in regulation of neuronal morphology through different effectors, such as neurite branching with Rapostlin and neurite outgrowth inhibition with Pragmin.

Rnd1 and Rnd2 are predominantly expressed in neurons in brain (22, 23, 29). Rnd1 has been shown to promote neurite formation in PC12 cells (27). On the other hand, we here revealed that Rnd2 suppresses the neurite outgrowth through Pragmin in PC12 cells. In addition, knock-down of endogenous Pragmin by siRNA induces neurite elongation. Therefore, Rnd1 and Rnd2 induce opposite control of neurite morphology through differential regulation of RhoA activity, and Pragmin plays an important role in the Rnd2 signaling pathway.

In conclusion, we have identified a novel downstream effector of Rnd2, Pragmin, stimulating RhoA activity and regulating neurite outgrowth. We recently revealed that Rnd1 associates with FRS2, an adaptor of fibroblast growth factor receptor, and fibroblast growth factor releases Rnd1 from FRs2 and induces Rnd1-mediated down-regulation of RhoA activity, resulting in stimulating neurite outgrowth (38). On the other hand, molecules participating in the upstream signaling of Rnd2 have not yet been elucidated. Further studies focusing on the identification of Rnd2-interacting proteins will contribute to understanding of the upstream signaling pathway of Rnd2 and regulation of Rho signaling by Rnd subfamily GTPases.