Rapostlin Is a Novel Effector of Rnd2 GTPase Inducing Neurite Branching*

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Rho family GTPases are central regulators of neuronal morphology. Recently, Rho proteins, Rnd1, Rnd2, and Rnd3/RhoE, have been identified as new members of Rho family GTPases. Of these, Rnd2 is specifically expressed in neurons in brain; however, the signaling pathways of Rnd2 are not known. Here we have performed a yeast two-hybrid screen using Rnd2 as a bait and identified a novel Rnd2-effector protein, expressed predominantly in brain. We named it Rapostlin (apostle of Rnd2). Rapostlin has two functional domains, FCH (FvCIP4 homology) and SH3 (Src homology 3) domain at the amino terminus and SH3 (Src homology 3) domain at the carboxyl terminus. In vitro binding assays, Rapostlin specifically binds to Rnd2 among the Rho family GTPases in a GTP-dependent manner, and the Rnd2-binding domain of Rapostlin is localized between FCH and SH3 domains. Rapostlin directly binds to microtubules, and the amino-terminal region containing the FCH domain of Rapostlin is essential for this interaction. In PC12 cells, Rapostlin induces neurite branching in response to Rnd2, and at least the amino-terminal region of Rapostlin is necessary for this activity. Therefore, Rapostlin is the first effector of Rnd2, regulating neurite branch formation.

The organization of the nervous system is a complex and orchestrated process. Neurons migrate to their characteristic locations, extend axons and dendrites toward proper target regions, and form synaptic connections with appropriate partners. These dynamic morphological changes of neurons are largely decided by the cytoskeletal organization.

Rho family GTPases, consisting of Rho, Rac, and Cdc42, have been implicated in the regulation of the cytoskeleton and subsequent morphological changes in various cells, such as formations of actin stress fibers, lamellipodia, and filopodia, respectively in fibroblasts (1). These actions of Rho family GTPases are mediated by a variety of downstream effectors (2). In neuronal cells, Rho family GTPases have been shown to be involved in the regulation of neuronal cell morphology including neuritogenesis. Rho activation induces the inhibition of neuritogenesis, the collapse of the growth cone, and the retraction of neurites (3–5). These morphological actions of Rho have been shown to be mediated by Rho-kinase, a downstream effector of Rho (6, 7). On the other hand, Rac and Cdc42 are involved in the formation of filopodia and lamellipodia of the growth cone and in the outgrowth of neurites (4). Effectors of Rac and Cdc42, PAK1 and neural Wiskott-Aldrich syndrome protein (N-WASP), have been shown to play roles in these actions (8, 9). The less studied members of Rho family GTPases, such as RhoG and TC10, are also involved in the regulation of neuritogenesis (10, 11), suggesting that a variety of Rho family GTPases participate in the regulation of neuronal morphology.

Recently, a new branch of Rho family GTPases, the Rnd subfamily, consisting of Rnd1, Rnd2, and Rnd3, has been identified (12). Rnd1 is mainly expressed in brain and liver, whereas Rnd3 is expressed ubiquitously (12, 13). Unlike other Rho family GTPases, Rnd1 and Rnd3 possess very low intrinsic GTPase activity and constitutively bind to GTP (12–14). Expression of Rnd1 or Rnd3 in fibroblasts results in loss of actin stress fibers and focal adhesions, indicating the antagonistic effect on the Rho-regulated signaling pathway (12). We have demonstrated that Rnd1 induced neurite process formation, probably due to the inhibition of the Rho-regulated signaling pathway (15), and then we have identified a novel Rnd GTPase-interacting protein, Socius, which is involved in the Rnd1-induced disassembly of actin stress fibers (16). In contrast to Rnd1 and Rnd3, little is known about Rnd2, although Rnd2 is expressed specifically in neurons in brain (17), suggesting that Rnd2 plays an important role in neuronal functions. We have recently demonstrated that Vps4-A, involved in the endosomal vesicle trafficking, is the first Rnd2-binding protein but is not an effector of Rnd2 since it binds to both active and negative forms of Rnd2 (18).

To dissect the molecular mechanism of neuronal functions of Rnd2, it is urgent to identify downstream effectors of Rnd2. Here we performed a yeast two-hybrid screen to identify effectors of Rnd2 and found a novel effector protein of Rnd2, Rapostlin. Rapostlin specifically binds to Rnd2 among the Rho family GTPases in a GTP-dependent manner. Rapostlin directly binds to microtubules, and the amino-terminal region of Rapostlin is essential for this interaction. In PC12 cells, Rapostlin in concert with constitutively active Rnd2 induces neurite branching, and at least the amino-terminal region of Rapostlin is necessary for this activity. Therefore, this is the first protein to be identified as an effector of Rnd2.

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids—Wild-type and mutant forms of Rnd2, wild-type Rnd1, Rnd3, RhoA, Rac1, and Cdc42 were obtained as described previously (10, 16, 19). For the yeast two-hybrid screen, the cDNA encoding Rnd2wt–234, lacking a farnesylation site in the carboxy-terminal CAAX motif by a substitution of cysteine to serine, was fused to the GAL4-DNA binding domain in the yeast expression vector pAS2-1 (Clontech). For purification of recombinant proteins, cDNA of Rnd2 was subcloned into pAcG2T (Pharmingen), whereas cDNAs of
Dot-blot assays were performed as described previously (21). GST-fused deletion mutants of Rapostlin (GST-Rapostlin) were spotted onto nitrocellulose membranes and allowed to dry, and the specific antibody was purified by elution with 5 μl of cell lysate by using the Dual-Luciferase reporter assay system (Promega) and a Luminoskan luminometer (Labsystems, Helsinki, Finland). Relative activity was defined as the ratio of firefly luciferase activity to Renilla luciferase activity.

**Microtubule Binding Assay—Co-sedimentation of Rapostlin and microtubules was performed as described previously (23). Briefly, 293T cells in 100-mm dishes at a density of 2 × 10⁵ cells/dish were transiently transfected with Myc-tagged Rapostlin or Myc-tagged Rapostlin ΔN1 and lysed with general tubulin buffer (Cytoreskeleton) containing 1% Triton X-100 and 0.5% sodium deoxycholate after the transfection. Lysates were centrifuged at 100,000 × g for 1 h at 4 °C in a Micro Ultracentrifuge (Hitachi). Clear supernatant fractions were incubated with 5 μg tubulin, 4 °C 1 h with 10 μg of rabbit anti-GST Rnd2 antibody, which were generated using the Microtubule/Tubulin Biochem kit (Cytoreskeleton). Microtubules were then pelleted by centrifugation at 100,000 × g for 1 h. Supernatant and pellet fractions were subjected to 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Millipore Corporation). The membrane was blocked with 3% low fat milk in Tris-buffered saline and incubated with mouse anti-Myc monoclonal antibody (mAb) E5-1-2 (Sigma; 1:1000 dilution) or mouse anti-Myc mAb 9E10 (Santa Cruz Biotechnology; 2 μg/ml). These antibodies were detected by using horseshadish peroxidase-conjugated anti-mouse IgG (DAKO) and an ECL detection kit.

**Immunofluorescence Microscopy—**HeLa and PC12 cells were seeded in 24-well plates at a density of 5 × 10⁴ cells/well onto glass coverslips (traditional, 13 mm in diameter) and in 12-well plates at a density of 8 × 10⁴ cells/well onto poly-l-lysine-coated glass coverslips (circular, 18 mm in diameter), respectively. Forty h after the transfection, cells on coverslips were washed with phosphate-buffered saline (PBS) and then 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, cells were permeabilized with 0.2% Triton X-100 in PBS for 30 min and incubated in anti-Myc antibody (0.2 μg/ml) for 30 min. Cells were then incubated with rabbit anti-Rnd2 pAb (1 μg/ml) in PBS for 1 h followed by incubation with a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Chemicon; 1:250 dilution) in PBS for 1 h. For detection of Myc-tagged Rapostlin or Rapostlin ΔN1, cells were incubated with either mouse anti-Myc mAb 9E10 (2 μg/ml) or rabbit anti-Myc mAb 9E10 (2 μg/ml) in PBS for 1 h followed by incubation with a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Chemicon; 1:250 dilution) and a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:250 dilution), respectively, in PBS for 1 h. Microtubules were stained with mouse anti-α-tubulin mAb (Oncogene Science; 0.2 μg/ml) followed by incubation with a rhodamine-conjugated goat anti-rabbit IgG (1:250 dilution) in PBS for 1 h. Filamentous actin (F-actin) was visualized with rhodamine-conjugated phalloidin (Molecular Probes; 0.4 units/ml) in PBS for 1 h. Cells on coverslips were mounted in 90% glycerol containing 0.1% p-phenylenediamine dihydrochloride in PBS and photographed by an MRC-1024 laser scanning confocal imaging system (Bio-Rad) equipped with a Nikon Eclipse E800 microscope and a Nikon Plan Apo 40 × 1.4 oil immersion objective lens.

**Branching Activity—**Branching activity was determined as described previously (24). Briefly, PC12 cells bearing neurites longer than 5 μm were counted. Only processes longer than 5 μm were considered as branches. The branching activity was defined as the ratio of the total number of the branches to the total length of the neurites from 20 cells, that is, the average number of branches per region. We adopted this criterion because neurites had generally more branches as neurites were longer.
RESULTS

Cloning of Rapostlin, a Novel Rnd2 Effector—To gain insight into the downstream signaling of Rnd2, we carried out a yeast two-hybrid screen using human Rnd2 as a bait. Several positive clones were isolated from a rat brain cDNA library. Sequence analyses revealed that one of these encodes a protein almost identical to human formin-binding protein 17 (FBP17). In the yeast two-hybrid system, this clone specifically interacted with Rnd2 within the Rnd subfamily and with the well-studied Rho GTPases, RhoA, Rac1, and Cdc42. Then, we named it Rapostlin (apostle of Rnd2) (Fig. 1A). The context of the putative Met codon (ACCAUGA) (where underlining represents initiator codon) of Rapostlin is closer to the Kozak’s consensus translation initiation sequence (ACCAUGG) (25). Rapostlin and hFBP17 proteins share 93% sequence identity and have well conserved regions, a Fer-CIP4 homology (FCH) domain at the amino terminus and an SH3 domain at the carboxyl terminus (Fig. 1B). Rapostlin shares 47% sequence identity with CIP4, which was isolated previously as a Cdc42 effector protein (26). However, Rapostlin has a unique insert region, which is absent in CIP4. A coiled-coil region is found between FCH and the insert region of Rapostlin. To examine the tissue distribution of Rapostlin, we carried out a Northern hybridization analysis. Rapostlin mRNAs were predominantly expressed in brain, and weaker expression was detected in lung and spleen (Fig. 2).

To confirm the interaction of Rapostlin and Rnd2, we carried out dot-blot assays. Rapostlin lacking the amino-terminal region two-hybrid screen using human Rnd2 as a bait. Several positive clones were isolated from a rat brain cDNA library. Sequence analyses revealed that one of these encodes a protein almost identical to human formin-binding protein 17 (FBP17). In the yeast two-hybrid system, this clone specifically interacted with Rnd2 within the Rnd subfamily and with the well-studied Rho GTPases, RhoA, Rac1, and Cdc42. Then, we named it Rapostlin (apostle of Rnd2) (Fig. 1A). The context of the putative Met codon (ACCAUGA) (where underlining represents initiator codon) of Rapostlin is closer to the Kozak’s consensus translation initiation sequence (ACCAUGG) (25). Rapostlin and hFBP17 proteins share 93% sequence identity and have well conserved regions, a Fer-CIP4 homology (FCH) domain at the amino terminus and an SH3 domain at the carboxyl terminus (Fig. 1B). Rapostlin shares 47% sequence identity with CIP4, which was isolated previously as a Cdc42 effector protein (26). However, Rapostlin has a unique insert region, which is absent in CIP4. A coiled-coil region is found between FCH and the insert region of Rapostlin. To examine the tissue distribution of Rapostlin, we carried out a Northern hybridization analysis. Rapostlin mRNAs were predominantly expressed in brain, and weaker expression was detected in lung and spleen (Fig. 2).

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transiently transfected with pcDNA3 encoding Rnd2Val-16 (B). The results shown were the means ± S.E. for triplicate determinations.

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Fig. 5. Rapostlin binds to microtubules in vitro. 293T cells were transiently transfected with pcDNA3 encoding Myc-tagged Rapostlin (A) or Myc-tagged Rapostlin ΔN1 (B). Cells were lysed for 40 h after the transfection and centrifuged to prepare cytosolic extracts. These cytosolic extracts were incubated with (+) or without (−) taxol-stabilized microtubules, and then microtubules were pelleted by high speed centrifugation. Supernatant (s) and pellet (p) fractions were subjected to SDS-PAGE and immunoblotting using anti-Myc mAb (A and B) and anti-α-tubulin mAb (C).

Fig. 4. Interaction of Rapostlin with Rnd2 and effects of Rapostlin and Rnd2 on cytoskeletons in mammalian cells. A, in vivo interaction of Rapostlin with Rnd2 in mammalian two-hybrid system. COS-7 cells were transiently transfected with pBIND vector encoding both the Renilla luciferase and GAL4-fused small GTPases, pACT vector encoding VP16-fused Rapostlin, and the reporter pG5luc vector encoding the firefly luciferase. Thirty-six h after the transfection, cells were solubilized, and luciferase activities were measured as described under “Experimental Procedures.” Relative activity was defined as the ratio of firefly luciferase activity to Renilla luciferase activity. The results shown were the means ± S.E. for triplicate determinations. B, colocalization of Rapostlin and Rnd2 in HeLa cells. HeLa cells were transiently transfected with pcDNA3 encoding Rnd2Val-16 (lane a), Myc-tagged Rapostlin (lane b), or Rnd2Val-16 + Myc-tagged Rapostlin (lane c). Forty h after the transfection, cells were fixed and stained with anti-Rnd2 pAb (Rnd2Val-16, anti-Myc pAb (lane b, Rapostlin), or anti-Myc mAb (lane c, Rapostlin). The right panel (Merged) shows the superposition of left and middle images. The scale bar represents 10 μm. C, effects of Rapostlin and Rnd2 on cytoskeletons in HeLa cells. HeLa cells were transiently transfected with pcDNA3 encoding Myc-tagged Rapostlin (lanes a and b) or Rnd2Val-16 + Myc-tagged Rapostlin (lane c).

Forty h after the transfection, cells were fixed and stained with anti-Myc pAb (Rapostlin) and either anti-α-tubulin mAb (Tubulin) or rhodamine-conjugated phalloidin (F-actin). The right panels (Merged) show the superposition of left and middle images. The scale bars represent 10 μm.
Rd2<sup>Val-16</sup> was mainly localized in the particulate fraction (data not shown). In the Rapostlin-expressing cells, Rapostlin was localized in the cytoplasmic linear structures (Fig. 4B, lane b). When HeLa cells were cotransfected with Rd2<sup>Val-16</sup> and Rapostlin, they were colocalized in a perinuclear condensed region accompanied with emanated linear structures (Fig. 4B, lane c). These results suggest that Rapostlin binds to constitutively active Rd2 in mammalian cells. We next examined the effects of Rd2 and Rapostlin on cytoskeletons in HeLa cells. In the Rapostlin-expressing cells, Rapostlin was partially localized in microtubules (Fig. 4C, lane a), whereas Rapostlin neither overlapped with F-actin nor induced a prominent change in the F-actin structure (Fig. 4C, lane b). When HeLa cells were cotransfected with Rd2<sup>Val-16</sup> and Rapostlin, they induced reorganization of F-actin to the perinuclear condensed region, where Rd2<sup>Val-16</sup> and Rapostlin were colocalized (Fig. 4C, lane c). These results suggest that the association of Rd2<sup>Val-16</sup> with Rapostlin induces the drastic reorganization of F-actin.

**Binding of Rapostlin to Microtubules**—The amino-terminal region of CIP4 has been reported to bind to microtubules (27). To examine whether the amino-terminal region of Rapostlin is essential for the interaction of Rapostlin to microtubules, we transiently expressed Rapostlin or Rapostlin ΔN1 in 293T cells and carried out microtubule binding assays (Fig. 5). Since microtubules can be pelleted by high speed centrifugation, binding of Rapostlin to microtubules can be tested in these co-sedimentation assays. In the presence of taxol-stabilized microtubules, Rapostlin bound to microtubules during high speed centrifugation (Fig. 5A), but Rapostlin ΔN1 did not (Fig. 5B). These results suggest that Rapostlin binds to microtubules, either directly or indirectly, and that the amino-terminal region of Rapostlin is essential for the interaction.

**Rnd2 and Rapostlin Induce Neurite Branching in PC12 Cells**—Rnd2 and Rapostlin are predominantly expressed in brain, and in situ hybridization analyses revealed that Rnd2 was expressed specifically in neurons in brain (17). To examine whether Rnd2 and Rapostlin were involved in the regulation of neuronal cell morphology, we transiently expressed Rnd2, Rapostlin, or both in the nerve growth factor-differentiated PC12 cells. GFP-expressing control cells extended a few long unbranched neurites bearing microtubules (Fig. 6A, lane a). Expression of Rnd2<sup>Val-16</sup> induced a few branches (Fig. 6A, lane b), but Rnd2<sup>Asn-21</sup>-expressing cells had unbranched neurites (Fig. 6A, lane c). Expression of Rapostlin or Rapostlin ΔN1 induces no or few branches from neurites (Fig. 6A, lanes d and e). On the other hand, coexpression of Rnd2<sup>Val-16</sup> and Rapostlin dramatically induced many branches from neurites (Fig. 6A, lane f). Fig. 6B shows the quantitative analyses of the effects of Rnd2 and Rapostlin on the branching activity. Rnd2<sup>Val-16</sup> alone significantly displayed the branching activity. This may be mediated by endogenous Rapostlin since we detected the expression of Rapostlin mRNA in PC12 cells by reverse transcriptase-PCR (data not shown). Active Rnd2, Rnd2<sup>Val-16</sup>, with Rapostlin (lane d), Myc-tagged Rapostlin ΔN1 (lane e), Rnd2<sup>Val-16</sup> + Myc-tagged Rapostlin (lane f), Rnd2<sup>Asn-21</sup> + Myc-tagged Rapostlin (lane g), or Rnd2<sup>Val-16</sup> + Myc-tagged Rapostlin ΔN1 (lane h). After the transfected cells had been differentiated with nerve growth factor for 40 h, they were fixed and stained with anti-a-tubulin mAb (Tubulin, middle panels) and either anti-Rnd2 pAb (Rnd2<sup>Val-16</sup> and Rnd2<sup>Asn-21</sup>) or anti-Myc pAb (Rapostlin and Rapostlin ΔN1). GFP labeling was shown in the panel labeled GFP. Right panels (Merged) show the superposition of left and middle images. The scale bars represent 10 μm. B, PC12 cells were transiently transfected with cDNA(s) encoding the indicated proteins and differentiated with nerve growth factor for 40 h. Branching activities of the cells were determined as described under “Experimental Procedures.” The results shown were the means ± S.E. for triplicate determinations.
Rapostlin strongly stimulated the branching activity, but negative Rnd2, Rnd2\(^{Asn-21}\), failed to stimulate the activity in the presence of Rapostlin (Fig. 6, A lane g and B). Therefore, Rnd2\(^{Val-16}\) associates with Rapostlin and induces the branching activity. Furthermore, to analyze the function of the amino-terminal region of Rapostlin, we transiently expressed Rnd2\(^{Val-16}\) with Rapostlin 3N1 (Fig. 6A, lane h, and B). These cells did not induce neurite branching, suggesting that the amino-terminal region of Rapostlin was at least necessary for the neurite branching.

**DISCUSSION**

Rnd2 is a new member of Rho family GTPases, which is specifically expressed in neurons in brain. To identify downstream effectors of Rnd2, we performed a yeast two-hybrid screen with Rnd2 as a bait and isolated a novel effector of Rnd2, Rapostlin, which induces neurite branching in neuronal cells. Rapostlin has a highly homologous protein, human FBP17, which was identified previously as one of the formin-binding proteins (28). Recently, FBP17 was reported to interact with sorting nexin 2 (29), suggesting that FBP17 and Rapostlin are involved in receptor trafficking. Rapostlin shows high similarity to CIP4, a Cdc42 effector protein (26), and has similar domain structures to CIP4, an FCH domain at the amino terminus and SH3 domain at the carboxyl terminus. A previous study showed that the region of CIP4 responsible for binding to Cdc42 is localized to amino acids 383–417 (27). FBP17 has a domain (amino acids 475–537) with significant homology to the Rho binding domains of Rhotekin and Rhophilin, and this domain is also present in Rapostlin. In this study, we showed that Rapostlin does not interact with Cdc42 or RhoA and that the unique insert region is critical for the Rnd2 binding to Rapostlin. These results indicate that Rapostlin is a specific effector of Rnd2 but not of Cdc42 or RhoA. The SH3 domain of CIP4 interacts with Wiskott–Aldrich syndrome protein, a regulator of the actin cytoskeleton, whereas the amino-terminal region including the FCH domain binds to microtubules, suggesting that CIP4 is a potential linker between the actin cytoskeleton and microtubules (27). Actually, CIP4 has been shown to be involved in microtubule-dependent formation of F-actin-rich podosomal adhesion in macrophages (30). Rapostlin has two functional domains, FCH-containing amino-terminal region and SH3 domain, those of CIP4 being involved in the reorganization of both microtubules and actin filaments. In this study, we showed that Rapostlin binds to microtubules in the microtubule-binding assay and that Rapostlin induces reorganization of F-actin in response to Rnd2 in HeLa cells. Thus, Rapostlin may act as a regulatory linker between the actin cytoskeleton and microtubules.

Rho family GTPases are central regulators of neuronal morphology, including neurite formation, elongation, retraction, and branching (31). The process of neurite branching is a dynamic combination of branch addition and branch elimination as well as branch extension and retraction. In the beginning of branch formation, F-actin-rich filopodia and lamellipodia are extended from the neurites. Cortical protrusions enlarge from the filopodial and lamellipodial area, and then branches emerge. While branches are formed, the protrusion sites are invested with microtubules, indicating that branch formation is closely accompanied by the reorganization of both the actin cytoskeleton and microtubules. It has been shown that dominant negative forms of Rac1 and Cdc42 lead to a reduction in the dendritic complexity of \textit{Xenopus} retinal ganglion cells (32). In addition, constitutively active Rac1 leads to increased branch dynamics (33). In contrast to Rac1 and Cdc42, RhoA activation leads to a reduction in dendritic branching (34). Therefore, Rac and Cdc42 exert a positive effect on dendritric remodeling and remodeling, whereas Rho is a negative regulator for branch formation. However, molecular mechanisms of branch formation regulated by these Rho family GTPases are not well understood. In this study, we revealed that Rapostlin induces neurite branching in response to Rnd2 in neuronal cells. The binding of active Rnd2 to Rapostlin triggers the branch formation because negative Rnd2, Rnd2\(^{Asn-21}\), fails to stimulate the Rapostlin-mediated branch formation. Therefore, Rapostlin is a downstream effector of Rnd2 for the branch formation. Moreover, we showed here that the amino-terminal region of Rapostlin is necessary for the neurite branching induced by Rapostlin in concert with Rnd2 in PC12 cells. In the microtubule-binding assay, this amino-terminal region of Rapostlin is essential for the binding of Rapostlin to microtubules, suggesting that the interaction of Rapostlin with microtubules is crucial for the neurite branching induced by Rnd2 and Rapostlin.

Most of Rho family GTPases cycle between inactive GDP-bound and active GTP-bound forms. Unlike GTPase-cycling Rho family GTPases, Rnd1 and Rnd3 have been shown to be constitutively active forms due to point substitutions at the residue equivalent of Ras Gly-12 to valine and serine, respectively, decreasing intrinsic GTPase activity (12–14). In contrast, this residue of Rnd2 is replaced by alanine. Here we showed that Rapostlin binds to GTP\(^{S}\)-bound Rnd2, but not to GDP-bound Rnd2, in a dot-blot assay and that Rapostlin strongly binds to Rnd2\(^{Val-16}\) (equivalent to a constitutively active Val-12 mutation in Ras), whereas Rapostlin does not bind to Rnd2\(^{Asn-21}\) (equivalent to a dominant negative Asn-17 mutation in Ras). Furthermore, we showed that Rapostlin induces neurite branching in response to Rnd2\(^{Val-16}\) but not to Rnd2\(^{Asn-21}\) in PC12 cells. Therefore, Rnd2 has an ability to become either GTP-bound active or GDP-bound negative form, and the only active form of Rnd2 binds to Rapostlin, inducing neurite branching. Biochemical characterization of GTPase activity of Rnd2 is currently in progress in our laboratory.

In conclusion, we have identified a novel downstream effector of Rnd2, Rapostlin, involved in the neurite branch formation. Rapostlin is the first to be identified as an effector of Rnd2. However, many questions have not yet been elucidated about Rnd2 signaling, for example, what molecules participate in the upstream signaling of Rnd2. Further studies focusing on the identification of Rnd2-interacting proteins will contribute to understanding of the Rnd2-signaling pathway and the regulation of neuronal morphology.

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