Identification of Splicing Variants of Rapostlin, a Novel Rnd2 Effector that Interacts with Neural Wiskott-Aldrich Syndrome Protein and Induces Neurite Branching

Received for publication, November 21, 2003, and in revised form, January 16, 2004
Published, JBC Papers in Press, January 19, 2004, DOI 10.1074/jbc.M312763200

Tetsuhiro Kakimoto, Hironori Katoh, and Manabu Negishi

From the Laboratory of Molecular Neurobiology, Graduate School of Biostudies, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

Rho family GTPases regulate neuronal morphology. Rnd subfamily is a new branch of Rho family GTPases. Of these GTPases, Rnd2 is specifically expressed in brain. We recently identified Rapostlin as a novel effector of Rnd2. Rapostlin induces neurite branching in response to Rnd2 in PC12 cells. During the cloning of Rapostlin, we have found two mainly expressed splicing variants of Rapostlin (renamed as RapostlinL, RapostlinM and RapostlinS, lacking 29 residues and 61 residues within the unique insert region at the center, respectively, and three minor variants, RapostlinLd, RapostlinMd, and RapostlinSd, each with the identical five-amino acid deletion from RapostlinL, RapostlinM, and RapostlinS, respectively. RapostlinL is predominantly expressed in brain, whereas RapostlinS is expressed ubiquitously. In a dot-blot assay, all splicing variants bind to Rnd2 in a GT-dependent manner. However, RapostlinM and RapostlinS induce less neurite branching when coexpressed with Rnd2 in PC12 cells, indicating that the insert region is important for the branching activity of RapostlinL. All splicing variants bind to N-WASP in vitro and in vivo through the SH3 domain at the carboxyl terminus, and the SH3 domain is essential for branching activity of RapostlinL. In immunoprecipitation experiments, Rnd2 reduces RapostlinL-N-WASP interaction, whereas it has little effect on the interaction of RapostlinM or RapostlinS with N-WASP. Therefore, we found that functionally different splicing variants of Rapostlin have different responses to Rnd2 in association with N-WASP.

Neurons form a complex neural network to function properly. Formation of this network includes many steps: neuronal migration to proper regions, neurite outgrowth, formation of polarity, guidance of axons and dendrites to proper targets, dendritic maturation, and synapse formation with appropriate partners. Rho family GTPases are involved in the regulation of all these steps by regulating cytoskeleton (1, 2).

Rho family GTPases are implicated in morphological changes of various cells by reorganizing cytoskeleton (3). Among these GTPases, Rho, Rac, and Cdc42 have been extensively investigated, and they are known to induce stress fibers, lamellipodia, and filopodia in fibroblasts, respectively. In neurons, Rac and Cdc42 stimulate extension of lamellipodia and filopodia at advancing growth cones, respectively (4), whereas Rho triggers growth cone collapse and neurite retraction (5). Rho family GTPases serve as molecular switches by cycling between GDP-bound inactive state and GTP-bound active state, and once activated, they transduce signals to a variety of specific downstream effectors, leading to morphological changes (6). For example, a well studied Rho effector, Rho-kinase is involved in stress fiber formation and focal adhesion assembly in fibroblasts by regulating myosin phosphorylation; it is also involved in Rho-induced neurite retraction in neurons (7-9). Rac and Cdc42 also activate actin cytoskeleton by their effectors such as p21-activated kinase (1, 2). p21-Activated kinase is involved in the induction of both filopodia and membrane ruffles in fibroblasts and neurite outgrowth in neurons by inducing dynamic reorganization of actin cytoskeleton (1, 2).

Neuronal Wiskott-Aldrich syndrome protein (N-WASP), another effector of Cdc42, stimulates actin polymerization through the activation of Arp2/3 complex, inducing filopodia in fibroblasts and neurite extension in neurons (10, 11). In addition to Cdc42, phosphorylformisin, 4,5-bisphosphate (PIP2) and several SH3 domain-containing proteins, such as Grb2/Ash, Nck, and the WASP-interacting SH3 protein (WISH) activate N-WASP. N-WASP is also activated by phosphorylation from Src-family kinases. In addition to these three well characterized Rho family GTPases, other members, including RhoG (12, 13), Rnd (14-16), and TC10, have been shown to play important roles in neuronal network formation.

The Rnd subfamily, consisting of Rnd1, Rnd2, and Rnd3/RhoE, is a new branch of the Rho family, whose function is largely unknown (17). Rnd1 and Rnd2 are predominantly expressed in brain, whereas Rnd3 is expressed ubiquitously (17-19). Rnd1 and Rnd3 inhibit the formation of stress fibers and focal adhesions, suggesting that they inhibit Rho-regulated signaling pathway. Rnd1 inhibits the formation of stress fibers by its effector, Soscius (20), whereas Rnd3 antagonizes Rho-signaling pathway by inhibiting the Rho effector, ROCK I (21), and activating Rho inactivator, p190RhoGAP (22). In contrast,
little is known about Rnd2, although the predominant neuronal expression of Rnd2 suggests its important role in neural function (19). We recently identified Rapostlin as a novel effector of Rnd2 (23). Rapostlin has a similar domain structure to a Cdc42 effector, Cdc42-interacting protein-4 (CIP4; Ref. 24), with a Fer-CIP4 homology (FCH) domain at the amino-terminal region, including a SH3 domain. Rapostlin regulates neurite branching in the presence of Rnd2 in PC12 cells, and the amino-terminal region of Rapostlin is necessary for this branching activity, suggesting that Rapostlin induces neurite branching through interaction with microtubules (23).

While the isolation of Rapostlin was in process, we cloned the splicing variants of Rapostlin (renamed as RapostlinL: RapostlinM, RapostlinS, RapostlinLd, RapostlinMd, and RapostlinSd, which differ only in the size of the insert region. We report here that all splicing variants bind to active Rnd2, but that RapostlinM and RapostlinS induce less neurite branching in the presence of Rnd2 in PC12 cells, indicating that the insert region is important for the branching activity. We also report that all variants bind to N-WASP through the SH3 domain, which is essential for branching activity, and that Rnd2 releases N-WASP from RapostlinL, but releases less from RapostlinM and RapostlinS.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—For expression in mammalian cells, cDNAs encoding the full-length RapostlinL (amino acids 2–620), RapostlinM (amino acids 2–591), RapostlinS (amino acids 2–591), and RapostlinL ΔN (amino acids 2–552) were subcloned into mammalian expression vector pcDNA3 (Invitrogen), encoding an initiating Met followed by the Myc epitope tag sequence at the amino terminus. cDNA encoding Rnd2V16 was subcloned into pcDNA3, encoding an initiating Met followed by the hemagglutinin (HA) epitope tag sequence at the amino terminus, for expression in PC12 cells, while it was subcloned into pEF-BOS for expression in 293T cells. cDNA encoding green fluorescent protein (GFP) was subcloned into pcDNA3. N-WASP cDNA was a generous gift from Dr. T. Takenawa (University of Tokyo); it was subcloned into pCMV-HA (Clontech). For purification of recombinant proteins, cDNA of Rnd2 was subcloned into pGEX-2T (Pharmingen), whereas cDNAs of Rnd1, Rnd3, RapostlinL ΔN (amino acids 329–620), RapostlinM ΔN (amino acids 329–615), RapostlinS ΔN (amino acids 329–591), RapostlinL ΔN (amino acids 329–595), RapostlinL (amino acids 2–620), RapostlinMd (amino acids 2–615), RapostlinM (amino acids 2–591), RapostlinS (amino acids 2–559), Rapostlin SH3 domain (amino acids 553–620 of RapostlinL), and Plexin-B1 (amino acids 1724–1915) were subcloned into pGEX-4T-2 (Amersham Biosciences).

Cloning and RT-PCR of Rapostlin-Splicing Variants—To initially clone Rapostlin-splicing variants, two primers, 5′-gaatctaccaacasaanagaagtgc-3′ and 5′-aataagacttcagcttggaagggca-3′, were used in RT-PCR with rat brain RNA as a template. The PCR products obtained were cloned into pcRII.1 vector (Invitrogen) and sequenced completely. For determination of the complete coding sequences, two pairs of primers, 5′-aggagtccagagggctttttca-3′ and 5′-tggctggtgggctggaggtg-3′ for the 5′-terminal region, and another pair of 5′-tcctctggctaaggagggcttttca-3′ and 5′-aagttctggtgggctggaggtg-3′ for the 3′-terminal region were used in RT-PCR. The PCR products were cloned and sequenced as above.

To investigate the expression pattern of the splicing variants, two primers, 5′-agtggaggggtcagactggctgt-3′ and 5′-tctctgtgtggtggtggtggt-3′, were used in RT-PCR from various adult rat tissues.

Cell Culture and Transfection—293T cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 4 mM glutamine, 100 units/ml of penicillin, and 0.1 mg/ml of streptomycin under humidified air containing 5% CO2 at 37 °C. Rat pheochromocytoma (Rat-1) cells were grown in Dulbecco’s modiﬁed Eagle’s medium containing 10% horse serum, 5% fetal bovine serum, 4 mM glutamine, 100 units/ml of penicillin, and 0.1 mg/ml of streptomycin under humidified air containing 5% CO2 at 37 °C. Cells were transfected with test plasmids using LipofectAMINE 2000 (Invitrogen) for PC12 cells, or LipofectAMINE Plus (Invitrogen) for 293T cells, according to the manufacturer’s instructions. PC12 cells were differentiated with 50 ng/ml of nerve growth factor (NGF) (Promega) in serum-free Dulbecco’s modiﬁed Eagle’s medium after the transfection.

Immunofluorescence Microscopy—PC12 cells were seeded in 24-well plates at a density of 2 × 104 onto poly-l-lysine-coated glass coverslips (13 mm in diameter). Forty-eight hours after the transfection, cells on coverslips were washed with phosphate-buffered saline (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, cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min and incubated with 10% fetal bovine serum in PBS for 30 min. Cells were incubated with rabbit anti-HA pAb (Santa Cruz Biotechnology) in PBS for 1 h for detection of HA-tagged Rnd2V16, or incubated with rabbit anti-Rapostlin pAb for detection of GST-fused Rapostlin variant (RapostlinL ΔN, RapostlinL ΔN, RapostlinM ΔN, RapostlinS ΔN), full-length splicing variants (RapostlinL, RapostlinM, RapostlinL ΔN, RapostlinM, and RapostlinS), and SH3 domain were prepared from Escherichia coli, as described previously (8, 23, 26). For a dot-blot assay, recombinant GST-fused Rnd protein was loaded with 1 mM GDP or guanosine 5′-S-O-(thio)triphosphate (GTP·S) in previn (50 μM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM MgCl2, and 1 mM dithiothreitol) at 30 °C for 15 min, and the reaction was started by the addition of MgCl2 to a ﬁnal concentration of 55.5 mM (23). A dot-blot assay was performed as described previously (23, 27). GST (5 μg), GST-fused carboxyl-terminal region of Rapostlin-splicing variants (RapostlinL ΔN, RapostlinL ΔN, RapostlinL ΔN, or RapostlinS ΔN) (5 μg), or GST-fused Plexin-B1 (5 μg) was spotted onto a nitrocellulose membrane and allowed to dry for 1 h at room temperature. The membrane was blocked in buffer A containing 5% low fat milk in Tris-buffered saline and then incubated with primary antibodies. The primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies (DAKO) and an ECL detection kit (Amersham Biosciences).

In Vitro Binding Assays—Glutathione S-transferase (GST)-fused Rnd2 protein was prepared from Sf9 cells, whereas GST and GST-fused Rnd1, Rnd3, Plexin-B1, carboxyl-terminal regions of Rapostlin-splicing variants (RapostlinL ΔN, RapostlinL ΔN, RapostlinM ΔN, and RapostlinS ΔN), full-length splicing variants (RapostlinL, RapostlinM, RapostlinL ΔN, RapostlinM, and RapostlinS), and SH3 domain were prepared from Escherichia coli, as described previously (8, 23, 26). For a dot-blot assay, recombinant GST-fused Rnd protein was loaded with 1 mM GDP or guanosine 5′-S-O-(thio)triphosphate (GTP·S) in previn (50 μM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM MgCl2, and 1 mM dithiothreitol) at 30 °C for 15 min, and the reaction was started by the addition of MgCl2 to a ﬁnal concentration of 55.5 mM (23). A dot-blot assay was performed as described previously (23, 27). GST (5 μg), GST-fused carboxyl-terminal region of Rapostlin-splicing variants (RapostlinL ΔN, RapostlinL ΔN, RapostlinL ΔN, or RapostlinS ΔN) (5 μg), or GST-fused Plexin-B1 (5 μg) was spotted onto a nitrocellulose membrane and allowed to dry for 1 h at room temperature. The membrane was blocked in buffer A containing 5% low fat milk in 1 h at 4 °C. The membrane was then incubated for 1.5 h at 4 °C in buffer A containing 24 μg of GST-fused Rnd protein preloaded with GDP or GTP·S. The membrane was washed with buffer A and then incubated with 3% low fat milk in Tris-buffered saline containing rabbit anti-Rnd2 pAb (previously described previously; Ref. 26), rabbit anti-Rapostlin L pAb (previously described previously; Ref. 16), or goat anti-Rnd3 pAb (Santa Cruz Biotechnology). This antibody was detected by using horseradish peroxidase-conjugated secondary antibody (DAKO) and an ECL detection kit (Amersham Biosciences).

For a pull-down assay, 293T cells (1 × 106 cells) transfected with HA-tagged N-WASP were rinsed with PBS and lysed with the ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM MgCl2, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 2 μg/ml leupeptin). Cell lysates were then centrifuged for 10 min at 16,000 × g at 4 °C. The supernatants were incubated with 2 μg of GST, GST-fused full-length splicing variant (RapostlinL, RapostlinLd, RapostlinMd, RapostlinSd, or RapostlinSd) or GST-fused SH3 domain and then with glutathione-Sepharose beads for 1 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, analyzed by SDS-PAGE, and immuno- blotted with rat anti-HA mAb (Roche Applied Science).

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RESULTS

Cloning of Rapostlin-splicing variants—When we performed RT-PCR from rat brain to clone Rapostlin, Rapostlin-splicing variants were identified by RT-PCR with rat brain RNA as a template. The splicing regions overlap with the unique insert region. FCH, Fer CIP4 homology domain; CC, coiled-coil region; IN, insert region; SH3, Src homology 3 domain. B, expression pattern of Rapostlin-splicing variants in various rat tissues. RT-PCR amplification of the splicing regions from the adult rat brain, lung, spleen, testis, or thymus was performed. Total RNAs were isolated from the respective rat tissues. RNA (0.5 µg) was reverse-transcribed with random primers, followed by amplification with the primers, 5′-agtgaaggccggcagttactattga-3′ and 5′-tctctgctcaggcgggaagttgc-3′.

Immunoprecipitation—293T cells (1 × 106 cells) cotransfected with HA-tagged N-WASP, Rnd2V16, and Myc-tagged Rapostlin-splicing variant (RapostlinL, RapostlinM, or RapostlinS) or RapostlinL ΔSH3 were rinsed with PBS and lysed with the ice-cold lysis buffer. Cell lysates were then centrifuged for 10 min at 16,000 × g at 4 °C. The supernatants were diluted 1:4 with lysis buffer without Triton X-100 and incubated with mouse anti-Myc mAb (Upstate Biotechnology) for 1 h at 4 °C. They were subsequently incubated with protein A-Sepharose (Amersham Biosciences) for 1 h at 4 °C. After the beads were washed with the ice-cold cell lysis buffer with 0.2% Triton X-100, the bound proteins were analyzed by SDS-PAGE and immunoblotted with rat anti-HA mAb, mouse anti-Myc mAb, or rabbit anti-Rnd2 pAb.

All Splicing Variants Bind to Rnd2 in a GTP-dependent Manner—We reported previously that RapostlinL specifically binds to GTPγS-loaded GST-Rnd2 but not to GDP-loaded GST-Rnd2 through its carboxyl-terminal region in dot-blot assays (23). To examine the interaction of Rapostlin-splicing variants with Rnd2, we carried out a dot-blot assay (Fig. 2). GST-fused splicing variant lacking the amino-terminal region was spotted onto a nitrocellulose membrane, and the membrane was overlaid with GDP- or GTPγS-preloaded GST-Rnd2. All splicing variants bound to GSTγS-loaded GST-Rnd2 but not to GDP-loaded GST-Rnd2. Considering that SH3 domain does not associate with Rnd2 (23), these results suggest that the Rnd2 binding site is located between the insert region and SH3 domain. We also examined the interaction of Rapostlin-splicing variants with GST-Rnd1 and GST-Rnd3 preloaded with GTPγS. All splicing variants did not bind to GTPγS-loaded GST-Rnd1 or GST-Rnd3, although Plexin-B1 bound to both of them as reported previously (Fig. 2; Ref. 15). These results indicate that all splicing variants specifically bind to Rnd2 among Rnd subfamily GTPases in a GTP-dependent manner.

Only RapostlinL Induces Neurite Branching in Response to Rnd2 in PC12 Cells—We showed previously that RapostlinL induces neurite branching in response to a constitutively active Rnd2,Rnd2V16 (equivalent to a constitutively active V12 mutation in Ras) but not to a negative Rnd2,Rnd2N21 (equivalent to a dominant-negative N17 mutation in Ras) in PC12 cells (23). Therefore, we examined the branching activity of Rapostlin-splicing variants with or without coexpressed Rnd2V16 (Figs. 3 and 4). We transiently expressed Myc-tagged Rapostlin-splicing variant and/or HA-tagged Rnd2V16 in the NGF-differentiated PC12 cells and analyzed the morphological changes of the cells. GFP-expressing control cells and Rnd2V16-expressing cells extended a few unbranched neurites bearing microtubules, as we reported previously (23). Without coexpressed...
Rnd2V16, all of the splicing variants examined, namely, RapostlinL, RapostlinM, and RapostlinS, induced few branches from neurites. On the other hand, RapostlinL induced neurite branching when coexpressed with Rnd2V16 as in our previous report (23). However, RapostlinM and RapostlinS induced less branching when coexpressed with Rnd2V16. These results indicate that the deletion in the insert region makes a functional difference in inducing neurite branching.

Rapostlin Binds to N-WASP via the SH3 Domain

The SH3 domain of CIP4, a Cdc42 effector with a similar domain structure to Rapostlin, associates with WASP (29). WASP is a Cdc42 effector regulating actin cytoskeleton, and CIP4 mediates the association of WASP with microtubules. This interaction of CIP4 with WASP is implicated in the microtubule-dependent formation of podosomes, unique actin-rich adhesion structures of monocyte-derived cells (30). Because WASP is expressed exclusively in hematopoietic cells, we speculated that Rapostlin interacts with N-WASP, a WASP-related protein expressed predominantly in brain-like RapostlinL and Rnd2. Therefore, we carried out a pull-down assay (Fig. 5A). HA-tagged N-WASP was expressed in 293T cells, and the cell lysates were incubated with GST, GST-fused full-length splicing variant, or GST-fused SH3 domain. All splicing variants and SH3 domain bound to N-WASP in vitro. These results indicate that Rapostlin interacts with N-WASP through its SH3 domain, and all splicing variants bind to N-WASP, because they all have the SH3 domain.

We next performed an immunoprecipitation experiment to verify that Rapostlin interacts with N-WASP through its SH3 domain in vivo (Fig. 5B). Myc-tagged RapostlinL or RapostlinL mutant lacking SH3 domain (RapostlinLΔSH3) and HA-tagged N-WASP were expressed in 293T cells, and the cell lysates were immunoprecipitated with anti-Myc pAb. HA-tagged N-WASP was communoprecipitated with Myc-tagged RapostlinL, but we could not detect the interaction between N-WASP and RapostlinLΔSH3 regardless of Rnd2V16. These results confirm that Rapostlin interacts with N-WASP via its SH3 domain. Interestingly, Rnd2V16 released N-WASP from RapostlinL.

Furthermore, to analyze the function of this SH3 domain of Rapostlin, we transiently expressed RapostlinLΔSH3 with Rnd2V16 in PC12 cells (Fig. 6). These cells failed to induce neurite branching, suggesting that the SH3 domain is essential for the RapostlinL-mediated neurite branching.

Rnd2 Releases N-WASP from RapostlinL, but Releases Less from RapostlinM and RapostlinS

To investigate the difference in the in vivo association with N-WASP between Rapostlin-splicing variants, we performed an immunoprecipitation study (Fig. 7). 293T cells were transfected with HA-tagged N-WASP and Rnd2. After the transfected cells had been differentiated with NGF for 44 h, they were fixed and stained with anti-α-tubulin mAb (tubulin, middle panels) and either anti-HA pAb (HA, left panels) or anti-Myc pAb (Myc, left panels). GFP labeling is shown in panel labeled GFP. Right panels (Merged) show the superfposition of left (green) and middle (red) images. Bars, 10 μm.
and the cell lysates were immunoprecipitated with anti-Myc mAb. HA-tagged N-WASP was communoprecipitated with Myc-tagged RapostlinL in the absence of Rnd2V16, but this interaction was significantly reduced by coexpression of Rnd2V16. On the other hand, the interaction between N-WASP and RapostlinM or RapostlinS was less affected by coexpression of Rnd2V16. These results indicate that the insert region is important for Rnd2 to reduce the association of RapostlinL with N-WASP.

Fig. 4. Quantification of neurite branching activity in PC12 cells. PC12 cells were transiently transfected with expression vectors encoding the indicated proteins and differentiated with NGF for 44 h. Branching activities (branch points/mm neurite) of the cells were determined as described under “Experimental Procedures.” The results shown are means ± S.E. for triplicate determinations.

Fig. 5. In vivo and in vitro interaction of Rapostlin with N-WASP. A, interaction of Rapostlin with N-WASP via SH3 domain in vitro. Twenty-four hours after transfection, cell lysates from 293T cells transfected with HA-tagged N-WASP were incubated with GST, GST-fused full-length Rapostlin-splicing variant (RapostlinL, RapostlinMd, RapostlinM or RapostlinS), or GST-fused SH3 domain of Rapostlin. Then they were immobilized on glutathione-Sepharose beads, and bound proteins and lysate input were analyzed by immunoblotting with anti-HA mAb. B, interaction of Rapostlin with N-WASP through SH3 domain in vivo. 293T cells were transfected with expression vectors encoding HA-tagged N-WASP, Rnd2V16, and Myc-tagged RapostlinL (FL, full-length RapostlinL) or RapostlinL mutant lacking SH3 domain (ASH3, RapostlinL ΔSH3). Twenty-four hours after transfection, cell lysates were immunoprecipitated with anti-Myc mAb. The immunoprecipitates and the total cell lysates were analyzed by immunoblotting with anti-HA mAb, anti-Myc mAb, or anti-Rnd2 pAb.

Fig. 6. Requirement of SH3 domain for the RapostlinL-induced neurite branching. A, morphological changes of PC12 cells. PC12 cells were transiently transfected with expression vectors encoding HA-tagged Rnd2V16 + Myc-tagged RapostlinL ΔSH3. They were differentiated, fixed, and stained as in Fig. 3. Bar, 10 μm. B, quantification of neurite branching activity in PC12 cells. PC12 cells were transiently transfected with vectors encoding the indicated proteins and differentiated with NGF for 44 h. Branching activities of the cells were determined as in Fig. 4. The results shown are means ± S.E. for triplicate determinations.
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**Fig. 7.** In vivo interaction of Rapostlin-splicing variants with N-WASP. 293T cells were transfected with expression vectors encoding HA-tagged N-WASP, Rnd2V16, and Myc-tagged Rapostlin-splicing variant (RapostlinL, RapostlinM, or RapostlinS). Twenty-four hours after transfection, cell lysates were immunoprecipitated with anti-Myc mAb. The immunoprecipitates and the total cell lysates were analyzed as in Fig. 5B.

**DISCUSSION**

Rho family GTPases are central regulators of neuronal morphology, including neurite formation, elongation, retraction, and branching (1, 2). They regulate such a wide variety of morphological changes by transducing signals to cytoskeleton, especially actin cytoskeleton (3). Rnd2 belongs to the Rnd subfamily, a new member of the Rho family GTPases. It is specifically expressed in neurons in brain, which suggests its important role in neuronal function (19). Nevertheless, little is known about Rnd2. We showed previously that a novel Rnd2 effector, RapostlinL, induces neurite branching in response to Rnd2 in PC12 cells (23). We also showed that the aminoterminal region of RapostlinL, which is essential for the binding of RapostlinL to microtubules, is necessary for the neurite branching induced by RapostlinL in concert with Rnd2. These results suggest that the interaction of RapostlinL with microtubules is crucial for the neurite branching induced by Rnd2 and RapostlinL. Here, we have reported that RapostlinL, through the carboxyl-terminal SH3 domain, binds to N-WASP. N-WASP is known to stimulate actin polymerization-inducing filopodia and neurite extension (10, 11). A RapostlinL mutant lacking the SH3 domain (RapostlinL ΔSH3) fails to induce neurite branching. In addition, we showed previously that RapostlinL induces reorganization of actin filaments in response to Rnd2 in HeLa cells (23). These results suggest that RapostlinL may regulate actin reorganization through N-WASP. Well studied effectors of Rho family GTPases usually regulate either actin cytoskeleton or microtubules. Thus, RapostlinL is a novel effector, linking actin cytoskeleton and microtubules.

Neurite branching requires coordinate reorganization of not only actin cytoskeleton, but also microtubules (31). In the beginning of branch formation, actin filament-rich filopodia and lamellipodia are extended from the neurites. Bundled microtubules splay apart, with coincident accumulation of actin filaments, and microtubules invade lamellipodia. Then, branches emerge with new growth cones at their tips. We suggest that RapostlinL may function as a linker between actin cytoskeleton and microtubules, eventually causing the induction of neurite branching.

In this study, we have cloned Rapostlin-splicing variants and investigated their functional difference. We cloned two major variants, RapostlinM and RapostlinS. RapostlinM lacks 29 residues, the amino-terminal half of exon 10 of human homolog FBP17 within the insert region, whereas RapostlinS lacks 61 residues, the entire exon 10 of the human homolog. We also cloned three minor variants, RapostlinLd, RapostlinMd, and RapostlinSd, with the identical five-amino acid deletion, exon 11 of human homolog, from RapostlinL, RapostlinM, and RapostlinS, respectively. From the results of immunohistochemistry of human tissues (32), FBP17 has been reported to be expressed ubiquitously. However, Fuchs et al. (32) used polyclonal antibody raised against the coiled-coil region, which is present in all splicing variants. Therefore, they have confused human RapostlinL, FBP17, with other splicing variants. Based upon our RT-PCR results, Fuchs et al. seem to have detected RapostlinS in various tissues. Here, we have shown, by RT-PCR, that RapostlinL is predominantly expressed in brain just like Rnd2, but that RapostlinS is expressed ubiquitously. Considering relatively weak expression of RapostlinM, it is speculated that RapostlinL mainly mediates downstream signals of Rnd2 as an effector in the brain. Indeed, RapostlinM and RapostlinS induce little neurite branching in response to Rnd2. However, all splicing variants specifically bind to Rnd2 among Rnd subfamily GTPases in a GTP-dependent manner. They also bind to N-WASP. Consistent with this functional difference, Rnd2 reduces RapostlinL-N-WASP interaction, whereas it has little effect upon the interaction of RapostlinM or RapostlinS with N-WASP. Therefore, to induce neurite branching, it seems essential to release N-WASP when Rnd2 binds to RapostlinL. In light of the result that the ability of RapostlinL to interact with N-WASP via SH3 domain is also essential for the induction of neurite branching, it may be reasonable to speculate that N-WASP is recruited by RapostlinL from cytosol to RapostlinL-bound microtubules and that Rnd2 releases N-WASP, regulating actin cytoskeleton. At present, how Rnd2 is activated or recruited is unknown (33). Therefore, elucidation of the upstream regulation mechanism of Rnd2 will be required to further dissect how Rnd2 and RapostlinL regulate actin cytoskeleton via N-WASP.

In conclusion, we have shown that RapostlinL interacts with N-WASP through the SH3 domain and that the SH3 domain is essential for neurite branching. We cloned Rapostlin-splicing variants, which induce less neurite branching and have different association mechanisms with N-WASP in response to Rnd2. However, many questions remain unsolved concerning Rnd2 signaling and Rnd2-RapostlinL-induced neurite branching. Further studies focusing on upstream regulators and downstream effectors will help to elucidate Rnd2 signaling pathways and the regulation of neuronal morphology.

**Acknowledgment**—We thank Dr. T. Takenawa of University of Tokyo for supplying cDNA of N-WASP.

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Identification of Splicing Variants of Rapostlin, a Novel Rnd2 Effector that Interacts with Neural Wiskott-Aldrich Syndrome Protein and Induces Neurite Branching
Tetsuhiro Kakimoto, Hironori Katoh and Manabu Negishi

J. Biol. Chem. 2004, 279:14104-14110.
doi: 10.1074/jbc.M312763200 originally published online January 19, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M312763200

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