Direct Interaction of Rnd1 with Plexin-B1 Regulates PDZ-RhoGEF-mediated Rho Activation by Plexin-B1 and Induces Cell Contraction in COS-7 Cells*

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Plexins are receptors for the axon guidance molecule semaphorins, and several lines of evidence suggest that Rho family small GTPases are implicated in the downstream signaling of Plexins. Recent studies have demonstrated that Plexin-B1 activates RhoA and induces growth cone collapse through Rho-specific guanine nucleotide exchange factor PDZ-RhoGEF. Here we show that Rnd1, a member of Rho family GTPases, directly interacted with the cytoplasmic domain of Plexin-B1. In COS-7 cells, coexpression of Rnd1 and Plexin-B1 induced cell contraction in response to semaphorin 4D (Sema4D), a ligand for Plexin-B1, whereas expression of Plexin-B1 alone or coexpression of Rnd1 and a Rnd1 interaction-defective mutant of Plexin-B1 did not. The Sema4D-induced contraction in Plexin-B1/Rnd1-expressing COS-7 cells was suppressed by dominant negative RhoA, a Rho-associated kinase inhibitor, a dominant negative form of PDZ-RhoGEF, or deletion of the carboxyl-terminal PDZ-RhoGEF-binding region of Plexin-B1, indicating that the PDZ-RhoGEF/RhoA/Rho-associated kinase pathway is involved in this morphological effect. We also found that Rnd1 promoted the interaction between Plexin-B1 and PDZ-RhoGEF and thereby dramatically potentiated the Plexin-B1-mediated RhoA activation. We propose that Rnd1 plays an important role in the regulation of Plexin-B1 signaling, leading to Rho activation during axon guidance and cell migration.

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The actin cytoskeleton mediates a variety of essential cellular processes, including cell migration, cytokinesis, and axon and dendrite formation, and it is already well known that Rho family small GTPases are key regulators of the actin cytoskeleton in various cell types (1). Like other GTPases of the Ras superfamily, they serve as molecular switches by cycling between an inactive GDP-bound state and an active GTP-bound state, and once activated, they can interact with their specific effectors, leading to a variety of biological functions. Activation of the Rho family proteins requires GDP-GTP exchange catalyzed by various guanine nucleotide exchange factors (GEFs), whereas the activation of the GTPases is down-regulated by GTPase-activating proteins, which stimulate the intrinsic GTPase activities. In addition, guanine nucleotide dissociation inhibitors interact with the GDP-bound form of Rho GTPases and inhibit the exchange of GDP for GTP. Presently, at least 14 mammalian Rho family proteins have been identified: Rho (A, B, and C), Rac (1, 2, and 3), Cdc42, RhoD, RhoG, RhoH/TTF, TC10, and Rnd (1, 2, and 3). Among them, the functions of Rho, Rac, and Cdc42 have been extensively characterized. In neuronal cells, activation of Rac and Cdc42 induces the formation of lamellipodia and filopodia of the growth cone, respectively, whereas activation of Rho causes the collapse of the growth cone, and recent studies have revealed their involvement in the downstream signaling pathways of several guidance molecule receptors to regulate the actin cytoskeleton during axon outgrowth and guidance (2).

Semaphorins are a large family of secreted and transmembrane molecules that play a central role in axon guidance in developing nervous system (3–5). In addition to the nervous tissues and are thought to mediate diverse processes such as cardiac and skeletal development (6), tumor growth and metastasis (7), and the immune response (8, 9). The function of semaphorins in the nervous system is mediated by Plexins, which can be classified into four subfamilies: Plexin-A1–4, Plexin-B1–3, Plexin-C1, and Plexin-D1 (10, 11). The most characterized member of the semaphorins is semaphorin 3A (Sema3A), and a variety of molecules have been shown to be involved in the intracellular signaling pathway for the actions of Sema3A (12, 13). Although most other semaphorins appear to bind to and directly activate Plexins, Sema3A requires receptor complexes consisting of Plexin-A1/2 and neuropilins (10, 14). Plexin-B1 has been identified as a receptor for semaphorin 4D (Sema4D, also known as CD100) (10), and recent studies indicate that Plexin-B1 directly interacts with PDZ-RhoGEF/leukemia-associated Rho GEF (LARG) through its carboxy-terminal PSD-95/Dlg/ZO-1 (PDZ) domain-binding motif to induce RhoA activation and growth cone collapse in response to Sema4D (15–19). PDZ-RhoGEF was originally identified as a Rho-specific GEF with the PDZ domain in its amino terminus that interacts with activated α subunits of the G12 family of heterotrimeric G proteins and can link G protein-coupled receptors to RhoA activation (20). In addition to RhoA activation, Plexin-B1 directly interacts with Rac in a GTP-dependent manner (21, 22), and this interaction appears to inhibit Rac-de-
pendent actions by sequestering active GTP-bound Rac (23, 24). However, precise mechanisms by which the activity of Plexin-B1 is regulated remain unclear.

Rnd GTPases, Rnd1, Rnd2, and Rnd3 (also known as RhoE), comprise a distinct branch of Rho family GTPases in that they have a low affinity for GDP and very low intrinsic GTPase activities (25–27). In fibroblasts, transient expression of Rnd1 or Rnd3 leads to loss of stress fibers, retraction and rounding of the cell body, and production of extensively branching processes (27), and a part of these morphological effects of Rnd1 or Rnd3 is mimicked by Y27632. Rnd GTPase-interacting proteins (RIPs) include Coilin and Tart (28). In Madin-Darby canine kidney epithelial cells, Rnd3 regulates cell migration speed and is involved in the alteration of the actin cytoskeleton associated with oncogenic transformation (26, 29). Among Rnd GTPases, Rnd1 and Rnd2 are abundantly expressed in the nervous system (27, 30), and Rnd2 induces neurite branching through its novel effector Rapostin (31). Recently, Rnd1 has been shown to interact with Plexin-A1 and appears to be involved in the signaling pathway of Plexin-A1 (32, 33). However, the precise role of Rnd1 in the Plexin-mediated actions remains unknown. Here we provide evidence that Rnd1 directly interacts with the cytoplasmic region of Plexin-B1 and that this interaction potentiates RhoA activation by Plexin-B1 and induces contraction of COS-7 cells. We propose a role of Rnd1 in the Plexin-B1 signaling pathway leading to Rho activation and cytoskeletal rearrangements.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions and Antibodies**—Plexin-A1 and Plexin-A2 cDNAs, Plexin-B1 and Plexin-B2 cDNAs, and PDZ-RhoGEF (KIAA0888) cDNA were kindly provided by Dr. H. Fujisawa (Nagoya University, Nagoya, Japan), Dr. Y. Hara (Tokyo Women’s Medical University, Tokyo, Japan), and Dr. K. Nagata (Aichi Cancer Center Research Institute, Japan). The expression plasmid encoding a soluble form of Sema4D fused to human IgG1 Fc fragment was a generous gift from Dr. H. Kikutani (Osaka University). To generate recombinant proteins, the cytoplasmic domains of Plexin-A1 (Plexin-A1-cyt, amino acids 1296–1894) and Plexin-A2 (Plexin-A2-cyt, amino acids 1281–1884) were subcloned into pGEX-4T-2 (Amersham Biosciences), and the cytoplasmic domains of Plexin-B1 (Plexin-B1-cyt, amino acids 1543–2136) and Plexin-B2 (Plexin-B2-cyt, amino acids 1255–1841) were subcloned into pGEX-4T-1. To generate glutathione S-transferase (GST)-fused Plexin-B1-cyt1 and Plexin-B1-cyt2, PCR-amplified fragments of Plexin-B1 (cyt1, amino acids 1724–1916; cyt2, amino acids 1916–2132) were subcloned into pGEX-4T-2. Plexin-B1-GGA (L1849G, V1850G, P1851A) was generated by PCR-mediated mutagenesis (34). Plexin-B1-ΔC (lacking the last seven carboxyl-terminal amino acids) was generated by PCR amplification. All Plexin-B1 constructs for expression in mammalian cells included a Myc tag at the amino terminus, and were subcloned into pcDNA3 (Invitrogen). Hemagglutinin (HA)-tagged Rnd1, Rnd2, Rnd3, Rnd1ADD, Rhosh119, Racs1NT, and green fluorescent protein (GFP)-tagged Rnd1 were obtained as described previously (28, 35). Rac1L50 was generated by PCR-mediated mutagenesis and subcloned into pEGFP-C1 (Clontech). The PDZ domain of PDZ-RhoGEF (amino acids 1–231) was obtained by PCR amplification, and the full length and the PDZ domain of PDZ-RhoGEF were subcloned into pGEX-CMV (University), and M131, horse-radish peroxidase-conjugated secondary antibodies (Dako), and Alexa 594-conjugated secondary antibodies (Molecular Probes, Inc., Eugene, OR).

**Yeast Two-hybrid Screening**—A rat brain cDNA library fused to the GAL4 activation domain of the aCT2 vector (Clontech) was screened using pAS2-1/Rnd3M4 as a bait in the yeast strain Y190 according to the manufacturer’s instructions. Interaction between the brain library proteins activates transcription of the reporter gene HIS3 and lacZ. From 10⁶ transformants, 218 colonies grew on selective medium lacking histidine and were also positive for β-galactosidase activity. One of these, clone 179, was found to encode the carboxyl-terminal 438 amino acids of Plexin-B2.

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

**Cell Culture and Transfections**—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 4% glutamine, 100 units/ml penicillin, and 0.2 mg/ml streptomycin under humidified conditions in 95% air and 5% CO2 at 37 °C. Transient transfections were carried out with LipofectAMINE 2000 (Invitrogen) 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 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 (28). Nonfused Rnd1 was recovered by incubation with 10 units/ml thrombin (Sigma) for 4 h at 4 °C, and then thrombin was removed by absorption to p-aminobenzamidine-agarose beads (Sigma). Protein concentration was determined by comparing absorbance standards after SDS-PAGE and staining with Coomassie Brilliant Blue.

For pull-down assays, COS-7 cells (7 × 10⁵ cells) transfected with HA-tagged Rnd GTPases were rinsed once with phosphate-buffered saline (PBS) and lysed with the ice-cold cell lysis buffer (20 mm Tris-HCl, pH 7.4, 2 mm MgCl₂, 1 mm dithiothreitol (DTT), 0.2% Triton X-100, 1 mm phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Cell lysates were then centrifuged for 15 min at 18,000 × g at 4 °C. The supernatants were incubated for 10 min at 4 °C with 10 μg of GST fusion proteins and subsequently incubated with glutathione-Sepharose beads for 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 and analyzed by SDS-PAGE and immunoblotting with anti-HA antibody.

To examine direct interaction between the cytoplasmic domains of Plexins and Rnd GTPases, an overlay assay was performed according to the modified method of Manser et al. (36). E. coli cell lysates expressing GST-fused cytoplasmic domains of Plexins were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was soaked for 5 min in 6 M guanidinium hydrochloride dissolved in buffer A (25 mm Hepes-NaOH, pH 7.0, 0.5 mM MgCl₂, 0.05% Triton X-100) at 4 °C, and the buffer was diluted with an equal volume of buffer B and agitated for a further 5 min. This process was repeated three times. The membrane was then agitated for 10 min five times in buffer A, transferred to PBS containing 1% bovine serum albumin, 0.1% Triton X-100, 0.5 mM MgCl₂, and 5 mM DTT, and incubated in GAP buffer (25 mm Hepes-NaOH, pH 7.0, 5 mM MgCl₂, 0.05% Triton X-100, 2.5 mM DTT, and 100 μM GTP) containing [γ-32P]GTP (6000 Ci/mmol; PerkinElmer Life Sciences)-loaded Rnd1. After washing with wash buffer (25 mm Hepes-NaOH, pH 7.0, 5 mM MgCl₂, and 0.05% Triton-X-100), the membrane was dried, and bound radioactivity was visualized with an FLA-3000 image analyzer (Fuji). Nonfused Rnd1 (1 μg) were loaded with [γ-32P]GTP by incubation in loading buffer (20 mm Tris-HCl, pH 7.4, 2.5 mm NaCl, 0.1 mM DTT, 0.5 μg/ml bovine serum albumin, and 2 mM MgCl₂) at 30 °C for 10 min.

**Immunoprecipitation**—COS-7 cells (2 × 10⁶ cells) cotransfected with Myc-tagged Plexin-B1 and GFP-Rnd1 were lysed with ice-cold cell lysis buffer (10 mm Tris-HCl, pH 7.4, 100 mm NaCl, 5 mM MgCl₂, 1% DTT, 1% Nonidet P-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). After centrifugation, the supernatants were incubated with anti-Myc polyclonal antibody for 1 h and then with protein A-Sepharose (Amersham Biosciences) for 1 h. The beads were washed with the lysis buffer, and bound proteins were analyzed by SDS-PAGE and immunoblotting.

**Immunofluorescence Microscopy**—COS-7 cells (2 × 10⁶ cells) were seeded onto round 13-mm glass coverslips, and then they were transfected with expression vectors encoding Myc-tagged Plexin-B1 and GFP-Rnd1. Sixteen hours after transfection, cells on coverslips were fixed in 4% paraformaldehyde in PBS for 15 min. 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 to block nonspecific antibody binding. Then cells were incubated with anti-Myc
antibody in PBS for 1 h, followed by the incubation with Alexa 594-conjugated secondary antibody in PBS for 1 h. Cells on coverslips were mounted in 90% glycerol containing 0.1% p-phenylenediamine dihydrochloride in PBS.

For the COS-7 cell contraction assay, a soluble form of Sema4D expressed as a fusion protein with the Fc fragment of human IgGl (9) was harvested from the medium of transiently transfected COS-7 cells. Stimulation with Sema4D was performed by incubation of the cells with Sema4D-Fc-containing medium for 5 min at 37°C. A specific inhibitor for Rho-associated kinase (Rho-kinase) Y-27632 (10 μM; a generous gift from Welfide Corp.) was added immediately after transfection. The size of transfected cells was determined from digital images acquired at × 20 magnification by using a Leica DC550F digital camera system equipped with a Nikon Eclipse E800 microscope and an Image-Pro Plus image analysis software (Media Cybernetics).

Measurement of RhoA Activity—Measurement of RhoA activity was performed as described previously (37, 38). Briefly, COS-7 cells (7 × 10⁵ cells) were transfected with an expression vector encoding HA-tagged wild-type RhoA together with Myc-tagged Plexin-B1 and GFP-tagged Rnd1 and were serum-starved for 16 h. Then they were stimulated with Sema4D-Fc for 5 min at 37°C, and the cells were washed with the ice-cold Tris-buffered saline (20 mM Tris-HCl, pH 7.4, 300 mM NaCl). After centrifugation, the cells were lysed with the cell lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 30 mM MgCl₂, 0.2% Triton X-100, 10% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Cell lysates were then centrifuged for 5 min at 10,000 × g at 4°C, and the supernatants were incubated with 16 μg of GST-fused Rho-binding domain of mouse Rhotekin pre-bound to glutathione-Sepharose beads for 60 min at 4°C. The beads were washed with the lysis buffer, and bound proteins were analyzed by SDS-PAGE and immunoblotting.

RESULTS

Rnd1 Directly Interacts with the Cytoplasmic Domain of Plexin-B1—To isolate proteins that interact with Rnd GTPases, we performed a yeast two-hybrid screening with a rat brain cDNA library by using Rnd3S241, a mutant of Rnd3 lacking the carboxyl-terminal CAXX motif, as a bait. Approximately 10⁷ clones were screened, and one of the positive clones encoded a carboxy-terminal 425 amino acids of Plexin-B2. Plexin-B2, together with Plexin-B1 and Plexin-B3, constitutes a subfamily of Plexins, transmembrane receptors for semaphorins (10). This clone also interacted with Rnd1 and Rnd2 in yeast (data not shown). To examine whether the interaction between the Plexin-B family and Rnd GTPases is observed in vitro, we performed an overlay assay by using recombinant Rnd1 preloaded with [γ-³²P]GTP as a probe. As shown in Fig. 1A, the GST-fused cytoplasmic domain of Plexin-B1 (GST-Plexin-B1-cyt) showed strong binding to [γ-³²P]GTP-loaded Rnd1 on a nitrocellulose membrane, indicating that the interaction between Rnd1 and Plexin-B1 is direct. The interaction between Rnd1 and GST-Plexin-B2-cyt was not observed in the same condition, but a very weak signal was seen after a longer exposure (data not shown). A previous study showed the interaction between Rnd1 and Plexin-A1 (32), but we failed to detect the interaction of Rnd1 with GST-Plexin-A1-cyt or GST-Plexin-A2-cyt in this assay even after a much longer exposure. Similar results were obtained by using [γ-³²P]GTP-loaded Rnd3 as a probe (data not shown). Therefore, we further examined the interaction between Rnd1 and Plexin-B1. By using the overlay assay, the Rnd1 binding site in the cytoplasmic domain of Plexin-B1 was shown to be located between amino acids 1724 and 1915 (Plexin-B1-cyt1; Fig. 1A). To confirm the interaction between Plexin-B1 and Rnd GTPases, HA-tagged wild-type Rnd1, Rnd2, or Rnd3 was expressed in COS-7 cells, and a pull-down assay was performed with purified GST-fused Plexin-B1-cyt1. All Rnd subfamily GTPases interacted with GST-fused Plexin-B1-cyt1, whereas Rnd1A45, a mutant of Rnd1 containing a T45A substitution in the effector domain, did not (Fig. 1B), indicating that the effector domain of Rnd1 is important for the interaction with Plexin-B1. It has been reported that Rac directly interacts with the cytoplasmic domain of Plexin-B1 and that mutation of three amino acid residues, L1849G, V1850G, and P1851A, in Rac completely abolishes the interaction of Plexin-B1 with Rac (21, 22). Plexin-B1-cyt1 used in this study includes this region. Therefore, we created GST-fused Plexin-B1-cyt1 containing L1849G, V1850G, and P1851A substitutions (Plexin-B1-cyt1-GGA) and performed a pull-down assay. HA-tagged Rnd1 expressed in COS-7 cells was precipitated with GST-fused Plexin-B1-cyt1 but not with GST (lane 1), GST-fused Plexin-B1-cyt1-GGA (Fig. 1C), indicating that this mutation completely abolishes the interaction of Plexin-B1 with Rnd1.

The in vitro interaction studies were performed with the cytoplasmic domain of Plexin-B1. Therefore, we next examined whether Rnd1 interacts with the full length of Plexin-B1 in vivo in the presence of Sema4D, a ligand for Plexin-B1. Myc-tagged full-length Plexin-B1 and HA-tagged Rnd1 were expressed in COS-7 cells, and the cell lysate was immunoprecipitated with anti-Myc antibody. Stimulation of the cells
with Sema4D was performed by the incubation with the medium from cells expressing the extracellular region of Sema4D fused with the Fc fragment of human IgG1 (9). As shown in Fig. 2, HA-tagged Rnd1 was coimmunoprecipitated with Myc-tagged Plexin-B1, and this interaction was not affected by the addition of Sema4D, indicating that Rnd1 shows a constitutive, ligand-independent interaction with Plexin-B1 in vivo. On the other hand, we could not detect the interaction between Rnd1 and the full length of Plexin-B1-GGA or between Plexin-B1 and Rnd1ΔC by immunoprecipitation studies (Fig. 2). These results were consistent with those of in vitro binding studies.

**Interaction of Rnd1 with Plexin-B1 Induces COS-7 Cell Contraction in Response to Sema4D**—To investigate the role of the interaction of Rnd1 with Plexin-B1, COS-7 cells were transfected with Myc-tagged full-length Plexin-B1 together with GFP or GFP-tagged Rnd1, and morphological changes in transfected cells were analyzed by the fluorescence of GFP after the addition of medium from mock-transfected (lanes 1, 3, 5, and 7) or Sema4D-transfected cells (lanes 2, 4, 6, and 8) for 5 min, and cell lysates were immunoprecipitated with anti-Myc antibody. The immunoprecipitates and the total cell lysates were analyzed by immunoblotting with anti-HA or anti-Myc antibody.

Fig. 2. Interaction between Rnd1 and Plexin-B1 in COS-7 cells. COS-7 cells were transfected with expression vectors encoding Myc-tagged Plexin-B1 (WT; wild type; GGA, a mutant containing L1849G, V1850G, and P1851A substitutions; ΔC, a mutant lacking the last seven carboxyl-terminal amino acids) and HA-tagged Rnd1 (WT; wild type; A45, Rnd1ΔA45). Sixteen hours after transfection, cells were incubated with medium from mock-transfected (lanes 1, 3, 5, and 7) or Sema4D-transfected cells (lanes 2, 4, 6, and 8) for 5 min, and cell lysates were immunoprecipitated with anti-Myc antibody. The immunoprecipitates and the total cell lysates were analyzed by immunoblotting with anti-HA or anti-Myc antibody.

RacRL61, did not induce the COS-7 cell contraction in response to Sema4D (Fig. 3), this result being consistent with a previous report (24). Thus, the ability to induce the Plexin-B1-mediated COS-7 cell contraction is specific to Rnd1 or Rnd3. The majority of the contracted cells expressing Plexin-B1 and Rnd1 after stimulation with Sema4D took again a spread morphology when the cells were incubated in Sema4D-free medium for an additional 12 h (data not shown), indicating that this morphological effect is reversible and is not part of a cell death response but reflects a cytoskeletal event, as observed in the Sema3A-induced morphological change (39). In these experiments, immunostaining with anti-Myc antibody showed that expression levels and subcellular localizations of various constructs of Plexin-B1 were similar in the presence and the absence of GFP-Rnd1 (data not shown).

**Plexin-B1- and Rnd1-mediated COS-7 Cell Contraction Involves PDZ-RhoGEF, RhoA, and Rho-kinase**—Several lines of evidence indicate that activation of RhoA is involved in the downstream signaling pathway of Plexin-B1 (15–18, 22). In hippocampal neurons, Plexin-B1-mediated growth cone collapse in response to Sema4D was inhibited by C3 exoenzyme, a Rho inhibitor, or by Y-27632, an inhibitor for its downstream effector Rho-kinase (15). Therefore, we next examined the involvement of RhoA and Rho-kinase in the Plexin-B1 and Rnd1-mediated COS-7 cell contraction. The Sema4D-induced contraction in the cells expressing Myc-tagged Plexin-B1 and GFP-tagged Rnd1 was suppressed by coexpression with HA-tagged dominant-negative RhoA (RhoAN19) or by pretreatment of the cells with Y-27632 (Fig. 4). Although dominant negative Rac blocked the Plexin-B1-mediated Rho-dependent stress fiber formation in Swiss 3T3 fibroblasts (22), coexpression of HA-tagged dominant negative Rac1 (Rac1ΔN17) had no effect on the Sema4D-induced contraction in Plexin-B1/Rnd1-expressing cells (Fig. 4). Recent studies have shown that activation of RhoA by Plexin-B1 is mediated by PDZ-RhoGEF or LARG, members of Rho-specific GEFs. PDZ-RhoGEF contains a PDZ domain in its amino-terminal region, and the interaction between the PDZ domain of PDZ-RhoGEF and the PDZ domain-binding motif at the carboxyl terminus of Plexin-B1 is required for the RhoA activation by Plexin-B1 (15–18). To determine whether PDZ-RhoGEF is involved in the Plexin-B1 and Rnd1-mediated COS-7 cell contraction, we coexpressed GFP-tagged Rnd1 and Myc-tagged Plexin-B1 lacking the carboxyl-terminal PDZ domain-binding motif (Plexin-B1-ΔC) in COS-7 cells. Plexin-B1-ΔC could interact with Rnd1 in vivo, as shown by immunoprecipitation studies (Fig. 2). However, morphological changes were not detected in the cells coexpressing Rnd1 and Plexin-B1-ΔC after Sema4D stimulation (Fig. 4). Overexpression of the PDZ domain of PDZ-RhoGEF has been shown to inhibit the Plexin-B1-mediated cytoskeletal effect, and the PDZ domain of PDZ-RhoGEF therefore serves as the dominant negative form probably by dissociating endogenous PDZ-RhoGEF or LARG from Plexin-B1 (18). We showed that expression of the FLAG-tagged PDZ domain of PDZ-RhoGEF also suppressed the Sema4D-induced contraction in the Plexin-B1/Rnd1-expressing cells (Fig. 4). These results indicate that the PDZ-RhoGEF/RhoA/Rho-kinase pathway participates in the Plexin-B1 and Rnd1-mediated COS-7 cell contraction. In these experiments, expressions of wild-type Plexin-B1 and Plexin-B1-ΔC were almost the same levels, and more than 80% of Plexin-B1 and Rnd1 double positive cells also expressed RhoAN19, Rac1ΔN17, or the PDZ domain of PDZ-RhoGEF (data not shown).

**Interaction of Rnd1 with Plexin-B1 Potentiates Sema4D-induced RhoA Activation**—We have shown that RhoA and PDZ-RhoGEF are involved in the Plexin-B1 and Rnd1-mediated cell contraction.
contraction. This result led us to examine whether the interaction of Rnd1 with Plexin-B1 affects the PDZ-RhoGEF-mediated RhoA activation by Plexin-B1 in COS-7 cells. To measure the level of active GTP-bound RhoA in the cells, we used GST-fused Rho-binding domain of Rhotekin to precipitate GTP-bound active RhoA from the cell lysates (37). We could not detect endogenous RhoA in COS-7 cells with anti-RhoA antibody. Therefore, we cotransfected HA-tagged wild-type RhoA with Myc-tagged Plexin-B1 and GFP-tagged Rnd1 in COS-7 cells, and precipitated GTP-bound RhoA was detected with anti-HA antibody. When COS-7 cells were cotransfected with Plexin-B1 and HA-tagged wild-type RhoA, a slight increase in RhoA activity was observed after stimulation with Sema4D. In contrast, coexpression of Rnd1 with Plexin-B1 dramatically increased the GTP loading of RhoA in the cells in response to Sema4D (Fig. 5). On the other hand, the potentiation of RhoA activity was not observed in the cells coexpressing Rnd1 and Plexin-B1-GGA or coexpressing Rnd1 and Plexin-B1-ΔC. These results suggest that the interaction of Rnd1 with Plexin-B1 potentiates PDZ-RhoGEF-mediated RhoA activation by Plexin-B1 in vivo. Thus, the abilities of Plexin-B1 mutants to enhance the RhoA activity correlated with their abilities to induce the cell contraction.

Rnd1 Promotes the Interaction between Plexin-B1 and PDZ-RhoGEF—Finally, we examined the possibility that Rnd1 regulates the interaction between Plexin-B1 and PDZ-RhoGEF. Myc-tagged Plexin-B1 and FLAG-tagged PDZ-RhoGEF were expressed in COS-7 cells, and the cell lysate was immunoprecipitated with anti-Myc antibody. Plexin-B1 was communoprecipitated with PDZ-RhoGEF, and this interaction was significantly enhanced by coexpression with Rnd1 (Fig. 6). Deletion of the carboxyl-terminal PDZ domain-binding motif of Plexin-B1 completely abolished the interaction between Plexin-B1 and PDZ-RhoGEF in the presence of Rnd1, indicating that the increased interaction of Plexin-B1 with PDZ-RhoGEF is also mediated through its carboxyl-terminal PDZ domain-binding motif. On the other hand, Plexin-B1-GGA also interacted with PDZ-RhoGEF, but coexpression of Rnd1 did not promote this interaction (Fig. 6). These results suggest that the interaction of Rnd1 with the cytoplasmic domain of Plexin-B1 promotes the interaction between PDZ-RhoGEF and the carboxyl terminus of Plexin-B1. We also tested whether active Rac regulates the interaction of Plexin-B1 with PDZ-RhoGEF and the Plexin-B1-mediated RhoA activation, but both of them were not influenced by expression of Rac1L61 (data not shown).

DISCUSSION

Plexin-B1 has been shown to activate RhoA through PDZ-RhoGEF or LARG to induce cytoskeletal reorganization such as growth cone collapse (15–18). Here we show that Rnd1 directly interacts with the cytoplasmic domain of Plexin-B1 and that this interaction is required for the Plexin-B1-mediated, PDZ-RhoGEF/RhoA/Rho-kinase-dependent contraction of COS-7 cells. In COS-7 cells, association of Rnd1 with Plexin-B1 promotes the interaction between Plexin-B1 and PDZ-RhoGEF, and the Plexin-B1-mediated RhoA activation, but both of them were not influenced by expression of Rac1L61 (data not shown).
and when Plexin-B1 is activated by Sema4D, probably through the receptor clustering, activation of a large amount of RhoA and its effector Rho-kinase occurs at the cell surface through PDZ-RhoGEF, resulting in an increase in contractile force at the cell surface area and subsequent cell contraction. The interaction of Rnd1 with Plexin-B1 may trigger a conformational change in the cytoplasmic domain of Plexin-B1, which facilitates the interaction of its carboxyl terminus with PDZ-RhoGEF and thereby enhances the ability of Plexin-B1 to activate PDZ-RhoGEF. Thus, our results suggest a crucial role of Rnd1 in the regulation of RhoA activation and cytoskeletal rearrangement by Plexin-B1. Class 3 semaphorins, such as Sema3A, induce the contraction of COS-7 cells when they are cotransfected with both Plexin-A1 and neuropilin, functional class 3 semaphorin receptors, and the COS-7 cell contraction by Sema3A appears to reflect cytoskeletal changes observed during the growth cone collapse, because the downstream signaling molecules are similar to those involved in Sema3A-induced growth cone collapse in dorsal root ganglion neurons (14, 39, 39, 40).
40). We found that Sema4D-induced contraction of Plexin-B1/Rnd1-expressing COS-7 cells requires PDZ-RhoGEF, RhoA, and Rho-kinase, which are also involved in the Sema4D-induced growth cone collapse in hippocampal neurons (15). Considering that Rnd1 is expressed in most regions of brain, including hippocampal neurons (27), Sema4D-induced growth cone collapse in hippocampal neurons may involve endogenous Rnd1. We are currently investigating this possibility by using cultured hippocampal neurons.

Rac in its active GTP-bound state has been shown to interact directly with Plexin-B1 (21, 22). We showed that the Rnd1 binding region in Plexin-B1 overlaps with the region responsible for the interaction with Rac and that mutation of three amino acid residues in Plexin-B1 (L1849G, V1850G, and P1851A), which abolishes the interaction of Plexin-B1 with Rac, also suppressed the interaction with Rnd1, suggesting that Rnd1 and Rac use a similar or an identical binding site in Plexin-B1. In contrast, coexpression of Plexin-B1 with constitutively active Rac1 in COS-7 cells had no effect on the RhoA activation by Plexin-B1 and did not induce the cell contraction even after stimulation with Sema4D. This may be due to the lack of ability of constitutively active Rac1 to facilitate the interaction of Plexin-B1 and PDZ-RhoGEF. These observations suggest that effects of the interaction of Plexin-B1 with Rnd1 and Rac on the Rnd1 signaling are largely different and that potentiation of PDZ-RhoGEF-mediated RhoA activation is an essential step for the induction of cell contraction by Plexin-B1.

It has been recently reported that Rnd1 also interacts with Plexin-A1 and induces COS-7 cell collapse when cotransfected with Plexin-A1 (32, 33). Considering the lack of the PDZ domain-binding motif at the carboxyl terminus of Plexin-A1 and inability of Plexin-A1 to associate with PDZ-RhoGEF or LARG (16), the intracellular signaling pathways of Plexin-A1 and Plexin-B1 for the morphological changes in COS-7 cells are likely to be different. In addition, the cell collapse induced by Rnd1 and full-length Plexin-A1 in COS-7 cells does not require ligand stimulation (33), whereas the cell contraction induced by Rnd1 and full-length Plexin-B1 requires Sema4D stimulation. At present, it is not known why there is a difference in ligand dependence between Plexin-A1 and Plexin-B1 for the induction of the morphological changes in COS-7 cells in the presence of Rnd1, but further elucidation of signal transductions of Plexin family receptors will help us to understand precise mechanisms by which Rnd1 regulates Plexin-mediated cellular functions.

Previous studies have shown that Rnd1 possesses an antagonistic effect on G protein-coupled receptor-mediated RhoA activation signals, such as lysophosphatidic acid-induced formation of actin stress fibers and focal adhesions in fibroblasts (26, 27, 35, 41). In contrast, we have demonstrated that Rnd1 promotes the Plexin-B1-mediated RhoA activation, suggesting that Rnd1 oppositely regulates the RhoA activation induced by G protein-coupled receptors and Plexin-B1. PDZ-RhoGEF, which interacts with Plexin-B receptors through the PDZ domain, is also known to associate with activated G_{12} or G_{13} through the regulators of G protein signaling-like domain (RGS domain, also known as Lsc homology domain) and to mediate RhoA activation induced by certain G protein-coupled receptors (20, 42). In the light of the Rnd1-facilitated interaction of Plexin-B1 and PDZ-RhoGEF, Rnd1 may inhibit RhoA activation by G protein-coupled receptors due to sequestration of PDZ-RhoGEF to Plexin-B-like receptors. Thus, Rnd1 may act as a key regulator of the signal transductions of both Plexin-B receptors and G protein-coupled receptors.
