Plexin B Regulates Rho through the Guanine Nucleotide Exchange Factors Leukemia-associated Rho GEF (LARG) and PDZ-RhoGEF*

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Plexins represent a novel family of transmembrane receptors that transduce attractive and repulsive signals mediated by the axon-guiding molecules semaphorins. Emerging evidence implicates Rho GTPases in these biological events. However, Plexins lack any known catalytic activity in their conserved cytoplasmic tails, and how they transduce signals from semaphorins to Rho is still unknown. Here we show that Plexin B2 associates directly with two members of a recently identified family of Dbl homology/pleckstrin homology containing guanine nucleotide exchange factors for Rho, PDZ-RhoGEF, and Leukemia-associated Rho GEF (LARG). This physical interaction is mediated by their PDZ domains and a PDZ-binding motif found only in Plexins of the B family. In addition, we show that ligand-induced dimerization of Plexin B is sufficient to stimulate endogenous RhoA potently and to induce the reorganization of the cytoskeleton. Moreover, overexpression of the PDZ domain of PDZ-RhoGEF but not its regulator of G protein signaling domain prevents cell rounding and neurite retraction of differentiated PC12 cells induced by activation of endogenous Plexin B1 by semaphorin 4D. The association of Plexins with LARG and PDZ-RhoGEF thus provides a direct molecular mechanism by which semaphorins acting on Plexin B can control Rho, thereby regulating the actin-cytoskeleton during axonal guidance and cell migration.

The Rho family of small GTP-binding proteins, which includes Rho, Rac, and Cdc42, participate in a number of key cellular events, such as the regulation of cell morphology, cell aggregation, tissue polarity, cytokinesis, cell motility, smooth muscle contraction, and cell growth control (reviewed in Refs. 1 and 2). In vivo, the activity of small GTP-binding proteins of the Rho family is tightly regulated. Guanine nucleotide exchange factors (GEFs) act as activators by promoting the conversion of the inactive GDP-bound to the active GTP-bound species. All known GEFs for GTP-binding proteins have in common a 250-amino acid stretch of significant similarity with Dbl, called Dbl homology (DH) domain, that is critical for their ability to stimulate nucleotide exchange toward GTPases of the Rho family and is often adjacent to a pleckstrin-homology (PH) domain (reviewed in Ref. 3). In turn, these GEFs link a variety of cell surface receptors to the activation of Rho proteins, thereby regulating the dynamic remodeling of actin-containing cytostructures and gene expression.

Recently, a new family of DH-domain containing RhoGEFs, including PDZ-RhoGEF, leukemia-associated Rho GEF (LARG), and p115RhoGEF, has been identified (4–6). In addition to their DH/PH domains, these GEFs exhibit a number of structural motifs suggestive of a role in signal transduction. Indeed, these GEFs associate with G_{12} and G_{13} through a regulators of G protein signaling (RGS)-like domain (5, 7, 8), thus providing a direct link from these heterotrimeric G protein α-subunits and their coupled cell surface receptors to Rho. Of interest, PDZ-RhoGEF and LARG, but not p115RhoGEF, exhibit in their amino-terminal part a PDZ (PSD-95/Dlg/ZO-1) domain, a modular protein-protein interaction domain that binds to a consensus motif (S/TXV) in the carboxyl terminus of partner proteins or, alternatively, to other PDZ domains (9). As the precise nature of the GEFs by which most cell surface receptors promote Rho activation is still largely unknown, we hypothesized that PDZ-RhoGEF and LARG may utilize their PDZ domain to interact with novel signaling molecules, thereby leading to Rho activation in response to extracellular stimuli in addition to those mediated by heterotrimeric G proteins. In the present study, we found that LARG and PDZ-RhoGEF bind through their PDZ-domain to all members of the B class of Plexins, which are a recently identified family of transmembrane proteins that participate in axon guidance and cell migration in response to semaphorins (10–13). Indeed, an emerging body of information indicates that Rho-dependent pathways are essential for Plexin B1 function (14–17). However, how Plexin B stimulates Rho downstream targets is still unknown. Our findings indicate that the association of Plexin B with PDZ-RhoGEF and LARG provides a direct mechanism by which Plexins and semaphorins can promote RhoA activation, thereby regulating the cytoskeleton during axonal and dendritic guidance, as well as in an array of biological process that involve cell migration.

MATERIALS AND METHODS

Yeast Two-hybrid Screening—A human bone marrow cDNA library was screened using the PDZ domain of PDZ-RhoGEF as a bait using the Matchmaker system III (Clontech), following the manufacturer’s instructions. Transformants were obtained under high stringency conditions using growth media lacking –Ade/–His/–Leu/–Trp. The specificity of the interaction was confirmed using a Gβ2-subunit of heterotrimeric G proteins and phosphducin, which binds Gβ strongly (18), as a control bait and prey, respectively.

DNA Constructs—The cDNAs corresponding to Plexin B1 (KIAA 0407), B2 (KIAA 0315), B3 (KIAA 1206), and D1 (KIAA 0620) were kindly provided by Dr. T. Nagase (Kazusa DNA Research Institute, Kisarazu, Japan). Their cytoplasmic domain were amplified by PCR

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† The abbreviations used are: GEF, guanine nucleotide exchange factors; LARG, leukemia-associated Rho GEF; DH, Dbl homology; PH, pleckstrin homology; RGS, regulator of G protein signaling; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; NGF, nerve growth factor; Sema4D, semaphorin 4D.

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and ligated into the NheI and NotI restriction sites of pCEFL-EGFP. The extracellular and transmembrane regions of a myc-tagged TrkA (19), kindly provided by Dr. D. Martin-Zanca (University of Salamanca, Salamanca, Spain), was amplified and subcloned in the expression vector pCEFL. Chimeric receptors containing the extracellular and transmembrane domains of TrkA and the cytoplasmic part of Plexin B1, B2, or B2 lacking its PDZ-binding region were prepared by conventional DNA recombination techniques. An expression plasmid for semaphorin 4D was obtained from ResGen (Invitrogen).

Cell Lines and Western Blotting—Human kidney 293T and mouse fibroblasts Swiss 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum and calf serum, respectively. Rat pheochromocytoma PC12 cells, kindly provided by Dr. H. Kleinman (National Institute of Dental and Craniofacial Research, National Institutes of Health), were cultured in DMEM supplemented with 7.5% equine serum and 7.5% fetal bovine serum. Cell lysis, immunoprecipitation, and immunoblotting were performed as described (5). Specific antibodies to AU1, GFP, myc (9E10), and HA tags were purchased from Covance.

Stress Fiber Formation and in Vivo Rho Activation Assay—Swiss 3T3 cells were co-transfected with expression vectors for GFP-tagged clones 4 and 91 together with the AU1-tagged PDZ domain of PDZ-RhoGEP (+) (B), or the AU1-tagged full-length PDZ-RhoGEP (+) (C), or a control expression vector (−). The expression levels of GFP- and AU1-tagged proteins were examined by immunoblotting total cellular lysates (TL) with anti-GFP and anti-AU1 antibodies, respectively. AU1-tagged proteins were immunoprecipitated (IP) with anti-AU1 antibody followed by immunoblotting (WB) with anti-GFP antibody.

After fixation with paraformaldehyde and permeabilization with Triton X-100, cells were stained using Texas red X-labeled phalloidin (Molecular Probes). In vivo Rho activity was assessed according to Ref. 20 after modifications (21) using the Rho-binding domain (RBD) of rhotekin bound to glutathione-Sepharose beads to isolate the GTP-bound forms of Rho. Western blot analysis using a monoclonal antibody against RhoA (26C4, Santa Cruz Biotechnology).

Neurite Retraction Assay—Rat pheochromocytoma PC12 cells plated on poly-L-lysine and transfected with EGFP or EGFP-PDZ-RhoGEP-RGS domains of PDZ-RhoGEP using LipofectAMINE Plus™ were treated with NGF (100 ng/ml) for 5 days to induce their differentiation. Differentiated PC12 cells were then co-cultured with HEK-293T cells previously transfected with red fluorescent protein with or without semaphorin 4D. After 24 h, cells were fixed with paraformaldehyde and visualized using a confocal microscope. The number of transfected (green) PC12 cells in contact with 293T cells (red) exhibiting neurite extensions or lacking neurites and displaying a round morphology were counted in a series of independent experiments and expressed as a percentage of total transfected PC12 cells.

RESULTS AND DISCUSSION

To identify proteins that may interact with the PDZ domain of RGS-containing Rho GEFs, we performed a yeast two-hybrid

FIG. 1. The PDZ domain of PDZ-RhoGEP interacts with Plexin B2 in yeast and in eukaryotic cells. A, a yeast two-hybrid screen using the PDZ domain of PDZ-RhoGEP identified three clones from a human bone marrow cDNA library that encode a fusion protein including the carboxy terminus of Plexin B2. The intracellular region of Plexin B2 is depicted, and sequences corresponding to clones 4 and 91 are underlined. Clone 4 and clone 91, or a control encoding phosducin as a prey, were tested for interaction with the bait (PDZ domain) or a Gα-subunit of heterotrimeric G protein as a control. All yeast grew under low stringency condition (LT, left panel), but only those displaying strong interaction grew under high stringency conditions (AHLT, right panel). Positive interactions were visualized by the secretion of α-galactosidase. B and C, 293T cells were co-transfected with expression vectors for GFP-tagged clones 4 and 91 together with the AU1-tagged PDZ domain of PDZ-RhoGEP (+) (B), or the AU1-tagged full-length PDZ-RhoGEP (+) (C), or a control expression vector (−). The expression levels of GFP- and AU1-tagged proteins were examined by immunoblotting total cellular lysates (TL) with anti-GFP and anti-AU1 antibodies, respectively. AU1-tagged proteins were immunoprecipitated (IP) with anti-AU1 antibody followed by immunoblotting (WB) with anti-GFP antibody.
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FIG. 2. Specificity of the interaction between Plexin and PDZ-RhoGEF and LARG. A, the intracellular domain of Plexins B1, B2, and B3 but not Plexin D1 interact specifically with the PDZ domain of PDZ-RhoGEF in yeast under high stringency condition (−AHLT, right panel). Positive interactions were visualized by the secretion of α-galactosidase. All yeast grew up under low stringency condition (−LT, left panel), and phosducin and Gα were used as controls for the prey and bait, respectively. B, 293T cells were cotransfected with the AU1-tagged form of PDZ-RhoGEF together with the GFP-tagged intracellular region of Plexin B1, B2, B3, and D1. The expression level of different tagged proteins was examined by immunoblotting analysis using anti-AU1 and anti-GFP antibodies, as indicated. Immunoprecipitation with anti-AU1 antibody was followed by immunoblotting with anti-GFP antibody. The identity of immunoreacting bands is indicated. C, cartoon depicting the major structural features of the cytoplasmic domain of the different Plexin constructs used. D, 293T cells were transfected with AU1-PDZ-RhoGEF together with wild-type GFP-tagged intracytoplasmic region of Plexin B1, B2, B3, and D1, or their corresponding mutants lacking the PDZ-binding region (ΔPDZ) or where this motif was added (PDZ). The expression level of different tagged proteins was examined by immunoblotting analysis using anti-AU1 and anti-GFP antibodies. Immunoprecipitation with anti-AU1 antibody was followed by immunoblotting with anti-GFP antibody.

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FIG. 3. Plexin B interacts specifically with LARG and PDZ-RhoGEF. A, 293T cells were co-transfected with the GFP-tagged intracellular region of Plexin B1 or B2 together with AU1-tagged forms of PDZ-RhoGEF, LARG, p115RhoGEF, HA-tagged Tiam 1, or their control expression vectors. The expression level of each protein was examined by immunoblotting the same membranes containing total cell extracts successively with anti-AU1, anti-HA, and anti-GFP antibodies. The asterisks represent the immunoreactive p115RhoGEF that was recognized by the anti-AU1 antibody (see WB: anti-AU1 panel) and is still detected after reprobing the same membrane with the anti-GFP serum. Immunoprecipitation with anti-AU1 or anti-HA antibodies was followed with immunoblotting analysis with anti-GFP antibody. B, the PDZ domain of LARG and PDZ-RhoGEF is critical for their binding to Plexin B. 293T cells were co-transfected with GFP-tagged Plexin B2 together with AU1-tagged wild-type, ΔN, ΔC, DH/PH domain of LARG, and PDZ-RhoGEF or their control expression vector, as indicated. Expression of each tagged-protein was examined by immunoblotting sequentially with anti-AU1 and anti-GFP antibodies (WB: anti-HA panel). The asterisks represent the DH/PH domains of LARG and PDZ-RhoGEF that were immunodetected after reprobing the anti-AU1 treated membrane with the anti-GFP serum. Immunoprecipitation with anti-AU1 antibody was followed by immunoblotting with anti-GFP antibody.
FIG. 4. Plexin B stimulates Rho through PDZ-RhoGEF and LARG. Evidence for a role in Sema4D-induced neurite retraction. A, the extracellular and transmembrane domains (ETM) of myc-tagged TrkA were fused to the cytoplasmic domain of PlexinB2 wild-type or ΔPDZ. Expression of the chimeric receptors was detected by blotting with an anti-myc antibody upon expression in 293T cells. B, Swiss-3T3 cells were...
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–Trp), we identified 3 clones corresponding to the last 61 (clone 4) and 96 (clones 12 and 91) amino acids of the cytoplasmic domain of Plexin B2, a member of a novel family of cell surface receptors for semaphorins (22, 23) (Fig. 1A). As shown in Fig. 1A, clones 4 and 91 interact specifically with the PDZ domain of PDZ-RhoGEF in yeast, as judged by their ability to support yeast growth under high stringent conditions and to promote the expression of α-galactosidase from an integrated reporter system. The interaction of Plexin B2 with the PDZ domain of PDZ-RhoGEF observed in yeast was confirmed in eukaryotic cells. Indeed, as shown in Fig. 1, B and C, clone 4 and 91 when expressed as EGFP chimeras in 293T cells were co-immunoprecipitated with both a tagged PDZ domain of PDZ-RhoGEF and the full-length PDZ-RhoGEF, respectively.

Plexin B2 belongs to a recently discovered large family of transmembrane proteins that is organized into four subfamilies, known as Plexin A, B, C, and D (12). Thus, we next examined whether PDZ-RhoGEF could bind to other members of this family. As shown in Fig. 2A, in addition to Plexin B2, PDZ-RhoGEF was also able to interact with Plexin B1 and B3, but not with Plexin D1, both in yeast and in eukaryotic cells (Fig. 2B).

The cytoplasmic domain of Plexins is composed of two conserved regions separated by a variable linker, named Sex-Plexin domain, which is unique and not related to any other protein domain. It lacks signaling domains except for a PDZ-binding motif present at the carboxyl terminus of only members of the Plexin B subfamily. To determine whether PDZ-RhoGEF binds to the PDZ-binding motif of Plexin, we generated Plexin mutants in which this motif has been deleted (Plexin-B1ΔPDZ and Plexin-B2ΔPDZ) or added (Plexin D1-PDZ) (Fig. 2C). As seen in Fig. 2D, the interaction of PDZ-RhoGEF with Plexin B1 and B2 was lost when the PDZ-binding motif was removed. In contrast, PDZ-RhoGEF interacted with Plexin D1 upon addition of this motif. These results indicate that the PDZ-binding motif of Plexin B is required for its ability to associate with PDZ-RhoGEF. As the PDZ-binding motif is only present in B-type Plexins, this observation may provide a structural base for their signaling selectivity.

Based on these findings, we next examined the specificity of the interaction of Plexin B1 and B2 for other GEFs. As shown in Fig. 3A, in addition to PDZ-RhoGEF, Plexin B1 and B2 were also able to interact with LARG but not with p115RhoGEF, which lacks a PDZ domain. Furthermore, this interaction was specific, as Plexins did not interact with the Rac GEF, Tiam 1, which also contains a PDZ domain. Because PDZ-RhoGEF and LARG are multidomain-containing proteins, we asked which region of these GEFs participates in the binding to Plexin B by co-expressing the EGFP-Plexin chimeras with epitope-tagged constructs corresponding to wild-type, Δ-N (which lacks the PDZ domain), Δ-C (which lacks the carboxyl-terminal part), and the isolated DH/PH domain of PDZ-RhoGEF and LARG. As shown in Fig. 3B, removal of the PDZ-containing aminoterminal part of PDZ-RhoGEF and LARG abolished the binding to Plexin B2. However, neither the carboxyl-terminal nor the DH/PH domain of these GEFs was required for interaction with this Plexin. Similar results were obtained with Plexin B1 (data not shown). Together, these data suggest that B-type Plexins bind specifically to PDZ-RhoGEF and LARG and that the amino-terminal region of these GEFs, which includes the PDZ domain, is critical for this interaction.

The nature of the signaling molecules acting downstream of Plexins has not yet been characterized. Plexins lack any demonstrable catalytic activity in their intracellular region, but a direct association between active Rac (14, 15, 24) to the cytoplasmic domain of Plexin B1 was recently demonstrated. However, Plexin B1 does not promote Rac activation (Ref. 25 and data not shown), and this association has a regulatory function; binding of GTP-Rac to Plexin B1 enhances the binding of Sema4D to its receptor and prevents the activation of Rac by p21-activated kinase (PAK). Instead, recent data suggest that Plexin B1 may stimulate Rho-dependent pathways (14), although the nature of the intervening molecular events leading to Rho activation by Plexins still remains unknown.

To begin exploring whether Plexin B can induce the activation of Rho, and whether this process involves the association to PDZ-RhoGEF and LARG, we first examined whether activation of Plexin B can lead to cytoskeletal changes that are characteristic of those elicited by this small GTPase. As the ligand for Plexin B1, semaphorin 4D (Sema4D, also known as CD100) (12), is a membrane-bound semaphorin, and the ligand for Plexin B2 is still unknown, we took advantage of the recent observation that signaling by Plexins can be initiated by ligand-dependent dimerization of their intracellular tails (14) to engineer a cellular system in which Plexins can be readily activated in response to a soluble ligand. As an approach, we constructed a chimeric receptor in which the extracellular and transmembrane domains of the myc-tagged TrkA (19) was fused to the intracellular domain of wild-type Plexin B2 or its ΔPDZ deletion mutant (Fig. 4A). As shown in Fig. 4B, although the activation of a truncated TrkA itself did not affect the actin cytoskeleton, the addition of NGF was sufficient to induce the rapid formation of stress fibers in Swiss 3T3 cells expressing the chimeric Plexin-B2 construct, a cytoskeletal response that is characteristic of RhoA activation. This remarkable effect required an intact PDZ-binding motif, as NGF did not promote stress fiber formation in TrkA-Plexin B2ΔPDZ-expressing cells.

These observations prompted us to ask whether Plexin B can indeed activate Rho using a recently developed highly sensitive in vivo Rho guanine nucleotide exchange assay that allows the detection of the GTP-bound form of endogenous Rho in 293T cells (20, 21). As shown in Fig. 4C, activation of a truncated TrkA by NGF did not stimulate Rho, even if co-expressed with PDZ-RhoGEF. In contrast, addition of NGF to TrkA-Plexin B2-expressing cells led to the rapid activation of Rho, which peaked at 1 min and remained elevated for up to 10 min (Fig. 4C and data not shown). This response was prevented by the EGFP-PDZ domain of PDZ-RhoGEF (not shown) and enhanced dramatically when the full-length of PDZ-RhoGEF was over-expressed with TrkA-ETM, TrkA-Plexin B2 wild-type, or ΔPDZ and EGFP expression vector. After serum-starvation, cells were either left untreated (−NGF) or treated with NGF (+NGF) (100 ng/ml) for 10 min, fixed, and polymerized actin-stained with labeled phalloidin. Transfected cells were visualized by direct fluorescence. C, 293T cells transfected with TrkA-ETM, TrkA-Plexin B2 wild-type, or ΔPDZ together with or without PDZ-RhoGEF were incubated with NGF (100 ng/ml) for the indicated times and lysed. Active Rho was affinity-precipitated (AP) with GST-rhotekin-Rho-binding domain (GST-RBD), and the level of the GTP-bound form of Rho in vivo was visualized by immunoblotting analysis using a mouse monoclonal RhoA antibody. The expression level of RhoA in total cellular lysates was examined by immunoblotting using an anti-RhoA antibody. D, PC12 cells (green) transfected with GFP (a–c) and GFP-tagged PDZ domain (d and e) or RGS domain (f) of PDZ-RhoGEF were differentiated for 5 days using NGF (100 ng/ml) before co-culturing with 293T cells transfected with red fluorescent protein, alone (red, b), or together with an expression vector for Sema4D (c–f). Cells were examined by confocal microscopy. White arrows point to neurites. E, quantification of the inhibition of Sema4D-induced neurite retraction and cell rounding in PC12 cells. PC12 cells and 293T cells were transfected with the indicated DNA and processed as described in D, and the percentage of PC12 cells with neurites in contact with transfected 293T cells was counted. Data are the average ± S.E. of three experiments. Note that the Semaphorin 4D effect is only reversed by the PDZ domain of PDZ-RhoGEF (black bar).
expressed (Fig. 4C). Moreover, when its PDZ-binding motif was deleted, TrkA-Plexin B2 was no longer able to activate Rho, even when PDZ-RhoGEF was overexpressed. Together, these data provide evidence that Plexin B2 can stimulate RhoA and that this process may involve the recruitment of PDZ-RhoGEF through its PDZ-binding motif.

Recent work (26) has provided evidence that Plexins B provide attractive and repulsive cues necessary for axon guidance. Of interest, Rho can promote neurite retraction and growth cone collapse in neuronal cells (reviewed in Ref. 26). Thus, we examined the biological significance of the interaction between PDZ-RhoGEF and LARG with B-type Plexins using differentiated rat pheochromocytoma (PC12) cells, which exhibit dendritic extensions that collapse in response to Rho activation (27). We first confirmed by Western-blotting that Plexin B1 is endogenously expressed in PC12 cells and that these cells also express LARG (data not shown). As neuronal Plexin B1 is activated upon interaction with membrane-bound Sema4D, we next developed a cellular assay system in which 293T cells expressing Sema4D or control plasmids together with red fluorescent protein were co-cultured with differentiated PC12 cells expressing Sema4D or control plasmids (Fig. 4D). We first confirmed by Western-blotting that Plexin B1 is endogenously expressed in PC12 cells and that these cells also express LARG (data not shown). As neuronal Plexin B1 is activated upon interaction with membrane-bound Sema4D, we next developed a cellular assay system in which 293T cells expressing Sema4D or control plasmids together with red fluorescent protein were co-cultured with differentiated PC12 cells transfected with EGFP or EGFP fusion proteins. As shown in Fig. 4D, differentiated PC12 cells (green) exhibit extensive neurites, which were not affected after making contact with untransfected (Fig. 4D, a) or control transfected 293T cells (red) (Fig. 4D, b). In contrast, contact with Sema4D-expressing 293T cells induced the retraction of neurites and cell rounding in more than 85% of differentiated PC12 cells (Fig. 4D, c). Remarkably, expression of the EGFP-PDZ domain of PDZ-RhoGEF in PC12 cells was sufficient to block cell rounding induced by Sema4D (Fig. 4D, d–e), as only 22% of cells expressing this construct retracted when in contact with Sema4D-expressing 293T cells (Fig. 4E). Expression of its EGFP-RGS domain did not have any effect on neurite retraction (Fig. 4, D, f, and E). Together, these observations indicate that the interaction of Plexin B with PDZ-RhoGEF and LARG provides a molecular mechanism by which these cell surface receptors transduce signals to Rho, thus promoting neurite retraction and cell rounding.

Although the precise mechanism by which Plexin B activates the in vivo GEF activity of PDZ-RhoGEF and LARG is still unknown, we can postulate that the formation of a molecular complex at the plasma membrane through the interaction of the PDZ-binding motif of Plexin B with the PDZ-domain of LARG and PDZ-RhoGEF results in Rho activation, thus causing the retraction of growth cones and extending neurites in response to semaphorins. Moreover, semaphorins and Plexins have been found in a large variety of tissues in addition to the central nervous system and have been recently implicated in a variety of biological functions including cellular and humoral immune response (28), cell migration (28), and tumor growth (29). Thus, our findings indicating that PDZ-RhoGEF and LARG link Plexin B to Rho activation may now provide a molecular understanding of the role of Plexin B in axonal and dendritic guidance and likely in a growing array of normal and pathological biological processes initiated by semaphorins that involve rapid changes in the actin-cytoskeleton and cell migration.

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