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Interaction of plexin-B1 with PDZ domain-containing Rho guanine nucleotide exchange factors

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Abstract

The Rho family GTPase has been implicated in plexin-B1, a receptor for Semaphorin 4D (Sema4D), mediating signal transduction. Rho may also play a function in this signaling pathway as well as Rac, but the mechanisms for Rho regulation are poorly understood. In this study, we have identified two kinds of PDZ domain-containing Rho-specific guanine nucleotide exchange factors (RhoGEFs) as proteins interacting with plexin-B1 cytoplasmic domain. These PDZ domain-containing RhoGEFs showed significant homology to human KIAA0380 (PDZ-RhoGEF) and LARG (KIAA0382), respectively. Both KIAA0380 and LARG could bind plexin-B1 and a deletion mutant analysis of plexin-B1, KIAA0380 and LARG revealed that KIAA0380 and LARG bound plexin-B1 cytoplasmic tail through their PDZ domains. The tissue distribution analysis indicated that plexin-B1 was co-localized with KIAA0380 and LARG in various tissues. Immunocytochemical analysis showed that LARG was recruited to plasma membrane by plexin-B1. These results suggest that PDZ domain-containing RhoGEFs play a role in Sema4D-plexin-B1 mediating signal transduction.

Keywords: Semaphorin; Semaphorin 4D; Plexin-B; PDZ domain; RhoGEF; KIAA0380; LARG

Semaphorins are a large family of secreted and transmembrane molecules showing many physiological functions [1–3]. Semaphorin 4D (Sema4D), also known as CD100, was recently identified as a transmembrane member of semaphorin family of the vertebrate [4,5]. Sema4D is enriched in the neuronal and immune tissue, but the function of Sema4D in neuronal tissue is poorly understood. A recent study shows that Sema4D binds to mammalian cells transfected with plexin-B1, suggesting that plexin-B1 is a receptor for Sema4D [6]. The cytoplasmic domain of plexin-B1 (~600 amino acids) is highly conserved among plexin-B family but has no homology with any other proteins. The cytoplasmic domain of plexin-B1 may play an important role in signal transduction by Sema4D-plexin-B1. The Rho family of small GTPase, which includes Rho, Rac, and Cdc42, is involved in regulation of cytoskeleton and, thus, regulated many actin-driven processes, including cell migration and axon guidance [7,8]. Recent reports have shown that the cytoplasmic domain of plexin-B1 contains CRIB (Cdc42/Rac interactive binding) motif and interacts with active Rac1 and the interaction inhibits p21-activated kinase (PAK) activation [9–12]. On the other hand, plexin-B1 clustering induces assembly of actin and myosin filaments and cell contraction in fibroblasts, suggesting that Rho is also involved in plexin-B1 signaling [10]. Genetic analysis in *Drosophila* shows that reducing RhoA gene dosage suppresses the plexin-B gain-in-function phenotypes, suggesting RhoA interacts with plexin-B signaling [11]. However, signal transduction mechanisms of Rho in plexin-B1 signaling are poorly understood.

In this study, based on yeast two-hybrid screening on rat brain cDNA library, we have isolated two kinds of PDZ domain-containing RhoGEFs, as proteins interacting with plexin-B1 cytoplasmic tail. These proteins

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have significant homology to human KIAA0380 and LARG, respectively. Both KIAA0380 and LARG function as RhoA-specific guanine-nucleotide exchange factors (GEFs) and induce actin re-organization in the transfected cells [13–18]. KIAA0380 and LARG also contain RGS (regulating G protein signaling) homology domain suggesting that these proteins are involved in regulating not only RhoA but also heterotrimeric G proteins. Thus, it is possible that Sema4D-plexin-B1-mediating signal may be modified by heterotrimeric G proteins coupled receptors (GPCR) through such PDZ-containing RhoGEFs.

Materials and methods

Yeast two-hybrid screening and β-galactosidase assay. The bait vector, pBTM116-plexin-B1-cytoplasmic domain (cd) (amino acids [aa] 1489–2135), was constructed by subcloning the inserts encoding the respective aa residues of plexin-B1. The yeast two-hybrid library constructed from an adult rat brain cDNA was screened using a pBTM116-plexin-B1-cd as bait as described [19]. β-Galactosidase assay was also performed as described [19].

Preparation of soluble Sema4D-AP. The conditioned medium containing Sema4D-AP was obtained from HEK293 cells expressing them. The concentration of the proteins was determined from AP activity at 405 nm [20].

Construction of expression vectors. The cDNAs of KIAA0380, provided from Kazusa DNA Research Institute (Japan), were subcloned into the pCMV-Myc expression vector [21,22]. A deletion mutant of KIAA0380ΔPDZ (aa 125–1533) was generated by PCR using primers gcgaattcatgggcatctctgggctc and tagtcgacttatggtcctggtgacgc and subcloned into the pCMV-Myc expression vector. The cDNAs of LARG, gifted from Dr. Caligiuri, were subcloned into the pCMV-Myc expression vector. A deletion mutant of LARGΔPDZ (aa 185–1547) was generated by PCR using primers atacgcgtatgagtggcacacag and gcgtcgactaacttttatctgagtg and subcloned into the pCMV-Myc expression vector.

The cDNAs of human plexin-B1 (KIAA0407), -B2 (KIAA0315) provided from Kazusa DNA Research Institute (Japan) were subcloned into the pEF-HA expression vectors. A deletion mutant of plexin-B1ΔC (aa 1–2132) was generated by PCR using primers atgtcgacaatgcctgctctgggc and atgcggccgctagaccttgttttccac and subcloned into the pEF-HA expression vector.

Antibodies. Rabbit polyclonal anti-KIAA0380 and LARG antibodies were raised against GST-KIAA0380 (aa 1216–1521) and LARG (aa 1203–1547), respectively. The antiserum was affinity purified with each GST fusion protein covalently coupled to NHS-activated Sepharose (Amersham Pharmacia). The specificity of the antibodies was confirmed by Western blotting of the pCMV-Myc-KIAA0380 or -LARG-transfected HEK293 cells. Mouse monoclonal antibody to HA was obtained from Boehringer–Mannheim (Tokyo) and mouse monoclonal antibody to Myc (9E10) was purchased from American Type Culture Collection. For Western blot analysis, primary antibodies to KIAA0380, LARG, Myc, and HA were used at a 1:2000 dilution. Immunoreactive proteins were incubated with HRP-conjugated goat anti-rabbit IgG or HRP-conjugated goat anti-mouse IgG (New England Bio Labs) at a 1:2000 dilution and then visualized using the ECL systems (Amersham Pharmacia).

In vitro binding of plexin-B1 to KIAA0380 and LARG. HEK293 cells were transfected by calcium phosphate method with pEF-HA-plexin-B1, or various mutant plasmids of plexin-B1. After 48 h, the cells were lysed in 0.5 ml extract buffer (20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1 mM PMSF, and 1% (w/v) Triton X-100) and centrifuged at 100,000*g* for 30 min. Five hundred μl supernatant was incubated with the various GST-fused proteins that were fixed on 20 μl glutathione–Sepharose 4B beads (Amersham Pharmacia). After the beads were washed with the extract buffer, proteins on the beads were subjected to the immunoblot analysis.

Immunoprecipitation. pCMV-Myc-KIAA0380, -LARG or pCMV-Myc-KIAA0380ΔPDZ, -LARGΔPDZ was co-transfected with pEF-HA-plexin-B1 into HEK293 cells by calcium phosphate method [20]. After 48 h of culture, the cells were lysed with extract buffer and centrifuged. The supernatant was incubated with anti-Myc or anti-HA antibodies fixed on protein A–Sepharose beads. After the beads were washed with the extract buffer, proteins on beads were detected with the immunoblottings by the indicated antibodies.

RT-PCR. RT-PCR experiments with specific primers of mouse plexin-B1 (atccacatctggaagacc and gaccttgttttccacagc) were performed using cDNA prepared from mouse each tissue as template [23]. PCR was performed using the Taq DNA polymerase (Sigma) under the following conditions: denaturation at 94 °C for 5 min, 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. Aliquots (20 μl) of PCR products were analyzed by 1.2% agarose gel electrophoresis.

Immunofluorescence. Immunofluorescence microscopy of transfected cells was done as described [19]. Briefly, cells were cultured on a cover glass and fixed with 4% formaldehyde in PBS. The fixed sample was treated with 0.2% Triton X-100 in PBS and washed three times with PBS. After the sample was soaked with PBS containing 1% BSA, the sample was treated with the indicated antibodies and washed with PBS containing 1% BSA, followed by incubation with the rhodamine-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG antibodies. After the incubation, the sample was washed with PBS, embedded in PBS containing 95% glycerol–PBS, and viewed with the confocal imaging system (Bio-Rad MRC-1024).

Result

Identification of PDZ domain-containing RhoGEFs, as a plexin-B1-binding protein

In an effort to study the functional mechanisms of the cytoplasmic domain (cd) of plexin-B1, we searched for proteins that can bind to plexin-B1-cd. We used the entire cytoplasmic domain of plexin-B1 inserted into the GAL4–DNA-binding domain in pBTM116 vector as bait to screen an adult rat brain cDNA library in a yeast two-hybrid system. Eight independent clones were obtained. Of these eight clones, one clone had PDZ domain showing significant homology to the human KIAA0380 PDZ domain. Another two clones also had PDZ domain showing significant homology to the human LARG PDZ domain. KIAA0380 and LARG were known as RhoA-guanine nucleotide exchange factor. We use KIAA0380 and LARG for subsequent experiments, because these clones are likely to encode a rat counterpart of human KIAA0380 and LARG, respectively.

Specific interaction of plexin-B1 with PDZ domain of KIAA0380 and LARG

Plexin-B1 has cytoplasmic regions with a C-terminal motif of three amino acid residues, T–D–L, and these aa

Bait (pBTM116) \ Prey (pVP16)	PDZ domains						
	0380	LARG	PSD95 (PDZ 1-3)	PSD93 (PDZ 1-3)	ZO-1 (PDZ 1-3)	dlgA (PDZ 1-3)	NOS (PDZ 1)
Plexin-B1cd	++	+++	–	–	–	–	–
Plexin-B2cd	+	+++	–	–	–	–	–
Plexin-B1cdAAA	–	–					
Plexin-B2cdAAA	–	–					
Plexin-A1cd	–	–			(β–galactosidase activity on filter)		

Fig. 1. Specific interaction of plexin-B1 C-terminus with the PDZ domain of KIAA0380 and LARG. Yeast clones co-transfected with the prey pVP16 and the bait pBTM116 vectors in the indicated combinations were selected and grown on the selection plates. β-Galactosidase activities were measured on the filters after 2 h and intensity of color was scored as the following: (−) no color (background); (+) faint color (but above background); (++) strong color; and (+++) very strong color.

sequences are conserved among plexin-B family, plexin-B1, plexin-B2, and plexin-B3. The C-terminal motifs of three amino acid residues of another plexin family such as plexin-A and plexin-C are different from those of plexin-B family. We next examined whether plexin-B1, specifically bound KIAA0380 and LARG through PDZ domain and the C-terminal motif. For this purpose, we tested several other members of PDZ-containing proteins, PSD-95, SAP102, PSD-93, ZO-1, dlgA, and NOS, for their ability to interact with plexin-B1, -B2 C-terminus in the yeast two-hybrid system (Fig. 1). None of those tested clones interacted with C-terminus of plexin-B1, -B2. Plexin-A1, having the C-terminal motif was L–S–S and the mutants of plexin-B1, -B2 which were substituted of three amino acid residues with three alanines at C-terminus did not interact. Thus, there appears to be a specific interaction of the C-terminus of plexin-B1 and -B2 with PDZ domain of KIAA0380 and LARG.

In vitro and in vivo binding of plexin-B1 to KIAA0380 and LARG

We next examined the in vitro direct binding of plexin-B1 to KIAA0380 and LARG by affinity chromatography (Fig. 2). Cell extracts from HEK293 cells transfected with full-length plexin-B1, or C-terminus deletion mutants lacking C-terminus three amino acids (plexin-B1ΔC), were incubated with GST-fusion protein of the PDZ domain of KIAA0380 or LARG. Full-length of plexin-B1 bound GST-PDZ domains of KIAA0380 or LARG but no interaction of plexin-B1ΔC with GST-PDZ domains was observed. These results indicated that C-terminal of plexin-B1 interacts with PDZ domains of KIAA0380 and LARG and a motif of three amino acid residue, T–D–L, of plexin-B1 C-terminus is essential for binding. Similar results were also obtained for plexin-B2 (data not shown).

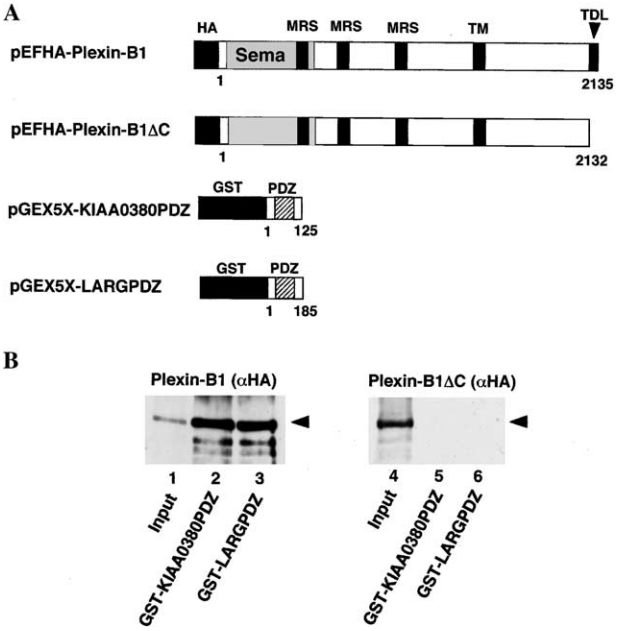

Fig. 2. In vitro binding of plexin-B1 to the PDZ domain of KIAA0380 or LARG. (A) Construction of plexin-B1 and plexin-B1ΔC with proteins having Sema-region, MRS-region, and the transmembrane region (TM) and PDZ domains of KIAA0380 and LARG. (B) The cell lysates from pEF-HA-plexin-B1 or plexin-B1ΔC-transfected HEK293 cells were applied to glutathione–Sepharose beads containing GST-PDZ (KIAA0380 or LARG). The beads were washed extensively and eluted with SDS–PAGE sample buffer. Each fraction was subjected to SDS–PAGE (7.5% polyacrylamide gel) followed by Western blotting with the anti-HA antibody.

Next, we examined the binding of plexin-B1 to KIAA0380 and LARG in vivo. HEK293 cells were co-transfected with pEF-HA-plexin-B1 and pCMV-Myc-KIAA0380 or the mutant lacking PDZ domain (KIAA0380ΔPDZ). The cell extracts were immunoprecipitated with anti-HA or anti-Myc antibodies on protein A–Sepharose beads and analyzed by Western blotting with anti-HA or anti-Myc antibodies (Fig. 3). Anti-Myc antibody precipitated both the Myc-KIAA0380 and Myc-KIAA0380ΔPDZ from the transfected cells, and Myc-KIAA0380 protein co-immunoprecipitated with HA-plexin-B1 and vice versa. Myc-KIAA0380ΔPDZ failed to precipitate HA-plexin-B1. Similar results were obtained with plexin-B1 and LARG (data not shown). These results indicated that PDZ domain of KIAA0380 and LARG is essential for binding to plexin-B1.

Constitutive interaction of plexin-B1 with KIAA0380

Next, to investigate whether the interaction of plexin-B1 with KIAA0380 was modulated by ligand-binding to plexin-B1, we examined soluble Sema4D, plexin-B1 ligand, treatment effects on the interaction. Both pEF-HA-plexin-B1 and pCMV-Myc-KIAA0380-transfected HEK293 cells were further incubated with or without 1 nM soluble Sema4D-AP. The cell extracts were

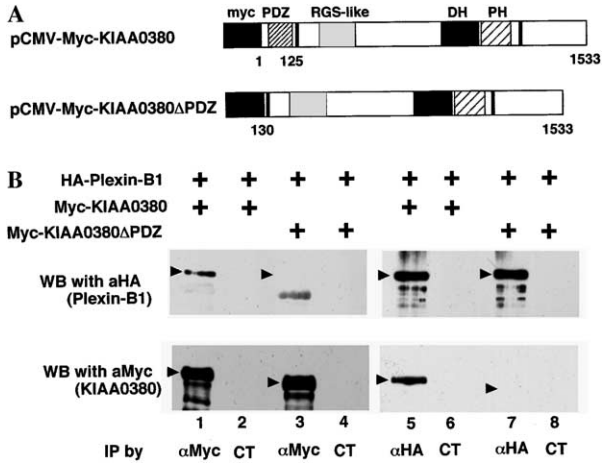

Fig. 3. Co-immunoprecipitation of plexin-B1 with KIAA0380. (A) Construction of KIAA0380 and KIAA0380ΔPDZ with proteins having PDZ domain, RGS-like domain, and the DH/PH-domain. (B) The cell lysates from pEF-HA-plexin-B1 and pCMV-Myc-KIAA0380 or KIAA0380ΔPDZ were incubated with anti-HA or anti-Myc antibodies or normal mouse serum. Protein A–Sepharose bead associated immunocomplexes were subjected to SDS–PAGE (7.5% acrylamide gel) followed by Western blotting with anti-HA or Myc antibodies.

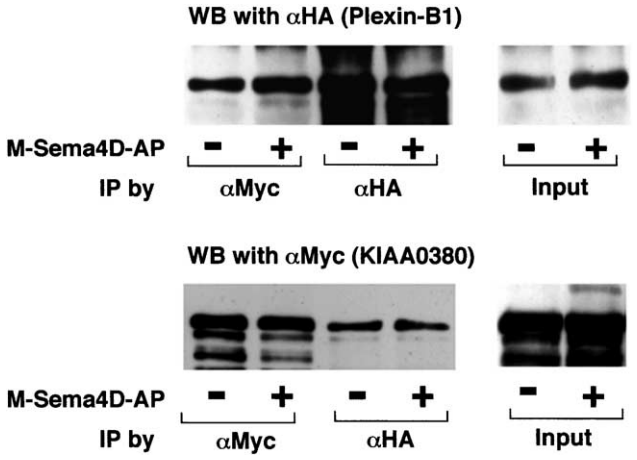

Fig. 4. Constitutive interaction of plexin-B1 with KIAA0380. The cell lysates from both pEF-HA-plexin-B1 and pCMV-Myc-KIAA0380-transfected HEK293 cells with or without treatment of soluble Sema4D (1 nM) were incubated with anti-HA or anti-Myc antibodies. Protein A–Sepharose bead associated immunocomplexes were subjected to SDS–PAGE (7.5% acrylamide gel), followed by Western blotting with anti-HA or Myc antibodies.

immunoprecipitated with anti-HA or anti-Myc antibodies on protein A–Sepharose beads and analyzed by Western blotting with anti-HA or anti-Myc antibodies (Fig. 4). There was no significant difference in the interaction of plexin-B1 with KIAA0380 in the presence or absence of soluble Sema4D. These results indicated that the interaction of plexin-B1 with KIAA0380 was not modulated by treatment with soluble Sema4D.

Overlap expression of plexin-B1, KIAA0380 and LARG

We examined tissue distribution of KIAA0380 and LARG in mouse using Western blotting with anti-KIAA0380 and LARG antibodies (Fig. 5A). KIAA0380 was widely expressed in various tissues except for heart and skeletal muscle but LARG was enriched in brain

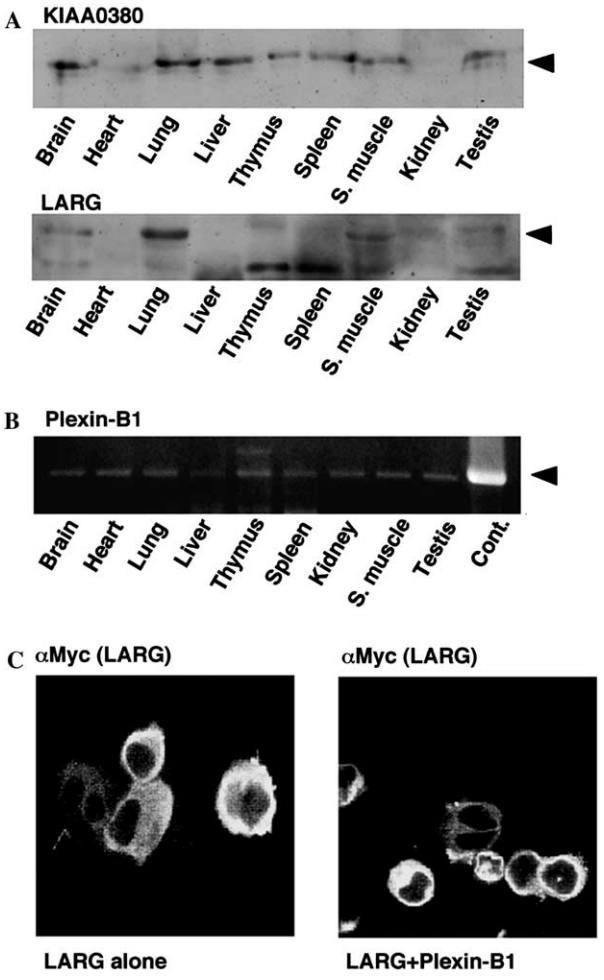

Fig. 5. Overlap expression of plexin-B1 and PDZ-containing RhoGEFs. (A) Tissue distribution of KIAA0380 and LARG in various mouse tissues. The homogenates of various mouse tissues (20 μg protein each) were subjected to SDS–PAGE (7.5% acrylamide gel) followed by Western blotting with the polyclonal anti-KIAA0380 or anti-LARG antibodies. (B) Tissue distribution of plexin-B1 in various mouse tissues. RT-PCR experiments with specific primers of mouse plexin-B1 were performed as described in Materials and methods. The expected size of the amplified plexin-B1 was 650 bp. Aliquot (20 μl) of PCR products was analyzed by 1.2% agarose gel electrophoresis. (C) Co-localization of LARG and plexin-B1 in HEK293 cells. HEK293 cells were transfected with and pCMV-Myc-KIAA0380 and/or pEF-HA-plexin-B1, fixed and stained with the monoclonal antibody against Myc (LARG) and polyclonal antibody against plexin-B1, and visualized with rhodamine-conjugeted anti-rabbit IgG and FITC-conjugated anti-mouse IgG. The left panel shows the immunofluorescence for LARG alone, the right panel shows that for KIAA0380 in the presence of plexin-B1.

and lung. Next, RT-PCR analysis with specific primers of mouse plexin-B1 was performed using cDNA prepared from mouse each tissue as template (Fig. 5B). Plexin-B1 was widely expressed in various tissues. These results indicated that the expression of plexin-B1 and KIAA0380, LARG was roughly overlapped in various tissues. Finally, to test the interactions of plexin-B1 with PDZ domain-containing RhoGEF in intact cells, we transfected HEK293 cells with pCMV-Myc-LARG and/or pEF-HA-plexin-B1 (Fig. 5C). LARG was diffusely localized in the cytoplasm, when expressed alone. On the other hand, plexin-B1 was localized in plasma membrane (data not shown). But, when LARG was co-expressed with plexin-B1, their overlapping staining was shown in the plasma membrane. These results suggested that plexin-B1 recruited LARG from cytoplasm to plasma membrane. KIAA0380 was localized in the plasma membrane in plexin-B1-independent manner (data not shown).

Discussion

The C-terminus of plexin-B1 binds to PDZ domain of PDZ domain-containing RhoGEFs

PDZ domains are known as modular domains that bind to specific C-terminus peptide sequences of many membrane proteins [24]. PDZ proteins were originally identified as mediators of clustering of neurotransmitter receptors and ion channels and then they were also involved in asymmetric distribution of receptors in epithelial cells [25–28]. The recent analysis of distinct ligand preference of the PDZ domain using peptide library technique shows that PDZ domains are divided into two classes according to their peptide-binding specificity [29]. The PDZ domains of PDZ domain-containing RhoGEFs are seen to be classified as the class I, selecting peptide with hydroxyl amino acid at position −2. The class I PDZ domain interacts with preferentially to C-terminal amino acid sequence, S/T–X–V (X presents any amino acid). Three amino acids of the C-terminus of plexin-B1 are T–D–L. In general, PDZ domains bind to the peptides that terminated in a hydrophobic amino acid such as V, I, or L. Our finding is consistent with this observation, but we have shown here by the yeast two-hybrid assay that plexin-B1 C-terminal did not bind to any another class I PDZ domains such as PSD-95 and the like. Recent reports indicated X residues at position −1 and the upstream amino acid residues of three amino acids at C-terminus and the specificity and affinity are determined [30]. It is possible that amino acids in these positions in plexin-B1 C-terminus are crucial for the specificity for the PDZ domain of KIAA0380 and LARG.

Plexin-B1 interacts with PDZ domain-containing RhoGEFs

Previous studies have suggested that not only Rac but also Rho may interact with downstream pathway of Sema4D-plexin-B1 signal transduction [10,11]. However, little is known about the mechanisms by which Sema4D-plexin-B1 signaling regulate Rho activation. How does PDZ domain-containing RhoGEFs play a function in regulation of Rho activation in Sema4D-plexin-B1 signaling? The cellular localization of RhoGEFs is seen to be important for their physiological activities. It has been reported that the proline-rich motif adjacent to DH/PH domain of KIAA0380 is required for plasma membrane localization and cortical actin reorganization [15]. In this study, we show that plexin-B1 recruited LARG, which does not contain such a proline-rich motif, from cytoplasm to plasma membrane. Consequently, the binding of LARG to plexin-B1 may be required to activate RhoA in plasma membrane. However, since KIAA0380 is localized in plasma membrane in plexin-B1-independent manner, the interaction of plexin-B1 with PDZ domain-containing RhoGEFs may have another meaning. It has been reported that clustering of CD2/plexin-B1 chimera protein by anti-CD2 antibodies cross-linking induced F-actin assembly and cell contraction [10]. This report suggests that plexin-B1 clustering is required for RhoA activation. Since PDZ proteins are essential for clustering of their binding protein as described above, it is likely that the clustering of plexin-B1 depends on PDZ domain-containing RhoGEF binding to plexin-B1, by a PDZ-mediated interaction. An alternative could be oligomerization of DH domain of PDZ domain-containing RhoGEFs. Recent report reveals that the oligomerization of dbl oncogene product (onco-Dbl), RhoGEF, through an intermolecular interaction between DH domain is essential for its efficient execution of RhoGEF function [31,32]. The oligomerization of onco-Dbl may allow the recruitment of multiple Rho substrates into one signaling complex. Since Sema4D may act as a homodimer [33], the binding of Sema4D to the plexin-B1 may trigger oligomerization of PDZ domain-containing RhoGEFs, leading to activation of them. Further analysis of interactions between plexin-B1 and PDZ domain-containing RhoGEFs is necessary for our understanding of the physiological function of Sema4D-plexin-B1 signaling in nervous system.

Acknowledgments

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