Plexin-B1 Utilizes RhoA and Rho Kinase to Promote the Integrin-dependent Activation of Akt and ERK and Endothelial Cell Motility*

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The semaphorins are a family of proteins originally identified as axon-guiding molecules in the developing nervous system that have been recently shown to regulate many cellular functions, including motility, in a variety of cell types. We have previously shown that in endothelial cells Semaphorin 4D acts through its receptor, Plexin-B1, to elicit a pro-angiogenic phenotype that involves the activation of the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway. Here we show through the use of a receptor chimeric approach, Plexin-B1 mutants, and dominant negative and pharmacological inhibitors that this response is dependent upon the activation of RhoA and its downstream target, Rho kinase (ROK). Indeed, we demonstrate that in endothelial cells, Semaphorin 4D promotes the formation of focal adhesion complexes, stress fibers, and the phosphorylation of myosin light chain, a response that was abolished by the use of ROK inhibitors and absent from cells expressing Plexin-B1 mutant constructs incapable of signaling to RhoA. Stress fiber polymerization and contraction are in turn necessary for RhoA-dependent pro-angiogenic signaling through Plexin-B1. Furthermore, we observed that in endothelial cells Plexin-B1 promotes the integrin-mediated activation of Pyk2, resulting in the stimulation of PI3K, Akt, and ERK. These findings provide evidence that Plexin-B1 promotes endothelial cell motility through RhoA and ROK by regulating the integrin-dependent signaling networks that result in the activation of PI3K and Akt.

The semaphorins are a family of transmembrane and secreted proteins originally identified as regulators of axonal growth and neurite extension in the developing nervous system (1, 2). Semaphorins are also expressed outside of the nervous system, where they play a role in regulating the morphology, migration, and proliferation in a variety of cell types. For example, semaphorins control the proliferation and activation of lymphocytes (3–7); development of the lungs (8), vasculature, and heart (9–12); and tumor growth and metastasis (13, 14). Currently, more than 20 semaphorins have been identified, which are grouped into eight classes; classes 1 and 2 are invertebrate semaphorins, classes 3–7 are found in vertebrates, and an eighth class, class V, has been identified in some non-neurotropic DNA viruses (1, 15).

Plexins, which are receptors for the semaphorins, share homology in their extracellular segment with the scatter factor receptors c-Met and RON but fail to exhibit significant homology with any other known receptor in their intracellular region (16). In humans, at least nine plexins have been identified and grouped into four families, A through D, each of which have been shown to mediate neuronal cell adhesion and contact, nerve fasciculation, and axon guidance (17). Secreted semaphorins, such as those in class 3, require co-receptors, the neuropilins, to bind and initiate intracellular signaling through the plexins (18, 19). In the case of the membrane-bound class IV semaphorin Semaphorin 4D (Sema4D),2 signaling is mediated directly through binding to its single-pass transmembrane receptor, Plexin-B1.

Ligation of plexins by semaphorins initiates a signaling cascade, which in many cases involves small GTPases of the Ras and Rho family and their signaling pathways. For example, Plexin-A1 and Plexin-B1 are known to act as R-Ras GAPs (GTPase-activating proteins) when bound by their respective semaphorins (20, 21). Plexin-B1 may also compete for Rac binding with p21-activated kinase; therefore, in addition to inhibiting R-Ras signaling, Plexin-B1 may sequester Rac and inhibit p21-activated kinase activation (22). Plexin-B1 can also inhibit RhoA signaling in certain cell culture conditions through the membrane trapping of p190 RhoGAP (23). In this regard, however, our group and others have found that the Rho-specific GAPs (guanine nucleotide exchange factors), leukemia-associated RasGAP (LARG), and PDZ-RhoGAP bind to the C terminus of Plexin-B1 and mediate activation of the small GTPase RhoA in response to Sema4D (24–29). In line with these findings, we have observed that the robust migratory and pro-angiogenic response elicited by Sema4D in endothelial cells is dependent upon the ability of Plexin-B1 to stimulate RhoA (25). We have also observed that Plexin-B1 stimulation in endothelial cells leads to the activation of an intracellular tyrosine kinase cascade resulting in the stimulation of phosphatidylinositol 3-kinase and Akt (30). Furthermore, a recent study

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2 The abbreviations used are: Sema4D, Semaphorin 4D; GAP, GTPase-activating protein; GEF, guanine nucleotide-exchange factor; ROK, Rho kinase; MLCK, myosin light chain; GFP, green fluorescent protein; EGFP, enhanced GFP; BES, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; PBS, phosphate-buffered saline; WT, wild type; mut, mutant; ERK, extracellular signal-regulated kinase; NGF, nerve growth factor.
suggests that Plexin-B1 can utilize RhoA to stimulate ERK (31). How Plexin-B1 activates ERK through RhoA and which of the signaling pathways controlled by Plexin-B1 result in phosphatidylinositol 3-kinase and Akt activation are at the present unknown.

In the present study, we used a chimeric receptor approach, Plexin-B1 mutants, and dominant negative and pharmacological inhibitors of RhoA signaling to show that in endothelial cells the Plexin-B1-mediated phosphorylation and activation of Akt, ERK, and Pyk2 are dependent upon its ability to activate RhoA and not on Plexin-B1 residues involved in its RasGAP activity. We show that in those cells capable of signaling to RhoA, Sema4D treatment results in phosphorylation of myosin light chain (MLC), as a consequence of ROK activation, and the downstream polymerization and contraction of actin/myosin stress fibers. We also provide evidence that, in response to Sema4D activation of Plexin-B1, integrin-mediated attachment to the extracellular matrix is required for pro-angiogenic signaling to phosphatidylinositol 3-kinase, Akt, and ERK in endothelial cells downstream from RhoA.

EXPERIMENTAL PROCEDURES

Cell Culture—Human umbilical vein endothelial cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin/amphotericin B (Sigma). Cells stably expressing Trk-A/Plexin-B1 fusion proteins (see below) were selected in 1 mg/ml G418 (Calbiochem, San Diego, CA).

Trk/Plexin Fusion Proteins—Trk-A/Plexin-B1 fusion proteins were made as previously described (28). Briefly, the intracellular portion of Plexin-B1 with and without the PDZ binding motif was cut out of the plasmid pCEFL EGFP Plexin-B1 with Nhel/NotI and cloned in-frame with the extracellular and transmembrane portion of the nerve growth factor (NGF) receptor Trk-A in the vector pCEFL-HA. The constructs were then transfected into endothelial cells using Superfect (Qiagen, Valencia, CA) and stable cells selected in G418. A Trk-A/Plexin-B1 mutant lacking key residues involved in RasGAP activity was generated as previously described (30) using the QuikChange II XL site-directed mutagenesis kit according to the manufacturer’s instructions (Stratagene, La Jolla, CA). Mutations were confirmed by sequencing.

Production of Soluble Semaphorin 4D—Semaphorin 4D was produced and purified as described previously (25). Briefly, the extracellular portion of mouse Semaphorin 4D was subjected to PCR and the resulting product cloned into the plasmid pSecTag2B (Invitrogen). This construct was transfected into 293T cells growing in serum-free medium using the calcium chloride (Fluka Chemika, Sigma Aldrich) BES (Fluka Chemika) method (32). Medium containing soluble Semaphorin 4D was collected 65 h post-transfection and purified with TALON metal affinity resin (Clontech Laboratories, Palo Alto, CA) according to the manufacturer’s instructions. Concentration and purity of the TALON eluates were determined by SDS-PAGE analysis with silver stain (Amersham Biosciences) and the Bio-Rad assay (Hercules, CA). In all cases, medium collected from parallel transfectants using the empty pSecTag2B vector was used as control.

Measurement of R-Ras Activity—Cells were maintained on plates coated with collagen (10 μg/ml; Roche Applied Science) or poly-L-lysine (1 μg/ml; Sigma) and treated with 100 ng/ml NGF (Upstate, Lake Placid, NY) for 10 min. The cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1% Triton X-100, 20 mM MgCl₂, and 40 mM β-glycerophosphate) supplemented with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 μl/ml aprotinin and leupeptin; Sigma) and phosphatase inhibitors (2 mM NaF and 0.5 mM sodium orthovanadate; Sigma). Aliquots were reserved for an immunoblot, while the rest was incubated with glutathione-Sepharose 4B beads (Amersham Biosciences) previously bound to glutathione S-transferase fused to the Ras binding domain of c-Raf-1 for 30 min at 4 °C to affinity precipitate GTP-bound R-Ras. The beads were washed in ice-cold lysis buffer and subjected to SDS-polyacrylamide gel electrophoresis along with aliquots of total protein and transferred onto a polyvinylidene difluoride membrane (Immobilon P; Millipore Corp., Billerica, MA). The membranes were then incubated with anti-R-Ras antibody (Santa Cruz Biotechnology). Proteins were detected using the ECL chemiluminescence system (Pierce).

Immunoblot Analysis—Cells were treated with soluble 400 ng/ml Sema4D (see above) or 100 ng/ml NGF (Upstate) and infected with lentivirus encoding the RGS or PDZ domains of PDZ-RhoGEF (see below) or co-treated with the following inhibitors, where indicated: 25 μM HA1077 (fasudil; Calbiochem), 100 μM blebbistatin (Calbiochem), 50 μM RGDS peptide (Sigma), 67 units of tenatolysin and C3 toxin (List Biological Laboratories, Campbell, CA) to inhibit ROK, stress fiber contraction, integrin-mediated adhesion to fibronectin, and RhoA, respectively. Cells were then lysed in buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40) supplemented with protease inhibitors and phosphatase inhibitors (see above) for 15 min at 4 °C. After centrifugation, protein concentrations were measured using the Bio-Rad assay. 100 μg of protein from each sample was subjected to SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Immobilon P; Millipore Corp.). The membranes were incubated with the appropriate antibodies. The antibodies used were as follows: Sema4D (BD Transduction Laboratories, BD Biosciences), phospho-Akt (Ser-473; Cell Signaling Technology, Beverly, MA), total Akt (Cell Signaling Technology), phospho-ERK (Cell Signaling Technology), total ERK (Cell Signaling Technology), phospho-Pyk2 (BIOSOURCE, Camarillo, CA), total Pyk2 (BD Biosciences), Myc (Santa Cruz C-33), GFP (Covance, Princeton, NJ), phospho-MLC (Cell Signaling Technology), paxillin (BD Transduction Laboratories), and tubulin (TU-02; Santa Cruz). Proteins were detected using the ECL chemiluminescence system (Pierce) and quantitation performed with NIH image software.

Immunoprecipitation—Cells were lysed in buffer (see above), and proteins were immunoprecipitated from the cleared lysates by incubation for 2 h at 4 °C with antibody against Pyk2 (BD Biosciences). Immunocomplexes were recovered with γ-bind-Sepharose beads (GE Healthcare). Lysates and anti-Pyk2 immunoprecipitates were analyzed by Western blotting after SDS-polyacrylamide gel electrophoresis (see above) and immunoblotted with anti-Pyk2, anti-phospho Pyk2, and anti-paxillin
**Results**

**Plexin-B1 Signaling Is Dependent upon Its Ability to Activate RhoA, but Not on Its Ras GAP Activity**—We have previously demonstrated that Sema4D induces polymerization of actin stress fibers and chemotaxis in endothelial cells that is dependent upon the ability of Plexin-B1 to activate RhoA (25, 30). Our group and others also have observed phosphorylation of Akt, Pyk2, (30), and ERK (31) in response to treatment with Sema4D, although the question remains whether or not this response is specifically dependent upon Rho. To address this question and to examine whether the ability of Plexin-B1 to act as a RasGAP is important in these responses, we generated endothelial cells stably expressing chimeric receptor constructs containing the extracellular portion of the NGF receptor, Trk-A, fused to the full-length intracellular portion of Plexin-B1, the intracellular portion of Plexin-B1 mutated at the arginine residues required for RasGAP activity (20, 34, 35), and a chimeric receptor where the C-terminal PDZ binding motif of Plexin-B1 necessary for recruiting PDZ-RhoGEF and leukemia-associated RhoGEF and activating RhoA was deleted (28). First, we confirmed the localization of Trk-A/Plexin-B1 wild-type control (WT), Ras GAP mutant (mut), and the C-terminal PDZ binding motif deletion chimera (ΔPBM) in 293T. As shown by confocal analysis after surface staining of living cells, all three chimeric molecules accumulated at the plasma membrane (Fig. 1A). We then examined whether the Trk-A/Plexin-B1 chimera functions as a ligand-dependent R-RasGAP protein, as shown for the endogenous Plexin-B1 in response to Sema4D (34). We performed a pull-down assay in NGF-treated 293T cells expressing the Trk-A/Plexin-B1 wild-type receptor (WT) grown on poly-l-lysine or on collagen, which increases endogenous R-Ras-GTP levels (Fig. 1B), as previously reported (34). Cells transfected with an active mutant of R-Ras were used as an internal control. The level of R-Ras-GTP was clearly diminished in response to NGF in Trk-A/Plexin-B1-expressing cells, consistent with the ligand-activated R-Ras-GAP activity of Plexin-B1 (Fig. 1B) (34). However, the Trk-A/Plexin-B1 R-RasGAP mutant failed to block R-Ras activation, whereas Trk-A/Plexin-B1 lacking its C-terminal PDZ binding motif (ΔPBM) was still able to function as a ligand-dependent R-RasGAP protein (Fig. 1C). We then began investigating how Plexin-B1 signals in endothelial cells, by expressing Trk-A/Plexin-B1 wild-type control (WT) and the R-Ras GAP mutant (mut) and the C-terminal PDZ-binding motif deletion (ΔPBM) chimeras (Fig. 1D). Endothelial cells were cultured on fibronectin-coated glass coverslips, treated with NGF to elicit Plexin-B1 signaling, and analyzed under immunofluorescence for development of focal adhesion complexes and polymerization of actin/myosin stress fibers. Wild-type and R-RasGAP mutant chimeras demonstrated robust stress fiber polymerization (Fig. 1, E and F, WT and R-RasGAPmut, respectively), indicating that the ability of Plexin-B1 to act as a R-RasGAP is not necessary for stress fiber polymerization, whereas cells expressing the Trk-A/Plexin-B1 ΔPBM mutant construct failed to do so (Fig. 1, E and F, ΔPBM). We also observed phosphorylation and activation of Akt and ERK1/2 in...
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FIGURE 1. Plexin-B1 signaling to Akt and ERK is dependent upon its ability to activate RhoA, and not on its R-Ras GAP function. A, 293T cells were grown on poly-L-lysine and transfected for the Myc-tagged chimeric receptors Trk-A/Plexin-B1 (Trk-A/PB1) wild-type (WT), Trk-A/Plexin-B1 R-RasGAP mutant (R-RasGAP mut), and Trk-A/Plexin-B1 ΔPBM (ΔPBM). Anti-Myc antibodies were incubated at 4°C for 1 h in living cells to allow the binding to plasma membrane-exposed chimeras. Cells were then rinsed and processed for immunostaining and confocal analysis. Scale bar, 10 μm. B, 293T were grown on collagen (coll) (10 μg/ml) or poly-L-lysine (PLL) (1 μg/ml) and transfected with Trk-A/Plexin-B1 chimera or R-RasQL constitutively activated mutant. 24 h later, cells were starved in serum-free medium for 2 h (−), stimulated by NGF 100 ng/ml for 10 min (+), and lysed. R-Ras-GTP was detected upon affinity precipitation using glutathione S-transferase-c-Raf1 binding domain and total R-Ras in the total cell lysates. The chimera expression was detected by anti-Myc immunoblot in the cell extracts. C, similarly, 293T were transfected by the chimeric receptor Trk-A/Plexin-B1 WT and its R-RasGAP mutant (R-RasGAP mut) and ΔPBM and analyzed by Western blots for R-RasGTP content. D, immunoblot analysis for Myc shows a band present in cells transfected and selected to express the Myc-tagged chimeric receptors Trk-A/Plexin-B1 wild-type (WT), Trk-A/Plexin-B1 R-RasGAP mutant (R-RasGAP mut), and Trk-A/Plexin-B1 ΔPBM (ΔPBM). Empty vector-transfected and selected cells fail to exhibit a band (C). Tubulin was used as a loading control. E, endothelial cells stably expressing the chimeric receptor Trk-A/Plexin-B1 wild-type (WT) and its ΔPBM and R-RasGAP mutant (R-RasGAP mut) were treated for 3 min with 100 ng/ml NGF and analyzed for stress fiber formation. F, the number of endothelial cells exhibiting stress fiber polymerization was counted and the results expressed as a percentage of the total number of endothelial cells counted. G, cells stably expressing the wild-type (WT) and the R-RasGAP mutant (mut) chimeras, but not cells expressing the Trk-A/both wild-type (WT) and R-RasGAP mutant (mut)-expressing cells in response to NGF (Fig. 1G, left two columns), but not in cells stably expressing the ΔPBM mutants incapable of signaling RhoA (Fig. 1G, right two columns).

Activation of RhoA Is Necessary for Plexin-B1 Signaling in Endothelial Cells—The PDZ domain of PDZ-RhoGEF has been shown to disrupt RhoA signaling in response to Plexin-B1 activation in a dominant negative manner by competing for endogenous PDZ binding domains of the Rho GEFs (28). This signaling pathway does not involve the RGS domain of the Rho GEFs, which links Gα12- and Gα13-coupled receptors to RhoA activation (36, 37). We thus decided to test the effect of PDZ and RGS overexpression on Plexin-B1 signaling. First, confocal analysis showed the correct membrane localization of the Trk-A/Plexin-B1 chimera in 293T cells overexpressing GFP and the PDZ or RGS domains fused to GFP (Fig. 2A). We also confirmed that the overexpression of these domains did not affect the ligand-dependent R-RasGAP activity of the Plexin-B1 intracellular domain (Fig. 2B). Vectors expressing these EGFP-tagged RGS and PDZ domains of PDZ-RhoGEF were then engineered and packaged into lentiviruses, which were used to infect endothelial cells. The expression of both EGFP-PDZ and EGFP-RGS constructs in infected cells was confirmed by Western blot for EGFP (Fig. 2C) and visualization of EGFP expression under the fluorescent microscope (Fig. 2D). Endothelial cells treated with Sema4D revealed phosphorylation of Akt, ERK, and Pyk2 when infected by viruses encoding EGFP-RGS, but not with EGFP-PDZ-expressing viruses (Fig. 2E, left two columns). Phosphorylation of these proteins in control-treated cells, but not in cells treated with the RhoA-specific inhibitor C3 toxin, provided further evidence for the importance of RhoA in Plexin-B1-mediated activation of Akt, ERK, and Pyk2 (Fig. 2E, right two columns).

The phosphorylation of MLC by ROK contributes to actin polymerization and increased myosin light chain contractility downstream from RhoA activation. Thus, the status of phosphorylation of MLC serve as a control for monitoring RhoA function (38). As expected, we observed loss of MLC phosphorylation, and therefore actin/myosin stress fiber contraction, in cells infected with the EGFP-PDZ virus but not in EGFP-RGS-infected populations (Fig. 2E, left two columns, P-MLC) and in cells treated with the C3 toxin, but not in the vehicle-treated controls (Fig. 2E, right two columns). Taken together, these results suggested that the phosphorylation and activation of Pyk2, Akt, and ERK1/2, as well as phosphorylation of MLC and hence contraction of the stress fibers that form in Sema4D-treated endothelial cells, are dependent upon Plexin-B1-mediated activation of Akt, ERK, and Pyk2. 

ROK Activity and Actin Tension Are Necessary for Plexin-B1-mediated Signaling—To determine the significance of ROK activation in Plexin-B1-mediated phosphorylation of Akt, ERK1/2, and Pyk2, we treated endothelial cells with Sema4D and either vehicle control or the ROK inhibitor fasudil and then analyzed cell lysates for the phosphorylated forms of these pro-
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FIGURE 2. Recruitment of GEFs and activation of RhoA are necessary for Plexin-B1 signaling in endothelial cells. A, 293T cells were grown on poly-L-lysine and transfected for the Myc-tagged chimeric receptors TrkA/Plexin-B1 wild-type (WT), together with EGFP, RGS-EGFP, or PDZ-EGFP fusion proteins. The anti-Myc staining was performed as described in Fig. 1A. B, 293T cells were grown on collagen (10 μg/ml) and transfected for TrkA/PB1 chimeras with GFP, RGS-EGFP, or PDZ-EGFP fusion proteins. 24 h later, cells were starved in serum-free medium for 2 h (−), stimulated by NGF 100 ng/ml for 10 min (+), and lysed. R-Ras-GTP was detected in the glutathione S-transferase-c-Raf1 binding domain and total R-Ras and GFP in the total cell lysates. C, immunoblot confirming the expression of both EGFP-PDZ and EGFP-RGS constructs in lentivirus-infected cells. D, EGFP expression in infected cells. E, endothelial cells infected with lentivirus coding for EGFP-RGS (RGS) exhibit phosphorylation of Akt, ERK, Pyk2, and MLC in response to treatment with 400 ng/ml Sema4D, whereas cells infected with lentivirus coding for EGFP-PDZ (PDZ) fail to do so (left panels). Total levels of these proteins and tubulin are used as a loading control. Vehicle control endothelial cells (V) exhibit phosphorylation of Akt, ERK, Pyk2, and MLC in response to treatment with 400 ng/ml Sema4D, whereas cells incubated with the ROK inhibitor fasudil fail to do so. Total levels of these proteins and tubulin are used as a loading control.

FIGURE 3. ROK activity and stress fiber polymerization and contraction are necessary for Plexin-B1-mediated signaling. A, vehicle control endothelial cells (V) exhibit phosphorylation of Akt, ERK, Pyk2, and MLC in response to treatment with 400 ng/ml Sema4D, whereas cells incubated with the ROK inhibitor fasudil fail to do so. Total levels of these proteins and tubulin are used as a loading control. B, vehicle control endothelial cells (V) exhibit phosphorylation of Akt, ERK, Pyk2, and MLC in response to treatment with 400 ng/ml Sema4D, whereas cells incubated with blebbistatin (blebb), an inhibitor of actin/myosin stress fiber contraction, fail to do so. Total levels of these proteins and tubulin are used as a loading control.

Binding of Integrins to the Extracellular Matrix at Focal Adhesions Is Necessary for Sema4D-induced Cell Responses—

The internal forces generated by stress fiber formation and contraction produce isometric tension in cells if they are adherent to the substrate. This tension can direct the assembly and composition of focal adhesion complexes and trigger adhesion-dependent signaling (40–44). Thus, based on our results we next explored whether adhesion-dependent signaling contributes to the propagation of the RhoA-dependent, Plexin-B1-mediated signal. For these experiments, endothelial cells were first cultured with and without the RGDS peptide as a competitor for integrin-extracellular matrix interaction. Cells were treated with Sema4D and analyzed under immunofluorescence for development of mature focal adhesion complexes and polymerization of actin filaments. Cells treated with Sema4D exhibited polymerization of actin/myosin stress fibers (Fig. 4A, upper right panel, red, and see below, higher magnification, Fig. 4C, upper right panel, in purple) and localization of paxillin into maturing focal adhesion complexes (Fig. 4A, in yellow, and Fig. 4C, in white), a phenotype lost in Sema4D-treated cells grown in RGDS peptide (Fig. 4A, middle right panel, and see below, Fig. 4C, lower right panel). Sema4D-treated cells incubated with fasudil also failed to exhibit significant stress fiber/focal adhesion formation (Fig. 4A, fasudil). The quantification of cells exhibiting focal adhesions clearly showed that both fasudil and RGDS blocked the actin polymerization-induced focal adhesions caused by Sema4D (Fig. 4B). These results demonstrated the importance of integrin-mediated adhesion in the RhoA-ROK-dependent assembly of focal adhesion complexes in endothelial cells upon Sema4D treatment.
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The cytoplasmic tyrosine kinase Pyk2 is known to function at a point of convergence of integrins and G-protein-coupled receptor signaling cascades (45). As we have previously observed that Pyk2 is necessary to propagate the signal from Plexin-B1, which is devoid of intrinsic tyrosine kinase activity (30), we therefore examined whether formation of focal adhesion complexes, and hence cell adhesion, is necessary for Pyk2 activation by Sema4D in endothelial cells. As described above, the addition of the RGDS peptide inhibited the formation of stress fibers and paxillin recruitment to focal adhesions upon Sema4D treatment of endothelial cells (Fig. 4C). We performed an immunoprecipitation for Pyk2 in endothelial cells growing on fibronectin, with and without the RGDS peptide. We observed association of phosphorylated Pyk2 with the focal adhesion protein paxillin in response to Sema4D in vehicle-treated controls but not in cells grown with RGDS (Fig. 4D, V and RGDS, respectively). We next examined lysates from endothelial cells treated with Sema4D growing on fibronectin-coated plates in the presence and absence of the RGDS peptide for phosphorylated forms of Akt, ERK1/2, and Pyk2. Not only is Pyk2 activation lost in cells incapable of forming secure attachment to the substrate due to the RGDS peptide but these cells also lose evidence of phosphorylation of paxillin, Akt, and ERK1/2 (Fig. 4E).

To determine the biological significance of the loss of substrate adhesion in Sema4D-mediated endothelial cell migration, chemotaxis toward wells containing Sema4D was measured in cells growing in control medium or medium containing RGDS peptide. Endothelial cells, which normally exhibit robust migration toward Sema4D (30), failed to migrate when integrin-adhesive function was prevented by the RGDS peptide (Fig. 4F). Taken together, these results demonstrate that in response to Sema4D, endothelial cells need to regulate the actomyosin cytoskeleton through RhoA and ROK to promote the integrin-dependent assembly of focal adhesions. In turn, focal adhesion complexes recruit and activate signaling molecules mediating the pro-migratory effects in response to Sema4D treatment.
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In the present study, we provide evidence that Plexin-B1 causes the RhoA-ROK-dependent activation of Pyk2, ERK1/2, and Akt and that this process requires integrin function. Indeed, both Plexin-B1 and integrins may act together to mediate endothelial cell migration in response to Sema4D.

Initially, through the use of Plexin-B1 mutants and dominant negative and pharmacological inhibitors of RhoA, we confirmed and extended earlier findings indicating that the pro-angiogenic phenotype observed in Sema4D-treated endothelial cells is dependent upon RhoA activation. We also observed that phosphorylation and activation of Akt and Pyk2, which are necessary for endothelial cell migration (30), as well as phosphorylation of ERK (31), were blocked by RhoA inhibition. Phosphorylation of MLC, a downstream target of the RhoA effector ROK involved in actin polymerization and stress fiber contraction (38), was also lost upon RhoA inhibition. As might be expected, the ROK inhibitor fasudil prevented phosphorylation of MLC by Sema4D, but it also inhibited the phosphorylation and activation of Akt, ERK, and Pyk2. This strongly supported the fact that Plexin-B1 can activate these downstream cascades through a RhoA-ROK-dependent signaling pathway. How in turn does ROK regulate Akt, ERK1/2, and Pyk2? Surprisingly, we observed that blebbistatin, an inhibitor of actin/myosin cross-linking and contraction (39), abolished the Sema4D-induced phosphorylation of Akt, ERK, and Pyk2, indicating that stress fiber contraction is necessary for the phosphorylation and activation of these signaling molecules downstream from RhoA and ROK.

There is a large body of evidence to suggest that integrins can regulate Rho GTPases (46–48). Reciprocally, RhoA-mediated contractility of stress fibers leads to the bundling of actin filaments, aggregation of integrins, and assembly of focal adhesion complexes (43, 49). These focal adhesion complexes can include the scaffold proteins paxillin and vinculin and cytoskeletal proteins such as talin, tensin, α-actinin, and F-actin and induce the recruitment and activation of focal adhesion kinase and Src family kinases (43, 50–52). When endothelial cells were pretreated with the peptide RGDS, which blocks the ability of integrins to bind to fibronectin (53, 54), activation of Plexin-B1 could no longer elicit the assembly of focal adhesions, phosphorylation and activation of Akt, ERK, and Pyk2, and endothelial cell chemotaxis toward Sema4D. Given the broad effects of the RGDS peptide in inhibiting the basal integrin-mediated adhesion, these observations imply that Plexin-B1 signal can only be transmitted and amplified when contracting stress fibers are capable of generating tension within the cell by anchoring to integrins bound to the extracellular matrix. This conclusion is also supported by the loss of Sema4D signaling in blebbistatin-treated cells, where stress fibers fail to reorganize. Overall, we conclude that Plexin-B1 requires the engagement of integrins through RhoA and ROK and that, in turn, integrins regulate the activation of Pyk2, Akt, and ERK1/2, similarly to the obligatory signaling cross-talk between tyrosine kinase receptors and integrins (50). Thus, we propose that Plexin-B1 can co-opt other cell surface-adhesive receptors, such as integrins, to propagate signal transmission.

A proposed model for Sema4D/Plexin-B1-mediated signaling is shown in Fig. 5, depicting how the sequential engagement of Plexin-B1 and integrins might be necessary for the downstream effects of activated Plexin-B1 in response to Sema4D. In this model, adhesive contacts in the form of clustered integrins are organized and stabilized with the actin cytoskeleton at focal adhesion complexes upon ligation of Plexin-B1 with Sema4D. As stress fibers contract, they generate tension at the focal adhesions, which is converted from a mechanical signal into a biochemical signal, resulting in the tyrosine phosphorylation of Pyk2, the
most apical tyrosine kinase activated upon Plexin-B1 stimulation in endothelial cells (30). Pyk2 then activates a tyrosine kinase-dependent signaling network resulting in the downstream stimulation of phosphatidylinositol 3-kinase, Akt, and ERK1/2, thereby controlling endothelial cell migration.

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