

Semaphorin 4D Signaling Requires the Recruitment of Phospholipase C γ into the Plexin-B1 Receptor Complex[∇]

Jakub M. Swiercz,^{1,2*} Thomas Worzfeld,^{1,2} and Stefan Offermanns^{1,2}

Institute of Pharmacology, University of Heidelberg, Im Neuenheimer Feld 366, D-69120 Heidelberg, Germany,¹ and Department of Pharmacology, Max Planck Institute for Heart and Lung Research, Parkstr. 1, D-61231 Bad Nauheim, Germany²

Received 22 January 2009/Returned for modification 3 April 2009/Accepted 18 September 2009

The semaphorin 4D (Sema4D) receptor plexin-B1 constitutively interacts with particular Rho guanine nucleotide exchange factors (RhoGEFs) and thereby mediates Sema4D-induced RhoA activation, a process which involves the tyrosine phosphorylation of plexin-B1 by ErbB-2. It is, however, unknown how plexin-B1 phosphorylation regulates RhoGEF activity. We show here that activation of plexin-B1 by Sema4D and its subsequent tyrosine phosphorylation creates docking sites for the SH2 domains of phospholipase C γ (PLC γ). PLC γ is thereby recruited into the plexin-B1 receptor complex and via its SH3 domain activates the Rho guanine nucleotide exchange factor PDZ-RhoGEF. PLC γ -dependent RhoGEF activation is independent of its lipase activity. The recruitment of PLC γ has no effect on the R-Ras GTPase-activating protein activity of plexin-B1 but is required for Sema4D-induced axonal growth cone collapse as well as for the promigratory effects of Sema4D on cancer cells. These data demonstrate a novel nonenzymatic function of PLC γ as an important mechanism of plexin-mediated signaling which links tyrosine phosphorylation of plexin-B1 to the regulation of a RhoGEF protein and downstream cellular processes.

Mammalian semaphorins were originally identified as axon guidance factors but are now recognized also as important regulators of morphogenesis and homeostasis in various organ systems, including the immune, cardiovascular, and renal systems (3–5, 7, 19, 23, 30, 35, 40, 56, 64, 76). Most effects of semaphorins are mediated by a group of large transmembrane proteins called plexins, of which four families exist in the mammalian system: plexin-A1 to -4, plexin-B1 to -3, plexin-C1, and plexin-D1 (60, 61). The four members of the plexin-A family in most cases require neuropilins as ligand binding partners to respond to semaphorins, whereas the three members of the plexin-B family are directly activated by semaphorins. While plexin-B1 binds Sema4D, plexin-B2 can be activated by Sema4C and Sema4D, and plexin-B3 has been shown to respond to Sema5A (31, 35).

The activation of plexins by semaphorins initiates a variety of signaling processes, which involve several small GTPases of the Ras and Rho families (31, 34, 43). All plexin family members possess an R-Ras GTPase-activating protein (GAP) domain (36). Activated plexin-B1 and -A1 have been shown to also interact with other small GTPases, including GTP-bound Rac1 and RhoD as well as Rnd1, Rnd2, and Rnd3 (14, 37, 48, 63, 67, 68, 74). Different from other plexin families, the C terminus of B-family plexins contains a PDZ domain-binding motif which mediates a stable interaction with the guanine nucleotide exchange factors PDZ-RhoGEF and LARG (1, 15, 26, 39, 57). Activation of the plexin-B1/PDZ-RhoGEF complex by semaphorin 4D (Sema4D) results in RhoA activation downstream of plexin-B1 (15, 39, 57). Members of the plexin-B

family also interact with and are phosphorylated by the receptor tyrosine kinases ErbB-2 and c-Met (12, 22, 58). ErbB-2-mediated phosphorylation of plexin-B1 is required for plexin-mediated RhoA activation and downstream cellular effects, including the promigratory effects of Sema4D on cancer cells and the induction of axonal growth cone collapse by Sema4D (58, 59). However, the molecular mechanisms linking ErbB-2-mediated phosphorylation of plexin-B1 to the regulation of RhoA activity and subsequent cellular effects are unknown.

Here we report that upon activation by Sema4D, plexin-B1 becomes phosphorylated by ErbB-2 at particular tyrosine residues on its intracellular portion. These phosphorylated tyrosine residues serve as docking sites for the SH2 domains of PLC γ . PLC γ is thereby recruited into the plexin-B1 receptor complex and through its SH3 domain mediates RhoA activation and downstream cellular effects.

MATERIALS AND METHODS

Antibodies and reagents. The following antibodies were used: mouse monoclonal anti-Myc (9E10), rabbit polyclonal anti-R-Ras, rabbit polyclonal anti-PLC γ 2, and goat polyclonal anti-plexin-B2 (Santa Cruz Biotechnology); mouse monoclonal anti-G protein of vesicular stomatitis virus (VSV), mouse monoclonal anti- α -tubulin, mouse monoclonal and rabbit polyclonal anti-FLAG, and rabbit polyclonal anti-glutathione *S*-transferase (anti-GST; all from Sigma-Aldrich); and rabbit polyclonal anti-ErbB-2[pY1248], anti-PLC γ 1, anti-Grb2, and anti-RhoA (Cell Signaling Technology); mouse monoclonal antiphosphotyrosine (4G10; Upstate Biotechnology); mouse monoclonal anti-Rac (23A8) and anti-ErbB-2 (Biosource Invitrogen); goat polyclonal anti-plexin-B1 (R&D Systems); and rabbit polyclonal anti-PDZ-RhoGEF (Imgenex). Human recombinant Sema3A was purchased from R&D Systems.

Plasmids. Eukaryotic expression plasmids carrying the cDNAs of PDZ-RhoGEF, Sema4D, Myc-RhoA, GST-RhoA, c-Met, and Rnd1, were described previously (29, 57, 58). VSV-plexin-B1 was kindly provided by L. Tamagnone (University of Torino, Turin, Italy). R-Ras was provided by Monique Dail (The Burnham Institute, La Jolla, CA). GST-RacL61 was provided by Dominique T. Brandt. Myc-PLC γ 1 was a kind gift from Peter Gierschik (Institute of Pharmacology and Toxicology, University of Ulm, Ulm, Germany). Specific mutations introduced in VSV-plexin-B1, Myc-PLC γ 1, and FLAG-PDZ-RhoGEF were carried out using the QuikChange site-directed mutagenesis procedure (Stratagene,

* Corresponding author. Mailing address: Department of Pharmacology, Max Planck Institute for Heart and Lung Research, Parkstr. 1, D-61231 Bad Nauheim, Germany. Phone: 49 (0) 6032 54-705-402. Fax: 49 (0) 6032 54-705-419. E-mail: jakub.swiercz@mpi-bn.mpg.de.

[∇] Published ahead of print on 5 October 2009.

La Jolla, CA) employing *Pfu Turbo* as a proofreading DNA polymerase. Correct mutagenesis was confirmed by DNA sequencing. Myc-PLC γ 1 lacking amino acids 1 to 142 (Δ PH1), 152 to 187 (Δ EF), 320 to 464 (Δ Xbox), 550 to 657 (Δ SH2.N), 668 to 756 (Δ SH2.C), 550 to 756 (Δ SH2), 791 to 851 (Δ SH3), 489 to 523 and 895 to 931 (Δ PH2), 953 to 1070 (Δ Ybox), or 1075 to 1177 (Δ C2) was generated using standard molecular biology methods. Bacterial expression constructs containing FLAG- and hemagglutinin-tagged C-terminal portions of PDZ-RhoGEF (amino acids 1080 to 1522), FLAG-tagged C-terminal portion of LARG (amino acids 1132 to 1544), and the SH3 domains of PLC γ 1 and Grb2 (amino acids 788 to 854 and 153 to 217, respectively) were generated using standard methods.

Cell culture, immunoprecipitation studies, and transfection. HEK 293, MCF-7, and MDA-MB-468 cells were cultured, and immunoprecipitations were performed as described previously (59). Primary hippocampal neurons were prepared and cultured as described previously (57). HEK 293 cells were transfected using the calcium phosphate method, primary cells were transfected using FuGENE HD (Roche), and cells were transfected with small interfering RNAs (siRNAs) using HiPerFect reagent according to the manufacturer's instructions (Qiagen). Cell migration was assayed using polystyrene Transwell inserts (Greiner) with pore sizes of 0.8 μ m (59).

Determination of activated RhoA and R-Ras. The amounts of activated cellular Rho and R-Ras were determined by precipitation with a fusion protein consisting of GST and the Rho-binding domain of Rhotekin (GST-RBD) or the Ras-binding domain of Raf1 (GST-Raf1) as described previously (45, 65). In vitro determination of RhoA activation was performed as described before (29).

siRNAs. The target sequences for siRNAs specific to ErbB-2 and c-Met used in this study were published before (16, 33). The sequence of the siRNA used to suppress plexin-B1 expression was GAAUCCGGGUGACCGUGG, and siRNAs for PLC γ 1 (AAGAAGUCGACGACCGAG), PLC γ 2 (CTCCGTGTTTCATCTAGGAAA), and Grb2 (TACAAATGTGTACACAT AAA) were purchased from Qiagen. A control siRNA, CGCGUAGAAGAUGAAGUUG, was purchased from IBA.

Production and purification of recombinant Sema4D. Generation of Lec3.2.8.1 CHO cells expressing the extracellular part of human Sema4D (residues 1 to 657 followed by a lysine residue and a carboxy-terminal histidine tag for purification) was described previously (32, 59).

Retroviral infections. In order to obtain siRNA-insensitive plexin-B1, silent mutations were introduced at positions 3855 (C \rightarrow T) and 3858 (G \rightarrow A) of the coding region of the cDNAs encoding wild-type and mutated (Y1708F/Y1732F) plexin-B1. In order to obtain siRNA-insensitive PLC γ 1, silent mutations were introduced at positions 123 (G \rightarrow T) and 126 (G \rightarrow T) of wild-type PLC γ 1 and its Δ Xbox and Δ SH3 mutants. The resulting sequences were subcloned into the retroviral vector pLNCX2 (Clontech). Selection and retroviral transfection were carried out as described before (59).

Peptide synthesis and isolation of binding partners. Peptides C-DSVGEPLYMLFRGIK and C-SVTGKAKYTLNDNRL corresponding to residues 1701 to 1715 and 1725 to 1739 of plexin-B1, respectively, were synthesized in the Analytical Laboratory of the University Hospital in Düsseldorf (Germany). For phosphorylated peptides, a phosphotyrosine was inserted in positions corresponding to amino acid residues 1708 and 1732 of plexin-B1. Peptides were coupled to the SulfoLink coupling resin (Thermo Scientific), nonbound peptide was removed by washing with radioimmunoprecipitation assay buffer, and the resin was incubated with 50 mM cysteine to reduce unspecific binding. HEK 293 cells were lysed in radioimmunoprecipitation assay buffer as described above, and cleared lysates were incubated with the peptide-coupled SulfoLink for 2 h at 4°C. The resin was then extensively washed and boiled in Laemmli buffer, and proteins bound to peptides were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were visualized using Coomassie blue staining.

Mass spectrometry. Mass spectrometry analysis was performed in the Core Facility for Mass Spectrometry and Proteomics, Centrum of Molecular Biology, University of Heidelberg, Heidelberg, Germany. Briefly, individual spots were excised from gels and digested with trypsin. Matrix-assisted laser desorption ionization of quadruple time-of-flight mass spectrometry was performed using an Ultraflex time-of-flight apparatus (Bruker Daltonik). For protein identification, the peptide mass fingerprint was run against the NCBI-nr database using Mascot (Matrix Science).

Reporter gene assays. For the SRE reporter gene assays, cells were grown in six-well plates and transfected with 100 ng 3D.ALuc, a reporter plasmid expressing firefly luciferase under the control of the mutant SRE (SRE.L) lacking a ternary complex factor-binding site (24), and with 100 ng of pRLTK (plasmid expressing *Renilla* luciferase under the control of the thymidine kinase promoter) together with the experiment-specific plasmids (as described in the figure leg-

ends). At 36 h after transfection, cells were starved for 12 h, and the activities of firefly and *Renilla* luciferases were measured using the Stop and Glow kit (Promega) according to the manufacturer's instructions. Relative luciferase activity was determined by normalizing firefly luciferase activity against *Renilla* luciferase activity.

Protein purification. Proteins were produced and purified in *Escherichia coli* strain DE3 or in HEK cells [(FLAG)-PDZ-RhoGEF]. GST fusion proteins were eluted from glutathione disulfide beads (Amersham), and FLAG-tagged proteins were purified using anti-FLAG antibody-coated Sepharose and eluted with the FLAG peptide (Sigma), followed by gel filtration chromatography, and were then concentrated by using Amicon Ultra centrifugal filter devices (Millipore).

Statistical analysis. Statistical significance was evaluated by Student's *t* test. Levels of significance were 0.05, 0.01, and 0.001.

RESULTS

Phosphorylation of specific tyrosine residues of plexin-B1 is required for Sema4D-induced RhoA activation. We have previously shown that tyrosine phosphorylation of plexin-B1 by ErbB-2 is required for plexin-B1-mediated RhoA activation (58). In order to identify the tyrosine residues of plexin-B1 phosphorylated by ErbB-2, we generated tyrosine-to-phenylalanine (Y/F) mutants of all 26 tyrosine residues of the cytoplasmic portion of plexin-B1 and tested whether they were still phosphorylated by ErbB-2 in response to Sema4D (Fig. 1A). The analysis of three independently performed experiments revealed that only the mutation of tyrosine residue 1708 or 1732 led to a significant decrease in Sema4D-induced phosphorylation of plexin-B1. The mutation of both tyrosine residues [plexin-B1(Y1708F/Y1732F)] completely abrogated Sema4D-induced phosphorylation of plexin-B1 by ErbB-2 (Fig. 2A), whereas c-Met-mediated phosphorylation remained unaffected (Fig. 1B). Consistent with the established role of plexin-B1 tyrosine phosphorylation in Sema4D-induced RhoA activation, plexin-B1(Y1708F/Y1732F) was not able to mediate Sema4D-induced activation of RhoA (Fig. 2A). In contrast, we found no difference in Sema4D-regulated R-Ras GAP activity or Rac1 association in cells expressing any of the tyrosine mutants of plexin-B1 (data not shown) or the Y1708F/Y1732F double mutant of plexin-B1 (Fig. 2B and C, respectively). This indicates that the Y1708F/Y1732F double mutant of plexin-B1 had not lost its ability to become activated by Sema4D and that the tyrosine phosphorylation of both residues is required for RhoA activation but not for the regulation of other plexin-B1 signaling pathways.

Sema4D-induced phosphorylation of plexin-B1 generates docking sites for PLC γ . Phosphorylated tyrosine residues can provide docking sites for SH2 or PTB domain-containing proteins (49). In order to test whether the phosphorylated tyrosine residues 1708 and 1732 of plexin-B1 serve as docking sites for other proteins, we synthesized peptides corresponding to 15-amino-acid-long sequences of the plexin-B1 cytoplasmic portion containing tyrosine residues 1708 or 1732 in a non-phosphorylated or a phosphorylated version. Peptides were then immobilized on agarose beads and incubated with lysates of HEK 293 cells. After washing of beads, bound proteins were eluted and separated using SDS-PAGE. Whereas no additional bands were obtained with the peptide containing phosphorylated Y1732 compared to the nonphosphorylated version (data not shown), several proteins specifically interacted with the peptide containing phosphorylated tyrosine residue 1708 (Fig. 3A). Bands detected by Coomassie blue staining were excised and analyzed by matrix-assisted laser desorption ion-

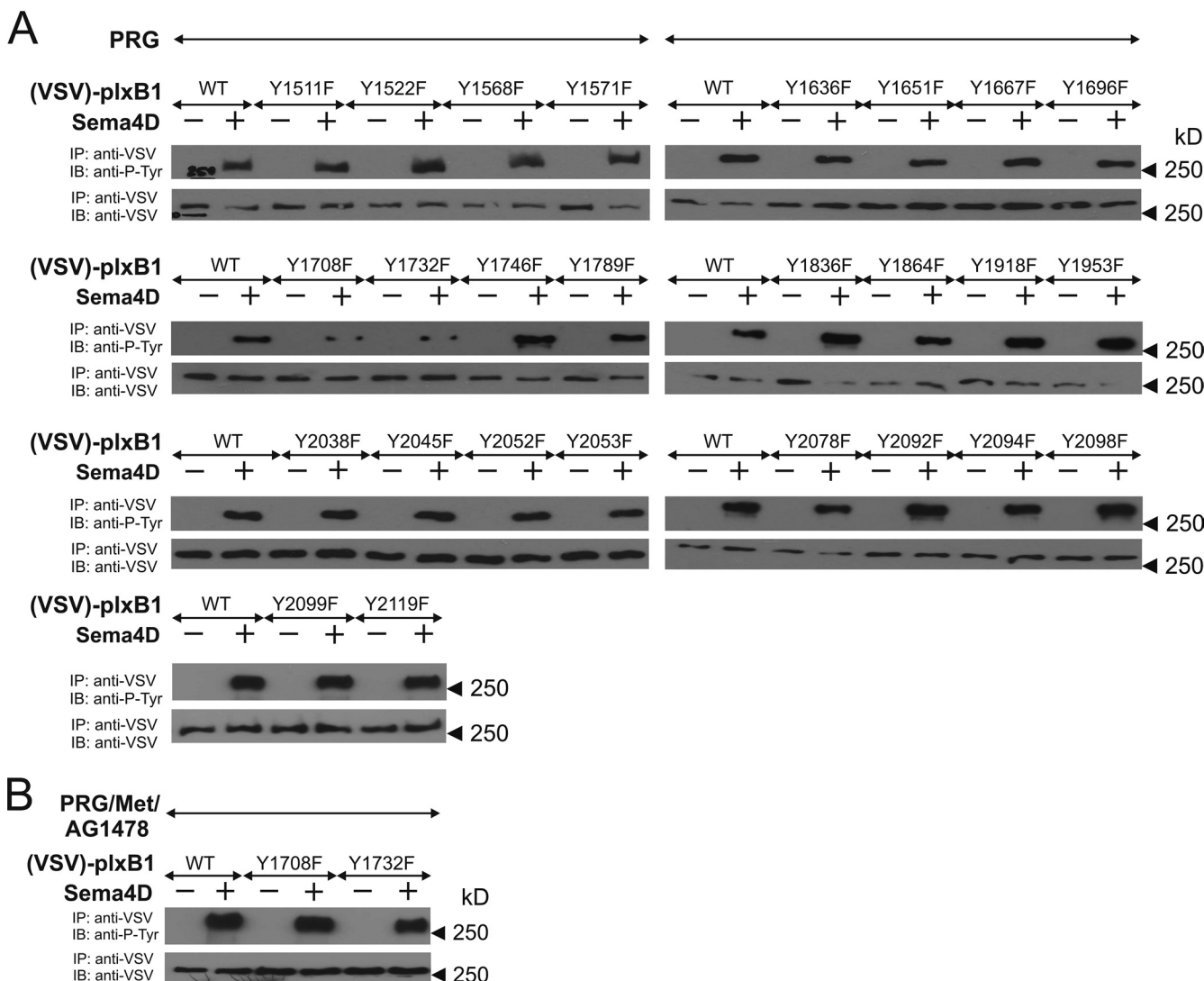


FIG. 1. Characterization of ErbB-2-mediated phosphorylation of plexin-B1. (A) HEK 293 cells were transfected with wild-type plexin-B1 or the indicated tyrosine to phenylalanine (Y/F) mutants and PDZ-RhoGEF (PRG). Cells were then incubated without (–) or with (+) 150 nM Sema4D and lysed, and plexin-B1 was immunoprecipitated (IP). Immunoprecipitates were subjected to SDS-PAGE and were then immunoblotted (IB) using anti-VSV and antiphosphotyrosine antibodies (anti-P-Tyr). (B) HEK 293 cells were transfected with wild-type plexin-B1 or the indicated Y/F mutants together with PDZ-RhoGEF (PRG) and c-Met (Met). Cells were preincubated with 1 μ M of the ErbB-2 inhibitor AG1478 and were then incubated without or with 150 nM Sema4D. Samples were processed as described above. Shown are representative autoluminograms.

ization mass spectrometry. In two independent experiments, 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase γ 1 (PLC γ 1) and growth factor receptor-bound protein 2 (Grb2) were identified as SH2 domain-containing proteins interacting with phosphorylated tyrosine 1708 (Fig. 3A, right panel). To test whether PLC γ 1 and Grb2 indeed bound only to the peptide containing phosphorylated tyrosine 1708, the material isolated with the different peptides was subjected to an immunoblot analysis using antibodies against PLC γ 1, its homologue PLC γ 2, and Grb2. All three proteins were identified in the material isolated with the phosphorylated peptide 1708 but not with the unphosphorylated peptide (Fig. 3B).

To test whether PLC γ and Grb2 can interact with plexin-B1 in a cellular context, we expressed VSV-tagged plexin-B1 in HEK 293 cells. Endogenous PLC γ 1 and Grb2 coimmunopre-

cipitated with wild-type plexin-B1 in a Sema4D-dependent manner (Fig. 3C). Mutation of the tyrosine residue 1708 or 1732 resulted in a decreased interaction, and the Y1708F/Y1732F plexin-B1 double mutant completely lost the ability to bind PLC γ 1 and Grb2 in response to Sema4D. Thus, Sema4D-dependent phosphorylation of tyrosine residues 1708 and 1732 induced interaction of plexin-B1 with PLC γ 1 and Grb2.

Endogenous plexin-B1 and plexin-B2 interact with PLC γ upon activation by semaphorins. In order to further evaluate the Sema4D-dependent interaction of plexin-B1 with PLC γ 1 and Grb2 we studied the human breast cancer cell line MCF-7. MCF-7 cells express both plexin-B1 and ErbB-2, and stimulation with Sema4D results in an ErbB-2-dependent phosphorylation of plexin-B1 (59). Upon stimulation of cells with Sema4D, endogenous plexin-B1 could be coimmunoprecipi-

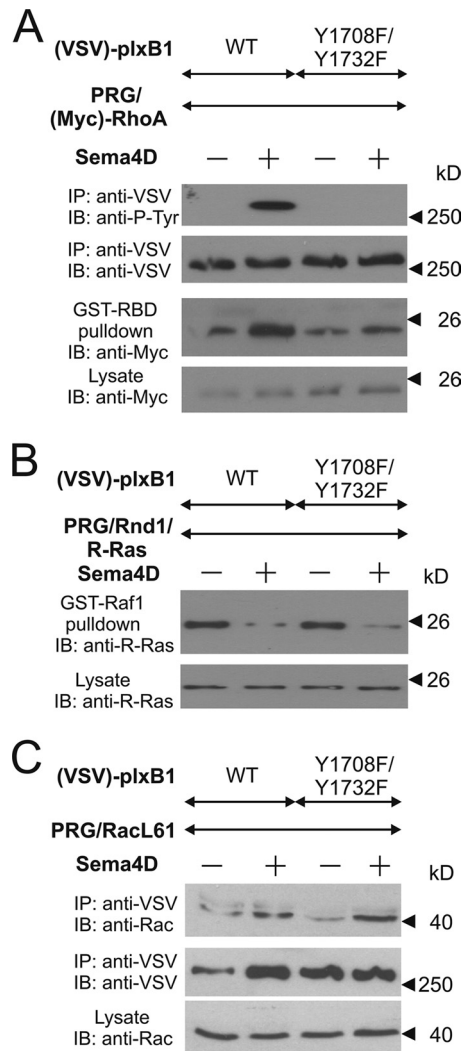


FIG. 2. Effects of the plexin-B1(Y1708F/Y1732F) mutant on plexin-B1 signaling. HEK 293 cells were transfected with cDNAs encoding wild-type (WT) VSV-tagged plexin-B1 [(VSV)-plxB1] or its mutant (Y1708/1732F) together with either Myc-tagged RhoA [(Myc)-RhoA] and wild-type PDZ-RhoGEF (PRG) (A), R-Ras, Rnd1, and PRG (B), or the constitutively active GST-tagged Rac1 mutant [Rac(Q61L)] and wild-type PRG (C). After 48 h cells were kept in 0.5% FBS (A) or 10% FBS (B and C) for 12 h followed by incubation without (-) or with (+) 150 nM Sema4D for 20 min. Thereafter, cells were lysed, and plexin-B1 was immunoprecipitated (IP) using anti-VSV-Sepharose (A and C). (A) Precipitated proteins were analyzed by immunoblotting using antiphosphotyrosine antibodies. In a parallel experiment active RhoA was precipitated from cell lysates as described in Materials and Methods. (B) Activated R-Ras was precipitated from the cell lysates with a GST-fused Ras-binding domain of Raf1. Shown are autoluminograms of immunoblots (IB) of lysed (lysate) or precipitated samples (IP/GST-RBD or GST-Raf1 pull-down) and the indicated antibodies.

tated with endogenous PLC γ 1, PLC γ 2, and Grb2 (Fig. 4A). Interestingly, the siRNA-mediated knockdown of PLC γ 1 and PLC γ 2 abrogated not only the interactions between plexin-B1 and both PLC γ proteins but also blocked the interaction of plexin-B1 and Grb2, whereas knockdown of Grb2 had no effect on the interaction between plexin-B1 and PLC γ (Fig. 4A). This

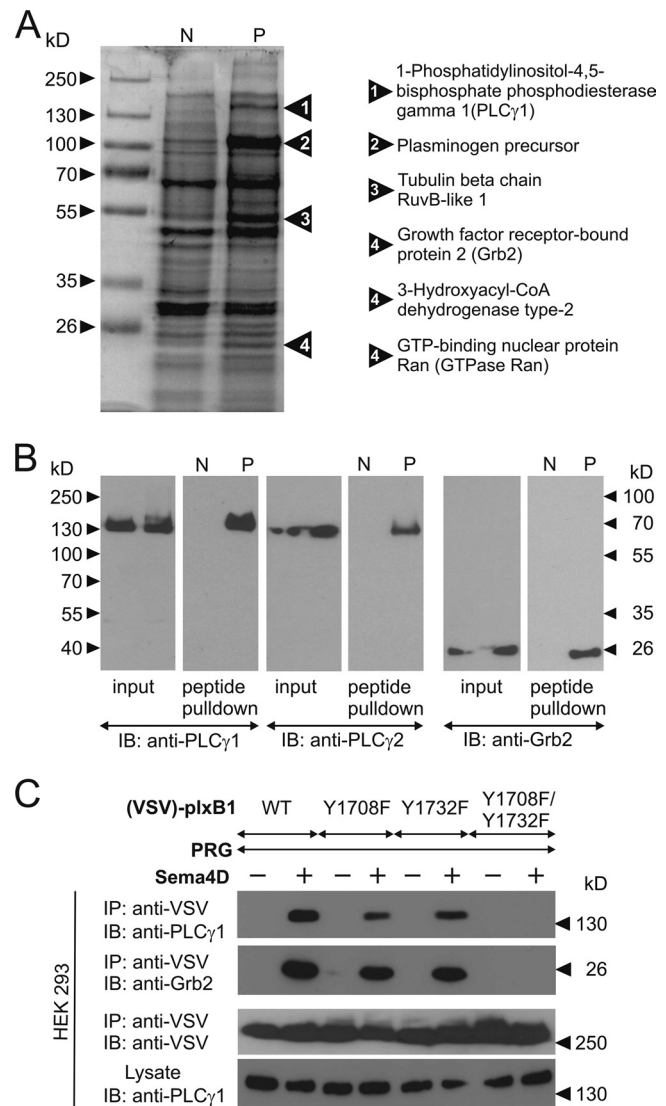


FIG. 3. Detection of proteins interacting with pY1708. (A) Peptides representing amino acids 1701 to 1715 of plexin-B1 containing a nonphosphorylated (N) or phosphorylated (P) tyrosine residue at 1708 were bound to agarose beads and incubated with lysates from HEK 293 cells for 2 h. Bound proteins were separated by SDS-PAGE and visualized by Coomassie blue staining. Bands appearing only in samples eluted from phosphorylated peptides were cut out and analyzed by mass spectrometry. Proteins identified in the corresponding band are shown on the right. (B) HEK 293 cell lysates (input) were incubated with immobilized peptide containing nonphosphorylated (N) or phosphorylated (P) tyrosine residue 1708 (peptide pull down). After washing and elution, proteins bound to the peptide were separated by SDS-PAGE, and immunoblotting (IB) was performed with the indicated antibodies. (C) HEK 293 cells were transfected with wild-type (WT) plexin-B1 or the indicated mutants. Cells were starved and incubated without (-) or with (+) Sema4D. Thereafter, WT or mutated plexin-B1 was immunoprecipitated from cell lysates using anti-VSV antibody. Immunoprecipitates were separated by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies.

strongly suggests that the interaction between plexin-B1 and Grb2 is indirect and dependent on the presence of PLC γ .

In contrast to MCF-7 cells, MDA-MB-468 cells do not contain ErbB-2 but express plexin-B1 together with the re-

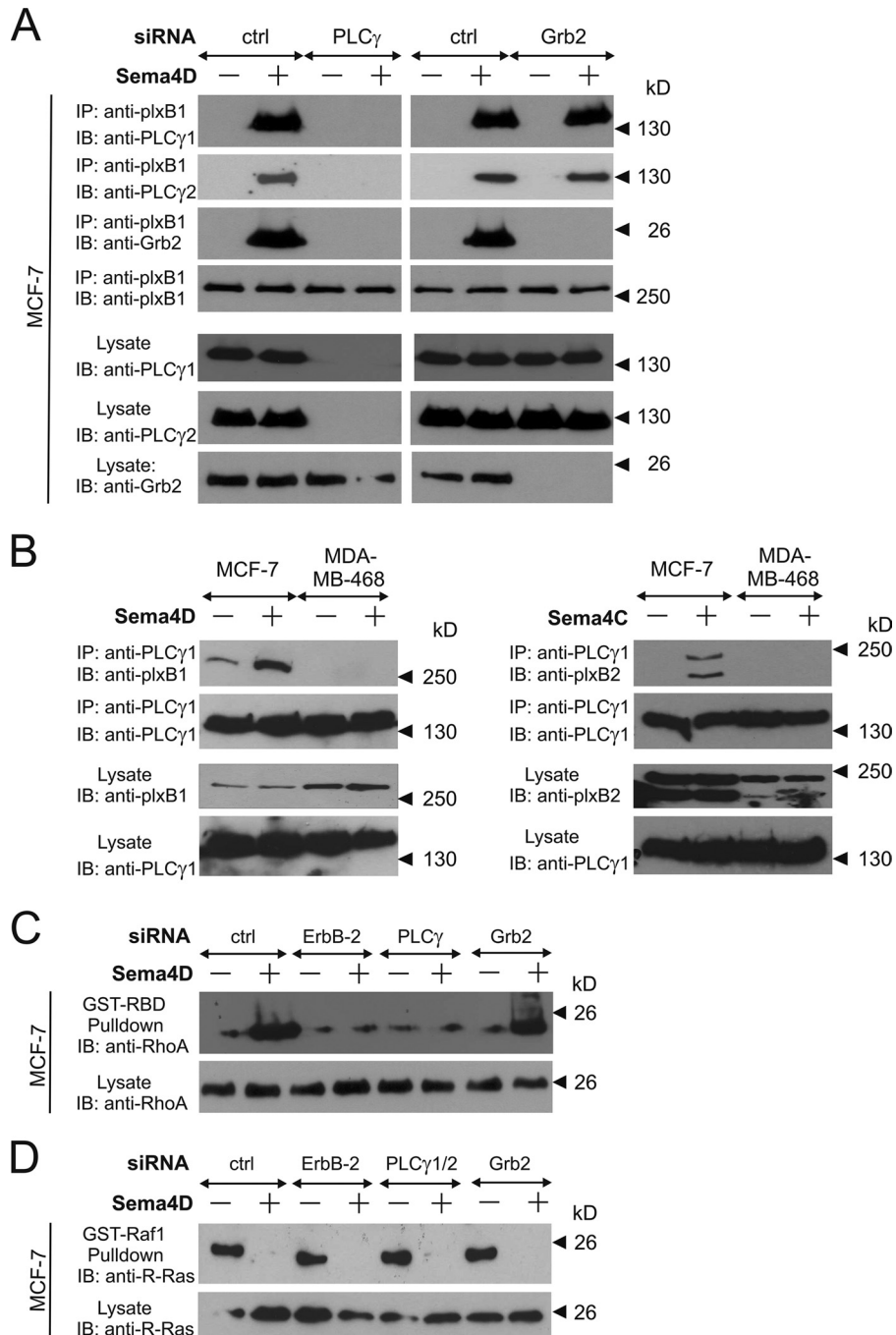


FIG. 4. PLC γ interacts with B-family plexins. MCF-7 cells or MDA-MB-468 cells were not transfected (B) or transfected (A, C, and D) with the indicated siRNAs (PLC γ represents PLC γ 1 plus PLC γ 2). At 48 h after transfection cells were starved (0.5% FBS) and incubated in the absence (-) or presence (+) of 150 nM Sema4D. Cells were then lysed, and the proteins interacting with plexin-B1 (A) or PLC γ 1 (B) were coimmunoprecipitated (IP) using anti-plexin-B1 or anti-PLC γ 1 antibodies. Lysates and immunoprecipitates were analyzed using the indicated antibodies (IB). (C and D) MCF-7 cells were transfected with siRNAs as indicated, and 48 h later cells were transferred to 0.5% FBS (C) or were kept in 10% FBS (D) and incubated in the absence or presence of 150 nM Sema4D for 20 min. Active RhoA (C) or R-Ras (D) was precipitated from cell lysates as described in Materials and Methods. Shown are autoluminograms of immunoblots (IB) analyzed with the indicated antibodies.

ceptor tyrosine kinase c-Met (59). Although PLC γ 1 is expressed in both cell lines, a Sema4D-dependent interaction with plexin-B1 can only be seen in the ErbB-2-expressing MCF-7 cell line (Fig. 4B). This supports the notion that ErbB-2-dependent phosphorylation of plexin-B1 is neces-

sary for this interaction. MCF-7 cells also express plexin-B2, and stimulation of cells with the plexin-B2 ligand Sema4C (13) induced interaction of PLC γ 1 with plexin-B2 (Fig. 4B right panel). We did not find any Sema4C-dependent interaction of PLC γ 1 and plexin-B2 in MDA-MB-468 cells.

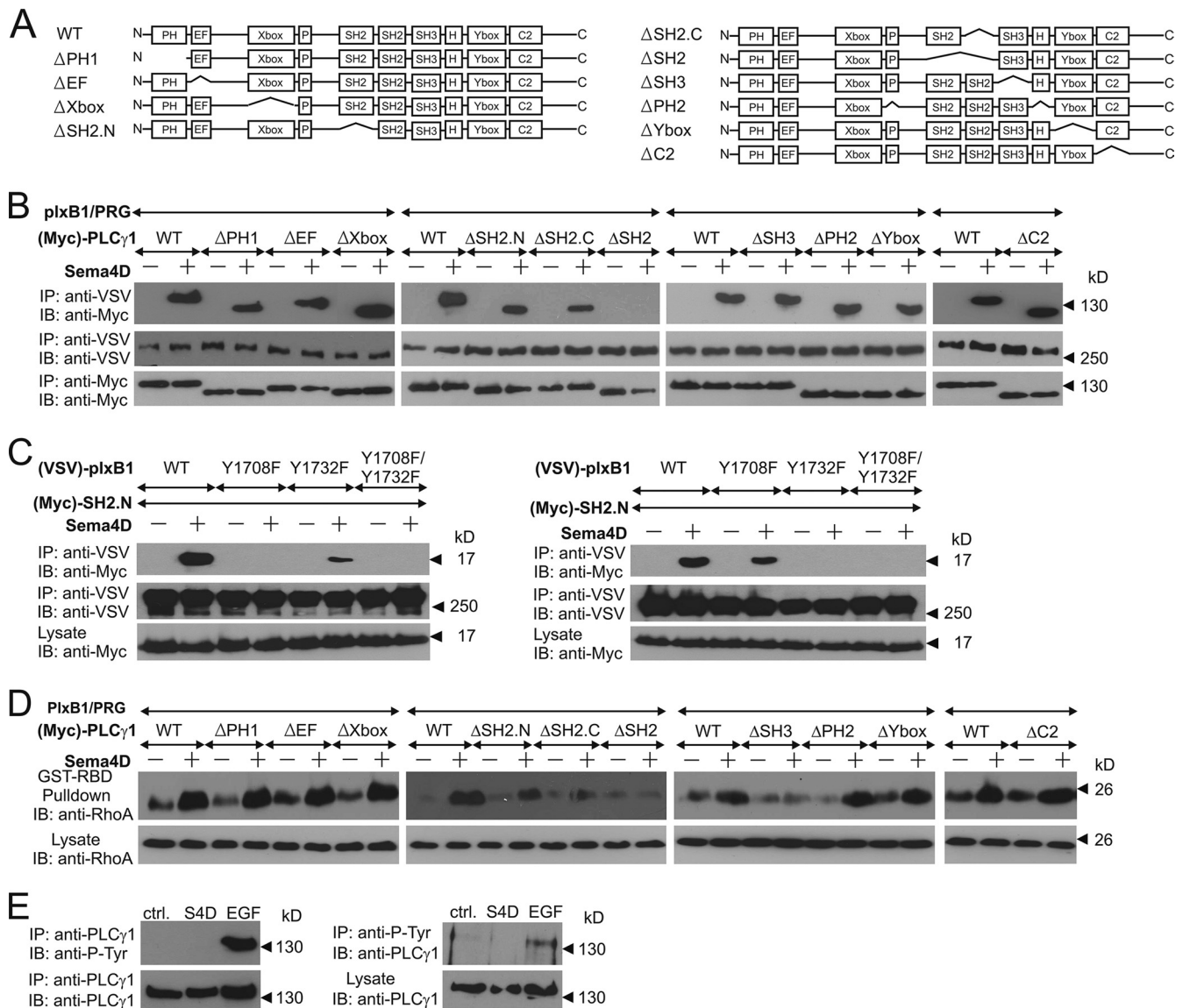


FIG. 5. Analysis of PLC γ domains required for plexin-B1-mediated signaling. (A) Graphical representation of the analyzed PLC γ mutants. (B and D) HEK 293 cells were transfected with plexin-B1, PDZ-RhoGEF (PRG), wild-type PLC γ 1 (WT), or its mutants. Cells were incubated without (-) or with (+) 150 nM Sema4D and lysed, and the interaction between plexin-B1 and PLC γ 1 and its mutants was analyzed by immunoprecipitation (B), or the amount of activated RhoA was analyzed as described in Materials and Methods (D). (C) Wild-type or Y/F mutants of plexin were cotransfected with the Myc-tagged N- or C-terminal SH2 domain of PLC γ 1 (SH2.N or SH2.C, respectively). Protein-protein interactions were analyzed by coimmunoprecipitation. (E) MCF-7 cells were treated with phosphate-buffered saline (ctrl.), 150 nM Sema4D, or 10 ng/ml EGF. Cells were lysed, and immunoprecipitation (IP) was performed using anti-PLC γ 1 or antiphosphotyrosine antibodies (left and right panels, respectively). Samples were then analyzed by immunoblotting (IB) using the indicated antibodies.

Plexin-B1-induced RhoA activation but not R-Ras inactivation requires PLC γ . Knockdown of PLC γ 1 or PLC γ 2 in MCF-7 cells decreases plexin-B1/PDZ-RhoGEF-mediated RhoA activation (data not shown), whereas the depletion of both isoforms blocked Sema4D-induced RhoA activation (Fig. 4C). Consistent with previous data (59), we observed also that siRNA-mediated knockdown of ErbB-2 abolished RhoA activation downstream of plexin-B1, whereas Grb2 knockdown had no effect on RhoA activation (Fig. 4C). In contrast to the Sema4D-induced RhoA activation, the regulation of R-Ras GAP activity of plexin-B1 did not depend on PLC γ or ErbB-2,

as indicated by the lack of any effect of PLC γ 1/2 or ErbB-2 knockdown on Sema4D-induced inhibition of R-Ras (Fig. 4D). Hence, the recruitment of PLC γ into the plexin-B1 receptor complex is necessary for RhoA activation but not for R-Ras function regulated by plexin-B1.

Analysis of the interaction between plexin-B1 and PLC γ 1. To obtain insight into the mechanism underlying the PLC γ -dependent RhoA activation through plexin-B1, PLC γ 1 mutants lacking functional domains (Fig. 5A) were expressed in HEK 293 cells. The removal of the N- or C-terminal SH2 domain decreased the interaction between Sema4D-acti-

vated plexin-B1 and PLC γ 1, whereas deletion of both SH2 domains blocked interaction of PLC γ 1 with plexin-B1 (Fig. 5B).

Consistent with these results we observed that the point mutation (R586K) (62) in one of the SH2 domains decreased the amount of PLC γ 1 that coimmunoprecipitates with plexin-B1 (data not shown). Thus, both SH2 domains of PLC γ 1 mediated the interaction with tyrosine-phosphorylated plexin-B1, while none of the other domains appeared to be critically involved. Interestingly, a remarkable specificity in the interaction of phosphorylated Y1708 and Y1732 and each of the two SH2 domains of PLC γ 1 was observed. While the more N-terminally localized SH2 domain (SH2.N) did not interact with plexin-B1(Y1708F) but it did with plexin-B1(Y1732F) (Fig. 5C, left panel), the more C-terminal SH2 domain (SH2.C) was unable to interact with the Y1732F mutant, while it did interact with plexin-B1(Y1708F) (Fig. 5C, right panel).

In order to study the role of different PLC γ domains in mediating downstream effects of plexin-B1 signaling, we determined RhoA activity in transfected HEK 293 cells (Fig. 5D). Removal of one of the SH2 domains led to an impaired RhoA activation in response to Sema4D, while removal of both SH2 domains completely blocked plexin-B1-mediated RhoA activation. Interestingly, PLC γ 1 mutants lacking the X or Y box, which are required for the enzymatic activity of the phospholipase (47), were still able to mediate Sema4D-induced RhoA activation, thereby indicating that PLC γ functions in plexin-B-mediated signaling independently of its lipase activity. Consistent with this, stimulation of cells with epidermal growth factor (EGF) but not with Sema4D resulted in tyrosine phosphorylation of PLC γ , a well-described prerequisite for the activation of its phospholipase activity (47) (Fig. 5E). Among other PLC γ domains, only the removal of the SH3 domain affected plexin-B1-mediated RhoA activation (Fig. 4D). While the Δ SH3 mutant was recruited into the plexin-B1 complex upon Sema4D stimulation (Fig. 5B), it was unable to promote Sema4D-induced RhoA activation. Consistent with the results obtained with the Δ SH3 mutant of PLC γ 1 we found that the point mutation (W828K) (62) in the SH2 domain of PLC γ 1 also blocks Sema4D-mediated RhoA activation, whereas the interaction between plexin-B1 and PLC γ 1 remains unaffected (data not shown). This indicates that the SH3 domain of PLC γ but not its enzymatic activity is required for Sema4D-induced RhoA activation.

The SH3 domain of PLC γ 1 mediates activation of PDZ-RhoGEF. Since plexin-B1 stably interacts with particular RhoGEF proteins to mediate RhoA activation and since the SH3 domain of PLC γ appeared to be crucial for plexin-B1-mediated RhoA activation, we tested the possibility that the SH3 domain of PLC γ 1 interacts with PDZ-RhoGEF. In co-transfected HEK 293 cells, we found that PDZ-RhoGEF coimmunoprecipitated with PLC γ 1 or its SH2 domain-lacking mutant, but not with a PLC γ 1 mutant lacking the SH3 domain (Fig. 6A), suggesting that PLC γ 1 interacts with PDZ-RhoGEF via its SH3 domain.

Among six proline-rich regions of the RhoGEF protein, the software SH3 Hunter (17) identified one region at positions 1332 to 1338 as a potential binding partner for the SH3 domain of PLC γ 1. Immunoprecipitation experiments with PDZ-RhoGEF carrying proline-to-alanine mutations in this region

(P1335A/P1338A) and two other regions (P510A/P513A and P554A/P557A) indicated that the proline-rich region at positions 1332 to 1338 of PDZ-RhoGEF was necessary for the interaction with the SH3 domain of PLC γ 1 (Fig. 6B). In order to test whether the SH3 domain of PLC γ 1 and the C terminus of PDZ-RhoGEF interact directly, we purified both proteins after expression in bacteria and tested their interaction *in vitro*. We found that the C terminus of PDZ-RhoGEF indeed interacted with the SH3 domain of PLC γ 1 but not with the SH3 domain of Grb2 (Fig. 7A).

Expression of PDZ-RhoGEF together with the SH3 domain of PLC γ 1 resulted in a synergistic activation of a mutated serum response element (SRE.L) which lacks the binding site for the ternary complex factor and is strongly activated by RhoA (25) (Fig. 6C, left panel). In contrast, the SH3 domain of Grb2 had no effect. Consistent with the results of the interaction studies, the mutation of the proline-rich regions 507 to 513 and 551 to 557 of PDZ-RhoGEF had no effect on the PLC γ -SH3 domain-dependent RhoA activation, whereas mutation of the proline-rich region 1332 to 1338 of PDZ-RhoGEF abolished the effect of the SH3 domain of PLC γ (Fig. 6C, left panel). This effect was specific for the SH3 domain of PLC γ , since wild-type PDZ-RhoGEF and all P/A mutants were equally activated by a constitutively active G α_{13} mutant (G α_{13} Q226L) (Fig. 6D, left panel). These data were confirmed by direct determination of RhoA activity using RhoA pull-down assays (Fig. 6C and D, right panels). In order to test whether the SH3 domain of PLC γ can directly stimulate the catalytic activity of PDZ-RhoGEF, we measured the effect of the purified SH3 domain of PLC γ on RhoA activation *in vitro*. We found that the PLC γ 1 SH3 domain increases the guanine nucleotide exchange activity of PDZ-RhoGEF toward RhoA (Fig. 7B). This effect was specific for the SH3 domain of PLC γ 1, since addition of the purified Grb2 SH3 domain to purified PDZ-RhoGEF had no effect (Fig. 7C).

Oligomerization of PDZ-RhoGEF has been suggested to be involved in the regulation of its catalytic activity. Since our data showed that the SH3 domain of PLC γ 1 directly influences the catalytic activity of PDZ-RhoGEF, we tested the possibility that binding of the SH3 domain may interfere with the oligomerization of PDZ-RhoGEF. In coimmunoprecipitation experiments with PDZ-RhoGEF and increasing amounts of the SH3 domain of PLC γ 1, we were unable to detect any influence of even a 100-fold molar excess of the SH3 domain on homooligomerization of PDZ-RhoGEF (Fig. 7D).

PLC γ regulates plexin-B1-mediated migration in MCF-7 cells. Activation of plexin-B1 by Sema4D has been shown to exert promigratory effects in various cells, including MCF-7 cells (2, 3, 21, 22, 59), a process requiring ErbB-2-dependent activation of RhoA (59). MCF-7 cells were stably transfected with cDNAs encoding wild-type or mutant (Y1708F/Y1732F) plexin-B1 RNAs carrying mutations which made them insensitive to particular siRNAs (see Materials and Methods) (data not shown). While an siRNA-induced knockdown of endogenous plexin-B1 resulted in the inability of MCF-7 cells to respond to Sema4D with migration and RhoA activation, the stable expression of siRNA-insensitive wild-type plexin-B1 fully restored the effect of Sema4D (Fig. 8A). However, cells expressing the siRNA-insensitive

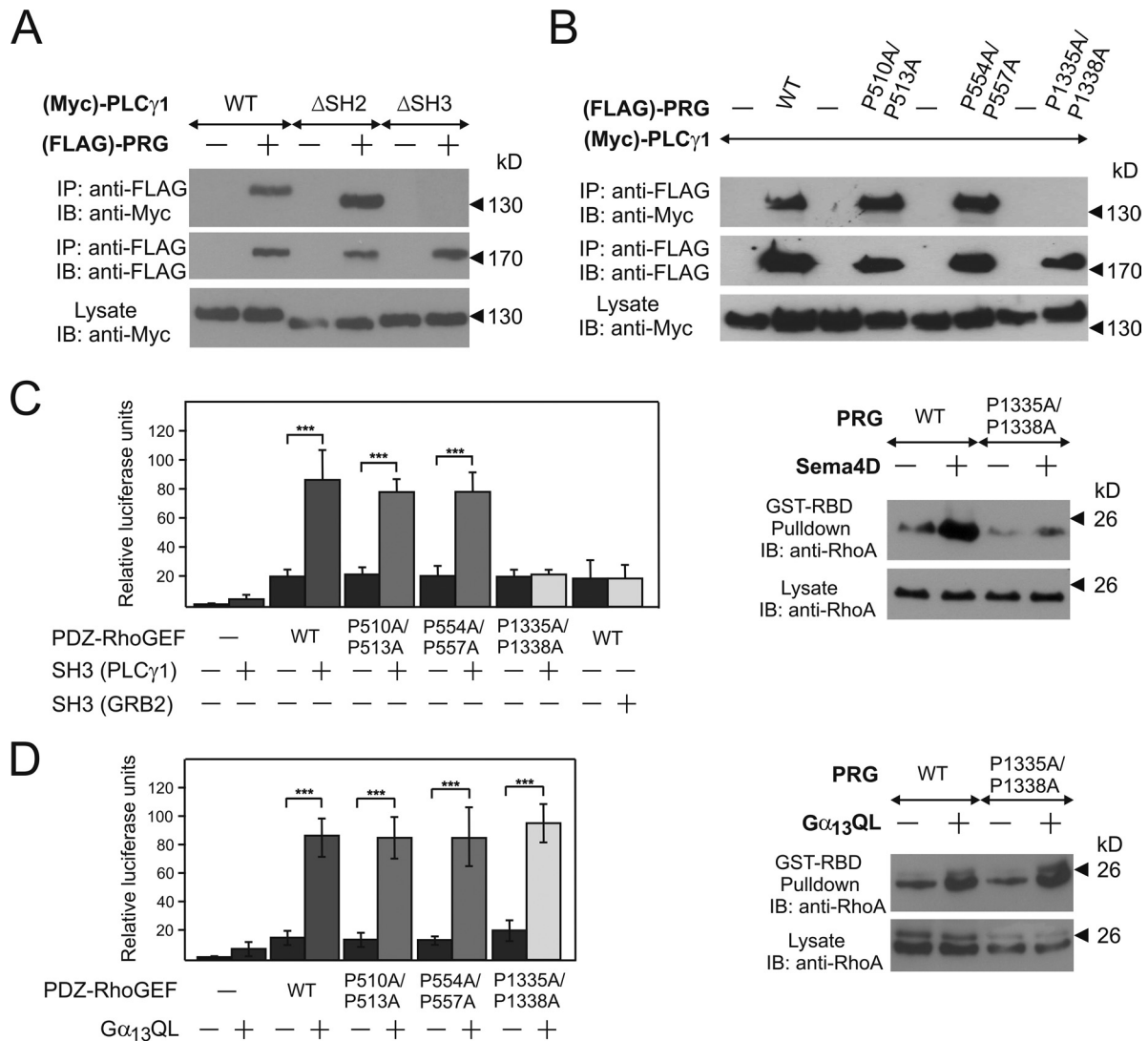


FIG. 6. PLC γ 1 interaction with PDZ-RhoGEF. (A) HEK 293 cells were transfected without or with FLAG-tagged PDZ-RhoGEF [(FLAG)-PRG] and Myc-tagged wild-type (WT) PLC γ 1 or its indicated mutants. From cell lysates, PDZ-RhoGEF was immunoprecipitated (IP) and precipitates were then separated and immunoblotted (IB) with the indicated antibodies. (B) HEK 293 cells transfected with Myc-tagged PLC γ 1 and FLAG-tagged wild-type PDZ-RhoGEF or its indicated mutants were lysed, and PLC γ 1 was immunoprecipitated. Precipitates were analyzed by immunoblotting using the indicated antibodies. (C and D, left panels) HEK 293 cells were cotransfected with SRE.L firefly luciferase reporter plasmid, *Renilla* luciferase expression plasmid without (-) or with various PDZ-RhoGEF versions, the SH3 domains of Grb2 and PLC γ , or constitutively active G α ₁₃ (G α ₁₃QL) as indicated, and relative luciferase activity was determined as described in Materials and Methods. Shown values are the means of three independent experiments \pm standard deviations. (Right panels) HEK 293 cells were transfected with wild-type PDZ-RhoGEF (WT) or its mutant (P1335A/P1338A) and were incubated without or with 150 nM Sema4D (C) or were cotransfected with control or with a constitutively active version of G α ₁₃ (G α ₁₃QL) (D). Thereafter, RhoA activity was determined as described in Materials and Methods section. ***, $P \leq 0.001$.

Y1708F/Y1732F mutant of plexin-B1 were unresponsive (Fig. 8A), indicating that the phosphorylation of the tyrosine residues 1708 and 1732 is crucial for Sema4D-induced cell migration and RhoA activation.

Given that these phosphorylated tyrosine residues serve as docking sites for PLC γ , we analyzed the relevance of PLC γ for plexin-B1-mediated regulation of cell migration. Depletion of PLC γ 1 and PLC γ 2, which are both expressed in MCF-7 cells, abolished Sema4D-stimulated cell migration, whereas the siRNA-mediated knockdown of Grb2 had no effect (Fig. 8B). The ability of cells to migrate was not generally affected by the

lack of PLC γ , as indicated by the insensitivity of fetal bovine serum (FBS)-stimulated cell migration to the knockdown of PLC γ (Fig. 8B).

We stably transfected MCF-7 cells with cDNAs encoding wild-type and mutant forms of PLC γ RNAs additionally carrying mutations which made them insensitive to particular siRNAs (see Materials and Methods). The expression of the recombinant proteins and the knockdown efficiency were proven by Western blotting (data not shown). In cells treated with anti-PLC γ siRNA, the expression of siRNA-resistant cDNAs of wild-type and Δ Xbox mutant PLC γ 1

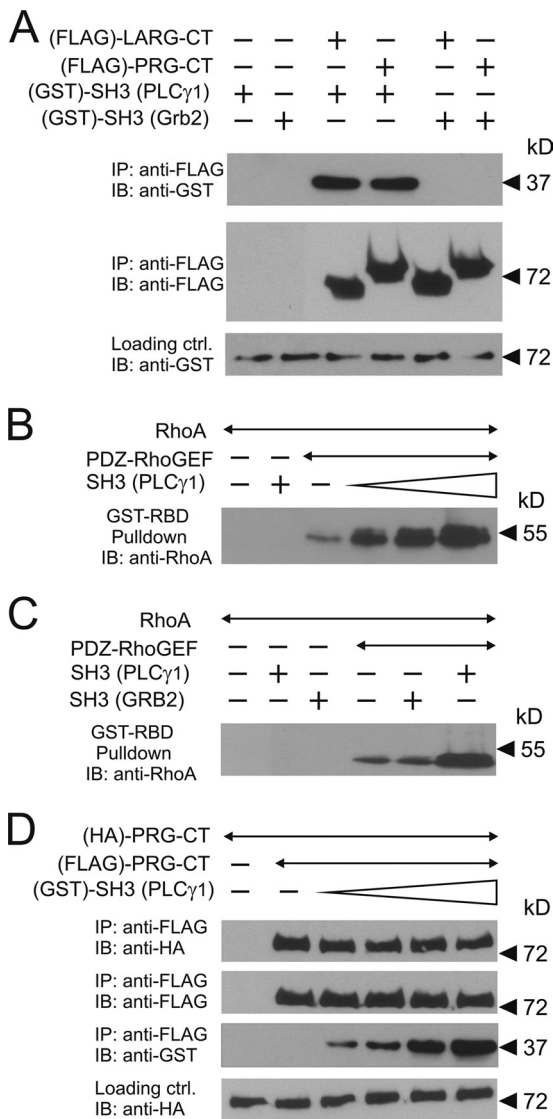


FIG. 7. In vitro characterization of the role of the SH3 domain of PLC γ in PDZ-RhoGEF signaling. (A) Equal amounts of purified GST-tagged SH3 domains of PLC γ 1 or Grb2 were incubated without (-) or with (+) purified FLAG-tagged C-terminal portions of LARG or PDZ-RhoGEF (LARG-CT and PRG-CT, respectively). The RhoGEF proteins were then immunoprecipitated (IP) using anti-FLAG antibody. Shown are immunoblots (IB) of the precipitates using the indicated antibodies. (B) One nanomole of FLAG-PDZ-RhoGEF was incubated without or with a 1-, 10-, or 100-fold molar excess of GST-SH3 of PLC γ 1. The reaction was started by addition of 100 nanomole of GDP-loaded RhoA. After 10 min of incubation, activated RhoA was precipitated as described in Materials and Methods. Shown are immunoblots using the anti-RhoA antibody. (C) FLAG-PDZ-RhoGEF was incubated with the SH3 domains of PLC γ 1 or Grb2. The experiment was performed as described in B. (D) Equal amounts of hemagglutinin (HA)- and FLAG-tagged C-terminal portions of PDZ-RhoGEF were incubated alone or together with increasing concentrations of the SH3 domain of PLC γ 1. FLAG-tagged PDZ-RhoGEF was immunoprecipitated, and precipitates were analyzed by immunoblotting with the indicated antibodies.

restored the Sema4D-induced promigratory effect and RhoA activation (Fig. 8C). However, expression of the siRNA-insensitive Δ SH3 mutant of PLC γ 1 did not rescue these Sema4D effects (Fig. 8C).

PLC γ is required for plexin-B1-mediated growth cone collapse. Sema4D has been shown to induce axonal growth cone collapse in hippocampal neurons via plexin-B1 (36, 57). In control siRNA-transfected hippocampal neurons, Sema4D decreased the number of growth cone-positive cells from 85% to 30%; however, siRNA-mediated knockdown of PLC γ 1 abolished this effect (Fig. 9A and B). In contrast, knockdown of PLC γ 1 had no effects on the ability of Sema3A to promote growth cone collapse of hippocampal neurons (Fig. 9B), indicating that depletion of PLC γ 1 did not affect the ability of neurons to respond with growth cone collapse in general.

To further analyze the potential role of plexin-B1-phosphorylation of tyrosine residues 1708 and 1732 in Sema4D-induced growth cone collapse, we studied hippocampal neurons isolated from plexin-B1-deficient mice (*plxnb1*^{-/-}) (13) and transfected them with either VSV-tagged wild-type plexin-B1 or the plexin-B1(Y1708F/Y1732F) mutant. The expression of wild-type or mutated plexin-B1 was visualized by staining with anti-VSV antibodies (Fig. 9D). While plexin-B1-deficient neurons had lost the ability to respond to Sema4D, expression of wild-type plexin-B1 in neurons isolated from *plxnb1*^{-/-} mice restored Sema4D-induced growth cone collapse (Fig. 9C and D). However, the expression of the Y1708F/Y1732F mutant of plexin-B1 was not able to rescue Sema4D-induced growth cone collapse in plexin-B1-deficient cells (Fig. 9C and D).

DISCUSSION

Plexins of the B-family serve as receptors for particular semaphorins and mediate their cellular effects. During recent years some of their downstream signaling mechanisms have been described. Besides the regulation of the small GTPase R-Ras through an inherent GAP function (36), plexin-B family members mediate the activation of the small GTPase RhoA by direct interaction with the RhoA guanine nucleotide exchange factors PDZ-RhoGEF and LARG (1, 39, 57). Activation of RhoA requires the tyrosine phosphorylation of plexin by ErbB-2 (58). However, the mechanism linking plexin-B1 tyrosine phosphorylation to the activation of RhoA is completely unclear. Here we have shown that upon activation by Sema4D, the intracellular tyrosine residues 1708 and 1732 of plexin-B1 become phosphorylated and serve as docking sites for SH2 domains of PLC γ 1 and -2. The recruitment of PLC γ into the plexin-B1 receptor complex is a requirement for plexin-B-mediated RhoA activation, as well as for downstream cellular effects of Sema4D, like plexin-B1-mediated promigratory effects and the Sema4D-induced growth cone collapse of hippocampal neurons.

The tyrosine residue 1708 of plexin-B1 is conserved among all three members of the plexin-B family, and tyrosine 1732 of plexin-B1 is also found in plexin-B2 but not in plexin-B3 (18, 61). Our data show that also plexin-B2 interacts with PLC γ upon activation by its ligand semaphorin 4C. Thus, the observed tyrosine phosphorylation-dependent recruitment of PLC γ is likely to be a common mechanism of plexin-B family members. The phosphorylation of plexin-B1 by c-Met was not affected by the mutations of Y1708 and Y1732, indicating that Erb-2 and c-Met regulate the function of plexin-B1 by phosphorylation of different tyrosine residues of plexin-B1. This is

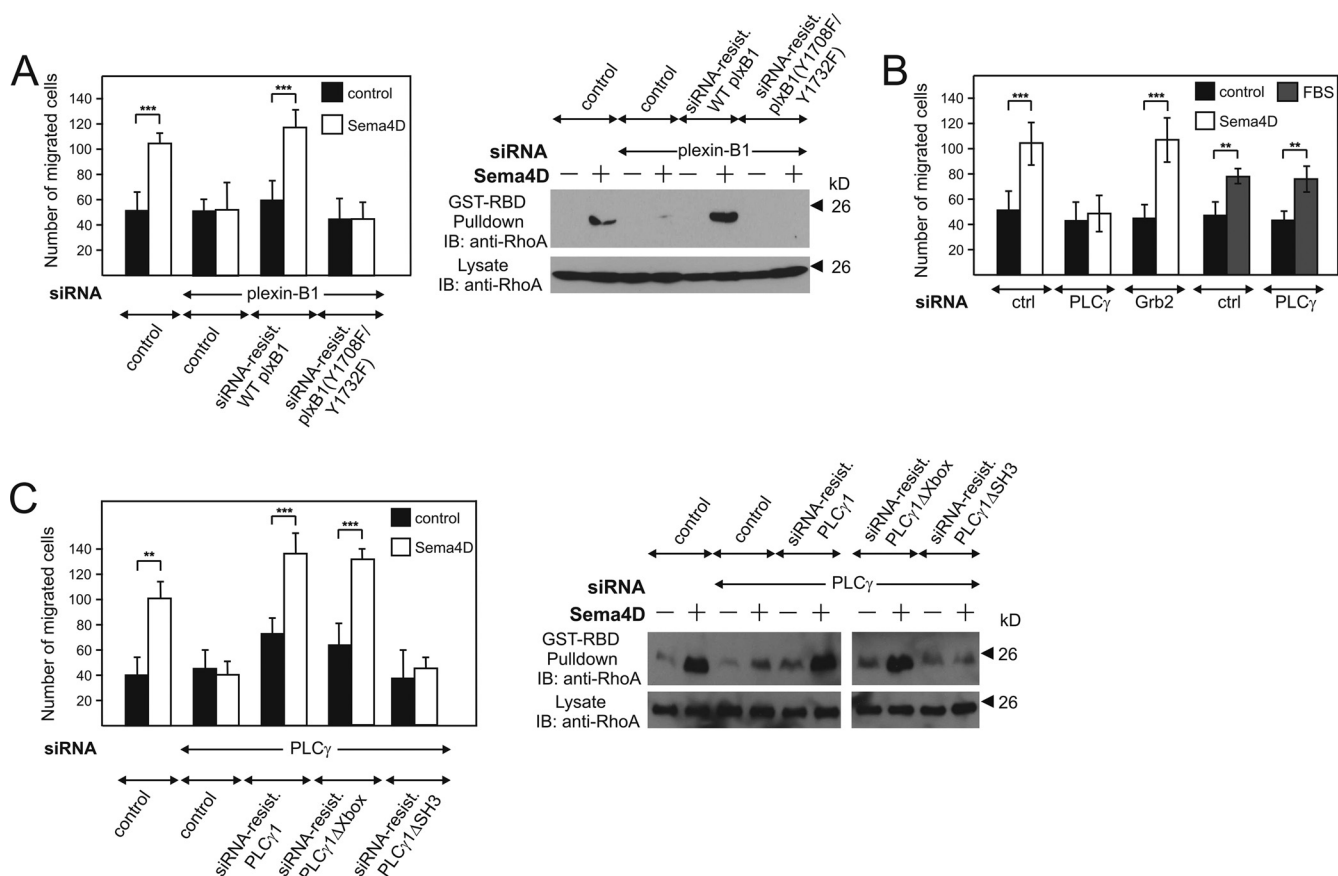


FIG. 8. PLC γ mediates plexin-B1-dependent cellular migration. Normal MCF-7 cells or MCF-7 cells stably expressing siRNA-insensitive mutants of wild-type plexin-B1 and PLC γ 1 or their indicated mutants (A and C) were transfected with siRNA directed against the indicated targets (PLC γ indicates PLC γ 1 plus PLC γ 2) and kept at 0.5% FBS. Cells were then incubated in the absence (control) or presence of 150 nM Sema4D or 10% FBS. Sema4D-induced cellular migration (B and left panels of A and C) and RhoA activation (right panels of A and C) were analyzed as described in Materials and Methods. Control cells were stably transfected with the viral vector pLNCX2 alone. Data presented are representative of at least three independently performed experiments. Data of the analysis of migration are shown as means of triplicates (\pm standard errors of the means). **, $P \leq 0.01$; ***, $P \leq 0.001$.

consistent with the observation that the phosphorylation of plexin-B1 by ErbB-2 and c-Met induces different and sometimes opposing downstream signaling and cellular effects (21, 22, 58, 59).

Phosphorylation of tyrosine residues results in the formation of docking sites for SH2 and PTB domain-containing proteins (49). Using a peptide-based as well as several functional approaches, we identified the SH2 domain-containing protein PLC γ as an interaction partner of ErbB-2-phosphorylated plexin-B1. The analysis of the amino acid sequences surrounding tyrosine residues 1708 and 1732 of plexin-B1, GEPLYMLFR and GKAKYTLND, respectively, shows that especially the region surrounding tyrosine 1708 fits the consensus sequence recognized by the SH2 domain of PLC γ 1 (pY-hydrophobic-X-hydrophobic) (52, 53). While in most receptor tyrosine kinases able to interact with PLC γ a single phosphorylated tyrosine residue has been shown to mediate interaction with the enzyme (47), at least two autophosphorylated tyrosine residues of the EGF receptor appear to interact with PLC γ 1 (9, 51, 66).

The individual role of the two SH2 domains of PLC γ in the interaction of the enzyme with tyrosine phosphorylated pro-

teins has been analyzed for the EGF and platelet-derived growth factor receptors (9, 28, 41). In both cases, a predominant role of the more N-terminally localized SH2 domain was observed. The C-terminally localized SH2 domain has recently been shown to be involved in the activation mechanism of lipase activity by intramolecular interaction with the phosphorylated Y783 residue of PLC γ 1 (42). Our data show that efficient binding of PLC γ to activated plexin-B1 requires phosphorylation of both Y1708 and 1732 as well as the presence of both SH2 domains of PLC γ . Since PLC γ does not become tyrosine phosphorylated after recruitment into the plexin receptor complex, the more C-terminal SH2 domain is free to interact with a pY residue of plexin-B1. Interestingly, mutagenesis of individual tyrosine phosphorylation sites of plexin-B1 and of each of the SH2 domains revealed a remarkable specificity in that the N-terminal SH2 domain of PLC γ interacted with pY1708, while the more C-terminally localized SH2 domain preferentially interacted with pY1732. This specificity suggests that the exact orientation of plexin-B1-bound PLC γ is critical for its correct function in plexin-B1-mediated signaling.

Our immunoprecipitation data indicate that phosphorylated plexin-B1 can interact with Grb2, which was also isolated by a

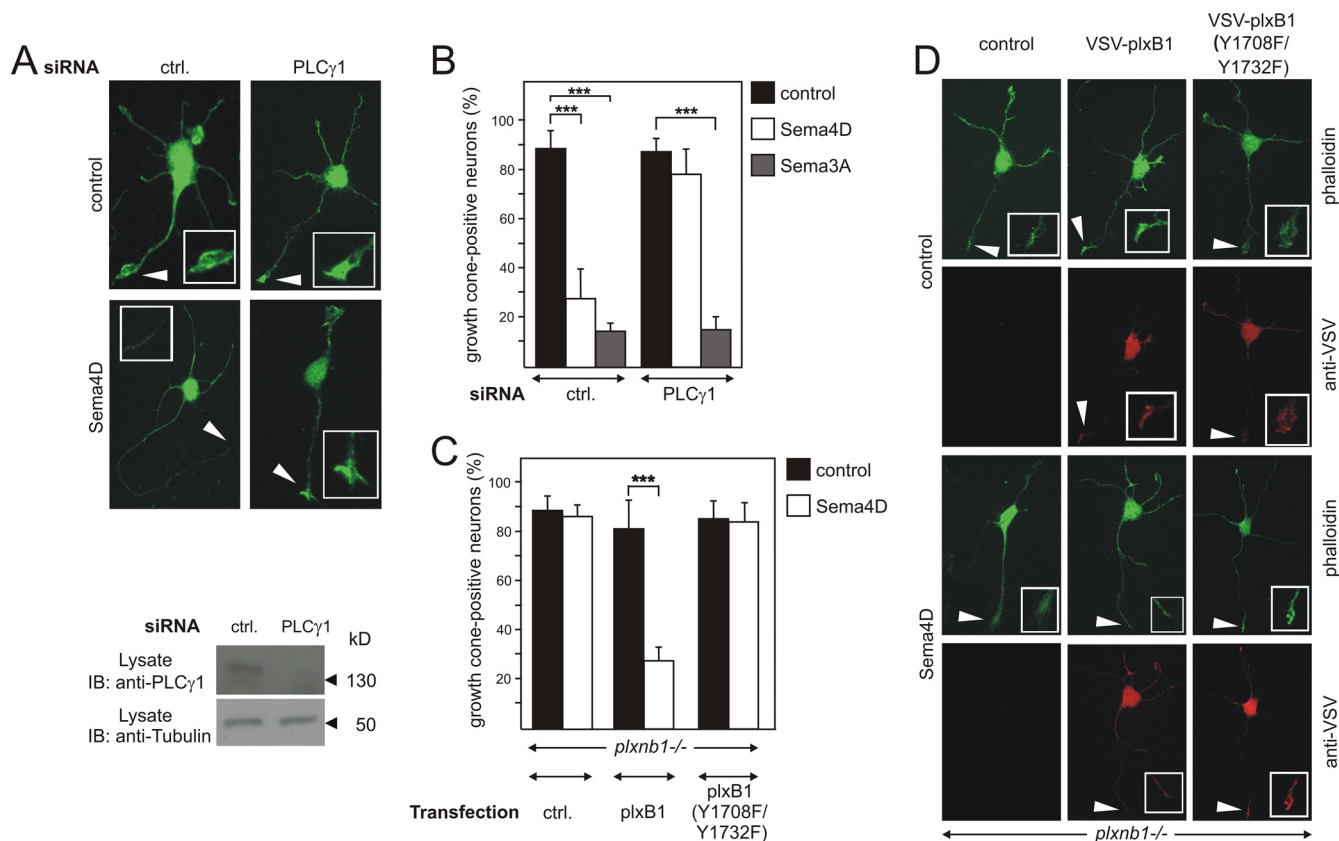


FIG. 9. Role of PLC γ 1 in plexin-B1-mediated growth cone collapse. Primary E17.5 hippocampal neurons from wild-type (A and B) or plexin-B1-deficient embryos (C and D) were transfected with control (ctrl.) siRNA or siRNA against PLC γ 1 (A and B) or with vector alone (ctrl.) or vectors carrying wild-type VSV-plexin-B1 or its mutant, [VSV-plxB1(Y1708F/Y1732F)]. At 48 h later, cells were incubated without (control) or with 150 nM Sema4D or 2 nM Sema3A. Knockdown of PLC γ 1 was verified by immunoblotting of cell lysates with an anti-PLC γ 1 antibody. Shown are representative hippocampal neurons incubated without or with Sema4D and stained with phalloidin-FITC (A and D) and counterstained with an anti-VSV antibody (D) to identify transfected cells. Insets show a magnified view of axonal ends or growth cones, which are indicated by arrows. (B) Hippocampal neurons were scored for growth cone collapse after treatment with control medium, 150 nM Sema4D, or 2 nM Sema3A. (C) Transfected neurons, identified by immunocytochemistry using anti-VSV antibodies, were scored for growth cone collapse. Shown are mean values of three independent experiments ($n = 300$; \pm standard errors of the means). ***, $P \leq 0.001$.

peptide containing phosphorylated Y1708. The fact that the siRNA-mediated knockdown of PLC γ 1 and PLC γ 2 abolished the interaction between plexin-B1 and Grb2 indicates, however, that Grb2 is brought to the plexin-B receptor complex through its interaction with PLC γ rather than through the direct interaction with the phosphorylated receptor. Grb2 could interact with PLC γ directly through an interaction of its SH3 domain with a proline-rich domain of PLC γ , as recently described (6). Alternatively, it is conceivable that Grb2 and PLC γ interact indirectly through a protein able to interact with both Grb2 and PLC γ like LAT (8). Our data showed that knockdown of Grb2 had no effect on Sema4D-induced RhoA activation or promigratory effects. Thus, the potential role of Grb2 in plexin-B-mediated signaling remains unclear.

Two types of mammalian PLC γ are encoded in mammalian genomes, PLC γ 1, which is ubiquitously expressed, and PLC γ 2, whose expression pattern is more restricted (47). Despite the fact that both PLC γ isoforms are homologous and share the domain organization, studies in cells expressing both isoforms have shown that PLC γ 1 and PLC γ 2 can have distinct functions (44, 71). Nevertheless, our data show that both enzymes can

interact with phosphorylated plexin-B1. According to their expression patterns PLC γ 1 may mediate plexin-B1-dependent RhoA activation in the nervous system, whereas PLC γ 2 may preferentially be involved in functions of plexin-B1 in the immune system (40).

The classic paradigm of PLC γ activation involves binding of the SH2 domain to activated receptor tyrosine kinases, resulting in the tyrosine phosphorylation of PLC γ and activation of its lipase function (55). Interestingly we did not observe phosphorylation of PLC γ upon treatment with Sema4D, and a mutational analysis revealed that the phospholipase catalytic domain was dispensable for mediation of plexin-B1 signaling. However, Sema4D-induced, plexin-B1-mediated effects required the presence of the SH3 domain of the PLC γ enzyme. There is evidence for PLC γ functions independent of its lipase activity (38, 50), and the SH3 domain of PLC γ 1 in particular has been shown to be necessary for downstream cellular effects of PLC γ 1, like the mitogenic response (27). The SH3 domain of PLC γ binds to multiple proteins, including SLP-76, tau, and Akt (46, 69, 72), and it can act as a guanine nucleotide exchange factor for the GTPases phosphatidylinositol-3-OH ki-

nase enhancer and dynamin-1 (11, 73). Since PLC γ and PDZ-RhoGEF interacted through the PLC γ SH3 domain and the C-terminal proline-rich region of the RhoGEF protein and since the SH3 domain of PLC γ alone was able to activate PDZ-RhoGEF but not a PDZ-RhoGEF version with a mutated proline-rich region 1332 to 1338, we think that binding of the PLC γ SH3 domain to the proline-rich region of PDZ-RhoGEF mediates RhoA activation once PLC γ has been recruited to the plexin-B1/RhoGEF receptor complex. Thus, our data identify a novel lipase-independent function of PLC γ involving its SH3 domain.

It has been shown that PDZ-RhoGEF and its homologue, LARG, oligomerize via a mechanism involving C-terminal parts of the RhoGEF proteins (10). Removal of a large C-terminal region of PDZ-RhoGEF results in constitutive RhoGEF activation, suggesting that the C terminus contains sequences which are involved in the inhibition of basal GEF activity. (10) Activation of PDZ-RhoGEF has been reported to also require the release of an autoinhibitory, intramolecular interaction involving the RGS domain and another region upstream of the DH domain of PDZ-RhoGEF and the catalytic surface of the DH domain (70, 75). However, the exact mechanism of PDZ-RhoGEF activation remains unclear (20, 54). Our data further support the regulatory role of the C-terminal region of PDZ-RhoGEF. Although we were able to rule out the influence of the SH3 domain of PLC γ 1 on homo-oligomerization of PDZ-RhoGEF, it is still possible that binding of the PLC γ SH3 domain to the C-terminal proline-rich region of PDZ-RhoGEF releases an inhibitory mechanism involving intra- or intermolecular interactions within the PDZ-RhoGEF oligomer, which then results in RhoGEF activation. One of the well-established functions of plexin-B1 is its role in regulating growth cone morphology in neurons. Both RhoA activation downstream of plexin-B1 and GAP activity toward R-Ras have been reported to be involved in plexin-B1-mediated axonal growth cone collapse (36, 57). Our data show that knockdown of PLC γ 1 abolishes the ability of Sema4D to induce growth cone collapse and that the expression of an ErbB-2 phosphorylation-resistant plexin-B1 mutant that retains its full R-Ras GAP activity does not rescue the Sema4D-induced growth cone collapse of hippocampal neurons isolated from plexin-B1-deficient mice. This further supports the concept that plexin-B1-mediated neuronal growth cone collapse requires signaling through R-Ras as well as RhoA and that the activation of RhoA depends on the recruitment of PLC γ 1 to ErbB-2-tyrosine-phosphorylated plexin-B1.

In summary, we have shown here that the activation of the plexin-B1/ErbB-2 receptor complex results in the phosphorylation of specific tyrosine residues of plexin-B1, thereby providing docking sites for the SH2-domains of PLC γ . After recruitment of PLC γ into the plexin-B1 receptor complex, PLC γ via its SH3 domain mediates the activation of PDZ-RhoGEF, which interacts constitutively with the C terminus of plexin-B1. This PLC γ -dependent signaling mechanism is required for Sema4D-induced, plexin-B1-mediated regulation of cell migration and neuronal growth cone morphology. Interestingly, the lipase activity of PLC γ is dispensable for its function in plexin-B-mediated signaling. These data identify an important new component of plexin-B-mediated signaling and reveal a novel

function of PLC γ , a versatile protein acting here as a regulator of RhoGEF proteins in plexin-B-mediated signaling.

ACKNOWLEDGMENTS

We thank Evelyne Jones (London) for kindly providing Lec3.2.8.1 CHO cells expressing part of human Sema4D and Peter Gierschik (Ulm) for kindly providing (Myc)-PLC γ 1 cDNA. We thank Martina Kurejova for support, H.-P. Gensheimer for technical assistance, and R. LeFaucheur for help with the preparation of the manuscript.

J. Swiercz and T. Worzfeld were recipients of a postdoctoral scholarship of the Medical Faculty Heidelberg.

REFERENCES

- Aurandt, J., H. G. Vikis, J. S. Gutkind, N. Ahn, and K. L. Guan. 2002. The semaphorin receptor plexin-B1 signals through a direct interaction with the Rho-specific nucleotide exchange factor, LARG. *Proc. Natl. Acad. Sci. USA* **99**:12085–12090.
- Basile, J. R., T. Afkhami, and J. S. Gutkind. 2005. Semaphorin 4D/plexin-B1 induces endothelial cell migration through the activation of PYK2, Src, and the phosphatidylinositol 3-kinase-Akt pathway. *Mol. Cell. Biol.* **25**:6889–6898.
- Basile, J. R., A. Barac, T. Zhu, K. L. Guan, and J. S. Gutkind. 2004. Class IV semaphorins promote angiogenesis by stimulating Rho-initiated pathways through plexin-B. *Cancer Res.* **64**:5212–5224.
- Basile, J. R., R. M. Castilho, V. P. Williams, and J. S. Gutkind. 2006. Semaphorin 4D provides a link between axon guidance processes and tumor-induced angiogenesis. *Proc. Natl. Acad. Sci. USA* **103**:9017–9022.
- Bielenberg, D. R., and M. Klagsbrun. 2007. Targeting endothelial and tumor cells with semaphorins. *Cancer Metastasis Rev.* **26**:421–431.
- Browaeys-Poly, E., I. Broutin, A. F. Antoine, M. Marin, A. Lescuyer, J. P. Vilain, A. Ducruix, and K. Cailliau. 2007. A non-canonical Grb2-PLC-gamma1-Sos cascade triggered by lipovitelin 1, an apolipoprotein B homologue. *Cell Signal.* **19**:2540–2548.
- Bussolino, F., D. Valdembrì, F. Caccavari, and G. Serini. 2006. Semaphoring vascular morphogenesis. *Endothelium* **13**:81–91.
- Cantrell, D. 1998. The real LAT steps forward. *Trends Cell Biol.* **8**:180–182.
- Chattopadhyay, A., M. Vecchi, Q. Ji, R. Mernaugh, and G. Carpenter. 1999. The role of individual SH2 domains in mediating association of phospholipase C- γ 1 with the activated EGF receptor. *J. Biol. Chem.* **274**:26091–26097.
- Chikumi, H., A. Barac, B. Behbahani, Y. Gao, H. Teramoto, Y. Zheng, and J. S. Gutkind. 2004. Homo- and hetero-oligomerization of PDZ-RhoGEF, LARG and p115RhoGEF by their C-terminal region regulates their in vivo Rho GEF activity and transforming potential. *Oncogene* **23**:233–240.
- Choi, J. H., J. B. Park, S. S. Bae, S. Yun, H. S. Kim, W. P. Hong, I. S. Kim, J. H. Kim, M. Y. Han, S. H. Ryu, R. L. Patterson, S. H. Snyder, and P. G. Suh. 2004. Phospholipase C- γ 1 is a guanine nucleotide exchange factor for dynamin-1 and enhances dynamin-1-dependent epidermal growth factor receptor endocytosis. *J. Cell Sci.* **117**:3785–3795.
- Conrotto, P., S. Corso, S. Gamberini, P. M. Comoglio, and S. Giordano. 2004. Interplay between scatter factor receptors and B plexins controls invasive growth. *Oncogene* **23**:5131–5137.
- Deng, S., A. Hirschberg, T. Worzfeld, J. Y. Penachioni, A. Korostylev, J. M. Swiercz, P. Vodrazka, O. Mauti, E. T. Stoekli, L. Tamagnone, S. Offermanns, and R. Kumer. 2007. Plexin-B2, but not plexin-B1, critically modulates neuronal migration and patterning of the developing nervous system in vivo. *J. Neurosci.* **27**:6333–6347.
- Driessens, M. H., H. Hu, C. D. Nobes, A. Self, I. Jordens, C. S. Goodman, and A. Hall. 2001. Plexin-B semaphorin receptors interact directly with active Rac and regulate the actin cytoskeleton by activating Rho. *Curr. Biol.* **11**:339–344.
- Driessens, M. H., C. Olivo, K. Nagata, M. Inagaki, and J. G. Collard. 2002. B plexins activate Rho through PDZ-RhoGEF. *FEBS Lett.* **529**:168–172.
- Faltus, T., J. Yuan, B. Zimmer, A. Kramer, S. Loibl, M. Kaufmann, and K. Strebhardt. 2004. Silencing of the HER2/neu gene by siRNA inhibits proliferation and induces apoptosis in HER2/neu-overexpressing breast cancer cells. *Neoplasia* **6**:786–795.
- Ferraro, E., D. Peluso, A. Via, G. Ausiello, and M. Helmer-Citterich. 2007. SH3-Hunter: discovery of SH3 domain interaction sites in proteins. *Nucleic Acids Res.* **35**:W451–W454.
- Franco, M., and L. Tamagnone. 2008. Tyrosine phosphorylation in semaphorin signalling: shifting into overdrive. *EMBO Rep.* **9**:865–871.
- Fujisawa, H. 2004. Discovery of semaphorin receptors, neuropilin and plexin, and their functions in neural development. *J. Neurobiol.* **59**:24–33.
- Fukuhara, S., H. Chikumi, and J. S. Gutkind. 2001. RGS-containing RhoGEFs: the missing link between transforming G proteins and Rho? *Oncogene* **20**:1661–1668.
- Giacobini, P., A. Messina, F. Morello, N. Ferraris, S. Corso, J. Penachioni, S. Giordano, L. Tamagnone, and A. Fasolo. 2008. Semaphorin 4D regulates

- gonadotropin hormone-releasing hormone-1 neuronal migration through PlexinB1-Met complex. *J. Cell Biol.* **183**:555–566.
22. **Giordano, S., S. Corso, P. Conrotto, S. Artigiani, G. Gilestro, D. Barberis, L. Tamagnone, and P. M. Comoglio.** 2002. The semaphorin 4D receptor controls invasive growth by coupling with Met. *Nat. Cell Biol.* **4**:720–724.
 23. **Gitler, A. D., M. M. Lu, and J. A. Epstein.** 2004. PlexinD1 and semaphorin signaling are required in endothelial cells for cardiovascular development. *Dev. Cell* **7**:107–116.
 24. **Hill, C. S., J. Wynne, and R. Treisman.** 1995. The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. *Cell* **81**:1159–1170.
 25. **Hill, C. S., J. Wynne, and R. Treisman.** 1994. Serum-regulated transcription by serum response factor (SRF): a novel role for the DNA binding domain. *EMBO J.* **13**:5421–5432.
 26. **Hirota, M., Y. Ohoka, T. Yamamoto, H. Nirasawa, T. Furuyama, M. Kogo, T. Matsuya, and S. Inagaki.** 2002. Interaction of plexin-B1 with PDZ domain-containing Rho guanine nucleotide exchange factors. *Biochem. Biophys. Res. Commun.* **297**:32–37.
 27. **Huang, P. S., L. Davis, H. Huber, P. J. Goodhart, R. E. Wegrzyn, A. Oliff, and D. C. Heimbrook.** 1995. An SH3 domain is required for the mitogenic activity of microinjected phospholipase C-gamma 1. *FEBS Lett.* **358**:287–292.
 28. **Ji, Q. S., A. Chattopadhyay, M. Vecchi, and G. Carpenter.** 1999. Physiological requirement for both SH2 domains for phospholipase C- γ 1 function and interaction with platelet-derived growth factor receptors. *Mol. Cell. Biol.* **19**:4961–4970.
 29. **Kitzing, T. M., A. S. Sahadevan, D. T. Brandt, H. Knieling, S. Hannemann, O. T. Fackler, J. Grosshans, and R. Grosse.** 2007. Positive feedback between Dia1, LARG, and RhoA regulates cell morphology and invasion. *Genes Dev.* **21**:1478–1483.
 30. **Korostylev, A., T. Worzfeld, S. Deng, R. H. Friedel, J. M. Swiercz, P. Vozdrak, V. Maier, A. Hirschberg, Y. Ohoka, S. Inagaki, S. Offermanns, and R. Kuner.** 2008. A functional role for semaphorin 4D/plexin B1 interactions in epithelial branching morphogenesis during organogenesis. *Development* **135**:3333–3343.
 31. **Kruger, R. P., J. Aurandt, and K. L. Guan.** 2005. Semaphorins command cells to move. *Nat. Rev. Mol. Cell Biol.* **6**:789–800.
 32. **Love, C. A., K. Harlos, N. Mavaddat, S. J. Davis, D. I. Stuart, E. Y. Jones, and R. M. Esnouf.** 2003. The ligand-binding face of the semaphorins revealed by the high-resolution crystal structure of SEMA4D. *Nat. Struct. Biol.* **10**:843–848.
 33. **Maeda, A., K. Nakashiro, S. Hara, T. Sasaki, Y. Miwa, N. Tanji, M. Yokoyama, H. Hamakawa, and R. Oyasu.** 2006. Inactivation of AR activates HGF/c-Met system in human prostatic carcinoma cells. *Biochem. Biophys. Res. Commun.* **347**:1158–1165.
 34. **Negishi, M., I. Oinuma, and H. Katoh.** 2005. R-ras as a key player for signaling pathway of plexins. *Mol. Neurobiol.* **32**:217–222.
 35. **Neufeld, G., and O. Kessler.** 2008. The semaphorins: versatile regulators of tumour progression and tumour angiogenesis. *Nat. Rev. Cancer* **8**:632–645.
 36. **Oinuma, I., Y. Ishikawa, H. Katoh, and M. Negishi.** 2004. The semaphorin 4D receptor plexin-B1 is a GTPase activating protein for R-Ras. *Science* **305**:862–865.
 37. **Oinuma, I., H. Katoh, A. Harada, and M. Negishi.** 2003. Direct interaction of Rnd1 with Plexin-B1 regulates PDZ-RhoGEF-mediated Rho activation by plexin-B1 and induces cell contraction in COS-7 cells. *J. Biol. Chem.* **278**:25671–25677.
 38. **Patterson, R. L., D. B. van Rossum, N. Nikolaidis, D. L. Gill, and S. H. Snyder.** 2005. Phospholipase C-gamma: diverse roles in receptor-mediated calcium signaling. *Trends Biochem. Sci.* **30**:688–697.
 39. **Perrot, V., J. Vazquez-Prado, and J. S. Gutkind.** 2002. Plexin B regulates Rho through the guanine nucleotide exchange factors leukemia-associated Rho GEF (LARG) and PDZ-RhoGEF. *J. Biol. Chem.* **277**:43115–43120.
 40. **Potiron, V., P. Nasarre, J. Roche, C. Healy, and L. Boumsell.** 2007. Semaphorin signaling in the immune system. *Adv. Exp. Med. Biol.* **600**:132–144.
 41. **Poulin, B., F. Sekiya, and S. G. Rhee.** 2000. Differential roles of the Src homology 2 domains of phospholipase C- γ 1 (PLC- γ 1) in platelet-derived growth factor-induced activation of PLC- γ 1 in intact cells. *J. Biol. Chem.* **275**:6411–6416.
 42. **Poulin, B., F. Sekiya, and S. G. Rhee.** 2005. Intramolecular interaction between phosphorylated tyrosine-783 and the C-terminal Src homology 2 domain activates phospholipase C- γ 1. *Proc. Natl. Acad. Sci. USA* **102**:4276–4281.
 43. **Püschel, A. W.** 2007. GTPases in semaphorin signaling. *Adv. Exp. Med. Biol.* **600**:12–23.
 44. **Regunathan, J., Y. Chen, S. Kutlesa, X. Dai, L. Bai, R. Wen, D. Wang, and S. Malarkannan.** 2006. Differential and nonredundant roles of phospholipase C γ 2 and phospholipase C γ 1 in the terminal maturation of NK cells. *J. Immunol.* **177**:5365–5376.
 45. **Ren, X. D., and M. A. Schwartz.** 2000. Determination of GTP loading on Rho. *Methods Enzymol.* **325**:264–272.
 46. **Reynolds, C. H., C. J. Garwood, S. Wray, C. Price, S. Kellie, T. Perera, M. Zvebil, A. Yang, P. W. Sheppard, I. M. Vardell, D. P. Hanger, and B. H. Anderton.** 2008. Phosphorylation regulates tau interactions with Src homology 3 domains of phosphatidylinositol 3-kinase, phospholipase C γ 1, Grb2, and Src family kinases. *J. Biol. Chem.* **283**:18177–18186.
 47. **Rhee, S. G.** 2001. Regulation of phosphoinositide-specific phospholipase C. *Annu. Rev. Biochem.* **70**:281–312.
 48. **Rohm, B., B. Rahim, B. Kleiber, I. Hovatta, and A. W. Puschel.** 2000. The semaphorin 3A receptor may directly regulate the activity of small GTPases. *FEBS Lett.* **486**:68–72.
 49. **Schlessinger, J., and M. A. Lemmon.** 2003. SH2 and PTB domains in tyrosine kinase signaling. *Sci. STKE* **2003**:RE12.
 50. **Smith, M. R., Y. L. Liu, N. T. Matthews, S. G. Rhee, W. K. Sung, and H. F. Kung.** 1994. Phospholipase C-gamma 1 can induce DNA synthesis by a mechanism independent of its lipase activity. *Proc. Natl. Acad. Sci. USA* **91**:6554–6558.
 51. **Soler, C., C. V. Alvarez, L. Beguinot, and G. Carpenter.** 1994. Potent SHC tyrosine phosphorylation by epidermal growth factor at low receptor density or in the absence of receptor autophosphorylation sites. *Oncogene* **9**:2207–2215.
 52. **Songyang, Z., S. E. Shoelson, M. Chaudhuri, G. Gish, T. Pawson, W. G. Haser, F. King, T. Roberts, S. Ratnoffsky, R. J. Lechleider, et al.** 1993. SH2 domains recognize specific phosphopeptide sequences. *Cell* **72**:767–778.
 53. **Songyang, Z., S. E. Shoelson, J. McGlade, P. Olivier, T. Pawson, X. R. Bustelo, M. Barbacid, H. Sabe, H. Hanafusa, T. Yi, et al.** 1994. Specific motifs recognized by the SH2 domains of Csk, 3BP2, fps/fes, GRB-2, HCP, SHC, Syk, and Vav. *Mol. Cell. Biol.* **14**:2777–2785.
 54. **Sternweis, P. C., A. M. Carter, Z. Chen, S. M. Danesh, Y. F. Hsiung, and W. D. Singer.** 2007. Regulation of Rho guanine nucleotide exchange factors by G proteins. *Adv. Protein Chem.* **74**:189–228.
 55. **Suh, P. G., J. I. Park, L. Manzoli, L. Cocco, J. C. Peak, M. Katan, K. Fukami, T. Kataoka, S. Yun, and S. H. Ryu.** 2008. Multiple roles of phosphoinositide-specific phospholipase C isozymes. *BMB Rep.* **41**:415–434.
 56. **Suzuki, K., A. Kumanogoh, and H. Kikutani.** 2008. Semaphorins and their receptors in immune cell interactions. *Nat. Immunol.* **9**:17–23.
 57. **Swiercz, J. M., R. Kuner, J. Behrens, and S. Offermanns.** 2002. Plexin-B1 directly interacts with PDZ-RhoGEF/LARG to regulate RhoA and growth cone morphology. *Neuron* **35**:51–63.
 58. **Swiercz, J. M., R. Kuner, and S. Offermanns.** 2004. Plexin-B1/RhoGEF-mediated RhoA activation involves the receptor tyrosine kinase ErbB-2. *J. Cell Biol.* **165**:869–880.
 59. **Swiercz, J. M., T. Worzfeld, and S. Offermanns.** 2008. ErbB-2 and met reciprocally regulate cellular signaling via plexin-B1. *J. Biol. Chem.* **283**:1893–1901.
 60. **Takahashi, T., A. Fournier, F. Nakamura, L. H. Wang, Y. Murakami, R. G. Kalb, H. Fujisawa, and S. M. Strittmatter.** 1999. Plexin-neuropilin-1 complexes form functional semaphorin-3A receptors. *Cell* **99**:59–69.
 61. **Tamagnone, L., S. Artigiani, H. Chen, Z. He, G. I. Ming, H. Song, A. Chedotal, M. L. Winberg, C. S. Goodman, M. Poo, M. Tessier-Lavigne, and P. M. Comoglio.** 1999. Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. *Cell* **99**:71–80.
 62. **Tanaka, M., R. Gupta, and B. J. Mayer.** 1995. Differential inhibition of signaling pathways by dominant-negative SH2/SH3 adapter proteins. *Mol. Cell. Biol.* **15**:6829–6837.
 63. **Tong, Y., P. Chugha, P. K. Hota, R. S. Alviani, M. Li, W. Tempel, L. Shen, H. W. Park, and M. Buck.** 2007. Binding of Rac1, Rnd1, and RhoD to a novel Rho GTPase interaction motif destabilizes dimerization of the plexin-B1 effector domain. *J. Biol. Chem.* **282**:37215–37224.
 64. **Tran, T. S., A. L. Kolodkin, and R. Bharadwaj.** 2007. Semaphorin regulation of cellular morphology. *Annu. Rev. Cell Dev. Biol.* **23**:263–292.
 65. **van Triest, M., and J. L. Bos.** 2004. Pull-down assays for guanosine 5'-triphosphate-bound Ras-like guanosine 5'-triphosphatases. *Methods Mol. Biol.* **250**:97–102.
 66. **Vega, Q. C., C. Cochet, O. Filhol, C. P. Chang, S. G. Rhee, and G. N. Gill.** 1992. A site of tyrosine phosphorylation in the C terminus of the epidermal growth factor receptor is required to activate phospholipase C. *Mol. Cell. Biol.* **12**:128–135.
 67. **Vikis, H. G., W. Li, and K. L. Guan.** 2002. The plexin-B1/Rac interaction inhibits PAK activation and enhances Sema4D ligand binding. *Genes Dev.* **16**:836–845.
 68. **Vikis, H. G., W. Li, Z. He, and K. L. Guan.** 2000. The semaphorin receptor plexin-B1 specifically interacts with active Rac in a ligand-dependent manner. *Proc. Natl. Acad. Sci. USA* **97**:12457–12462.
 69. **Wang, Y., J. Wu, and Z. Wang.** 2006. Akt binds to and phosphorylates phospholipase C-gy1 in response to epidermal growth factor. *Mol. Biol. Cell* **17**:2267–2277.
 70. **Wells, C. D., M. Y. Liu, M. Jackson, S. Gutowski, P. M. Sternweis, J. D. Rothstein, T. Kozasa, and P. C. Sternweis.** 2002. Mechanisms for reversible regulation between G13 and Rho exchange factors. *J. Biol. Chem.* **277**:1174–1181.
 71. **Wilde, J. I., and S. P. Watson.** 2001. Regulation of phospholipase C gamma isoforms in haematopoietic cells: why one, not the other? *Cell Signal.* **13**:691–701.

72. **Yablonski, D., T. Kadlec, and A. Weiss.** 2001. Identification of a phospholipase C- γ 1 (PLC- γ 1) SH3 domain-binding site in SLP-76 required for T-cell receptor-mediated activation of PLC- γ 1 and NFAT. *Mol. Cell. Biol.* **21**:4208–4218.
73. **Ye, K., B. Aghdasi, H. R. Luo, J. L. Moriarity, F. Y. Wu, J. J. Hong, K. J. Hurt, S. S. Bae, P. G. Suh, and S. H. Snyder.** 2002. Phospholipase C gamma 1 is a physiological guanine nucleotide exchange factor for the nuclear GTPase PIKE. *Nature* **415**:541–544.
74. **Zanata, S. M., I. Hovatta, B. Rohm, and A. W. Puschel.** 2002. Antagonistic effects of Rnd1 and RhoD GTPases regulate receptor activity in Semaphorin 3A-induced cytoskeletal collapse. *J. Neurosci.* **22**:471–477.
75. **Zheng, M., T. Cierpicki, K. Momotani, M. V. Artamonov, U. Derewenda, J. H. Bushweller, A. V. Somlyo, and Z. S. Derewenda.** 2009. On the mechanism of autoinhibition of the RhoA-specific nucleotide exchange factor PDZrhoGEF. *BMC Struct. Biol.* **9**:36.
76. **Zhou, Y., R. A. Gunput, and R. J. Pasterkamp.** 2008. Semaphorin signaling: progress made and promises ahead. *Trends Biochem. Sci.* **33**:161–170.