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Semaphorin 5A and Plexin-B3 Inhibit Human Glioma Cell Motility through RhoGDIα-mediated Inactivation of Rac1 GTPase*[S]

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Semaphorins and plexins are implicated in the progression of various types of cancer, although the molecular basis has not been fully elucidated. Here, we report the expression of plexin-B3 in glioma cells, which upon stimulation by its ligand Sema5A results in significant inhibition of cell migration and invasion. A search for the underlying mechanism revealed direct interaction of plexin-B3 with RhoGDP dissociation inhibitor α (RhoGDIα), a negative regulator of RhoGTPases that blocks guanine nucleotide exchange and sequesters them away from the plasma membrane. Glioma cells challenged with Sema5A indeed showed a marked reduction in Rac1-GTP levels by 60%, with a concomitant disruption of lamellipodia. The inactivation of Rac1 was corroborated to contribute to the impediment of glioma cell invasion by Sema5A, as supported by the abolishment of effect upon forced expression of a constitutively active Rac1 mutant. Furthermore, silencing the endogenous expression of RhoGDIα in glioma cells was found to be sufficient in abrogating the down-regulation of Rac1-GTP and the ensuing suppression of glioma cell motility induced by Sema5A. Mechanistically, we provide evidence that Sema5A promotes Rac1 recruitment to RhoGDIα and reduces its membrane localization in a plexin-B3-dependent manner, thereby preventing Rac1 activation. This represents a novel signaling of semaphorin and plexin in the control of cell motility by indirect inactivation of Rac1 through RhoGDIα.

Semaphorins represent one of the largest families of axon guidance molecules. To date, more than 30 semaphorins have been identified in vertebrates and invertebrates, which fall into eight subclasses of secreted, membrane glycosylphosphatidylinositol-anchored, and transmembrane proteins according to their structural features (1). Although initially identified as guidance cues, semaphorins and their receptor plexins have been implicated in a strikingly diverse set of biological processes ranging from cell migration, immune responses, and angiogenesis to organogenesis (2). Recent studies have reported the expression of semaphorins and plexins in various types of cancer (3–5), suggesting their emerging roles in cancer progression. The secreted class 3 semaphorins Sema3B and -3F, for instance, show potent anti-tumor effects in breast and lung cancers (6, 7). A loss of Sema3F protein is in fact significantly correlated with the advanced stage of cancer invasion (7). Furthermore, Sema3A was found to inhibit tumor cell invasive growth *in vitro* (8). In contrast to these inhibitory effects of semaphorins on cancer cells, there has been evidence indicating that members such as Sema3C and -3E instead promote tumorigenesis and tumor progression (9, 10). Similarly, transmembrane members of semaphorins can mediate both tumor progression and suppression effects in different cancer types. For instance, Sema4D is highly expressed in invading islands of head and neck squamous cell carcinoma, which when shed from the cell surface stimulates endothelial cell migration and promotes head and neck squamous cell carcinoma invasion through its receptor plexin-B1 (11). Nonetheless, gene microarray analysis of breast cancer specimens showed that low expression level of the receptor plexin-B1 correlates with a more aggressive tumor phenotype (12). In fact, it has been shown that activation of plexin-B1 signaling by the ligand Sema4D triggers its endogenous GTPase-activating protein activity toward R-Ras, thereby negatively regulating integrin functions, and may potentially suppress metastasis (13). Recently, several somatic missense mutations in the plexin-B1 gene have been identified in both primary and metastatic prostate tumor samples, which lead to a compromise of its GTPase-activating protein activity toward R-Ras, resulting in an increase in cancer cell motility and invasion (5, 14). Although these findings point to the importance of semaphorins and plexins in cancer development, the underlying mechanisms remain to be further elucidated.

The expression of Sema5A and its receptor plexin-B3 (15) has recently been shown to be correlated with the progression of pancreatic, prostate, and gastric cancers. Nonetheless, their functional roles in these cancers remain unclear because of contrasting results reported in different studies (16–19). Here, we report the expression of plexin-B3 in glioma cells of human and rat origin. Sema5A was found to impose inhibitory effect on glioma cell motility in a plexin-B3-dependent manner. We provide evidence that Sema5A suppresses glioma cell invasion by inhibiting the activation of Rac1 GTPase through its negative modulator RhoGDIα. This represents a novel mechanism

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through which semaphorins and plexins regulate cancer cell motility.

EXPERIMENTAL PROCEDURES

Antibodies—Antibodies directed against hemagglutinin (HA), glutathione *S*-transferase (GST), maltose-binding protein (MBP),[2] and RhoGDIα were purchased from Santa Cruz Biotechnology, Inc. Antibodies to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and human IgG-Fc were from Chemicon International. Rac1 antibody was obtained from BD Transduction Laboratories. Peroxidase-conjugated anti-human IgG-Fc was from Sigma. Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Pierce.

Cell Culture and Transfection—C6 and U87-MG glioma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The glioma cells and HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin (100 IU/ml)/streptomycin (100 μg/ml), at 37 °C in a humidified atmosphere of 5% CO_2. For transfection experiments, cells grown to ~80% confluence were transiently transfected with expression constructs using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions.

RT-PCR—Total RNA isolated from cells using the RNeasy mini kit (Qiagen) was reverse-transcribed by Superscript II reverse transcriptase (Invitrogen) and oligo(dT) primer. The resulting cDNA was then subjected to amplifications by PCR using the following primer pairs: rat plexin-B3 (5′-AACCCTGACCCTTCTCT-3′ and 5′-CATGACTAAGTGGAGCCAGG-3′), human plexin-B3 (5′-TCAACATTTCCGAGGCCTGC-3′ and 5′-TGCCTGGTGGCTGGCTCTTA-3′), rat GAPDH (5′-GTGCAGTGCCAGCCTCGTC-3′ and 5′-GGCAGCACCAGTGGATGCA-3′), and human GAPDH (5′-TTTGCGTCGCCAGCCGAGC-3′ and 5′-TTGGCAGCGCCAGTAGAGG-3′). PCR products were then analyzed by agarose gel electrophoresis.

Production of Soluble Sema5A-Fc and Fc Proteins—To produce soluble Sema5A, HEK293 cells were transfected with an expression construct, which encompasses a fusion cDNA between the extracellular domain of Sema5A and the Fc region of human IgG (Sema5A-Fc). An expression plasmid harboring the human IgG-Fc alone serves as the control (Fc control). Transfected cells were allowed to recover in DMEM containing 10% FBS for 20 h prior to incubation in serum-free DMEM for 2 days. Conditioned medium containing Sema5A-Fc or Fc was purified and concentrated using Centricon column (Millipore), followed by Western blot analysis using anti-human IgG-Fc antibodies. For all functional studies, Sema5A-Fc and Fc proteins were pretreated with anti-human IgG-Fc antibodies at a concentration of 7.5 μg/ml for multimerization.

Scratch Wound Assay—The migration of glioma cells was analyzed by wound-healing assay as described with modifications (20). Briefly, cells (4×10^5/well) were seeded into 6-well culture dishes and allowed to grow to near-confluency. Physical "scratch wounds" were created on the cell layer using a p-20-μl pipette tip to stimulate cell migration. After a brief wash in DMEM to remove dislodged cells, the culture was incubated in conditioned medium containing Sema5A-Fc or Fc protein for 24 h. "Wound closure" was monitored and documented by photomicrography for quantification of the number of migrated cells.

Invasive Growth Assays—Glioma cell invasion was analyzed by *in vitro* transfilter Matrigel assay. Briefly, Transwell insert (5 μm pore size, Costar) was pretreated with Matrigel solution (1 mg/ml in DMEM, BD Biosciences) at 37 °C for 2 h. An aliquot of 2×10^4 cells suspended in serum-free DMEM was plated in Matrigel-coated Transwell insert and allowed to migrate and invade toward either Sema5A-Fc or Fc control protein in the lower chamber for 24 h. Cells that remained on the inner side of the membrane were removed with a cotton swab, and those that have invaded through the Matrigel to the outer side of the membrane were stained with 0.2% crystal violet in 0.9% NaCl containing 10% ethanol. Cells were photographed, and the dye was solubilized in 10% acetic acid to measure absorbance at 590 nm in a microplate reader.

Gene Silencing—Glioma cells were transfected with siRNAs against plexin-B3, RhoGDIα, or control siRNAs in scrambled irrelevant sequences using the transfection reagent Lipofectamine RNAiMAX according to manufacturer's protocol (Invitrogen). Briefly, cells were plated in antibiotics-free DMEM supplemented with 10% FBS and 100 pmol of siRNA in transfection reagent and cultured in a CO_2 incubator to reach 30–50% confluence in 24 h. The efficiency and specificity of plexin-B3 and RhoGDIα down-regulation were assessed by Western blot analysis 24–72 h post-transfection.

Yeast Two-hybrid Library Screening—A GAL4-based two-hybrid interaction screen of adult mouse brain cDNA library (Clontech) was performed using the cytoplasmic domain of plexin-B3 as a bait. Briefly, yeast host AH109 harboring the bait expression plasmid pGBKT7/plexin-B3CD was mixed with Y187 yeast host pretransformed with the library according to the manufacturer's instructions. The transformants were screened on the quadruple dropout selection medium SD/−Ade/−His/−Leu/−Trp. Positive colonies that survive the selection were confirmed with X-α-Gal assay. Candidate plasmids were then isolated from the yeast and expanded in *Escherichia coli* for sequence determination. Interactions between plexin-B3CD and the positive clones were confirmed by co-transforming the bait plasmid and the respective AD/library plasmids into the yeast host AH109, or via reciprocal mating of the yeast hosts AH109 and Y187 transformed with the AD/library clone and the bait plasmid, respectively. The interaction was further verified in mammalian cells by pull-down and co-immunoprecipitation assays.

Recombinant Protein Expression—To express RhoGDIα as a fusion protein with MBP, full-length cDNA of RhoGDIα was amplified by RT-PCR from adult mouse brain and cloned into the pMALC2 vector via XbaI and EcoRI sites. The cytoplasmic domain of mouse plexin-B3 was expressed as a GST fusion protein by cloning the PCR-amplified cDNA fragment into the EcoRI and BamHI sites of pGEX-KG vector. p21-activated kinase 1 (PAK1) (amino acids 69–140) with an HA epitope at

[2] The abbreviations used are: MBP, maltose-binding protein; TRITC, tetramethylrhodamine isothiocyanate; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate.

the C terminus was cloned into the pGEX-KG vector via EcoRI and NotI sites. Each of these expression constructs was transformed into the BL21 strain of *E. coli,* and the expression of recombinant proteins was induced by 0.5 to 1 mM of isopropyl β-D-thiogalactopyranoside. Bacteria were subjected to sonication, and the GST or MBP fusion proteins in the soluble fraction of the resulting bacterial lysate were purified with GSH-Sepharose 4B beads (Amersham Biosciences) or amylose resin (New England Biolabs), respectively, following the manufacturers' instructions. Correct expression of these proteins was confirmed with Coomassie Blue staining and Western blot analysis.

GST Pulldown Assays—Purified MBP or MBP-RhoGDIα was incubated with 20 μl of GST or GST-plexin-B3CD prebound to GSH-Sepharose in a binding buffer (20 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT) containing 0.1% Triton X-100, 1 mM PMSF, and 1× protease inhibitor mixture (Roche Applied Science) for 2 h. Beads were collected by centrifugation, washed three times with 1× PBS, and eluted in SDS sample buffer, followed by SDS-PAGE and Western blot analysis. To verify the yeast two-hybrid screening results in mammalian cells, mouse brain lysate was subjected to pulldown assay with GST-plexin-B3CD. Briefly, postnatal day 7 mouse brain was homogenized in 1 ml of binding buffer supplemented with 0.1% Triton X-100, 1 mM PMSF, and 1× protease inhibitor mixture. After incubation for 30 min at 4 °C with gentle shaking, the brain lysate was precleared with 30 μl of GSH-Sepharose, followed by overnight incubation with 30 μl of GST or GST-plexin-B3CD prebound to GSH-Sepharose beads at 4 °C with gentle agitation. The beads were then washed three times with the binding buffer. Proteins were eluted in sample buffer and subjected to Western blot analysis using anti-RhoGDIα antibody and HRP-conjugated secondary antibody, followed by chemiluminescence detection with x-ray radiography.

Immunoprecipitation—To assay for the binding between RhoGDIα and Rac1, glioma cells at 50–70% confluence were treated with Sema5A-Fc or Fc control protein for 30 min. Cells were then lysed in ice-cold RIPA buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM $Na_2P_2O_7$, 1 mM NaF, 1 mM EDTA, 2 mM Na_3VO_4) containing 1× protease inhibitor mixture for 15 min. 200 μg of precleared lysate were incubated with 2 μg of anti-RhoGDIα antibody overnight with agitation at 4 °C. 20 μl of protein A-agarose (Santa Cruz Biotechnology) was then added and incubated for 2 h. The immunoprecipitates were washed three times with PBS before Western blot analysis using anti-Rac1 and anti-RhoGDIα antibodies. To confirm the interaction of plexin-B3 with RhoGDIα in glioma cells by immunoprecipitation, 200 μg of precleared cell lysates were incubated with antibodies specific for plexin-B3 or RhoGDIα overnight, followed by 20 μl of protein A-agarose for 2 h. The immunoprecipitates were washed twice with PBS and subjected to Western blot analysis.

RhoGTPase Activation Assays—Glioma cells stimulated with Sema5A-Fc or Fc control at predetermined time points were extensively washed with PBS prior to lysis in RIPA buffer containing 1× protease inhibitor. Lysates were clarified by centrifugation at 14,000 rpm for 10 min at 4 °C. Rac1-GTP level was then determined with a PAK1 binding domain pulldown assay previously described with modifications (21, 22). Briefly, 100 μg of lysates were precleared with GSH-Sepharose beads in a binding buffer (20 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 3 mM $MgCl_2$) containing 1× protease inhibitor mixture for 15 min at 4 °C. The lysate was then incubated for 45 min at 4 °C with GST-PAK1 binding domain (GST-PBD) fusion protein coupled to GSH-Sepharose. Bound complexes were pelleted and washed twice in PBS. The beads and total cell lysate were subjected to Western blot analysis using monoclonal anti-Rac1 and polyclonal anti-Cdc42 antibody. The intensity of immunoreactive signals was quantified with Quantity One software (Bio-Rad).

Immunocytochemical Staining of Actin Cytoskeleton—Glioma cells grown to 50% confluence on fibronectin-coated coverslips were stimulated with Sema5A-Fc or Fc protein for 30 min. Changes in the actin cytoskeleton were then revealed by phalloidin stain. Briefly, cells were fixed in 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 2 min, followed by incubation in a 2% bovine serum albumin (BSA) blocking solution for 1 h at room temperature. Cells were then stained with 200 ng/ml TRITC-conjugated phalloidin (Sigma) for 1 h at room temperature. Nucleus was stained with 4′,6-diamidino-2-phenylindole (DAPI). Coverslips were mounted on microscopic slides using an antifade mounting agent (Aqua PolyMount, Polysciences) and examined under a confocal microscope (LSM 510, Zeiss).

Subcellular Fractionation—Glioma cells treated with Sema5A-Fc or Fc control protein for 30 min were harvested and resuspended in ice-cold hypotonic lysis buffer (HBL) (10 mM Tris-Cl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA) supplemented with 1× protease inhibitor and 1 mM PMSF for 10 min to allow cell swelling. Cells were then lysed by Dounce homogenization (40 strokes) with a tight fitting pestle, and the lysates were cleared by low speed centrifugation at 10,000 rpm for 10 min at 4 °C to remove insoluble cell debris, nuclei, and intact cells. The post-nuclear supernatant was centrifuged at 150,000 × *g* for 1 h at 4 °C to obtain the cytosolic (soluble) and membrane (pellet) fractions. Equal amounts of proteins from these two fractions were subjected to Western blot analysis using anti-Rac1 antibody.

Statistical Analysis—All data are mean ± S.D. from at least three independent experiments. Differences between groups were analyzed by Student's *t* test. $p < 0.05$ is considered statistically significant.

RESULTS

Sema5A Inhibits Glioma Cell Migration and Invasion through Plexin-B3—The presence of plexin-B3 expression in glioma cells was first examined by RT-PCR analysis. As shown in Fig. 1*A*, plexin-B3 was found to be endogenously expressed in rat C6 glioma cells (*lane 1*). The same primer pair also successfully amplified plexin-B3 in rat oligodendrocyte precursor cells, which is known to express plexin-B3 (Fig. 1*A*, *lane 2*). Western blot analysis of both rat and human glioma cells (Fig. 1*B*, *lanes 1* and *2*, respectively) using a plexin-B3-specific antibody revealed a 260-kDa protein, consistent with that detected in HEK293 cells heterologously expressing full-length plexin-B3 (Fig. 1*B*, *lane 3*).

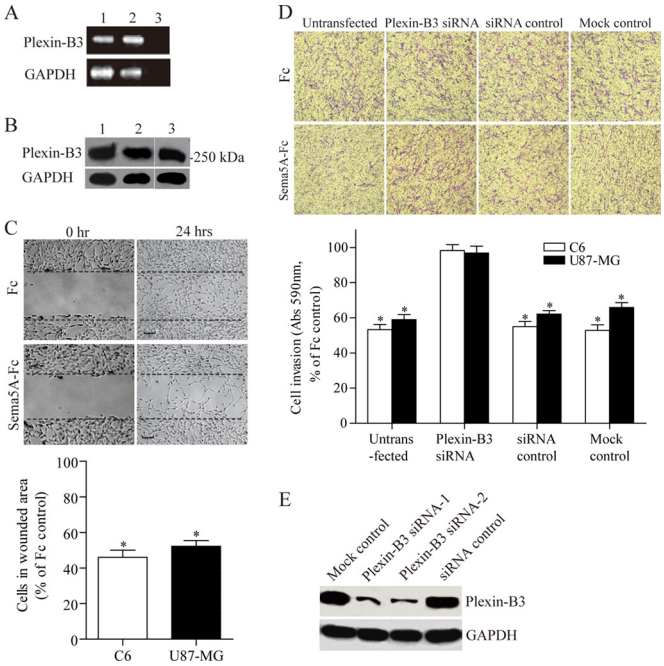

FIGURE 1. **Plexin-B3 is expressed in glioma cells and mediates the inhibitory signals of Sema5A in cell motility.** *A,* RT-PCR analysis of plexin-B3 expression in rat C6 glioma cells (*lane 1*). Rat oligodendrocyte precursor cells, which endogenously express plexin-B3, serve as a positive control (*lane 2*). *Lane 3* is the negative control in which RT product was excluded in the PCR mix. Amplification of GAPDH serves as an internal control. *B,* Western blot detection of endogenous plexin-B3 expression in rat C6 (*lane 1*) and human U87-MG (*lane 2*) glioma cells as an ~260-kDa protein using plexin-B3CD antibody, which is also detected in HEK293 cells heterologously expressing plexin-B3 (*lane 3*). *C,* scratch wound assay of glioma cell migration 24 h after Sema5A-Fc or Fc treatment. Photographs were taken for quantification of the number of cells migrated into the wounded area, and the results are summarized in the *bar chart*. Cell migration in the Sema5A-Fc group was expressed as a percentage to that in the Fc control. *, $p < 0.01$ *versus* Fc control. *Scale bar,* 100 μm. *D,* modified transwell assay of glioma cell invasion upon Sema5A-Fc or Fc stimulation for 24 h. Cells invaded through the Matrigel were stained with crystal violet. The degree of cell invasion was measured by the absorbance of dye eluted from the cells. Values of the Sema5A-Fc-treated groups were expressed as a percentage of those of the Fc controls. The dependence of plexin-B3 in relaying the effect of Sema5A on glioma cell invasion was assessed by siRNA-mediated silencing of endogenous plexin-B3 expression. Cells subjected to scrambled siRNA or mock transfection serve as controls. *, $p < 0.01$ *versus* Fc control. *E,* Western blot analysis of plexin-B3 expression in C6 glioma cells separately treated with two siRNAs that target different regions of plexin-B3 (plexin-B3 siRNA-1 and -2). siRNA of scrambled sequence and mock transfection serve as controls. Equal loading of protein across samples was verified with GAPDH antibody.

Given the high invasiveness of glioma cells, we asked whether plexin-B3 plays a role in the regulation of glioma motility. To begin with, rat C6 and human U87-MG glioma cells were challenged with Sema5A, the functional ligand for plexin-B3 (15), and monitored for changes in cell migration using scratch wound assay. Similar to untreated cells, glioma cells treated with Fc control protein showed active migration and attained a near-complete closure of the scratch wound in 24 h (Fig. 1*C*). Nonetheless, cells subjected to Sema5A-Fc stimulation displayed a significant reduction in migration by 50% as compared with the Fc control (Fig. 1*C*). Next, the effect of Sema5A on glioma cell invasion was evaluated by an *in vitro* transfilter Matrigel invasion assay. Glioma cells untreated or subjected to Fc control protein stimulation both actively invade through the Matrigel (Fig. 1*D*). In contrast, Sema5A-Fc significantly reduced the rate of cell invasion to 55% that in the Fc control. To ascertain the role of plexin-B3 in relaying the signal of Sema5A in inhibiting glioma cell invasion, the endogenous expression of plexin-B3 in glioma cells was subjected to down-regulation by RNAi prior to Sema5A stimulation. Western blot analysis of cells transfected with plexin-B3 siRNA-1 or -2 indicated a >80% reduction in plexin-B3 protein expression 48 h post-treatment, whereas scrambled siRNA control and mock transfection have no effect (Fig. 1*E*). When glioma cells transfected with siRNA control or mock transfected were challenged with Sema5A-Fc, cell invasion was significantly impeded to an extent similar to that in untransfected cells (Fig. 1*D*). Nonetheless, the effect was almost completely abolished when endogenous expression of plexin-B3 in glioma cells was silenced (Fig. 1*D*). These findings suggest that Sema5A inhibits glioma cell motility through plexin-B3. In addition, Sema5A was found to impose no appreciable effects on glioma cell proliferation throughout the course of motility assays (data not shown), further supporting its function in suppressing glioma cell migration and invasion.

Cytoplasmic Domain of Plexin-B3 Directly Interacts with RhoGDIα—Semaphorins and plexins are known to control cell motility by regulating cytoskeletal dynamics through various mechanisms (13, 23–25). To understand the way Sema5A and plexin-B3 inhibit glioma cell motility, we began by characterizing the signal transduction pathways mediated by plexin-B3. A yeast two-hybrid screening for molecules that interact with the cytoplasmic domain of plexin-B3 (plexin-B3CD) revealed candidate clones that showed complete sequence identity to the C-terminal 130 amino acids of RhoGDP dissociation inhibitor α (RhoGDIα), which is known to negatively regulate RhoGTPases by sequestering prenylated GTPases from membranes, blocking guanine nucleotide exchange, and maintaining them in the inactive form in cytosolic complexes (26–28). To confirm direct interaction between RhoGDIα and plexin-B3CD, they were expressed as MBP and GST fusion proteins, respectively. The purified recombinant proteins were then subjected to GST pulldown assays. The results showed that RhoGDIα co-precipitated with plexin-B3CD but not with GST protein alone (Fig. 2*A*). Interaction of plexin-B3CD and RhoGDIα in mammalian brain was also confirmed with *in vitro* binding assay by incubating mouse total brain lysate with GST-plexin-B3CD, as shown in Fig. 2*B*. To verify the interaction of endogenous plexin-B3 with RhoGDIα in glioma cells, C6 cells were subjected to three different treatments: (i) nonstimulated, (ii) challenged with Fc control protein, or (iii) challenged with Sema5A-Fc protein prior to immunoprecipitation. Plexin-B3 was found to co-precipitate with RhoGDIα in a Sema5A-dependent manner (Fig. 2*C*, *upper panel*). The result was corroborated in reciprocal immunoprecipitation assay using anti-RhoGDIα antibodies (Fig. 2*C*, *lower panel*). Plexin-B3 represents the first plexin member and is among the few cell surface receptors that directly interact with RhoGDIα. Next, the RhoGDIα-binding site was mapped by GST pulldown assay using various truncation mutants of plexin-B3CD. Although plexin-B3CD-A–C (which together cover the entire intracellular domain of plexin-B3) all interact with RhoGDIα, the N-terminal fragment A shows significantly stronger binding than B and C, and at a level well above that of full-length plexin-B3CD

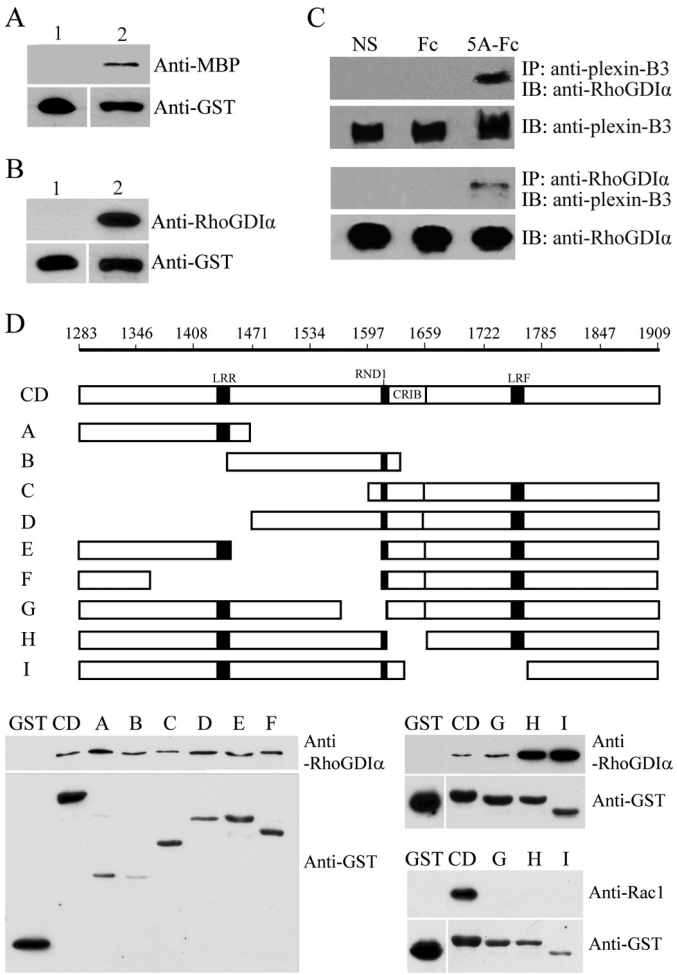

FIGURE 2. **RhoGDIα is an interacting partner of plexin-B3.** *A,* direct interaction of RhoGDIα and the cytoplasmic domain of plexin-B3 (plexin-B3CD) was confirmed by GST pulldown assays. GST (*lane 1*) or GST-plexin-B3CD (*lane 2*) prebound to GSH-Sepharose was incubated with purified MBP-RhoGDIα fusion protein. Pulldown samples were subjected to Western blot analysis using anti-MBP antibody. *B, in vitro* binding assay confirmed the interaction of RhoGDIα in mouse brain lysate with GST-plexin-B3CD (*lane 2*) but not with GST (*lane 1*) prebound to GSH-Sepharose. The pulldown samples were analyzed by Western blot using anti-RhoGDIα antibody. *C,* interaction of endogenous plexin-B3 and RhoGDIα in C6 glioma cells nonstimulated (*NS*), challenged with Fc or Sema5A-Fc (*5A-Fc*) was determined by immunoprecipitation (*IP*) using anti-plexin-B3 antibodies and analyzed by anti-RhoGDIα in Western blot (*upper panel*). Reciprocal immunoprecipitation was performed using anti-RhoGDIα antibody, and the immunoblot (*IB*) was detected with plexin-B3 antibody (*lower panel*). *D,* mapping RhoGDIα-binding site on plexin-B3CD. Various deletion mutants of plexin-B3 were expressed as GST fusion proteins and subjected to pulldown assay with RhoGDIα protein. The resulting pellet was analyzed by Western blot using anti-RhoGDIα antibody.

(Fig. 2*D*). Further analysis revealed that deletion mutants excluding either fragment A or B (plexin-B3CD-D and -E, respectively) still show stronger interaction than fragment C alone (Fig. 2*D*). In light of the former report of intramolecular interaction between the N and C terminus of the cytoplasmic domain of plexin-B1 (29), we proposed that plexin-B3CD-C might interact with -A and -B, thereby masking the major RhoGDIα binding regions on the latter. Evidence in support of this postulation includes the confirmation of direct binding of GST-plexin-B3CD-A and -B with MBP-plexin-B3CD-C (supplemental Fig. S1). Furthermore, the deletion mutants plexin-B3CD-H and -I (that lack the CRIB domain (30) or the N-terminal half of fragment C, respectively) allow robust binding to RhoGDIα (Fig. 2*D*). Notably, the deleted domains in these two mutants are critical for Rac1 binding. Nonetheless, the Rnd1-binding site is only essential for Rac1 but not RhoGDIα interaction (Fig. 2*D*). In short, the N-terminal half of plexin-B3CD shows preferential binding to RhoGDIα, which, however, can be impaired by the C-terminal Rac1-binding sites via intramolecular interaction. It is tempting to speculate that Sema5A stimulation plausibly promotes conformational changes in plexin-B3CD, which together with the recruitment of Rac1 to the CRIB region would facilitate the binding of RhoGDIα in a ligand-dependent manner.

Sema5A and Plexin-B3 Negate Rac1 Activation and Inhibit Lamellipodia Formation in Glioma Cells—GTPases of the Rho superfamily have been known to play pivotal roles in the control of cell motility through the regulation of F-actin cytoskeletal dynamics (31, 32). The finding of direct binding between plexin-B3 and RhoGDIα therefore prompted us to assay for the activation status of various RhoGTPases in glioma cells upon Sema5A stimulation. Using GST-PAK1 pulldown and Rhotekin G-LISA™ assays, the levels of active Rac1/Cdc42 and RhoA were measured accordingly. Sema5A stimulation was found to induce a significant reduction in the level of active Rac1 at a time point as early as 20 min post-treatment. A further reduction of Rac1-GTP to 40% that in the nonstimulated control was observed at 30 min and remained at that level 60 min post-treatment (Fig. 3*A*). Glioma cells similarly treated with Fc control protein, however, showed no major changes in Rac1-GTP level throughout the course of the experiment. Assays for active Cdc42 and RhoA in glioma cells challenged with Sema5A-Fc or Fc protein under the same conditions also revealed no significant changes (supplemental Fig. S2). To ascertain the inactivation of Rac1 by Sema5A is mediated through plexin-B3, the experiments were repeated in glioma cells in which the expression of endogenous plexin-B3 was silenced by RNAi. Although a significant reduction in Rac1-GTP levels was still observed in the siRNA control and mock transfection groups, the negative effect induced by Sema5A-Fc was abrogated when plexin-B3 expression in the cells was down-regulated (Fig. 3*B*).

Rac1 activation is known to organize the cytoskeleton into lamellipodia, which are important structures involved in cell migration (33). The significant reduction of Rac1-GTP level and the inhibition of cell motility in glioma cells induced by Sema5A point to potential alterations in the actin cytoskeleton. In fact, C6 glioma cells that are nonstimulated or treated with Fc control protein are rich in lamellipodia at cell edges (Fig. 4*A*, *panels i* and *ii, arrows*). When challenged with Sema5A-Fc, however, lamellipodia formation was compromised as early as 15 min. Remarkably, a significant reduction of lamellipodia to almost undetectable levels was observed at 30 min post-treatment (Fig. 4*A*, *panel iii*). The disruption of actin-based lamellipodia was abolished when endogenous plexin-B3 expression in glioma cells was silenced by siRNA (Fig. 4*A*, *panel iv*), which otherwise persisted in the scrambled siRNA or mock transfection controls (Fig. 4*A*, *panels v* and *vi*). Notably, these cytoskeletal changes temporally coincided with the suppression of Rac1 activation by Sema5A (Fig. 3*A*). In summary, Sema5A represses

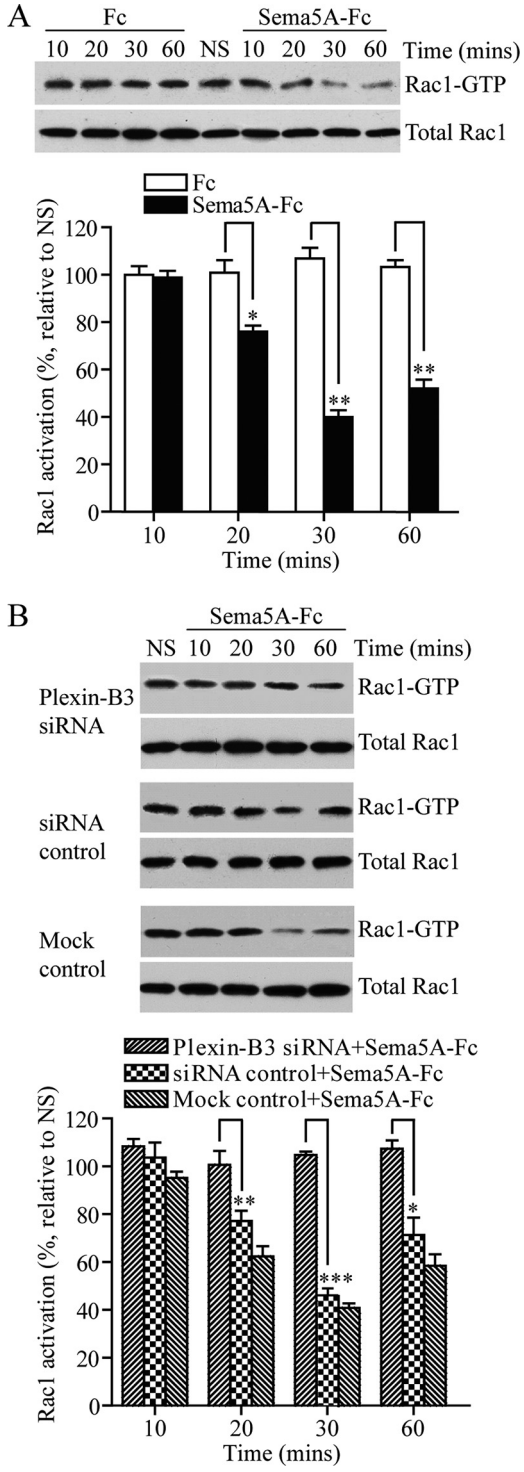

FIGURE 3. **Sema5A inhibits Rac1 activation in glioma cells via plexin-B3.** *A,* levels of Rac1-GTP in glioma cells at various time points after Fc or Sema5A-Fc stimulation were measured by GST-PBD pulldown assay and analyzed by Western blot using anti-Rac1 antibody. Band intensity of Rac1-GTP (*upper panel*) was normalized against that of total Rac1 (*lower panel*) in each sample. The level of active Rac1 in cells challenged with Fc or Sema5A-Fc was expressed as a percentage to that in the nonstimulated (*NS*) reference. *, $p < 0.05$; **, $p < 0.01$ *versus* Fc control. *B,* glioma cells transfected with siRNAs against plexin-B3 or in scrambled sequence were challenged with Sema5A-Fc or nonstimulated. Relative levels of active Rac1 in these cells were analyzed as described above. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *versus* plexin-B3 siRNA.

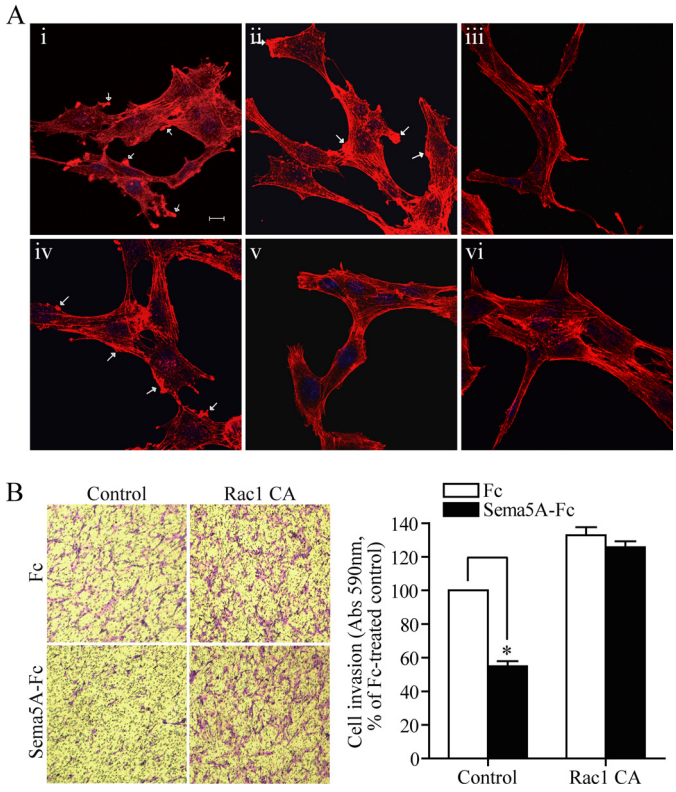

FIGURE 4. **Sema5A disrupts lamellipodia and inhibits cell invasion in glioma through Rac1 inactivation.** *A,* actin cytoskeleton of C6 glioma cells was stained with TRITC-conjugated phalloidin. Extensive lamellipodia were observed at the periphery of cells subjected to no treatment (*panel i*) or Fc stimulation (*panel ii*) (*arrows*) but not in cells challenged with Sema5A-Fc for 30 min (*panel iii*). The disruption of lamellipodia by Sema5A-Fc was abolished in cells transfected with an siRNA against plexin-B3 (*panel iv*). Cells subjected to scrambled siRNA (*panel v*) or mock transfection (*panel vi*) still exhibited significant regression of lamellipodia in response to Sema5A-Fc stimulation. *Scale bar,* 20 μm. *B,* glioma cells transfected with constitutively active Rac1 (*Rac1 CA*) or mock-transfected were treated with Fc or Sema5A-Fc and subjected to Transwell invasion assay. Cell invasion through Matrigel was measured by the absorbance of crystal violet dye eluted from the invading cells and expressed as a percentage of that in Fc-treated mock transfection control. *, $p < 0.01$ *versus* Fc-treated mock transfection control.

Rac1 activation and lamellipodia formation through plexin-B3 in C6 glioma cells.

Sema5A Inhibits Glioma Cell Invasion through Rac1 Inactivation—Rac1 activation is commonly observed in various cancers and is associated with increased invasiveness of glioblastomas (34, 35). It is tempting to speculate that the suppression of Rac1 activation by Sema5A might have contributed to the inhibition of glioma cell motility. To test this hypothesis, C6 glioma cells were transfected with a constitutively active Rac1-Q61L mutant and subjected to Transwell Matrigel invasion assays in the presence of Sema5A-Fc or Fc control protein. Although Sema5A-Fc still imposed the same degree of inhibition of cell invasion in the control, the inhibitory effect was completely abolished upon forced expression of active Rac1 (Fig. 4*B*). Although it can be argued that an overexpression of active Rac1 also promotes cell invasion in Fc-treated cells by 30%, the percentage increase in the Sema5A-Fc group is well beyond that and reaches over 70%. These findings suggest that the impairment of C6 glioma cell motility by Sema5A and plexin-B3 is mediated through Rac1 inactivation. Similarly, the inhibition of

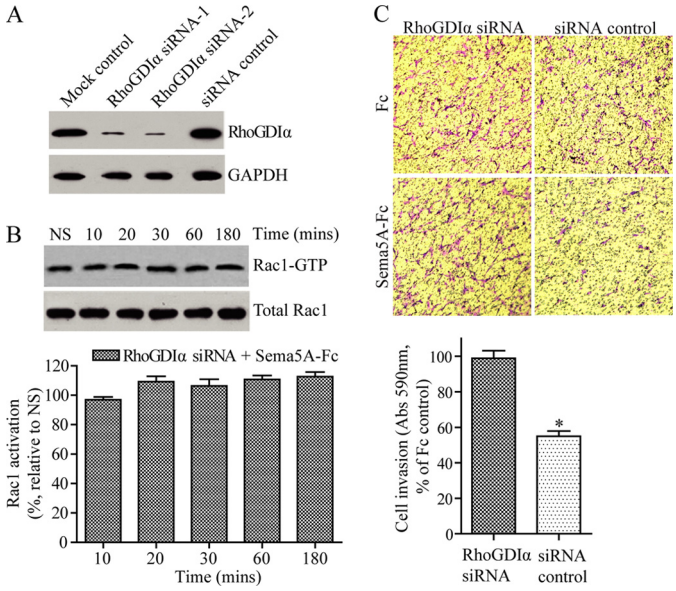

FIGURE 5. **RhoGDIα is required for Sema5A-induced Rac1 inactivation and inhibition of cell invasion in C6 glioma.** *A,* Western blot analysis of endogenous expression of RhoGDIα in C6 glioma cells separately treated with two siRNAs specific for different regions of RhoGDIα (RhoGDIα siRNA-1 and -2). siRNA of scrambled sequence and mock transfection serve as controls. Equal loading of protein across samples was verified by GAPDH antibody. *B,* silencing of RhoGDIα expression in C6 glioma abolished the down-regulation of active Rac1 by Sema5A. C6 cells transfected with RhoGDIα siRNA were challenged with Sema5A-Fc, Fc control (data not shown), or non-stimulated (*NS*). Rac1-GTP level in the cells was determined by PAK1 binding domain pulldown assay and Western blot using Rac1 antibody. The values were normalized against total Rac1 and expressed as a percentage to the nonstimulated reference. *C,* involvement of RhoGDIα in Sema5A-induced inhibition of invasion in C6 glioma cells was examined by modified Transwell assays. C6 cells transfected with RhoGDIα-specific or scrambled siRNA were plated onto the Matrigel-coated porous membrane in Transwell insert and were allowed to migrate toward Sema5A-Fc or Fc-conditioned medium in the lower chamber for 24 h. Cell invasion through the Matrigel was measured by the absorbance of crystal violet dye eluted from the invading cells. Values of the Sema5A-Fc group were expressed as a percentage to that of the Fc-treated control. *, $p < 0.001$ *versus* RhoGDIα siRNA group.

human glioma cell invasion can be rescued by forced expression of active Rac1 (data not shown).

Sema5A and Plexin-B3 Inhibit Rac1 Activation and Cell Invasion in C6 Glioma through RhoGDIα—Having confirmed the involvement of Rac1 inactivation in Sema5A-induced impediment of glioma cell motility, the mechanism through which plexin-B3 down-regulates Rac1-GTP was sought. To this end, RhoGDIα represents a promising candidate because it is a known negative modulator of GTPases, including Rac1, and can directly interact with plexin-B3 as shown above. We therefore proposed that RhoGDIα might act downstream of plexin-B3 to inactivate Rac1 in C6 glioma cells. Based on this hypothesis, a down-regulation of endogenous RhoGDIα expression in glioma cells would mitigate the effects of Sema5A in suppressing Rac1 activation and cell motility. To test this, we began by independently transfecting C6 cells with two siRNAs specific for rat RhoGDIα. Both siRNAs were found to suppress RhoGDIα protein expression by >90% 48–72 h post-treatment, whereas the scrambled siRNA and mock transfection controls showed no effects (Fig. 5*A*). When cells treated with RhoGDIα siRNA were challenged with Sema5A-Fc, no major change in Rac1-GTP level was observed throughout the course of study (Fig. 5*B*). This suggests that RhoGDIα is involved in Rac1 inactivation induced by Sema5A and plexin-B3. Because our data indicated that Sema5A impedes glioma cell motility through Rac1 inactivation, we next asked whether the knockdown of RhoGDIα would abolish Sema5A-induced inhibition of C6 glioma cell invasion. As shown in Fig. 5*C*, Sema5A-Fc indeed failed to induce any significant blockade of cell invasion when the endogenous expression of RhoGDIα in glioma cells was down-regulated. In contrast, Sema5A-Fc still imposed the same degree of inhibition of invasion in cells treated with scrambled siRNA control (Fig. 5*C*). Taken together, RhoGDIα functions to mediate the signals of Sema5A and plexin-B3 in inactivating Rac1 and inhibiting invasion in glioma cells.

Sema5A Promotes the Association of Rac1 with RhoGDIα and Reduces Its Membrane Localization—RhoGDIα is known to negatively regulate RhoGTPases by sequestering the prenylated form from membranes, blocking guanine nucleotide exchange, and maintaining them in the inactive form in cytosolic complexes (26–28). The role of RhoGDIα in the inactivation of Rac1 by Sema5A and plexin-B3 as shown above may involve an enhancement of its interaction with Rac1. To test this hypothesis, C6 glioma cells stimulated with Sema5A-Fc or Fc control protein were subjected to immunoprecipitation using anti-RhoGDIα antibodies, and the level of Rac1 in the immunocomplex was measured. The results showed that significantly more Rac1 co-immunoprecipitated with RhoGDIα in cells treated with Sema5A-Fc as compared with Fc and non-stimulated controls (Fig. 6*A*). This is attributable to an increased recruitment of Rac1 to RhoGDIα because Sema5A stimulation imposes no observable effect on the levels of total RhoGDIα and Rac1 (Figs. 6*A* and 3*A*). Furthermore, the increase in Rac1 binding to RhoGDIα induced by Sema5A is mediated through plexin-B3, as shown by the complete abrogation of effect when endogenous plexin-B3 expression is down-regulated (Fig. 6*A*). The binding affinity of RhoGDIα for Rac1 has previously been shown to be under the modulation of its phosphorylation state by kinases such as PAK1 (36), PKCα (37), and Src (38). We therefore examined the phosphorylation state of RhoGDIα in glioma cells stimulated with Sema5A. However, no significant change in the level of phosphorylated RhoGDIα was observed (data not shown).

Although accumulating the RhoGDIα-Rac1 complex is known to promote its shuttling back to cytosolic compartments (39), it can also be retained in the submembranous region because of the association of RhoGDIα with the cytoplasmic tail of plexin-B3. It is therefore of interest to assay for the distribution of Rac1 in C6 glioma cells challenged with Sema5A-Fc. As shown in Fig. 6*B*, the majority of Rac1 in nonstimulated and Fc-treated cells resides in the cytosolic fraction, with a small population being targeted to the membrane fraction. Nonetheless, Sema5A stimulation causes a precipitous reduction of membrane-associated Rac1 by 70% to a barely detectable level. This effect is dependent on plexin-B3 and can be rescued by silencing plexin-B3 expression (Fig. 6*B*). These findings suggest that Sema5A stimulation of plexin-B3 maintains Rac1 in the cytosol through an enhancement of RhoGDIα-Rac1 binding. Nonetheless, this observation is seemingly counterintuitive because Sema5A is known to promote the recruitment of RhoGDIα to plexin-B3 (Fig. 2*C*), which is a transmembrane

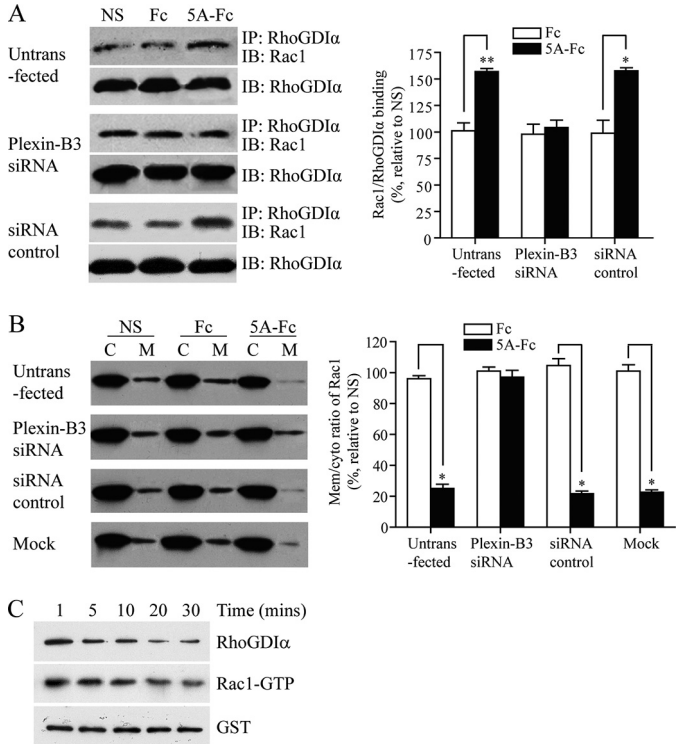

FIGURE 6. **Sema5A enhances RhoGDIα binding and reduces membrane association of Rac1 in glioma cells.** *A,* C6 glioma cells untransfected, transfected with plexin-B3-specific siRNA or scrambled siRNA control were nonstimulated (*NS*), challenged with Fc control (*Fc*) or Sema5A-Fc (5A-Fc) protein, and subjected to immunoprecipitation (*IP*) using RhoGDIα antibody to determine the level of Rac1 association with RhoGDIα. Band intensity of Rac1 was normalized against that of RhoGDIα in each sample. Values of the Fc and the Sema5A-Fc groups were expressed as a percentage to that of the nonstimulated reference. *IB*, immunoblot. *, $p < 0.05$; **, $p < 0.01$ *versus* Fc control. *B,* same set of experiments was repeated to determine the distribution of Rac1 in the cytosol *versus* membrane. Equal amounts of cell lysates from each treatment were subjected to subcellular fractionation. The resulting cytosolic (*C*) and membrane (*M*) fractions were analyzed by Western blot using anti-Rac1 antibody. Signal intensity of Rac1 immunoreactive bands in each fraction was quantified and expressed as a membrane/cytosolic (*Mem/cyto*) ratio. Values of the Fc and Sema5A-Fc groups were expressed as a percentage to those in the nonstimulated group. *, $p < 0.001$ *versus* Fc control. *C,* time course assay for the association of RhoGDIα and active Rac1 with plexin-B3CD. GST-plexin-B3CD prebound to GSH-Sepharose was incubated with Rac1-GTPγS for 30 min before RhoGDIα was added. The tripartite interaction at various time points was captured by GST pulldown and subjected to Western analysis to measure the level of bound RhoGDIα and Rac1.

protein. Furthermore, active Rac1 can also directly bind to the cytoplasmic domain of plexin-B3 (Fig. 2*D*). To better understand the underlying mechanism, the association of RhoGDIα and Rac1 with plexin-B3 over time was determined by *in vitro* binding assays. Although RhoGDIα is confirmed to bind plexin-B3CD, the level of interaction appears to be moderate (Fig. 2, *A* and *D*). In the presence of active Rac1, however, strong binding of RhoGDIα to plexin-B3CD was observed within 1 min of incubation (Fig. 6*C*). Nonetheless, this interaction is transient, which starts to decline from 5 min onward and stabilizes by 30 min. Intriguingly, reducing RhoGDIα binding is accompanied by a corresponding dissociation of active Rac1 from plexin-B3CD (Fig. 6*C*). Because RhoGDIα and Rac1-GTP bind to plexin-B3CD at different sites (Fig. 2*D*), the dissociation of Rac1 from plexin-B3CD is unlikely due to competitive displacement by RhoGDIα.

DISCUSSION

Accumulating evidence points to the implication of semaphorins and their receptor plexins in glioblastoma development and progression (4, 40, 41). Here we show that plexin-B3 is expressed in glioma cells of both human and rat origin. Sema5A stimulation of plexin-B3 in glioma cells results in an inhibition of cell migration and invasion through the promotion of Rac1 inactivation by RhoGDIα. These findings revealed a novel pathway through which plexin regulates Rac1, the actin cytoskeleton, and motility in glioma cells.

The suppression of glioma cell motility by Sema5A is concomitant with a down-regulation in the level of active Rac1 in a plexin-B3-dependent manner. Forced expression of constitutively active Rac1 in these cells successfully abrogated the inhibitory effect of Sema5A on glioma invasion, suggesting the mechanistic role of Rac1 inactivation in this process. Numerous studies have in fact reported a positive correlation between Rac1-GTP level and the invasiveness of glioblastomas (35, 42) and other cancers (43–46). An inactivation of Rac1 in glioblastomas was found to be sufficient to impede cell migration and invasion (34, 47). Classically, RhoGTPases are known to be key players in the control of cell motility through dynamic regulation of the actin cytoskeleton (48). Active Rac1 in particular, induces coordinated reorganization of actin filaments into lamellipodia and stimulates the formation of protrusions at the leading edge, giving rise to the locomotive force in migrating cells (49). The induction of both a down-regulation of Rac1-GTP and a disruption of lamellipodia in C6 glioma cells by Sema5A as reported here can therefore contribute to the impediment of glioma cell motility.

The inactivation of Rac1 by Sema5A and plexin-B3 was shown in this study to be dependent on RhoGDIα, as suggested by the absence of effect when endogenous RhoGDIα expression in glioma cells was down-regulated by RNAi. RhoGDIα is classically known to negatively regulate RhoGTPases by sequestration and blockade of guanine nucleotide exchange (26–28). Accumulating evidence also points to the role of RhoGDIα in extracting RhoGTPases from the membrane and preventing them from reactivation by GEFs before shuttling back to the cytosol (39, 50). The inactivation of Rac1 by Sema5A and plexin-B3 may therefore engage these functions of RhoGDIα. Because both RhoGDIα and active Rac1 are recruited to plexin-B3 by Sema5A in a noncompetitive manner, this would provide a docking site that promotes their interactions. Our results in fact revealed that Sema5A enhanced RhoGDIα-Rac1 binding in a plexin-B3-dependent fashion. Next, we provided evidence that RhoGDIα binding to plexin-B3 is transient, and it dissociates from the cytoplasmic domain of plexin-B3 together with Rac1-GTP. This is consistent with our observation of a significant decline in membrane localization of Rac1 in glioma cells challenged with Sema5A-Fc. Taken together, Sema5A stimulation of plexin-B3 might therefore silence Rac1 signaling by promoting its extraction from the cell membrane via RhoGDIα. Previous studies have shown that the phosphorylation of serine residues on Cdc42 and RhoA by protein kinase A (PKA) can promote their association with RhoGDIα, thereby facilitating the extraction from membranes (51). Phosphoryla-

tion of tyrosine residue on Cdc42 by Src kinase can also enhance its binding with RhoGDIα (52). Nonetheless, there has been no report on the mechanism that promotes RhoGDIα-mediated extraction of Rac1 from the membrane thus far. The effects of Sema5A and plexin-B3 in facilitating RhoGDIα-Rac1 interaction and reducing membrane localization of Rac1 therefore represent a potential candidate. Structural analysis of the plexin-B3-RhoGDI-Rac1 complex may provide mechanistic insight into this process.

The interaction of RhoGDIα with cell surface receptors like plexin-B3 is scarce but not unprecedented. The neurotrophin receptor $p75^{NTR}$ has been found to function as a RhoGDI displacement factor (RhoGDF), which sequesters RhoGDIα from RhoA, thereby relieving the inhibition of GDP/GTP exchange and facilitating subsequent activation of RhoA by RhoGEFs (53). Other RhoGDFs that bind to RhoGDI and displace GTPases include the ezin/radixin/moesin family of proteins (54) and the tyrosine kinase Etk (55). Intriguingly, although RhoGDFs facilitate the dissociation of GTPases from RhoGDI for ensuing activation, counteracting mechanisms that stabilize the complex and terminate GTPase signaling have not been reported. The enhancement of RhoGDIα-Rac1 association by Sema5A and plexin-B3 as shown in this study may represent a candidate mechanism to subserve this function. Importantly, RhoGDIα binding to plexin-B3 is transient in nature, enabling its dissociation together with Rac1. In fact, the dissociation constant for plexin-B1-RhoGTPase complex was found to be in the low micromolar range (56), whereas RhoGDIα-Rac1 can bind at a higher affinity in the nanomolar order (57). These findings therefore represent a novel way to inactivate Rac1 signaling by cell surface receptor through RhoGDIα. Notably, the involvement of both RhoGDIα and Rac1 docking on plexin-B3 to promote their interactions may limit the applicability of this model to Rac1 because other small GTPases such as RhoA and Cdc42 have not been shown to bind to plexin-B3.

Semaphorins and plexins have formerly been shown to regulate Rac1 activity. The binding of Sema3A to neuropilin-1-plexin-A1 receptor complex triggers the dissociation of FARP2 from plexin-A1, rendering this guanine nucleotide exchange factor available for Rac1 activation. Rac1-GTP facilitates the binding of Rnd1 GTPase to plexin-A1, thereby triggering its latent RasGAP activity to inactivate R-Ras, which in turn results in repulsive responses by inhibiting integrin functions (58). Sema4D stimulation promotes direct binding of active Rac1 to plexin-B1 instead (30), which facilitates the recruitment of Rnd1 to plexin-B1, leading to the inactivation of R-Ras, growth cone collapse, and inhibition of cell migration (13, 29, 59). It should be noted that the enhanced interaction of plexin-B1 with Rac1 has also been proposed to simply sequester active Rac1 from its downstream effector PAK1, thereby suppressing Rac1 signals in inducing membrane protrusions, which would otherwise counteract repulsive responses (60, 61). Here we describe for the first time that plexin-B3 negatively regulates Rac1 signaling by reducing their membrane targeting through RhoGDIα upon Sema5A stimulation. Although the present data suggest that this inactivation is sufficient in inhibiting glioma cell motility, it will be of great interest to study the potential contribution of RasGAP activity of plexin-B3.

As a modulator for GTPases of the Rho subfamily, RhoGDIα has been implicated in the regulation of cell motility. Forced expression of RhoGDIα in immortalized fibroblasts and human keratinocytes leads to disruption of the actin cytoskeleton and inhibition of cell motility (62). Accumulating evidence also points to the involvement of RhoGDIα in the development of various cancers, including glioblastomas. Underexpression of RhoGDIα is reported to be associated with heightened metastasis, invasion, and progression in breast cancer and melanoma (63–65). The progression to advanced grades and the malignancy of glioblastomas have also been found to be correlated with reducing expression of RhoGDIα (66). Nonetheless, the precise functions of RhoGDIα in cancer progression remain controversial. This study reveals RhoGDIα as a novel mediator of Sema5A and plexin-B3 signals in suppressing glioma motility through negative regulation of Rac1.

In conclusion, we show that Sema5A inhibits cell migration and invasive growth of glioma cells via its receptor plexin-B3. The specific interaction of plexin-B3 with RhoGDIα and the ensuing negative regulation of Rac1 activity represent a novel pathway through which signals of semaphorin and plexin are relayed to the actin cytoskeleton, thereby regulating glioma cell motility. These findings suggest that Sema5A and plexin-B3 may represent potential therapeutic targets in glioblastoma treatment. Further investigations of their functions in primary glioblastomas are warranted.

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