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Class IV Semaphorins Promote Angiogenesis by Stimulating Rho-Initiated Pathways through Plexin-B

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ABSTRACT

The semaphorins are a large family of secreted and cell surface proteins that provide attractive and repulsive cues for axon guidance during neuronal development. Semaphorins share a conserved NH_2-terminal Sema domain with their receptors, the plexins, which mediate neuronal cell adhesion, axon guidance, and maintenance of established neuronal pathways in the adult. Both semaphorins and plexins share structural homology with the extracellular domain of c-Met, a member of the scatter factor family of receptors. However, the highly conserved cytoplasmic region of plexins has no homology with the c-Met tyrosine kinase or with any other known protein. Using a recently developed antibody and RNA analysis, we found that high levels of plexin-B1 are expressed in endothelial cells. Whereas c-Met, with which plexin-B1 can interact, is known to be a potent promoter of angiogenesis, the effects of semaphorin-mediated plexin activation in endothelial cells are still poorly understood. Here, we examined the role of plexin-B1 activation in angiogenesis using a purified, secreted form of its ligand, Semaphorin 4D (Sema4D). Sema4D potently induced chemotaxis and tubulogenesis in endothelial cells and enhanced blood vessel formation in an *in vivo* mouse model. Interestingly, responses to Sema4D did not require c-Met activation. Instead, the use of chimeric plexin-B1 receptors, Rho inhibitors, and lentiviral gene delivery of interfering molecules revealed that these proangiogenic effects are dependent on a COOH-terminal PDZ-binding motif of plexin-B1, which binds two guanine nucleotide exchange factors for the small GTPase Rho, PDZ-RhoGEF and LARG, and are mediated by the activation of Rho-initiated pathways.

INTRODUCTION

The semaphorins represent a large family of phylogenetically conserved molecules, which were originally identified based on their ability to provide both attractive and repulsive axon guidance cues during neural development (1). The more than 30 semaphorins identified to date share a conserved NH_2-terminal Sema domain and have been classified into eight subgroups based on their species of origin and sequence similarity (see Refs. 2 and 3 for reviews). Classes I and II are found in invertebrates, classes III–VII are found in vertebrates, and class VIII is found in viruses (2, 3). Intense search for molecules mediating the biological responses to semaphorins led to the discovery of a highly conserved family of single pass transmembrane receptors known as plexins, which are essential components of the semaphorin-receptor complex (4, 5). In humans, there are at least nine plexins, most of which have been shown to mediate neuronal cell adhesion and contact, fasciculation, and axon guidance (5, 6). Plexin-semaphorin interactions now have been implicated in a host of responses, including loss of cell-cell contacts and branching morphogenesis in epithelium, regulation of angiogenesis, growth and metastasis of tumors, and immune responses (reviewed in Refs. 7 and 8). Based on their sequence similarities, the plexins have been divided into four families. Whereas members of the plexin-A group bind semaphorins of the III class, plexin-B1 binds Semaphorin 4D (Sema4D), and plexin-C1 binds Sema7A, the nature of the ligands for plexin-B2, -B3, and -D1 is still unknown (2).

Among the vertebrate semaphorins, the class III semaphorins, which are secreted semaphorins, are the most thoroughly investigated (9–11). These semaphorins bind to plexin-A, but only when associated in a complex with members of the neuropilin class of cell surface receptors, neuropilin-1 and neuropilin-2 (10, 11). Also first identified as regulators of axonal growth (12, 13), evidence points to neuropilins as having certain functions in angiogenesis (14). For example, neuropilin-1 binds not only the class III semaphorins (11) but also the proangiogenic factor vascular endothelial growth factor, a potent mitogenic and chemotactic cytokine whose receptors are expressed on the surface of endothelial cells and some tumor cells (14). Aligned with these observations, the class III semaphorins and their receptors, plexin-A and neuropilins, are now known to participate in vascular morphogenesis by promoting an autocrine chemorepulsive signal, in addition to their best-understood role in axon pathfinding (15–17).

In contrast to the class III semaphorins, Sema4D appears to bind plexin-B1 directly (5). Plexin-B1, as well as all plexins and semaphorins, exhibits in its extracellular segment an area of high homology to the proto-oncogene product c-Met and the Met-like protein tyrosine kinase RON, receptors collectively known as the scatter factor receptors (4, 18). c-Met activation by its ligand, scatter factor-1 [also called hepatocyte growth factor (HGF); Ref. 19], promotes branching morphogenesis and axonal guidance in neuronal tissues (20) and proliferation, enhanced cell motility, and metastasis in many tumor cells (reviewed in Refs. 7 and 21). In contrast, the cytoplasmic region of the plexins has no homology with the Met tyrosine kinase cytoplasmic domain or with any other known protein, but is highly conserved within and across species (4, 5). Nonetheless, plexins and scatter factor receptors may be functionally linked because it has been observed recently that on Sema4D ligation, plexin-B1 interacts through its extracellular domain with c-Met, which is then phosphorylated and activated in a step required for successful plexin-B1 signaling (22).

Plexin-B1 is highly expressed in nervous tissues, where it provides repelling cues for the guidance of axon growth during development of the nervous system and inhibition of axon growth after injury and maintenance of established neural pathways in the adult (23), but its gene expression profile suggests that plexin-B1 transcripts are detectable in numerous adult tissues. Of interest, while screening a panel of tissues with a recently developed antibody, we unexpectedly found high levels of plexin-B1 expressed in endothelial cells, results that were confirmed with RNA analysis. Thus, we hypothesized that activation of plexin-B1 by Sema4D might influence angiogenic responses in endothelial cells. To test this, we treated porcine aortic endothelial (PAE) cells with a purified, soluble form of Sema4D and looked for a proangiogenic phenotype. Indeed, PAE cells exhibited migration toward soluble Sema4D and developed a "tubulogenic" phenotype in the presence of Sema4D when growing on a reconstituted basement membrane substrate. Sema4D also promoted blood vessel formation in mice. Interestingly, c-Met was not required for the

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angiogenic responses provoked by Sema4D because c-Met tyrosine phosphorylation was not detectable in PAE cells treated with Sema4D, and expression of chimeric receptors containing the extracellular portion of the nerve growth factor (NGF) receptor Trk-A fused to the intracellular segment of plexin-B1 and thus lacking all elements of the c-Met and plexin-B1 extracellular domains was sufficient to promote angiogenic responses on NGF treatment. Furthermore, we provide evidence that the proangiogenic response to Sema4D through plexin-B1 is dependent on a COOH-terminal PDZ-binding motif of plexin-B1, which binds two guanine nucleotide exchange factors (GEFs) for Rho (24–27), and mediated by the activation of Rho-initiated pathways.

MATERIALS AND METHODS

Cell Culture. PAE cells were cultured in Ham's F-12 media (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin/amphotericin B (Sigma). Human umbilical vein endothelial cells (HUVECs) were cultured in EBM-2 (Clonetics, San Diego, CA) supplemented with 100 units/ml penicillin/streptomycin/amphotericin B. 293T embryonic kidney cells and HaCaT cells were grown and maintained in DMEM (Sigma) supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin/amphotericin B.

Production of Soluble Sema4D. PCR primers were designed for the extracellular portion of mouse Sema4D. The nucleotide sequences for the PCR primers (Sigma) were as follows: forward (*Bam*HI), 5′-GGATCCTAGATACGACTCACTATAGGG-3′; and reverse (*Not*I), 5′-TGCGCGGCCGCTTTATTCCTCACCCT-3′. PCR products were cut with *Bam*HI/*Not*I and cloned into the *Bam*HI/*Not*I site in plasmid pSecTag2B (Invitrogen, Carlsbad, CA). This construct was transfected into 293T cells growing in serum-free media using the calcium chloride N,N-bis[2-hydroxyethyl]-2-amino-ethane sulfonic acid-buffered saline (Fluka Chemika; Sigma Aldrich, St. Louis, MO) method (28). Media containing soluble Sema4D was collected 1 and 2 days after transfection and purified with TALON metal affinity resin (Clontech Laboratories, Palo Alto, CA) according to the manufacturer's instructions. Briefly, TALON resin was resuspended in a wash buffer of 50 mM sodium phosphate and 300 mM NaCl (pH 7.0) and centrifuged at 700 × *g* for 2 min. The buffer was removed, and conditioned media were added and allowed to mix with the resin for 20 min at room temperature. The mixture was centrifuged, washed twice, and transferred to a column, where the remaining wash buffer was eluted. The His_6-tagged protein bound to the resin was eluted in a buffer containing 150 mM imidazole, 50 mM sodium phosphate, and 300 mM NaCl. Purity and concentration of the TALON eluates were determined by SDS-PAGE analysis followed by silver stain (Amersham Life Science, Piscataway, NJ) and the Bio-Rad assay (Bio-Rad, Hercules, CA), respectively. In all cases, media collected from parallel transfectants using the empty pSecTag2B vector were used as control.

Immunoblot Analysis. Cells were lysed in EBC lysis buffer (50 mM Tris-HCl, 150 mM NaCl, and 1% NP40) supplemented with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 μl/ml aprotinin, and 1 μl/ml leupeptin) and phosphatase inhibitors (2 mM NaF and 0.5 mM sodium orthovanadate) for 15 min at 4°C. After centrifugation, protein concentrations were measured using the Bio-Rad assay (Bio-Rad). Protein from each sample (100 μg) was subjected to SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Immobilon P; Millipore Corp., Billerica, MA). The membranes were then incubated with the appropriate antibodies. The antibodies used were as follows: Sema4D (BD Transduction Laboratories, BD Biosciences, Palo Alto, CA); phospho-Met (Cell Signaling Technology, Beverly, MA); c-Met (Cell Signaling Technology); tubulin (PharMingen, BD Biosciences, Palo Alto, CA); myc (C-33; Santa Cruz Biotechnology, Santa Cruz, CA); and green fluorescence protein (Covance, Princeton, NJ). For plexin-B1, the complete intracellular domain of plexin-B1 was expressed as maltose-binding protein fusion MBP-B1. MBP-B1 was purified on amylose affinity column (BioLabs). Pocono Rabbit Farm & Laboratory used purified MBP-B1 (1.4 mg) to immunize two rabbits. The specificity of antiserum was determined by Western blotting using HEK293 cells transfected with plexin-B1. Proteins were detected using the enhanced chemiluminescence system (Pierce, Rockford, IL), and quantitation was performed with NIH image software.

Semiquantitative PCR. Total RNA was obtained from human brain tissue (Clontech) and harvested from 293T cells and HUVECs using the Rneasy Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. A 5-μg aliquot was reverse transcribed with SuperScript II reverse transcriptase (Invitrogen), and the resulting cDNA was combined with 5 pM of each primer pair in a final reaction volume of 50 μl. A PCR was carried out using RedTaq (Sigma) for 30 cycles (95°C melting temperature for 1 min, 55°C annealing temperature for 1 min, 72°C extension temperature for 1 min), with a 5-μl aliquot removed for agarose gel analysis after cycles 15, 18, 21, 24, 27, and 30. The nucleotide sequences for the PCR primers (Sigma) were as follows: plexin-B1, 5′-GCACGGGACATTCCCCGGTAC-3′ (forward) and 5′-CTATAGATCTGTGACCTTGTTTTC-3′ (reverse); and human 18S rRNA, 5′-CGCCGCTAGAGGTGAAATTC-3′ (forward) and 5′-TTGGCAAATGCTTTCGCTC-3′ (reverse).

Migration Assays. Serum-free Ham's F-12 containing the indicated chemoattractant was placed in the bottom well of a Boyden chamber while medium containing PAE cells was added to the top chamber. The two chambers were separated by a polyvinylpyrrolidone filter membrane (Osmonics; GE Water Technologies, Trevose, PA; 8-μm pore size) coated with 10 μg/ml fibronectin (GIBCO, Invitrogen, Carlsbad, CA). After 7 h, the chamber was disassembled, and the membrane was stained with Diff-Quick Stain (Diff-Quick; Dade Behring, Deerfield, IL), placed on a glass slide, and scanned. Densitometric quantitation was performed with NIH Image software. Where indicated, cells were transfected with pCEFL plexin-B1 using the calcium chloride N,N-bis[2-hydroxyethyl]-2-amino-ethane sulfonic acid-buffered saline method before migration or permeabilized with 67 units of tetanolysin (List Biological Laboratories, Campbell, CA) and incubated with C3 toxin (List Biological Laboratories) to inhibit Rho activity. The chemoattractants used were soluble Sema4D, either TALON-purified or present in conditioned media, HGF (R&D Systems, Minneapolis, MN), or NGF (Upstate Biotechnology, Lake Placid, NY). Basic fibroblast growth factor (bFGF; R&D Systems) was used as a positive control.

Immunofluorescence. Cells were grown on sterile glass coverslips in 35-mm, 6-well plates and treated with 500 ng/ml purified Sema4D for the times indicated. The cells were fixed in 4% paraformaldehyde, washed in PBS, and blocked in 3% fetal bovine serum for 30 min at room temperature. Coverslips were then incubated in a humidity chamber for 1 h at room temperature with anti-Semaphorin 4D antibody (BD Transduction Laboratories, BD Biosciences; 1:250 dilution in PBS with 3% fetal bovine serum), a nonspecific primary antibody (anti-TrpE; Oncogene Research Products; 1:250 dilution in PBS with 3% fetal bovine serum), or secondary antibody alone (1:100 dilution of rhodamine-conjugated donkey antimouse secondary antibody; Jackson Immunoresearch, West Grove, PA). After three washings with PBS, coverslips were again placed in a humidity chamber for 1 h at room temperature with a 1:100 dilution of rhodamine-conjugated donkey antimouse secondary antibody (Jackson Immunoresearch). Coverslips were then inverted and mounted onto glass slides with Vectashield containing 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) and viewed with fluorescence microscopy. Images were taken using a SPOT digital camera attached to a Zeiss Axiophot microscope (Carl Zeiss, Thornwood, NY).

Tubulogenesis Assays. PAE cells were grown on 35-mm plates coated with 150 μl of Cultrex basement membrane extract (Trevigen, Gaithersburg, MD) and incubated overnight in serum-free Ham's F-12 media containing the indicated factors. Cells were then fixed in 0.5% glutaraldehyde and photographed. Where indicated, cells were treated with soluble Sema4D, HGF, or NGF. bFGF was used as a positive control.

***In Vivo* Cultrex Assay.** Nine hundred μl of serum-free DMEM alone or media containing soluble Sema4D were added to 900 μl of Cultrex basement membrane extract (Trevigen) supplemented with bFGF (100 pg/ml) and injected s.c. into mice (29, 30). The animals were sacrificed after 10 days, and the polymerized Cultrex plugs were removed, frozen, and processed for microscopy. Slides were hydrated through graded alcohols and incubated in 3% hydrogen peroxide for 30 min to quench the endogenous peroxidase. The sections were then incubated in blocking solution (1% horse serum) for 1 h at room temperature followed by treatment with anti-CD31 primary antibody (anti-platelet/endothelial cell adhesion molecule; PharMingen; 1:100 dilution) overnight at 4°C. After washing with PBS, the slides were incubated with the biotinylated secondary antibody (Vector Laboratories; 1:300) for 1 h, followed by the avidin-biotin complex (Vector Stain Elite; avidin-biotin complex kit;

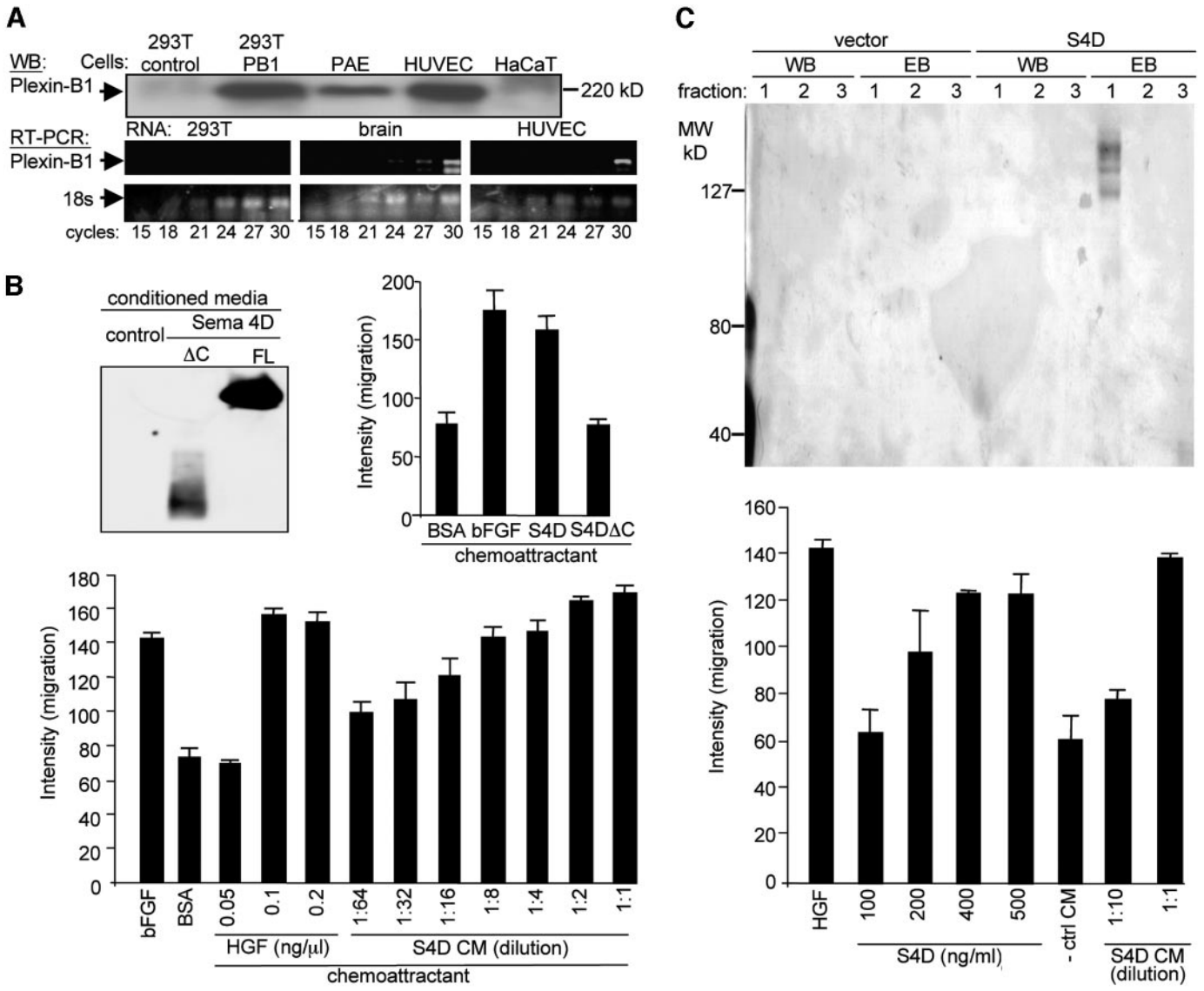

Fig. 1. Plexin-B1 is expressed in endothelial cells. *A*, SDS-PAGE immunoblot analysis for plexin-B1 shows a band at M_r 220,000 for an endogenously expressed protein in lysates from endothelial cells, human umbilical vein endothelial cells (HUVECs), and porcine aortic endothelial (PAE) cells (*top panel*). This band corresponds to that seen in lysates of 293T cells transiently transfected with a plexin-B1 expression plasmid (*PB1*). No band was detected in empty vector-transfected negative control or HaCaT cell lysates. Agarose gel shows the PCR product for plexin-B1 in reverse transcribed RNA from 293T cell, human brain cell, and HUVEC lysates (*bottom panel).* After completion of 15, 18, 21, 24, 27, and 30 cycles of PCR, no signal is detected in 293T cells, whereas brain cell and HUVEC reactions show a strong signal between 24 and 30 cycles. Human 18S reverse transcription-PCR product was used as a control. *B*, conditioned media from transfected 293T cells contain soluble Sema4D, which promotes chemotaxis and tubulogenesis in PAE cells. Conditioned serum-free media collected from 293T cells transiently transfected with vector control (*control*) or pSecTag2B Sema4D, full-length (*FL*) or truncated (*Sema4D ΔC*), exhibit high levels of secreted, soluble protein as judged by anti-His_6 Western blotting (*top left panel*). These media were used as chemoattractants in a cell migration assay, as indicated on the *X* axis (*top right panel*). Control media containing 0.1% BSA or BSA with 150 pg/ml basic fibroblast growth factor (bFGF) were used as negative and positive controls for migration, respectively. *S4D,* full-length Sema4D-containing conditioned media. The *bars* represent the degree of migration as determined by densitometry. Conditioned media containing successive dilutions of soluble Sema4D or hepatocyte growth factor were used as chemoattractants in a cell migration assay, as indicated on the *X* axis (*bottom panel*). Media containing 0.1% BSA or BSA with 150 pg/ml bFGF were used as negative and positive controls for migration, respectively. The *bars* represent the degree of migration as determined by densitometry. *C*, Sema4D was purified with metal affinity chromatography from conditioned media collected from subconfluent plates of 293T cells transfected with pSecTag2B Sema4D. Purified Sema4D was detected in the first fraction collected from elution buffer (*EB*) and was not found in any fractions from the wash buffer (*WB*) or from negative control cells (pSecTag2B, empty; *top panel*). Purified soluble Sema4D or successive dilutions of Sema4D conditioned media (*S4D CM*) were used as chemoattractants in a cell migration assay as indicated on the *X* axis (*bottom panel).* Both forms of Sema4D act as chemoattractants for PAE cells in a dose-dependent manner. Media containing 0.1% BSA or BSA with 150 pg/ml bFGF were used as negative and positive controls for migration, respectively. The *bars* represent the degree of migration as determined by densitometry.

Vector Laboratories) for 30 min at room temperature. The slides were developed in 3,3-diaminobenzidine (Sigma FASTDAB tablet; Sigma) and then counterstained with methyl green. Images were taken using a SPOT digital camera attached to a Zeiss Axiophot microscope (Carl Zeiss).

Immunoprecipitation. 293T cells maintained in DMEM supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin/amphotericin B were washed in PBS; treated for the indicated periods of time with serum-free media, media conditioned by 293T cells expressing pSecTag2B empty vector, serum-free media containing 100 ng/ml HGF, or serum-free media conditioned by 293T cells expressing pSecTag2B Sema4D; and then washed twice with PBS and lysed in EBC lysis buffer. Proteins were immunoprecipitated from the cleared lysates by incubation for 2 h at 4°C with antibodies against phospho-tyrosine [ICN Valeant Pharmaceuticals (Costa Mesa, CA) and Upstate Biotechnology; mixed 1:1]. Immunocomplexes were recovered with the aid of γ-bind Sepharose beads (Pharmacia, Pfizer, New York, NY). Lysates and anti-phospho-tyrosine immunoprecipitates were analyzed by Western blotting after SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Immobilon P; Millipore Corp.), and immunoblotted with anti-Met antibody (Cell Signaling Technology). Immunocomplexes were visualized by enhanced chemiluminescence detection (Amersham Life Science)

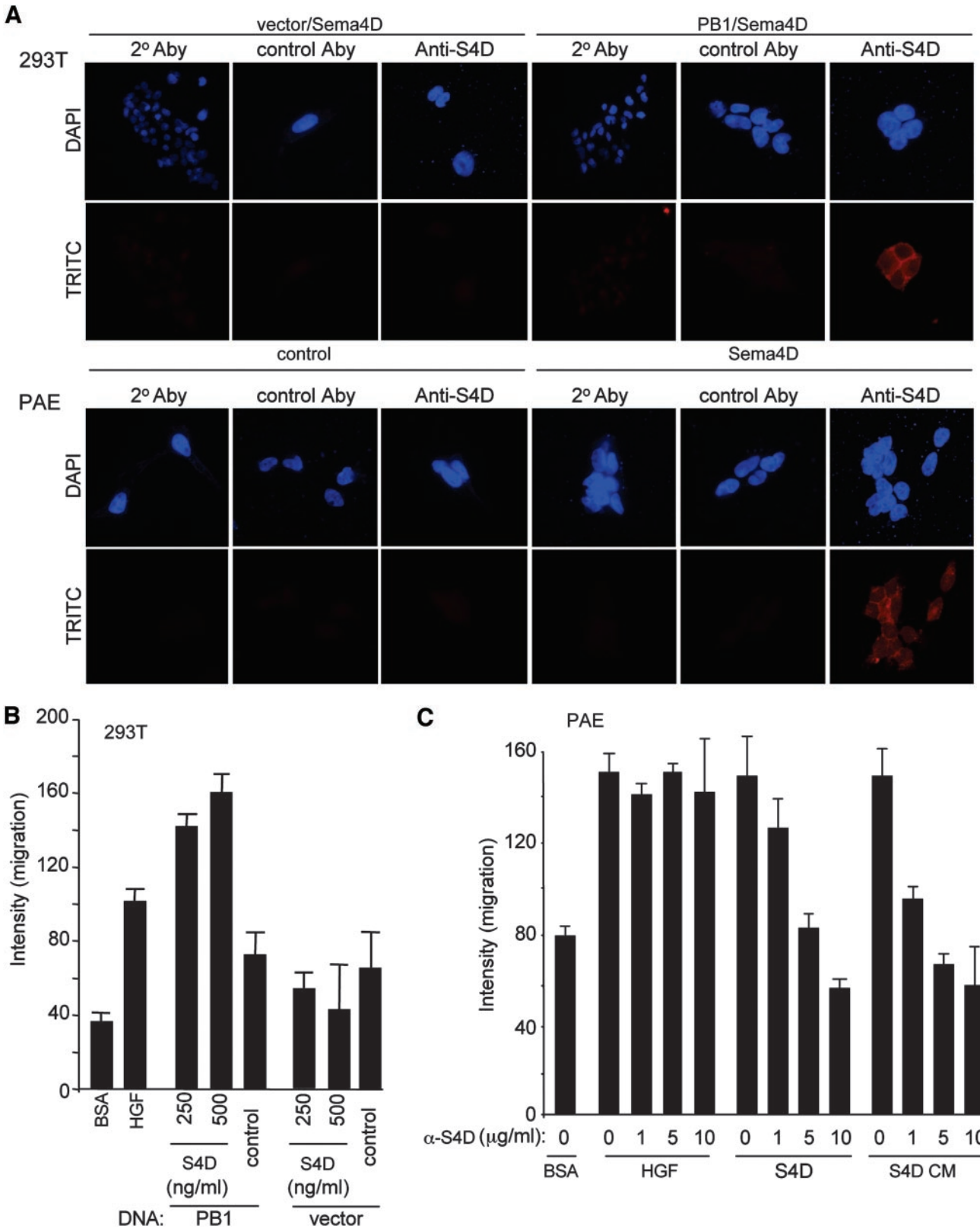

Fig. 2. Sema4D mediates cell migration through plexin-B1. *A*, Sema4D binds to porcine aortic endothelial (PAE) cells and 293T cells expressing plexin-B1. 293T cells transfected with plexin-B1 (*top right panels*) and vector control (*top left panels*) treated with 500 ng/ml Sema4D for 5 min were labeled with either secondary antibody alone (*2° Aby*), nonspecific primary antibody (*control Aby*), or anti-Sema 4D antibody (*Anti-S4D*). A fluorescent pattern consistent with cell surface labeling was observed only in the plexin-B1-transfected population labeled with anti-Sema4D antibody (*top right panels, last row).* PAE cells were left untreated (*bottom left panels*) or treated with 500 ng/ml Sema4D for 5 min (*bottom right panels*) and labeled as indicated above. A fluorescent pattern consistent with cell surface labeling was observed only in Sema4D-treated PAE cells labeled with anti-Sema4D antibody (*bottom right panels, last row). B,* 293T cells transfected with plexin-B1 (*PB1*) exhibit chemotaxis toward purified Sema4D in a dose-dependent manner, whereas vector-transfected negative controls (*vector*) do not. BSA (0.1%) and 150 pg/μl hepatocyte growth factor were used as negative and positive controls for migration, respectively. Media purified from pSecTag2B empty vector-transfected 293T cells were used as a negative control (*control*). *C,* PAE cell migration can be blocked by the indicated increasing concentrations of anti-Sema4D blocking antibody in a dose-dependent manner. This effect is observed in cells where either purified Sema4D (*S4D*) or Sema4D conditioned media (*S4D CM*) is used as the chemoattractant. Hepatocyte growth factor-mediated cell migration was not blocked by the antibody.

using goat antimouse coupled to horseradish peroxidase as a secondary antibody.

Trk/Plexin Fusion Proteins. Plasmids pCEFL EGFP (enhanced green fluorescence protein) plexin-B1, with and without the PDZ-binding motif, were digested with *Nhe*I/*Not*I and cloned in-frame with the COOH-terminal, extracellular, and transmembrane portion of the rat NGF receptor Trk-A into vector pCEFL-HA. pCEFL Trk-A HA, pCEFL Trk-A/plexin-B1, and pCEFL Trk-A/plexin-B1 ΔPDZ constructs were then transfected into PAE cells using Superfect (Qiagen), and stable cells were selected in 1 mM G418 (CalBiochem, San Diego, CA).

Detection of Stress Fibers. PAE cells were grown on sterile glass coverslips in 35-mm, 6-well plates and treated for 8 h with bFGF, HGF, Sema4D, or NGF, where indicated. The cells were fixed in 4% paraformaldehyde, stained with Texas Red-X phalloidin (Molecular Probes, Eugene, OR), mounted with Vectashield containing 4′,6-diamidino-2-phenylindole (Vector Laboratories), and viewed with fluorescence microscopy. Images were taken using a SPOT digital camera attached to a Zeiss Axiophot microscope.

Rho Pulldown Assay. PAE cells maintained in Ham's F-12 media supplemented with 10% fetal bovine serum were washed in PBS and returned to serum-free Ham's F-12 for 36 h. The cells were then washed in PBS and treated for the indicated periods of time with 500 ng/ml purified soluble Sema4D and 67 units of tetanolysin (List) or Sema4D, tetanolysin, and 1 μg/ml C3 toxin (List). Cells were washed with PBS and lysed in EBC lysis buffer. Rho activity was assessed using purified glutathione *S*-transferase-rhotekin-RBD previously bound to glutathione-Sepharose 4B (Amersham Pharmacia) to affinity precipitate GTP-bound RhoA. Western blot analysis of total and active Rho was performed using an antibody against RhoA (26C4; Santa Cruz Biotechnology). Immunocomplexes were visualized by enhanced chemiluminescence detection (Amersham Life Science) using goat antirabbit coupled to horseradish peroxidase as a secondary antibody (Santa Cruz Biotechnology).

Lentiviral Infections. CSCG-based retroviral vectors were prepared as reported previously (31) for EGFP-RGS and EGFP-PDZ expression using 293T cells as the packaging cells. PAE cells were infected with viral supernatants for 24 h at 37°C in the presence of 8 μg/ml Polybrene (hexadimethrine bromide; Sigma). The plates were washed twice with PBS and returned to serum-free Ham's F-12 media.

RESULTS

Plexin-B1 Is Expressed in Endothelial Cells. While screening for plexin-B expression with a recently developed antibody, we found that this plexin was highly expressed in human and porcine endothelial cells. 293T cells transfected with expression vectors for plexin-B1 or vector alone served as positive and negative controls for plexin expression, respectively. As shown in Fig. 1A, *top panel*, plexin-B1 was absent in empty vector-transfected control 293T cells, but a band of approximately M_r 220,000 was observed in 293T cells transfected with plexin-B1, as well as in HUVECs and PAE cells. In contrast, HaCaT, a keratinocyte cell line (32), does not express plexin-B. Because this antibody may cross-react with other plexin-B family members, we next obtained RNA from 293T cells, human brain tissue, and HUVECs, and we examined the relative abundance of plexin-B1 mRNA. Using primers specific to the intracellular portion of plexin-B1, the resulting PCR products revealed no signal for plexin-B1 in 293T cells after 30 cycles, whereas a strong signal for plexin-B1 was

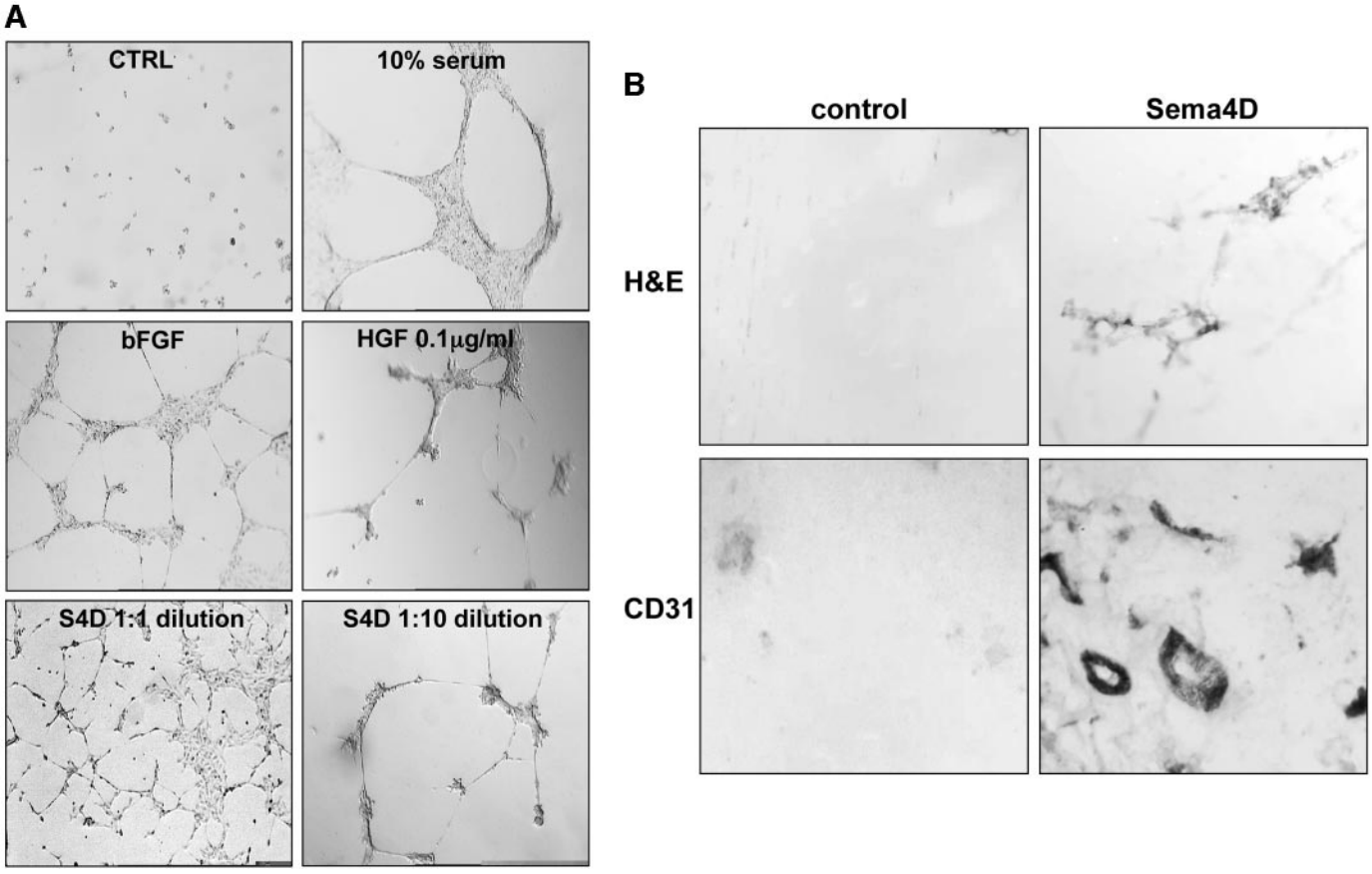

Fig. 3. Semaphorin promotes tubulogenesis in endothelial cells. *A*, porcine aortic endothelial cells were grown overnight in serum-free media with the indicated factors on a substrate of Cultrex basement membrane extract and then fixed and analyzed for tubule formation. Media containing 0.1% BSA were used as the negative control (*CTRL*). Media with BSA and 150 pg/ml basic fibroblast growth factor or 10% fetal bovine serum were the positive controls. *S4D*, Sema4D-containing media at the indicated dilution. *B*, reconstituted basement membrane implants containing soluble Sema4D exhibit enhanced blood vessel infiltration in an *in vivo* mouse model. Aliquots of Cultrex basement membrane extract containing conditioned media alone or media with Sema4D were injected s.c. into mice and harvested after 10 days. The polymerized plugs were frozen and processed for microscopy with either H&E staining or anti-CD31 immunoperoxidase staining, as indicated.

detected in human brain tissue extracts and HUVECs (Fig. 1A, *bottom panel*). PCR primers to human 18S rRNA were used as a control. Collectively, these results indicate that plexin-B1 is expressed in endothelial cells.

Sema4D Induces Cell Migration in Endothelial Cells. To produce soluble protein, Sema4D cDNA was subcloned into the plasmid pSecTag2B, which targets proteins for secretion (33). This soluble form of Sema4D activates plexin-B1, similar to its membrane-bound species (27). Fig. 1B, *top left panel*, shows that secreted Sema4D, both a full-length form and a COOH-terminal-truncated form, was present in the serum-free conditioned media collected from 293T cells transfected with pSecTag2B containing the appropriate cDNA, but not in media from empty vector-transfected control cells. These conditioned media were used as chemoattractants in a migration assay. Fig. 1B, *top right panel*, demonstrates significant chemotaxis toward wells of 293T conditioned media containing bFGF, a known chemoattractant for endothelial cells that was used as a control (34), and wells containing media with the full-length version of secreted Sema4D, but not toward media containing the truncated version of the protein. Next, we used increasing dilutions of Sema4D-containing media in a similar experiment and compared it with different concentrations of the scatter factor HGF, another well-known chemoattractant for endothelial cells (35, 36). Fig. 1B, *bottom panel*, demonstrates that migration toward Sema4D was dose dependent and that its maximal response was equivalent to that elicited by HGF. These results indicate that full-length Sema4D acts as a potent chemoattractant for endothelial cells.

Conditioned media collected from plates of 293T cells expressing pSecTag2B, which codes for a His_6 tag, or pSecTag2B Sema4D (33) were collected and purified with the TALON metal affinity resin and then analyzed with SDS-PAGE. Fig. 1C, *top panel*, shows purified Sema4D coming off of the resin column in the first elution buffer fraction from pSecTag2B Sema4D-transfected cells, but not in pSecTag2B-transfected controls or in any of the wash buffer elutions. The concentration of this purified Sema4D was determined and then used as a chemoattractant in a migration assay. Fig. 1C, *bottom panel*, demonstrates that in addition to migration toward conditioned media containing Sema4D, PAE cells migrated toward wells containing purified Sema4D in a dose-dependent manner, with a maximal response similar to that of HGF. Media collected from parallel transfectants of the empty pSecTag2B vector and subjected to the same protein purification steps were used as negative control.

Sema4D Mediates Cell Migration through Plexin-B1. To determine whether Sema4D can bind to the surface of endothelial cells, we first examined the binding of Sema4D to 293T cells transfected with plexin-B1. Cells were incubated with purified soluble Sema4D for 5 min, rapidly fixed, and processed for immunofluorescence to detect bound Sema4D (Fig. 2A, *top panels*). Strong immunofluorescence, in a pattern consistent with cell surface staining, was observed in plexin-B1-expressing 293T cells treated with Sema4D (*PB1/Sema4D*) and labeled with anti-Sema4D antibody (*Anti-S4D*), but not in treated cells labeled with nonspecific antibody (*control Aby*) or secondary antibody only (*2° Aby*) or in Sema4D-treated empty vector-transfected controls (Fig. 2A, *vector/Sema4D*). Sema4D-treated PAE cells also showed an almost identical pattern of immunofluorescence when labeled with Sema4D antibody (Fig. 2A, *bottom panels, Sema 4D*), but not when labeled with nonspecific primary antibody (*control Aby*) or secondary antibody only (*2° Aby*) or in cells that were not treated with Sema4D (*control*). These results are aligned with the findings that Sema4D specifically binds to plexin-B1 to exert its biological effects (5).

To confirm that Sema4D can induce cell migration specifically by binding to and activating plexin-B1, 293T cells, which do not express detectable levels of this receptor, were transfected with pCEFL (*vector*) or pCEFL plexin-B1 (*PB1*) and subjected to a migration assay in which Sema4D was used as the chemoattractant (Fig. 2*B*). Plexin-B1-transfected cells exhibited strong migration toward wells containing purified Sema4D in a dose-dependent manner, even above that observed in HGF-treated positive control populations, whereas the empty vector-transfected cells did not migrate (Fig. 2*B*). To further establish the specificity of Sema4D/plexin-B1 interactions in this response, migration assays were performed using Sema4D as a chemoattractant for PAE cells placed in media containing increasing concentrations of anti-Sema4D blocking antibody (*α-S4D*). Fig. 2*C* shows that whereas increasing concentrations of this antibody had no effect on HGF-induced migration, which thus served as a negative control, this blocking antibody could, in fact, inhibit chemotaxis toward both purified Sema4D (*S4D*) and Sema4D conditioned media (*S4D CM*) in a dose-dependent manner. Together, these results show that Sema4D/plexin-B1 interactions are required to mediate Sema4D-induced migration.

Sema4D Promotes Tubulogenesis in Endothelial Cells. Endothelial cells cultured on a reconstituted basement membrane material under the appropriate conditions can grow toward each other and create small tube-like structures that mimic blood vessel formation, a process called tubulogenesis (37). PAE cells were grown overnight on a reconstituted basement membrane substrate in conditioned serum-free media containing only 0.1% BSA or BSA with bFGF, 10% fetal bovine serum, HGF, or Sema4D and then examined the next day for the tubulogenic phenotype. Cell spreading and tubule formation were observed in bFGF- and serum-treated PAE cells, but not in cells incubated solely with BSA (Fig. 3*A*). Cells treated with HGF demonstrated tube formation (Fig. 3*A*, *middle right panel*). Of interest, PAE cells incubated with soluble Sema4D also exhibited this phenotype, even at the lowest dilutions used, indicating that this protein is a strong promoter of tubulogenesis (Fig. 3*A*, *bottom row*). Taken together, these findings indicate that Sema4D induces a proangiogenic response in PAE cells similar to the known proangiogenic cytokines bFGF and HGF.

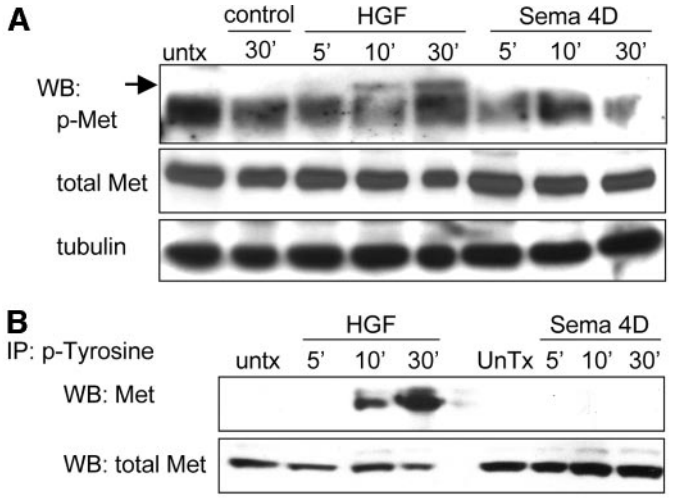

Fig. 4. Sema4D does not induce c-Met phosphorylation in endothelial cells. *A*, porcine aortic endothelial (PAE) cells were grown in serum-free media (*untx*), media conditioned by pSecTag2B empty vector-expressing 293T cells without (*control*) or with 100 ng/ml hepatocyte growth factor (*HGF*), or media conditioned by pSecTag2B Sema4D-expressing 293T cells (*Sema 4D*) for the indicated periods of time and then lysed and analyzed for the presence of tyrosine-phosphorylated c-Met. Phospho-c-Met (*p-Met*) was seen in HGF-treated cells, but not in negative control populations or in lysates from cells treated with Sema4D. Total c-Met levels and tubulin were used as loading controls. *B*, c-Met is not detected in phospho-tyrosine immunoprecipitations from Sema4D-treated cells. Lysates of PAE cells treated as indicated were immunoprecipitated for tyrosine-phosphorylated (*p-Tyrosine*) proteins and then immunoblotted for the presence of c-Met. As expected, c-Met was seen in HGF-treated PAE cell immunoprecipitates, but not in those from negative control or Sema4D-treated populations.

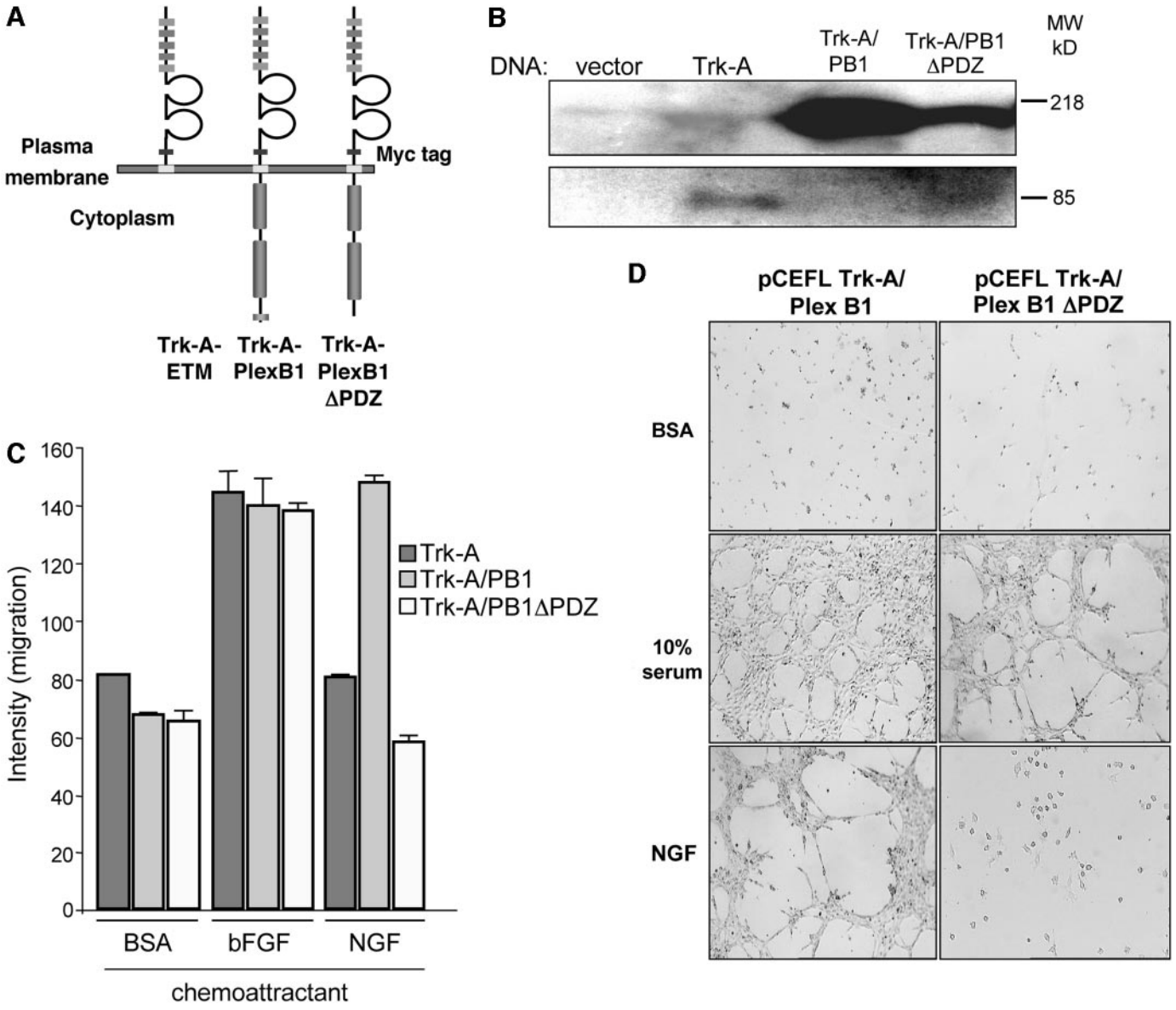

Fig. 5. Structure of myc-tagged Trk-A and Trk-A/plexin-B1 chimeric receptors. *A*, chimeras were made coding for the myc-tagged extracellular and transmembrane portions of the rat Trk-A receptor (*Trk-A-ETM*), alone or fused to the intracellular COOH-terminal portion of the plexin-B1 receptor, either full length (*Trk-A-PlexB1*) or lacking the PDZ-binding motif (*Trk-A-PlexB1ΔPDZ*). *B*, expression of chimeric receptor constructs in stably transfected and selected porcine aortic endothelial (PAE) cells. Lysates from PAE cells transfected with the above-mentioned constructs and selected in G418 for 2 weeks were subjected to SDS-PAGE analysis, and an immunoblot performed for myc demonstrates stable expression of these constructs. *C*, nerve growth factor (NGF) induces migration in cells stably expressing Trk/plexin chimeras containing an intact PDZ-binding motif. Conditioned medium containing 100 ng/ml NGF was used as a chemoattractant for Trk-A-, Trk-A/plexin-B1-, and Trk-A/plexin-B1 ΔPDZ-expressing PAE cells in a cell migration assay. Media containing 0.1% BSA or BSA with 150 pg/ml basic fibroblast growth factor were used as negative and positive controls for migration, respectively. The *bars* represent the degree of migration as determined by densitometry. *D*, NGF induces tubulogenesis in PAE cells stably expressing Trk/plexin chimeras with an intact PDZ-binding motif when growing on a reconstituted basement membrane substrate. PAE cells expressing Trk-A/plexin-B1 and Trk-A/plexin-B1 ΔPDZ were grown overnight in conditioned medium containing 100 ng/ml NGF on a substrate of Cultrex basement membrane extract and then fixed and analyzed for tubule formation. Media containing 0.1% BSA and BSA with 10% fetal bovine serum were used as negative and positive controls, respectively.

Reconstituted Basement Membrane Implants Containing Soluble Sema4D Exhibit Enhanced Blood Vessel Infiltration *in Vivo*. To test whether Sema4D can promote angiogenesis *in vivo*, conditioned serum-free media were collected from empty vector-transfected 293T cells or cells transfected with pSecTag2B Sema4D and mixed with reconstituted basement membrane extracts. Control extracts and extracts containing soluble Sema4D were then injected s.c. into the flanks of nude mice to form plugs (29, 30). After 10 days, the animals were sacrificed, and the plugs were removed and processed for microscopy. Fig. 3*B* shows enhanced blood vessel infiltration in the plugs enriched with Sema4D media, as demonstrated by staining for anti-CD31, an endothelial cell marker (38), when compared with the control plugs that did not exhibit blood vessel growth. These results provide biological evidence for the significance of Sema4D in *in vivo* angiogenesis assays.

Sema4D Treatment of Endothelial Cells Does Not Result in Detectable Phosphorylation and Activation of c-Met. Sema4D can promote the interaction of its receptor, plexin-B1, with the prototypical scatter factor receptor c-Met, resulting in tyrosine phosphorylation and activation of c-Met, which in turn phosphorylates and activates plexin-B1 (22). To determine the significance of this interaction for Sema4D-mediated signaling in endothelial cells, PAE cells were incubated with serum-free media containing HGF and media conditioned with 293T cells transfected with pSecTag2 empty vector

(*control*) or pSecTag2 Sema4D (*Sema 4D*), and levels of tyrosine-phosphorylated c-Met were analyzed by the use of a phospho-specific c-Met antiserum (Fig. 4*A*). We failed to detect c-Met phosphorylation and activation in Sema4D-treated PAE cells, whereas phospho-tyrosine-containing c-Met was clearly demonstrable in HGF-treated control cells (Fig. 4*A*, *arrow*). In an effort to enhance the sensitivity of this assay, phospho-tyrosine immunoprecipitations were performed on lysates of PAE cells treated with HGF or Sema4D for the indicated periods of time, and lysates were then immunoblotted for the presence of c-Met (Fig. 4*B*). Whereas we readily detected c-Met in phospho-tyrosine immunoprecipitates from cells treated with HGF, we did not detect c-Met in Sema4D-treated cells (Fig. 4*B*). Although it is possible that a plexin-B1/c-Met interaction occurs below our limit of detection, these results raised the possibility that c-Met activation may not be necessary for the Sema4D-mediated angiogenic responses that were observed in endothelial cells.

Plexin-B1-Mediated Angiogenic Responses Are Independent of Its Extracellular Region but Dependent on Its COOH-Terminal PDZ-Binding Motif. Members of the plexin-B family of receptors contain a PDZ-binding motif at their COOH-terminal cytoplasmic tail (26). The PDZ domain is a protein-protein interaction domain found in a diverse array of signaling and structural proteins (39). They include two recently identified RhoGEFs, PDZ-RhoGEF and LARG (40, 41), which promote the exchange of GDP bound to Rho for GTP, thereby activating Rho and its downstream effectors and eliciting a variety of cellular responses, the best known of which is increased actin polymerization and stress fiber formation (42). Because we and others have implicated this sequence in plexin-B1 signal transduction (24, 26, 27, 43, 44), we examined the significance of the interactions of plexin-B1 with PDZ domain-containing proteins in Sema4D-mediated angiogenesis. For these experiments, we took advantage of the observation that PAE cells do not respond to treatment with NGF (data not shown) to explore whether chimeric molecules including myc-tagged extracellular and transmembrane portions of the NGF receptor Trk-A, alone or fused to the cytoplasmic segment of plexin-B1, with and without the COOH-terminal PDZ-binding motif, could transduce angiogenic signals on NGF stimulation. In addition, because the extracellular domain of plexin-B1 mediates its interaction with c-Met (22), these Trk-A/plexin-B1 chimeric molecules would be expected to act independently of c-Met-initiated pathways.

Cells were transfected with these constructs (Fig. 5*A*) and selected in G418 to generate stable cell lines (Fig. 5*B*). PAE cells expressing truncated Trk-A, Trk-A/plexin-B1, or Trk-A/plexin-B1 ΔPDZ chimeric receptors were then subjected to a migration assay using serum-free media containing BSA or BSA with bFGF or NGF as chemoattractants. Fig. 5*C* demonstrates the expected chemotactic response for all three lines toward bFGF, but not BSA. Whereas PAE cells stably expressing only the truncated Trk-A receptor did not respond to NGF, cells expressing Trk-A/plexin-B1 chimeras exhibit potent migration nearly equal to that seen in bFGF wells (Fig. 5*C*). These results demonstrated that the intracellular domain of plexin-B1 is sufficient to induce endothelial cell migration. Interestingly, this response was lost in PAE cells expressing the Trk-A/plexin-B1 ΔPDZ chimeras, indicating that the COOH-terminal PDZ-binding motif is critical for the chemotaxis induced by plexin-B1 receptor activation (Fig. 5*C*).

PAE cells expressing Trk-A/plexin-B1 and Trk-A/plexin-B1 ΔPDZ were also incubated overnight on a reconstituted basement membrane substrate in serum-free media containing only BSA or BSA with 10% fetal bovine serum or NGF and then examined for tubule formation. In both lines, tubulogenesis was observed in serum-treated cells, but

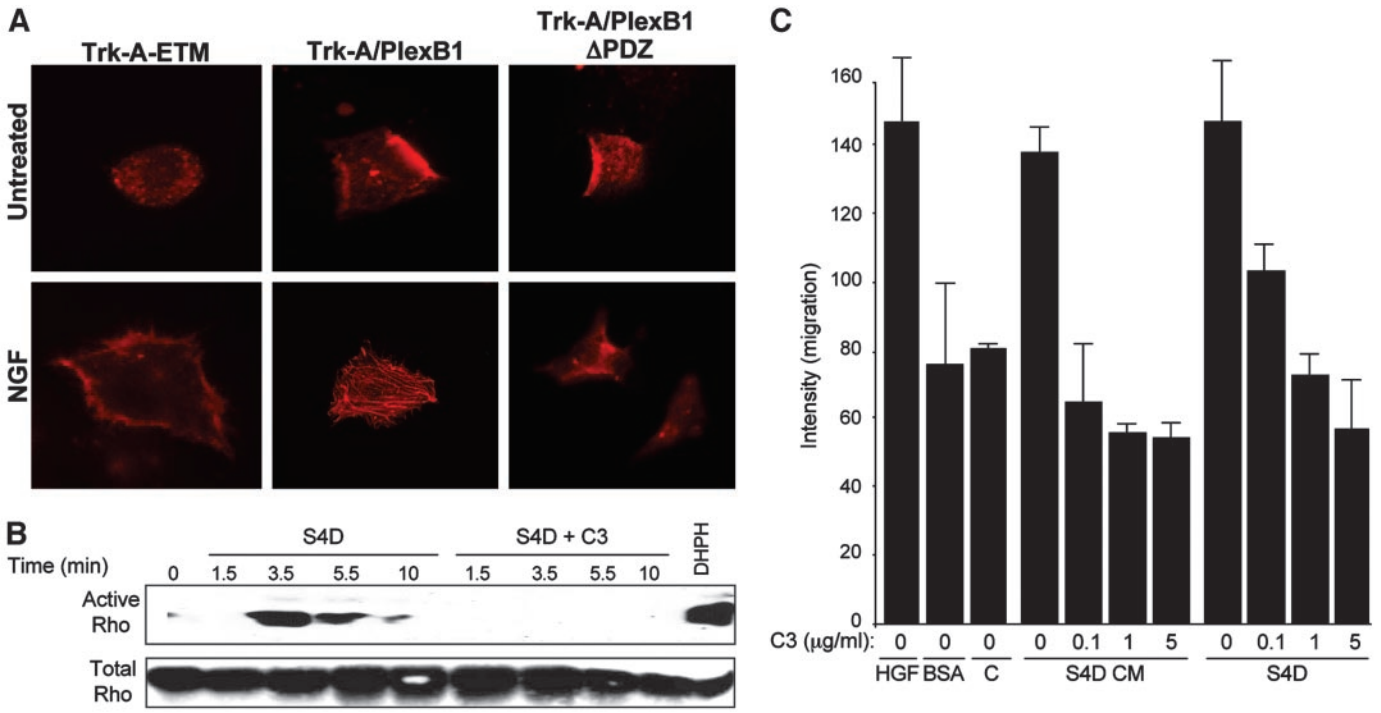

Fig. 6. Plexin-B1 signaling in endothelial cells activates Rho-initiated pathways in a PDZ-binding domain-dependent manner. *A*, porcine aortic endothelial (PAE) cells expressing Trk-A (extracellular and transmembrane segments only; *Trk-A-ETM*), Trk-A/plexin-B1 (*Trk-A/PlexB1*), and Trk-A/plexin-B1 ΔPDZ (*Trk-A/PlexB1 ΔPDZ*) were grown on glass coverslips and left untreated or treated for 8 h with 100 ng/ml nerve growth factor. Cells were fixed and stained with 4′,6-diamidino-2-phenylindole and Texas Red-X phalloidin and viewed under an immunofluorescence microscope. *B*, Sema4D induces Rho activation. PAE cells were treated with 500 ng/ml purified Sema4D for the times indicated, either with or without C3 toxin. Sema4D treatment resulted in the rapid accumulation of GTP-bound RhoA, which was completely blocked by C3. 293T cells transfected with a plasmid coding for the DH-PH domain of PDZ-RhoGEF were used as a positive control. *C*, Rho activation is necessary for Sema4D-mediated chemotaxis. PAE cell migration was blocked by the indicated increasing concentrations of C3 toxin. This effect was observed in cells where either purified Sema4D (*S4D*) or Sema4D conditioned media (*S4D CM*) was used as the chemoattractant. Media containing 0.1% BSA (*BSA*) and media conditioned from empty vector transfected 293T cells (*C*) were used as negative controls. Media containing 0.1% BSA with 10% fetal bovine serum were used as the positive control.

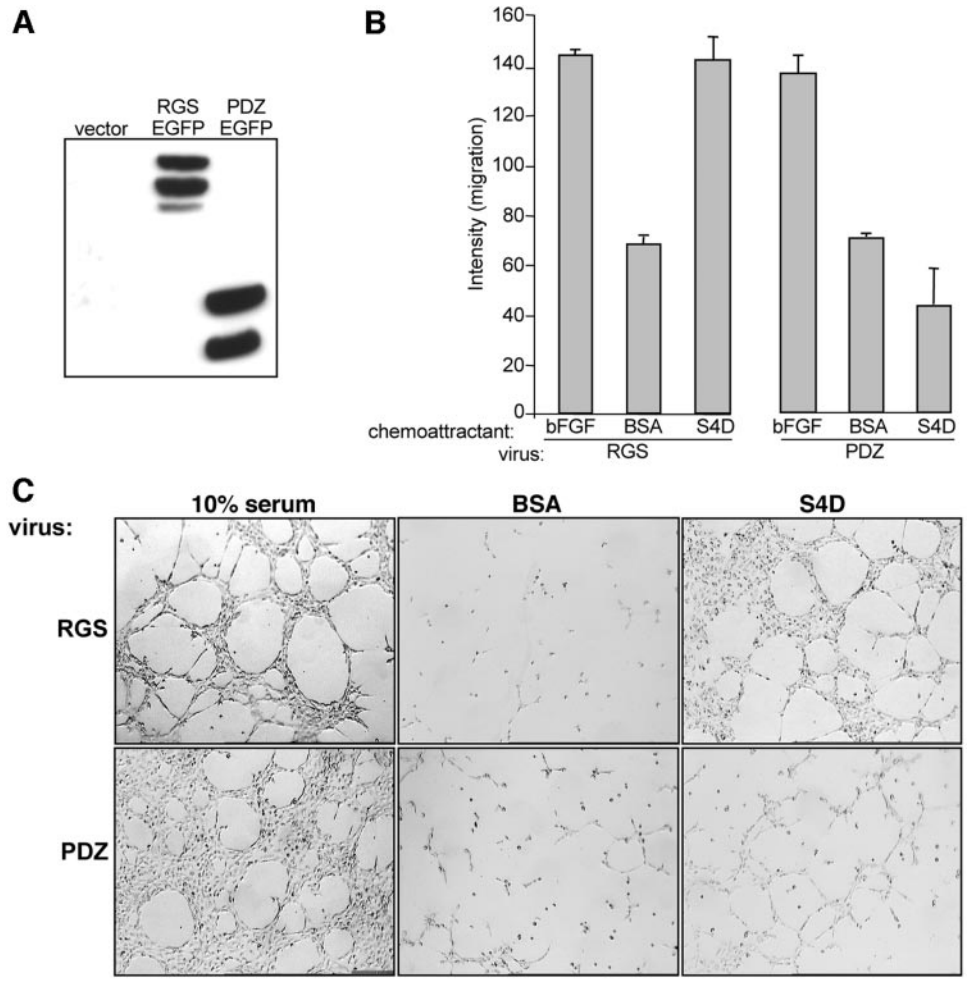

Fig. 7. Porcine aortic endothelial (PAE) cells express lentiviral constructs after infection. *A*, lysates from PAE cells infected with lentivirus coding for EGFP-RGS and EGFP-PDZ, as indicated, were subjected to SDS-PAGE analysis and an immunoblot performed for green fluorescence protein expression. PAE cells infected with virus containing an empty vector (*vector*) were used as negative controls. *B*, PAE cells infected with lentivirus expressing EGFP-PDZ fail to exhibit cell migration on treatment with Sema4D. Conditioned media containing Sema4D (*S4D*) were used as chemoattractants in a cell migration assay for PAE cells infected with EGFP-RGS (*RGS*)- or EGFP-PDZ (*PDZ*)-expressing lentivirus. Media containing 0.1% BSA or BSA with 150 pg/ml basic fibroblast growth factor were used as negative and positive controls for migration, respectively. The *bars* represent the degree of migration as determined by densitometry. *C*, Sema4D (*S4D*) failed to promote tubulogenesis in PAE cells infected with lentivirus expressing EGFP-PDZ. PAE cells infected with EGFP-RGS (*RGS*)- or EGFP-PDZ (*PDZ*)-expressing lentivirus were grown overnight in serum-free media with the indicated factors on a substrate of Cultrex basement membrane extract and then fixed and analyzed for tubule formation. Media containing 0.1% BSA and 10% fetal bovine serum were used as the negative and positive controls, respectively. *D*, PAE cells infected with lentivirus expressing EGFP-PDZ fail to exhibit stress fiber formation in response to Sema4D. PAE cells infected with lentivirus expressing EGFP-RGS (*RGS, top row, left two columns*) or EGFP-PDZ (*PDZ, top row, right two columns*) were grown on glass coverslips and treated for 8 h with conditioned media alone (*control, top row*) or media containing soluble Sema4D (*Sema 4D, bottom row*). Cells were fixed and stained with Texas Red-X phalloidin and viewed under an immunofluorescence microscope. *Green cells* (green fluorescence protein-expressing cells) represent PAE cells successfully infected with the indicated retrovirus and therefore expressing viral proteins. Uninfected cells indicated by the *white arrowheads* fail to exhibit green fluorescence protein fluorescence and therefore served as internal controls. Quantitation of these results reveals that after both 20- and 40-min incubation with 500 ng/ml purified Sema4D, most cells infected with RGS-expressing virus exhibited stress fiber formation but that this response could be almost completely eliminated by infection with virus expressing the PDZ domain (*bar graph*).

not in cells incubated with BSA alone (Fig. 5*D*). When treated with NGF, Trk-A/plexin-B1 stably transfected cells exhibited a tubulogenic phenotype, a response absent in NGF-treated Trk-A/plexin-B1 ΔPDZ lines (Fig. 5*D*). Taken together, these findings suggest that the proangiogenic phenotype induced by plexin-B1 signaling is dependent on an intact PDZ-binding domain.

Sema4D/Plexin-B1-Mediated Angiogenic Responses Require Rho-Initiated Pathways. Because PDZ domain interactions seem to be crucial for plexin-B1-mediated angiogenic responses and are known to play a role in Rho activation, we wanted to determine whether plexin-B1 activation induced stress fiber formation in PAE cells, thus implicating Rho activity in plexin-mediated angiogenesis. PAE cells stably expressing Trk-A, Trk-A/plexin-B1, and Trk-A/plexin-B1 ΔPDZ were grown on glass coverslips and incubated in serum-free media alone or in media containing NGF. The cells were then fixed, stained with phalloidin, and viewed under an immunofluorescence microscope (Fig. 6*A*). All cell lines incubated with media alone showed little to no stress fiber formation (Fig. 6*A*, *top row*). In contrast, Trk-A/plexin-B1-expressing cells show a dramatic induction of stress fibers after NGF treatment (Fig. 6*A*, *bottom row, middle panel*). No stress fibers are observed in identically treated cells expressing Trk-A or Trk-A/plexin-B1 ΔPDZ, however (Fig. 6*A*, *bottom row, left and right panels, respectively*). In line with these observations and consistent with those recently described in fibroblasts and 293T cells expressing plexin-B1 (26), treatment of PAE cells with purified Sema4D resulted in the rapid accumulation of GTP-bound RhoA, as judged by Rho pulldown assays, which could be prevented by the Rho-specific inhibitory C3 toxin (Fig. 6*B*). In fact, C3 toxin compound inhibited PAE migration toward both purified Sema4D and Sema4D conditioned media, even at very low concentrations, and in a dose-dependent manner (Fig. 6*C*). These results indicate that in endothelial cells, activation of plexin-B1 signaling by Sema4D causes

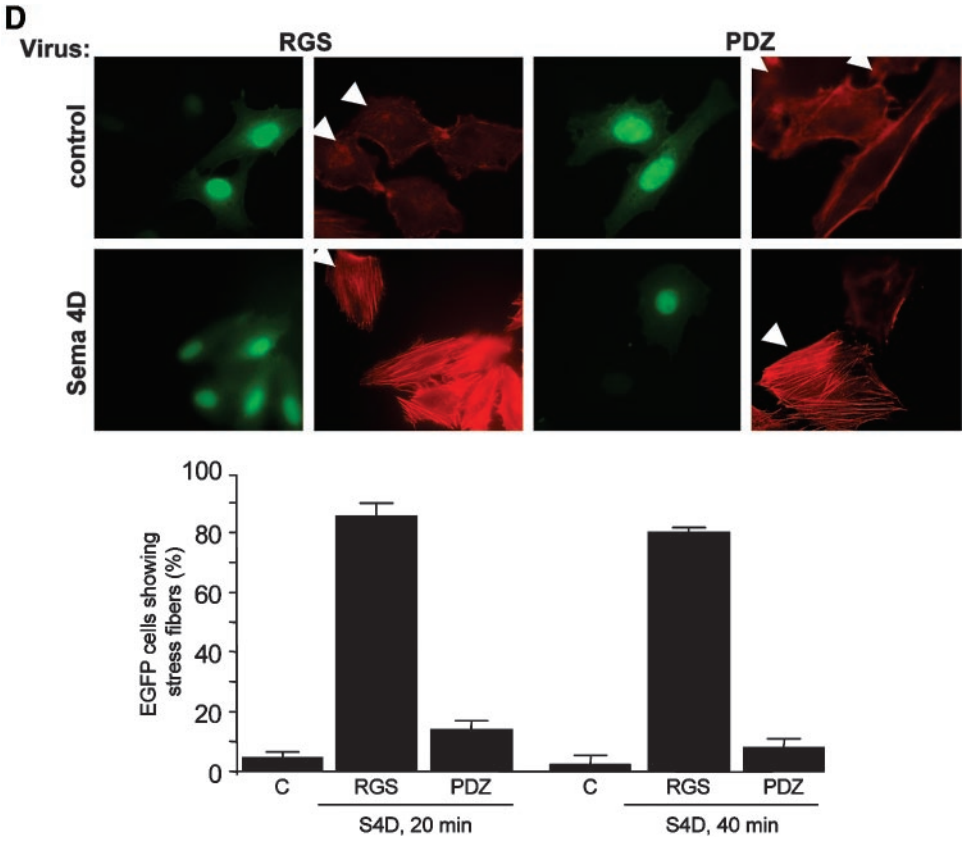

Fig. 7. *continued.*

Rho activation and actin stress fiber polymerization and that this response is likely necessary for the resulting proangiogenic phenotype.

Overexpression of the PDZ Domain of PDZ-RhoGEF Blocks the Sema4D-Mediated Proangiogenic Phenotype. The PDZ domain of PDZ-RhoGEF, when fused to EGFP, has been shown to disrupt Rho signaling in response to plexin-B1 in a dominant-negative manner by competing for endogenous PDZ-binding domains of Rho GEFs (26). Thus, to determine the significance of PDZ domain interactions in Sema4D-initiated plexin-B1 proangiogenic signaling, vectors expressing EGFP or the EGFP-tagged PDZ domain of PDZ-RhoGEF were engineered and packaged into lentiviruses, which were used to infect PAE cells. This signaling pathway does not involve another motif present in Rho GEFs, such as the RGS domain that links $G\alpha_{12}$- and $G\alpha_{13}$-coupled receptors to Rho activation (40, 41). Therefore, EGFP-RGS viruses were also produced, serving as a negative control. The expression of both EGFP-PDZ and EGFP-RGS constructs in infected PAE cells was confirmed by Western blot for EGFP (Fig. 7*A*). PAE cells were then infected with either EGFP-RGS- or EGFP-PDZ-containing viruses and subjected to a migration assay using serum-free media containing BSA or BSA with bFGF or Sema4D as the chemoattractant. Fig. 7*B* demonstrates the expected chemotactic response toward bFGF and Sema4D, but not BSA. Furthermore, EGFP-RGS infection of PAE cells did not affect the ability of the cells to migrate toward bFGF and Sema4D, and EGFP-PDZ did not prevent the migration of PAE cells toward bFGF. In contrast, the chemotactic response toward Sema4D was lost in EGFP-PDZ-infected PAE cells (Fig. 7*B*).

EGFP-RGS- and EGFP-PDZ-infected PAE cells were also subjected to a tubulogenesis assay in the indicated media (Fig. 7*C*). Although tubulogenesis was observed in all serum-treated cells, Sema4D promoted tubulogenesis in EGFP-RGS-infected PAE cells, but not in EGFP-PDZ-infected cells (Fig. 7*C*). These findings suggest that the proangiogenic phenotype induced by plexin-B1 signaling on Sema4D binding is dependent on an intact PDZ-binding motif.

Similarly, EGFP-PDZ abolished stress fiber formation in endothelial cells in response to Sema4D. As shown in Fig. 7*D*, PAE cells growing on glass coverslips were infected with a low titer of EGFP-RGS and EGFP-PDZ viruses and incubated in serum-free media alone or in media containing Sema4D to look for actin stress fiber formation under immunofluorescence. The *top row* of Fig. 7*D* demonstrates that all cells incubated with media alone, both infected (those expressing EGFP) and uninfected (those not expressing EGFP, indicated by the *white arrowhead*), showed little to no stress fiber formation. Cells incubated with EGFP-RGS lentivirus retained a dramatic induction of stress fibers after treatment with Sema4D-containing media (Fig. 7*D*, *bottom row*, *left two panels*), whether successfully infected with virus (*green cells*) or not (indicated by the *white arrowhead*). However, stress fiber formation in response to Sema4D was blocked in identically treated EGFP-PDZ-infected cells (Fig. 7*D*, *bottom row*, *right two panels*), although uninfected cells in the same field, indicated by the *white arrowhead*, exhibited strong stress fiber formation, thus serving as an internal control. These results were quantified and are displayed in a *bar graph* at the *bottom* of Fig. 7*D*, which demonstrates a >80% decrease in the number of cells forming stress fibers in response to Sema4D treatment in PDZ-infected cells, compared with RGS-infected controls. Taken together, these findings suggest that Sema4D requires the PDZ-binding motif of plexin-B1 to initiate the Rho-mediated cytoskeletal reorganization and, in turn, to promote a proangiogenic response.

DISCUSSION

Sema4D-plexin-B1 interactions provide attractive and repulsive cues for the navigation of axonal growth cones (27). Our present findings suggest that these proteins also play an unexpected role in the regulation of the biological functions of endothelial cells, specifically in the control of angiogenesis. Here we show that Sema4D-mediated activation of plexin-B1 induces tubulogenesis and cell migration in endothelial cells and that Sema4D is proangiogenic *in vivo*. Mechanistically, we provide evidence that the plexin-B1-mediated angiogenic response is independent of its extracellular region but is dependent on the integrity of its COOH-terminal PDZ-binding motif and the activation of Rho-GTPases.

The plexin family of cell surface proteins was originally identified through homology in their extracellular domains to the scatter factor receptors (4, 5), the prototype of which is c-Met, the receptor for HGF. In addition to sharing structural homology, plexins and scatter factor receptors mediate similar responses in target tissues, such as survival of sensory neurons (20), outgrowth of motor neuron axons (4), and the induction of cell migration, proliferation, and branching morphogenesis, known as the "scatter phenotype," in epithelium (reviewed in Refs. 7, 21, and 45). HGF itself exerts well-known proangiogenic effects on blood vessels, stimulating chemotaxis, protease production, proliferation, and capillary formation in endothelial cells in a vascular endothelial growth factor-independent manner (36, 46). HGF also induces proliferation of smooth muscle cells and pericytes, cells that participate in the maturation of developing microvasculature (46). Furthermore, there is evidence that class III semaphorins cooperate with HGF in endothelial cell motility and capillary sprouting and in the induction of invasive growth in tumor metastasis (47). For example, neuropilin-1 and neuropilin-2, which are coreceptors along with members of the plexin A family for secreted class III semaphorins, also act as coreceptors with the vascular endothelial growth factor receptor KDR to enhance endothelial cell chemotaxis induced by certain heparin-binding splice variants of vascular endothelial growth factor (14, 47–49). Thus, the finding that endothelial cells express plexin-B1 prompted us to explore a role for Sema4D and plexin-B1 in angiogenic signaling. Stimulation of plexin-B1 receptors with soluble Sema4D did, in fact, induce chemotaxis in PAE cells equal to that seen for bFGF and HGF, both strong inducers of angiogenesis, as well as formation of capillary-like tubules in cells growing on a reconstituted basement membrane substrate (37). We also observed *in vivo* evidence that plexin-B1-Sema4D interactions influence angiogenesis because a bolus of reconstituted basement membrane material containing Sema4D injected s.c. into mice demonstrated remarkable blood vessel infiltration compared with controls. The observations that only 293T cells expressing plexin-B1 could respond to Sema4D in a migration assay and that endothelial cell chemotaxis could be blocked by a specific anti-Sema4D blocking antibody provided further support to the specificity of the Sema4D-plexin-B1 interaction mediating these biological responses. To our knowledge, this represents the fist demonstration that class IV semaphorins can act on their endothelial-expressed receptors to promote an angiogenic response.

Emerging evidence suggests that for plexin-B1, ligation by Sema4D results in activation of RhoA (26, 27, 50). Indeed, our findings indicated that RhoA is potently activated by Sema4D in endothelial cells and that activation of RhoA is in turn required for the migratory activity of Sema4D. However, the nature of the signals generated by semaphorin/plexin binding is likely to be complex and is still poorly defined. In this regard, recent studies have suggested that activation of the scatter factor receptor c-Met is necessary in MLP29 liver progenitor cells for Pexin-B1 signaling (22). However, we did not detect c-Met phosphorylation and activation in Sema4D-treated endothelial cells at biologically active levels of this semaphorin. Intrinsic differences in these cell types, such as differences in the level of expression of plexins and c-Met or their downstream targets, may account for this distinct ability of Sema4D to stimulate c-Met. Nonetheless, the available data do not support the requirement for c-Met activation in the proangiogenic response to Sema4D. For example, PAE cells stably expressing fusion receptors with the extracellular and transmembrane elements of plexin-B1 replaced with Trk-A, thus lacking any extracellular plexin-B1 elements that may engage c-Met, successfully induced cell migration, tubulogenesis, and stress fiber formation in response to NGF. Instead, our results implicate the direct involvement of PDZ-binding domain interactions and activation of Rho-dependent pathways in plexin-B1-mediated angiogenic signaling.

PDZ-containing proteins are typically involved in the assembly of supramolecular complexes that perform localized signaling functions at particular subcellular locations (39). PDZ domains associate with other protein-binding motifs to form multidomain scaffolds that enhance the rate and fidelity of signal transduction within a signaling complex (39). Two Rho GEFs, PDZ-RhoGEF and LARG, are linked through such PDZ-binding domain interactions to transmembrane receptors, coupling receptor ligation to Rho-mediated cytoskeletal changes (24–27, 43, 44, 51–53). Indeed, plexin-B subfamily members, while lacking intrinsic catalytic activity in their intracellular region, contain a COOH-terminal PDZ-binding motif (26) and in fact have been shown to directly interact with Rho GEFs and stimulate Rho (44) while simultaneously inhibiting Rac (27, 54). In line with these observations, endothelial cells stably expressing the receptor chimeras of Trk-A fused to the full-length plexin-B1 intracellular segment (but not the truncated chimera lacking the PDZ-binding motif) and treated with the Trk-A ligand NGF exhibited cell migration, tubulogenesis, and actin stress fiber polymerization. The latter is a cytoskeletal change characteristic of that elicited by Rho activation (26). In fact, we observed activation of RhoA in PAE cells treated with Sema4D in a Rho pulldown assay and were able to block this response with the Rho-specific inhibitor C3 toxin. Interestingly, the concentrations of C3 toxin sufficient to prevent the formation of GTP-bound Rho were sufficient to block endothelial migration. Together, these results suggest that whereas other pathways may also be stimulated and thus play a role plexin-B1-mediated angiogenesis, the ability to stimulate Rho-regulated pathways is necessary for this novel biological response.

Because plexin-B1 requires its PDZ-binding motif to stimulate Rho, we took advantage of the observation that overexpression of the isolated PDZ domain of PDZ-RhoGEF blocks Rho activation by plexin-B1 to examine the contribution of Rho-initiated pathways to the angiogenic response of Sema4D (26). Therefore, we engineered lentiviruses expressing EGFP fused to either the PDZ domain of PDZ-RhoGEF or to its RGS domain as a control, and we used these viruses to infect endothelial cells. Cells infected with the EGFP-PDZ virus failed to migrate to wells containing Sema4D and did not form tubes when grown in the presence of Sema4D. In contrast, EGFP-RGS infection did not impair any of these responses to Sema4D. This inhibition presumably resulted from the ability of the virally transduced PDZ domain to compete with endogenous Rho GEFs for the PDZ-binding domain of plexin-B1. Indeed, the overexpression of this domain resulted in a 6-fold decrease in the formation of stress fibers caused by treatment with Sema4D. These findings suggest that whereas other pathways may be activated and play a role in plexin-B1-mediated angiogenesis, the activation of Rho GEFs and Rho GTPases downstream from plexin-B1 is an integral component of the angiogenic response initiated by Sema4D.

Our current observations may have significant implications in tumor and inflammation-induced angiogenesis. Sema4D is highly expressed on T cells and weakly expressed on B cells, and its expression is increased in both cell types after activation, as is the proteolytic cleavage and shedding of soluble Sema4D (8). Lymphocytes are often a prominent component of the connective tissue stroma in areas of tumor angiogenesis, and they are known to produce a large panel of inflammatory mediators that exert a strong influence on many phases of angiogenesis (55). Because plexin-B1 is apparently not expressed in T cells or B cells and therefore cannot be the receptor for Sema4D in these cell types (8), our observations raise the possibility of the existence of a proangiogenic mechanism whereby the lymphocytes that are recruited to areas of inflammation and tumor growth, once activated, can facilitate angiogenesis by expressing membrane-bound as well as soluble Sema4D, which in turn promotes the recruitment of plexin-B1-expressing endothelial cells to the tumor microenvironment. Sema4D may also be expressed by certain tumor cells. Indeed, while screening cancer cells for expression of semaphorins, we have recently found that Sema4D message and protein are highly expressed in squamous carcinoma of the head and neck,[3] suggesting that Sema4D may directly promote angiogenesis in this and other tumor types. These possibilities are under current investigation.

In summary, plexins and semaphorins are expressed in a variety of tissues outside the nervous system, which suggests that, together with the scatter factors and their receptors, these proteins may comprise a system that controls cell migratory events in numerous cell types (3). The data presented in this study demonstrate that the stimulation of plexin-B1 in endothelial cells by the class IV semaphorin Sema4D leads to the activation of Rho-initiated signaling pathways and promotes a proangiogenic response. These observations, together with the existence of receptors such as the neuropilins, which bind both angiogenic and neuronal factors (56), and the fact that in embryonic development there is a close spatial relationship between growing neurons and blood vessels (57) provide an intriguing link between the motility events that occur during axon guidance and those involved in angiogenesis and further support the possibility that a similar molecular mechanism dependent on Rho GTPases controls both processes (47, 58).

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