Robo4 Signaling in Endothelial Cells Implies Attraction Guidance Mechanisms

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Roundabouts (robo) are cell-surface receptors that mediate repulsive signaling mechanisms at the central nervous system midline. However, robo5 may also mediate attraction mechanisms in the context of vascular development. Here, we have performed structure-function analysis of roundabout4 (Robo4), the predominant robo expressed in embryonic zebrafish vasculature and found by gain of function approaches in vitro that Robo4 activates Cdc42 and Rac1 GTPases in endothelial cells. Indeed, complementary robo4 gene knockdown approaches in zebrafish embryos show lower amounts of active Cdc42 and Rac1 and angioblasts isolated from these knockdown embryos search actively for directionality and guidance cues. Furthermore, Robo4-expressing endothelial cells show morphology and phenotype, characteristic of Robo GTPase activation. Taken together, this study suggests that Robo4 mediates attraction-signaling mechanisms through Rho GTPases in vertebrate vascular guidance.

Roundabouts (Robos)4 are class of neural guidance receptors that bind to the slit family of guidance cues and primarily mediate axon repulsion signals (1, 2). Outside the nervous system, slit-robo signaling has been implicated in inhibition of leukocyte migration (3), kidney induction (4), and vascular system (5, 6). In vertebrates, three Robo receptor family members were identified, all with prominent neural induction (4), and vascular system (5, 6). In vertebrates, three Robo receptor family members were identified, all with prominent neural induction (4), and vascular system (5, 6). The on-line version of this article (available at http://www.jbc.org) contains supplemental material 1–4.

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4 The abbreviations used are: Robo, roundabout; ISV, intersomitic vessel; MO, morpholino; robo4, roundabout4; som, somite; WT, wild type; YFP, yellow fluorescent protein; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; PAE, porcine aortic endothelial; aa, amino acid(s); HEK, human embryonic kidney; BSA, bovine serum albumin; GTPase activating protein; GAP, GTPase-activating protein; WASP, Wiskott-Aldrich syndrome protein.

Received for publication, August 11, 2005, and in revised form, February 15, 2006. Published, JBC Papers in Press, February 15, 2006, DOI 10.1074/jbc.M508853200

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Zebrafish Stocks and Reagents—Zebrafish were grown and maintained at 28.5°C (11). Mating was routinely carried out at 28.5°C, and the embryos were staged according to established protocols (12). Zygogen Inc. (Atlanta) provided the Tg(vegfr2: G-RCFP) fish (13). The dominant negative and constitutive active Robo GTPase constructs have been reported before (14). All zebrafish studies were performed under NCI and National Institutes of Health animal institutional guidelines (animal protocol no. LP-020). Antibodies used in this study include GFP (Santa Cruz Biotechnology, Santa Cruz, CA), Cdc42 and Rac (BD Transduction Laboratories, Rho (Santa Cruz), Actin (Sigma), Tubulin (abcam), anti-rabbit horseradish peroxidase (Amersham Biosciences), pan-cadherin (abcam), and anti-rabbit fluorescein isothiocyanate (Jackson ImmunoResearch). Mounting media with 4’,6-diamidino-2-phenylindole were purchased from Vector Laboratories Inc.

Cloning and Constructs—For fusion constructs F, N, C, C1, C2, and C3, fragments were amplified from previously cloned full-length Robo4 construct using upstream CCACCATGCGTGTCTCGATGATGTTGT (F and N), CCACCATGAGGTGACATGCACATGTGC (C), CCACCATATGGTGTCTCGAATAGGGCGCCGTT (C1, C2, and C3), and downstream GTGGGTGAGGGGCTCTTCCAGATGTCCTC (F, C, and C1), GACTCCAGGCTTGTTGTGATTCAG (C2), GCTTTTAGCGGTAATAGGCAGATGTG (C3), and TGCCACAGGGTTGGTGTGATGTGATGTTGC (N) primers using cycling parameters: 94°C 2 min, 94°C 30 s, 58°C 30 s, 72°C 2 min (34 cycles), and 72°C 5 min.
as described before. The PCR products were cloned in-frame into CT-GFP fusion Topo TA expression vectors (Invitrogen), and all clones were sequenced. Expression constructs were as follows. PAKN: N-terminal 150 aa non-catalytic domain of PAK1, which contains the Cdc42 and Rac binding regions was cloned into pCEFL backbone containing an au1 tag. PAKNL2: same region of PAK1 with mutations in aa residues Leu-83 and Leu-86 (15). WASP: 201–321 aa residues of WASP were cloned into pCEFL backbone.

Cell Lines and Transfections—Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin/amphotericin B. Tissue culture plates were treated with phosphate-buffered saline containing 20 μg/ml poly-d-lysine for 15 min before seeding the cells to prevent them from detaching from the plates when in serum-free conditions. HEK-293T cells were transiently transfected with 5 μg of plasmid in a 10-cm plate using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s instructions. Porcine aortic endothelial cells (PAE) were maintained in Ham’s F-12 media (Sigma) supplemented with 10% fetal bovine serum and 100 units/ml penicillin-streptomycin. Transient transfections in PAE cells were performed using the Lipofectamine Plus reagent (Invitrogen) in conjunction with Magnetofection™ Combimag-2000 (OZ biosciences) to increase transfection efficiency. For co-transfection experiments, equal amounts of the two plasmids were used.

Western Blots—Cells were lysed at 4°C in radioimmune precipitation assay buffer containing 25 mM Tris (pH 8.0), 150 mM KCl, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. For pull-down analysis Rho lysis buffer was used. For GFP westerns, radioimmune precipitation assay buffer, or Rho lysis buffer were used. Solubility of the different mutant proteins varies in radioimmune precipitation assay buffer and Rho lysis buffers.

In vivo Rac1 and Cdc42 activity were assessed by a modified method described before (16). Briefly, after serum starvation for 24 h, the cells were lysed with ice-cold Rho-lysis buffer containing 20 mM HEPES, pH 7.4, 0.1 mM NaCl, 1% Triton X-100, 10 mM EGTA, 40 mM β-glycerophosphate, 20 mM MgCl2, 1 mM NaVO4, 1 mM dithiothreitol, mixture of protease inhibitors, and 1 mM phenylmethylsulfonyl fluoride. The lysates were incubated for 20 min with a purified, bacterially expressed GST fusion protein containing the CRIB domain of PAK1 (p21 activated kinase), previously bound to glutathione-Sepharose beads, followed by three washes with Rho-lysis buffer. The GTP-bound forms of Rac1 or Cdc42 associated with GST-CRIB were quantified by Western blot analysis using a monoclonal antibody against Rac1 or Cdc42. For Rho pull-down assays, GST-Rhotekin-RBD beads were used and assay was performed as described before (17). For in vivo embryo pull-down assays, most of the procedure remained the same except that lysates were made from 75 utilization embryos per sample using 500 μL of Rho-lysis buffer. The fixed cells were permeabilized with 0.5% Triton X-100 in 1× PBS for 10 min, blocked with 0.5% bovine serum albumin (BSA), and incubated for 20 min with phalloidin conjugated to Texas Red (Molecular Probes, Inc.). For membrane staining, transfected cells were stained with primary anti-pan cadherin followed by secondary anti-rabbit fluorescein isothiocyanate antibody. The coverslips were mounted, and confocal images were acquired at room temperature using a Zeiss LSM 510 confocal system mounted on a Zeiss Axiovert 100M microscope equipped with an oil immersion Plan-Apochromat 63×/1.4 differential interference contrast (DIC) objective lens.

Morpholino and RNA Microinjections—Microinjections of one-cell stage zebrafish embryo with RNA or MO were carried out as described before (11). MOs were purchased from Gene Tools, Inc. and reconstituted in nuclelease-free water to a 2 mM stock concentration (16 ng/ml). Appropriate dilutions were made in 5× injection dye (100 mM HEPES, 1 mM KCl, 1% phenol red), and ~2–3 nl of MOs (8–12 ng) were injected at the one-cell stage. Sequences of MO1 and MO2 were described previously (9) except that MO1 and control MO (CTTCTACCTCATG-TACAATTATA) used in this study were fluorescein-tagged. For RNA injections, 150 ng was injected per embryo of wt-Robo4, untagged zebrafish Robo4(zfR4), and YFP-capped RNA transfected by T7 polymerase from linearized vectors containing the respective inserts.

Embryo Dissociation Protocol—Embryos from Tg(vegfr2: G-RCFP) were dechorionated manually at 21 som in egg water. Dechorionated embryos were washed three times in 1× PBS before incubating the embryos in Tm1 buffer (100 mM NaCl, 5 mM KCl, 5 mM HEPES, 1% polyethylene glycol 200000). The cells were dissociated in 1× Tryptsin containing Proteinase K (0.08 mg/ml) with a narrow bore pipette tip and observed under a stereomicroscope until a single cell suspension was obtained. The dissociated cells were filtered with 70-μm nylon cell strainer and immediately transferred into a LabTek glass chamber along with media containing 10% sheep serum.

Ex Vivo Time-lapse and Image Analysis—Dissociated cells from 21 som embryos were observed for GFP and RFP fluorescence using argon gas laser for GFP at 488 nm and helium neon laser for Rhodamine at 546 nm. Once a GFP+/RFP+ cell was detected, initial confocal time series acquisitions was collected one frame (sequentially) every 5 s for 30 min using the Zeiss AIM software version 3.2 sp2 (Carl Zeiss GmbH, Heidelberg, Germany). All original confocal time series datasets were of frame size 532 pixels by 532 pixels, scan zoom of 3 and ~94 to 394 megabytes. Post-processing of the time series datasets was analyzed using Bitplane’s Imaris software version 4.1.1 (Bitplane AG, Zurich, Switzerland). Datasets were cropped to an approximate frame size of 225 pixels by 190 pixels and resampled using a subsampling factor of 2 for x and y. Each dataset was then volume-rendered with a 5-μm grid, and coordinate axes were calculated from the image properties used during image acquisition. After all modifications were completed, the time series datasets were recorded using MPEG file format at 25 frames per second and with a compression factor of 50. For time track analysis, each time series was collected using a Plan-Apochromat 63×/1.4 numerical aperture differential interference contrast oil immersion objective with a scan zoom ranging from 2.5 to 5. For panels B–E in Fig. 2, Imaris 3D (Bitplane AG, Basel, Switzerland) software (version 4.2.0) was used to create a volume rendering of each time series where a spot with a diameter of 4–5 μm represented the cell and a time track reflecting the movement of the spot was generated with the auto-regressive motion gap close function. Time tracks were color-coded with blue for early time points and white for late time points (see color-coded time bar, bottom right of each image). For panels F–P in Fig. 2, a 40-frame subset of each time series was made with the Zeiss AIM software, keeping the midpoint the same as the full 360 frame (5 s/frame) time series. Regions of interest were drawn around the cell perimeter using Image-Pro Plus (MediaCybernetics Inc., Silver Spring, MS) software (version 5.1.2). The green and/or red fluorescence plus phase contrasts were used as a guide to draw regions of interest around each cell at each time point. The new tif series was imported into the Zeiss AIM software to modify the z-step size from 1 mm to 15 mm to create the cylindrical effect for visual clarity. Using Imaris 3D software, the modified z-step
FIGURE 1. Robo4 structure-function mutants and functional analysis in vivo. A, Robo1 and Robo4 structural domain comparisons are shown. Robo1 contains four conserved cytoplasmic intracellular domains, two of which are conserved in Robo4 (CCD and C2C). Octagons represent immunoglobulin repeats; pentagons represent fibronectin type III repeats. TM, transmembrane, small circles represent CCD, CC1, CC2, and CC3 domains, respectively, from left to right. B, the structure-function mutants tagged with GFP at C terminus designed for this study are depicted. F is full-length (wt-Robo4), N is extracellular N terminus (n-Robo4), C is partial extracellular region with C terminus Robo4 (c-Robo4), and C1 is exclusively intracellular C terminus zebrafish Robo4 (c1-Robo4). Hexagons represent GFP tag. C, Western blot with GFP antibody detects the fusion proteins from 293 lysates. D–G, the trunk region of 22- to 24-som Tg(vegfr2: GRCFP) embryos injected with RNAs (lower left corner). H, quantitation of the defective embryos displaying ISVs defects; WT, wild type (n = 93); YFP, yellow fluorescent protein (n = 86); zfR4, zebrafish Robo4 untagged (n = 93); F, wt-Robo4 (n = 106).

RESULTS

Roundabouts are a class of cell-surface receptors originally discovered to mediate repulsion guidance decisions that target axons to their final destination (2). Robo1 and Robo4 are the only two Robos of the robo family that are implicated to play a role in vascular signaling (Fig. 1A) (5, 6, 18). Previously, we have shown that removing endogenous rob4 in zebrafish results in temporal and spatial disruption of ISVs sprouting from dorsal aorta (9). To investigate structure-function correlation for Robo4, we initially generated four deletion mutants tagged with GFP at their C terminus. A full-length zebrafish Robo4 tagged with GFP (wt-Robo4: 1–1134AA), N-terminal that lacks the entire C terminus (n-Robo4: 1–624AA), and two C-terminal GFP fusion constructs with one lacking partial N terminus (c-Robo4: 531–1134AA) while another lacking the N terminus completely (c1-Robo4: 585–1134AA) were generated (Fig. 1B). The constructs were transiently transfected into HEK-293T cells and lysates were immunoblotted with GFP antibody. Western blot showed protein above 160 kDa for wt-Robo4 (Fig. 1C, lane F), slightly above 105 kDa for n-Robo4 (Fig. 1C, lane N), and below 105 kDa for c-Robo4 (Fig. 1C, lane C) and c1-Robo4 (Fig. 1C, lane C1) constructs. Interestingly, wt-Robo4 and n-Robo4 fusion proteins migrate higher in molecular mass than their expected size (wt-Robo4: 150 kDa, n-Robo4: 91 kDa) reflecting probable post-translational modification in the extracellular domain of Robo4. The difference between c-Robo4 and c1-Robo4 is 54 aa, which amounts to a ∼6-kDa size difference that is observed in gradient gel (data not shown).

To check if addition of GFP tag altered the function of the native protein, we used RNA overexpression readouts that was previously shown to phenocopy gene knockdown experiments. To observe vessels, we utilized Tg(vegfr2: GRCFP) transgenic fish carrying a 6.5-kb

Statistical Analysis and Quantitation—A two-way $\chi^2$ test was performed to determine $p$ value of RNA injected samples in Fig. 1. The RNA injection experiments showed statistical significance across samples for YFP and wt-Robo4 or zfR4 ($p < 0.0005$), whereas no significance was attributed between WT and YFP or wt-Robo4 and zfR4 samples. Statistical significance for Fig. 8 was determined by Student two-sample test assuming equal variances. For Fig. 8 (A and B), $p$ values for GFP and wt-Robo4 or wt-Robo4 and wt-Robo4 plus Cdc42DN are $p < 0.005$ in migration assay (stimulus), and $p < 0.0005$ for adhesion assay (fibronecin). For Fig. 8C, $p = 0.16$ for wt-Robo4 and wt-Robo4 plus WASP, $p < 0.001$ for wt-Robo4 and wt-Robo4 plus PAKN. For Fig. 8D, $p < 0.003$ for c-Robo4 and GFP, $p = 0.63$ for n-Robo4 and GFP, $p = 0.75$ for c1-Robo4 and GFP. For Fig. 8E, $p = 3.9e-08$ for GFP and c-Robo4, $p = 0.05$ for GFP and c1-Robo4, and $p = 0.009$ for GFP and n-Robo4. Quantitation of Western blots was performed using Quantity One software. A volumetric analysis tool was used to generate values and, after background subtraction, percentage activation was calculated by GTP pull-down values divided by total lysate values multiplied by 100. The fold activation was determined by dividing all values to untransfected or GFP values.
Robo4 Signaling in Endothelial Cells

FIGURE 2. Ex vivo angioblast assay. A, depicts the steps in the ex vivo angioblast assay. B–E, still images from the supplemental angioblast movies shown with colored time tracks. B, WT; C, MO1; D, MO2; and E, CMO. F–I, cylindrical projection of the movies. F–I are uninjected angioblast cylindrical projections, F–‘F are corresponding angioblast projections for the MO2-injected angioblast, and F–‘F are projections for control the MO-injected angioblast.

FIGURE 3. Confocal microscopy of transfected endothelial cells with robo4 fusion constructs. A–I, coverslips containing transfected porcine aortic endothelial (PAE) cells stained for membrane marker cadherin (red). A, D, G, and J are GFP panels; B, E, H, and K are cadherin-stained; and C, F, I, and L are merged panels. The nomenclatures of F, N, C, and C1 are similar to Fig. 1.

Robo4 Knockdown Angioblasts ex Vivo Search Actively for Guidance Cues—Because rob04 knockdown zebrafish embryos display ISVs sprouting defects (9), we checked the behavior of angioblasts isolated from these embryos. We developed an ex vivo approach (Fig. 2A) where fluorescein-tagged splicing MO was injected at one-cell stage into Tg(vegfr2: GRCPF) embryos. The embryos were developed until desired stage, and angioblasts were isolated by the embryo dissociation protocol explained under “Materials and Methods.” Two different splicing MOs were used that were previously shown to target vegfr2 promoter fragment driving GRCPF (green reef coral fluorescent protein) expression in angioblasts. We injected capped RNA for untagged zebrafish Robo4 (zfR4), wt-Robo4, and control yellow fluorescent protein (YFP) into Tg(vegfr2: GRCPF) embryos and checked for ISVs sprouting from dorsal aorta at 22–24 som (Fig. 1, D–G). Both untagged zebrafish Robo4 (zfR4) (Fig. 1F) and wt-Robo4 RNA (Fig. 1G) disrupted ISVs sprouting from dorsal aorta when compared with YFP (Fig. 1E) or uninjected embryos (Fig. 1D) suggesting that the GFP tag did not alter the native functionality of Robo4. Quantitation shows a 3-fold increase in defective embryos in wt-Robo4 and zfR4 injected samples when compared with wild type (WT) uninjected or YFP-injected embryos (Fig. 1H) and are statistically significant ($p < 0.0005$).

Robo4 Fusion Proteins Activate Rho GTPases—Because Robo4 knockdown zebrafish embryos display ISVs sprouting defects (9), we checked the behavior of angioblasts isolated from these embryos. We developed an ex vivo approach (Fig. 2A) where fluorescein-tagged splicing MO was injected at one-cell stage into Tg(vegfr2: GRCPF) embryos. The embryos were developed until desired stage, and angioblasts were isolated by the embryo dissociation protocol explained under “Materials and Methods.” Two different splicing MOs were used that were previously shown to target rob04 transcript (9). Live time-lapse pictures were captured of angioblasts plated on glass cover chambers, and all movies were made during the period from 24 to 26 som stage where rob04 expression is seen in the vasculature (9). Unexpectedly, angioblasts isolated from rob04 MO1 (supplemental movie 3) and MO2 (movie 4) embryos show movement that resemble cells actively searching for guidance and continue rolling (movie 4) in a non-directional manner. On the other hand, control MO injected (movie 2) or uninjected (movie 1) angioblasts display less activity and appear stationary. Moreover, cells that were not endothelial in origin (GFP−) but were injected with MO did not show any difference in behavior when compared with control MO or uninjected embryos (data not shown). Time tracks in the still images (Fig. 2, B–E) show the track of angioblast movement. The MO-injected embryos (Fig. 2, C and D) show extended time tracks from the original position in comparison to uninjected (Fig. 2B) or control MO-injected embryos (Fig. 2E), which show compacted tracks. In addition to time tracks, four different cylindrical projections (Fig. 2, F–I) showing different cell topology angle were generated for each sample. Comparing the F panels, the MO sample (F) shows numerous deviations from concentric patterns seen in angioblast from uninjected (F) and control MO (F) injected samples. Moreover, the well defined path traversed by the angioblast in both uninjected (G–I) and control MO (G–I) is lost in random movements seen in MO (G–I) cylindrical projections. These results suggest that angioblast in the absence of Robo4 seem to search and move in a random trajectory.

To determine whether the absence of Robo4 had any correlation to the angioblast behavior and movements, we investigated whether Rho GTPases that play a role in actin cytoskeletal rearrangement leading to directional migration were perturbed in the presence of Robo4.

Robo4 Fusion Proteins Activate Rho GTPases—Structure-function fusion constructs described previously were transfected into endothelial cells (Fig. 3, A–L), and protein expression was visualized under GFP.
fluorescent microscopy. Because all deletion proteins contained the transmembrane domain we checked for membrane targeting for fusion proteins by performing immunostaining with pan-cadherin marker on the transfected cells. Confocal analysis shows co-localization patterns for wt-Robo4 (Fig. 3, A–C), n-Robo4 (Fig. 3, D–F), c-Robo4 (Fig. 3, G–I), and c1-Robo4 (Fig. 3, J–L) proteins with cadherin suggesting that all the proteins are appropriately targeted to the plasma membrane. We also checked for co-localization with markers for nucleus, golgi, ER, mitochondria, and lysosome, and none of them co-localized with the fusion proteins (data not shown).

Initially, we performed overexpression analysis in HEK-293T cells, because biochemical studies are relatively easier to perform due to their high transfection efficiency. We transfected the fusion constructs into HEK-293T cells and performed an endogenous Rho GTPase pull-down assay with PAK beads that recognized both activated Cdc42 and Rac1, followed by Western blot with specific antibodies to Cdc42 (Fig. 4A) or Rac1 (Fig. 4B). Both wt-Robo4 (Fig. 4, A and B, lane F) and c-Robo4 (Fig. 4, A and B, lane C) activated Cdc42 or Rac1, but n-Robo4 and c1-Robo4 did not (Fig. 4, A and B, lanes N and C1). Moreover, mouse Robo4 (mRobo4) (Fig. 4, A and B, lane M) and rat Robo1 (rRobo1) (Fig. 4, A and B, lane R1) also activated Cdc42 and Rac1. In contrast, Rho pull-down assays with lysates from wt-Robo4, c-Robo4, mRobo4, or rRobo1 (Fig. 4C, lanes F, C, M, and R1, respectively) showed no active Rho. Quantitation of the blots show 70–80% activation for Cdc42 in wt-Robo4, and 80% Cdc42 or Rac1 activation for mRobo4-transfected cells. Cross-species activation differences were not statistically significant (Cdc42: p = 0.2, Rac: p = 0.3). Taken together, these results suggest that extracellular ligand binding region of Robo4 is necessary for activation of Cdc42 and Rac1 but not Rho and that this activation is mediated by the cytoplasmic region, and is conserved across family members, and in evolution.

Robo4 Activates Cdc42 and Rac1 in Endothelial Cells and Induces Actin Bundles That Resemble Filopodia and Lamellipodia.—To determine if the Rho GTPase activation was conserved in endothelial cells, we repeated the pull-down experiments in PAE cells. Endothelial cells were transfected with wt-Robo4, n-Robo4, and c-Robo4 constructs by magnetofection method, and pull-down assays were subjected to western blotting with Cdc42 (Fig. 4D, top gel) or Rac1 (Fig. 4D, bottom gel) specific antibody. Again, similar to HEK-293T cells, wt-Robo4 and c-Robo4 (Fig. 4D, lanes F and C) activate Cdc42 when compared with untransfected
TABLE 1

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Robo4 Signaling in Endothelial Cells

FIGURE 5. Robo4 induces filopodia and lamellipodia in endothelial cells. Confocal microscopy of endothelial cells transfected with constructs (A, untransfected wild-type cells; B, Rac; C, Cdc42; D, C construct; E, C construct) and stained for actin cytoskeleton are depicted. All panels are merged 40× images of endothelial cells showing actin filaments (red), total quantification of the pull-down assays for different mutants are depicted

FIGURE 6. Gain and loss of function of Robo4 reciprocate Rho GTPase activation state. A, schematic representation of C2 and C3 constructs in comparison to F, C, and C1 are shown. B, pull-down assays of C2 and C3 lysates for Cdc42 and Rac1 in HEK-293T cells is depicted. C, total quantification of the pull-down assays for the different mutants are depicted graphically and explained under “Materials and Methods.” D, pull-down assays for Cdc42 (top gel), Rac1 (middle gel), and Rho (bottom gel) performed with lysates from embryos injected with morpholino1 (MO) or control morpholino (CMO) in comparison to uninjected (UI) embryos are shown and quantitation with ± S.D. are depicted. Error bars represent ± S.D. G, GFP; UT, untransfected.
the small extracellular region that is required for Rho GTPase activation but they lack the CC2 domain in c2-Robo4 and both CC0 and CC2 domains in c3-Robo4 (Fig. 6A). Essentially, c2-Robo4 and c3-Robo4 encompass 531–807 aa and 531–688 aa in Robo4 and, when transfected into endothelial cells, localize to the plasma membrane (data not shown) and produce proteins of expected sizes (data not shown). Further, Rho GTPase pull-down assays in HEK-293T cells show that both c2-Robo4 and c3-Robo4 activate Cdc42 and Rac when compared with c1-Robo4 (Fig. 6B, lanes C2 and C3). However, the c3–Robo4 lysate shows attenuated levels (50%) of Cdc42-GTP when compared with c2-Robo4 (80%) and c-Robo4 (80%). Rac activation, however, was not much affected with 80% for c2-Robo4 and 70% for c3-Robo4. These results when taken together with previous deletion mutants suggest that Robo4 mediates Rho GTPase activation through the intracellular C-terminal domain and that the 54-amino acid extracellular ligand region is necessary for this activation. Further, the intracellular CC0 domain partially mediates Cdc42 activation but is not necessary for Rac activation. Fig. 6C shows quantitative differences in Cdc42 and Rac activation between all the mutants. wt-Robo4, c–Robo4, c2–Robo4, and c3–Robo4 show comparable Rac activation levels when compared with n–Robo4 and c–Robo4-expressing 293 cells. For Cdc42, a marked decrease in activation levels is noted for c3-Robo4-expressing cells, although it is still higher than n-Robo4 and c1–Robo4-expressing cells.

**Gene Knockdown of Robo4 in Zebrafish Embryos Results in Lower Amounts of Active Rho GTPases in Vivo**—Based on the findings that overexpression of Robo4 activates Cdc42 and Rac in HEK-293T and endothelial cells, we asked whether knockdown of Robo4 in vivo in zebrafish embryos would lower the activation state of Rho GTPases. For these experiments, we performed pull-down assays with lysates from MO-injected embryos (Fig. 6D). Robo4 knockdown embryos showed 80% decrease in Cdc42-GTP levels (Fig. 6D, top gel, lane MO) when compared with uninjected (Fig. 6D, top gel, lane UI) and control MO (Fig. 6D, top gel, lane CMO) injected embryos, whereas the Rac-GTP pull-down assays showed a decrease of 40% in MO samples when compared with uninjected. No difference was observed in Rho pull-down assays across uninjected and MO samples (Fig. 6D, bottom gel). The in vivo pull-down results suggest that robod knockdown in zebrafish embryos results in lesser amounts of active Cdc42 and Rac but no difference in Rho and reciprocates with gain of function analysis in endothelial cells.

**Robo4-induced Morphological Effects in Endothelial Cells Are Blocked by Cdc42 Dominant Negative—**Dominant negative (DN) Rho GTPase constructs are extensively used in Rho GTPase biology to address whether blocking specific Rho GTPase activation results in changes in downstream functional consequences measured by phenotypic readouts (19). Initially, we determined by biochemical pull-down assays in HEK-293T cells whether the Cdc42DN and Rac1DN constructs did indeed block Robo4-activated Cdc42 and Rac1 proteins. Interestingly, only Cdc42DN blocks the Robo4-activated Cdc42-GTP (Fig. 7A, top gel, compare lanes F and F+DN), whereas Rac1DN did not (Fig. 7A, bottom gel, compare lanes F and F+DN). One explanation for this result is that Robo4 might activate Rac indirectly by activating Cdc42, and precedence for this cascade mechanism exists at least in fibroblasts (20). To investigate whether activation of Rac is a consequence of Cdc42 activation by Robo4, we checked Rac-GTP levels in wt-Robo4 plus Cdc42DN-transfected cells (Fig. 7B). The pull-down assays show that Rac-GTP level is indeed affected (compare lanes F and F+cDN) and suggest that Robo4-induced Rac activation is not direct. However, whether Robo4-mediated Rac activation is exclusively indirect is an open question.

Because Robo4 also activates both Cdc42 and Rac1 in endothelial cells, we determined whether Robo4-induced Rac activation was indirect in endothelial cells by confocal microscopy of phalloidin-stained actin cytoskeletal changes in endothelial cells transfected with wt-Robo4, wt-Robo4 plus Cdc42DN, and wt-Robo4 plus RacDN. wt-Robo4-transfected cells show thick actin bundles and filopodia that are short and thick (Fig. 7C) in comparison to wt-Robo4 plus Cdc42DN (Fig. 7E) co-transfected PAE cells that show minimal filopodia and loss of actin bundles in the cytoskeleton. In high power, the outer edges of the wt-Robo4-transfected cells clearly shows filopodia and membrane protrusions (Fig. 7D) that disappear when cells are co-transfected with Cdc42DN (Fig. 7F), and instead the outer edge appears smooth and uniform. When wt-Robo4 plus Cdc42DN was compared with wt-Robo4 plus RacDN, Cdc42DN blocked all actin structures induced by Robo4 (Fig. 7E), and RacDN did not (Fig. 7G), suggesting that Robo4-induced Rac activation is most likely indirect in endothelial cells as well. In addition to Robo4, we had earlier shown that Robo1 also activates Cdc42 and Rac in HEK cells (Fig. 4A, lane R1) and endothelial cells (data not shown). Interestingly, Robo1-transfected endothelial cells were constricted (Fig. 7F) and showed retraction fibers that are long and thin suggesting a level of specificity in the actin cytoskeleton rearrangement induced by robos.

**Robo4-induced Phenotypic Effects Are Rescued by Dominant Negative Cdc42**—To investigate the biological consequence of Rho GTPase activation, we performed migration (Fig. 8A) and adhesion (Fig. 8B) assays in HEK-293T cells, because interference with endogenous Robo4 in endothelial cells could hinder interpretation of results. wt-Robo4-expressing cells showed a 2-fold increase in migration to serum when compared with GFP-transfected cells (Fig. 8A). We have also performed migration in HEK-293T cells in response to epidermal growth factor and found no difference between GFP- and Robo4-transfected cells (data not shown) suggesting either epidermal growth factor is not the ligand for Robo4 or that some factor in the serum induces the pro-migratory signal. The angioblast movies (supplemental movie 4) and the structural features of Robo4s extracel-
Robo4 Signaling in Endothelial Cells

FIGURE 8. Robo4-mediated phenotypic readouts are ligand dependent. A, C, and D, graphical representation of HEK293T migration to serum-free media (SF) or 10% fetal bovine serum (FBS) for 5 h at 37 °C. B and E, HEK-293T adhesion assay on BSA- or fibronectin (FN)-coated plates for 5 h. The sample nomenclature include UT: untransfected; G: GFP; F: full-length Robo4; N, C, and C1: mutants; F+cDNA: F plus Cdc42DN; cDNA: Cdc42DN alone; WASP: blocks only active Cdc42; PAKN: blocks both active Cdc42 and Rac; F+ WASP: F plus WASP; F+PAKN: F plus PAKN; PAKNL2: mutant of PAKN. Error bars represent ± S.D., and each experiment was repeated at least three times. The data shown here are from a representative experiment. Occasionally, we noticed an increase in baseline migration of Robo4-transfected cells in the absence of serum.

lular domain (Fig. 1B) suggest adhesion properties for Robo4. We performed adhesion assay in the presence of BSA or fibronectin, because Robo4 contains fibronectin type III domains in its extracellular region. wt-Robo4-transfected HEK-293T cells adhere 4-fold better when compared with GFP-transfected HEK-293T cells (Fig. 8B) on fibronectin matrix, but no significant change was observed on BSA. Because adhesion is one component of chemotaxis, these data together suggest that increased adhesion of Robo4-transfected cells may contribute to the enhanced migratory activity of these cells.

We next investigated whether blocking Robo4-induced filopodia with Cdc42DN would also block the migration and adhesion readouts. Cdc42DN alone does not alter the basal migration status of the cells but blocked Robo4-induced HEK-293T migration to serum (Fig. 8A, panel F+cDNA). In addition, Robo4-induced adhesion in HEK-293T cells was also partially blocked by Cdc42DN (Fig. 8B, panel F+cDNA). Because Cdc42DN blocks Robo4-induced Rac and Cdc42 activation and their downstream effector functions, we asked whether blocking only downstream events from Cdc42 activation specifically by WASP (21) would rescue Robo4-mediated migration responses. WASP-mediated blocking of serum-induced migration of wt-Robo4 co-transfected cells (Fig. 8C, panel F+ WASP) is not statistically significant (p = 0.16) when compared with wt-Robo4-transfected cells (Fig. 8C, panel F). Further, PAKN (another blocker of downstream effectors of both Cdc42 and Rac) blocked serum-induced migration of Robo4-transfected 293 cells (Fig. 8C, compare black bars, F+PAKN and F). Also, PAKNL2, a control for PAKN, was unable to block the activation, and this state indeed promoted migration of HEK293T cells to serum (Fig. 8C, panel PAKNL2). Taken together, these results suggest that blocking downstream effector activation from both activated Rac and Cdc42 is necessary for attenuating Robo4-mediated phenotypic responses.

To determine whether Robo4-induced phenotypic responses are ligand-dependent, we checked for the phenotypic responses of c-Robo4- and c1-Robo4-transfected 293 cells to serum. As expected, wt-Robo4- and c-Robo4-transfected cells migrated in response to serum, but c1-Robo4- and n-Robo4-transfected cells did not (Fig. 8D). In addition, the trend in adhesion assays is similar to the migration assay (Fig. 8E). Previously, we have shown that c-Robo4 activates Rho GTPase while c1-Robo4 did not (Fig. 4A). The differing activities of c-Robo4 and c1-Robo4 mutant in Rho GTPase activation assay along with their expected phenotypic readouts suggest that Robo4-induced Rho GTPase activation is ligand-dependent.

DISCUSSION

Robos in general have been reported to transduce negative guidance cues (22). However, in the case of Robo4, removal of Robo4 in vivo, a presumptive negative regulator, results in fewer sprouts. We had previously proposed two mechanisms to explain this finding (9). First, a non-productive sprouting mechanism where in the absence of guidance a vascular cell throws many sprouts in different directions and eventually regresses and dies and second, an attraction mechanism, where robos mediate attractive cues and removal of such cues results in collapse of vessels. Here, we provide evidence that suggests both mechanisms are
not mutually exclusive and implies that intracellular Rho GTPase signaling molecules play a role in Robo4-mediated attractive guidance mechanism in vertebrates.

In general, axon guidance molecules are bi-functional. Slits were originally discovered as attractants (23) and more recently have been implicated in repulsion signaling (22). Whether Robos mediate attraction or repulsion cues in terms of endothelial guidance has remained controversial (5, 6, 24). Our study reports a pathway that can be explained by both attraction and repulsion mechanisms and suggests, perhaps, that this pathway may be dynamically utilized to relay both signals arising from different ligands. Robos mediate signals through four conserved cytoplasmic (CC) domains (25). Robo4 has diverged from other members of the Robo family in that it contains two of the four CC domains, namely CC0 and CC2 (Fig. 1A). We initially made four deletion mutants of Robo4 and found that the extracellular ligand-binding region is essential for Robo4-mediated signaling. A minimal region of 54 amino acids in the extracellular region is sufficient to activate intracellular signaling pathways, and deletion of this region in construct c1-Robo4 results in no signal transmission. Additional mutants c2-Robo4 and c3-Robo4 focusing on the CC0 and CC2 domains suggest that CC0 domain is partially involved in Robo4-mediated signaling events.

One of the well studied signaling mechanisms induced by robo in neurons involves the Rho family of GTPases (including Cdc42, Rac1, and RhoA) (25). Rho family GTPases regulate several actin-dependent cellular processes such as cell migration, adhesion, morphogenesis, and axon guidance in addition to other aspects of cell biology such as cell polarity, microtubule cytoskeleton, gene expression, and vesicle formation (19). Most small Rho GTPases are regulated by the combined activity of guanine nucleotide exchange factors (GEFs) that exchanges GDP for GTP and GTPase-activating proteins (GAPs) that hydrolyze GTP. Robo1 is established as a repulsive guidance cue for axons and slit binding to Robo1 recruits srGAP1 (GTPase-activating protein) to the CC3 domain, which results in local inactivation of Cdc42 (26). Recently, Vilsé, a RhoGAP, has been implicated in robo-mediated repulsion mechanisms through Rac1 (27, 28). The result of inactivation of Rho GTPases is to reorganize the cytoskeletal components such as actin and microtubules leading to directed cell migration (29). The absence of CC3 domain in Robo4 would suggest that either Robo4 inactivates Cdc42 through alternate mechanisms or that Robo4 does not inactivate Cdc42.

Here, we find that Robo4 when overexpressed, activates both Cdc42 and Rac1 in HEK-293T and endothelial cells, and this activation results in induction of filopodia and lamellipodia in endothelial cells, consequently enhancing cell adhesion and migration. Further, lysates from robol knockout embryos show lower amounts of Cdc42 and Rac in vivo with no difference in Rho levels. Moreover, angioblasts isolated from the knockout embryos display behavior characteristic of cells searching for guidance. Taken together, results from this study imply a role for attraction mechanisms for Robo4 in vascular guidance.

Most of the phenotypic and morphological readouts observed in this study can be explained by a dominant negative mechanism where overexpressing a receptor leads to sequestering components through the intracellular C-terminal domain such as Cdc42GAP and hence leading to Cdc42 activation. However, the strongest argument against this mechanism arises from evidence that two mutants made in this study, namely c-Robo4 and c1-Robo4, share the entire C terminus domain, but only c-Robo4 activates Rho GTPases and c1-Robo4 does not. This suggests that simply sequestering GAPs may not be an exclusive mechanism of Robo4-mediated signaling and argues that ligand-mediated activation of Rho GTPase occurs either by recruitment of GEFs that activate Cdc42/Rac or of GAPs that inactivate Cdc42/Rac.

This brings us to the ligand question. Slits are ligands for Robos (1). However the slit2-Robo4 interaction remains controversial (6, 30). Further, at least three independent lines of evidence suggest that ligands other than slits may be involved in Rho GTPase activation. First, c-Robo4 activates Rho GTPases in the absence of N-terminal Ig domains, which are known interacting domains for slits (24), and HEK-293T cells do express slit2 and slit3 (microarray data not shown). Second, Robo4 dimerizes via the cytoplasmic tail independent of slit binding. Third, during development none of the slits are temporally or spatially expressed during robo4 expression in vascular development (31, 32). Because most assays here were performed in the absence of serum, we suggest here that ligand is secreted in a cell autonomous manner, and a recent report by Gönn et al. (33) suggests this possibility in cell culture supernatants of proliferating endothelial cells.

Overexpression experiments can also be interpreted as ligand-independent signaling, and recent reports suggest that Robo4 overexpression in endothelial cells can trigger other signaling cascades, such as FAK (10). In addition, overexpression in cells has been previously proposed for Robo4 (6) to induce ligand-independent dimerization and for Robo1 and robo2 to promote axonal outgrowth through homophilic and heterophilic mechanisms (34). Unpublished data show that Robo4 does dimerize through intracellular C-terminal domain, but whether dimerization is ligand-dependent or necessary for Rho GTPase activation is not known. However, in our study the differential Rho GTPase activation observed when overexpressing c-Robo4 and c1-Robo4 argues that Rho GTPase activation by Robo4 is ligand-dependent. Until now, relatively little has been known about the role of cell adhesion in the function of axon guidance molecules. Our data suggest that, in the absence of adhesion, Rho GTPase is not activated and, consequently, cells do not move (compare C and CJ in Figs. 6B, 8D, and 8E). Further, because n-Robo4-expressing cells do not adhere, the extracellular Ig domains in Robo4 do not exclusively mediate adhesion. Moreover, wt-Robo4- and c-Robo4-expressing cells adhere well to fibronectin suggesting that inside-out signaling is in effect with Robos.

Angioblast movies suggest that Robo4 knockdown cells appear lost and continue to actively search for guidance cues in several directions. Although the ex vivo data seem counterintuitive at first, it suggests either that alternate mechanisms exist for filopodia generation other than Cdc42 in vivo or simply that we are observing the end stage of a confused angioblast in the movies. The ex vivo data correlate well with in vivo data where vessels collapse in robol knockdown embryos suggesting that, when the guidance cue is lost, cells do not have a general direction to migrate and resort to a default mechanism of collapse.

Experiments with Cdc42DN dominant negative construct suggest that Rac activation is not direct but is mediated through activated Cdc42. Confocal microscopy of endothelial cells stained for actin suggesting that inside-out signaling is in effect with Robos.

This study can be explained by a dominant negative mechanism where overexpressing a receptor leads to sequestering components through the intracellular C-terminal domain such as Cdc42GAP and hence leading to Cdc42 activation. However, the strongest argument against this mechanism arises from evidence that two mutants made in this study, namely c-Robo4 and c1-Robo4, share the entire C terminus domain, but only c-Robo4 activates Rho GTPases and c1-Robo4 does not. This suggests that simply sequestering GAPs may not be an exclusive mechanism of Robo4-mediated signaling and argues that ligand-mediated activation of Rho GTPase occurs either by recruitment of GEFs that activate Cdc42/Rac or of GAPs that inactivate Cdc42/Rac.

5 D. Li, University of Utah, personal communication.
Robo4 mediates vascular guidance. GAPs involved specifically in Robo4 signaling and downstream mechanisms that shut down guidance signals in the absence of guidance molecules are essential for the understanding of robo guidance mechanisms by including attraction to or away from a target. The decisions to move depending on cues from surrounding milieu. Our study extends the current fashion, the robos have evolved an exquisite cross-talk mechanism with slit to perhaps vascular guidance as well and whether these signals arise from common or separate ligand is unclear.

Vascular guidance involves integrating multiple signaling cues both positive and negative to direct the endothelial tip through complex environment. Based on results here, we suggest Robo4 functions as a one of the molecular rheostats in endothelial cells that regulate critical signals for steering the cell to its appropriate target. Because the decisions to move away or toward a target have to be made temporally and spatially in a rapid fashion, the robos have evolved an exquisite cross-talk mechanism with Rho GTPases (Cdc42 and Rac1), which results in an active competition for these proteins resulting in triggering attraction or repulsion mechanisms depending on cues from surrounding milieu. Our study extends the current understanding of robo guidance mechanisms by including attraction to repulsion mechanisms and suggests that vertebrates have evolved mechanisms that shut down guidance signals in the absence of guidance molecules like robos, by simply collapsing the growth cone for axon guidance or endothelial tip in the case of vascular guidance. Identifying the GEFS or GAPS involved specifically in Robo4 signaling and downstream players that induce Cdc42 and Rac1 signaling is a future goal that will shed light on how Robo4 mediates vascular guidance.

Acknowledgments—We thank members of the David Roberts laboratory at NCI for their valuable scientific input, Raymond Stock and Lyndsay Field for maintaining and taking care of our fish stocks, Ruth Lyons from Silvio Gutkind’s laboratory for assistance with pull-down assay, and Susan Garfield and Steven Wincovitch of the Laboratory of Experimental Carcinogenesis for confocal analysis and angioblast movie production and editing.

REFERENCES

Additional resources and further reading may be found through the reference list provided in the document.