β₃ Integrin Promotes Long-Lasting Activation and Polarization of Vascular Endothelial Growth Factor Receptor 2 by Immobilized Ligand

Cosetta Ravelli, Elisabetta Grillo, Michela Corsini, Daniela Coltrini, Marco Presta, Stefania Mitola

**Objective**—During neovessel formation, angiogenic growth factors associate with the extracellular matrix. These immobilized factors represent a persistent stimulus for the otherwise quiescent endothelial cells (ECs), driving directional EC migration and proliferation and leading to new blood vessel growth. Vascular endothelial growth factor receptor 2 (VEGFR2) is the main mediator of angiogenesis. Although VEGFR2 signaling has been deeply characterized, little is known about its subcellular localization during neovessel formation. Aim of this study was the characterization and molecular determinants of activated VEGFR2 localization in ECs during neovessel formation in response to matrix-immobilized ligand.

**Approach and Results**—Here we demonstrate that ECs stimulated by extracellular matrix–associated gremlin, a noncanonical VEGFR2 ligand, are polarized and relocate the receptor in close contact with the angiogenic factor–enriched matrix both in vitro and in vivo. GM1 (monosialotetrahexosylganglioside)-positive planar lipid rafts, β₃ integrin receptors, and the intracellular signaling transducers focal adhesion kinase and RhoA (Ras homolog gene family, member A) cooperate to promote VEGFR2 long-term polarization and activation.

**Conclusions**—A ligand anchored to the extracellular matrix induces VEGFR2 polarization in ECs. Long-lasting VEGFR2 relocation is closely dependent on lipid raft integrity and activation of β₃ integrin pathway. The study of the endothelial responses to immobilized growth factors may offer insights into the angiogenic process in physiological and pathological conditions, including cancer, and for a better engineering of synthetic tissue scaffolds to blend with the host vasculature. (Arterioscler Thromb Vase Biol. 2015;35:2161-2171. DOI: 10.1161/ATVBAHA.115.306230.)

**Key Words:** angiogenesis • gremlin • integrins • lipid rafts • VEGFR2

Tumor neovascularization and angiogenesis-dependent diseases are characterized by the uncontrolled release of angiogenic growth factors, leading to endothelial cell (EC) activation. Numerous angiogenesis inducers, including vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) family members, are produced by tumor or inflammatory cells, accumulate in extracellular matrix (ECM), and interact with tyrosine kinase (TK) receptors expressed on EC surface. In most of the in vitro studies, the characterization of angiogenic growth factor responses is performed after their addition to EC culture medium, implying their interaction with the luminal aspect of endothelium. However, angiogenic growth factors bind different ECM components, including heparan-sulfate proteoglycans (HSPGs), leading to the formation of immobilized ECM-bound complexes. Accordingly, they are found associated with endothelial ECM in vitro and basal membranes in vivo. Even though various proteases and glycosidases may mobilize ECM-anchored angiogenic mediators, stratum-immobilized growth factors induce EC activation by interacting with the basal aspect of the endothelium, leading to long-term EC stimulation. This implies that angiogenic growth factor signaling is likely to be driven by abluminal rather than luminal receptors in ECs. Nevertheless, the fate and subcellular localization of angiogenic TK receptors during neovessel formation triggered by ECM-associated growth factors have not been described yet.

Gremlin is a bone morphogenetic protein antagonist produced by human tumors and expressed by FGF2-activated ECs in vitro and tumor endothelium in vivo. Similar to other heparin-binding angiogenic factors, gremlin binds HSPGs and accumulates on cell surface and ECM rather than in culture medium. Gremlin stimulates EC intracellular signaling, sprouting, migration, and 3-dimensional gel invasion in vitro, leading to a potent angiogenic response in vivo. This is because of the capacity of gremlin to bind and activate VEGF receptor 2 (VEGFR2), as well as HSPGs that act as functional co-receptors.

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Although gremlin does not interact with neuropilin 1, it exerts a VEGFR2-dependent activation of ECs, leading to a tight VEGFR2/αvβ3 integrin crosstalk with mechanisms that are, at least in part, similar to VEGFs.

These bases prompted us to assess the capacity of ECM-associated gremlin to induce a proangiogenic, VEGFR2-dependent response in ECs and to investigate the subcellular fate of VEGFR2 during this process.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Matrix-Bound Gremlin Recruits VEGFR2 at the Basal Aspect of ECs

Gremlin is a proangiogenic, heparin-binding VEGFR2 ligand produced by human tumors. It accumulates on cell surface– and ECM-associated HSPGs of producing cells in vitro and in the perivascular stroma of human tumor grafts in vivo (Figure IA in the online-only Data Supplement). This supports the notion that angiogenic growth factors released by tumor or inflammatory cells accumulate in ECM, thus attracting ECs from preexisting blood vessels to generate a new vascular network. It is therefore possible to hypothesize that these factors interact with their receptors at the basal aspect of ECs. However, limited experimental evidences are available to support this assumption.

To this aim, the subcellular localization of endothelial VEGFR2 was analyzed during in vivo neovascularization induced by ECM-bound gremlin in a murine Matrigel matrix plug angiogenesis assay. Matrigel is a tumor-derived basement membrane matrix composed of a variety of ECM proteins able to retain and slowly release ECM-binding growth factors, mimicking what occurs in tumor stroma. Moreover, at variance with tumor models, the Matrigel plug assay allows the analysis of the effect of a single, selected angiogenic factor. On this basis, gremlin (1.0 mg/mL) was dissolved in Matrigel, and plugs were implanted subcutaneously in C57BL/6 mice. In parallel, VEGF-A and FGF2 plugs were used as a further VEGFR2-dependent and VEGFR2-independent stimulus, respectively. After 7 days, plugs were harvested, and VEGFR2 localization was investigated by immunohistochemical analysis of newly formed blood vessels.

Independent from the angiogenic stimulus, ECs of functional vessels grown in Matrigel plugs were polarized as demonstrated by luminal podocalyxin staining (Figure 1A).

In polarized gremlin-induced vessels, VEGFR2 localized at the basal side of ECs, whereas the luminal side was characterized by CD31 and podocalyxin immunoreactivity only (Figure 1A). At variance, EC sprouts invading the gremlin-enriched Matrigel plug showed a homogeneous distribution of VEGFR2. Similar results were obtained for VEGF-A–induced vessels (data not shown). Specificity of VEGFR2 polarization was supported by the homogeneous, nonpolarized distribution of the receptor in FGF2-induced vessels (Figure II in Nonstandard Abbreviations and Acronyms

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<td>ECD</td>
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<td>HSPG</td>
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Figure 1. Vascular endothelial growth factor receptor 2 (VEGFR2) relocate at the basal aspect of endothelial cells (ECs) during in vivo vessel formation induced by extracellular matrix (ECM)–bound gremlin. Matrigel plugs containing 1.0 μg/mL of gremlin, vascular endothelial growth factor A, or fibroblast growth factor 2 (FGF2) were implanted subcutaneously in C57BL/6 mice. After 1 week, plugs were stained for CD31 or podocalyxin (red), VEGFR2 (green), and nuclei (blue) and analyzed. Images of 0.3 μm sections were collected using a Zeiss Axiovert 200 mol/L epifluorescence microscope equipped with a Plan-Apochromat 63×/1.4 NA oil objective and ApoTome system. A, VEGFR2 (arrowheads) and CD31 or podocalyxin (arrows) immunolocalization and DAPI staining in gremlin induced EC sprout or neovessel in gremlin-enriched Matrigel plugs. Asterisks indicate vessel lumens (Bar, 10 μm). B, Percentage of VEGFR2 staining, respect to the total VEGFR2 positivity, at the basal aspect of ECs in gremlin-, VEGF-A– or FGF2-stimulated lumen-equipped neovessels (mean±SD, n=4 with 2 vessels per experiment; *P<0.001, Student t test).
In a second set of experiments, a haptotactic migration assay was performed to confirm the ability of ECM-associated gremlin to induce VEGFR2 relocation in ECs co-cultured with gremlin-expressing cells. To this aim, mock and gremlin-overexpressing VEGF−/− fibroblasts were seeded on the lower face of 8-μm-pore filters and allowed to deposit their own ECM for the next 48 hours (Figure III in the online-only Data Supplement). Then filters were inserted into a Boyden chamber, and GM7373 (transformed fetal bovine aortic endothelial GM7373 cells) ECs overexpressing the enhanced yellow fluorescent protein (EYFP)–tagged extracellular domain (ECD) of VEGFR2 (ECD-VEGFR2-EYFP GM7373 cells) were seeded on the upper face of the filter and allowed to migrate for 1 hour through the membrane pores in response to the haptotactic stimulus (Figure 2A). Digital cross-sections showed the recruitment of the ECD-VEGFR2-EYFP receptor at the basal side and in cytoplasmic protrusions extending into the filter pores of ECs haptotactically attracted by the ECM deposited by gremlin-overexpressing VEGF−/− fibroblasts (Figure 2B).

In contrast, despite a similar cell adhesion to the filter (data not shown), little amount of VEGFR2 was at the basal side and in the protrusions of ECs seeded on filters conditioned by mock VEGF−/− fibroblasts (Figure 2B–2D). β3 integrin staining was used to unequivocally stain cells and migrative protrusions. Of note, ECD-VEGFR2-EYFP expression did not vary under the different experimental conditions tested (Figure 2E).

We next assessed the capacity of VEGFR2 to relocate at the basal aspect of human umbilical vein endothelial cells (HUVECs) when challenged by gremlin bound to the substratum. To this purpose, HUVECs were seeded on tissue culture plastic irreversibly adsorbed with gremlin and fibrinogen (Figure IV in the online-only Data Supplement), used as a negative control here. No differences in cell adhesion were observed on the 2 substrata (Figure 3A).

As expected, confocal Z-stack imaging of adherent cells showed a significant polarization of VEGFR2 at the basal aspect of cells 4 hours after seeding on immobilized gremlin. This was paralleled by the decrease of VEGFR2 immunoreactivity at the apical side of adherent cells. No VEGFR2 polarization was observed in ECs seeded on fibrinogen (Figure 3A).

**Figure 2.** Substrate-bound gremlin recruits vascular endothelial growth factor receptor 2 (VEGFR2) at the basal aspect and the leading protrusions of migrating endothelial cells (ECs). Mock and gremlin-overexpressing vascular endothelial growth factor (VEGF)−/− fibroblasts were seeded on the lower face of 8-μm-pore chemotaxis filters and incubated for 48 hours to allow them to deposit their own ECM containing gremlin or not. Filters were inserted in a Boyden chamber and enhanced yellow fluorescent protein (EYFP)–tagged extracellular domain (ECD) of VEGFR2 (ECD-VEGFR2-EYFP GM7373 cells) were seeded on the upper face of the filter and allowed to migrate for 1 hour through the membrane pores in response to the haptotactic stimulus (Figure 2A). Digital cross-sections showed the recruitment of the ECD-VEGFR2-EYFP receptor at the basal side and in cytoplasmic protrusions extending into the filter pores of ECs haptotactically attracted by the ECM deposited by gremlin-overexpressing VEGF−/− fibroblasts (Figure 2B).
Figure 3. Vascular endothelial growth factor receptor 2 (VEGFR2) is recruited and activated at the basal portion of endothelial cells (ECs) on immobilized gremlin. A, Human umbilical vein endothelial cells (HUVECs) seeded on substrate-bound fibrinogen (FG) or gremlin show, after 4 hours, a similar adhesive capacity (brightfield images; Bar, 20 μm). Adherent ECs were stained for VEGFR2 (green) and actin (red) and analyzed using a LSM510 Meta confocal microscope equipped with Plan-Apochromat 63×/1.4 NA oil objective. Images show the basal portion of adherent cells with the orthogonal z reconstruction of the whole cell (Bar, 10 μm). B, Ventral plasma membranes (VPMs) from VEGFR2-overexpressing GM7373 (transformed fetal bovine aortic endothelial GM7373 cells) ECs (VEGFR2-GM7373) adherent on immobilized FG, gremlin, fibroblast growth factor 2 (FGF2), or uncoated coverslips for 4 hours were stained for VEGFR2, actin, and nuclei. Samples were analyzed with epifluorescence microscope equipped with Plan-Apochromat 63×/1.4 NA oil objective (Bar, 10 μm). Total VEGFR2 was quantified in 30 cells/sample using Image-Pro Plus software. Data are expressed as percentage±SEM of VEGFR2-positive area in respect to the total VPM area, as defined by actin staining (*P<0.01, Student t test).

B′, After 4 hours of adhesion, VEGFR2-GM7373 cells seeded on immobilized FG or gremlin were lysed, and lysates were probed by VEGFR2 Western blotting analysis. Uniform loading was confirmed by focal adhesion kinase (FAK) Western blotting. C, 50 μg of cell extracts of HUVECs seeded for the indicated time on substrate-bound FG or gremlin or on uncoated cell culture plates were probed by phospho-VEGFR2 (pVEGFR2 [phospho-vascular endothelial growth factor receptor 2]; pTyr 1175) or total VEGFR2 Western blotting. Uniform loading was confirmed by FAK Western blotting. D, HUVECs were left adhered on substrate-bound FG or gremlin for 4 hours and stained for pVEGFR2 (pTyr 1175; green) and actin (red) and analyzed using a LSM510 Meta confocal microscope equipped with Plan-Apochromat 63×/1.4 NA oil objective. Images show the basal portion of adherent cells with the orthogonal z reconstruction of the whole cell (Bar, 10 μm). C′, 50 μg of cell extracts of HUVECs seeded for the indicated time on substrate-bound FG or gremlin or on uncoated cell culture plates were probed by phospho-p85 (P-p85; red) and nuclei (blue) and analyzed with epifluorescence microscope equipped with Plan-Apochromat 63×/1.4 NA oil objective. Images show the basal portion of adherent cells with the orthogonal z reconstruction of the whole cell (Bar, 20 μm). E, VPMs from VEGFR2-GM7373 cells left adhered on immobilized FG or gremlin for 4 hours in the absence or in the presence of SU5416 (Sugen5416) were stained for VEGFR2, actin, and nuclei. Samples were analyzed with epifluorescence microscope equipped with Plan-Apochromat 63×/1.4 NA oil objective (Bar, 10 μm). F, VEGFR2 was quantified in 30 cells/sample using Image-Pro Plus software. Data are expressed as percentage±SEM of phospho-p85–positive area in respect to the total cell area (*P<0.001, Student t test). F′, Cells were left adhered in the absence or presence of SU5416. Phospho-p85 was quantified in 30 cells/sample using Image-Pro Plus software. Data are expressed as percentage±SEM of phospho-p85–positive area in respect to the total cell area (*P<0.001, Student t test). G, HUVECs were treated or not with SU5416 and seeded on immobilized gremlin or FG. Cell motility was assessed by time-lapse video microscopy using an inverted microscope (Zeiss Axiovert 200 mol/L). Phase-contrast snap photographs (one frame every 10 minutes) were digitally recorded for 8 hours. Cell paths (40–50 cells per experimental point) were generated from centroid positions, and migration parameters were analyzed with the Chemotaxis and Migration Tool of ImageJ Software (http://rsbweb.nih.gov/ij). Graph shows the accumulated distances (in μm) of HUVECs seeded on immobilized gremlin or fibrinogen in the absence or presence of SU5416 (*P<0.05, Student t test).
Finally, VEGFR2-overexpressing GM7373 ECs\textsuperscript{10} were seeded on immobilized gremlin, fibrinogen, or uncoated coverslips for 4 hours. Ventral plasma membranes (VPMs) were prepared from adherent cells by osmotic shock\textsuperscript{17} and decorated for the presence of immunoreactive VEGFR2. In all the experiments, the absence of DAPI (4′,6-diamidino-2-phenylindole) staining and the persistence of actin filaments were used to unequivocally identify the VPM remnants bound to the substrate. As shown in Figure VA in the online-only Data Supplement and Figure 3B, VEGFR2 was specifically recruited in VPMs of cells seeded on immobilized gremlin but not in cells seeded on fibrinogen or uncoated coverslips. This occurred in the absence of any change in VEGFR2 expression (Figure 3B).

Taken together, these data provide in vivo and in vitro experimental evidence about a directional relocation of VEGFR2 after receptor engagement by its substratum-associated ligand.

**Matrix-Bound Gremlin Induces VEGFR2 Activation at the Basal Aspect of ECs**

To assess the ability of substratum-immobilized gremlin to exert a directional stimulation of ECs through a productive interaction with VEGFR2, VEGFR2 phosphorylation was analyzed by Western blotting performed on the total cell lysates of HUVECs adherent to immobilized gremlin at 20 minutes, 2 hours, and 5 hours after seeding. Immobilized gremlin induces a rapid and long-lasting phosphorylation of VEGFR2, whereas no receptor activation was detectable in HUVECs seeded on fibrinogen or uncoated wells. Of note, total VEGFR2 expression did not vary during the timing of the experiment (Figure 3C).

Immunofluorescence analysis of HUVECs seeded on immobilized gremlin confirmed that VEGFR2 recruited at the basal aspect was phosphorylated (Figure 3D). Similarly, VEGFR2 was activated in VPMs of VEGFR2-overexpressing GM7373 ECs seeded on immobilized gremlin (Figure VB in the online-only Data Supplement and Figure 3E). We also observed a significant increase in the phosphorylation of the PI3-kinase regulatory subunit p85\textsuperscript{18} (Figure 3F). VEGFR2 and p85 activations were both inhibited by the VEGFR2 TK inhibitor SU5416 (Signet5416; Figure 3E and 3F).

Finally, we analyzed EC motility as a biological response to VEGFR2 activation. As shown in Figure 3G, time-lapse video microscopy of individual cells demonstrates that immobilized gremlin induces an increase of cell migration when compared with fibrinogen. As anticipated, SU5416 reduced the cellular response to immobilized gremlin.

**Matrix-Bound Gremlin Induces VEGFR2/β\textsubscript{3} Integrin Complex Formation in Planar Lipid Rafts**

VEGFR2/β\textsubscript{3} integrin complex formation plays a pivotal role in mediating the EC response to free gremlin.\textsuperscript{14} On this basis, we assessed the capacity of immobilized gremlin to induce a direct VEGFR2/β\textsubscript{3} integrin interaction at the basal portion of ECs. To this purpose, β\textsubscript{3}-enhanced cyan fluorescent protein/ECD-VEGFR2-EYFP co-transfected GM7373 cells were allowed to adhere on immobilized gremlin or fibrinogen. A cytoplasmic cyan fluorescent protein–yellow fluorescent protein fusion was used as a control of fluorescence resonance energy transfer efficiency.\textsuperscript{19} After 4 hours of adhesion, fluorescence resonance energy transfer acceptor photobleaching analysis showed a direct interaction between VEGFR2 and β\textsubscript{3} integrin in VPMs of ECs seeded on gremlin but not in cells adherent to fibrinogen (Figure 4A and 4A′).

Engagement by ECM proteins causes the recruitment of integrin receptors into paxillin\textsuperscript{*} focal adhesions.\textsuperscript{19} Accordingly, immobilized fibrinogen induced the enrichment of β\textsubscript{3} integrin in paxillin\textsuperscript{*} focal adhesions at the basal aspect of β\textsubscript{3}-EGFP–overexpressing GM7373 cells and HUVECs (Figure VIA in the online-only Data Supplement). At variance, ECs seeded on immobilized gremlin showed small paxillin\textsuperscript{*} focal adhesions and a widespread distribution of β\textsubscript{3} integrin at the basal side of the cell (Figure VIB in the online-only Data Supplement). Indeed, triple co-localization experiments demonstrated that 2 β\textsubscript{3} integrin subpopulations exist at the basal aspect of HUVECs seeded on immobilized gremlin: one subpopulation involved in the formation of small paxillin\textsuperscript{*} focal adhesions and a second VEGFR2-associated subpopulation recruited in distinct paxillin-negative cell membrane structure(s) (Figure 4B).

Lipid rafts are cholesterol- and sphingolipid-rich plasma membrane microdomains in which multiple signaling molecules and receptors are assembled to provide the molecular proximity for activation of downstream signaling.\textsuperscript{21} To identify the structure(s) involved in VEGFR2/β\textsubscript{3} integrin complex formation at the basal aspect of cells seeded on immobilized gremlin, we compared the immunolocalization of VEGFR2 and β\textsubscript{3} integrin to the distribution of GM1 (monosialotetrahexosylganglioside) ganglioside, a lipid raft marker,\textsuperscript{22} highlighted by fluorescently labeled cholera toxin B subunit.\textsuperscript{19} Triple co-localization analysis\textsuperscript{20,23} at the basal portion of HUVECs seeded on immobilized gremlin revealed that VEGFR2 co-localizes with β\textsubscript{3} integrin and GM1 in paxillin-negative structures with a frequency 6× higher than that on fibrinogen (Figure 4C and Figure VII in the online-only Data Supplement). Of note, VEGFR2 polarized at the basal aspect of ECs is not recruited in caveoleae, as ruled out by VEGFR2 and Caveolin 1 double-immunostaining, thus suggesting its presence in planar lipid raft structures\textsuperscript{24} (Figure VIII in the online-only Data Supplement). Importantly, GM1-associated VEGFR2 is activated, as shown by the immunolocalization of pVEGFR2 (phospho-vascular endothelial growth factor receptor 2) in GM1-positive membrane microdomains (Figure 5A). As expected, no pVEGFR2 is associated with GM1 ganglioside at the apical side of cells adhered to immobilized gremlin (Figure 5A). On these bases, to assess the role of lipid rafts in VEGFR2 activation by immobilized gremlin, HUVECs were treated with the lipid raft–disrupting agent 2,6-di-O-methyl-β-cycloexodextrin (MβCD).\textsuperscript{19} Pretreatment with MβCD prevented VEGFR2 relocation and phosphorylation at the basal aspect of ECs seeded on immobilized gremlin, and its effect was fully reversed by cholesterol repletion of MβCD-treated cells (Figure 5A and 5B). Accordingly, MβCD treatment caused a significant reduction in the motogenic response of HUVECs to immobilized gremlin, fully reverted by cholesterol repletion (Figure 5C).

**Long-Lasting Retention of VEGFR2 at the Basal Aspect of ECs Requires β\textsubscript{3} Integrin Signaling**

The data above prompted us to assess the role, if any, of β\textsubscript{3} integrin in VEGFR2 recruitment at the basal aspect of ECs,
To this purpose, \( \beta_3 \)-enhanced cyan fluorescent protein and ECD-VEGFR2-EYFP GM7373 cells were cultured on glass coverslips that were flipped upside-down on gremlin- or fibrinogen-coated microslides (Figure 6A). Time-lapse analysis of Z-stack sections was performed to follow the recruitment of VEGFR2 and \( \beta_3 \) integrin at the basal side of cells during cell adhesion to the substratum. As shown in Figure 6A’ and 6A”, VEGFR2 rapidly moved to the membrane portion in close contact with immobilized gremlin but not with fibrinogen. VEGFR2 recruitment was already detectable 6 to 8 minutes after EC/gremlin interaction and preceded the slow relocation of \( \beta_3 \) integrin that occurred 60 to 120 minutes thereafter. In contrast, higher levels of \( \beta_3 \) integrin were rapidly recruited in cells in contact with fibrinogen in the absence of any VEGFR2 polarization (Figure 6A”).

Next, the effect of the neutralizing anti-\( \beta_3 \), BV4 antibody on VEGFR2 recruitment and phosphorylation induced by immobilized gremlin was assessed at 30 minutes and at 2 to 4 hours after seeding. Of note, the BV4 antibody is known to inhibit \( \beta_3 \) integrin at the dose tested without affecting EC adhesion.14,18 As shown in Figure 6B and 6C, VEGFR2 ventral localization and phosphorylation observed at 2 and 4 hours after seeding of ECs on immobilized gremlin was reduced by the BV4 antibody, whereas no effect was observed at 30
Together, these data point to a nonredundant role of β3 integrin in the long-lasting phosphorylation of VEGFR2 and in its retention at the basal side of ECs engaged by immobilized gremlin, being instead dispensable during the early phases of receptor relocalization and activation.

Phosphorylation (pTyr759 [phospho-tyrosine 759]) of β3 integrin is a crucial event for its direct interaction with VEGFR2 after stimulation by free VEGF-A.25 We found that immobilized gremlin induces a transient phosphorylation of β3 integrin with a peak at 30 minutes after EC seeding (Figure IX in the online-only Data Supplement). At variance, in agreement with previous observations,25 only a limited β3 integrin Tyr759 phosphorylation was observed in ECs adherent to fibrinogen. On this basis, to assess whether focal adhesion kinase (FAK)/Ras homolog gene family, member A (RhoA) β3 integrin downstream signaling26 may mediate VEGFR2 long-lasting retention at the basal membrane, VEGFR2-overexpressing GM7373 ECs were stably transfected with the cDNA encoding for the FAK C-terminal domain (focal adhesion kinase-related nonkinase [FRNK]) or with the FRNK L1034S mutant.26 FRNK exerts a dominant negative effect on FAK activation, promoting its dephosphorylation at Tyr397, effect that is abolished by the L1034S point mutation preventing FRNK localization to focal contact sites.27 As shown in Figure 6D, FRNK overexpression inhibited VEGFR2 retention, whereas FRNK L1034S was ineffective. In addition, similar to BV4 antibody treatment or FRNK overexpression, VEGFR2 polarization driven by immobilized gremlin was prevented also by the Rho inhibitor exoenzyme C3 (Figure 6D).

At variance, SU5416, although inhibiting the phosphorylation

Figure 5. Lipid raft integrity is essential for vascular endothelial growth factor receptor 2 (VEGFR2) activation and relocation on immobilized gremlin. A, Human umbilical vein endothelial cells (HUVECs) were treated with 10 mmol/L 2,6-di-O-methyl-β-cyclodextrin (MβCD) followed or not by 400 mg/mL of cholesterol, left adhered on immobilized fibrinogen (FG) or gremlin, and stained for phospho-VEGFR2 (pTyr 1175) (green) and GM1 (monosialotetrahexosylganglioside) ganglioside (red). Samples were analyzed using a LSM510 Meta confocal microscope equipped with Plan-Apochromat 63×/1.4 NA oil objective. Images of the basal and apical aspect of cells were then analyzed for fluorescence signal co-localization (white dots; Bar, 10 μm). All image pixels are displayed in the scattergrams below; co-localized pixels are represented in the upper right quadrant. B, Ventral plasma membranes (VPMs) of VEGFR2-GM7373 (transformed fetal bovine aortic endothelial GM7373 cells) cells treated with 10 mmol/L MβCD followed or not by 400 mg/mL of cholesterol and left adhered on immobilized gremlin or FG for 4 hours were stained for VEGFR2 and actin. Stained VPMs were photographed under an epifluorescence microscope equipped with Plan-Apochromat 63×/1.4 NA oil objective (Bar, 10 μm). Total VEGFR2 was quantified in 30 cells/sample using Image-Pro Plus software. Data are expressed as percentage±SEM of VEGFR2-positive area in respect to the total VPM area, as defined by actin staining (*P<0.01, Student t test). C, HUVECs were treated or not with 10 mmol/L MβCD followed or not by 400 mg/mL of cholesterol and seeded on immobilized gremlin or FG. Cell motility was assessed by time-lapse video microscopy using an inverted microscope (Zeiss Axiovirt 200 mo/L). Phase-contrast snap photographs (one frame every 10 minutes) were digitally recorded for 8 hours. Cell paths (40–50 cells per experimental point) were generated from centroid positions, and migration parameters were analyzed with the Chemotaxis and Migration Tool of ImageJ Software (http://rsbweb.nih.gov/ij). Graph shows the accumulated distances (in μm) of HUVECs seeded on immobilized gremlin or fibrinogen in the absence or presence of MβCD, cholesterol, or MβCD plus cholesterol (*P<0.05, Student t test).
of recruited VEGFR2 (Figure 3E), did not affect the amount of total VEGFR2 detected in VPMs of ECs stimulated by immobilized gremlin (Figure 6D). This is in keeping with the observation that VEGFR2 was able to rearrange on the plasma membrane and complex with β3 integrin in response to gremlin stimulation also when devoid of its intracellular TK moiety.
(Figure 2 and 4A). Finally, we investigated the effect of BV4 antibody and exoenzyme C3 on the motility of ECs seeded on immobilized gremlin. Time-lapse video microscopy of individual cells showed that the increase of cell motility induced by immobilized gremlin was significantly reduced by treatment with BV4 antibody or exoenzyme C3 (Figure 6E).

Discussion
The anchorage of growth factors to ECM represents an important event of the angiogenic switch that drives neovessel formation. Even though proteases and glycosidases may mediate the spatial distribution of these factors by generating freely diffusible forms, in vitro and in vivo evidences show that ECM-immobilized angiogenic growth factors are bioactive and retain the capacity to engage their signaling receptors.

Gremlin is a heparin-binding factor produced by tumors and retained in the stromal ECM surrounding CD31-positive domains. In agreement with our previous findings and the growth factors. Also, VEGF isoforms result in different VEGFR2/β3 integrin interaction occurs in the absence of any direct binding of gremlin to β3 integrins. Further experiments will be required to define the molecular bases of gremlin-mediated VEGFR2/β3 integrin interaction.

Under our experimental conditions, VEGFR2/β3 integrin complexes are localized in paxillin- and Caveolin 1–negative cell membrane structures identified as GM1-positive lipid rafts. Lipid rafts are cholesterol- and sphingolipid-rich plasma membrane microdomains that favor the interaction between transmembrane receptors and the recruitment/activation of downstream second messengers. Accordingly, EC pretreatment with the lipid raft–disrupting agent MJβCD prevents VEGFR2 phosphorylation and recruitment and inhibits the motogenic response of ECs to immobilized gremlin, both rescued by cholesterol repletion. These data are in keeping with the role of lipid rafts in mediating VEGFR2/β3 integrin interaction by immobilized HIV-Tat (Human Immunodeficiency Virus trans-activator) protein and VEGFR2 activation by free VEGF. Of note, ECs exposed to collagen-bound VEGF organize VEGFR2/β3 integrin complexes, suggesting that integrin partnership and receptor complex localization after VEGFR2 engagement are contextual and may depend on the ECM microenvironment surrounding angiogenic vessels.

Our observations demonstrate that the kinetics of recruitment by immobilized gremlin of VEGFR2 and β3 integrin at the basal aspect of ECs differs between the 2 receptors. Indeed, VEGFR2 relocation largely precedes β3 integrin activation and its interaction with VEGFR2. Accordingly, the neutralizing anti–β3 integrin antibody BV4 has no effect on the early recruitment of VEGFR2 and its phosphorylation. At variance, this antibody hampers the long-lasting phosphorylation and basal membrane retention of VEGFR2 and inhibits the motogenic activity exerted by the substratum-bound angiogenic factor on adherent ECs.

The role of β3 integrin in the long-term relocation and activation of VEGFR2 by immobilized gremlin is further supported by the capacity of FAK and Rho inhibitors to suppress these responses in adherent ECs. The FAK residue Leu1034 acts as a docking site for p190RhoGEF, a Rho-specific GDP/GTP exchange factor, and abrogation of FAK activity hampers the capacity of ECs to activate RhoA in response to immobilized Tat. RhoA is a monomeric GTPase protein known to regulate actin polymerization in controlling cell shape, polarity, and locomotion. A tight crosstalk exists between actin cytoskeleton and lipid rafts: rafts control actin cell shape, polarity, and locomotion. A tight crosstalk exists between actin cytoskeleton and lipid rafts: rafts control actin cytoskeleton, regulating focal adhesion turnover in migrating cells; on the contrary, an intact actin cytoskeleton is required for several structural and functional properties of lipid rafts, such as the formation of large rafts macrodomains and the co-clustering of raft-associated proteins through the condensation of the plasma membrane. Here we demonstrate that the downregulation of FAK activity by FRNK overexpression and the inhibition of Rho by C3 exoenzyme efficiently blocks VEGFR2 retention at the basal aspect of ECs and cell motility induced by immobilized gremlin, highlighting the importance of integrin signaling in the long-lasting functionality of lipid rafts–associated VEGFR2.
Overall, these results indicate that immobilized gremlin retains its biological activity, representing a long-lasting stimulus that induces VEGFR2 polarization at the basal aspect of ECs in vivo and in vitro. Recruited VEGFR2 forms complexes with β integrin in EC lipid rafts, and this organization is essential for long-lasting VEGFR2 retention, phosphorylation, and activation of biological responses in ECs.

Our results highlight the notion that accumulation of angiogenic growth factors in ECM may represent a receptor-polarizing stimulus for the otherwise quiescent endothelium, leading to directional migration and proliferation and, eventually, neovessel formation. The study of the EC responses to immobilized growth factors may offer insights into the angiogenic process in physiological and pathological conditions, including cancer, and for a better engineering of synthetic tissue scaffolds to blend with the host vasculature.

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**Disclosures**

None.

**References**


Significance

Engagement by extracellular matrix–associated angiogenic factors drives cognate receptors to the basal aspect of neovascular endothelium.
β3 Integrin Promotes Long-Lasting Activation and Polarization of Vascular Endothelial Growth Factor Receptor 2 by Immobilized Ligand
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SUPPLEMENTAL FIGURES

Beta3 integrin promotes long-lasting activation and polarization of Vascular Endothelial Growth Factor Receptor 2 by immobilized ligand

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FIGURE S. I

Figure S. I: Gremlin accumulates in the stroma surrounding CD31\(^+\) vessels of human adenocarcinoma xenografts. Human endometrial adenocarcinoma HEC-1-B-derived Tet-FGF2 cells were injected s.c. in nude mice. (A) Sections from 8 weeks tumors were double-immunostained with the rat monoclonal anti-mouse CD31 antibody MEC 13.3 and with goat anti-murine gremlin antibody (R&D Systems) followed by biotin-conjugated rabbit anti-rat secondary antibody (DAKO) and Alexa-fluor 488 rabbit anti-goat secondary antibody (Molecular Probes) followed by incubation with Texas red Avidin D (Vector). Samples were analysed using a Zeiss Axiovert 200M epifluorescence microscope equipped with a Plan-Apochromat 63x/1.4 NA oil objective (Bar, 100 μm). (B) Image shows secondary antibodies only staining.
Figure S. II: VEGFR2 is equally distributed between apical and basal aspects of ECs in vivo vessel formation induced by ECM-bound FGF2. Matrigel plugs containing 1.0 µg/mL of FGF2 were implanted s.c. in C57BL/6 mice. After 1 week, plugs were stained for CD31 or podocalyxin (red), VEGFR2 (green) and nuclei (blue) and analyzed. Images of 0.3 µm sections were collected using a Zeiss Axiovert 200M epifluorescence microscope equipped with a Plan-Apochromat 63x/1.4 NA oil objective and ApoTome system (Zeiss). Asterisks indicate vessel lumens (Bar, 10 µm).
Figure S. III: Gremlin produced by gremlin-overexpressing VEGF−/− fibroblast is bound to extracellular ECM. 80% confluent mock- and gremlin-overexpressing VEGF−/− fibroblasts were starved in DMEM without FCS. After 24 hours the conditioned medium was harvested and cells were washed once with PBS and once with PBS containing 1.5 mol/L NaCl. Cells were then lysed. Conditioned medium and 1.5 mol/L NaCl wash were concentrated 10 times using Centricon devices (Millipore). Finally, lysate and 20 μL of concentrated conditioned medium and concentrated 1.5 mol/L NaCl wash were analysed by Western blotting using a specific anti-gremlin antibody (R&D System).
Figure S. IV: Substrate-bound gremlin does not elute from tissue culture plastic. Aliquots (100 µL) of sterile PBS containing gremlin (0.2-2-10 µg/mL) were added to polystyrene tissue culture plates. After 16 hours of incubation at 4°C wells were washed 3 times with cold PBS. Uncovered plastic was blocked with 1 mg/mL BSA for 1 hour at room temperature and immobilized gremlin was measured by ELISA. Plastic-immobilized gremlin resists extraction with 2 mol/L NaCl and detergent treatment following incubation for 1 hour at 37°C with 0.2% Triton X-100.
Figure S. V: Substrate-bound gremlin recruits and activates VEGFR2 in EC VPMs. (A) Ventral plasma membranes (VPMs) from VEGFR2-GM7373 cells seeded on uncoated coverslips, immobilized FG, gremlin or FGF2 were stained for VEGFR2, actin and nuclei. (B) VPMs from VEGFR2-GM7373 cells seeded on immobilized FG or gremlin and stained for phospho-VEGFR2 (pTyr1175), actin and nuclei. Samples were analyzed with epifluorescence microscope equipped with a Plan-Apochromat 63x/1.4 NA oil objective epifluorescence microscope (Bar, 10 μm). Note the absence of nuclear DAPI staining and the persistence of actin filaments used to unequivocally identify the VPM remnants bound to the substratum.
Figure S. VI: Immobilized gremlin does not recruit $\beta_3$ integrin in EC VPMs. (A) $\beta_3$-EGFP-overexpressing GM7373 cells were seeded on substrate-bound gremlin, FG or uncoated coverslips. A similar cell adhesion was observed under all the experimental conditions (not shown). After 4 hours VPMs were prepared from ECs, fixed and stained with TRITC-phalloidin. Then, cells were photographed under a Zeiss Axiovert 200M epifluorescence microscope equipped with a
Plan-Apochromat 63x/1.4 NA oil objective and ApoTome system (Bar 20 μm). β3-EGFP was quantified in 30 cells/sample using Image-Pro Plus software. Data are expressed as percentage ± SEM of β3 integrin positive area in respect to the total VPM area, as defined by actin staining (*, P<0.01, Student's t test). (B) HUVECs were seeded on substrate-bound FG or gremlin. After 2 hours cells were fixed and immunostained with anti-paxillin and anti-β3 integrin antibodies followed by AlexaFluor 488-conjugated anti-mouse IgG and Cy5-conjugated anti-rabbit IgG. Samples were analysed using a LSM510 Meta confocal microscope equipped with Plan-Apochromat 63x/1.4 NA oil objective (Zeiss). Panels show the double immunostaining for paxillin and β3 integrin at the basal portion of adherent cells (Bar, 20 μm). Areas highlighted by white boxes are shown at higher magnification for β3 integrin and paxillin immunolocalization (arrows indicate paxillin-positive focal adhesions).
Figure S. VII: Recruited VEGFR2 co-localizes with β3 integrin and GM1 ganglioside. HUVECs were seeded on immobilized FG or gremlin, stained for VEGFR2 (green), β3 integrin (blue) and GM1 ganglioside (red) and acquired using a LSM510 Meta confocal microscope equipped with Plan-Apochromat 63x/1.4 NA oil objective (Zeiss). Images show the basal portion of adherent ECs (Bar, 10 μm). Pictures were analyzed using BlobProb ImageJ plugin [10]: white blobs represent areas in which respectively VEGFR2 co-localizes with GM1 co-localizes with β3 integrin and VEGFR2 co-localizes with β3 integrin. (B) Double co-localization values are the number of voxels in the co-localizing objects divided by the total number of voxels for that molecule (*, P<0.001, n=10). (C) Co-localization between VEGFR2, β3 integrin and GM1 ganglioside was calculated three times,
each one with respect to the first molecule listed. Values are number of voxels in the colocalizing objects divided by the total number of voxels for that molecule (*, \( P<0.001, n=10 \)).
Figure S. VIII: Recruited VEGFR2 does not co-localize with Caveolin 1. HUVECs were seeded on substrate-bound FG or gremlin. After 2 hours cells were fixed and immunostained with anti-VEGFR2 and anti-Caveolin 1 antibodies followed by AlexaFluor 594-conjugated anti-rabbit IgG and AlexaFluor 488-conjugated anti-mouse IgG. Samples were analysed using a LSM510 Meta confocal microscope equipped with Plan-Apochromat 63x/1.4 NA oil objective. Panels show the double immunostaining for VEGFR2 and Caveolin 1 at the basal portion of adherent cells (Bar, 10 μm). Area highlighted by white box is shown at higher magnification. Co-localization coefficients were calculated for both FG and gremlin and they do not significantly differ (FG: 0.17±0.11; gremlin: 0.19±0.14).
**FIGURE S. IX**

Figure S. IX: Immobilized gremlin induces β3 integrin phosphorylation. (A) 50 μg of cell extracts of HUVECs seeded for the indicated time on substrate-bound FG or gremlin were probed by phospho-β3 integrin (pTyr759) Western blotting. Uniform loading was confirmed by FAK Western blotting. (B) HUVECs were seeded on substrate-bound FG or gremlin for the indicated time. Cells were fixed and immunostained with anti-phospho-β3 integrin (pTyr759) followed by AlexaFluor 594-conjugated anti-mouse IgG and DAPI staining. Samples were analysed using a Zeiss Axiovert 200M epifluorescence microscope equipped with a Plan-Apochromat 63X/1.4 NA oil objective and ApoTome system (Zeiss. Bar, 10 μm).
METHODS AND MATERIAL

Beta3 integrin promotes long-lasting activation and polarization of Vascular Endothelial Growth Factor Receptor 2 by immobilized ligand

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Cell cultures

Human umbilical vein endothelial cells (HUVECs) were grown in M199 medium (Gibco, Life Technologies, Grand Island, NY) supplemented with 20% foetal calf serum (FCS, Gibco, Life Technologies), endothelial cell growth factor (100 µg/mL) (Sigma Chemical Co., St. Louis, MO) and porcine heparin (Sigma) (100 µg/mL). HUVECs were used at early passages (I-IV) and grown on plastic surface coated with porcine gelatin (Sigma). Foetal bovine aortic endothelial GM7373 cells 1 were grown in Dulbecco’s modified Eagle medium (DMEM, Gibco, Life Technologies) containing 10% FCS, vitamins, essential and non-essential amino acids. GM7373 cells were transfected with a pcDNA3.1 expression vector harbouring the mouse VEGFR2 cDNA (provided by G. Breier, Max Planck Institute, Bad Nauhein, Germany) to generate stable GM7373-VEGFR2 transfectants 2. Also, GM7373 cells were co-transfected with a pcDNA3/Enhanced Yellow Fluorescent Protein (EYFP) vector harbouring the extracellular domain of human VEGFR2 (ECD-VEGFR2) cDNA (provided by K. Ballmer-Hofer, PSI, Villigen, Swiss) and with the pcDNA3/β3-ECFP vector to generate β3-ECFP/ECD-VEGFR2-EYFP GM7373 cells. β3-Enhanced Green Fluorescent Protein-overexpressing (β3-EGFP) GM7373 cells were obtained as described 3. Finally, GM7373 cells were also transfected with the expression vectors pcDNA 3.1 FRNK or pcDNA 3.1 FRNK-Ser1034 encoding for the FAK C-terminal domain (FRNK) or the inactive FRNKL1034S mutant, respectively 4. Mock and gremlin-expressing VEGF/β fibroblasts (provided by K. Alitalo, Haartman Institute, Helsinki, Finland) were grown in DMEM containing 10% FCS, vitamins, essential and nonessential amino acids.

Analysis of gremlin expression in human tumor xenografts

Human endometrial adenocarcinoma HEC-1-B-derived Tet-FGF2 cells 5 were injected s.c. in nude mice. After 8 weeks, animals were sacrificed and tumors (400-500 mg) were snap-frozen in liquid nitrogen. Frozen sections were incubated overnight with anti-murine PECAM1 antibody MEC 13.3 (kindly provided by A. Vecchi, Istituto Humanitas, Milano) and anti-murine gremlin antibody (R&D Systems, Minneapolis, MN, USA) followed by 1 hour incubation with biotin-conjugated rabbit anti-rat secondary antibody (DAKO, Glostrup, Denmark) and Alexa fluor 488 rabbit anti-goat secondary antibody (Molecular Probes, Life Technologies) followed by 45 minutes incubation with Texas red Avidin D (Vector, Burlingame, CA).

Murine Matrigel plug assay

C57BL/6 mice (Charles River, Calco, Italy) were injected subcutaneously with 400 µL of growth factor reduced Matrigel (Cultrex BME, Gaithersburg, MD) containing 400 ng of gremlin, VEGF-A or FGF2 6. After 7 days, plugs were collected after whole animal perfusion fixation 7. Upon appropriate antigen retrieval (boiling in 1 mmol/L EDTA pH 8 followed by 15 minutes at sub-boiling temperature), 7 µm-paraffin embedded sections were incubated overnight with rabbit anti-VEGFR2 (Cell Signaling Technology) and goat anti-PECAM1 (Santa Cruz Biotechnology) or goat anti-Podocalyxin (R&D Systems) antibodies followed by 1 hour incubation with AlexaFluor 594-conjugated anti-goat IgG and AlexaFluor 488-conjugated anti-rabbit IgG. Finally, sections were incubated with 0.1% Sudan Black in 70% ethanol for 30 minutes at room temperature and washed 8 times with PBS. Samples were photographed under a Zeiss Axiovert 200M epifluorescence microscope equipped with a Plan-Apochromat 63x/1.4 NA oil objective; Z-stack images were acquired using ApoTome imaging system and elaborated through AxioVision Extended Focus module (Carl Zeiss). Quantification of VEGFR2 fluorescence within each apical and basal region of interest belonging to the same EC was carried on acquired images. Values obtained were divided by the total fluorescence to give a percentage of distribution between apical and basal aspects (n=4, 2 vessels per experiment).
Western blotting

24 hours and 10 times concentrated conditioned media, 1.5 mol/L NaCl washes and cell lysates from mock and gremlin-overexpressing VEGF-/- fibroblasts were probed with anti-gremlin antibody (R&D System) in a Western blot.

Confluent HUVECs were detached from culture plates, resuspended in M199 medium containing 5% FCS, allowed to adhere on immobilized gremlin or FG for the indicated time in the absence or in the presence of 10 μg/mL of BV4 antibody (Immunological Sciences, Rome, Italy). Cells were then lysed in lysis buffer [50 mmol/L Tris-HCl buffer (pH 7.4) containing 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L Na3VO4, and protease and phosphatase inhibitors (Sigma)]. Next, 50 μg of total cell lysate were separated by SDS-PAGE and probed with anti-phospho-VEGFR2 antibody (pTyr1175, Cell Signaling Technology, Beverly, MA), anti-FAK antibody (Santa Cruz Biotechnology), anti-VEGFR2 antibody (Santa Cruz Biotechnology) or anti-phospho-β3 integrin (pTyr759, Santa Cruz Biotechnology) in a Western blot.

Boyden chamber haptotactic assay

Mock and gremlin-overexpressing VEGF-/- fibroblasts were seeded on the lower face of gelatin-coated PVP-free polycarbonate filters (8 μm pore size, Costar, Cambridge, MA). Filters were incubated for 48 hours in DMEM 10% FCS to allow attached cells to deposit their own ECM and inserted in the Boyden chamber. Then, ECD-VEGFR2-EYFP-overexpressing GM7373 cells were seeded in the upper compartment of the Boyden chamber at 1x10^6 cells/mL. After 1 hour of incubation at 37°C, cells adherent to both sides of the filter were fixed and immunostained for β3 integrin as described below. Then, cells were analysed using a LSM510 Meta confocal microscope equipped with Plan-Apochromat 63x/1.4 NA oil objective (Carl Zeiss). Z-Stack sections and orthogonal z reconstitution for VEGFR2 were analysed by confocal microscopy, while 3D reconstruction images were obtained through AxioVision Inside 4D module (Carl Zeiss).

Immobilization of proteins to tissue culture plastic or glass coverslip

Aliquots (100 μL) of sterile PBS containing 2 μg/mL of gremlin or FG were added to polystyrene tissue culture plates or glass coverslips. After 16 hours of incubation at 4°C, the solution was removed and wells were washed 3 times with cold PBS. Uncovered plastic/glass was blocked with 1 mg/mL bovine serum albumin (BSA) for 1 hour at room temperature. Under these conditions gremlin binds to tissue culture plastic in a dose-dependent manner, with maximal binding at coating concentrations ≥ 2 μg/mL, as assessed by ELISA. Also, substratum-immobilized gremlin is resistant to high molar salt (2 mol/L NaCl) and detergent (0.2% Triton X-100) washes (Figure S3).

Immobilized gremlin ELISA

Ninety-six-well tissue culture plates were coated for 16 hours at 4°C with 0.2, 2 or 10 μg/mL of gremlin as described above. Then, plastic-immobilized gremlin was washed with 2 mol/L NaCl or with 0.2% Triton X-100 for 15 minutes at 37°C. An anti-gremlin goat polyclonal antibody (R&D Systems) diluted in PBS containing 0.1% BSA, 5 mmol/L EDTA, 0.004% Tween 20 (PBET buffer) was added to the wells at 500 ng/mL and incubated for 2 hours at room temperature. Finally, wells were incubated for 1 hour at room temperature with a secondary anti-goat horseradish peroxidase (HRP)-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Ventral plasma membrane (VPM) preparation

VPMs were prepared by osmotic shock using a modification of the squirting lysis technique. Briefly, cells were washed twice with ice-cold water; after 1 minute cells were squirted over by using a jet of ice-cold water and immediately fixed for immunocytochemistry analysis. In all the experiments, the absence of DAPI staining and the persistence of actin filaments were used to unequivocally identify the VPM remnants bound to the substratum.

Glass coverslips were coated with gremlin, FG or FGF2 (2 μg/mL) as described above. Mock cells (75.000/cm² in cell culture medium containing 1% FCS) or cells expressing FRNK and FRNLK1034S mutant and were allowed to adhere to the coverslips for 4 hours in absence or presence of different inhibitors used in pre-treatment or not: VEGFR2 inhibitor SU5416 (5 μmol/L,
Calbiochem, La Jolla, CA), RhoA inhibitor exoenzyme C3 from Clostridium botulinum (1 µg/mL, Calbiochem), β3 integrin inhibitor BV4 monoclonal antibody (10 µg/mL, Immunological Sciences). For lipid rafts disruption experiments, cells were incubated in suspension (1 hour at 37°C) with 2,6-di-O-methyl-β-cyclodextrin (MβCD, 10 mmol/L, Sigma). VPMs were acquired under an Axiovert 200 fluorescence microscope equipped with a Plan-Apochromat 63x/1.4 NA oil objective and ApoTome system (Carl Zeiss). VEGFR2-positive areas and total VPM areas, defined by actin staining, were quantified using Image-Pro Plus software.

**Time-lapse adhesion assay**

β3-ECFP and ECD-VEGFR2-EYFP co-expressing GM7373 cells were seeded and cultured on coverslips for 24 hours in FCS-free Endothelial Cell Basal Medium (Lonza, Basel, Swiss). Coverslips were then flipped on immobilized gremlin or FG coated µslides (IBIDI). Z-stack images in time-lapse were recorded for 120 minutes using a Zeiss Axiovert 200M epifluorescence microscope equipped with a Plan-Apochromat 63x/1.4 NA oil objective and ApoTome system. Collected images were then used to quantify the fluorescence of ECD-VEGFR2-EYFP and β3-ECFP normalized to the background.

**Fluorescence resonance energy transfer (FRET) analysis**

FRET experiments were performed as previously described. Briefly, β3-ECFP/ECD-VEGFR2-EYFP GM7373 cells were seeded on immobilized gremlin or FG (both at 2 µg/mL) and allowed to adhere for 4 hours. After adhesion, VPMs were isolated, fixed with 4% paraformaldehyde and subjected to FRET analysis at LSM510 Meta confocal microscope (Carl Zeiss). To this purpose, a region of interest was selected and photobleached by applying 100% intensity of a 514 nm laser. FRET efficiency was calculated using the formula: FRET = (Dpost - Dpre)/Dpre, where Dpre and Dpost represent the donor (ECFP) emission intensities before and after photobleaching, respectively. FRET efficiency was also measured in a non-photobleached region of the same cell as an in situ control. In all experiments, cells transfected with ECFP-EYFP fusion protein were used as FRET positive controls.

**Cell motility assay and lipid raft disruption experiments**

EC motility was assessed by time-lapse video-microscopy. To this purpose, ECs were seeded on immobilized gremlin or FG (both at 2 µg/mL) at 150 cells per mm² in 24 well plates in the absence or in the presence of SU5416 (5µmol/L, Calbiochem), BV4 antibody (10 µg/mL, Immunological Sciences) or exoenzyme C3 (1 µg/mL, Calbiochem). For lipid rafts disruption experiments, cells were incubated in suspension (1 hour at 37°C) with MβCD (10 mM, Sigma) followed by 1 hour incubation with cholesterol (chol, 400 mg/mL, Sigma). Constant temperature (37°C) and 5% CO2 were maintained throughout the experimental period by means of a heatable stage and climate chamber. Cells were observed under an inverted photomicroscope (Zeiss Axiovert 200M) and phase-contrast snap photographs (one every 10 minutes) were digitally recorded for 8 hours. Cell paths (40-50 cells per experimental point) were generated from centroid positions and migration parameters were analysed with the “Chemotaxis and Migration Tool” of ImageJ Software (http://rsbweb.nih.gov/ij).

**Immunocytochemistry**

Fixed whole cells or VPMs were permeabilized with 0.5% Triton-X100 and saturated with 3% BSA in PBS. Adherent cells, migrating cells or VPMs underwent the following staining procedures: i) actin staining: samples were incubated for 30 minutes with TRITC-phalloidin (0.9 mg/mL in PBS, Sigma); ii) GM1 ganglioside staining: samples were incubated for 30 minutes with AlexaFluor 594-conjugated Cholera Toxin B subunit (CTB, Sigma); iii) paxillin immunostaining: samples were incubated overnight with a monoclonal anti-paxillin antibody (Transduction Laboratories, Lexington, KY) followed by a 45 minutes incubation with AlexaFluor 488-conjugated anti-mouse IgG (Molecular Probes, Life Technologies); iv) total VEGFR2 immunostaining: samples were incubated overnight with a rabbit polyclonal anti-VEGFR2 antibody (Santa Cruz Biotechnology) followed by 1 hour incubation with AlexaFluor 594 and AlexaFluor 488-conjugated anti-rabbit IgG or with a goat polyclonal anti-VEGFR2 antibody (Santa Cruz Biotechnology) followed by 1 hour incubation with...
Alexa594-conjugated anti-goat IgG (Molecular Probes, Life Technologies); v) phospho-VEGFR2 immunostaining: samples were incubated overnight with a rabbit monoclonal anti-phospho-VEGFR2 antibody (pTyr1175, Cell Signaling Technology) followed by 1 hour incubation with AlexaFluor 488-conjugated anti-rabbit IgG; vi) β3 integrin immunostaining: samples were incubated overnight with a polyclonal anti-β3 integrin antibody (Immunological Sciences) followed by 1 hour incubation with Cy5-conjugated anti-rabbit IgG; vii) phospho-p85 immunostaining: samples were incubated overnight with a polyclonal anti-phospho-p85 antibody (Santa Cruz Biotechnology) followed by 1 hour incubation with AlexaFluor 594-conjugated anti-goat IgG. viii) Caveolin 1 immunostaining: samples were incubated overnight with a mouse monoclonal anti-Caveolin 1 antibody (BD Transduction Laboratories, Franklin Lakes, New Jersey) followed by 1 hour incubation with AlexaFluor 488-conjugated anti-mouse IgG. ix) Phospho-β3 integrin immunostaining: samples were incubated overnight with a rabbit polyclonal anti-phospho-β3 integrin (pTyr759, Santa Cruz Biotechnology) followed by 1 hour incubation with AlexaFluor 594-conjugated anti-rabbit IgG. All antibody dilutions were in PBS containing 3% BSA. Cells and VPMs were photographed under a Zeiss Axiovert 200M epifluorescence microscope equipped with a Plan-Apochromat 63x/1.4 NA oil objective. In some experiments the extent of antigen immunoreactivity was quantified by computerized image analysis (Image-Pro Plus; Media Cybernetics, Rockville MD). When indicated cells were photographed using a Zeiss LSM510 Meta confocal microscope equipped with a Plan-Apochromat 63x/1.4 NA oil objective. VEGFR2 and Caveolin 1 co-localization analysis was performed using Axiovision Co-localization module (Zeiss) while VEGFR2, β3 integrin and GM1 triple co-localization was analyzed using BlobProb ImageJ plugin 10, 11.

Data representation
Data are expressed as mean ± SD or mean ± SEM. Statistical analyses were performed using the Student's t-test.

SUPPLEMENTAL REFERENCES

