The Scaffolding Adapter Gab1 Mediates Vascular Endothelial Growth Factor Signaling and Is Required for Endothelial Cell Migration and Capillary Formation*

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Vascular endothelial growth factor (VEGF) is involved in the promotion of endothelial cell proliferation, migration, and capillary formation. These activities are mainly mediated by the VEGFR2 receptor tyrosine kinase that, upon stimulation, promotes the activation of numerous proteins including phospholipase Cγ (PLCγ), phosphatidylinositol 3-kinase (PI3K), Akt, Src, and ERK1/2. However, the VEGFR2-proximal signaling events leading to the activation of these targets remain ill defined. We have identified the Gab1 adapter as a novel tyrosine-phosphorylated protein in VEGF-stimulated cells. In bovine aortic endothelial cells, Gab1 associates with VEGFR2, Grb2, PI3K, SHP2, Shc, and PLCγ, and its overexpression enhances VEGF-dependent cell migration. Importantly, silencing of Gab1 using small interfering RNAs leads to the impaired activation of PLCγ, ERK1/2, Src, and Akt; blocks VEGF-induced endothelial cell migration; and perturbs actin reorganization and capillary formation. In addition, co-expression of VEGFR2 with Gab1 mutants unable to bind SHP2 or PI3K in human embryonic kidney 293 cells and bovine aortic endothelial cell mimics the defects observed in Gab1-depleted cells. Our work thus identifies Gab1 as a novel critical regulatory component of endothelial cell migration and capillary formation and reveals its key role in the activation of VEGF-evoked signaling pathways required for angiogenesis.

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is a highly regulated process that is essential for the development of multicellular organisms (1–3). In the adult, angiogenesis is normally restricted and predominantly associated with female reproductive functions and wound healing (2, 4). Loss of this tight regulation, resulting in uncontrolled and excessive neovascularization, contributes to the development of many pathologies, including retinopathies, rheumatoid arthritis, and tumor growth (2, 5–7). Vascular endothelial growth factor (VEGF or VEGF-A) is a key regulator of normal and pathological angiogenesis (8–10). At the cellular level, VEGF stimulation drives multiple responses including endothelial cell proliferation, migration, survival, and permeability (7, 11, 12). Recent reports however suggest that increased permeability is not required per se for normal or tumor-associated angiogenesis, but that it is essential for tumor cells to metastasize (13, 14).

VEGF binds to two distinct receptor tyrosine kinases (RTKs), VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1), but numerous studies suggest that VEGFR2 is the main receptor conveying the mitogenic, chemotactic, and survival effects of VEGF in endothelial cells (12, 15). VEGFR2 is composed of an extracellular domain containing seven immunoglobulin-like domains, a transmembrane domain, and an intracellular kinase domain typical of the PDGF receptor subclass family of RTKs (15, 16). Following VEGF binding, VEGFR2 undergoes conformational changes leading to autophosphorylation on several sites, including Tyr1054 and Tyr1059 in the activation loop, Tyr951 and Tyr996 in the kinase insert domain, and Tyr1214 in the C terminus tail (17–21). Activated VEGFR2 then associates with and/or phosphorylates/activates numerous targets, including phospholipase Cγ (PLCγ), phosphatidylinositol 3-kinase (PI3K), Akt, the adapter proteins Grb2, Shc, and SRCAD/VRAP, the tyrosine phosphatase SHP2, Src (13, 22), and the Ser/Thr kinases ERK1/2 and p38 (11, 15, 23–25). Activation of PLCγ and the ERK1/2 pathway has been associated with VEGF-induced cell proliferation (12, 24, 25). Accordingly, mutagenesis studies have demonstrated that phosphorylation of Tyr1175 of VEGFR2 is required for the activation of PLCγ and ERK1/2 and for endothelial cell proliferation (17, 26).

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3 The abbreviations used are: VEGF, vascular endothelial growth factor; RTK, receptor tyrosine kinase; PLCγ, phospholipase Cγ; PI3K, phosphatidylinositol 3-kinase; ERK1/2, extracellular signal-regulated kinases 1/2; HRP, horseradish peroxidase; HEK 293, human embryonic kidney 293; BAEC, bovine aortic endothelial cell; HMVEC, human microvascular endothelial cells; EBM, endothelial basal medium; EGM, endothelial growth medium; HA, hemagglutinin; siRNA, small interfering RNA; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; DMEM, Dulbecco’s modified Eagle’s medium; WT, wild type; HGF, hepatocyte growth factor.
work has further demonstrated that signaling downstream of this tyrosine residue is essential for vasculogenesis (26). In some circumstances, ERK1/2 activity also correlates with the stimulation of cell migration (27). On the other hand, PI3K is mainly involved in the promotion of endothelial cell migration and cell survival, through AKT and AKT-dependent phosphorylation of eNOS (28–31). However, despite these and many other cellular targets identified so far, the VEGFR2-proximal signaling events involved in cell migration and proliferation in response to VEGF stimulation are not fully understood.

Recent evidence has revealed that many signaling molecules are recruited to growth factor- or cytokine-activated receptors via scaffolding/adapter proteins, such as Gab1 (Grb2-associated binder-1), Gab2, and Gab3 (32, 33). Gab1, which is the most characterized in nonhematopoietic cells, was first identified as a Grb2-associated protein downstream of EGF and insulin-mediated signaling involved in cell growth and transformation (34) and as a direct Met/HGF receptor-interacting protein involved in epithelial morphogenesis (35). Gab1 encompasses a pleckstrin homology domain that mediates its interaction with specific membrane lipids (phosphatidylinositol 3,4,5-triphosphate derived from PI3K activity) (36). It also contains a large number of tyrosines and proline-rich regions that allow its interaction with the Src homology 2 and 3 domains of signaling molecules, including Grb2, PI3K, PLCγ, and SHP2 (32, 33). Following receptor activation, Gab1 becomes tyrosine-phosphorylated and acts as a docking center for the assembly of multi-protein complexes. Thus, the diverse signaling pathways and biological activities induced downstream of various receptors might in part rely on the receptor’s ability to signal to specific adapter proteins in a time- and space-defined fashion.

Gab1 has been described as a critical activator of the PI3K/Akt and SHP2/ERK1/2 pathways in many cell systems and has been shown to play important roles in several biological processes promoted by RTKs, including cell survival, differentiation, and morphogenesis (32, 33, 37, 38). In particular, Gab1 has been shown to mediate HGF-induced PI3K and ERK1/2 activation as well as tubule formation following HGF stimulation of Madin-Darby canine kidney cells grown in collagen gels (39–41). Given the critical role played by the PI3K and ERK1/2 pathways in VEGF-evoked angiogenic responses, we postulated that Gab1 could be involved in the mediation of VEGF-dependent biochemical and biological events. Here, we show that Gab1 associates with VEGFR2 and proteins previously reported to be activated in response to VEGF stimulation of endothelial cells. We also provide evidence that Gab1 acts as a VEGFR2-proximal signaling center and promotes the optimal activation of the PLCγ, ERK1/2, Src, and Akt pathways shown to be essential for the angiogenic responses of endothelial cells. Consistent with these findings, our data reveal a critical role for Gab1 in VEGF-dependent endothelial cell migration, actin reorganization, and capillary formation.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—Gab1, Gab2, p85, Grb2, Shc, PLCγ, and Src antibodies were obtained from Upstate (Cedercane Laboratories Ltd., Hornby, Canada). Anti-phosphotyrosine P199, SHP2, and VEGFR2 (clone C-1158 for immunoprecipitation and A3 for blotting) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A third VEGFR2 polyclonal antibody (for immunoprecipitation) was also purchased from R&D Systems (Minneapolis, MN). Mouse monoclonal anti-VEGFR1 was from Sigma. HA antibody was obtained from BAbCO (Richmond, CA). The β-tubulin (E7) monoclonal antibody developed by Michael W. Klymkowsky was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health, and maintained by the Department of Biological Sciences, University of Iowa (Iowa City, IA). Anti-Tyr(P)783PLC γ and Tyr(P)418Src antibodies were obtained from BioSource Inc. (Medicorp, Montreal, Canada). Anti-Ser(P)673Akt, -Akt, -Thr(P)202/Tyr(P)204ERK1/2, and -ERK1/2 antibodies were from Cell Signaling Technology Inc. and were purchased from New England Biolabs Ltd. (Pickering, Canada). Rabbit IgG was obtained from Pierce. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories (Bio/Can Scientific, Mississauga, Canada), and HRP-linked protein A was supplied by Amersham Biosciences. The HRP-conjugated anti-rabbit IgG from Cell Signaling Technology Inc. was used to detect Ser(P)673Akt. PD98059 and LY294002 were purchased from Cell Signaling Technology Inc. PP2 and U73122 were obtained from Biomol (Plymouth Meeting, PA). VEGF, EGF, FG, and CSF-1 were purchased from R&D Systems. HGF was a kind gift of Pascal Reboul (Research Center of the Centre Hospitalier de l’Université de Montréal).

**Cell Culture**—Bovine aortic endothelial cells (BAECs) (Clonetics/Cambrex, purchased from Cederlane Laboratories Ltd.) were cultured (passages 3–10) in DMEM low glucose (Invitrogen) containing 10% calf serum (Invitrogen), 2 ng/ml FGF, and 50 μg/ml gentamycin. Human microvascular endothelial cells (HMVEC) (passages 3–7; Clonetics/Cambrex) were maintained in EBM-2 supplemented with 5% fetal bovine serum, EGF, VEGF, FGF, heparin, IGF-1, hydrocortisone, and ascorbic acid (EGM-2-MV bullet kit; Clonetics/Cambrex). HEK 293 cells were grown in DMEM high glucose (Invitrogen) containing 10% fetal bovine serum (Invitrogen) and 50 μg/ml gentamycin.

**Expression Plasmids and Site-directed Mutagenesis**—Constructs encoding HA-tagged mouse WT Gab1 (in pCNA1.1) and the HA-Gab1ΔPI3K (Y448F/Y473F/Y590F) and HA-Gab1Y628F mutants (kindly provided by Morag Park, McGill University, Montreal, Canada) have been described elsewhere (40, 42). The HA-Gab1ΔSHP2 mutant (Y628F/Y660F) was generated from the HA-Gab1Y628F mutant using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The sequence of the overlapping oligonucleotides used was 5′-GAGAGAGGTTTCACTTCCGGTGTTGGTGG-3′ in the sense and antisense orientation. The mutated nucleotides are underlined; the first two are silent mutations that generate a novel SalI restriction site. The Gab1ΔGrb2 (A341–348/P518A/R522A) mutant was generated by deleting and mutating the two proline-rich sequences previously shown to bind Grb2, GBS2 and GBS1 (Grb2-binding sequences 2 and 1), respectively (41, 43). The sequence of the oligonucleotides used to generate the GBS2 mutant (deletion of
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amino acids 341–348) was the following: 5’-CACCATTCTCTGACATCCATCCAACACTGAC-3’. The underlined nucleotide is a silent mutation that eliminates an EcoRV restriction site. The GB51 mutations (P518A/R522A) were sequentially generated on the GB52 mutant using two sets of oligonucleotides overlapping the following sequences: 5’-CTGACTGTGAAACCAGCGCTGGTGGATGGAAC-3’ (encompassing the P518A mutation) and 5’-CCAGCGCGGTGGATGGAACCTAAGCCAGAC-3’ (encompassing the P518A and R522A mutations). The P518A and R522A mutations generate new HaeII and BstF1I restriction sites, respectively. The pCR3-KDR (VEGFR2), pRK5-Flt1 (VEGFR1), and pCDNA3-Grb2 cDNA constructs were generously provided by Cam Patterson (University of North Carolina, Chapel Hill, NC), Jean-Philippe Grathey (University of Montreal), respectively.

Construction of the Chimeric CSF-VEGFR2 Expression Vector—The full-length cDNA of human VEGFR2 was used as a template to generate the VEGFR2 transmembrane and cytoplasmic domain by PCR. PCR primers were designed to generate a new XhoI site at the 5’ end. The upper primer (5’-GACGAACCTCCTGAGATCATTATTCTCAGGTAG-3’) corresponds to nucleotides 2583–2612, and the lower primer (5’-TTGGGTTAGATCCTTCTTATACAGGA-3’) is complementary to nucleotides 4365–4395 in the VEGFR2 sequence. To create a new XhoI site, nucleotides +2590 T, +2592 G, and +2595 A were converted to nucleotides C, C, and G, respectively, in the upper primer.

The conversion does not lead to modification of the VEGFR2 amino acid sequence. To create a new XbaI site, the nucleotides outside of the VEGFR2 coding sequence, +4386 C and +4388 C, were converted to T and G, respectively, in the lower primer. The VEGFR2 PCR product was digested with XhoI and XbaI and cloned into the adenovirus transfer vector pShuttle-CMV (Qbiogene). The full-length cDNA of human CSF-1R (a kind gift of Martine Roussel, St. Jude Children’s Research Hospital, Memphis, TN) was used as a template to generate the extracellular domain of CSF-1R by PCR. PCR primers were designed to generate a new NotI site at the 5’ end and XhoI site at the 3’ end. The upper primer (5’-ACCTGGGCGCGCGGCTTTCCGCACCCGGAGG-3’) corresponds to nucleotides −273 to −298, and the lower primer (5’-TGAAGAGGAACCTCGACGCGGGGATTGCCTGACG-3’) is complementary to nucleotides −1816 to −1846 in the CSF-1R sequence. To create a new NotI site, nucleotides −278 C, −280 T, and −284 A in the CSF-1R sequence were all converted to G in the upper primer. To create a new XhoI site, nucleotides +1831 G, +1832 A, and +1833 T were converted to C, T, and T, respectively, thus causing a substitution of amino acid Asp511 to Leu511 in the CSF-1R. The CSF-1R PCR product and the adenovirus transfer vector pShuttle-CMV encompassing the VEGFR2 PCR product were both digested with NotI and XhoI and ligated together to generate the chimeric CSF-VEGFR2 expression vector (pSCMV.CSF-VEGFR2).

DNA Transfection, Cell Stimulation, and Lysis—BAECs were plated (1.5 × 10⁶ cells/100-mm dish; Falcon) and transfected 24 h later with 6 µg of plasmid DNA using Lipofectin (6 µl/µg of DNA) in serum-free DMEM low glucose according to the manufacturer’s recommendations (Invitrogen). The cells were washed the next day, and fresh medium was added for another 24 h, at which time the cells were trypsinized and used in a migration assay. For growth factor stimulation of BAECs, 5.4 × 10⁵ cells/100-mm dish were grown for 3 days and then serum-starved (DMEM plus gentamycin) for 16 h. Cells were next incubated with VEGF (50 ng/ml) at 37 °C for the indicated times, washed, and incubated in PBS containing sodium vanadate (1 mM) for 30 min on ice. Cells were next lysed in 1 ml of buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% (v/v) glycerol, 0.5% (v/v) Triton X-100, 0.5% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 5 mM sodium fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin (Roche Applied Science). HEK 293 cells (9 × 10⁵ to 1 × 10⁶) were plated and transfected 24 h later with 10 µg of VEGFR2 or VEGFR1 cDNA plasmid and 5 µg of either empty vector (pCDNA 1.1) or Gab1 plasmids using the calcium phosphate precipitation method (44). For experiments with Grb2, 5 µg of cDNA construct were transfected in all conditions. The medium was changed the next morning, and the cells were serum-starved at night for another 16 h, after which time the cells were stimulated with VEGF as described above.

siRNA Transfections—HMVECs (5.8 × 10⁶) were seeded in 60-mm dishes the day before transfection. Pools of four nontargeting, control siRNAs and four specific Gab1 siRNAs (Smart Pools) were purchased from Upstate/Dharmacon (Lafayette, CO). siRNA transfections (100 nM final concentration) were performed in DMEM high glucose using Targefect reagents according to the manufacturer’s recommendations (Targeting Systems Inc., Santee, CA). After a 2-h incubation, cells were rinsed twice in DMEM and then incubated in complete EB-2 (supplemented with the EGM-2-MV bullet kit) for an additional 48 h. Next, cells were either starved in EBM for 6 h and processed for biochemical analysis or subjected to the migration and capillary formation assays as described. For rescue experiments, cells were electroporated with both siRNA and cDNA using the Nucleofector system from Amaxa Biosystems according to the manufacturer’s recommendations. Briefly, HMVECs (1 × 10⁶) were suspended in 100 µl of prewarmed nleofection solution for microvascular cells (HMVEC-L) containing 23.3 µl of siRNAs (the amount equivalent to a concentration of 100 nM for a 60-mm dish) and 3 µg of plasmid DNA. Immediately following nucleofection, 500 µl of prewarmed complete EB-2 was added to the cells, which were then transferred to 60-mm dishes containing 2.5 ml of the same prewarmed medium. Twelve hours later, medium was replaced (8 ml/60 mm), and cells were further incubated up to 48 h after nucleofection, at which time they were trypsinized and subjected to a migration assay.

Immunoprecipitation and Western Blotting—BAEC lysates (1–2 mg for co-immunoprecipitation experiments) and HEK 293 cell lysates (0.75–1 mg) were incubated with antibodies for 2 h at 4 °C (or overnight for co-precipitations), followed by incubation with 25 µl of a 50% (v/v) slurry of protein A- or protein G-conjugated Sepharose beads (Amersham Biosciences) for an additional 2–4 h. Beads were washed three times with lysis buffer and then denatured in 1 × Laemmli sample buffer (45). Immunoprecipitated proteins or total protein
extracts (40–50 μg) were resolved by SDS-PAGE, transferred to nitrocellulose Hybrid ECL membranes (Amersham Biosciences), and blocked in 3% (w/v) bovine serum albumin in TBST (10 mM Tris-HCl (pH 7.4), 2.5 mM EDTA, 150 mM NaCl, 0.1% Tween 20) for 1 h (or according to the recommended protocol for the phospho-specific antibodies). Proteins were detected by Western blotting with the appropriate antibodies and HRP-conjugated anti-mouse antibody (Jackson Immunoresearch Laboratories), HRP-conjugated protein A (Amersham Biosciences), or HRP-conjugated anti-rabbit antibody (for anti-Ser(P)473-akt antibody; Cell Signaling) using the ECL detection kit according to the manufacturer’s recommendations (Amersham Biosciences).

Migration Assay—Forty-eight hours after transfection or electroporation, BAECs or HMVECs (1 × 10⁴ in 200 μl of serum-free low glucose DMEM) were seeded on Transwell filters (polycarbonate membrane, 8-μm pore size; Corning Brand, Fisher) precoated with 0.2% (w/v) gelatin (overnight at 4 °C) and inserted in 24-well plates. After a 30-min incubation (for BAECs only), VEGF (10 ng/ml), FGF (10 ng/ml), EGF (10 ng/ml), or HGF (10 ng/ml) were added to the lower chamber containing 800 μl of the same medium for 3 h (BAECs plus VEGF), 6 h (BAECs plus other growth factors), or 16 h (HMVECs). At the end of the assay, cells were fixed with phosphate-buffered formalin for 20 min and stained with crystal violet (0.1% in 20% (v/v) methanol) for a minimum of 60 min. Cells remaining on the upper surface of the filter were wiped off, and those that had migrated through the filter pores were visualized and counted (a minimum of 6 fields/insert at ×40 magnification).

Proliferation Assay—Thirty hours after transfection of control and Gab1 siRNAs, HMVECs (7.5 × 10⁴) were seeded in 24-well plates and incubated for 10 h in EBM-2 supplemented with the EGM-2-MV bullet kit. The cells were next rinsed twice in PBS (t = 0) and then incubated with or without VEGF (10 ng/ml) for 42 h (t = 1) in EBM containing 1% fetal bovine serum. Cell counts were determined using a hemacytometer at t = 0 and t = 1.

In Vitro Angiogenesis Assay—Capillary tube formation by endothelial cells grown between layers of fibrin gels was evaluated using a “fibrin gel in vitro angiogenesis” assay kit (Chemicon International). Briefly, HMVECs (7 × 10⁴ cells/well of a 24-well plate) were seeded on the first layer of fibrin gel (generated by mixing fibrinogen with thrombin) and incubated overnight in fully complemented EBM-2 medium. The medium was next aspirated, and a second layer of gel was overlaid on the apical surface of the cells and allowed to polymerize before basal EBM containing VEGF (10 ng/ml) was added to each well. Capillaries were photographed after 12 h of incubation using a Leica DM IL microscope (magnification of ×10) and a Leica DFC 320 camera. Total capillary tube length was measured in arbitrary units in two view fields, which covered most of the well, and the values were averaged. Data are representative of four independent experiments performed in duplicate.

Actin Filament Staining—HMVECs were transfected with siRNAs as described above. Cells (7.5 × 10⁶) were seeded on gelatin-coated glass coverslips 40 h after transfection. Twenty-four hours later, cells were serum-starved in EBM for 3 h before stimulation with VEGF (10 ng/ml). Cells were fixed in 3.7% formaldehyde (in PBS) for 10 min, permeabilized with 0.05% Triton X-100 (in PBS) for 15 min, and then incubated for 30 min with Alexa Fluor 546-conjugated phalloidin (Molecular Probes, Invitrogen) (1:120) to detect actin filaments. Coverslips were washed and mounted using Pro long Gold antifade reagents with 4′,6-diamidino-2-phenylindole (Molecular Probes). Cells were photographed with an Olympus Q-Color5 CCD camera using the QCapture imaging system software with an Olympus BX51 microscope.

RESULTS

Gab1 Is Tyrosine-phosphorylated and Associates with Signaling Proteins upon VEGF Stimulation—Gab1 is a ubiquitously expressed scaffolding adapter that upon phosphorylation associates with signaling proteins in response to the activation of growth factor and cytokine receptors (32, 33). To determine whether Gab1 recruits signaling proteins in response to VEGF stimulation in endothelial cells, we first investigated if VEGF was able to induce its phosphorylation. Fig. 1A shows that treatment of BAECs for 2 min with increasing concentrations of VEGF led to the stimulation of Gab1 tyrosine phosphorylation, which was maximal at a concentration of 50 ng/ml. Stimulation of BAECs (Fig. 1B) as well as of HMVECs and the microvascular cell line HMEC-1 (data not shown) with VEGF (50 ng/ml) over a 60-min period led to a peak of Gab1 tyrosine phosphorylation 2–5 min after the addition of VEGF. These data indicate that Gab1 is part of the VEGF-induced phosphorylation cascade in various endothelial cell types.

Since Gab1 is tyrosine-phosphorylated downstream of VEGF stimulation, we postulated that it could participate in VEGF-induced signaling in endothelial cells. To test this possibility, we examined the ability of Gab1 to associate with proteins previously reported to be part of the VEGF-dependent signaling pathways (15). We observed that upon VEGF stimulation of BAECs, immunoprecipitated Gab1 associated with PLCγ, a
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Gab1 Associates with VEGF Receptor Complexes—Gab1 is tyrosine-phosphorylated and associates with signaling proteins previously reported to be involved in the mediation of VEGF-dependent signaling and biology in endothelial cells. We assessed if Gab1 was present in VEGFR2 complexes. VEGFR2 was immunoprecipitated from BAECs, stimulated or not with VEGF (Fig. 2A). We found that Gab1 co-precipitated with VEGFR2 in unstimulated BAECs and that this interaction was slightly increased upon stimulation with VEGF (Fig. 2A). Conversely, a phosphorylated protein potentially corresponding to VEGFR2, since it had the same gel mobility, co-precipitated with Gab1 in VEGF-stimulated BAECs (Fig. 2B, compare the Gab1 IP lanes with the VEGFR2 IP lane). To confirm this interaction, reconstitution experiments were performed in HEK 293 cells transfected with VEGFR2 along with HA-tagged Gab1 and Grb2, which has been shown to link Gab1 to membrane receptors (41, 43, 46, 50). As observed in BAECs, HA-Gab1 co-precipitated with immunoprecipitated VEGFR2, whereas a tyrosine-phosphorylated band with the same gel mobility as VEGFR2 co-precipitated with HA-Gab1 upon VEGF stimulation (Fig. 2C). In addition, VEGF was also able to induce a strong phosphorylation of Gab1. To determine the specificity of this interaction, we investigated the ability of VEGFR1 to associate with Gab1. Since BAECs express very little endogenous VEGFR1, reconstitution experiments were conducted in HEK 293 cells. The results showed that VEGFR1 could associate with Gab1 and increase its phosphorylation upon VEGF stimulation (Fig. 2D). Altogether, our results show that phosphorylated Gab1 recruits signaling proteins to VEGFR2 complexes upon VEGF stimulation of endothelial cells, suggesting that Gab1 might contribute to the activation of proximal VEGF2-dependent signaling. Moreover, our findings suggest that VEGFR1 might also contribute with VEGFR2 to the recruitment and “activation” of Gab1 in endothelial cells.

Grb2 Is Required for Gab1-VEGFR2 Association and Optimal VEGF-dependent Gab1 Phosphorylation and Signaling—Grb2 mediates the association of Gab1 with several RTKs, such as Met, EGF, PDGF, and RET. The Grb2 SRC homology 2 domain binds to tyrosine-phosphorylated receptors, whereas the Grb2 C-terminal SRC homology 3 domain is constitutively associated with Gab1 proline-rich sequences, GBS1 and GBS2 (41, 43). To find out if a similar mechanism is involved in the recruitment of Gab1 to VEGFR2, we investigated the ability of VEGFR2 to associate with and phosphorylate a Gab1 mutant unable to bind Grb2 (Gab1ΔGrb2). In this experiment, a chimeric CSF-VEGFR2 receptor comprising the CSF-1R extracellular domain fused to the transmembrane and intracellular domains of VEGFR2 was co-transfected with Grb2 and either WT HA-Gab1 or the Gab1ΔGrb2 mutant in HEK 293 cells. As expected, immunoprecipitated Gab1ΔGrb2 mutant failed to associate with Grb2 and the phosphorylated receptor compared with WT Gab1 (Fig. 3A). Conversely, the immunoprecipitated receptor failed to interact with the Gab1ΔGrb2 mutant, although it could still associate with Grb2 (Fig. 3B). Consistent with this, tyrosine phosphorylation of the Gab1ΔGrb2 mutant in response to stimulation was greatly attenuated, and this mutant was unable to associate with phosphorylated SHP2 and to activate ERK1/2 as WT Gab1 (Fig. 3A). Overall, these results suggest that the association of Gab1 with VEGFR2 complexes via Grb2 is required for optimal Gab1 tyrosine phosphorylation, Gab1 association with phosphorylated SHP2, and the promotion of Gab1-dependent ERK1/2 activation.

Gab1 Overexpression Enhances VEGF-dependent Cell Migration—The proteins found to associate with Gab1 upon VEGF stimulation of BAECs have previously been shown to major VEGF-induced phosphorylated protein, as well as with the Shc adapter protein and the SHP2 tyrosine phosphatase (Fig. 1C). Gab1 also constitutively associated with the p85 subunit of PI3K and with Grb2, but VEGF stimulation had little or no effect on these associations. These results demonstrate that upon VEGF stimulation, Gab1 is tyrosine-phosphorylated and associates with signaling proteins previously reported to be activated downstream of VEGF receptors.

Gab1 Associates with VEGFR2 and VEGFR1—Gab1 has been found to associate in a direct or indirect fashion with a number of receptor tyrosine kinases, including the Met/NGF, EGF, PDGF, and RET receptors (35, 41, 43, 46–50). Since VEGFR2 has been reported to be the main receptor tyrosine kinase involved in the mediation of VEGF-dependent signaling and biology in endothelial cells, we assessed if Gab1 was present in VEGFR2 complexes. VEGFR2 was immunoprecipitated from BAECs, stimulated or not with VEGF (Fig. 2A). We found that...
promote cell migration downstream of several activated growth factor receptors. As a first approach to evaluate if Gab1 participates in the promotion of VEGF-induced cell migration, empty vector or HA-tagged Gab1 were transiently transfected in BAECs, and the cells were tested 48 h later in a Boyden chamber migration assay (Fig. 4). These experiments show that modest overexpression of Gab1 potentiated VEGF-induced cell migration without altering the basal level of migration observed in the absence of VEGF (Fig. 4, A and B). To determine the specificity of this effect, the ability of Gab1 to potentiate the migration of BAECs in response to other growth factors, such as EGF, HGF, and FGF, was also evaluated. Interestingly, Gab1 overexpression was also able to increase to various extents the migration of BAECs in response to EGF and HGF stimulation but not FGF (Fig. 4C). Thus, these results show that Gab1 contributes to VEGF-induced signaling pathways involved in endothelial cell migration. Moreover, Gab1 may also represent a common mediator of endothelial cell migration downstream of other proangiogenic growth factors that were also observed to induce Gab1 tyrosine phosphorylation in BAECs (data not shown).

Knockdown of Gab1 Expression Impairs VEGF-dependent Signaling, Migration, and Capillary Formation—To investigate the importance of endogenous Gab1 in VEGF-dependent signaling and angiogenic activities, HMVECs were transfected with a pool of four Gab1 siRNAs or control siRNAs (Figs. 5 and 6). We observed a strong inhibition of Gab1 expression in HMVECs 48 h post-transfection with the Gab1 siRNAs (Fig. 5A). The specificity of this inhibition was demonstrated by the unaltered expression of a close family member (Gab2) and of tubulin in the various conditions (Fig. 5A). The signaling response of the Gab1-depleted cells to VEGF stimulation was evaluated using phosphospecific antibodies recognizing

FIGURE 3. Gab1-associated Grb2 is required for optimal Gab1 phosphorylation, VEGFR2 association, and downstream signaling. HEK 293 cells were transiently transfected with various combinations of the pCDNA1.1 empty vector, WT Gab1, or the ΔGrb2 mutant together with vectors encoding Grb2 and the chimeric CSF-VEGFR2. Cells were serum-starved for 16 h and stimulated or not with CSF-1 (50 ng/ml) for 5 min. Cell lysates from each condition were incubated with anti-HA (A) or anti-VEGFR2 (B) antibodies, and the immune complexes were collected using protein G- and protein A-Sepharose beads, respectively. The amount of immunoprecipitated HA-Gab1 and CSF-VEGFR2 and their association with specific proteins were determined using the corresponding antibodies. The levels of phosphorylated Gab1 and of CSF-VEGFR2 co-precipitating with HA-Gab1 were detected by immunoblotting using an anti-phosphotyrosine antibody (PY99). TCL, total cell lysates. IP, immunoprecipitation; IB, immunoblotting.

FIGURE 4. Gab1 overexpression potentiates endothelial cell migration. A, BAECs were transfected with empty vector pCDNA1.1 (pCDNA) or the HA-Gab1 construct. Forty hours later, cells (10⁵/well) were plated in gelatin-coated wells of Boyden migration chambers, allowed to adhere for 30 min, and then incubated or not with VEGF (10 ng/ml) for 3 h. At the end of the assay, cells were fixed and stained with crystal violet. Cells remaining on the upper surface of the filter were wiped off, and only those that had migrated through the filter pores were visualized and counted (a minimum of six fields at ×40 magnification). Cell counts were compared with those of unstimulated pCDNA-transfected cells. The results are representative of four independent experiments performed in duplicate. B, the remaining cells were lysed to determine the expression level of endogenous and transfected Gab1 by immunoblotting using anti-Gab1 and anti-HA antibodies, respectively. C, BAECs transfected with empty vector (pCDNA) or HA-Gab1 were submitted to a migration assay as described above but incubated for 6 h in the presence of EGF (10 ng/ml), HGF (10 ng/ml), or FGF (10 ng/ml). The results are representative of two independent experiments performed in duplicate. IB, immunoblotting.
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Gab1-associated PI3K and SHP2 Are Mediators of VEGF-induced Signaling and Cell Migration—Gab1-associated PI3K and SHP2 have been identified as mediators of Akt and ERK1/2 activation, respectively, downstream of several growth factor receptors (32). Recently, SHP2 was also shown to regulate Src and PLCγ phosphorylation/activation in response to EGF, FGF, and PDGF (51, 52). Since Gab1 is associated with PI3K and SHP2 in VEGF-stimulated endothelial cells (Fig. 1C) and optimal activation of PLCγ, ERK1/2, Src, and Akt requires Gab1 expression (Fig. 5), we next investigated whether Gab1-associated PI3K and SHP2 were involved in VEGF-dependent signaling and migration. HEK 293 cells were transfected with VEGFR2 together with either the empty vector, WT Gab1, or Gab1 mutants unable to recruit PI3K (ΔPI3K; Y448F/Y473F/Y590F) or SHP2 (ΔSHP2; Y628F/Y660F) (Fig. 7). The loss of PI3K and SHP2 binding to their respective mutants was confirmed following immunoprecipitation (Fig. 7A). SHP2 association to the ΔPI3K mutant was also slightly decreased. This was perhaps due to the weak tyrosine phosphorylation of the ΔPI3K mutant (Fig. 7A). Treatment of BAECs with the PI3K inhibitor LY294002 also led to attenuated tyrosine phosphorylation of Gab1 upon VEGF stimulation (data not shown), suggesting that PI3K activity could enhance Gab1 tyrosine phosphorylation by increasing the binding of the Gab1 pleckstrin homology domain to the membrane via PI3K-derived phospholipids, as previously demonstrated downstream of the EGF and B cell antigen receptors (46, 53). The ΔSHP2 mutant was also less phosphorylated than WT Gab1, possibly due to the loss of two major tyrosine phosphorylation sites. In contrast, its ability to associate with PI3K was similar to that of WT Gab1 (Fig. 7A). HEK 293 cells expressing the Gab1ΔSHP2 mutant had impaired activation of PLC, ERK1/2, and Src, but not of Akt, in response to VEGF stimulation when compared with WT Gab1-expressing cells (Fig. 7B). In contrast, cells expressing the Gab1ΔPI3K mutant had impaired Akt activation, in addition to reduced PLCγ and ERK1/2 phosphorylation. Interestingly, the reduced AKT phosphorylation was more pronounced at an early time (5 min poststimulation), similar to what was observed in Gab1-depleted cells (Fig. 5). These data demonstrate that Gab1-associated SHP2 and PI3K are involved in the promotion of the PLCγ, ERK1/2, Src, and Akt signaling pathways induced by VEGFR2.

To determine the contribution of Gab1-associated PI3K and SHP2 to VEGF-induced endothelial cell migration, BAECs transiently overexpressing WT Gab1 or the ΔPI3K or ΔSHP2 mutants were tested using the Boyden chamber assay (Fig. 8, A and B). Whereas overexpression of Gab1 potentiated the

the activated forms of VEGF-induced enzymes. Upon VEGF stimulation of Gab1-depleted cells, the activation/phosphorylation of PLCγ and of ERK1/2 was greatly reduced compared with that seen in control cells (Fig. 5, B and C). In contrast, the modulation of Src was more modest, whereas Akt Ser473 phosphorylation was mostly inhibited at an early time (Fig. 5). Total cell extracts (40 μg) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with the indicated antibodies (A–E). The results are representative of a minimum of four independent experiments. CTL, control.

FIGURE 5. Knockdown of Gab1 expression in HMVECs impairs VEGF-induced signaling. HMVECs were transfected with pools of four nonspecific control siRNAs or four Gab1 siRNAs (100 nM) using Targefect reagents as described under “Experimental Procedures.” After a 2-h incubation, complete medium was added, and cells were further incubated for 42 h, at which time they were serum-starved for 6 h prior to stimulation or not with VEGF (50 ng/ml). Total cell extracts (40 μg) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with the indicated antibodies (A–E). The results are representative of a minimum of four independent experiments. CTL, control.
migration of VEGF-stimulated BAECs (to a 4-fold induction over basal level compared with a 2.4-fold induction for pCDNA-transfected cells), expression of the ΔPI3K or the ΔSHP2 mutants in BAECs impaired their ability to move in response to VEGF stimulation (down to 1.9- and 1.3-fold inductions, respectively), below levels attained by VEGF-stimulated control cells (2.4-fold induction) (Fig. 8B). Thus, these results demonstrate that Gab1-associated PI3K, but most importantly SHP2, are mediating VEGF-dependent migratory signals. These results are also in agreement with the observed impaired migration of BAECs treated with chemical inhibitors of the PI3K, ERK1/2, Src, and PLCγ pathways (Fig. 8C).

Impaired Actin Reorganization in Gab1-depleted HMVECs—Cell migration requires the ability of cells to reorganize their actin cytoskeleton and to extend lamellipodial protrusions. To define the role of Gab1 in these events, staining of the actin filaments was performed on HMVECs transfected with control and Gab1 siRNAs and then stimulated or not with VEGF. Fig. 9 shows that the depletion of Gab1 led to an increase of stress fibers in unstimulated and VEGF-stimulated cells compared with control cells. Moreover, the Gab1-depleted cells were defective in their ability to form lamellipodia and membrane ruffles as observed on control cells at 10 min post-VEGF stimulation. These data therefore reveal the important role played by Gab1 during the reorganization of the actin cytoskeleton, which underlies the ability of endothelial cells to move in response to VEGF.

DISCUSSION

Uncontrolled and excessive neovascularization is associated with the development of a number of pathologies, including tumor growth and metastatic spreading (6). VEGF is a key regulator of normal and tumor-associated angiogenesis. However, despite the identification of several signaling pathways critical for the proliferation and migration of endothelial cells in response to VEGF, little is known about the VEGFR2-proximal signals regulating these downstream events. In this study, we have identified and characterized the biochemical and biological functions of the scaffolding adapter Gab1 in VEGF-stimulated endothelial cells. Gab1 was found to associate with VEGFR2 and several signaling proteins known to be recruited/activated in response to VEGF, including Grb2, Shc, PI3K, SHP2, and PLCγ. Importantly, our findings reveal that Gab1 and associated SHP2 and PI3K are essential components of the VEGFR2-dependent signaling cascade contributing to the phosphorylation/activation of PLCγ, ERK1/2, Src, and Akt pathways, which have been reported to play critical roles in various aspects of VEGF-dependent biological activities in vitro and in vivo (11). Consistently, we show that Gab1-associated PI3K, and most importantly SHP2, are crucial mediators of VEGF-induced endothelial cell migration, and that Gab1 is required for actin reorganization and in vitro capillary formation. Our results thus demonstrate that Gab1 actively participates in the signaling events required for cell migration and morphogenesis and uncover new VEGFR2-mediated molecular mechanisms triggering the proangiogenic signaling cascade in endothelial cells.

Gab1 and other Gab proteins have been found to associate with a number of membrane receptors, although generally in an indirect fashion via Grb2 and to a lesser extent via Shc (32, 33). Since we found that Gab1 was constitutively associated with Grb2 in BAECs and since Grb2 was reported to associate with VEGFR2 (54), we postulated that Gab1 could be recruited to VEGFR2 signaling complexes. We found that both in endothelial...
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FIGURE 7. Gab1-associated PI3K and SHP2 are involved in the mediation of VEGF-induced signaling. A, HEK 293 cells were transfected with a VEGFR2 encoding plasmid and either the empty vector (pcDNA) or constructs encoding HA-tagged WT Gab1 or the ΔPI3K or the ΔSHP2 mutants. Twenty-four hours later, cells were serum-starved for 16 h and then stimulated or not with VEGF (50 ng/ml) for 2 min. HA-Gab1 was immunoprecipitated from cell lysates (1 mg) using anti-HA antibody. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes, and the upper part of the membrane was immunoblotted with an anti-phosphotyrosine antibody (PY99) to detect phosphorylated Gab1 and then stripped to detect the level of immunoprecipitated Gab1 using anti-HA antibody. The lower part of the membrane was immunoblotted with anti-p85 and anti-SHP2 antibodies. The expression level of transfected VEGFR2 was determined on total cell lysates (50 μg) (TCL) using the anti-VEGFR2 antibody. B, HEK 293 cells were transfected and stimulated as described above for 5 and 15 min. Proteins from total cell lysates (50 μg) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with phosphospecific antibodies recognizing activated PLCγ, ERK1/2, Src, and Akt, as well as with anti-HA and anti-VEGFR2 antibodies to determine the expression level of the transfected constructs. Membranes were then stripped to determine protein levels using anti-PLCγ, anti-ERK1/2, anti-Src, and anti-Akt antibodies. These results are representative of three independent experiments. IP, immunoprecipitation; IB, immunoblotting.

PCLγ-ERK1/2 axis. However, since the activation of this pathway is not completely abrogated in Gab1-depleted cells, other upstream activators must also be involved. TSAd/VRAP, which has recently been shown to associate with Src and mediate VEGF-induced cell migration, could then also contribute to the activation of this pathway (21).

The PCLγ-ERK1/2 pathway has been recognized as a major VEGF proliferative pathway downstream of Tyr1175 of VEGFR2 (15, 17). However, ERK1/2 activity is also associated with the ability of endothelial cells to move in response to VEGF (Fig. 8C) (27), and the activation of PCLγ was shown in one study to correlate with tubulogenesis and the ability of endothelial cells to differentiate but not to proliferate (58). The explanation for these discrepancies is not known. However, intrinsic variations between endothelial cell populations, perhaps differing in the abundance of specific proteins, might account for the oscillation of PCLγ between having a proliferative versus a migratory function. Nevertheless, consistent with these broad biological functions, we show for the first time that, similarly to cells treated with the PI3K, MEK1/2, or Src inhibitors, treatment of BAECs with the PLC inhibitor U73122 strongly blocks VEGF-induced cell migration. In the context of the reported involvement of Src and of PI3K in PCLγ activation (22, 57) and of PCLγ in ERK1/2 activation (15, 17), our results suggest that VEGF-dependent cell migration and capillary formation are in part mediated via a Src-PLCγ-ERK1/2 signal-
ing pathway downstream of Gab1 and associated SHP2 and PI3K.

The VEGF-induced association of Gab1 with PLCγ further suggests that Gab1 might contribute to the recruitment of PLCγ to VEGFR2. Another interpretation of this result is that Gab1-associated PLCγ is in fact co-precipitated via VEGFR2. In this scheme, the contribution of Gab1 to PLCγ activation would then strictly depend on the Gab1-dependent activation of PI3K and Src. This is an interesting issue that could be further investigated in Gab1-depleted cells.

Gab1-associated PI3K has also and mainly been reported to mediate the activation of Akt downstream of several activated receptors, including those of EGF and FGF (37, 38). In addition, Gab1 was shown to be essential for the activation of Akt in response to shear stress in human umbilical vein endothelial cells or BAECs (59, 60). We did not detect a similar extensive block of Akt phosphorylation in Gab1-depleted HMVECs in response to VEGF stimulation, and the maximal activation level detected in control cells was often observed in Gab1-depleted cells, although with delayed kinetics (Fig. 5E). This demonstrates that the specificity of Gab1-derived signaling pathways may vary depending on the cellular context and/or stimulus used. While this manuscript was in revision, Dance et al. (61) reported that Gab1 was a primary actor in coupling VEGFR2 to the PI3K-Akt pathway in response to VEGF stimulation of human umbilical vein endothelial cells and porcine aortic endothelial cells. Although their study consisted of a very thorough analysis of the molecular mechanism involved in the ability of Gab1 to promote activation of this pathway, all of their studies were performed at an early time post-stimulation (5 min). At an initial time, we also observed a strong inhibition of Akt activation in HMVECs treated with Gab1 siRNAs, but this was not maintained. Whether Gab1 is as crucial for Akt activation in human umbilical vein endothelial cells and porcine aortic endothelial cells at later times thus remains to be shown.

Consistent with these biochemical results, overexpression experiments of WT Gab1, Gab1ΔPI3K, and Gab1ΔSHP2 mutants in BAECs, together with the siRNA studies in HMVECs, clearly establish a role for Gab1 in endothelial cell migration and capillary formation. Interestingly, the ΔSHP2 mutant was more potent than the ΔPI3K mutant at blocking VEGF-induced migration of BAECs, although PI3K is essential for endothelial cell migration (Fig. 8C) (29, 30). One explana-
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...tion might be that Gab1 is not the sole mediator of PI3K activation in response to VEGF stimulation, as our results regarding the phosphorylation of Akt suggest. Alternatively, it may also mean that other Gab1-associated proteins can partly rescue a PI3K binding defect, but not an SHP2 binding defect, possibly by activating common or collaborative downstream pathways required for cytoskeletal reorganization and cell migration.

PI3K is a well known activator of the Rac1 GTPase that induces the formation of membrane ruffles and lamellipodia that are essential for the motile behavior of cells (62). Consistent with this, the Gab1-depleted cells failed to extend lamellipodia at 10 min post-VEGF stimulation, demonstrating that at a time that coincides with the block of the PI3K-downstream Akt pathway, Rac1 activation also seems to be defective. In agreement with our results, Gab1-deficient fibroblasts and neuroectodermal tumor cells expressing a Gab1ΔPI3K mutant were also unable to extend lamellipodia in response to PDGF and glial cell line-derived neurotropic factor, respectively (49, 63). Thus, the early requirement for Gab1 in the activation of the PI3K pathway in response to VEGF may contribute, via PI3K-derived lipids, to the membrane recruitment of Rac1 guanine nucleotide exchange factors and lead to the activation of Rac1 and lamellipodia formation in endothelial cells (62). Moreover, in light of the reported association of Gab1 with other proteins, such as Crk, which can interact with the Rac1 guanine nucleotide exchange factor DOCK180, we anticipate that other Gab1-associated proteins regulate VEGF-dependent lamellipodia formation and cell migration (64). Along these lines, a Gab1ΔSHP2 mutant was unable to rescue lamellipodia formation in PDGF-stimulated Gab1-deficient cells, suggesting that SHP2 may also be involved in Rac1 activation (49). As well, several papers have reported the involvement of SHP2 in actin reorganization and cell adhesion, spreading, and migration (65–69). In particular, SHP2 inactivation or expression of a catalytically inactive mutant were shown to result in increased RhoA activation and increased focal adhesion and stress fiber formation, respectively (67, 68). Interestingly, an increase in stress fibers was similarly observed in fibroblasts overexpressing a Gab1ΔSHP2 mutant and in Gab1-depleted HMVECs (Fig. 9) (70). Moreover, Gab1-depleted cells were more difficult to detach from tissue culture plates, suggesting an increase in cell adhesion.4

These observations are consistent with the recently demonstrated ability of SHP2 to mediate Src activation and the Src-dependent phosphorylation/activation of p190RhoGap, a protein involved in the inactivation of RhoA, as well as of VAV2, a Rac1 guanine nucleotide exchange factor shown to be phosphorylated in VEGF-stimulated endothelial cells (51, 71). Conceivably, Gab1-associated SHP2 could then be involved in the down-regulation of actin stress fibers via Src-mediated activation of p190RhoGap and in allowing Rac-mediated lamellipodia formation via phosphorylation of VAV2. It is therefore tempting to speculate that disruption of the SHP2- and PI3K-dependent pathways, as a consequence of Gab1 silencing, leads to an increase in stress fiber formation and cell adhesion, together with a reduced ability to form cell protrusions, such as lamellipodia, thus explaining the reduced ability of Gab1-depleted cells to move and to form capillary-like structures.

In conclusion, the results presented here demonstrate for the first time that Gab1 forms a multimolecular complex with VEGFR2 and mediates VEGF signaling essential for microvascular endothelial cell migration and in vitro capillary formation (Fig. 10). The finding that Gab1 is required for the optimal activation of the well characterized PLCγ-ERK1/2 pathway and of Akt, potentially via associated SHP2 and PI3K, provides novel insights into our understanding of the molecular mechanisms that may regulate in vivo angiogenesis.

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