Heterotrimeric $\alpha_q/\alpha_{11}$ Proteins Function Upstream of Vascular Endothelial Growth Factor (VEGF) Receptor-2 (KDR) Phosphorylation in Vascular Permeability Factor/VEGF Signaling*

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Vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) functions by activating two receptor-tyrosine kinases, Flt-1 (VEGF receptor (VEGFR)-1) and KDR (VEGFR-2), both of which are selectively expressed on primary vascular endothelium. KDR is responsible for VPF/VEGF-stimulated endothelial cell proliferation and migration, whereas Flt-1 down-modulates KDR-mediated endothelial cell proliferation. Our recent works show that pertussis toxin-sensitive G proteins and G$\beta$ subunits are required for Flt-1-mediated down-regulation of human umbilical vein endothelial cell (HUVEC) proliferation and that G$\alpha_{q/11}$ proteins are required for KDR-mediated RhoA activation and HUVEC migration. In this study, we demonstrate that G$\alpha_{11}$ proteins are also required for VPF/VEGF-stimulated HUVEC proliferation. Our results further indicate that G$\alpha_{11}$ proteins specifically mediate KDR signaling such as intracellular Ca$^{2+}$ mobilization rather than Flt-1-induced CDC42 activation and that a G$\alpha_{q/11}$ antisense oligonucleotide completely inhibits MAPK phosphorylation induced by KDR but has no effect on Flt-1-induced MAPK activation. More importantly, we demonstrate that G$\alpha_{q/11}$ proteins interact with KDR in vivo, and the interaction of G$\alpha_{11}$ proteins with KDR does not require KDR tyrosine phosphorylation. Surprisingly, the G$\alpha_{11}$ antisense oligonucleotide completely inhibits VPF/VEGF-stimulated KDR phosphorylation. Expression of a constitutively active mutant of G$\alpha_{11}$ but not G$\alpha_q$ can cause phosphorylation of KDR and MAPK. In addition, a G$\beta$ minigene, h$\beta$ARK1(495), inhibits VPF/VEGF-stimulated HUVEC proliferation, MAPK phosphorylation, and intracellular Ca$^{2+}$ mobilization but has no effect on KDR phosphorylation. Taken together, this study demonstrates that G$\alpha_{q/11}$ proteins mediate KDR tyrosine phosphorylation and KDR-mediated HUVEC proliferation through interaction with KDR.

Pathological angiogenesis is a hallmark of cancer and various ischemic and inflammatory diseases. Many different cyto- kines and growth factors, such as vascular permeability factor/ vascular endothelial growth factor (VPF/VEGF),$^1$ basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and transforming growth factor-$\beta$, have an angiogenic activity (1–3). Among these, VPF/VEGF stands out because of its potency and selectivity for vascular endothelium. VPF/VEGF is not only involved in several steps of angiogenesis but is also the only angiogenic factor recognized to date that renders microvessels hyperpermeable to circulating macromolecules (4–8). VPF/VEGF extensively programs endothelial cell expression of proteases, integrins, and glucose transporters; stimulates endothelial cell migration and division; and protects endothelial cells from apoptosis and senescence (9–12).

Most VPF/VEGF biological activities are mediated by its interaction with two high affinity receptor tyrosine kinases, Flt-1 (VEGFR-1) and KDR (VEGFR-2, FLK-1 in mice) (13–17). A third receptor, neuropilin, which binds to VEGF165 but not VEGF121, has been recognized, but less is known about neuropilin’s capacity to initiate endothelial cell signaling (18, 19). A large body of work has demonstrated that KDR, not Flt-1, is responsible for VPF/VEGF-stimulated cell proliferation and migration in cultured EC and for microvascular permeability (20–24). However, Flt-1 functions to down-regulate KDR-mediated cultured EC proliferation as shown by two different VPF/VEGF receptor chimera fusion systems in which the N-terminal domains of KDR and Flt-1 were replaced by the N-terminal domain of either epidermal growth factor receptor or colony-stimulating factor-1 receptor (23, 24). Further studies indicated that the signaling pathway required for Flt-1-mediated down-regulation of EC proliferation involves activation of phosphatidylinositol 3-kinase, small GTPase Rac1, CDC42, and, surprisingly, pertussis toxin-sensitive G proteins (24, 25).

The signal transduction pathways mediated by KDR involve KDR phosphorylation (20–24), phospholipase C (PLC) activation (24, 26–30), inositol 1,4,5-trisphosphate accumulation (31), intracellular Ca$^{2+}$ mobilization (24, 32), and protein kinase C and MAPK activation (24, 27–30). Whereas PLC activation is involved in VPF/VEGF-induced HUVEC proliferation and migration, intracellular Ca$^{2+}$ mobilization and MAPK are required for VPF/VEGF-induced HUVEC proliferation but not migration (24). Our recent studies have shown that the G$\alpha_{q/11}$

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1 The abbreviations used are: VPF, vascular permeability factor; VEGF, vascular endothelial growth factor; HUVEC, human umbilical vein endothelial cell(s); EC, endothelial cell(s); MAPK, mitogen-activated protein kinase; PLC, phospholipase C; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; EGFR, epidermal growth factor; EGDR, the fusion receptor of EGFR and KDR; EGLT, the fusion receptor of EGFR and Flt-1.
family of the heterotrimeric GTP-binding proteins and Gβγ subunits mediates VPF/VEGF-induced HUVEC migration through the small GTPase RhoA (33). However, it is not clear whether G protein Gq₁₁ proteins are involved in VPF/VEGF-stimulated proliferation. In this study, we show for the first time that inhibition of Gq₁₁ protein expression by a Gq₁₁-specific antisense oligonucleotide blocked VPF/VEGF-stimulated proliferation and activation of signaling molecules in VPF/VEGF-stimulated HUVEC that are mediated by KDR, not by Flt-1. Moreover, Gq₁₁ proteins are not activated by tyrosine phosphorylation but through physical interaction with KDR. Surprisingly, the interaction of Gq₁₁ proteins with KDR does not require KDR tyrosine phosphorylation, and the Gq₁₁-specific antisense oligonucleotide blocks the interaction of Gq₁₁ with KDR and inhibits KDR phosphorylation. Furthermore, the Gα₁₁ constitutively active mutant, Gα₁₅(G209L), not the Gα₉₅ constitutively active mutant, Gα₉₅(G209L), activates phosphorylation of KDR and MAPK. However, expression of the Gβγ minigene, hβARK1(495), inhibits VPF/VEGF-stimulated HUVEC proliferation, MAPK phosphorylation, and intracellular Ca²⁺ mobilization but has no effect on KDR phosphorylation.

EXPERIMENTAL PROCEDURES

Materials—Recombinant VPF/VEGF was obtained from R&D Systems (Minneapolis, MN). The EGM-MV Bullet kit, trypsin-EDTA, and trypsin neutralization solution were obtained from Clonetics (San Diego, CA). Vitrogen 100 was purchased from Collagen Biomaterials (Palo Alto, CA). Rabbit polyclonal antibodies against the KDR C-terminal domain, Gα₉₅, and Gα₉₅ were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-phosphotyrosine antibody was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). The anti-phospho-p42/p44 MAPK antibody was obtained from New England Biolabs (Beverly, MA). PHAmydine was obtained from PerkinElmer Life Sciences. Fura-2/AM and Pluronic F-127 were obtained from Molecular Probes, Inc. (Eugene, Oregon). Pertussis toxin was obtained from Calbiochem.

Cell Culture—Primary human umbilical vein endothelial cells (HUVEC, obtained from Clonetics) were cultured with or without transduction with retroviruses as described (24). Only cells from passage 3 or 4 that were ~80% confluent were used for experiments. Transduction of HUVEC with EGD-resistant or -EGL-bearing retroviruses was carried out as described previously (24, 33).

Overexpression of Proteins in HUVEC—Retrovirus preparation and HUVEC infection with retrovirus were carried out as described (24, 33). Briefly, 293T cells were seeded at a density of 6 × 10⁶ cells/100-mm plate 24 h before transfection. DNA transfection was carried out with the Effectene™ transfection reagent (Qiagen, Valencia, CA). Two μg of each target gene (pMPG-EGDR, pMPM-LacZ, etc.) 1.5 μg of pMD.MLV gag.pol, and 0.5 μg of pMD.G DNA encoding the cDNAs of the proteins that are required for viral packaging (kindly provided by Dr. Mulligan), were mixed in 200 μl of E buffer. 32 μl of enhancer was added to the DNA mixture. After incubation at room temperature for 2 min, 30 μl of Effectene was added to the DNA mixture and incubated at room temperature for 5 min. The DNA mixture was added dropwise to 293T cells. The medium was changed after 16 h. The retrovirus was isolated 48 h after transfection and used immediately for infection or frozen at −70°C.

24 h before infection, HUVECs were seeded at a density of 0.3 × 10⁶ cells/100-mm plate. 1 ml of retrovirus solution (~2 × 10⁸ plaque-forming units/ml) and 5 ml of fresh medium were added to cells with 10 μg/ml polybrene. The medium was changed after 16 h, and cells were ready for experiments 48 h after infection.

Plasmids containing Gα₉₅, Gα₁₅(Q209L), Gα₉₅, and Gα₁₅(209L) cDNAs were obtained from Guthrie cDNA Resource Center, Guthrie Research Institute (Sayre, PA). Transfection of plasmids to HUVEC was carried out as described (34).

Synthesis and Transfection of Antisense Oligonucleotides—3′-End FITC-labeled phosphorothioated Gα₁₅ antisense oligonucleotide (ODN-G₉₅, 5′-CCATGGCAGTCTTGACTG-3′) and a 3′-end fluorescein isothiocyanate-labeled phosphorothioated random oligonucleotide (ODN-RD, 5′-CCCTATTACCTACTGCGC-3′ (35), were synthesized by Genemed Synthesis (Genemed Synthesis, San Francisco, CA).

Proliferation Assays—Assays were carried out as described (24, 33). Briefly, HUVEC (with or without oligonucleotide transfection) were serum-starved (0.1% serum) for 24 h and then stimulated with 10 ng/ml VEGF for 20 h. 1 μCi/ml [3H]thymidine was added to each well, and 4 h later the cells were washed, fixed, and lysed. Data were expressed as fold-activation with the stimulated cells compared with control treated cells. The data shown represent the means with S.D. of triplicate determinations per experimental condition, and the experiments were repeated at least three times.

Intracellular Ca²⁺ Release—Serum-starved HUVEC with or without transfection were loaded with Fura-2 AM and stimulated with 10 ng/ml VPF/VEGF. The assay was carried out as described (24, 33). All experiments were repeated at least three times.

CDC42 Activity Assays—The CDC42 activity assay was carried out as described (25). Briefly, 24-h serum-starved HUVEC with or without oligonucleotide transfection were stimulated with 10 ng/ml VEGF for 1 min. Cells were washed and lysed. Cell lysates were centrifuged at 14,000 rpm for 3 min. The supernatant was incubated with 50 μl of freshly prepared GST-Pak-CRIB beads at 4°C for 45 min. The proteins bound to beads were washed and analyzed by SDS-PAGE with antibodies against CDC42. All experiments were repeated at least three times.

Immunoprecipitation and Immunoblotting—HUVEC, with or without virus infection or oligonucleotide transfection, were serum-starved for 24 h and stimulated with 10 ng/ml VPF/VEGF or EGF as indicated for various time periods. Stimulation was halted by the addition of ice-cold phosphate-buffered saline, and cells were washed three times with ice-cold phosphate-buffered saline and lysed with cold radiomuneprecipitation buffer (20 mM Tris-HCl, pH 7.5, 0.15 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaVO₄, 1 mM EGTA, 1 μg/ml leupeptin, 0.5% aprotinin, 2 μg/ml pepstatin A). Cell lysates were collected after centrifugation at 14,000 × g for 15 min at 4°C. One mg of lysate protein was incubated with antibodies as indicated for 1 h, and with 50 μl of protein A-conjugated agarose beads at 4°C for another 1 h. For immunoprecipitation with phosphorylated tyrosine, cellular extracts were incubated with agarose-conjugated anti-phosphorylated tyrosine for 2 h. Beads were washed with radiomuneprecipitation buffer three times, and immunoprecipitates were resuspended in 2× SDS sample buffer for Western blot analysis. For the experiments with pertussis toxin, this was added 16 h before stimulation. All experiments were repeated at least three times.

RESULTS

Requirement of Gq₁₁ Proteins for DNA Synthesis in VPF/VEGF-stimulated HUVEC—In order to examine whether Gq₁₁ is involved in VPF/VEGF-induced HUVEC DNA synthesis, we utilized a Gq₁₁-specific oligonucleotide that has been previously shown by us to inhibit VPF/VEGF-induced HUVEC migration (33). The Gq₁₁ antisense oligonucleotide specifically blocks expression of both Gα₉₅ and Gα₁₅, as shown by Western blot analysis using an antibody that recognizes both Gα₉₅ and Gα₁₅ (Fig. 1a, top panel). However, this has no effect on the expression of the Gα₉₀ family of G proteins (Fig. 1a, middle panels). To rule out the possibility that any effect of the Gq₁₁ antisense on KDR signaling is due to inhibition of KDR expression, equal amounts of cellular extracts from HUVEC with or without transfection of ODN-Gq₁₁ or ODN-RD were subjected to immunoblot analysis with an antibody against KDR. The data show that ODN-Gq₁₁ has no effect on KDR expression (Fig. 1a, bottom panel). Then we determined the effect of the Gq₁₁ antisense oligonucleotide on KDR-mediated DNA synthesis in HUVEC. HUVEC were transfected with fluorescein isothiocyanate-labeled phosphorothioate ODN-Gq₁₁ with a method that gave out almost 100% transfection yield as described before (36) and subjected to a DNA synthesis assay with 10 ng/ml VPF/VEGF or bFGF stimulation. As shown in Fig. 1b, antisense oligonucleotide ODN-Gq₁₁ almost completely inhibits VPF/VEGF-stimulated but has no effect on bFGF-stimulated DNA synthesis in HUVEC. However, the control oligonucleotide, OND-RD (36), has no effect. These data clearly indicate that Gq₁₁ proteins are required for VPF/VEGF-stimulated DNA synthesis in HUVEC.

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MAPK. Surprisingly, ODN-Gq/11 partially inhibits VPF/VEGF-analysis using an antibody specifically against phosphorylated for 10 min. Cellular extracts were subjected to Western blot analysis with antibodies against Gq/11 (top panel) or Gq/11 (bottom panel). The blot of the top panel was stripped and reprobed with an antibody against Gq/11. Lane 1, HUVEC without transfection; lane 2, HUVEC transfected with ODN-Gq/11; lane 3, HUVEC transfected with ODN-RD. b, serum-starved HUVEC transfected with ODN-Gq/11 and ODN-RD were stimulated with 10 ng/ml VPF/VEGF or bFGF. [3H]Thymidine incorporation into DNA was measured as described under “Experimental Procedures” (n = 3).

**FIG. 1.** Effect of a Gq/11 antisense oligonucleotide on VPF/VEGF-stimulated HUVEC proliferation. a, HUVEC was transfected with an antisense oligonucleotide of Gq/11 (ODN-Gq/11) or control oligonucleotide (ODN-RD). Equal amounts of cellular extracts were immunoblotted (IB) with antibodies against Gq/11 or Gq/11. The blot of the top panel was stripped and reprobed with an antibody against Gq/11. Lane 1, HUVEC without transfection; lane 2, HUVEC transfected with ODN-Gq/11; lane 3, HUVEC transfected with ODN-RD. b, serum-starved HUVEC transfected with ODN-Gq/11 and ODN-RD were stimulated with 10 ng/ml VPF/VEGF or bFGF. [3H]Thymidine incorporation into DNA was measured as described under “Experimental Procedures” (n = 3).

Gq/11 Proteins Are Required for the KDR-mediated, Not Flt-1-mediated, Signaling Pathway in VPF/VEGF-stimulated HUVEC—Next, we examined the effect of ODN-Gq/11 on the activation of signaling molecules mediated by KDR and Flt-1. As shown in Fig. 2a, ODN-Gq/11, not the control ODN-RD, completely inhibits intracellular Ca2+ mobilization that is mediated by KDR but not Flt-1 (24). We have previously shown that Flt-1, but not KDR, can activate CDC42, a member of the Rho family of small GTPases (25). Therefore, we examined the effect of ODN-Gq/11 on CDC42 activation. To do this, serum-starved HUVEC that were transfected with ODN-Gq/11 or nontransfected were stimulated with VPF/VEGF for 1 min, and cellular extracts were subjected to a CDC42 activation assay as described under “Experimental Procedures.” The data indicate that ODN-Gq/11 has no effect on CDC42 activation (Fig. 2b).

It is known that MAPK phosphorylation is required for VPF/VEGF-stimulated HUVEC proliferation (24, 27–30). Therefore, we tested whether Gq/11 proteins are required for VPF/VEGF-stimulated MAPK phosphorylation in HUVEC. Serum-starved HUVEC, which were transfected with either ODN-Gq/11 or ODN-RD or nontransfected, were stimulated with VPF/VEGF for 10 min. Cellular extracts were subjected to Western blot analysis using an antibody specifically against phosphorylated MAPK. Surprisingly, ODN-Gq/11 partially inhibits VPF/VEGF-stimulated MAPK phosphorylation in HUVEC, whereas ODN-RD has no effect (Fig. 3a). Similar results were also obtained when cells were treated with VPF/VEGF for 15 and 30 min (data not shown). Because both KDR and Flt-1 are present in HUVEC, in order to further characterize the partial inhibition of MAPK phosphorylation by the Gq/11 antisense oligonucleotide, we used the recently developed chimeric receptors, EGDR and EGLT, in which the extracellular domains of KDR and Flt-1 were replaced with that of epidermal growth factor receptor. Serum-starved HUVEC with or without transduction of LacZ as a control, EGDR, or EGLT was stimulated with VPF/VEGF or EGF for the indicated time. Cellular extracts were used to determine the levels of phosphorylated MAPK. As shown in Fig. 3b, both EGDR and EGLT can mediate MAPK phosphorylation; however, HUVEC transduced with LacZ do not show any detectable MAPK phosphorylation. Furthermore, we used this receptor chimera system to further confirm whether ODN-Gq/11 inhibits only KDR-mediated and not Flt-1-mediated MAPK phosphorylation. EGDR/HUVEC and EGLT/HUVEC, transfected with either ODN-Gq/11 or pertussis toxin, were stimulated with EGF for 10 min. Cellular extracts were used to determine MAPK phosphorylation. The data show that ODN-Gq/11 completely inhibits EGDR-mediated MAPK phosphorylation but has no effect on MAPK phosphorylation mediated by EGLT (Fig. 3c). Moreover, our data also demonstrate that pertussis toxin completely inhibits Flt-1-mediated MAPK phosphorylation but has no effect on KDR-mediated MAPK phosphorylation (Fig. 3c). These results indicate that Gq/11 proteins are required for KDR-mediated signaling pathways and that pertussis toxin-sensitive Gq/11 proteins are required for Flt-1 signaling.

**FIG. 2.** Effect of the Gq/11 antisense oligonucleotide ODN-Gq/11 on the activation of signaling molecules stimulated with VPF/VEGF. a, HUVEC with or without transfection of ODN-Gq/11 or ODN-RD were stimulated with 10 ng/ml VPF/VEGF, and intracellular Ca2+ response was determined. b, serum-starved HUVEC with or without transfection of ODN-Gq/11 or ODN-RD were stimulated with VPF/VEGF for 1 min. Cellular extracts were incubated with GST-Pak-CRIB beads. The bound proteins (activated form of CDC42) were subjected to immunoblot analysis using an antibody against CDC42. Equal amounts of cellular extracts were immunoblotted with the CDC42 antibody to indicate similar levels of CDC42 in each sample (bottom panel).

### Gq/11 Proteins Are Required for the KDR-mediated, Not Flt-1-mediated, Signaling Pathway in VPF/VEGF-stimulated HUVEC

- **Fig. 1.** Effect of a Gq/11 antisense oligonucleotide on VPF/VEGF-stimulated HUVEC proliferation. 
  - **a:** HUVEC was transfected with an antisense oligonucleotide of Gq/11 (ODN-Gq/11) or control oligonucleotide (ODN-RD). Equal amounts of cellular extracts were immunoblotted (IB) with antibodies against Gq/11 or Gq/11. The blot of the top panel was stripped and reprobed with an antibody against Gq/11. Lane 1, HUVEC without transfection; lane 2, HUVEC transfected with ODN-Gq/11; lane 3, HUVEC transfected with ODN-RD. 
  - **b:** Serum-starved HUVEC transfected with ODN-Gq/11 and ODN-RD were stimulated with 10 ng/ml VPF/VEGF or bFGF. [3H]Thymidine incorporation into DNA was measured as described under “Experimental Procedures” (n = 3).

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  - **b:** Serum-starved HUVEC with or without transfection of ODN-Gq/11 or ODN-RD were stimulated with VPF/VEGF for 1 min. Cellular extracts were incubated with GST-Pak-CRIB beads. The bound proteins (activated form of CDC42) were subjected to immunoblot analysis using an antibody against CDC42 (top panel). Equal amounts of cellular extracts were immunoblotted with the CDC42 antibody to indicate similar levels of CDC42 in each sample (bottom panel).

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  - **a:** HUVEC was transfected with an antisense oligonucleotide of Gq/11 (ODN-Gq/11) or control oligonucleotide (ODN-RD). Equal amounts of cellular extracts were immunoblotted (IB) with antibodies against Gq/11 or Gq/11. The blot of the top panel was stripped and reprobed with an antibody against Gq/11. Lane 1, HUVEC without transfection; lane 2, HUVEC transfected with ODN-Gq/11; lane 3, HUVEC transfected with ODN-RD. 
  - **b:** Serum-starved HUVEC transfected with ODN-Gq/11 and ODN-RD were stimulated with 10 ng/ml VPF/VEGF or bFGF. [3H]Thymidine incorporation into DNA was measured as described under “Experimental Procedures” (n = 3).

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  - **a:** HUVEC with or without transfection of ODN-Gq/11 or ODN-RD were stimulated with 10 ng/ml VPF/VEGF, and intracellular Ca2+ response was determined. 
  - **b:** Serum-starved HUVEC with or without transfection of ODN-Gq/11 or ODN-RD were stimulated with VPF/VEGF for 1 min. Cellular extracts were incubated with GST-Pak-CRIB beads. The bound proteins (activated form of CDC42) were subjected to immunoblot analysis using an antibody against CDC42 (top panel). Equal amounts of cellular extracts were immunoblotted with the CDC42 antibody to indicate similar levels of CDC42 in each sample (bottom panel).
pling receptor. Therefore, we tested whether $G_{q/11}$ proteins interact with KDR in VPF/VEGF-stimulated HUVEC. HUVEC were stimulated with 10 ng/ml VPF/VEGF for 10 min. Phosphorylated MAPK in cellular extracts was detected with an antibody against phosphorylated MAPK (top panel). The blot was stripped and reprobed with an antibody against nonphosphorylated MAPK to confirm equal protein loading (lower panel). Serum-starved HUVEC with or without transfection of different receptors were stimulated with 10 ng/ml EGF for the indicated times. Cellular extracts were subjected to immunoblot analysis with antibodies against phosphorylated MAPK (A) and nonphosphorylated MAPK (B). Serum-starved EGDR/HUVEC or EGLT/HUVEC transfected with ODN-$G_{q/11}$ or pretreated with pertussis toxin (PTX; 100 ng/ml) for 16 h were stimulated with 10 ng/ml EGF for 10 min. Cellular extracts were subjected to immunoblot analysis with antibodies against phosphorylated MAPK (A) and nonphosphorylated MAPK (B). IB, immunoblot.

**FIG. 3.** Different pathways mediate MAPK phosphorylation in VPF/VEGF-stimulated HUVEC. A, Serum-starved HUVEC with or without oligonucleotide transfection were stimulated with 10 ng/ml VPF/VEGF for 5, 10, and 20 s. Cellular extracts were immunoprecipitated with an antibody against nonphosphorylated MAPK to confirm equal protein loading (lower panel). Serum-starved HUVEC with or without transfection of different receptors were stimulated with 10 ng/ml EGF for the indicated times. Cellular extracts were subjected to immunoblot analysis with antibodies against phosphorylated MAPK (A) and nonphosphorylated MAPK (B). IB, immunoblot.

**FIG. 4.** VPF/VEGF cannot induce $G_{q/11}$ phosphorylation. Serum-starved HUVEC were stimulated with 10 ng/ml VPF/VEGF for 20, 40, 60, and 120 s. Cellular extracts were immunoprecipitated with an agarose-conjugated phosphorylated tyrosine antibody. The immunoprecipitated complex was resolved on a 4–15% polyacrylamide gel. The blot was cut into two pieces at a molecular mass of 80 kDa. The lower and the upper portions of the blot were analyzed with antibodies against KDR and $G_{q/11}$, respectively. Cellular extract from HUVEC without stimulation was used as control.

**FIG. 5.** VPF/VEGF stimulates physical interaction of KDR with $G_{q/11}$. A, serum-starved HUVEC were stimulated with 10 ng/ml VPF/VEGF for 5, 10, and 20 s. Cellular extracts were immunoprecipitated (IP) with an antibody against $G_{q/11}$. The presence of KDR in the immunoprecipitates was detected with an antibody against KDR. IB, immunoblot. B, serum-starved HUVEC transfected with ODN-$G_{q/11}$ or ODN-RD were stimulated with 10 ng/ml VPF/VEGF for 5 s. Cellular extracts were immunoprecipitated with an antibody against $G_{q/11}$. The presence of KDR in the immunoprecipitates was detected with an antibody against KDR.

**Interaction of KDR and $G_{q/11}$ Does Not Require KDR Phosphorylation—**Next, we tested whether KDR phosphorylation is required for KDR and $G_{q/11}$ interaction. We utilized the chimeric receptor, EGDR. In HUVEC transduced with LacZ, EGF did not stimulate the interaction of KDR with $G_{q/11}$ (Fig. 6a). However, when serum-starved HUVEC transduced with EGDR were stimulated with EGF for various times, the interaction of EGDR and $G_{q/11}$ was found to be in a similar time course to that of VPF/VEGF-stimulated HUVEC (Fig. 6b). Previously, we showed that EGDR(Y915F), a mutated receptor with partial phosphorylation activity, cannot induce HUVEC migration but maintains its ability to induce cell proliferation. However, EGDR(Y1059F), a mutated receptor that could not be tyrosine-phosphorylated, cannot stimulate HUVEC proliferation but still induces HUVEC migration (33). Therefore, we overexpressed these two EGDR mutants in HUVEC to test their effect on KDR and $G_{q/11}$ protein interaction. The results show that like wild type EDGR, both EGDR(Y915F) and EGDR(Y1059F) interact with $G_{q/11}$ (Fig. 6b). Our data clearly demonstrate that tyrosine phosphorylation of KDR is not required for interaction of KDR with $G_{q/11}$ protein.
G₁₁α Protein Activation Is Required for KDR Phosphorylation—So far, our data indicate that, after VPF/VEGF stimulation in HUVEC, 1) Gq₁₁ are not tyrosine-phosphorylated in response to VPF/VEGF; 2) Gq₁₁ proteins physically interact with KDR; and 3) Gq₁₁ and KDR interaction does not require Gq₁₁ phosphorylation. Therefore, we further characterized the correlation between Gq₁₁ activation and KDR phosphorylation. First we examined whether Gq₁₁ proteins are required for KDR phosphorylation. To do this, serum-starved HUVEC transfected with wild type Gq₁₁, Gq₁₁(Q209L) and immunoblotted with an antibody against the C-terminal KDR. We found that serum-starved HUVEC infected with viruses expressing LacZ and hARK1(495) on VPF/VEGF-induced phosphorylation of KDR and MAPK and whether expression of constitutively active G₁₁α mutants alone could stimulate phosphorylation of KDR and MAPK. For this purpose, serum-starved HUVEC that were nontransfected or transfected with wild type Gαq, Gq₁₁, and their constitutively active mutants, Gαq(Q209L) and Gq₁₁(Q209L) were stimulated with 10 ng/ml VPF/VEGF or vehicle for 1 min for KDR phosphorylation or for 10 min for MAPK phosphorylation. The similar levels of the Gq₁₁ protein expression were first confirmed by immunoblot analysis using an antibody that recognized both Gαq and Gq₁₁ (data not shown). The results show that in nonstimulated cells, only Gαq(Q209L) caused phosphorylation of KDR (Fig. 7b) and MAPK (Fig. 7c). However, in VPF/VEGF-stimulated HUVEC, transfection of Gαq, Gα₁₁, or Gq₁₁(Q209L) did not change the levels of phosphorylation of KDR (Fig. 7b) and MAPK (Fig. 7c). These data clearly demonstrate that Gα₁₁, not Gαq, is required, and activated Gq₁₁ is sufficient for VPF/VEGF-stimulated KDR phosphorylation and the downstream signaling pathway.

Gβγ Is Not Required for KDR Phosphorylation—In G protein-coupling receptor systems, heterotrimeric G proteins, after interaction with activated receptors, dissociate into α and βγ subunits that trigger different downstream pathways. Recently, we demonstrated that Gβγ subunits are required for VPF/VEGF-stimulated KDR-mediated migration and Flt-1-mediated antiproliferation in HUVEC by utilizing a Gβγ minigene, hβARK1(495) (25, 36). Here, we further tested whether Gβγ is required for the signaling pathway that regulates VPF/VEGF-stimulated HUVEC proliferation. Serum-starved HUVEC transduced with LacZ and hARK1(495) were stimulated with 10 ng/ml VPF/VEGF or bFGF and subjected to a DNA synthesis assay. As shown in Fig. 8a, overexpression of hARK1(495) completely inhibited DNA synthesis in HUVEC stimulated with VPF/VEGF but had no effect on bFGF-stimulated DNA synthesis. We also examined the effect of hARK1(495) on VPF/VEGF-induced MAPK phosphorylation. Serum-starved HUVEC infected with viruses expressing LacZ or hARK1(495) were stimulated with 10 ng/ml VPF/VEGF for 10 min. Equal amounts of cellular protein were subjected to immunoblot analysis with an antibody against phosphorylated MAPK (Fig. 8b, top panel) as well as an antibody against nonphosphorylated MAPK to confirm equal protein loading (Fig. 8b, bottom panel). Overexpression of hARK1(495) completely inhibited VPF/VEGF-stimulated MAPK phosphorylation (Fig. 8b, bottom panel). Since VPF/VEGF stimulates intracel-
VEGF with or without transduction of LacZ or hARK1(495) were stimulated with 10 ng/ml VPF/VEGF for 1 min. Cellular extracts were subjected to immunoprecipitate with an antibody against KDR and then immunoblotted with an antibody against phosphorylated tyrosine. Surprisingly, hARK1(495) had no effect on VPF/VEGF-stimulated KDR phosphorylation (Fig. 8d). Our data indicated that Gβγ subunits are not required for VPF/VEGF-stimulated KDR phosphorylation but mediate the downstream signaling pathway after KDR phosphorylation.

**DISCUSSION**

VPF/VEGF is an important, multifunctional angiogenic cytokine that has a variety of biological activities on the vascular endothelium. These activities include induction of microvascular hyperpermeability, stimulation of proliferation and migration, reprogramming of gene expression, endothelial cell survival, and prevention of senescence (for reviews, see Refs. 10–12 and 27). All of these functions are thought to be mediated by two receptor tyrosine kinases, KDR and Flt-1, that are expressed on the vascular endothelium and are up-regulated at sites of VPF/VEGF overexpression as in the case of tumors, healing wounds, chronic inflammation, etc. (for a review, see Ref. 10). Both KDR and Flt-1 belong to the receptor tyrosine kinase superfamily. Unlike the epidermal growth factor receptor that directly binds and activates the Grb2-Sos-Ras-Raf-1-MAPK cascade, a number of other members of this superfamily including insulin receptor, insulin-like growth factor receptors, and PDGF receptor (37, 40–42) have been shown to utilize heterotrimeric G proteins for their signaling. For instance, Gq/11 proteins were found to be involved in insulin receptor signaling (40), and pertussis toxin-sensitive G proteins participate in the signaling of the insulin-like growth factor (42) and PDGF receptors (37) as well as Flt-1 (25, 43). Our recent study demonstrates that Gq/11 proteins also play a critical role in VPF/VEGF-stimulated HUVEC migration, and the effect is mediated via RhoA activation (36). In the present study, we further establish that Gq/11 proteins are required for VPF/VEGF-stimulated HUVEC proliferation and that Gq/11 proteins regulate the activation of signaling molecules that are stimulated only by KDR, such as intracellular Ca2+ mobilization, but not CDC42 activation that is mediated by Flt-1 (25). Furthermore, in the case of MAPK phosphorylation that can be stimulated by both KDR and Flt-1, a Gq/11 antisense oligonucleotide specifically inhibits KDR-mediated but not Flt-1-mediated MAPK phosphorylation. Therefore, the involvement of Gq/11 in VPF/VEGF response is specific for KDR.

It is well known that G protein-coupled receptors can transduce their signaling through heterotrimeric G protein-mediated EGF receptor transactivation (38, 39). Recently, a number of studies have indicated that receptor tyrosine kinases can also transduce their signaling through heterotrimeric G proteins. For example, platelet-derived growth factor β receptor stimulates MAPK activation via direct tyrosine phosphorylation of pertussis toxin-sensitive G protein (37). Overexpression of Goq(Q209L), a constitutively active form of Goq, that cannot promote DNA synthesis by itself, stimulates DNA synthesis in platelet-derived growth factor-mediated NIH-3T3 cells (44). Another receptor tyrosine kinase, insulin-like growth factor-1 receptor, stimulates MAPK and cell proliferation also via physical interaction with Gq and Gβγ subunits (41, 42). Interestingly, insulin-stimulated glucose transport is through Gq/11-dependent PI-3 kinase rather than protein Gq and Gβγ subunit pathways (40). In this study, we present evidence that VPF/VEGF stimulates KDR-mediated HUVEC proliferation and MAPK phosphorylation via induction of rapid but transient interaction between Gq/11 proteins and KDR. In order to deter-
mine whether the Gq/11 and KDR interaction require the tyrosine kinase activity of KDR, we used two EGDR mutants, EGDR(Y951F) and EGDR(Y1059F), in which each of the auto-phosphorylation sites of KDR was mutated to phenylalanine (33). As compared with wild type EGDR, EGDR(Y951F) shows a partial defect in its kinase activity, whereas EGDR(Y1059F) completely loses its kinase activity (33). In terms of ability to stimulate HUVEC proliferation, EGDR(Y951F) is similar to wild type EGDR, whereas EGDR(Y1059F) completely loses this activity when HUVEC transduced with the respective mutants are treated with EGF (33). With these mutant receptors, our current study indicates that these kinase-defective EGDR(Y951F) and kinase-deficient EGDR(Y1059F) receptors still interact with Gq/11 proteins to a similar extent as wild type EGDR. The results suggest that the tyrosine kinase activity of KDR is not essential for Gq/11 and KDR interaction. Surprisingly, our results show that the Gq/11 antisense oligonucleotide blocks KDR phosphorylation and a constitutively active form of Gq/11, Gq11(Q209L), activates KDR phosphorylation in the absence of VPF/VEGF stimulation. Thus, our conclusion is that interaction of KDR and Gq11 occurs upstream of KDR phosphorylation in VPF/VEGF-stimulated HUVEC, which is supported by our data indicating that Gq11 cannot be tyrosine-phosphorylated by VPF/VEGF stimulation. Our finding is in contrast with two recent studies by Alderton et al. (37) and De Vivo and Iyengar (44) showing that PDGF receptor stimulates MAPK activation via direct tyrosine phosphorylation of pertussis toxin-sensitive Gq protein, or activated Gq potentiates PDGF-stimulated DNA synthesis.

Recently, Thuringer et al. (45) have reported that stimulation of Gq/11-coupled bradykinin B2 receptor induces tyrosine phosphorylation of KDR, resulting in increased endothelial nitric-oxide synthase activity. These results are consistent with our current observations that upon stimulation, KDR rapidly binds and activates heterotrimeric G proteins Gq11, which in turn increases the intrinsic kinase activity and autophosphorylation of KDR. Interestingly, Tanimoto et al. (46) showed that sphingosine 1-phosphate, via binding to its Gq-coupled receptor endothelial differentiation 1, stimulates tyrosine phosphorylation of KDR and that inhibition of KDR expression or intrinsic kinase activity decreases sphingosine 1-phosphate-induced activation of Akt and eNOS. The results of Tanimoto et al. (46) indicate that sphingosine 1-phosphate-induced KDR transactivation is independent of metalloproteinase and VPF/VEGF release but requires intracellular calcium release and Src tyrosine kinase. Although it is not clear how the B2 receptors and sphingosine 1-phosphate stimulate KDR transactivation, their molecular mechanisms seem to be different. Therefore, the questions remain to be solved regarding how KDR binds and activates Gq11 and how activated Gq11 interacts and stimulates the tyrosine activity of KDR. The answer to the latter point may provide a common mechanism whereby Gq/11-coupled receptors transactivate KDR.

In the G protein-coupled receptor system, a ligand interacts with the receptor to stimulate G protein interaction with the ligand-bound receptors. The release of Gβγ subunits will activate Src kinase proteins, which, in turn, transactivate protein kinase receptors, such as epidermal growth factor receptor (38, 39). However, this mechanism does not apply to VPF/VEGF signaling pathways. Our data indicate that VPF/VEGF binds to KDR and induces the G protein to interact with KDR, which, in turn, stimulates KDR phosphorylation. However, the Gβγ minigene, hβARK1(495), does not inhibit KDR phosphorylation; rather, it inhibits intracellular Ca2+ mobilization and MAPK phosphorylation, which are required for VPF/VEGF-stimulated HUVEC proliferation (24, 33). Overexpression of hβARK1(495) also inhibits RhoA activation, which is required for VPF/VEGF-stimulated HUVEC migration (36). Therefore, there are two possibilities: 1) KDR phosphorylation is required for Gβγ dissociation from Gaα subunits, or 2) both Gβγ and KDR phosphorylation are required for activation of a downstream signaling molecule. The first assumption seems unlikely, because kinase-deficient receptor, EGDR(Y1059F), is still able to interact with Gq/11 proteins and mediate VPF/VEGF-stimulated HUVEC proliferation (33), and free Gβγ subunits are required for HUVEC migration (36). These data indicate that Gβγ subunits function without KDR phosphorylation to regulate HUVEC migration. It is known that, unlike bFGF, which directly binds and activates the Grb2-Sos-Ras-Raf-1-MAPK cascade, VPF/VEGF utilizes a pathway of PLC activation, intracellular Ca2+ mobilization, and MAPK phosphorylation (24, 33, 47). However, the mitogenic activity of VEGF is much weaker than bFGF (Fig. 1b), and the intracellular Ca2+ mobilization is much weaker responding to VPF/VEGF than to the ligands of the G protein-coupled receptor. Therefore, it is possible that neither released Gβγ nor KDR phosphorylation is enough to regulate the downstream signaling pathways, whereas both Gβγ and KDR phosphorylation are required to activate different PLC members to regulate intracellular Ca2+ mobilization. This hypothesis can be supported by the facts that 1) KDR phosphorylation is required for PLCγ phosphorylation (48); 2) a recent study show that leukotriene D4, a G protein-coupled receptor ligand, can stimulate association between Gβγ subunits, c-Src, and PLCγ; and 3) Gβγ-activated c-Src may phosphorylate and activate PLCγ (49). Therefore, our assumption is that maximal activation of PLCγ by VPF/VEGF and subsequent intracellular calcium mobilization in endothelial cells may require two components: KDR tyrosine phosphorylation and released Gβγ subunits. The future study will need to be carried out to further characterize how the Gq11 subunit, KDR, Gβγ subunits, and PLCγ interact with one another and characterize the role of each component in KDR signaling to cell proliferation and migration. Also, the Gq11-binding sites within the 1.8-kDa intracellular portion of KDR and the KDR-interacting sites within the Gq11 subunits will need to be identified.

In summary, our studies have identified a novel signaling pathway in which, upon VPF/VEGF binding, KDR interacts with and activates a G11α, family protein, resulting in KDR phosphorylation, MAPK activation, and cell proliferation. Released Gβγ subunits are not required for KDR phosphorylation but may function together with KDR phosphorylation to trigger downstream signaling pathways. Together with our previous reports that Gq/11 proteins are also required for KDR-mediated RhoA activation, which leads to HUVEC migration (36), and that pertussis toxin-sensitive G proteins are involved in Fli-1-mediated down-regulation of HUVEC proliferation (25), we conclude that KDR and Fli-1 employ heterotrimeric G proteins G11α and G11γ in their signaling pathways, respectively. This study not only significantly contributes to our understanding of the molecular mechanism of VPF/VEGF-induced vasculogenesis and angiogenesis but also provides a novel tool for cancer therapy by targeting the specific heterotrimeric G protein, G11α, to inhibit VPF/VEGF-induced angiogenesis.

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Heterotrimeric $\alpha_q/\alpha_{11}$ Proteins Function Upstream of Vascular Endothelial Growth Factor (VEGF) Receptor-2 (KDR) Phosphorylation in Vascular Permeability Factor/VEGF Signaling

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