Vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) promotes its function primarily by activating two receptor tyrosine kinases, Flt-1 (VEGFR-1) and KDR (VEGFR-2). Recently, it has been shown that KDR is responsible for VPF/VEGF-stimulated endothelial cell (EC) proliferation and migration, whereas Flt-1 activation down-modulates KDR-mediated EC proliferation. Although KDR-mediated EC proliferation and migration have been extensively studied, much less is known about Flt-1-mediated antiproliferation. Here, we demonstrate that Flt-1-mediated antiproliferative activity can be blocked completely by the dominant negative mutant of CDC42 (CDC42-17N) and partially by a Rac1 dominant negative mutant (Rac1-17N) but is not affected by a RhoA dominant negative mutant (RhoA-19N). Both CDC42-17N and Rac1-17N increase the intracellular Ca\(^{2+}\) mobilization in response to VPF/VEGF but have no effect on KDR and MAPK phosphorylation. Using the chimeric-receptor EGLT in which the extracellular domain of epidermal growth factor receptor was fused to the transmembrane and intracellular domains of Flt-1, we also demonstrate that CDC42 and Rac1 are activated by EGLT. Previously, we showed that phosphatidylinositol 3-kinase is required for Flt-1-mediated antiproliferative activity, but phospholipase C is not required. As expected, CDC42 and Rac1 activation mediated by EGLT can be completely inhibited by PI3K inhibitors, wortmannin and LY294002, and the p85 dominant negative mutant but not by either the phospholipase C inhibitor, U73122, or a intracellular Ca\(^{2+}\) chlilor BAPTA/AM. Surprisingly, pertussis toxin and overexpression of the free G\(\gamma\)-specific sequestering minigene h\(\gamma\)ARK1(495) also inhibit EGLT-mediated CDC42 and Rac1 activation completely. Moreover, pertussis toxin treatment also increases the intracellular Ca\(^{2+}\) mobilization and inhibits the antiproliferative activity, thus suggesting that pertussis toxin-sensitive G proteins and the G\(\beta\)\(\gamma\) subunits are involved in the signaling pathway of Flt-1 that down-regulates EC proliferation. Taken together, these results further expand our understanding of Flt-1-mediated antiproliferative activity in VPF/VEGF-stimulated endothelium.

VPF/VEGF\(^1\) is a multifunctional cytokine that is required for angiogenesis as well as vasculogenesis (1). It stimulates endothelial cell (EC) proliferation and migration and increases microvascular permeability (1–4). VPF/VEGF functions are mediated for the most part by two receptor tyrosine kinases, Flt-1 (VEGFR-1) and KDR (VEGFR-2) (2–6). A large body of work has demonstrated that KDR instead of Flt-1 is responsible for VPF/VEGF-stimulated proliferation and migration of endothelial cells, HUVEC (7–11). Interestingly, Rahimi et al. (10) and our laboratory show that one of the Flt-1 functions was to down-modulate KDR-mediated proliferation using two different VPF/VEGF receptor chimeric fusion systems (11). Our results further showed that the antiproliferative activity of Flt-1 was mediated through phosphotidylinositol-3 kinase (PI3K), and that this activity was correlated with its effect on the intracellular calcium mobilization in VPF/VEGF-stimulated HUVEC (11). Barleon et al. (1) show that pretreatment with pertussis toxin inhibited VPF/VEGF-stimulated macrophage migration. Based on the fact that macrophages express Flt-1 but not KDR, it was suggested that pertussis toxin-sensitive G proteins were involved in Flt-1-mediated macrophage migration in response to VPF/VEGF (1). Because Flt-1 functions to down-modulate KDR-stimulated HUVEC proliferation instead of inducing HUVEC migration (11), the involvement of pertussis toxin-sensitive G proteins in Flt-1-mediated antiproliferative activity remains to be examined.

The Rho family of the small GTPase superfamily has been shown to play an important role in cell growth, migration, transformation, and gene expression (12). The Rho family includes Rho (RhoA, RhoB, RhoC, RhoE, and RhoG), Rac (Rac1, Rac2, Rac3, and RhoG), CDC42 (CDC42Hs, G25K, and TC10), nud (RhoE/Rnd3, Rnd1/Rho6, and Rnd2/Rho7), RhoD, and TTF (12). Among them, RhoA, Rac1, and CDC42 are the most extensively studied members of this family. RhoA primarily induces the formation of stress fibers, whereas Rac1 and CDC42 promote the formation of lamellipodia and filopodia, respectively, when they are expressed in cells (13, 14). It was reported that VPF/VEGF induces actin-based mobility (15), suggesting that Rho family proteins might be involved in this response. However, there is no evidence of Rho family proteins playing a role in VPF/VEGF-induced cellular responses and whether VPF/VEGF activates these GTPases.

In this study, we examined whether Rho GTPases are involved in the antiproliferative function of Flt-1. We found that

\(^{1}\) The abbreviations used are: VPF/VEGF, vascular permeability factor/vascular endothelial growth factor; EC, endothelial cells; HUVEC, human umbilical vein endothelial cells; Flt-1, VEGFR-1; CDC42, RhoA, Rac1, CDC42, and Rac1 dominate negative mutant; PI3K, phosphatidylinositol-3 kinase; PMSF, phenylmethylsulfonyl fluoride; EGF, epidermal growth factor; GST, glutathione S-transferase; FMLP, formylmethionylleucylphosphylalanine.
a blockade of endogenous functions of CDC42 and Rac1 but not RhoA by their respective dominant negative mutants (CDC42-17N, Rac1-17N, and RhoA-19N) increases VPF/VEGF-induced HUVEC proliferation, indicating that CDC42 and Rac1 are involved in Fli-1-antiproliferative effect. In addition, overexpression of CDC42-17N and Rac1-17N increases VPF/VEGF-stimulated intracellular calcium release but not KDR and MAPK phosphorylation. Our results further demonstrate that Fli-1-stimulation activates CDC42 and Rac1, and that the activation of CDC42 and Rac1 is mediated through PI3K, free Gβγ subunits, and pertussis toxin-sensitive G proteins. Furthermore, the pretreatment with pertussis toxin increases VPF/VEGF-induced calcium mobilization and proliferation. Moreover, our data also indicate that there are two pathways for CDC42 activation, Rac1-dependent and Rac1-independent. The inhibition of the Rac1-dependent pathway by Rac1-17N partially inhibits CDC42 activation, resulting in partial inhibition of antiproliferation, but the inhibition of CDC42 completely prevented antiproliferation in VPF/VEGF-stimulated HUVEC.

EXPERIMENTAL PROCEDURES

Materials—Recombinant VPF/VEGF was obtained from R&D Systems (Minneapolis, MN). EGM-MV BulletKit, trypsin-EDTA, and trypsin neutralization solution were obtained from Clonetics (San Diego, CA). Vitrogen 100 was purchased from Collagen Biotmaterials (Palo Alto, CA). Mouse monoclonal antibodies against the KDR C-terminal domain and rabbit polyclonal antibodies against CDC42 and Rac1 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). [3H]Thymidine was obtained from PerkinElmer Life Sciences. Fura-2 AM and Phoronic F-127 were obtained from Molecular Probes (Eugene, Oregon). Pertussis toxin was obtained from Calbiochem.

Cell Culture—Primary HUVEC were obtained from Clonetics. Cells were grown on plates coated with 30 μg/ml vitrogen in EGM-MV BulletKit (5% fetal bovine serum in endothelial basal medium with 12 μg/ml bovine brain extract, 1 μg/ml hyrdroxortisone, 1 μg/ml GA-1000, and human EGF). HUVEC transduced with EGLT were grown to 80% confluence were used for most experiments. Cells were serum-starved (0.1% serum) for 24 h and then stimulated with 10 ng/ml of VPF/VEGF or EGF at different time intervals. Stimulation was stopped by the addition of ice-cold PBS. Cells were washed with PBS three times and lysed with lysis buffer (150 μM NaCl, 0.8 mM MgCl2, 5 μg/ml EGTa, 1% IGEPAL, 50 μM HEPES, pH 7.5, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Cell lysates were centrifuged at 14,000 rpm for 3 min. The supernatant was incubated with 50 μl of GST-Pak-CRIB beads at 4 °C for 45 min. Protein bound to beads was washed three times with AP wash buffer (50 mM Tris-HCl, pH 7.2, 1% Triton X-100, 150 mM NaCl, 10m M MgCl2, 1 μM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) and analyzed by SDS-PAGE with antibodies against CDC42 or Rac1 as indicated. For inhibitor experiments, different concentrations of inhibitors as indicated and VPF/VEGF were added 30 min before treatment.

Overexpression of Proteins in HUVEC—CDC42-17N, Rac1-17N, and RhoA-19N were kindly provided by Margaret M. Chou (University of Pennsylvania, Philadelphia, PA). These data indicate that CDC42 and Rac1 are involved in VPF/VEGF-mediated Signaling

Effect of the Dominant Negative Mutants of CDC42, Rac1, and RhoA on VPF/VEGF-stimulated HUVEC Proliferation—To examine whether the Rho family proteins CDC42, Rac1, and RhoA are involved in VPF/VEGF-stimulated HUVEC proliferation, we overexpressed the dominant negative mutants of CDC42 (CDC42-17N, Rac1-17N), or RhoA (RhoA-19N) using a recently developed retroviral gene-delivery system, which demonstrated an almost 100% infection yield in HUVEC to block the function of endogenous CDC42, Rac1, or RhoA (11, 17). HUVEC transduced with LacZ as a control, CDC42-17N, Rac1-17N, or RhoA-19N were serum-starved and stimulated with 10 ng/ml of VPF/VEGF. Proliferation assay was measured by the incorporation of [3H]thymidine. Data are expressed as fold increase as compared with the non-stimulated control. Surprisingly, as shown in Fig. 1, the overexpression of CDC42-17N and Rac1-17N increases VPF/VEGF-stimulated [3H]thymidine incorporation by 50 and 25%, respectively, as compared with LacZ-expressed cells stimulated with VPF/VEGF. However, [3H]thymidine incorporation is not affected in VPF/VEGF-stimulated HUVEC transduced with RhoA-19N. The different effects of CDC42-17N, Rac1-17N, and RhoA-19N are not attributed to the different levels of expression, because same amount of overexpressed CDC42, Rac1, and RhoA are detected by Western blot analysis (data not shown). These data indicate that CDC42 and Rac1 are involved in

RESULTS
Flt-1-mediated antiproliferative effect in VPF/VEGF-stimulated HUVEC.

Effect of CDC42-17N and Rac1-17N on VPF/VEGF-induced Activation of Intracellular Signaling Molecules—It is known that VPF/VEGF stimulates KDR phosphorylation, intracellular Ca\(^{2+}\) mobilization, and MAPK phosphorylation in EC (7–11). Therefore, we tested whether CDC42 and Rac1 play any role in the activation of these signaling molecules. Serum-starved HUVEC transduced with LacZ, CDC42-17N, and Rac1-17N were stimulated with VPF/VEGF for 0, 1, 5, and 10 min. Cellular extracts were immunoprecipitated with an antibody against KDR and immunoblotted with an antibody against phosphotyrosine. Fig. 2a clearly indicates that neither CDC42-17N nor Rac1 has any effect on KDR phosphorylation. Surprisingly, in a VPF/VEGF-stimulated intracellular Ca\(^{2+}\) mobilization assay, the kinetic slope and the maximum response of intracellular Ca\(^{2+}\) mobilization was increased in HUVEC transduced with CDC42-17N compared to that of those transduced with LacZ (Fig. 2b). The overexpression of Rac1-17N also increases the kinetic slope and maximum response of intracellular Ca\(^{2+}\) mobilization, however, the effects of Rac1-17N are less than that of CDC42-17N (Fig. 2b). However, MAPK was phosphorylated at the same level in HUVEC transduced with LacZ, CDC42-17N, or Rac1 after treatment with VPF/VEGF for different time intervals (Fig. 2c).

Flt-1 Mediates Activation of both CDC42 and Rac1—Because the dominant negative mutants of CDC42 and Rac1 inhibit VPF/VEGF-stimulated HUVEC proliferation and intracellular Ca\(^{2+}\) mobilization, we tested whether VPF/VEGF directly activates CDC42 and Rac1. The activity of CDC42 and Rac1 was measured using a pull-down assay of GST-Pak-CRIB fusion proteins that bound only to the GTP-bound forms of CDC42 and Rac1 to indicate a similar concentration of CDC42 and Rac1 in each sample (panel B). Cellular extracts were immunoprecipitated with an antibody against phosphorylated MAPK (P-MAPK) (panel A). Cellular extracts were immunoprecipitated with an antibody against KDR and immunoblotted with an antibody against phosphotyrosine (pTyr) (panel A). The blot was stripped and reprobed with an antibody against KDR to confirm equal protein loading (panel B).

Fig. 1. Effect of dominant negative mutants of CDC42-17N, Rac1-17N, and RhoA-19N on VPF/VEGF-stimulated HUVEC proliferation. HUVEC transduced with LacZ, CDC42-17N, Rac1-17N, and RhoA-19N were serum-starved for 24 h and stimulated with 10 ng/ml of VPF/VEGF for 20 h. [\(^{3}H\)]Thymidine was pulsed for an additional 4 h. Cells were washed, lysed, and precipitated as described under “Experimental Procedures” (n = 4).

Fig. 2. Effect of dominant negative mutants of CDC42-17N and Rac1-17N on signaling molecule activation in VPF/VEGF-stimulated HUVEC. a, KDR phosphorylation. Serum-starved HUVEC transduced with LacZ, CDC42-17N, and RhoA-19N were stimulated with 10 ng/ml VPF/VEGF for 0, 1, 5, and 10 min. Cellular extracts were immunoprecipitated with an antibody against KDR and immunoblotted with an antibody against phosphotyrosine (pTyr) (panel A). The blot was stripped and reprobed with an antibody against KDR to confirm equal protein loading (panel B). b, intracellular Ca\(^{2+}\) mobilization assay. Serum-starved HUVEC transduced with LacZ, CDC42-17N, and Rac1-17N were stimulated with 10 ng/ml of VPF/VEGF. c, phosphorylation of MAPK. Serum-starved HUVEC transduced with LacZ, CDC42-17N, and Rac1-17N were stimulated with 10 ng/ml of VPF/VEGF for 0, 1, 5, and 10 min. Cellular extracts were immunoprecipitated with an antibody against phosphorylated MAPK (P-MAPK) (panel A). Cellular extracts were immunoprecipitated with an antibody against MAPK to confirm equal protein loading (panel B).

Fig. 3. VPF/VEGF-stimulated activation of CDC42 in HUVEC. As described under “Experimental Procedures,” serum-starved HUVEC were stimulated with VPF/VEGF for different lengths of time as indicated. Cellular extracts were incubated with GST-Pak-CRIB beads. The bound proteins (activated form of CDC42 or Rac1) were analyzed with SDS-PAGE and immunoblotted with antibodies against CDC42 and Rac1 as indicated (panel A). Cellular extracts were immunoprecipitated with the CDC42 or Rac1 to indicate a similar concentration of CDC42 and Rac1 in each sample (panel B).

PI3K Mediates CDC42 and Rac1 Activation—To identify the
signaling molecules that mediate CDC42 and Rac1 activation, EGLT-expressing HUVEC were pretreated with an inhibitor of the phospholipase C family (U73122), PI3K inhibitors (wortmannin and LY294002), or BAPTA/AM, an intracellular Ca\(^{2+}\)/H\(^{+}\) chelator, for 5 min and treated with EGF for 1 min. Cellular extracts were used to measure the activation of CDC42 and Rac1. The data show that wortmannin and LY294002 completely inhibited both CDC42 and Rac1 activation, but neither U73122 nor BAPTA/AM inhibited CDC42 and Rac1 activation in EGF-stimulated EGLT/HUVEC (Fig. 5a). To further confirm that PI3K is required for EGF-stimulated CDC42 and Rac1 activation in EGLT-transduced HUVEC, HUVEC were transduced with EGLT together with LacZ as control or a dominant negative mutant of PI3K, p85DN. Serum-starved cells were stimulated with EGF, and cellular extracts were subjected to CDC42 and Rac1 activation assay. As shown in Fig. 5b, p85DN completely inhibited EGF-stimulated EGLT-mediated CDC42 and Rac1 activation. These results indicate that PI3K is a key mediator of EGLT-mediated CDC42 and Rac1 activation.

Pertussis Toxin-sensitive G proteins and G\(\alpha\)\(\gamma\) Subunits Mediate CDC42 and Rac1 Activation—It has been shown that pertussis toxin inhibited Flt-1-mediated microphage migration stimulated by VPF/VEGF (1) and suggested that pertussis toxin-sensitive G proteins may participate in Flt-1 signaling. Therefore, we examined whether pertussis toxin has any effect on CDC42 and Rac1 activation mediated by EGLT. Serum-starved HUVEC transduced with EGLT were pretreated with pertussis toxin overnight followed by stimulation with EGF for 1 min. Cellular extracts were subjected to CDC42 and Rac1 activation mediated by EGF. These data suggest that pertussis toxin-sensitive G proteins are involved in EGLT-mediated CDC42 and Rac1 activation. It is known that after activation heterotrimeric G proteins disso-

\[ \text{FIG. 5. Effect of different inhibitors on CDC42 and Rac1 activation in EGLT/HUVEC-stimulated by EGF.} \]
a. serum-starved EGLT-transduced HUVEC were incubated with a different concentration of inhibitors for 5 min as indicated before the addition of 10 ng/ml of EGF for 1 min. Proteins bound on GST-Pak-CRIB beads (panel A) and cellular extracts (panel B) were analyzed with antibodies against CDC42 (a, panel A) and Rac1 (b, panel A). a and b, panel B, cellular extracts were immunoblotted with antibodies against CDC42 or Rac1 to indicate a similar concentration of CDC42 and Rac1 in each sample.

\[ \text{FIG. 4. EGLT-mediated activation of CDC42 in HUVEC.} \]
As described under “Experimental Procedures,” serum-starved HUVEC transduced with LacZ or EGLT were stimulated with EGF for different lengths of time as indicated. Cellular extracts were incubated with GST-Pak-CRIB beads. The bound proteins (activated form of CDC42 or Rac1) were analyzed with SDS-PAGE and immunoblotted with antibodies against CDC42 (a, panel A) and Rac1 (b, panel A). a and b, panel B, cellular extracts were immunoblotted with antibodies against CDC42 or Rac1 to indicate a similar concentration of CDC42 and Rac1 in each sample.
Gβγ subunits are required for CDC42 and Rac1 activation, we overexpressed the hARK1(495) peptide to sequester free Gβγ. hβARK1(495) corresponds to the C-terminal domain of human βARK1 that physically interacts with free Gβγ and therefore acts as a specific intracellular Gβγ antagonist inhibiting Gβγ-mediated downstream events (22, 23). HUVEC were transduced with EGLT-expressing viruses together with LacZ- or hβARK1(495)-expressing viruses. The transduced cells were then stimulated with EGF for 1 min. Cellular extracts were subjected to a CDC42 or Rac1 activation assay. The data show that overexpression of hARK1(495) completely inhibits the activation of CDC42 and Rac1 (Fig. 7), indicating that Gβγ subunits are required for Flt-1-mediated CDC42 and Rac1 activation.

Rac1-17N Partially Inhibited CDC42 Activation—Currently, our data indicate that both CDC42 and Rac1 activation are regulated by pertussis toxin-sensitive G proteins, free Gβγ subunits, and PI3K but not by phospholipase C or intracellular Ca2+. However, the effect of CDC42-17N on VPF/VEGF-stimulated HUVEC proliferation and intracellular Ca2+ mobilization is almost twice that of Rac1-17N (Figs. 1 and 2b). Therefore, we examined whether there was any cross-talk between CDC42 and Rac1. ECLT/HUVEC transduced with LacZ or Rac1-17N was stimulated with EGF for 1 min. Cellular extracts were subjected to a CDC42 activation assay. As shown in Fig. 8a, Rac1-17N partially inhibits CDC42 activation. However, when ECLT/HUVEC transduced with CDC42-17N was stimulated with EGF and cellular extracts were subjected to Rac1 activation assay, CDC42-17N had no effect on Rac1 activation (Fig. 8b). These data indicate that Flt-1-stimulated CDC42 activation is mediated by Rac1-dependent as well as independent pathways.

Effect of Pertussis Toxin on VPF/VEGF-induced Intracellular Ca2+ Mobilization and EC Proliferation—Next, we tested whether pertussis toxin has any effect on Flt-1-stimulated HUVEC proliferation and intracellular calcium mobilization in response to VPF/VEGF. Serum-starved HUVEC were pretreated with 100 ng/ml of pertussis toxin for 16 h and stimulated with 10 ng/ml of VEGF/VEGF. The data show that pretreatment with pertussis toxin increases the kinetic slope and the maximum response of VEGF/VEGF-induced intracellular Ca2+ mobilization (Fig. 9a). We then examined the effect of pertussis toxin on Flt-1-mediated down-regulation of HUVEC proliferation. Serum-starved HUVEC were pretreated with 100 ng/ml of pertussis toxin for 16 h and stimulated with 10 ng/ml of VPF/VEGF for 24 h. As expected, the pretreatment with pertussis toxin increases the rate of VPF/VEGF-induced HUVEC proliferation (Fig. 9b) at the same rate as that in HUVEC-transduced CDC42-17N (Fig. 1a) and p85DN (11).

**DISCUSSION**

It is known that KDR mediates cell proliferation and migration of EC in response to VPF/VEGF (7–11), whereas Flt-1 down-modulates KDR-mediated cell proliferation (10, 11). Recently, with the chimeric receptors EGDR and ECLT, in which the extracellular domains were replaced with that of epidermal growth factor receptor, respectively, we found that ECLT co-transduction inhibited EGDR-stimulated HUVEC proliferation in response to EGF of up to 50% (11), and that both the inhibition of PI3K activity by wortmannin and p85 dominant negative mutant (11) and the inhibition of Flt-1 function with an antibody against Flt-1 increased the proliferation rate of approximately 50%. In this study, we show that VPF/VEGF-induced HUVEC proliferation is increased approximately 50 and 25% in CDC42-17N- and Rac1-17N-transduced cells, respectively. These data clearly indicate that CDC42-17N completely inhibits Flt-1-mediated HUVEC antiproliferative activity, but Rac1-17N partially inhibits this activity.

Whereas the downstream pathway of CDC42 and Rac1 regulation of actin rearrangement has been extensively studied (24–26), much less is known about how CDC42 and Rac1 are activated (27). fMLP has been reported to activate CDC42 through a pertussis toxin-sensitive pathway (28), whereas cytokines tumor necrosis factor-α and interleukin-1 activate CDC42 through c-Jun N-terminal kinase in some cells (29–32).
but not in others (33, 34). Recently, Jimenez et al. (35) reported that platelet-derived growth factor-stimulated CDC42 activation is independent of PI3K enzymatic activity but is dependent on the p85 regulatory subunit of PI3K. In this study, with the results of a pull-down assay we show that VPF/VEGF activates CDC42 and Rac1 in HUVEC, and that this activation is mediated by EGLT (i.e., Flt-1). Furthermore, CDC42 and Rac1 activation through EGLT (Flt-1) can be inhibited by PI3K inhibitors (wortmannin and LY294002) and p85DN, but not by U73122 nor by intracellular calcium chelator BAPTA/AM. Taken together, our findings demonstrate that Flt-1-stimulated PI3K is upstream of CDC42 and Rac1 activation that modulates KDR-stimulated intracellular calcium mobilization and cell proliferation.

It was reported that pertussis toxin inhibited VPF/VEGF-stimulated microphase migration (1). Because microphages express Flt-1 and not KDR, it was suggested that the Gi family proteins might play a role in Flt-1-mediated signaling (1). Our result that pretreatment with pertussis toxin inhibits EGLT-mediated CDC42 and Rac1 activation further confirms the involvement of Gi family proteins in Flt-1 signaling. Pretreatment with pertussis toxin also increased the kinetic slope and maximum response of VPF/VEGF-stimulated intracellular Ca$^{2+}$ mobilization in a similar way to the effect of overexpression of dominant negative mutants of CDC42 or PI3K (11) in the intracellular Ca$^{2+}$ response was measured as described under “Experimental Procedures.” b, serum-starved HUVEC with or without pretreatment of 100 ng/ml pertussis toxin were stimulated with 10 ng/ml VPF/VEGF, and the intracellular Ca$^{2+}$ response was measured as described under “Experimental Procedures.” n = 4.

**Fig. 9.** Effect of pertussis toxin on intracellular Ca$^{2+}$ mobilization and cell proliferation in HUVEC stimulated with VPF/VEGF. a, serum-starved HUVEC with or without pretreatment of 100 ng/ml pertussis toxin were stimulated with 10 ng/ml VPF/VEGF, and the intracellular Ca$^{2+}$ response was measured as described under “Experimental Procedures.” b, serum-starved HUVEC with or without pretreatment of 100 ng/ml pertussis toxin were stimulated with 10 ng/ml VPF/VEGF for 20 h. Proliferation assay was carried out as described under “Experimental Procedures” (n = 4).

antiproliferative effect of Rac1 is mediated through its effect on CDC42 activation. This also correlates with the effect of Rac1-17N and CDC42-17N in VPF/VEGF-stimulated HUVEC proliferation and intracellular Ca$^{2+}$ mobilization. Meanwhile, these data suggest that VPF/VEGF-induced CDC42 activation can be mediated through Rac1-dependent as well as independent pathways. Our results have demonstrated that both CDC42 and Rac1 activation in response to VPF/VEGF requires pertussis toxin-sensitive G proteins, free Gβγ subunits, and PI3K activation, indicating that VPF/VEGF-induced Rac1 and CDC42 activation is mediated by a common upstream signaling event. Although it has been shown that PI3K binds to Rac1 and CDC42 (27), it is not clear how PI3K regulates Rac1 and CDC42 activity and how Rac1 triggers CDC42 activation.

In summary, the current study demonstrates that CDC42 and Rac1 rather than RhoA mediate the antiproliferative effect of Flt-1. The effect of CDC42 and Rac1 is through reducing VPF/VEGF-induced intracellular calcium mobilization but not KDR and MAPK phosphorylation. Our results further demonstrate that Flt-1 stimulation activates CDC42 and Rac1, and that the activation of CDC42 and Rac1 is mediated through PI3K, free Gβγ subunits, and pertussis toxin-sensitive G proteins. Furthermore, pretreatment with pertussis toxin increases VPF/VEGF-induced calcium mobilization and proliferation. Together, our results extend the pathway that mediates Flt-1 down-modulated VPF/VEGF-induced HUVEC proliferation. The findings in this study significantly contribute to our understanding of how Flt-1 functions as an antiproliferative modulator during VPF/VEGF-induced vasculogenesis and angiogenesis.

**REFERENCES**

1. Barleon, B., Sozzani, S., Zhou, D., Weich, H. A., Mantovani, A., and Marone, D. (1996) **Blood** 87, 3336–3343

2. Millauer, B., Witzgamm-Voss, S., Schnurch, H., Martinez, R., Mellor, N. P. H., Reiss, W., and Ullrich, A. (1993) **Cell** 72, 835–846

3. Fong, G. H., Rossant, J., Gertsenstein, M., and Breitman, M. L. (1995) **Nature** 376, 66–70

4. Shalaby, F., Ha, J., Stanford, W. L., Fischer, K. D., Schuch, A. C., Schwartz, L., Bernstein, A., and Rossant, J. (1997) **Cell** 89, 981–990

5. Quinn, T. P., Peters, K. G., De Vries, C., Ferrara, N., and Williams, L. T. (1993) **Proc. Natl. Acad. Sci. U. S. A.** 90, 7535–7537

6. Terman, B., Dougher-Vermazen, M., Carrion, M., Dimitrov, D., Arnellino, D., Gospodarowicz, D., and Bohlen, P. (1992) **Biochem. Biophys. Res. Commun.** 187, 1579–1586

7. Walehnerger, J., Claessian-Welch, L., Sieghahn, A., Shibuya, M., and Heldin, C. H. (1994) **J. Biol. Chem.** 269, 26988–26995

8. Bernatchez, P. N., Soker, S., and Sirios, M. G. (1999) **J. Biol. Chem.** 274, 31047–31054

9. Gille, H., Kowalski, J., Li, B., LeCourtier, J., Moffat, B., Ziecheck, T. F., Pelletier, N., and Ferrara, N. (2001) **J. Biol. Chem.** 276, 3232–3230

10. Rahimi, N., Dayanir, V., and Lashkari, K. (2000) **J. Biol. Chem.** 275, 16986–16992

11. Zeng, H., Dvorak, F., and Mukhopadhyay, D. (2001) **J. Biol. Chem.** 276, 26969–26979

12. Aspenson, P. (1999) **Curr. Opin. Cell Biol.** 11, 95–102

13. Hall, A. (1998) **Science** 279, 509–514

14. Machesky, L. M., and Insall, R. H. (1999) **J. Biol. Chem.** 274, 22731–22737

15. Hamm, H. E. (1998) **Science** 280, 179–180

16. Zhao, D., Keates, A. C., Kuhnt-Moore, S., Moyer, M. P., Kelly, C. P., and Risau, W., and Ullrich, A. (1993) **J. Biol. Chem.** 269, 22731–22737

17. Koch, W. J., Hawes, B. E., Inglese, J., Luttrel, L. M., and Lefkowitz, R. J. (2000) **Biochem. Biophys. Res. Commun.** 274, 32714–32719

18. Ren, X. D., Klosses, W. B., and Schwartz, M. A. (1999) **EMBO J.** 18, 578–585

19. Bagrodia, S., Taylor, S. J., Creasy, C. L., Chernoff, J., and Cerione, R. A. (1995) **J. Biol. Chem.** 270, 22731–22737

20. Gilman, A. G. (1987) **Ann. Rev. Biochem.** 56, 615–649

21. Hamm, H. E. (1999) **J. Biol. Chem.** 274, 669–672

22. Ackert, S. A., Luttrell, L. M., Rockman, H. A., Iaccarino, G., Lefkowitz, R. J., and Koch, W. J. (1998) **Science** 280, 574–577

23. Koch, W. J., Hawes, B. E., Inglese, J., Luttrell, L. M., and Lefkowitz, R. J. (1994) **J. Biol. Chem.** 269, 6193–6197

24. Matsuzaki, T., Nakashishi, H., and Takai, Y. (2000) **Cell. Signalling** 12, 515–524

25. Evers, E. E., Zondag, G. C., Malliri, A., Price, L. S., ten Klooster, J. P., van der Kamen, R. A., and Collard, J. G. (2000) **Eur. J. Cancer** 36, 1269–1274

26. Bishop, A. L., and Hall, A. (2000) **Biochem. J.** 348, 241–255

27. Kjoller, L., and Hall, A. (1999) **Exp. Cell Res.** 253, 166–179

28. Benard, V., Bohl, B. P., and Bokoch, G. M. (1999) **J. Biol. Chem.** 274, 13196–13204
29. Kieser, A., Kaiser, C., and Hammerschmidt, W. (1999) *EMBO J.* 18, 2511–2521
30. Coso, O. A., Chiaruoli, M., Yu, J. C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gukkind, J. S. (1995) *Cell* 81, 1137–1146
31. Bagrodia, S., Derijard, B., Davis, R. J., and Cerione, R. A. (1995) *J. Biol. Chem.* 270, 27995–27998
32. Auer, K. L., Contessa, J., Brenz-Verca, S., Pirola, L., Rusconi, S., Cooper, G., Aba, A., Wymann, M. P., Davis, R. J., Birrer, M., and Dent, P. (1998) *Mol. Biol. Cell* 9, 561–573
33. Puls, A., Eliopoulos, A. G., Nobes, C. D., Bridges, T., Young, L. S., and Hall, A. (1999) *J. Cell Sci.* 112, 2983–2992
34. Davis, W., Stephens, L. R., Hawkins, P. T., and Saklatvala, J. (1999) *Biochem. J.* 338, 387–392
35. Jimenez, C., Portela, R. A., Mellado, M., Rodriguez-Frade, J. M., Collard, J., Serrano, A., Martinez, A. C., Avila, J., and Carrera, A. C. (2000) *J. Cell Biol.* 151, 249–262
36. Servant, G., Weiner, O. D., Herzmark, P., Balla, T., Sedat, J. W., and Bourne, H. R. (2000) *Science* 287, 1037–1040
37. Hirsch, R., Katanaev, V. L., Garlanda, C., Azzolino, O., Pirola, L., Silengo, L., Sozzani, S., Mantovani, A., Altruda, F., and Wymann, M. P. (2000) *Science* 287, 1049–1053
38. Li, Z., Jiang, H., Xie, W., Zhang, Z., Smrcka, A. V., and Wu, D. (2000) *Science* 287, 1046–1049
39. Sasaki, T., Irie-Sasaki, J., Jones, R. G., Oliveira-dos-Santos, A. J., Stanford, W. L., Bolon, B., Wakeham, A., Itie, A., Bouchard, D., Koziardzki, I., Jez, N., Mak, T. W., Ohashi, P. S., Suzuki, A., and Penninger, J. M. (2000) *Science* 287, 1040–1046
Flt-1-mediated Down-regulation of Endothelial Cell Proliferation through Pertussis Toxin-sensitive G Proteins, βγ Subunits, Small GTPase CDC42, and Partly by Rac-1
Huiyan Zeng, Dezheng Zhao and Debabrata Mukhopadhyay

J. Biol. Chem. 2002, 277:4003-4009.
doi: 10.1074/jbc.M110842200 originally published online November 28, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M110842200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 39 references, 28 of which can be accessed free at http://www.jbc.org/content/277/6/4003.full.html#ref-list-1