Distinct role of PLCβ3 in VEGF-mediated directional migration and vascular sprouting

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Summary

Endothelial cell proliferation and migration is essential to angiogenesis. Typically, proliferation and chemotaxis of endothelial cells is driven by growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). VEGF activates phospholipases (PLCs) – specifically PLCγ1 – that are important for tubulogenesis, differentiation and DNA synthesis. However, we show here that VEGF, specifically through VEGFR2, induces phosphorylation of two serine residues on PLCβ3, and this was confirmed in an ex vivo embryoid body model. Knockdown of PLCβ3 in HUVEC cells affects IP3 production, actin reorganization, migration and proliferation; whereas migration is inhibited, proliferation is enhanced. Our data suggest that enhanced proliferation is precipitated by an accelerated cell cycle, and decreased migration by an inability to activate CDC42. Given that PLCβ3 is typically known as an effector of heterotrimeric G-proteins, our data demonstrate a unique crosstalk between the G-protein and receptor tyrosine kinase (RTK) axes and reveal a novel molecular mechanism of VEGF signaling and, thus, angiogenesis.

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Key words: Migration, Proliferation, Endothelial signaling, PLCβ3

Introduction

Endothelial cell proliferation and migration are essential for angiogenesis. Typically, proliferation and chemotaxis of endothelial cells is driven by growth factors such as vascular endothelial growth factor (VEGF; also known as vascular permeability factor, VPF) and basic fibroblast growth factor (bFGF) (Kanda et al., 2004; Kanda et al., 2006; Matsumoto et al., 2005; Zeng et al., 2001b; Zeng et al., 2002).

VEGF is a multifunctional cytokine with important roles in both vasculogenesis and angiogenesis (Dvorak et al., 1979; Guidi et al., 1995; Koch et al., 1994; Senger et al., 1983; Senger et al., 1996). The activities of VEGF are mediated primarily by its interaction with two high-affinity receptor tyrosine kinases (RTKs), VEGFR2 (KDR in humans, Flk-1 in mice) and VEGFR1 (Flt-1), which selectively but not exclusively expressed on vascular endothelium. They are also expressed by a variety of tumor cells and tumor endothelial cells (Hamlyn et al., 1991; Terman et al., 1992; Vaisman et al., 1990). Moreover, neuropilin has recently been identified as a VEGF-binding protein and has been shown to function as a co-receptor for VEGFR2 (Soker et al., 2002; Soker et al., 1998). VEGF induces a cascade of phosphorylation reactions, beginning with autophosphorylation of both receptors on tyrosine (Risau, 1997). Subsequent steps in the signaling cascade are only partially understood, but VEGF is known to induce increased intracellular calcium ($[Ca^{2+}]_i$), accumulation of inositol-1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG), activation of protein kinase C (PKC), and tyrosine phosphorylation of both phospholipase Cγ (PLCγ) and phosphatidylinositol 3-kinase (PI3K) (Brock et al., 1991).

We have previously shown that Gq/11 and Gβγ proteins are required for VEGF-stimulated intracellular Ca^{2+} mobilization as well as migration and proliferation of human umbilical vein endothelial cells (HUVECs) (Mukhopadhyay and Zeng, 2002; Zeng et al., 2003). Recently, others have also shown that specific Gβγ protein knockdown in zebrafish causes disruption of VEGF-induced angiogenesis and a vascular phenotype (Leung et al., 2006). Moreover, we have demonstrated that of the PLC isoforms expressed in endothelial cells, VEGF is able to induce a significant increase in the catalytic PIP2 hydrolysis activities of only PLCβ3 and PLCγ1 (Mukhopadhyay and Zeng, 2002). PLCγ1 has been rigorously studied, and is involved in VEGF-induced DNA synthesis (Mukhopadhyay and Zeng, 2002; Takahashi et al., 2001). By contrast, the role of PLCβ3 in endothelial signaling and its physiological significance in VEGF biology remains largely undetermined.

PLC hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2) to generate IP3 and DAG, which are implicated in the mobilization of $[Ca^{2+}]_i$ and PKC activation, respectively (Rhee and Bae, 1997). PLCβ3 contains a PH domain in the N-terminal region, followed by an EF-hand domain. Following this are the X and Y regions, which are tightly associated and separated by a short sequence of 50-70 amino acids. Near the C-terminus, a Ca^{2+}-dependent C2 domain is present (Rhee and Bae, 1997). The α subunits (αq, α11, α14 and α16) of all four members of the Gq subfamilies of heterotrimeric G-proteins activate PLCβ isozymes but not PLCγ1 or PLCδ1 (Berridge, 1993; Cockcroft and Thomas, 1992; Noh et al., 1995; Rhee and Choi, 1992; Sternweis and Smrcka, 1992). The Gβγ dimer also activates PLCβ isozymes (Berridge, 1993; Cockcroft

and Thomas, 1992; Nakamura et al., 1995; Noh et al., 1995; Rhee and Choi, 1992). The receptors that activate this Gαq-PLCβ pathway include those for thromboxaneA2, bradykinin, bombesin, angiotensin II, histamine, vasopressin, acetylcholine (muscarinic m1 and m3), a1-adrenergic agonists, thyroid-stimulating hormone, C-C and C-*X*-C chemokines and endothelin-1 (Noh et al., 1995).

As described earlier, our previous studies indicate that VEGF mediates endothelial cell proliferation and migration through VEGFR2 via distinct signaling pathways (Zeng et al., 2001b). Although VEGFR2 is an RTK, it can activate both PLCγ1 and PLCβ3 lipase activities (Mukhopadhyay and Zeng, 2002). In fact, this is the first report of an RTK activating both PLCγ1 and PLCβ3. Two independent PLCβ3 mouse mutants have markedly different phenotypes (Wang et al., 1998; Xie et al., 1999). Xie and colleagues (Xie et al., 1999) report that PLCβ3-deficient mice are homozygous viable with skin tumors and have enhanced sensitivity to morphine; whereas Wang and co-workers (Wang et al., 1998) report that PLCβ3-deficient mutants are early embryonic lethal. These discrepancies in the PLCβ3 mutant mouse phenotypes might be due to differences in the deletion constructs used to assemble the founders. However, homozygous PLCβ3 zebrafish mutants develop defects in the patterning of the pharyngeal arch (Walker et al., 2007).

Here, we have taken several approaches to elucidate the role of PLCβ3 in VEGF-mediated endothelial cell function. Overall, our work identifies a novel role of PLCβ3 in VEGF-mediated directional migration and proliferation.

Results

Measurement of activity and changes in phosphorylation of PLCβ3

To test the hypothesis that PLCβ3 contributed towards production of IP3 after VEGF treatment, we transfected HUVECs with control scrambled or PLCβ3 siRNA. After 48 hours, the cells were treated with 10 ng/ml VEGF for 1 hour and IP1 levels determined using an ELISA kit (Cisbio-US, Bedford, MA). IP1 is a downstream metabolite of IP3, which accumulates in cells following activation of PLCs. IP1 production significantly increased in response to VEGF in control cells; however, in PLCβ3-knockdown cells, levels of IP1 were significantly lower (Fig. 1A). Hence, PLCβ3 promotes IP3 production in response to VEGF.

We examined the phosphorylation of two serine residues in PLCβ3 in response to VEGF. Serum-starved HUVECs were treated with 10 ng/ml VEGF and immunoblotted with antibodies against Ser537-*P* and Ser1105-*P* of PLCβ3. We observed a time-dependent increase in phosphorylation of these serine residues. Phosphorylation started to increase at 30 seconds and reached a maximum by 1 minute for Ser537 (Fig. 1B). However, for Ser1105, this phosphorylation was sustained throughout the course of the experiment (5 minutes). Both Ser537 and Ser1105 residues on PLCβ3 are phosphorylated upon stimulation with G-protein-coupled receptor agonists such as thrombin and PAR. Phosphorylation of PLCβ3 at Ser1105 is reported to cause inhibition of its enzyme activity (Strassheim et al., 1998; Strassheim and Williams, 2000; Xia et al., 2001). Mutation at Ser1105 removes the inhibitory effect on the activation of PLCβ3 by G-protein subunits (Xia et al., 2001). We also observed phosphorylation of PLCγ1 at Tyr783 upon stimulation with VEGF, as has been previously reported (Fig. 1B) (Jia et al., 2004).

PLCβ3 is localized and phosphorylated in sprouting endothelial cells upon VEGF stimulation

We next examined whether VEGF would induce PLCβ3 phosphorylation in an ex vivo mouse embryoid body model (Jakobsson et al., 2007), where, in response to VEGF, angiogenic sprouts are formed in a 3D collagen gel matrix. Endothelial structures were detected by immunostaining for CD31 antibody, an endothelial cell marker. Since no sprouts were formed in the absence of VEGF in the control, we analysed the central core of the embryoid body instead (Fig. 2A,B). Weak staining for PLCβ3 (green) was observed; however, immunostaining for PLCβ3-*P* (Ser1105) was essentially negative (Fig. 2A,B). By contrast, in the presence of VEGF, sprouting of angiogenic vessel-like structures was observed, as evident from CD31 (red) staining (Fig. 2C,D). Distinct colocalization of both PLCβ3 (green) and PLCβ3-*P* (green) (Ser1105) with CD31-stained endothelial cells was also observed (Fig. 2C,D), indicating that VEGF induces activation of PLCβ3 in endothelial cells.

VEGFR2 induces phosphorylation of PLCβ3

To determine whether the serine phosphorylation was specific to the VEGF-VEGFR pathway, we treated HUVECs with a VEGFR2 kinase IV inhibitor (100 nM) that acts as a potent ATP-competitive inhibitor of VEGFR2 (IC_{50}=19 nM) and displays a tenfold greater selectivity for VEGFR2 over VEGFR1 (Flt-1). The effect of the VEGFR2 kinase IV inhibitor was further confirmed by a significant decrease in tyrosine phosphorylation of Y951 on VEGFR2 when HUVECs were pretreated with the inhibitor before stimulation with VEGF (Fig. 3A). Phosphorylation of PLCβ3 at Ser537 and Ser1105 was completely inhibited in samples pretreated with kinase inhibitor compared with the control (Fig. 3A). To confirm our results, we used a genetic approach using the retroviral chimeric receptors EGDR and EGLT, as previously described (Zeng et al., 2001b). EGDR and EGLT possess the extracellular domain of EGFR, and

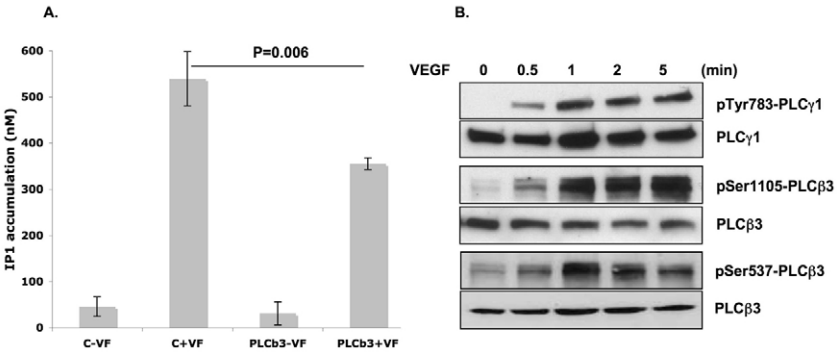


Fig. 1. IP1 production and phosphorylation of PLCs upon stimulation with VEGF. (A) HUVECs transfected with control scrambled siRNA or PLCβ3 siRNA were serum starved and stimulated with VEGF (10 ng/ml) for 1 hour, lysed and IP1 levels determined by ELISA assay. Results are means ± s.d. (B) Serum-starved HUVECs were stimulated with VEGF (10 ng/ml) for 0-5 minutes and immunoblotted with respective antibodies as shown.

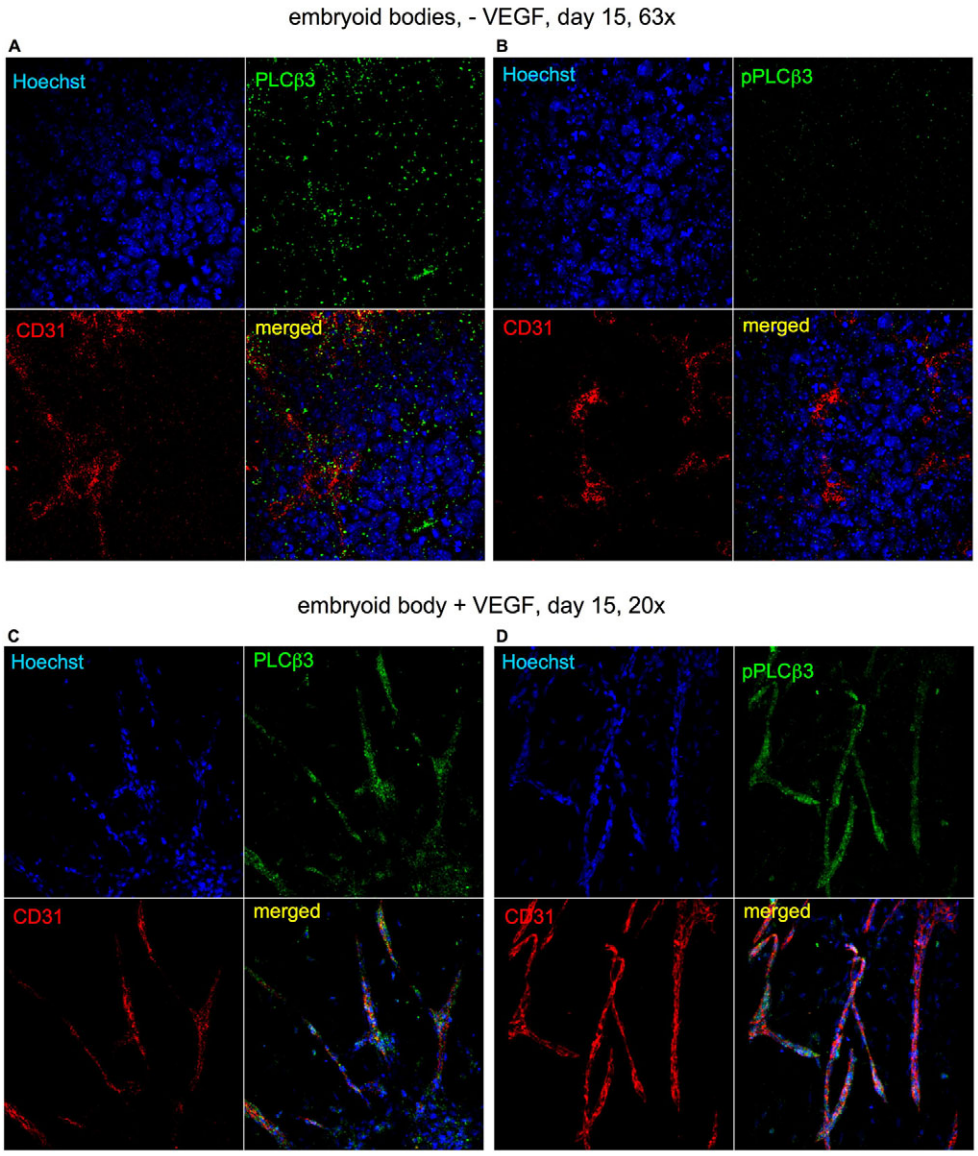


Fig. 2. Immunofluorescent staining of embryoid bodies. (A-D) Embryoid bodies differentiated for 15 days were immunostained to detect CD31 (red), PLCβ3 or SerPLCβ3-*P* (green). Hoechst 3342 was used to detect nuclei (blue). Since no sprouts were formed in the absence of VEGF (A,B), in this instance, analysis was carried out on the core of the embryoid body. Weak immunostaining for PLCβ3 was detected, but there was no immunostaining for pPLCβ3. (A,B) Sprouting of angiogenic vessel-like structures, induced by VEGF treatment of embryoid bodies. (C,D) Colocalization of PLCβ3 and pPLCβ3 with CD31-positive endothelial cells in the presence of VEGF.

transmembrane and intracellular domains of VEGFR2 or VEGFR1, respectively (Zeng et al., 2001b). Expression of EGDR and EGLT and their subsequent tyrosine phosphorylation in response to 10 ng/ml EGF was first confirmed in HUVECs by western blot (Fig. 3B). We observed that PLCβ3 was phosphorylated at Ser537 and Ser1105 upon stimulation with 10 ng/ml EGF in HUVECs expressing EGDR, but not EGLT (Fig. 3C). These data confirm that VEGF-mediated VEGFR2 activation resulted in phosphorylation of PLCβ3 at the serine residues.

Role of PLCβ3 in VEGF-mediated migration

To evaluate the role of PLCβ3 in VEGF-mediated endothelial cell function, HUVECs were transfected with siRNA targeting PLCβ3, or a scrambled control siRNA. As shown in Fig. 4A, we observed more than 90% knockdown of the PLCβ3 protein by treatment with specific siRNA, whereas the expression of other PLCβ family members did not change. Expression of PLCγ1 or its phosphorylation at Tyr783 was also not affected. Similarly, as shown in Fig. 4B, we observed more than 75% knockdown of the PLCγ1 protein by treatment with specific siRNA whereas other PLC family members were not affected. Expression of PLCβ3 or its phosphorylation at Ser537 was also not affected.

To determine the contribution of PLCβ3 to VEGF-induced endothelial signaling, we next studied the ability of HUVECs transfected with PLCβ3 siRNA to migrate in response to VEGF. We performed migration assays using two different established methods: the scratch migration assay (Matsumoto et al., 2005), and the Boyden-Chamber-based Transwell migration assay (Zeng et al., 2002). In both these assay systems, migration of HUVECs was significantly inhibited in cells treated with PLCβ3 siRNA compared with that of the scrambled siRNA control. Importantly, these data suggest an essential role of PLCβ3 in VEGF-mediated migration (Fig. 4C,D,F,H). By contrast, we did not observe any significant difference in migration (scratch migration assay) in PLCγ1-knockdown HUVECs compared with the control (Fig. 4E,G). This is in accordance with a previous report that showed that the requirement of PLCγ1 for motility was not significant when cells were plated on collagen I (Jones et al., 2005).

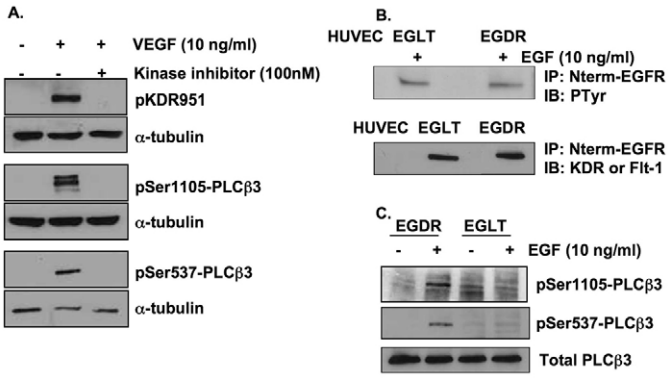


Fig. 3. Specificity of VEGFR induces serine phosphorylation of PLCβ3. (A) Serum-starved HUVECs were pretreated with or without kinase inhibitor (100 nM) and then stimulated with or without 10 mg/ml VEGF for 5 minutes. Immunoblotting was performed with antibodies as shown. (B) HUVECs were infected with or without retrovirus expressing EGDR or EGLT for 48 hours. Tyrosine phosphorylation of EGDR or EGLT following EGF treatment was confirmed by immunoprecipitation with an N-terminal EGFR antibody followed by immunoblotting with antibody against Tyr-*P*. Expression of EGDR and EGLT was confirmed by immunoprecipitation with an N-terminal EGFR antibody followed by immunoblotting with antibodies against KDR or Flt-1. (C) HUVECs were infected with retrovirus expressing EGDR or EGLT for 48 hours, followed by treatment with EGF (10 ng/ml) and immunoblotting as shown.

Quantitative statistical evaluation of the role of PLCβ3 in VEGF-induced migration

The scratch migration experiments were recorded on a time-lapse Apotome imaging system for 20 hours. Cells migrating from the wounded edge in response to VEGF were quantitatively characterized (n=30) by five descriptors of cell trajectory and velocity: tortuosity, time lag, direction changes, average speed and velocity component. Their precise definitions are provided in supplementary material Fig. S1. Table 1 shows that the medians of all descriptors for C–V and C+V were significantly different (P<0.05). According to these result, cells in C+V migrated more directionally and faster toward the final point than in C–V (smaller tortuosity, fewer direction changes, lower average speed and higher average velocity component). A comparison between β3–V and β3+V (Table 1) show no significant difference among descriptors, except the average speed. The difference between medians of average speed is in fact barely significant (P=0.0489). We also used the Kruskal-Wallis rank-sum test (MLAB implementation) for each of the comparisons. This yielded the same results, except for the average speed compared between β3–V and β3+V, with P=0.052, suggesting that there is no difference between considered average speeds.

A comparison between C+V and β3+V suggests that cell migration for C+V is somewhat more directional (higher velocity component) with a longer time lag and lesser tortuosity (Table 1). Therefore, we conclude that loss of PLCβ3 results in a loss of directional migration induced by VEGF in HUVECs.

Knockdown of PLCβ3 affects actin reorganization

VEGF is known to stimulate DNA synthesis and cell migration, involving actin stress fiber reorganization (Matsumoto et al., 2005). HUVECs were infected with lentiviral supernatant bearing control-scrambled-GFP or PLCβ3-GFP shRNA. Next, GFP-positive cells were selected by treatment with 2 μg/ml puromycin for 48 hours. These cells, which were 100% positive for GFP, were then plated onto culture slides, serum-starved and treated with 10 ng/ml VEGF for 30 minutes at 37°C and stained with phalloidin to visualize the actin cytoskeleton. More than 90% knockdown of PLCβ3 was confirmed by western blot (Fig. 5D). VEGF treatment induced formation of stress fibers in control-scrambled and PLCβ3-shRNA-transfected cells. However, the percentage of stress-fiber-forming cells decreased from 85% in the control to 67% in cells transfected with PLCβ3 shRNA (Fig. 5A,B,C,E). Furthermore, in VEGF-treated but not untreated HUVECs, pPLCβ3-Ser1105 colocalized with F-actin at the cell periphery (supplementary material Fig. S2).

Effect of PLCβ3 knockdown on activation of small GTPases by VEGF

It has been previously reported that the small GTPases RhoA, Rac-1 and CDC42 are involved in migration signaling in response to different ligands (Cascone et al., 2003; Lamalice et al., 2004; Lamalice et al., 2007; Pankov et al., 2005; van Nieuw Amerongen et al., 2003; Zeng et al., 2001a; Zeng et al., 2001b; Zeng et al., 2002). Therefore, we sought to determine whether VEGF-mediated activation of any of these small GTPases was disrupted in the absence of PLCβ3 signaling. Serum-starved HUVECs were treated with or without VEGF for 1 minute and active GTP-bound RhoA, Rac-1 or CDC42 was immunoprecipitated from the cellular lysates using respective kits from Millipore. In PLCβ3-downregulated cells, activation of RhoA and Rac1 was induced by VEGF to the same extent as in the control cells. By contrast, activation of CDC42 was markedly inhibited by the introduction of PLCβ3 siRNA (Fig. 6). These results suggest that VEGF-mediated endothelial cell migration is modulated by PLCβ3 through activation of CDC42.

Interacting partners of CDC42

To determine the possible partners that interface with PLCβ3 and CDC42, we studied the interaction of two proteins, PKCε and IQGAP1, both known to be involved in migration in a number of different cell systems. We found that PKCε was phosphorylated in a VEGF-dependent manner and phosphorylation increased in PLCβ3-knockdown cells even without VEGF stimulation (Fig. 7A). Moreover, IQGAP1 coprecipitated with both PKCε and CDC42, and this interaction increased further in PLCβ3-knockdown cells with or without VEGF (Fig. 7B). Thus, it is reasonable to speculate

Table 1. Migration descriptors determined from time-lapse images of cells in the scratch migration assay

	C–V median*	C+V median*	*P* value*	b3–V median†	b3+V median†	*P* value†
Tortuosity	9.47	2.57	<0.0001	5.53	5.20	0.834
Time lag for D/5 (seconds)	9.83	25.2	0.0030	15.9	10.3	0.108
Direction ± 9/12	1.5	0	<0.0001	0	0	0.099
Average speed (μm/second)	0.014	0.009	<0.0001	0.0092	0.0090	0.049
Velocity component (μm/second)	0.0014	0.0037	0.0012	0.0016	0.0013	0.502

*Data from cells transfected with control scrambled siRNA; †data from cells transfected with PLCβ3 siRNA. Each group represents data from at least 30 cells.

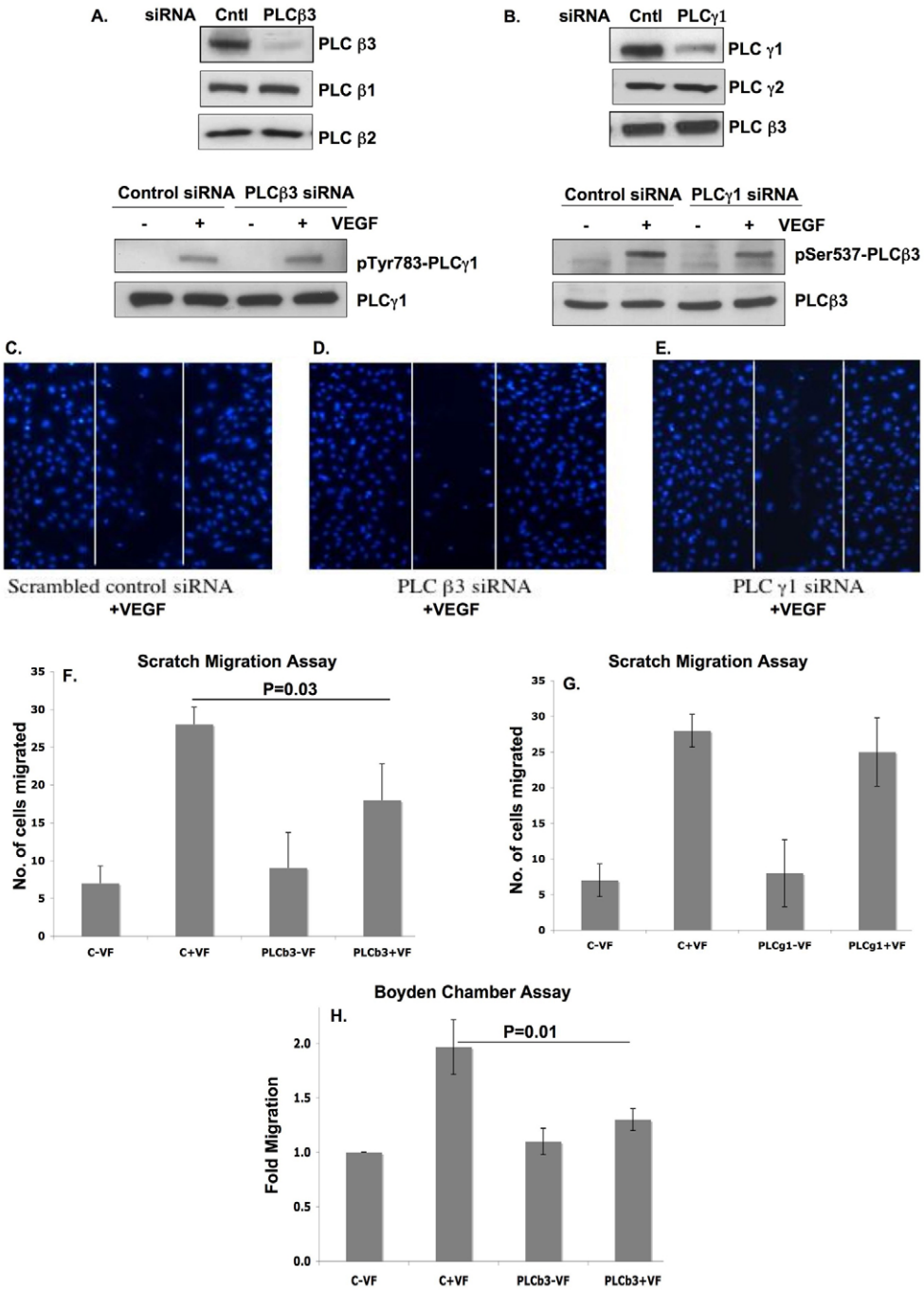


Fig. 4. Effect of knockdown of PLCs in HUVECs on migration assays. (A) HUVECs were transfected with control scrambled or PLCβ3 siRNA using oligofectamine for 48 hours. Samples were then immunoblotted for PLCβ1, PLCβ2 and PLCβ3. Expression of PLCγ1 and its phosphorylation at Tyr783 are also shown. (B) HUVECs were transfected with control scrambled or PLCγ1 siRNA using oligofectamine for 48 hours. Samples were then immunoblotted for PLCγ1, PLCγ2 and PLCβ3. Expression of PLCβ3 and its phosphorylation at Ser537 are also shown. (C-E) HUVECs were transfected with control scrambled or PLCβ3 or PLCγ1 siRNA using oligofectamine for 48 hours. Scratch migration assay was performed and Hoechst-33342-stained nuclei migrating into the scratched region are shown. (F) Quantitative representation of scratch migration assay performed on HUVECs transfected with control scrambled or PLCβ3 siRNA. (G) Quantitative representation of scratch migration assay performed on HUVECs transfected with control scrambled or PLCγ1 siRNA. (H) Boyden Chamber migration assay performed on HUVEC transfected with control scrambled or PLCβ3 siRNA. Data represent fold change in migration. All of the above results show the mean ± s.d. of three measurements from three experiments repeated at least three times.

that in PLCβ3-knockdown cells, active PKCε phosphorylates IQGAP1, which then sequesters CDC42 in the nucleotide-free form, thereby preventing activation and subsequent migration.

Role of PLCβ3 in VEGF-mediated proliferation

To determine the contribution of PLCβ3 to VEGF-induced endothelial signaling, we next studied the ability of HUVECs transfected with PLCβ3 siRNA to proliferate in response to VEGF. Relative proliferation was measured using the MTT assay in control versus PLCβ3-knockdown HUVECs in response to VEGF. We observed a significant increase (P=0.0001) in proliferation of HUVECs without VEGF treatment compared with the control (Fig. 8A). This result suggests that PLCβ3 is a negative regulator of endothelial cell proliferation. In accordance with previous reports, VEGF-induced proliferation was decreased in PLCγ1-knockout cells compared with the control (data not shown) (Meyer et al., 2003; Takahashi et al., 2001).

To determine whether this increase in proliferation was due to an increase in MAP kinase (MAPK) phosphorylation, we next tested the status of MAPK-*P* (42/44 kDa) in these cells. To our surprise VEGF induced phosphorylation of MAPK to the same extent in both control and PLCβ3-siRNA-transfected cells. We also performed a time-course experiment to determine whether the effect was perhaps more sustained in the PLCβ3-siRNA-transfected cells, but no significant differences were observed (Fig. 8B). Phosphorylation of the stress-activated protein kinase Jun N-

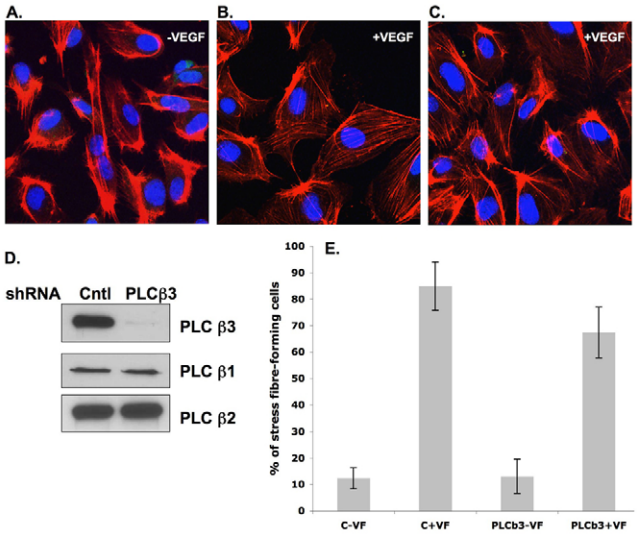


Fig. 5. Effect of PLCβ3 knockdown on actin reorganization. HUVECs bearing control-GFP or PLCβ3-GFP shRNA were selected with puromycin. HUVECs grown in culture slides were stimulated with 10 ng/ml VEGF for 30 minutes and then fixed and stained with phalloidin and DAPI. (A) HUVECs expressing control scrambled shRNA without any stimulation. (B) Cells from A stimulated with 10 ng/ml VEGF. (C) HUVECs expressing PLCβ3 shRNA stimulated with 10 ng/ml VEGF. (D) The percentage of stress-fiber-forming cells was calculated from five fields per well. Results show means ± s.d.

terminal kinase (SAPK/JNK) and p38MAPK was also determined, and although stimulated in response to VEGF, no significant differences between the control and the PLCβ3-siRNA-transfected cells were observed (data not shown).

PLCβ3 affects cell cycle in HUVECs

Next, we determined whether knockdown of PLCβ3 had any effect on the cell cycle in HUVECs. Since we observed increased proliferation but did not observe any difference in MAPK phosphorylation in PLCβ3-knockdown versus control cells, we hypothesized that this could be due to changes in the cell cycle.

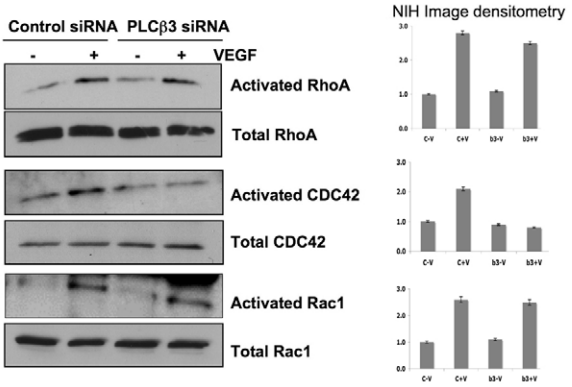


Fig. 6. Effect of knockdown of PLCβ3 on activation of small GTPases. HUVECs were transfected with siRNA for PLCβ3 for 48 hours followed by starvation for 12-14 hours, and then stimulated with VEGF at 10 ng/ml. Lysates were immunoprecipitated with respective substrate GST-beads, and GTP-bound RhoA, Rac1 or CDC42 was detected by immunoblotting. Experiments were repeated at least three times. Normalized fold-change for each blot is shown as determined by NIH image densitometry.

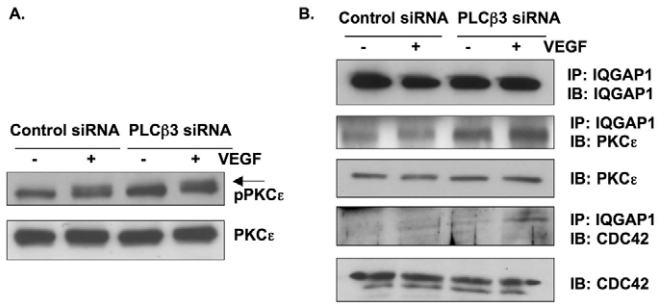


Fig. 7. Effect of knockdown of PLCβ3 on activation and coprecipitation of PKCε and IQGAP1, two migration related proteins. (A) HUVECs transfected with control scrambled or PLCβ3 siRNA were treated with or without 10 ng/ml VEGF for 5 minutes. Cell lysates were immunoblotted against antibodies for phosphorylated Ser729 PKCε and PKCε. Arrow indicates the phospho-PKCε band. (B) HUVECs transfected with control scrambled or PLCβ3 siRNA were treated with or without 10 ng/ml VEGF for 5 minutes. Cell lysates were immunoprecipitated with antibody against IQGAP1 and immunoblotted with antibodies against PKCε, CDC42 and IQGAP1. Total levels of PKCε and CDC42 in the lysates are also shown.

According to previous reports, passage 3 HUVECs have a doubling time of ~19 hours (Kalashnik et al., 2000; Marin et al., 2001). Hence, cells transfected with control scrambled or PLCβ3 siRNA were treated with or without VEGF for 10 and 24 hours, respectively, and then subjected to propidium iodide staining for cell cycle determination. Cell lysates were also collected for western blot. At 10 hours, presumably before synchronized HUVECs could undergo one full cell cycle, we observed significant increases in the percentage of cells in S phase, in both the control and PLCβ3-knockdown cells treated with VEGF (Fig. 9A). However, at 24

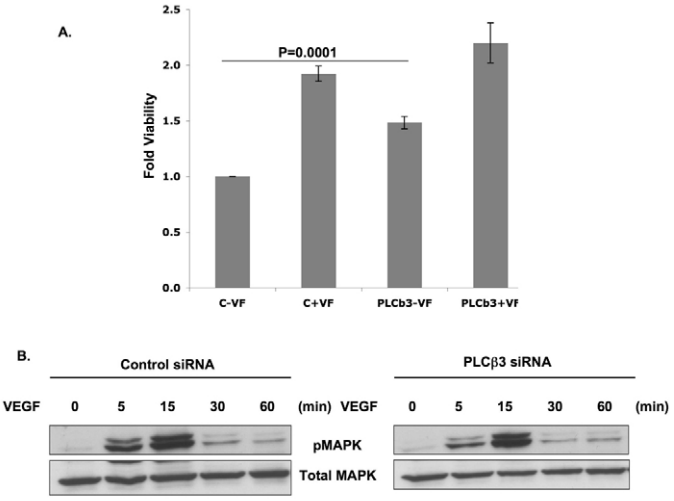
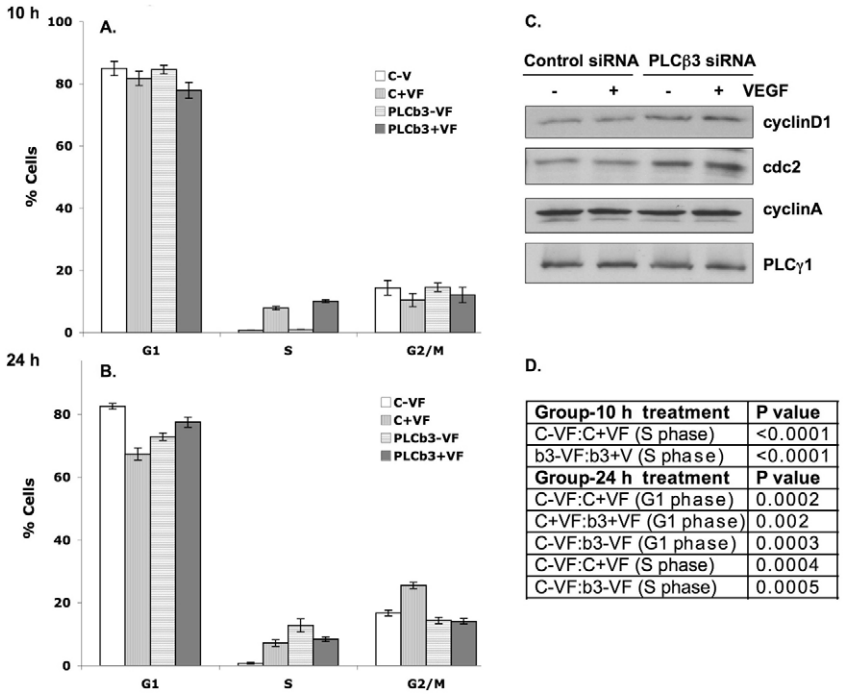


Fig. 8. Effect of knockdown of PLCβ3 on VEGF-mediated proliferation and MAPK phosphorylation. (A) HUVECs were transfected with control scrambled or PLCβ3 siRNA using oligofectamine for 48 hours. 2×10^4 cells were plated in a 96-well plate, serum starved overnight and treated with 10 ng/ml VEGF for 48 hours before adding MTT. Proliferation assay was then performed. Data represent relative fold changes in absorbance at 490 nm ± s.d. Experiments were repeated at least three times, each time in triplicate (B) HUVECs transfected with control scrambled or PLCβ3 siRNA were serum starved overnight and treated with 10 ng/ml VEGF for the respective times, as described. Cell lysates were collected and western blotted with antibodies against MAPK-*P* or total MAPK.



Group-10 h treatment	P value
C-VF:C+VF (S phase)	<0.0001
b3-VF:b3+V (S phase)	<0.0001
Group-24 h treatment	**P value**
C-VF:C+VF (G1 phase)	0.0002
C+VF:b3+VF (G1 phase)	0.002
C-VF:b3-VF (G1 phase)	0.0003
C-VF:C+VF (S phase)	0.0004
C-VF:b3-VF (S phase)	0.0005

Fig. 9. Effect of PLCβ3 knockdown on cell cycle. (A) HUVECs transfected with control scrambled or PLCβ3 siRNA were serum starved overnight and treated with VEGF for 10 hours (A) or 24 hours (B). The cells were then fixed and stained with PI and analyzed by FACS. The mean percentage of cells with DNA content in each of the three phases of the cell cycle is shown over three independent determinations. Results are means ± s.d. (C) HUVECs transfected with control scrambled or PLCβ3 siRNA were serum starved overnight and treated with 10 ng/ml VEGF for 5 minutes. Cyclin D1, CDC2, cyclin A and PLCγ1 were then detected by western blot of cell lysates. (D) Two-sided Student's *t*-test was performed on the data in A and B, and the corresponding *P* values are shown.

hours, when these cells had just completed one cycle, even without VEGF, the PLCβ3-knockdown cells showed a significantly higher percentage of cells in S phase, and a concomitant lower percentage of cells in G1 phase compared with control cells without VEGF. Upon the addition of VEGF, however, both control and PLCβ3-knockdown cells showed a significantly higher percentage of cells in the S phase compared with control cells without VEGF (Fig. 9B). The percentage of cells in G1 in the VEGF-treated samples (C+V) was significantly lower than that of untreated control; however, compared with C+V, a moderate increase in G1 in the VEGF-treated PLCβ3-knockdown cells indicated a faster cycling time and thus a return past the G2-M phase back to G1 (Fig. 9B). In accordance with the cell cycle data, western blot analysis revealed an increase in the cell cycle proteins cyclin D1 and CDC2, but not cyclin A. PLCγ1 was used as a loading control (Fig. 9C). Statistical analysis performed on data from Fig. 9A,B is shown in Fig. 9D.

Discussion

Since endothelial cells are the key component of new sprouting blood vessels, their ability to migrate and proliferate is essential to angiogenesis. We found that knockdown of PLCβ3 in HUVECs affects both migration and proliferation; although migration is inhibited, proliferation is enhanced. Our data suggest that enhanced proliferation is precipitated by an accelerated cell cycle, and decreased migration by an inability to activate CDC42. Therefore, in the context of blood vessel formation, PLCβ3 has an important role in that its presence makes the cell 'pro'-migration and 'anti'-proliferation.

In this report, we demonstrated that VEGF not only modulates the activity of RTK-coupled PLCγ1 but also G-protein-coupled PLCβ3. In support of this argument, we show that serine phosphorylation of PLCβ3 is dependent on the VEGF-VEGFR2 axis. These findings support our previous observations that PLCβ3 and PLCγ1 were the only two phospholipases significantly stimulated upon addition of VEGF to the endothelium (Mukhopadhyay and Zeng, 2002). It also corroborates our previous observation that, upon stimulation with VEGF, the Gαq axis is also stimulated in the endothelium (Zeng et al., 2003). We also showed that PLCβ3 contributes significantly towards IP3 production in VEGF-stimulated cells. Here, our main focus was to determine the role of PLCβ3 in the VEGF-VEGFR2 signaling axis in HUVECs with respect to migration and proliferation phenotypes. In two different assay systems, migration in response to VEGF was significantly decreased in PLCβ3-knockdown cells compared with that of the control. Furthermore, actin reorganization induced by VEGF was also inhibited in PLCβ3-knockdown cells compared with that of the control cells.

Mathematical analysis of our migration data reveals that medians of all descriptors for VEGF-treated endothelial cells were significantly different with respect to untreated control ($P<0.05$). According to these analyses, we conclude that the cells treated with VEGF could migrate faster and more directionally than untreated ones (smaller tortuosity, fewer direction changes, higher velocity component). Interestingly, in PLCβ3-knockdown cells, no significant changes could be observed in any of the five descriptors suggesting that loss of PLCβ3 resulted in the loss in directionality of migration after VEGF treatment.

To further evaluate the directionality of endothelial cells in response to VEGF, we tested the role of small GTPases in this process. Interestingly, we identified CDC42 as a downstream target involved in regulating endothelial migration in the VEGF-VEGFR2-PLCβ3 axis. The activation of the small GTPases of the Rho family is centrally involved in regulating endothelial cell migration in response to activation of VEGFR2. In particular, CDC42 is involved in the formation of filopodia; these structures act as sensors, underlying the 'guidance migratory mechanism' shown in early postnatal angiogenesis in the retina (Gerhardt et al., 2003). Interestingly, endothelial sprouts also extend multiple filopodia at their distal tips, indicating that growing vascular sprouts

are endowed with specialized tip structures with potential functions in guidance and migration in response to a VEGF gradient (Gerhardt et al., 2003). However, to establish the link between PLCβ3 and CDC42, we studied the interaction of two proteins, PKCε and IQGAP1, which are both known to be involved in migration in a number of different cell systems. That PKCε can be activated by PLC signaling has previously been shown (Joseph et al., 2007; Moriya et al., 1996). Activated PKCε can then phosphorylate IQGAP1 at Ser1443 (Grohmanova et al., 2004). IQGAP1 is also known to coprecipitate with both PKCε and CDC42 (Grohmanova et al., 2004). We found increased phosphorylation of PKCε in PLCβ3-knockdown cells compared with the control. Also, coprecipitation of CDC42 and PKCε with IQGAP1 increased in PLCβ3-knockdown cells compared with the control. It has recently been reported that two C-terminal domains of IQGAP1 exclusively bind nucleotide-free CDC42. The interaction of nucleotide-free CDC42 with IQGAP1 is favored upon phosphorylation at Ser1443 (Grohmanova et al., 2004). Therefore, we speculate that in PLCβ3-knockdown cells, PKCε is active and phosphorylates IQGAP1, which then sequesters CDC42 in the nucleotide-free from, thereby preventing activation and thus migration.

Previous reports suggest that activation of PLCγ1 through VEGFR2 in endothelial cells is required for tubulogenesis and differentiation of cells (Meyer et al., 2003). The integrin-Src-PLCγ1 pathway is important for cell motility on extracellular matrix (ECM). Our results on scratch migration are in accordance with previous reports that did not find any significant change in motility of PLCγ1-knockdown cells when plated on collagen I (Jones et al., 2005). However, our findings that knockdown of PLCγ1 inhibits VEGF-induced proliferation are also in accordance with previously published reports (data not shown) (Meyer et al., 2003; Takahashi et al., 2001). Interestingly, we have shown that PLCβ3 is not only an important regulator of migration, but also regulates proliferation in endothelial cells. A significant increase in proliferation and S phase of the cell cycle at 24 hours is observed, even in the absence of VEGF in the PLCβ3-knockdown cells, and in its presence the effect is further enhanced. However, we demonstrate that this effect on proliferation is probably due to upregulation of cell cycle proteins CDC2 and cyclinD1 and is not dependent on the canonical MAPK pathways.

Overall, our work challenges the existing dogma that heterotrimeric G-proteins solely promote signals from the seven-transmembrane-motif-containing cell surface receptors, also known as G-protein-coupled receptors (GPCRs). Our previous work specified that Gq/11 and Gβγ, subunits of the G-proteins, acquire unconventional signaling pathways by sending out signals from RTKs, such as VEGFR2. Hence, demonstrating a novel pathway involving VEGFR2-PLCβ3-CDC42 for VEGF-induced directional migration in endothelial cells indeed reaffirms our original hypothesis. The emerging crosstalk between G-proteins and RTK signaling is expected to have a wider impact in the way we think about cell signaling.

Materials and Methods

Reagents

VEGF-A was obtained from R&D Systems, Minneapolis, MN. The antibodies to VEGFR1, VEGFR2, VEGFR2-*P* (951), PLCβ1, PLCβ2, PLCβ3, PLCγ1, PLCγ2 and EGFR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); PLCβ3-*P* (S537, S1105), PKCε-*P* (S729) and PLCγ1-*P* (Y783) were obtained from Cell Signaling Technology (Danvers, MA). Lentiviral shRNA constructs were from Open Biosystems (Huntsville, AL). Kinase Inhibitor IV was purchased from EMD Biosciences (San Diego, CA). Small GTPase activation assay kits were obtained from Millipore (Lake Placid, NY); as were the CDC42 activation assay kit and RhoA activation assay kit.

Immunoprecipitation and western blot analysis

HUVECs were infected with retroviral supernatants prepared in HEK293T cells expressing EGDR or EGLT. After 48 hours, cells were starved overnight in EBM medium without serum. Subsequently the cells were stimulated with EGF 10 ng/ml for 5 minutes. 500 μg cell lysate was immunoprecipitated with N-terminal EGFR antibody and protein-A/G-conjugated agarose beads, and immunoblotted with antibodies against VEGFR1 or VEGFR2.

Serum-starved HUVECs were pretreated with kinase inhibitor IV (100 nM) for 15 minutes before treatment with or without VEGF (10 ng/ml). Cell lysates were prepared in RIPA buffer supplemented with protease inhibitor cocktail. The lysates were collected after centrifugation at 14,000 ***g*** for 10 minutes at 4°C and separated by SDS-PAGE. Experiments were repeated at least three times.

siRNA transfection

HUVECs were transfected with control scrambled (sc37007), PLCβ3 (sc36272) or PLCγ1 (sc29452) siRNA obtained from Santa Cruz Biotechnology (Santa Cruz, CA) using Oligofectamine reagent in Opti-MEM medium. The PLCβ3 siRNA was a pool of four duplex siRNA: (1) 513 5′ sense, UCAAGAACAUCCUGAAGAUtt and 5′ anti-sense, AUCUUCAGGAUGUUCUUGAtt; (2) 968 5′ sense, AGUGCCUACUU-CAUCAACUtt and 5′ anti-sense, AGUGCCUACUUCAUCAACUtt; (3) 1383 5′ sense, GUAUCCUGGUGAAGAACAAtt and 5′ anti-sense, UUGUUCUUCAC-CAGGAUACtt; (4) 1839 5′ sense, GCUUCGAGAUGUCGUCCUUtt and 5′ anti-sense, AAGGACGACAUCUCGAAGCtt.

Following transfection for 48 hours, the cells were starved overnight and stimulated with 10 ng/ml VEGF to collect lysates for western blot analysis to detect small GTPase activation. Alternatively, siRNA-transfected cells were subjected to migration assays as described below.

Measurement of IP1 as an indicator of IP3 production

2×10^4 HUVECs were plated in 24-well plates and after 18 hours of starvation, they were stimulated with 10 ng/ml VEGF for 1 hour. Following lysis, the supernatant was transferred into an ELISA plate and the IP1 produced was detected by addition of the ELISA reagents according to manufacturer's protocol (Cisbio-US, Bedford, MA).

Detection of stress-fiber formation

HUVECs were infected with lentiviral supernatant prepared in HEK293T cells bearing control-scrambled-GFP or PLCβ3-GFP shRNA. Next, GFP-positive cells were selected by treatment for 48 hours with 2 μg/ml puromycin. These cells, 100% positive for GFP, were then plated onto culture slides. HUVECs on culture slides were serum starved and treated with 10 ng/ml VEGF for 30 minutes at 37°C. Cells were fixed in 3% paraformaldehyde in PBS, and permeabilized in 0.2% Triton X-100 in PBS for 10 minutes. Cells were incubated in blocking buffer (10% goat serum in PBS), and subsequently incubated with 5 μg/ml Alexa Fluor 563-labeled phalloidin (Molecular Probes) for 45 minutes at room temperature, washed again, and incubated with 5 μg/ml Hoechst 33342 (Invitrogen). Confocal microscopy was performed using a Zeiss LSM 510 confocal laser-scanning microscope with C-Apochromat ×63/NA 1.2 water-immersion lens. Absence of signal crossover was established using single-labeled samples. The percentage of stress-fiber-forming cells/total cells was calculated from five fields (×63 objective) per well.

Small GTPase pull-down assay

RhoA or CDC42 activation assay kits from Millipore were used to perform these assays. Magnesium lysis buffer (MLB, Mg^{2+} lysis-wash Buffer) was made by diluting 5× MLB by adding sterile distilled water containing 10% glycerol. To the 1× MLB diluted buffer, 10 μg/ml aprotinin and 10 μg/ml leupeptin were added. The cells were rinsed twice with ice-cold PBS and an appropriate amount of ice-cold 1× MLB was added. The lysates were transferred to microfuge tubes. Protocols as described by the manufacturer (Millipore) were followed for CDC42, Rac and Rho-A. Immunoblotting was performed with anti-CDC42, anti-Rac1 and anti Rho-A antibodies.

Proliferation assay

HUVECs transfected with control scrambled or PLCβ3 siRNA (2×10^4) were seeded in 96-well plates in EGM. After 24 hours, the cells were serum starved (0.1%) overnight and then treated with VEGF at 10 ng/ml for 48 hours Proliferation was measured by using the thiazolyl blue tetrazolium bromide (MTT) colorimetric assay according to the manufacturer's recommendation (Promega). The absorbance at 490 nm was determined using Spectra Fluor PLUS (Molecular Devices, Sunnyvale, CA). Experiments were repeated at least three times.

Cell cycle analysis

DNA content was measured after staining cells with propidium iodide (PI). HUVECs transfected with control scrambled or PLCβ3 siRNA were serum starved for 18 hours.

Following starvation, the cells were treated with or without 10 ng/ml VEGF and collected either 10 or 24 hours after treatment. The cells were trypsinized, washed in PBS and fixed in 95% ethanol for 1 hour. Cells were rehydrated, washed in PBS and treated with RNaseA (1 mg/ml) followed by staining with PI (100 mg/ml). Flow cytometric quantification of DNA was performed with a FACScan (Becton Dickinson, San Jose, CA) and data analysis was carried out by using the Modfit software. Experiments were repeated at least three times.

Scratch migration assay

Monolayers of HUVECs transfected with respective siRNAs were scratched with a universal blue pipette tip and incubated for 24 hours in the presence of 20 ng/ml VEGF. Thymidine (10 mM; Sigma, St Louis, MO) was included during the incubation to inhibit cell proliferation. Cells were fixed in 4% paraformaldehyde, stained with 5 μg/ml Hoechst 33342 to visualize nuclei, and photographed. Hoechst-positive nuclei that had moved into the wounded area were counted in five fields per well. The means and s.d. from triplicate wells were determined.

For the mathematical modeling experiments, the scratch migration assay was repeated exactly as described above, except the cells were imaged using bright field optics with a ×5 objective lens every 10 minutes up to 20 hours after VEGF treatment on a heated stage maintained at 37°C with 5% CO_2 using an Axiovert 200M microscope from Carl-Zeiss (Munich, Germany). At the end of the experiment, 30 cells from each well were randomly analyzed using Axiovision software with tracking module (Carl-Zeiss, Munich, Germany). Only the cells from the edge of the scratch facing the vacant scratched area were considered.

Boyden Chamber migration assay

Serum-starved HUVECs (siRNA-transfected) were stained with calcein-AM (25 μg calcein-AM dissolved in 5 μl DMSO and then added to 4 ml EBM containing 0.1% BSA per 100 mm plate) at 37°C for 30 minute. The cells were then detached from tissue culture plates using 4 ml collagenase solution (0.2 mg/ml collagenase, 0.2 mg/ml soybean trypsin inhibitor, 1 mg/ml BSA and 2 mM EDTA in PBS). Then cells were seeded at 1×10^5/well in 500 μl EBM with 0.1% fetal bovine serum into Transwells coated with vitrogen (30 μg/ml). Transwells were placed in a 24-well plate containing 750 μl of the same medium. The cells were incubated at 37°C for 45 minutes to allow them to attach. Next, VEGF was added at a final concentration of 10 ng/ml, and an additional 4 hour incubation was performed. The migrated, stained cells were counted in a spectrofluorometer (Spectrafluor; TECAN) with Delta Soft 3 software. Data are expressed as fold change over control cells without VEGF ± s.d. of triplicate values. All experiments were repeated at least three times.

Immunofluorescent staining of embryoid bodies

Induction of angiogenesis in mouse embryoid bodies was described previously (Jakobsson et al., 2006). Briefly, dissociated ES cells were aggregated in hanging drops (1200 cells/20 μl medium without LIF; denoted day 0) to form embryoid bodies. After 4 days, embryoid bodies were seeded into a matrix of collagen I and thereafter maintained in the absence and presence of 30 ng/ml VEGF-A_{165} (PeproTech, Rocky Hill, NJ). On day 15, embryoid bodies were fixed and incubated with primary antibodies as indicated, followed by appropriate secondary antibodies. Endothelial cells were detected using a rat anti-mouse CD31 antibody (Becton Dickinson, San Jose, CA). Samples were examined using an LSM 510 META confocal microscope (Carl Zeiss, Munich, Germany).

Statistical analysis

All values are expressed as means ± s.d. Statistical significance was determined using two-sided Student's *t*-test and a value of $P<0.05$ was considered significant, unless stated otherwise.

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References

Berridge, M. J. (1993). Inositol trisphosphate and calcium signalling. *Nature* **361**, 315-325.

Brock, T. A., Dvorak, H. F. and Senger, D. R. (1991). Tumor-secreted vascular permeability factor increases cytosolic Ca^{2+} and von Willebrand factor release in human endothelial cells. *Am. J. Pathol.* **138**, 213-221.

Cascone, I., Audero, E., Giraudo, E., Napione, L., Maniero, F., Philips, M. R., Collard, J. G., Serini, G. and Bussolino, F. (2003). Tie-2-dependent activation of RhoA and Rac1 participates in endothelial cell motility triggered by angiopoietin-1. *Blood* **102**, 2482-2490.

Cockcroft, S. and Thomas, G. M. (1992). Inositol-lipid-specific phospholipase C isoenzymes and their differential regulation by receptors. *Biochem. J.* **288**, 1-14.

Dvorak, H. F., Dvorak, A. M., Manseau, E. J., Wiberg, L. and Churchill, W. H. (1979). Fibrin gel investment associated with line 1 and line 10 solid tumor growth, angiogenesis, and fibroplasia in guinea pigs: role of cellular immunity, myofibroblasts, microvascular damage, and infarction in line 1 tumor regression. *J. Natl. Cancer Inst.* **62**, 1459-1472.

Gerhardt, H., Golding, M., Fruttiger, M., Ruhrberg, C., Lundkvist, A., Abramsson, A., Jeltsch, M., Mitchell, C., Alitalo, K., Shima, D. et al. (2003). VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J. Cell Biol.* **161**, 1163-1177.

Grohmanova, K., Schlaepfer, D., Hess, D., Gutierrez, P., Beck, M. and Kroschewski, R. (2004). Phosphorylation of IQGAP1 modulates its binding to Cdc42, revealing a new type of rho-GTPase regulator. *J. Biol. Chem.* **279**, 48495-48504.

Guidi, A. J., Abu-Jawdeh, G., Berse, B., Jackman, R. W., Tognazzi, K., Dvorak, H. F. and Brown, L. F. (1995). Vascular permeability factor (vascular endothelial growth factor) expression and angiogenesis in cervical neoplasia. *J. Natl. Cancer Inst.* **87**, 1237-1245.

Hamlyn, J. M., Blaustein, M. P., Bova, S., DuCharme, D. W., Harris, D. W., Mandel, F., Mathews, W. R. and Ludens, J. H. (1991). Identification and characterization of a ouabain-like compound from human plasma. *Proc. Natl. Acad. Sci. USA* **88**, 6259-6263.

Jakobsson, L., Kreuger, J., Holmborn, K., Lundin, L., Eriksson, I., Kjellén, L. and Claesson-Welsh, L. (2006). Heparan sulfate in trans potentiates VEGFR-mediated angiogenesis. *Dev. Cell* **10,** 625-634.

Jakobsson, L., Kreuger, J. and Claesson-Welsh, L. (2007). Building blood vessels-stem cell models in vascular biology. *J. Cell Biol.* **177**, 751-755.

Jia, H., Bagherzadeh, A., Bicknell, R., Duchen, M. R., Liu, D. and Zachary, I. (2004). Vascular endothelial growth factor (VEGF)-D and VEGF-A differentially regulate KDR-mediated signaling and biological function in vascular endothelial cells. *J. Biol. Chem.* **279**, 36148-36157.

Jones, N. P., Peak, J., Brader, S., Eccles, S. A. and Katan, M. (2005). PLCgamma1 is essential for early events in integrin signalling required for cell motility. *J. Cell Sci.* **118**, 2695-2706.

Joseph, E. K., Bogen, O., Alessandri-Haber, N. and Levine, J. D. (2007). PLC-beta 3 signals upstream of PKC epsilon in acute and chronic inflammatory hyperalgesia. *Pain* **132**, 67-73.

Kalashnik, L., Bridgeman, C. J., King, A. R., Francis, S. E., Mikhalovsky, S., Wallis, C., Denyer, S. P., Crossman, D. and Faragher, R. G. (2000). A cell kinetic analysis of human umbilical vein endothelial cells. *Mech. Ageing Dev.* **120**, 23-32.

Kanda, S., Miyata, Y. and Kanetake, H. (2004). Role of focal adhesion formation in migration and morphogenesis of endothelial cells. *Cell. Signal.* **16**, 1273-1281.

Kanda, S., Miyata, Y., Kanetake, H. and Smithgall, T. E. (2006). Fibroblast growth factor-2 induces the activation of Src through Fes, which regulates focal adhesion disassembly. *Exp. Cell Res.* **312**, 3015-3022.

Koch, A. E., Harlow, L. A., Haines, G. K., Amento, E. P., Unemori, E. N., Wong, W. L., Pope, R. M. and Ferrara, N. (1994). Vascular endothelial growth factor. A cytokine modulating endothelial function in rheumatoid arthritis. *J. Immunol.* **152**, 4149-4156.

Lamalice, L., Houle, F., Jourdan, G. and Huot, J. (2004). Phosphorylation of tyrosine 1214 on VEGFR2 is required for VEGF-induced activation of Cdc42 upstream of SAPK2/p38. *Oncogene* **23**, 434-445.

Lamalice, L., Le Boeuf, F. and Huot, J. (2007). Endothelial cell migration during angiogenesis. *Circ. Res.* **100**, 782-794.

Leung, T., Chen, H., Stauffer, A. M., Giger, K. E., Sinha, S., Horstick, E. J., Humbert, J. E., Hansen, C. A. and Robishaw, J. D. (2006). Zebrafish G protein gamma2 is required for VEGF signaling during angiogenesis. *Blood* **108**, 160-166.

Marin, V., Kaplanski, G., Gres, S., Farnarier, C. and Bongrand, P. (2001). Endothelial cell culture: protocol to obtain and cultivate human umbilical endothelial cells. *J. Immunol. Methods* **254**, 183-190.

Matsumoto, T., Bohman, S., Dixelius, J., Berge, T., Dimberg, A., Magnusson, P., Wang, L., Wikner, C., Qi, J. H., Wernstedt, C. et al. (2005). VEGF receptor-2 Y951 signaling and a role for the adapter molecule TSAd in tumor angiogenesis. *EMBO J.* **24**, 2342-2353.

Meyer, R. D., Latz, C. and Rahimi, N. (2003). Recruitment and activation of phospholipase Cgamma1 by vascular endothelial growth factor receptor-2 are required for tubulogenesis and differentiation of endothelial cells. *J. Biol. Chem.* **278**, 16347-16355.

Moriya, S., Kazlauskas, A., Akimoto, K., Hirai, S., Mizuno, K., Takenawa, T., Fukui, Y., Watanabe, Y., Ozaki, S. and Ohno, S. (1996). Platelet-derived growth factor activates protein kinase C epsilon through redundant and independent signaling pathways involving phospholipase C gamma or phosphatidylinositol 3-kinase. *Proc. Natl. Acad. Sci. USA* **93**, 151-155.

Mukhopadhyay, D. and Zeng, H. (2002). Involvement of G proteins in vascular permeability factor/vascular endothelial growth factor signaling. *Cold Spring Harb. Symp. Quant. Biol.* **67**, 275-283.

Nakamura, F., Kato, M., Kameyama, K., Nukada, T., Haga, T., Kato, H., Takenawa, T. and Kikkawa, U. (1995). Characterization of Gq family G proteins GL1 alpha (G14 alpha), GL2 alpha (G11 alpha), and Gq alpha expressed in the baculovirus-insect cell system. *J. Biol. Chem.* **270**, 6246-6253.

Noh, D. Y., Shin, S. H. and Rhee, S. G. (1995). Phosphoinositide-specific phospholipase C and mitogenic signaling. *Biochim. Biophys. Acta* **1242**, 99-113.

Pankov, R., Endo, Y., Even-Ram, S., Araki, M., Clark, K., Cukierman, E., Matsumoto, K. and Yamada, K. M. (2005). A Rac switch regulates random versus directionally persistent cell migration. *J. Cell Biol.* **170**, 793-802.

Rhee, S. G. and Choi, K. D. (1992). Regulation of inositol phospholipid-specific phospholipase C isozymes. *J. Biol. Chem.* **267**, 12393-12396.

Rhee, S. G. and Bae, Y. S. (1997). Regulation of phosphoinositide-specific phospholipase C isozymes. *J. Biol. Chem.* **272**, 15045-15048.

Risau, W. (1997). Mechanisms of angiogenesis. *Nature* **386**, 671-674.

Senger, D. R., Galli, S. J., Dvorak, A. M., Perruzzi, C. A., Harvey, V. S. and Dvorak, H. F. (1983). Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* **219**, 983-985.

Senger, D. R., Ledbetter, S. R., Claffey, K. P., Papadopoulos-Sergiou, A., Peruzzi, C. A. and Detmar, M. (1996). Stimulation of endothelial cell migration by vascular

permeability factor/vascular endothelial growth factor through cooperative mechanisms involving the alphavbeta3 integrin, osteopontin, and thrombin. *Am. J. Pathol.* **149**, 293-305.

Soker, S., Takashima, S., Miao, H. Q., Neufeld, G. and Klagsbrun, M. (1998). Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* **92**, 735-745.

Soker, S., Miao, H. Q., Nomi, M., Takashima, S. and Klagsbrun, M. (2002). VEGF165 mediates formation of complexes containing VEGFR-2 and neuropilin-1 that enhance VEGF165-receptor binding. *J. Cell Biochem.* **85**, 357-368.

Sternweis, P. C. and Smrcka, A. V. (1992). Regulation of phospholipase C by G proteins. *Trends Biochem. Sci.* **17**, 502-506.

Strassheim, D. and Williams, C. L. (2000). P2Y2 purinergic and M3 muscarinic acetylcholine receptors activate different phospholipase C-beta isoforms that are uniquely susceptible to protein kinase C-dependent phosphorylation and inactivation. *J. Biol. Chem.* **275**, 39767-39772.

Strassheim, D., Law, P. Y. and Loh, H. H. (1998). Contribution of phospholipase C-beta3 phosphorylation to the rapid attenuation of opioid-activated phosphoinositide response. *Mol. Pharmacol.* **53**, 1047-1053.

Takahashi, T., Yamaguchi, S., Chida, K. and Shibuya, M. (2001). A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-A-dependent activation of PLC-gamma and DNA synthesis in vascular endothelial cells. *EMBO J.* **20**, 2768-2778.

Terman, B. I., Dougher-Vermazen, M., Carrion, M. E., Dimitrov, D., Armellino, D. C., Gospodarowicz, D. and Bohlen, P. (1992). Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. *Biochem. Biophys. Res. Commun.* **187**, 1579-1586.

Vaisman, N., Gospodarowicz, D. and Neufeld, G. (1990). Characterization of the receptors for vascular endothelial growth factor. *J. Biol. Chem.* **265**, 19461-19466.

van Nieuw Amerongen, G. P., Koolwijk, P., Versteilen, A. and van Hinsbergh, V. W. (2003). Involvement of RhoA/Rho kinase signaling in VEGF-induced endothelial cell migration and angiogenesis *in vitro*. *Arterioscler. Thromb. Vasc. Biol.* **23**, 211-217.

Walker, M. B., Miller, C. T., Swartz, M. E., Eberhart, J. K. and Kimmel, C. B. (2007). phospholipase C, beta 3 is required for Endothelin1 regulation of pharyngeal arch patterning in zebrafish. *Dev. Biol.* **304**, 194-207.

Wang, S., Gebre-Medhin, S., Betsholtz, C., Stalberg, P., Zhou, Y., Larsson, C., Weber, G., Feinstein, R., Oberg, K., Gobl, A. et al. (1998). Targeted disruption of the mouse phospholipase C beta3 gene results in early embryonic lethality. *FEBS Lett.* **441**, 261-265.

Xia, C., Bao, Z., Yue, C., Sanborn, B. M. and Liu, M. (2001). Phosphorylation and regulation of G-protein-activated phospholipase C-beta 3 by cGMP-dependent protein kinases. *J. Biol. Chem.* **276**, 19770-19777.

Xie, W., Samoriski, G. M., McLaughlin, J. P., Romoser, V. A., Smrcka, A., Hinkle, P. M., Bidlack, J. M., Gross, R. A., Jiang, H. and Wu, D. (1999). Genetic alteration of phospholipase C beta3 expression modulates behavioral and cellular responses to mu opioids. *Proc. Natl. Acad. Sci. USA* **96**, 10385-10390.

Zeng, H., Dvorak, H. F. and Mukhopadhyay, D. (2001a). Vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF) peceptor-1 down-modulates VPF/VEGF receptor-2-mediated endothelial cell proliferation, but not migration, through phosphatidylinositol 3-kinase-dependent pathways. *J. Biol. Chem.* **276**, 26969-26979.

Zeng, H., Sanyal, S. and Mukhopadhyay, D. (2001b). Tyrosine residues 951 and 1059 of vascular endothelial growth factor receptor-2 (KDR) are essential for vascular permeability factor/vascular endothelial growth factor-induced endothelium migration and proliferation, respectively. *J. Biol. Chem.* **276**, 32714-32719.

Zeng, H., Zhao, D. and Mukhopadhyay, D. (2002). KDR stimulates endothelial cell migration through heterotrimeric G protein Gq/11-mediated activation of a small GTPase RhoA. *J. Biol. Chem.* **277**, 46791-46798.

Zeng, H., Zhao, D., Yang, S., Datta, K. and Mukhopadhyay, D. (2003). Heterotrimeric G alpha q/G alpha 11 proteins function upstream of vascular endothelial growth factor (VEGF) receptor-2 (KDR) phosphorylation in vascular permeability factor/VEGF signaling. *J. Biol. Chem.* **278**, 20738-20745.