Requirement of Protein Kinase D Tyrosine Phosphorylation for VEGF-A<sup>165</sup>-induced Angiogenesis through Its Interaction and Regulation of Phospholipase Cγ Phosphorylation<sup>*</sup>

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Vascular endothelial cell growth factor-A<sup>165</sup> (VEGF-A<sup>165</sup>) is critical for angiogenesis. Although protein kinase C-mediated protein kinase D (PKD) activation was implicated in the response, the detailed mechanism remains unclear. In this study, we found that VEGF-A<sup>165</sup>-stimulated tyrosine phosphorylation of PKD and the dominant negative mutant of PKD, PKD(Y463F), inhibited VEGF-A<sup>165</sup>-induced human umbilical vein endothelial cell (HUVEC) proliferation. In addition, PKD(S738A/S742A) overexpression inhibited VEGF-induced HUVEC migration. Furthermore, knockdown of PKD by its specific small interfering RNA inhibited VEGF-induced HUVEC proliferation and migration. Moreover transfection of PKD(Y463F), PKD(S738A/S742A), or PKD-small interfering RNA blocked VEGF-induced angiogenesis in vivo. Our signaling experiments show that KDR not Flt-1 mediated PKD tyrosine phosphorylation and KDR tyrosine residues 951 and 1059 were required for VEGF-A<sup>165</sup>-stimulated PKD serine and tyrosine phosphorylation, respectively. Whereas G protein G<sub>βγ</sub> subunits were required for both PKD serine phosphorylation and tyrosine phosphorylation, intracellular Ca<sup>2+</sup> mobilization was required for VEGF-A<sup>165</sup>-stimulated PKD tyrosine phosphorylation and phospholipase C (PLC) activity was required for PKD serine phosphorylation. Surprisingly, the PLC inhibitor did not inhibit PKD tyrosine phosphorylation. Instead, PKD tyrosine 463 was required for VEGF-A<sup>165</sup>-stimulated PLCγ tyrosine phosphorylation. Moreover, PKD interacted with PLCγ even in unstimulated cells, and PKD tyrosine 463 phosphorylation was not required for this interaction. Together, we demonstrate that PKD interacts with PLCγ and becomes tyrosine phosphorylated upon VEGF stimulation, leading to PLCγ activation and angiogenic response of VEGF-A<sup>165</sup>.

Pathological angiogenesis is a hallmark of cancer and various ischemic and inflammatory diseases. Many different cytokines and growth factors, such as vascular endothelial growth factor (VEGF-A<sup>165</sup>),<sup>4</sup> basic fibroblast growth factor; platelet-derived growth factor, and transforming growth factor-α, have an angiogenic activity (1–3). Among these, VEGF-A<sup>165</sup> stands out because of its potency and selectivity for vascular endothelium. VEGF-A<sup>165</sup> 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). VEGF-A<sup>165</sup> extensively reprograms 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 VEGF-A<sup>165</sup> 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). KDR, not Flt-1, is responsible for VEGF-A<sup>165</sup>-stimulated cell proliferation and migration in cultured endothelial cells and for microvascular permeability (18–22). However, Flt-1 functions to down-regulate KDR-mediated cultured endothelial cell proliferation (20, 23). The signal transduction pathways mediated by KDR involve KDR phosphorylation (18–22), phospholipase C (PLC) activation (22, 24–29), inositol 1,4,5-trisphosphate accumulation (30), intracellular Ca<sup>2+</sup> mobilization (23, 31), protein kinase C and MAPK activation (23, 25, 27–29). Whereas PLC activation is involved in VEGF-A<sup>165</sup>-induced human umbilical vein endothelial cell (HUVEC) proliferation and migration, intracellular Ca<sup>2+</sup> mobilization and MAPK are required for VEGF-A<sup>165</sup>-induced HUVEC proliferation but not migration (23). Our studies have shown that the G<sub>q/11</sub> family of heterotrimeric GTP-binding proteins and G<sub>βγ</sub> subunits mediate VEGF-A<sup>165</sup>-induced HUVEC migration and proliferation through interacting with KDR (32).

PKD, also known as protein kinase C<sub>μ</sub> (PKC<sub>μ</sub>), is a serine/threonine protein kinase with structure, enzymology, and regulatory properties different from the PKC family members. Its most unique feature includes the presence of a Ca<sup>2+</sup>-independ-
ent catalytic domain, a regulatory pleckstrin homology region, and a highly hydrophobic stretch of amino acids in its N-terminal region (33, 34). PKD can be activated in intact cells in response to numerous extracellular stimuli such as growth factors and ligands for G protein-coupled receptors (35–43). In all these cases, rapid PKD activation is believed to be mediated by PKC-dependent phosphorylation of Ser738 and Ser742 within the activation loop of the catalytic domain of PKD (44–46). PKD activation is associated with its translocation to the plasma membrane and subsequent transient accumulation in the nucleus (44–46). PKD overexpression markedly potentiates DNA synthesis induced by the G protein-coupled receptor agonist bombesin and vasopressin in Swiss 3T3 cells by increasing the duration of MAP kinase activation (47). On the other hand, PKD can also be tyrosine phosphorylated at tyrosine 463 in response to oxidative stress in HeLa cells, leading to activation of PKD (40, 48–50). Therefore, we set out to study the role of PKD in VEGF-stimulated angiogenesis.

In this study, we found that VEGF-stimulated tyrosine phosphorylation of PKD in addition to the previously reported serine 738/serine 742 phosphorylation (51). By overexpressing the dominant negative mutants, PKD(S738A/S742A) and PKD(Y463F), we found that tyrosine 463 phosphorylation was required for VEGF-A165-stimulated HUVEC proliferation but not migration and that VEGF-induced HUVEC migration involved PKD Ser738/Ser742 phosphorylation. Both tyrosine phosphorylation and serine phosphorylation of PKD were required for VEGF-A165-induced angiogenesis in vitro, but they were regulated by different signaling pathways. Furthermore, our results indicate that PKD tyrosine phosphorylation was required for PLCγ tyrosine phosphorylation in VEGF-A165-stimulated HUVEC. More importantly, immunoprecipitation experiments show that PKD physically interacted with PLCγ even in non-stimulated HUVEC.

EXPERIMENTAL PROCEDURES

Materials—Recombinant VEGF-A165 was obtained from R&D Systems (Minneapolis, MN). 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). Mouse monoclonal antibody against KDR and rabbit polyclonal antibody against PKD were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phosphotyrosine antibody (PY20) was obtained from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-phosphoserine PKD and mouse monoclonal antibody against PLCγ were obtained from Cell Signaling (Beverly, MA) and Transduction Laboratory (San Jose, CA), respectively. [3H]Thymidine was obtained from PerkinElmer Life Sciences. 

Cell Culture—Primary HUVEC were obtained from Clonetics (San Diego, CA). Cells were grown on plates coated with 30 μg/ml vitrogen in EGM-MV Bullet Kit (5% fetal bovine serum in endothelial basal medium with 12 μg/ml bovine brain extract, 1 μg/ml hydrocortisone, 1 μl/ml GA-1000, and EGF). HUVEC transduced with EGDR, EGLT, or EGDR mutants were grown in the same medium without EGF. HUVEC (passages 3 or 4) that were ~80% confluent were used for most experiments. Cells were serum starved in 0.1% fetal bovine serum in EBM for 24 h prior to treatment.

Overexpression of Proteins in HUVEC—PKD(S738A/S742A), PKD(S738D/S742D), and PKD(Y463F) (48) were subcloned to retrovirus vector pCMBP (52). A PKD-siRNA fragment isolated from pSuper-PKD siRNA plasmid (kindly provided by Dr. Toker at our institution) (49) was subcloned into pSuper-retro vector ( oligoEngine Inc. Seattle, WA). Retrovirus preparation and HUVEC infection with retrovirus were carried out as described (22, 23).

Proliferation Assays—Assays were carried out as described (22, 23). 2 × 10^3 HUVEC/well (infected or not with retrovirus) were seeded in 24-well plates. After 2 days, cells were serum starved (0.1% serum) for 24 h and then stimulated with 10 ng/ml VEGF-A165 for 20 h. 1 μCi/ml of [3H]thymidine was added to each well and 4 h later, cells were washed 3 times with cold phosphate-buffered saline, fixed with 100% cold methanol for 15 min at 4°C, precipitated with 10% cold trichloroacetic acid for 15 min at 4°C, washed 3 times with water, and lyed with 200 μl of 0.1 N NaOH for 30 min at room temperature. [3H]Thymidine incorporation was measured in scintillation solution. Data were expressed as the values for stimulated cell relative to the mean for its control group. Data are expressed as the mean ± S.D. of triplicate values.

Migration Assays—Assays were carried out as described (22, 23). Serum-starved HUVECs (infected with retrovirus) were detached from tissue culture plates as described (22, 23) and washed twice with endothelial basal medium containing 0.1% fetal bovine serum, and seeded (1 × 10^5 cells per well) into the transwells coated with vitrogen (30 μg/ml) and the transwells were inserted in a 24-well plate containing 1 ml of the same medium. Cells over a range of 3 × 10^3 to 1 × 10^6 per well were seeded in a 96-well plate for standard curve. Cells were incubated at 37°C for 1 h to allow the cells to attach, then VEGF-A165 was added at a final concentration of 10 ng/ml. After incubation for an additional 2 h, cells remaining on the upper surface of the transwell filter membrane were wiped off with a cotton tip. The whole transwell membrane was cut out and placed in an individual well of the 96-well plate, which contains the cells for standard curve. Two hundred μl of CyQuant DNA stain was added to each well containing cells or membrane and the plate was kept at 4°C overnight. After warming to room temperature, stained cells were counted in a spectrofluorometer (SpectraFluor; TECAN) with Delta Soft 3 software. Data were expressed as the mean ± S.D. of quadruplicate values. All experiments were repeated at least three times.

Protein Kinase Phosphorylation—Serum-starved HUVECs transduced with different plasmids as indicated were treated with 10 ng/ml VEGF-A165, or 10 ng/ml EGF in the case of EGDR, EGLT, or mutants, for different time intervals as indicated. Cell lysates were either subjected to immunoblot analysis using an antibody specific for phosphoserine PKD, or immunoprecipitated with antibodies against PY20 or PKD followed by immunoblotting with antibodies as indicated. All experiments were repeated 3 times.
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Matrigel Angiogenesis Assays—Matrigel angiogenesis assays were carried out as described (53). SKMEL/VEGF cells (1 × 10⁷), alone or mixed with 1 × 10⁷ PT67 cells infected with retroviruses expressing various PKD mutants, were suspended in 0.5 ml of growth factor reduced Matrigel (BD Biosciences) and injected subcutaneously into Nu/Nu mice. Tissues were harvested, photographed, and fixed with 4% paraformaldehyde for immunohistochemistry. Each experiment was replicated on 8 mice.

Quantitative Analysis of Plasma Volumes in Matrigel Assays—These assays were carried out as described (53). Mice (4 per group) implanted with various cell combinations in Matrigel were anesthetized with Avertin (tribromoethanol, 200 mg/kg) and injected intravenously via the tail vein with 0.2 ml of Evans blue dye (5 mg/ml in saline). After 5 min, blood was collected in heparin by cardiac puncture and centrifuged at 14,000 g for 10 min to obtain platelet poor plasma that was diluted in formamide for measurement of Evans blue dye concentration. Animals were euthanized by CO₂ narcosis and Matrigel plugs were dissected free by cautery to prevent blood loss, weighed, and extracted with 2 ml of formamide at room temperature for 3 days. Dye in plasma or extracted from Matrigels was measured at 620 nm in a Thermo Max microplate reader (Molecular Devices, Menlo Park, CA) using Softmax 881 software. Standard curves were generated by measurement of serial dilutions of Evans blue dye in formamide (µg/ml). Intravascular plasma volumes (microliters per g of Matrigel) were calculated on the basis of Evans blue dye concentrations in blood plasma to provide an absolute measure of the volume of plasma in the vascular bed.

Immunohistochemistry— Implanted Matrigel plugs were dissected free, fixed in 4% paraformaldehyde for 4 h, changed to 30% sucrose overnight, and embedded in OCT compound. Frozen sections were then blocked with 5% goat serum and stained with the rat anti-mCD31 antibody (1:50 dilution, BD Biosciences) at room temperature for 1 h. Sections were then washed 3 times with phosphate-buffered saline and incubated for 1 h with biotinylated polyclonal anti-rat IgG antibody (1:500 dilution). Sections were then washed 3 times with phosphate-buffered saline, reacted with the ABC peroxidase kit (Vector Laboratories, Inc. Burlingame, CA) at room temperature for 45 min, and washed twice with phosphate-buffered saline prior to mounting for light microscopy and photography.

Animal Welfare—All animal experiments were performed in compliance with the Beth Israel Deaconess Medical Centers Animal Care and Use Committee.

Statistics—Analysis of variance and the Tukey-Kramer multiple comparisons test were used to determine statistical significance.

RESULTS

VEGF-A165-stimulated PKD Phosphorylation at Serine and Tyrosine Residues—HUVEC was treated with VEGF-A165 for the indicated times. Equal amounts of cellular extracted proteins were immunoblotted with an antibody against serine 738/742-phosphorylated PKD. Our data show that VEGF-A165 induced PKD serine 738/742 phosphorylation (Fig. 1A) as reported most recently (51). To further examine whether VEGF-A165 induced tyrosine phosphorylation of PKD, HUVEC were treated with VEGF-A165 for similar period of times. Equal amounts of total cell proteins were immunoprecipitated with antibodies against phosphotyrosine antibody (PY20) or PKD, and the immunoprecipitates were then subjected to immunoblot analysis using an antibody against total PKD or PY20. The results show that VEGF-A165-stimulated PKD tyrosine phosphorylation in HUVEC in a time-dependent manner (Fig. 1B).

Role of PKD Tyrosine Phosphorylation and Serine Phosphorylation in VEGF-A165-stimulated HUVEC Proliferation and Migration—Previously, Wong and Jin (51) showed that PKCα-mediated PKD serine 738/742 phosphorylation is involved in VEGF-stimulated HUVEC proliferation. Because PKD tyrosine phosphorylation was shown to be required for PKD activation in response to oxidative stress, we also examined whether PKD tyrosine phosphorylation is involved in VEGF-stimulated HUVEC proliferation. To do this, we overexpressed a dominant negative mutant of PKD, PKD(Y463F), in HUVEC with our retrovirus expression system that gave almost 100% infection yield in HUVEC (22). As expected, VEGF-A165-stimulated thymidine incorporation in HUVEC transduced with LacZ (Fig. 2A, lane 2 versus lane 1, p < 0.001). Baseline thymidine incorporation was unaffected in HUVEC transduced with PKD(Y463F) (lane 3 versus lane 1, p > 0.05) but the response to VEGF-A165 was strikingly inhibited (lane 4 versus lane 2, p < 0.001). To further confirm that PKD is required for VEGF-A165-stimulated HUVEC proliferation, we used PKD-siRNA to knockdown the expression of PKD in HUVEC (Fig. 2B). Our data show that VEGF-A165-stimulated HUVEC proliferation was blocked in HUVEC expressing PKD-siRNA (Fig. 2A, lane 6 versus lane 2, p < 0.001). However, negative control siRNA (SiNEG) had no effect (Fig. 2A, lane 8 versus lane 2, p > 0.05).
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We further tested whether PKD played an important role in VEGF-A\textsuperscript{165}-induced angiogenesis in vivo with the Matrigel angiogenesis assay (53). To elucidate the mechanisms of angiogenesis, it would be desirable to modulate the expression of individual vascular genes in vivo using the gene overexpression and silencing approaches that have proved to be so powerful in vitro. Recently a novel system has been developed that allowed us to introduce DNA into endothelial cells in vivo (53). SK-MEL-2 tumor cells transfected to overexpress VEGF-A\textsuperscript{165} (SK-MEL/VEGF cells) were mixed with PT67 cells packaging retroviruses that expressed PKD(S738A/S742A) and PKD(F463F). The cell mixtures were incorporated into Matrigels that were implanted in the subcutaneous space of nude mice. As previously reported (53), VEGF-A\textsuperscript{165} secreted by SK-MEL/VEGF-A\textsuperscript{165} cells induces nearby vascular endothelial cells to divide and therefore to become susceptible to infection with retroviruses secreted by PT67 packaging cells.

The angiogenic response that developed after implantation of Matrigel plugs containing various cell mixtures was evaluated on day 3 (Fig. 3A). Angiogenesis was assessed by microscopy (top panels) and by histology and immunohistochemistry for the endothelial cell marker CD31 (bottom panels). Matrigel plugs containing only PT67 cells packaging LacZ-expressing retroviruses (PT67/LacZ cells) induced minimal angiogenesis (lane 1). However, strong angiogenesis with typical “mother” vessels was induced in plugs containing SK-MEL/VEGF-A\textsuperscript{165} cells (lane 2). Mother vessels are enlarged, thin-walled, peri- genotype-poor vessels that are the first new vessel type induced by VEGF-A\textsuperscript{164} in vivo (54). The angiogenic response and mother vessel formation were strikingly depressed by inclusion of PT67/PKD(Y463F) cells (Fig. 3A, lane 3), PT67/PKD(S738A/S742A) cells (Fig. 3A, lane 4), and PT67/PKD-siRNA (Fig. 3A, lane 5). As expected, no effect was seen on the angiogenic response and the mother vessel formation in Matrigel containing PT/SiNEG (Fig. 3A, lane 6).

We next used the intravascular plasma volume of Matrigel plug-associated blood vessels as a novel measure to quantitate the angiogenic response (53). Intravascular plasma volume is an appropriate measure as enlarged mother vessels are a signature property of the early angiogenic response to VEGF-A (54, 55). Evans blue dye was injected intravenously into mice 3 days after implanting Matrigel plugs containing various cell mixtures. Evans blue dye binds to plasma proteins and therefore the amount of plasma within the Matrigel-associated vasculature can be calculated from simultaneous measurements of dye concentration in peripheral blood plasma. Matrigel plugs were harvested 5 min after intravenous dye injection, when blood vessels were filled with dye-plasma protein complexes but before there was time for significant extravasation. In Matrigel plugs con-
containing VEGF-A\textsuperscript{165}-expressing SKMEL/VEGF cells (alone or with PT67/LacZ cells), intravascular plasma volume as measured by Evans blue dye accumulation increased >2-fold above baseline levels (Fig. 3B, lane 2 versus lane 1, \( p < 0.001 \)). The presence of PT67/PKD(Y463F) cells, PKD(S738A/S742A) cells, or PT67/PKD-siRNA strikingly inhibited the angiogenic response expected from SKMEL/VEGF cells (Fig. 3B, lanes 3–5 versus lane 2, all \( p < 0.001 \)). Inclusion of PT67/SiNEG had no effect (Fig. 3B, lane 6 versus lane 2, \( p > 0.05 \)). The quantitative measurements of vascular plasma volumes presented in Fig. 3B therefore confirm the qualitative measures of angiogenesis presented in Fig. 3A. In \textit{in situ} hybridization indicated that the expression levels of VEGF-A\textsuperscript{165} were similar in Matrigels containing these different cell mixtures (see Ref. 53); Also, transfection of SKMEL and PT67 cells with PKD(Y463F) and PKD(S738A/S742A) has no effect on these cells proliferation (data not shown). Therefore, the results obtained cannot be attributed to effects on VEGF-A expression and the effect on SKMEL and PT67 cells.

**Signaling Pathways That Regulated VEGF-stimulated PKD Serine Phosphorylation and Tyrosine Phosphorylation**—To identify which VEGF receptor mediates PKD tyrosine phosphorylation and serine phosphorylation, we used the recently developed receptor chimera (EGDR and EGLT) in which the N-terminal domains of KDR or Flt-1 were replaced with that of EGFR to dissect the signaling pathways mediated by KDR or Flt-1 (22). It was previously shown that HUVECs were not responsive to EGF treatment in the experimental conditions used (22). As expected, in the HUVEC transduced with LacZ-expressing viruses, EGF did not stimulate PKD serine phosphorylation (Fig. 4A, first panel). When serum-starved HUVEC transduced with EGDR- or EGLT-expressing viruses were stimulated with EGF for different time intervals, it is EGDR but not EGLT that mediated PKD serine phosphorylation in a similar time course as in VEGF-A\textsuperscript{165}-stimulated HUVEC (Figs. 1A and 4A, second and third panels). Recently, we demonstrated that tyrosines 1059 and 951 of KDR are required for VEGF-induced proliferation and migration, respectively.
The data show that PKD tyrosine phosphorylation was completely blocked by overexpression of hβ2AK1(495) and pretreatment with the general PLC inhibitor U73122, however, pretreatment with BAPTA/AM or U733443 did not inhibit PKD serine phosphorylation in VEGF-A165-stimulated HUVEC (Fig. 4B). These results indicate that the Gβγ subunit of G protein and PLC activity are key intermediaries for VEGF-A165-induced PKD serine phosphorylation. However, intracellular Ca2+ mobilization is not required.

**Signaling Pathways That Regulated VEGF-stimulated PKD Tyrosine Phosphorylation**—We further studied the signaling pathway that regulated PKD tyrosine phosphorylation. As expected, in the HUVEC transduced with LacZ-expressing viruses, EGF did not stimulate PKD tyrosine phosphorylation (Fig. 5A, first panel). When serum-starved HUVEC transduced with EGDR- or EGLT-expressing viruses were stimulated with EGF for different time intervals, it was EGDR but not EGLT that mediated PKD tyrosine phosphorylation in a similar time course as in VEGF-A165-stimulated HUVEC (Figs. 1B and 5A, second and third panels). Furthermore, EGDR(Y1059F) could not mediate PKD tyrosine phosphorylation in response to EGF stimulation as shown in Fig. 5A (fourth panel). Therefore, we examined whether the EGDR mutants, EGDR(Y951F) and EGDR(Y1059F), are required for VEGF-stimulated PKD serine phosphorylation in HUVEC. As shown in Fig. 4A (fourth panel), EGDR(Y951F) cannot mediate PKD serine phosphorylation in response to EGF stimulation. However, EGDR(Y1059F) has no effect (Fig. 4A, fifth panel). These results indicate that tyrosine 951, not tyrosine 1059, is essential for PKD serine phosphorylation. It was shown that the Gβγ subunits of G protein and PLCγ tyrosine phosphorylation, but not intracellular Ca2+ mobilization were required for VEGF-stimulated HUVEC migration (22, 24, 25, 27–29, 31, 32). Therefore, we examined whether these molecules were required for VEGF-A165-stimulated PKD serine phosphorylation. Serum-starved HUVEC that were transduced with the Gβγ minigene, hβAPK1(495), or pretreated with U73122 (an inhibitor of the PLC family), U733443 (the negative control of U73122), or BAPTA/AM (an intracellular Ca2+ chelator) for 5 min were stimulated with VEGF-A165 for different times as indicated. Cellular extracts were immunoblotted with antibodies against serine-phosphorylated PKD (pSer-PKD) (left panel) and PKD to confirm equal protein loading (right panel). The experiments were repeated 3 times.
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**A**  

| VEGF-A\textsuperscript{165} | 0 | 1 | 5 | 10' | 0 | 1 | 5 | 10' |
|-----------------------------|---|---|---|-----|---|---|---|-----|
| LacZ                        |   |   |   |     |   |   |   |     |
| PKD(Y463F)                  |   |   |   |     |   |   |   |     |
| PKD(S738A/S742A)            |   |   |   |     |   |   |   |     |
| PKD-siRNA                   |   |   |   |     |   |   |   |     |
| PKD (S738A/S742A)           |   |   |   |     |   |   |   |     |

**B**  

| VEGF-A\textsuperscript{165} | 0 | 1 | 5 | 10 min |
|-----------------------------|---|---|---|--------|
| HUVEC                       |   |   |   |        |
| PKD(Y463F)                  |   |   |   |        |
| PKD(S738A/S742A)            |   |   |   |        |

FIGURE 6. PKD physically interacted with PLC\textgamma and regulated PLC\textgamma tyrosine phosphorylation.  

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Our data clearly show that either PKD dominant negative mutants or PKD siRNA had no effect on VEGF-A\textsuperscript{165}–stimulated KDR tyrosine phosphorylation (Fig. 6A, panel II). The same cellular extracts were immunoblotted with an antibody against MAPK to confirm the equal amount of proteins in all samples (Fig. 6A, panel III).

**PKD Physically Interacts with PLC\textgamma Even in the Quiescent State**—To further study the mechanism by which PKD regulates PLC\textgamma activation, we first tested whether VEGF-A\textsuperscript{165}–stimulated PKD interaction with PLC\textgamma. Serum-starved HUVEC were stimulated with VEGF-A\textsuperscript{165} for different times as indicated. Cellular extracts were immunoprecipitated with an antibody against PKD and then immunoblotted with an antibody against PLC\textgamma. Unfortunately, PKD interacted with PLC\textgamma in the absence of VEGF-A\textsuperscript{165} stimulation (Fig. 6B). VEGF-A\textsuperscript{165} treatment did not further stimulate PKD and PLC\textgamma interaction (Fig. 6B). To further confirm the PKD and PLC\textgamma interaction, we tested the effect of PKD dominant negative mutants on this interaction. Serum-starved HUVEC that were transduced with PKD(Y463F) or PKD(S738A/S742A) were stimulated with VEGF-A\textsuperscript{165}. Cellular extracts were immunoprecipitated with an antibody against PKD and then immunoblotted with an antibody against PLC\textgamma. As shown in Fig. 6B, overexpression of PKD(Y463F) and PKD(S738A/S742A) had no effect on the PKD and PLC\textgamma interaction.

**DISCUSSION**

The unique serine/threonine protein kinase PKD is typically activated through protein kinase C–dependent phosphorylation of serine 738 and serine 742 at the activation loop in response to stimulation of growth factor receptors and G-protein-coupled receptors (35–43). Consistent with this pathway of PKD activation, it was very recently shown that PKD can be phosphorylated at Ser\textsuperscript{738}/Ser\textsuperscript{742} by protein kinase Ca in VEGF-A\textsuperscript{165}–stimulated HUVEC and knock-down of PKCa or PKD expression by their respective siRNA inhibited VEGF-A\textsuperscript{165}–stimulated HUVEC proliferation (51). Interestingly, Storz et al. (48) reported a novel pathway for PKD activation, in which phosphorylation of PKD tyrosine 463 at the pleckstrin homology domain is essential for its activation by the Src-Abl pathway in response to oxidative stress. In this study, we found that PKD is not only phosphorylated at Ser\textsuperscript{738}/Ser\textsuperscript{742}, but also tyrosine phosphorylated presumably at tyrosine 463 in HUVEC stimulated with VEGF-A\textsuperscript{165}. Importantly, overexpression of PKD(Y463F) markedly inhibited VEGF-A\textsuperscript{165}–stimulated HUVEC proliferation, suggesting that in addition to serine phosphorylation, tyrosine 463 phosphorylation of PKD is also important for the VEGF response. Our data further indicate that Ser\textsuperscript{738}/Ser\textsuperscript{742} phosphorylation, not tyrosine 463 phosphorylation of PKD was required for VEGF-A\textsuperscript{165}–stimulated HUVEC migration. These results are consistent with our previous findings that VEGF-A\textsuperscript{165}–stimulated HUVEC proliferation and migration by different signaling pathways (22, 23, 32, 56, 57).

It was previously shown that VEGF-A\textsuperscript{165} stimulates HUVEC proliferation through the G\textbeta\gamma subunits of G protein and PLC activity (22, 23, 32, 56). Our data clearly show that the G\textbeta\gamma subunit of the G protein was required for PKD tyrosine phosphorylation. Surprisingly, the general PLC inhibitor U73122
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could not inhibit VEGF-A165-stimulated PKD tyrosine phosphorylation, suggesting that PLC\(\gamma\) activation may be downstream of PKD tyrosine phosphorylation (Fig. 7). Indeed, overexpression of PKD(Y463F) completely inhibited VEGF-A165-stimulated PLC\(\gamma\) tyrosine phosphorylation. Tyrosine phosphorylation of PLC\(\gamma\) is known to be necessary for VEGF-A165-stimulated MAP kinase activation and endothelial cell growth (28, 58). Although PLC\(\gamma\) tyrosine phosphorylation was believed to be mediated by the tyrosine kinase activity of VEGF receptor 2 (KDR) (58), our new findings show that another KDR autophosphorylation site, Tyr1059, mediates PKD tyrosine phosphorylation and subsequent PLC\(\gamma\) phosphorylation. The detailed mechanism about how these molecules interact with and activate each other remains to be investigated. Further studies indicate that PKD physically interacted with PLC\(\gamma\) even in the absence of VEGF-A165 stimulation.

The molecular mechanism by which oxidative stress induces the tyrosine phosphorylation of PKD in HeLa cell was shown to be mediated the Src-Abl pathway (49). This ROS-Src pathway may also be involved in VEGF-A165-induced PKD-mediated PLC\(\gamma\) activation based on several lines of evidence. First, reac-

tive oxygen species was known to play an essential role in VEGF-A165-stimulated endothelial cell proliferation (59, 60). Second, Src is required for VEGF-A165-induced PLC\(\gamma\) activation and angiogenesis (61, 62). Because our data also show that there is a constitutive association between PKD and PLC\(\gamma\), we speculate that PKD might act as a scaffold to recruit SH2 domain-containing Src to this complex to phosphorylate PLC\(\gamma\).

In summary, we have uncovered a novel mechanism in which PKD interacts with PLC\(\gamma\) and upon KDR stimulation, PKD become serine phosphorylated and tyrosine phosphorylated by different pathways as described in the legend to Fig. 7. The phosphorylated tyrosine of PKD likely acts as a co-docking site together with the phosphorylated KDR to activate PLC\(\gamma\) that in turn results in VEGF-A165-stimulated HUVEC proliferation and angiogenesis (Fig. 7).

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