Identification of Protein Kinase D2 as a Pivotal Regulator of Endothelial Cell Proliferation, Migration, and Angiogenesis*

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Angiogenesis, the fundamental physiological process by which new blood vessels are generated from pre-existing vascular. It plays a crucial role in embryonic development in addition to numerous normal physiological processes. Angiogenesis is critically involved in a wide variety of human diseases including ischemic vascular diseases, tumor growth and metastasis, diabetic retinopathy, age-related macular degeneration, and rheumatoid arthritis (1–3). Emerging evidence indicates that angiogenesis also plays a critical role in the development and progression of atherosclerosis, a chronic inflammatory disease of the vessel wall (4–7). For these reasons, an understanding of the complex mechanisms that regulate angiogenesis is seen not only as a fundamental problem in human biology but also critical in fulfilling the important goal of biomedical research to develop more specific and efficacious pro- and anti-angiogenic therapies.

The process of angiogenesis depends on endothelial cell (EC) proliferation, migration, and differentiation. It is controlled by a variety of positive and negative signals. Vascular endothelial growth factor (VEGF, also termed VEGF-A) is the most critical and potent of all the known proangiogenic factors, and its proangiogenic effect is mediated through the VEGF receptor 2 (VEGFR2, also termed Flk-1 or KDR) that is selectively expressed in vascular ECs (8). Besides VEGF, fibroblast growth factors (FGFs) and their receptors that are expressed on ECs also play a crucial role in angiogenesis (9). ECs express the FGF receptor 1 (FGFR1), and under some circumstances, FGFR2. Activation of FGFR1 or FGFR2 promotes EC proliferation, migration, and angiogenesis (9). Additionally, angiopoietins, transforming growth factors, cytokines including interleukins and chemokines, and the extracellular matrix also regulate angiogenesis (9). It has been demonstrated that many cell types including leukocytes, vascular cells, epithelial cells, tumor cells, and stromal cells can produce various proangiogenic factors such as VEGF and FGF in response to stimuli. The regulation of those proangiogenic factors has been well studied (8–11), and we have learned much about VEGFR2 and FGFR1 signal transduction. However, comparatively less is known about the regulation of VEGFR2 and FGFR1 expression in ECs. Identification of targetable (drugable) molecules or pathways regulating the expression of these key growth factor receptors on ECs may lead to more specific and efficacious pro- and anti-angiogenic therapies.

Protein kinase D2 (PKD2) is a member of a newly described serine/threonine protein kinase family that includes PKD1 (also known as protein kinase C-\(\mu\)) and PKD3 (protein kinase C-\(\nu\)) (12, 13). PKD contains a tandem repeat of zinc finger-like cysteine-rich motifs at its N terminus that display high affinity for diacylglycerol or phorbol ester, a pleckstrin homology domain, and a C-terminal catalytic domain that shares homology with the calmodulin-dependent kinases (12, 13). Although PKD family kinases exhibit a homologous catalytic domain, they vary with respect to their subcellular localization, expression, and regulation (12–15). It has been demonstrated that...
PKD2 has unique N-terminal and C-terminal domains that determine its nucleocytoplasmic shuttling, activation, and substrate targeting (14, 16, 17). To date, PKD1 is the best characterized isoform of this family, and it has been implicated to be functionally involved in cell migration, survival/apoptosis, membrane trafficking, and the expression of gene sets modulated by histone deacetylases (12). In contrast, relatively little is known about the biological functions of PKD2 and PKD3. It has been shown that overexpression of PKD2 potentiates DNA synthesis by bombesin in Swiss 3T3 cells (18). PKD2s also regulate the activation of nuclear factor κB evoked by the Bcr-Abl tyrosine kinase and by lysophosphatidic acid in human myeloid leukemia cells and human colonic epithelial cells, respectively (19, 20). Moreover, a recent report has shown that PKD2 regulates EC migration by VEGF, although the mechanism is unclear (21). Despite these intriguing observations, the specific physiological functions of PKD2 in EC biology, including the identity of PKD2-regulated genes and of PKD2 protein substrates in ECs, are largely unknown.

In this report, we have identified PKD2 as a pivotal regulator of EC proliferation, migration, and angiogenesis. We show that PKD2 is expressed in primary human ECs from different tissues and is an important PKD isoform mediating the phosphorylation of PKD substrates in ECs. We further show that PKD2, but not its isoform PKD1, is required for EC expression of both VEGF2 and FGF1, two key growth factor receptors involved in angiogenesis. Lastly, we have accumulated substantial lines of evidence indicating that PKD2 is crucial for EC proliferation, migration, and angiogenesis.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Recombinant human VEGF165 and protein tyrosine phosphatase (PTP)-MEG2 antibody were from R&D Systems (Minneapolis, MN). Phorbol-12-myristate-13-acetate (PMA) was from Calbiochem. CellTiter 96 AQ-supus one solution reagent for cell proliferation assay was from Promega (Madison, WI). The growth factor-reduced Matrigel was from BD Biosciences. Antibodies against protein kinase C-μ/ PKD1/2 (C-20), VEGF2, FGF1, intercellular adhesion molecule-1 (ICAM-1), platelet-endothelial-cell adhesion molecule-1 (PECAM-1), transforming growth factor β type-II receptor (TGFB-RII), p38α, and vinculin were from Santa Cruz Biotechnology (Santa Cruz, CA). Insulin-like growth factor-1 receptor-β (IGF1-RB), phospho-extracellular signal-regulated kinase (ERK) (Thr-202/Tyr-204), and phospho-(Ser/Thr) PKD substrate antibodies and reagents for chemiluminescence detection were from Cell Signaling (Beverly, MA). Antibodies against PKD2 and phospho-PKD2 (Ser-876) were from Upstate Biotechnology (Lake Placid, NY). Human endoglin monoclonal antibody (SN6h) was from DakoCytomation (Carpinteria, CA).

**Cell Culture**—Human coronary artery EC, human aortic EC, human umbilical vein EC (HUVEC), human lung microvascular EC, and human pulmonary artery EC were obtained from Lonza (Walkersville, MD) and cultured in EGM-2 complete medium containing 2% (v/v) fetal bovine serum (FBS). multiple recombinant human growth factors including VEGF, FGF, IGF1, and epidermal growth factor, as well as hydrocortisone, heparin, and ascorbic acid. Experiments were performed using cells up to passage 8. Where appropriate, cells were starved in serum-free basal EBM-2 medium for 2 h prior to agonist treatment. WI-38 human normal lung fibroblasts, H441 and A549 human lung cancer cell lines, were from American Type Culture Collection (Manassas, VA) and cultured in the appropriate media according to the manufacturer’s instructions.

**Small Interference RNA (siRNA) and siRNA Transfection**—AllStars non-targeting negative control siRNA (catalog number 1027280) and the validated human PKD1 siRNA-1 (PKD1-S1, catalog number SI00301350), PKD1 siRNA-2 (PKD1-S2, catalog number SI00042378), PKD2 siRNA-1 (PKD2-S1, catalog number SI0224768), PKD2 siRNA-2 (PKD2-S2, catalog number SI0224775), p38α siRNA (catalog number SI00605157), and PTP-MEG2 siRNA (catalog number SI02759253) were from Qiagen (Valencia, CA). The ON-TARGETplus SMART-pool duplexes of predesigned human PKD1 siRNA-3 (PKD1-S3, catalog number 005028) and human PKD2 siRNA-3 (PKD2-S3, catalog number 004197) were from Dharmaco (Chicago, IL). For siRNA transfection, HUVECs were seeded into different plates for 24 h to reach 50–70% confluence, and then siRNA was transfected into HUVECs in a final concentration of 20 nM by using Lipofectamine 2000 and Opti-MEM reduced serum medium according to the manufacturer’s protocols (Invitrogen). The medium was replaced with fresh EGM-2 medium 12 h after transfection. The silencing effects of siRNAs were then confirmed by Western blotting.

**Western Blotting and Immunoprecipitation**—Western blotting and immunoprecipitation were performed as described previously (22).

**EC Proliferation Assay**—HUVECs were seeded into 96-well plate at 1.2 × 10^4 cells/well or 24-well plate at 6 × 10^4 cells/well for 24 h to reach 50–70% confluence and then transfected with 20 nM siRNA duplexes for 24 h. The cells were then washed twice with Hanks’ balanced salt solution and incubated for additional 72 h in basal EBM-2 medium containing 0.1% FBS, EBM-2 containing 0.1% FBS and VEGF (100 ng/ml), or EGM-2 complete medium as indicated. The proliferation of the transfected cells in 96-well plate was assessed by CellTiter 96 AQ-supus one solution reagent according to the manufacturer’s protocols (Promega, Madison, WI). In addition, the cells in 24-well plates were trypsinized, and viable cells were counted with a Z1 cell counter (Beckman). Alternatively, HUVECs were first seeded into 6-well plates at a density of 2.5 × 10^3 cells/well, transfected with 20 nM siRNA duplexes, and grown in EGM-2 medium for 60 h. After transfection, HUVECs were trypsinized, and viable cells were replated into 96-well plates at 1.2 × 10^4 cells/well and grown in basal EBM-2 medium containing 0.1% FBS, EBM-2 containing 0.1% FBS and VEGF (100 ng/ml), or EGM-2 complete medium for an additional 72 h. The proliferation of the transfected cells was then assessed by CellTiter 96 AQ-supus one solution reagent (Promega).

**Cell Migration Assay**—HUVEC migration was measured essentially by a modified Boyden chamber assay using 24-well plate Transwells (Corning, Lowell, MA) with an 8.0-μm pore size polycarbonate membrane as we described previously (23). Polycarbonate membranes of Transwell inserts were coated with or without a thin layer of growth factor reduced Matrigel (1:10 dilution) (BD Biosciences) for 1 h at room temperature.
The inserts were placed over the lower chambers that have been filled with serum-free basal EBM-2 or EGM-2 complete medium. HUVECs were transfected with siRNAs and grown in EGM-2 medium for ~90 h. The cells then were collected by trypsinization, washed, and resuspended in serum-free EBM-2 medium supplemented with 0.15% FBS. Viable cells (5 × 10^5) in 0.15 ml were added to the inside of each insert and incubated for 24 h at 37 °C. HUVECs remaining on the upper side of the insert membrane were mechanically removed with cotton tips, and the filter membranes were fixed in methanol. Cells that had migrated to the under side of filters were stained with Giemsa stain (Sigma) and enumerated under a microscope at a final magnification of ×200. Five random microscopic fields were counted.

**Cell Adhesion and Spreading Assays**—HUVECs were transfected with siRNAs and grown in EGM-2 medium for ~90 h. The cells were collected by trypsinization, washed, and resuspended in serum-free EBM-2 medium supplemented with 0.15% FBS. The cells were then replated on fibronectin-coated 24-well plates, and the adhesion of cells to fibronectin and their spreading were measured as we described previously (23).

**RNA Isolation and Reverse Transcription (RT)-PCR**—Total RNA was isolated using the RNeasy RNA isolation kit, and semiquantitative RT-PCR was performed according to the manufacturer’s protocols (Qiagen). RT-PCR primers were designed with the Oligo6 software (Molecular Biology Insights, Cascade, CO) as follows: VEGFR2 (expected product of 406 bp), 5′-GCC TGC AAC AAA GTC GG-3′ (forward) and 5′-TGT CCA CAC GCT CTA GGA CTG-3′ (reverse); FGFR1 (418 bp), 5′-CTC TGA CAA GGG CAA CTA CAC-3′ (forward) and 5′-GGC TTC CAG AAC GGT CAA C-3′ (reverse); and internal control glyceraldehyde-3-phosphate dehydrogenase, 5′-CGC TGA CTG GTT GGA G-3′ (forward) and 5′-GAG GAG TGG GTG TCG CTG TG-3′ (reverse). Relative change in mRNA was measured by densitometric analysis and normalized to glyceraldehyde-3-phosphate dehydrogenase.

**In Vitro Angiogenesis Assay**—The in vitro angiogenesis was determined by measuring the formation of capillary-like structures of ECs in growth factor-reduced Matrigel (BD Biosciences). HUVECs were transfected with siRNAs and grown in EGM-2 medium for ~90 h. The cells were collected by trypsinization, washed, and resuspended in serum-free EBM-2 medium supplemented with 0.15% FBS. Viable cells were then replated at a density of 5 × 10^4 cells/well on growth factor-reduced Matrigel (10 mg/ml)-coated 48-well plates and incubated at 37 °C in basal EBM-2 medium with 0.1% FBS, EBM-2 with 0.1% FBS and VEGF (100 ng/ml), or EGM-2 medium for 8–14 h as indicated. The formation of EC tube-like structures was viewed by phase-contrast microscopy, and random fields were photographed using a Nikon TS-100F microscope equipped with a DXM 1200 digital camera and fitted with a CFI plan achromat ×4 objective (Nikon Instruments, Lewisville, TX). ACT-1 software was used for image processing.

**RESULTS**

**PKD2 Is Expressed in Primary Human ECs from Different Tissues**—We determined the expression level of PKD2 in five primary human ECs at passage 4. As shown in Fig. 1A (top panel), PKD1 (115 kDa) and PKD2 (105 kDa) were detected in Western blots of cell extracts prepared from all five of the primary human ECs from different tissues using a PKD1/2 (C-20) antibody that recognizes both PKD1 and PKD2 (13). It appears that HUVECs and human lung microvascular EC expressed more PKD2 than PKD1, whereas artery ECs such as human coronary artery ECs and human aortic ECs had more PKD1 than PKD2. A single band corresponding to PKD2 molecular mass (105 kDa) was detected in the ECs when an antibody specific against PKD2 was used (Fig. 1A, second panel). These findings indicate that PKD2 is expressed in a range of primary human ECs. In non-ECs, we found that WI-38 human lung fibroblasts predominantly expressed PKD1, whereas A549 human lung carcinoma cells expressed an abundant amount of PKD2, and H441 human lung adenocarcinoma cells mainly expressed PKD2 isoform (Fig. 1B).

**Activation of PKD2 by VEGF and PMA in HUVECs**—There are no published studies of the activation of PKD2 in ECs, although the activation of PKD1 by VEGF has been reported in ECs (24). The activation of PKD2 is believed to be mediated by an initial protein kinase C-dependent phosphorylation of two key serine residues in the activation loop followed by an auto-phosphorylation of Ser-876 in the enzyme (12, 13). Thus it is feasible to monitor PKD2 activation using a phospho-specific antibody against Ser-876-phosphorylated PKD2 (13). As shown in Fig. 2A (top panels), both VEGF and PMA activated PKD2 by inducing a strong autophosphorylation of PKD2 on Ser-876. Knockdown of PKD2 with validated human PKD2 siRNAs abolished the phosphorylation of PKD2 Ser-876 induced by

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**FIGURE 1. PKD2 expression in human ECs and non-ECs.** Cell lysates from five primary human ECs (A) or non-ECs (B) as indicated were subjected to Western blotting with a PKD1/2 antibody that recognizes both PKD1 and PKD2 or with a PKD2-specific antibody. The blots were stripped and reprobed with vinculin antibody to show the equal loading of lysates. Results are representative of Western blots of three independent experiments.

**FIGURE 2. Activation of PKD2 by VEGF and PMA in ECs.** HUVECs were transfected with 20 nM AllStars non-targeting negative control siRNA (con.) or the validated human PKD1 siRNA-1 or PKD2 siRNA-1, grown in EGM-2 medium for 72 h, and then starved for 2 h and left untreated (−) or treated for 10 min with VEGF165 (25 ng/ml) or PMA (30 nM). Immunoblotting with Ser-876-phosphorylated PKD2 antibody or antibodies against PKD2 or vinculin as indicated. A, HUVECs were starved for 2 h and then stimulated for 10 min with VEGF165 (25 ng/ml). PKD2 was immunoprecipitated (IP) with a PKD2-specific antibody and subjected to Western blotting with antibodies against Ser-876-phosphorylated (pS876) PKD2 or PKD2 as indicated. Results are representative of Western blots of three independent experiments.

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**PKD2-mediated Angiogenesis**

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**TABLE 1.** Western blots of cell extracts prepared from all five of the primary human ECs from different tissues using a PKD1/2 (C-20) antibody that recognizes both PKD1 and PKD2 (13). It appears that HUVECs and human lung microvascular EC expressed more PKD2 than PKD1, whereas artery ECs such as human coronary artery ECs and human aortic ECs had more PKD1 than PKD2. A single band corresponding to PKD2 molecular mass (105 kDa) was detected in the ECs when an antibody specific against PKD2 was used (Fig. 1A, second panel). These findings indicate that PKD2 is expressed in a range of primary human ECs. In non-ECs, we found that WI-38 human lung fibroblasts predominantly expressed PKD1, whereas A549 human lung carcinoma cells expressed an abundant amount of PKD2, and H441 human lung adenocarcinoma cells mainly expressed PKD2 isoform (Fig. 1B).

**Activation of PKD2 by VEGF and PMA in HUVECs**—There are no published studies of the activation of PKD2 in ECs, although the activation of PKD1 by VEGF has been reported in ECs (24). The activation of PKD2 is believed to be mediated by an initial protein kinase C-dependent phosphorylation of two key serine residues in the activation loop followed by an auto-phosphorylation of Ser-876 in the enzyme (12, 13). Thus it is feasible to monitor PKD2 activation using a phospho-specific antibody against Ser-876-phosphorylated PKD2 (13). As shown in Fig. 2A (top panels), both VEGF and PMA activated PKD2 by inducing a strong autophosphorylation of PKD2 on Ser-876. Knockdown of PKD2 with validated human PKD2 siRNAs abolished the phosphorylation of PKD2 Ser-876 induced by...
PKD2-mediated Angiogenesis

VEGF and PMA in HUVECs, confirming the specificity of the phospho-specific antibody. In contrast, PKD1 silencing with a validated human PKD1 siRNA virtually did not affect the phosphorylation of PKD2 on Ser-876. Moreover, both VEGF and PMA induced a mobility shift of PKD2 in HUVECs, supporting the activation of PKD2 by these agonists (Fig. 2, second panels). Lastly, immunoprecipitation of PKD2 with a PKD2-specific antibody confirmed that PKD2 was activated and phosphorylated by VEGF in ECs (Fig. 2B). Taken together, these data demonstrate that PKD2 is activated by proangiogenic factors VEGF and PMA in ECs.

PKD2 Is An Important PKD Isoform Mediating the Phosphorylation of PKD Substrates in HUVECs—It has been demonstrated that PKD family kinases preferentially phosphorylate substrates with a LXRXX(pS/T) consensus motif, where pS/T denotes the phospho-serine or -threonine (25). However, the identity of specific PKD2 substrates in cells is largely unknown. By using a phospho-Ser-Thr PKD substrate antibody that specifically recognizes the PKD consensus motif LXRXX(pS/T), we found that, in resting HUVECs, at least nine protein bands were detected by the PKD substrate antibody (Fig. 3, top panel under control). The phosphorylation of four proteins indicated with * (bands 4, 5, 7, and 9) was reduced by silencing PKD2 but not PKD1 in resting HUVECs. Interestingly, PKD2 silencing increased the phosphorylation of two proteins around 60 kDa indicated with † (bands 12 and 13). Remarkably, PMA, a direct activator of PKD family kinases, strongly induced the phosphorylation of at least 13 proteins detected by the PKD substrate antibody (Fig. 3, top panel under PMA). It should be noted that the PKD-mediated phosphorylation of 9 out of the 13 proteins (indicated by *) was markedly reduced by PKD2 silencing, especially for protein bands 2, 3, 4, 5, 6, 8, 9, and 10. Similar inhibitory effects were observed in HUVECs transfected with other two different PKD2 siRNAs (data not shown). However, only the phosphorylation of three proteins, bands 2, 3, and 15 (indicated by †), was inhibited by PKD1 silencing and only slightly. Similarly, two other different PKD1 siRNAs had very minor effects on PKD-dependent protein phosphorylation induced by PMA (data not shown). Stimulation of HUVECs with VEGF also induced the phosphorylation of at least 14 proteins detected by the PKD substrate antibody (Fig. 3, top panel under VEGF), and the phosphorylation of protein band 5 (~120 kDa) and protein bands 11 and 12 (~60 kDa) induced by VEGF was higher than that induced by PMA. Again, PKD2 silencing markedly reduced the VEGF-induced phosphorylation of all the proteins except for protein band 14 in HUVECs. Furthermore, the VEGF-induced activation of ERK was abolished by PKD2 silencing in HUVECs (Fig. 3, second panel under VEGF). PKD2 knockdown also inhibited the ERK activation by PMA. The activation of ERK was determined using a phospho-ERK (Thr-202/Tyr-204) antibody. In contrast, PKD1 silencing only slightly inhibited the VEGF-induced phosphorylation of two proteins, band 5 (~120 kDa) and band 6 (~110 kDa) in HUVECs. In addition, the signals of three proteins (bands 11, 12, and 14) seem to be increased by PKD1 silencing in VEGF-treated HUVECs. The knockdown of PKD2 or PKD1 was shown in Fig. 3, third and fourth panels, respectively. It should be noted that the phosphorylation of PKD1 and PKD2 interferes with their recognition by the antibody, which is against a C-terminal epitope of PKD1/2 (13). These novel findings indicate that PKD2 is an important PKD isoform mediating the phosphorylation of PKD substrates in HUVECs.

PKD2, but Not PKD1 Isoform, Is Critical for EC Proliferation—We next utilized the loss of function approach by silencing PKD1 and PKD2 with specific siRNAs to determine their roles in EC morphology and proliferation. Because angiogenesis is mediated by a variety of factors, we studied the role of PKD2 in HUVECs cultured in EGM-2 complete growth medium instead of in medium containing single growth factor. EGM-2 complete growth medium contains FBS, multiple recombinant human growth factors including VEGF, FGF, IGF1, and epidermal growth factor, as well as hydrocortisone, heparin, and ascorbic acid. Fig. 4A shows that PKD1 or PKD2 was almost completely knocked down by its respective isoform-specific siRNA in HUVECs and that the knockdown of each isoform had only a minor effect on the expression of the other PKD isoforms. Interestingly, we found that silencing PKD2, but not PKD1, significantly slowed the rate at which HUVECs reach confluence. The PKD2-silenced ECs adhered well but grew slowly and spread more, thus appearing relatively large in size (Fig. 4B). In addition, we measured the histone-associated DNA fragments with an enzyme-linked immunosorbent assay kit (Roche Applied Science) and found that PKD2 silencing did not induce apoptosis of HUVECs as compared with cells transfected with control siRNA or PKD1 siRNA (data not shown). These observations suggest that PKD2 may regulate EC proliferation.

FIGURE 3. PKD2 is a critical PKD isoform mediating the phosphorylation of PKD substrates in HUVECs. HUVECs were transfected with 20 nm non-targeting control (con.) siRNA or the validated human PKD2 siRNA-1 or PKD1 siRNA-1 and grown for 72 h and then starved for 2 h and stimulated for 15 min with either VEGF165 (25 ng/ml) or PMA (30 nM). Lysates (20 μg) were subjected to Western blotting (WB) with phospho-Ser/Thr PKD substrate antibody (p-Ser/Thr PKD substrate Ab), phospho-ERK (Thr-202/Tyr-204) antibody (p-ERK1/2), or antibodies against PKD2, PKD1/2, or vinculin as indicated. Please note that the phosphorylation of PKD1 and PKD2 interferes with their recognition by the antibody, which is against a C-terminal epitope of PKD1/2 (13). Results are representative Western blots of three independent experiments.
PKD2-mediated Angiogenesis

We next determined the role of PKD2 in EC proliferation using three different siRNAs that target different regions in PKD2 mRNA. Human PKD2 siRNA-1 (catalog number SI02224768) and PKD2 siRNA-2 (catalog number SI02224775) are validated siRNAs obtained from Qiagen. The human PKD2 siRNA-3 (catalog number 004197) was ON-TARGET plus SMARTpool duplexes containing a mixture of four SMARTs—electro-designed siRNAs targeting PKD2 on different sequences and was from Dharmacon. As shown in Fig. 5A, PKD2 was markedly knocked down by the PKD2 siRNAs in HUVECs. Furthermore, we found that PKD2 silencing by the three different PKD2 siRNAs significantly and markedly inhibited the proliferation of HUVECs cultured in EGM-2 complete medium (Fig. 5, B and C). In contrast, PKD1 silencing by three PKD1 siRNAs targeting different mRNA regions had only a minor effect on the proliferation of HUVECs. As experimental controls, we found that cell proliferation was not significantly affected by knocking down p38α or a tyrosine phosphatase PTP-MEG2 in HUVECs (Fig. 5). These data indicate that PKD2 plays an important role in regulating EC proliferation.

PKD2 Silencing Markedly Inhibits EC Migration and Angiogenesis in Vitro—We next examined the role of PKD2 in EC migration, an important step in angiogenesis. As shown in Fig. 6A, the migration of HUVECs through the plain polycarbonate membrane (8.0-μm pore size) or the growth factor-reduced Matrigel-coated membrane induced by EGM-2 medium was greatly inhibited by two different PKD2 siRNAs, as compared with control siRNA-transfected cells. In contrast, PKD1 silencing had only a minor effect on HUVEC migration induced by EGM-2 medium. Moreover, we found that cell adhesion to fibronectin and spreading were not significantly affected by silencing either PKD1 or PKD2 isoform in HUVECs (Fig. 6, B and C). Because PKD2 appears to be essential for EC migration, which in turn is a critical step for angiogenesis, we utilized an in vitro model of angiogenesis to study this process further. In the model used, HUVECs were induced to form a network of capillary-like tubes in growth factor-reduced Matrigel, and the role of PKD2 in the process was examined. As shown in Fig. 6D (left panel), a clear and complete network of capillary-like structure was formed in EGM-2-cultured HUVEC transfected with control siRNA. However, in HUVEC transfected with two different human PKD2 siRNAs (Fig. 6D, right two panels), the capillary-like tubes were only partially formed, and the process seems blocked at an early step. These data indicate that PKD2 is required for EC migration and angiogenesis in vitro.

PKD2 Silencing Suppresses the Expression of VEGFR2 and FGFR1 in HUVECs—The findings described above indicate that PKD2 silencing markedly inhibits the proliferation, migration, and in vitro angiogenesis of HUVECs cultured in EGM-2 complete growth medium. Based on these observations, we postulate that the mechanism by which PKD2 silencing causes these effects might be through the inhibition of the expression of receptors for proangiogenic factors on HUVECs, thereby reducing EC growth and migration responses. Through a series of studies, we have found that PKD2 silencing markedly reduced the expression of VEGFR2 and FGFR1, two key proangiogenic growth factor receptors in HUVECs (Fig. 7, second and third panels), but did not affect the expression of IGFl-R2, TGFβRII, and TGFβ co-receptor endoglin in HUVECs (Fig. 7A, second and third panels).
PKD2-mediated Angiogenesis

FIGURE 6. PKD2 silencing markedly inhibits EC migration and angiogenesis in vitro. HUVECs were transfected with 20 nM non-targeting control siRNA (Con. siRNA), human PKD1 siRNA-1 (PKD1-S1), PKD2 siRNA-1 (PKD2-S1), or PKD2 siRNA-2 (PKD2-S2) and grown in EGM-2 medium for 90 h. The resuspended cells were then subjected to a cell migration assay in plain (Control) and growth factor-reduced Matrigel-coated (Matrigel) transwells (A) and to the assays of cell adhesion to fibronectin (B) and spreading (C). Data are means ± S.E. (n = 4). **, p < 0.01 versus control siRNA. D, HUVECs transfected with control siRNA (con. siRNA), human PKD2 siRNA-1 (PKD2-S1), or PKD2 siRNA-2 (PKD2-S2) were seeded on growth factor-reduced Matrigel, and their capacities to form the capillary-like tubes in EGM-2 medium for 8 h were assessed visually at a magnification of ×40 (n = 3).

7A, sixth through eighth panels). Interestingly, PKD2 silencing significantly up-regulated the expression of cell adhesion molecule ICAM-1 but not PECAM-1 in HUVECs (Fig. 7A, fourth and fifth panels). The protein level of VEGFR2 was also reduced in HUVECs by a second PKD2 siRNA targeting a different sequence (Fig. 7B) and by a third PKD2 siRNA (PKD2-S3) (data not shown). In contrast to the results seen with PKD2 silencing, PKD1 silencing generally did not affect the expression of VEGFR2 (Fig. 7B) and other growth factor receptors (data not shown). To determine whether PKD2 regulates the mRNA levels of VEGFR2 and FGFR1, we performed semiquantitative RT-PCR. We found that the mRNA levels of VEGFR2 and FGFR1 were markedly reduced by PKD2 knockdown with two different validated siRNAs (Fig. 7C). As a consequence, the VEGF-induced proliferation of HUVECs (Fig. 7D) and in vitro angiogenesis in growth factor-reduced Matrigel (Fig. 7F) were significantly and markedly inhibited by PKD2 silencing. These findings indicate that PKD2 regulates angiogenesis at least in part through modulation of the expression of VEGFR2 and FGFR1 key players in EC proliferation, migration, and angiogenesis.

DISCUSSION

In this study, we have investigated the physiologic function of a newly described serine/threonine kinase PKD2 in the aspects of EC biology involved in angiogenesis. The major findings obtained from this study are that PKD2 controls the expression of VEGFR2 and FGFR1 in ECs and that PKD2 silencing markedly inhibits EC proliferation, migration, and in vitro angiogenesis. The down-regulation of VEGFR2 and FGFR1 by PKD2 silencing could be regulated at many different levels including the regulation of transcription, mRNA stability, or the degradation of receptor proteins after ubiquitinylation (26, 27). Because both the protein and the mRNA levels of VEGFR2 and FGFR1 were reduced by PKD2 silencing, it seems likely that PKD2 may regulate the expression of these key proangiogenic receptors at the levels of transcription or mRNA stability. Studies are ongoing in our laboratory to test this possibility. In contrast, PKD2 silencing did not affect the expression of IGF1-Rβ, TGFβ-RII, or the TGFβ co-receptor endoglin in HUVECs. Because VEGFR2 and FGFR1 play key roles in angiogenesis (8, 9), it appears that PKD2 regulates angiogenesis at least in part through modulation of the expression of these receptors in ECs. These findings are achieved with the loss of function approach by silencing PKD2 with three validated siRNAs that target different regions in PKD2 mRNA. RNA interference is a near-ubiquitous post-transcriptional gene regulatory pathway that is mediated by microRNAs and other small non-coding RNAs, including synthetic 19- or 21-bp siRNA (28). Although this technique has been widely adopted as a tool for functional genomics, it has been suggested that siRNA might have an off-target effect. Recent studies indicate that pairing between the hexamer seed region of an siRNA guide strand (nucleotides 2–7) and complementary sequences in the 3’ untranslated region of mature transcripts is a critical element in off-target gene regulation (29). In this study, we observed consistent down-regulation of VEGFR2 and FGFR1 in ECs by three different PKD2 siRNAs that contain distinct sequences in hexamer seed region (nucleotides 2–7). In addition, a low concentration (20 nM) of siRNA was used to transfected HUVECs. These results indicate that the down-regulation of VEGFR2 and FGFR1 by different PKD2 siRNAs is not attributable to an off-target effect.

Moreover, PKD2 silencing significantly up-regulated the expression of cell adhesion molecule ICAM-1 but not PECAM-1 in HUVECs. ICAM-1 is well known for its role in immunity and inflammation by mediating leukocyte-EC interaction and the subsequent leukocyte transmigration (30), and the role of ICAM-1 in angiogenesis is unclear but merits further investigation. It seems that PKD2 may also regulate the expression of other genes involved in angiogenesis, including cell cycle regulators. Identification of PKD2-regulated genes, in addition to VEGFR2 and FGFR1, could help us more fully understand the underlying mechanisms of PKD2-mediated angiogenesis.

Although PKD family kinases exhibit a homologous catalytic domain, it has been recently demonstrated that PKD2 has unique N-terminal and C-terminal domains that determine its nucleocytoplasmic shuttling, activation, and substrate targeting (14, 16, 17). These findings suggest a functional difference between PKD2 and other PKD isoforms. In this study, we found that PKD2, but not the PKD1 isoform, was required for EC expression of VEGFR2 and FGFR1, two key growth factor receptors involved in angiogenesis. We further showed that
PKD2 isoform was critical for EC proliferation, migration and angiogenesis evoked by the various components in EGM-2 medium. EGM-2 medium is a complete growth medium for ECs and contains FBS, multiple recombinant human growth factors including VEGF, FGF, IGF1, and epidermal growth factor, as well as hydrocortisone, heparin, and ascorbic acid. Given that angiogenesis is regulated by a variety of proangiogenic factors (31), our findings indicate that PKD2 could be a central mediator in the regulation of angiogenesis based on our observation that PKD2 silencing markedly suppressed the angiogenic process exerted by the many proangiogenic factors in EGM-2 medium. Although it has been recently shown that PKD1 regulates VEGF signaling and is involved in the VEGF-induced migration and in vitro tubular formation of HUVECs (21, 32), we found that PKD1 silencing by different siRNAs had little effect on the growth and migration of HUVECs cultured in EGM-2 medium. These data suggest that the VEGF signaling defect caused by PKD1 inhibition may be rescued by other proangiogenic factors or may be not sufficient to influence angiogenesis in the presence of multiple other proangiogenic growth factors. This contention is supported by the work of Liu et al. (33), who recently reported that knock out of PKD1 did not affect the proliferation of B lymphocytes cultured in growth medium. On the basis of these findings, PKD2 appears to be a more critical regulator of those aspects of EC biology involved in angiogenesis than PKD1. This notion was further supported by our finding that PKD2 (but not PKD1) was the primary PKD isoform mediating the phosphorylation of PKD substrates in HUVECs. The identity of specific PKD2 substrates in cells is largely unknown at this point. Identification and characterization of PKD2 substrates in ECs will reveal the PKD2-mediated signaling pathways regulating the expression of VEGFR2, FGFR1, and other critical genes involved in angiogenesis.

FIGURE 7. PKD2 silencing suppresses the expression of VEGFR2 and FGFR1 in HUVECs. A and B, HUVECs were transfected with 20 nM control (Con.) siRNA, human PKD2 siRNA-1 (PKD2-S1), PKD2 siRNA-2 (PKD2-S2), PKD1 siRNA-1 (PKD1-S1), or PKD1 siRNA-2 (PKD1-S2) and grown for 96 h in EGM-2 medium. Lysates (30 µg) were subjected to Western blot with the indicated antibodies, and relative changes in protein levels were measured by densitometric analysis and presented as the percentage of or fold over cells transfected with control siRNA. Representative Western blots of four independent experiments are shown. C, a representative RT-PCR showing the mRNA levels of VEGFR2 and FGFR1 in HUVECs transfected with control (Con.) or PKD2 siRNAs. Relative change in mRNA was measured by densitometric analysis and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (n = 3). **, p < 0.01 versus control siRNA. D, the VEGF-induced proliferation of the above transfected cells was assessed by the CellTiter 96 AQ one solution reagent. Data are means ± S.E. (n = 5). **, p < 0.01 versus control siRNA. E, HUVECs transfected with control siRNA or human PKD2 siRNA-1 (PKD2-S1) were seeded on growth factor-reduced Matrigel, and their capacities to form the capillary-like tubes by VEGF (100 ng/ml) for 14 h were determined (n = 3).
In summary, PKD2 is the primary PKD isoform mediating the phosphorylation of PKD substrates in ECs and plays a pivotal role in the aspects of EC biology involved in angiogenesis at least in part by modulating the expression of VEGFR2 and FGFR1, two key growth factor receptors in angiogenesis.

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