

Minireview

# Protein Kinase D1, a New Molecular Player in VEGF Signaling and Angiogenesis

Chang Hoon Ha, and Zheng Gen Jin\*

**Vascular endothelial growth factor (VEGF) is essential for many angiogenic processes both in normal and pathological conditions. However, the signaling pathways involved in VEGF-induced angiogenesis are incompletely understood. The protein kinase D1 (PKD1), a newly described calcium/calmodulin-dependent serine/threonine kinase, has been implicated in cell migration, proliferation and membrane trafficking. Increasing evidence suggests critical roles for PKD1-mediated signaling pathways in endothelial cells, particularly in the regulation of VEGF-induced angiogenesis. Recent studies show that class IIa histone deacetylases (HDACs) are PKD1 substrates and VEGF signal-responsive repressors of myocyte enhancer factor-2 (MEF2) transcriptional activation in endothelial cells. This review provides a guide to PKD1 signaling pathways and the direct downstream targets of PKD1 in VEGF signaling, and suggests important functions of PKD1 in angiogenesis.**

## INTRODUCTION

Protein kinase D1 (PKD1, also known as PKC- $\mu$ ), a novel serine/threonine protein kinase, is composed of two zinc finger-like cysteine-rich motifs at its N-terminus that have high affinity for diacylglycerol, a pleckstrin homology domain, and a C-terminal catalytic domain (Rozengurt et al., 2005). Although PKD family kinases (PKD1, PKD2, PKD3) have a homologous catalytic domain, they vary in respect of their expression level, function, and subcellular localization (Auer et al., 2005; Rey et al., 2003; Rozengurt et al., 2005). PKD1 has been implicated in the regulation of a variety of cellular functions, including signal transduction, cell migration, and membrane trafficking (Avkiran et al., 2008; Johannes et al., 1994; Rozengurt et al., 2005; Valverde et al., 1994). However, the direct downstream targets of PKD1 in VEGF signaling are not fully understood. Furthermore, few PKD1 targeting genes have been identified so far.

Angiogenesis, the formation of new blood capillaries, is essential for embryonic vascular development, wound healing, and organ regeneration, as well as pathological processes such as tumor growth, rheumatoid arthritis, diabetic retinopathy, and atherosclerosis (Carmeliet, 2003; Ferrara et al., 2003;

Folkman, 1995). Out of the many participants in the angiogenesis field, the vascular endothelial growth factors (VEGF) are by far the best characterized. VEGF (Folkman, 1995) is crucial for many angiogenic processes both in normal and pathological conditions (Carmeliet, 2003; Ferrara et al., 2003). VEGF receptors, VEGFR1 (Flt1) and VEGFR2 (mouse Flk1 or human KDR), are restricted in their tissue distribution primarily to endothelial cells (Yancopoulos et al., 2000). The binding of VEGF to its cognate receptors induces dimerization and subsequent intracellular signal cascades to mediate endothelial gene expression, cell migration, and angiogenesis (Claesson-Welsh, 2003; Sakurai et al., 2005; Takahashi et al., 2001; Zachary, 2003). In particular, the VEGF receptor 2 (VEGFR2)-mediated phospholipase C  $\gamma$  (PLC $\gamma$ )/protein kinase C (PKC) pathway regulates the activation of extracellular-regulated kinases and angiogenesis (Sakurai et al., 2005; Takahashi et al., 1999; 2001).

The importance of VEGF-induced signaling is demonstrated in that the genetic inactivation of either receptor leads to a complete lack of development of blood vessels in the embryo, and inactivation of VEGFR2 function dramatically impairs the growth of cancer cells *in vivo* (Carmeliet et al., 1996; Fong et al., 1995; Shalaby et al., 1995). However, the links from VEGF receptor's intracellular signaling cascades to gene regulation remain largely elusive. This review will focus on VEGF-mediated PKD1 activation in endothelial cells and the potential role of PKD in angiogenesis.

## VEGF signaling and angiogenesis

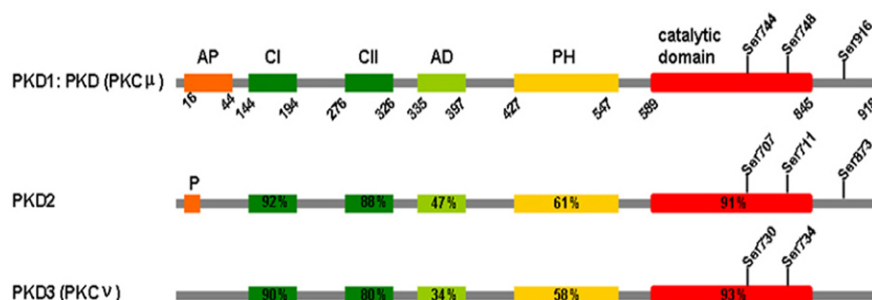
The functions of VEGF (VEGFA) in angiogenesis have been studied for many years (Ferrara, 2002). Recent data indicate that angiogenic processes are highly complex and coordinated processes, requiring the subsequent activation of a chain of receptors by various ligands, but VEGF signaling shows a critical role in physiological angiogenesis (Carmeliet, 2000; Ferrara and Alitalo, 1999; Yancopoulos et al., 2000). VEGF also seems to be important in pathological angiogenesis, such as tumor growth (Ferrara and Davis-Smyth, 1997). VEGF stimulates proliferation and migration of endothelial cells (Ferrara et al., 2003). In addition, VEGF is a potent survival factor for endothelial cells during angiogenesis and it has been also shown to

The Aab Cardiovascular Research Institute and Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York, USA

\*Correspondence: zheng-gen\_jin@urmc.rochester.edu

Received June 26, 2009; accepted June 29, 2009; published online July 20, 2009

**Keywords:** angiogenesis, CAMK, endothelial cells, HDAC, MEF2, migration, PKC, PKD, VEGF

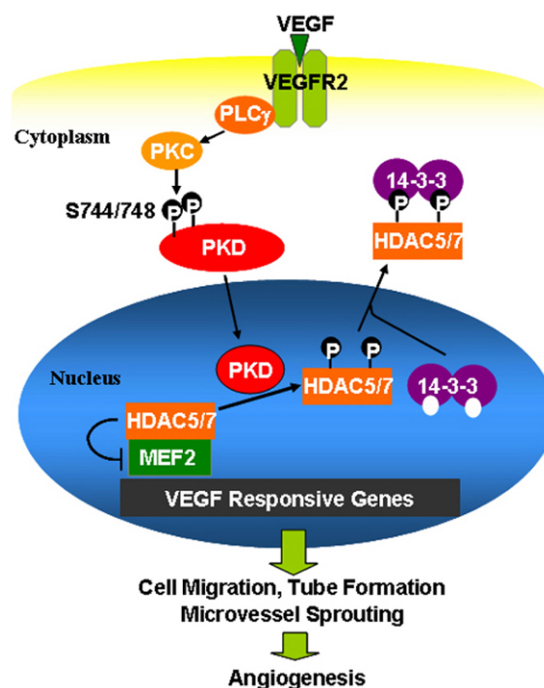


**Fig. 1.** Diagram of functional domains and conserved phosphorylation sites in PKD families. The percentages indicate amino acid sequence homology of PKD2 and PKD3 domains with corresponding mouse PKD1 domains. Abbreviations: AP, apolar region; C1 and C2, cysteine-rich and zinc finger-like domains (DAG-binding domain); AD, acidic domain; PH, pleckstrin homology domain; P, proline-rich region.

prevent apoptosis by inducing expression of the anti-apoptotic proteins such as Bcl-2 and A1 in the endothelial cells (Benjamin et al., 1999; Benjamin and Keshet, 1997; Gerber et al., 1998a; 1998b; Yuan et al., 1996). VEGF (VEGF-A), a key regulator of blood vessel growth, is affiliated with a gene family that includes placental growth factor (PLGF), VEGF-B, VEGF-C and VEGF-D (Ferrara and Davis-Smyth, 1997; Neufeld et al., 1999). The human VEGF-A gene is composed of eight exons (Houck et al., 1991; Tischer et al., 1991). Four different isoforms of variable amino acid number are produced through alternative exon splicing (VEGF121, VEGF165, VEGF189, VEGF206) (Ferrara et al., 2003; Houck et al., 1991; Tischer et al., 1991). VEGF121, VEGF165 and VEGF189 are the major forms secreted by most cell types (Robinson and Stringer, 2001). The divergent functions of VEGF isoforms were studied by isoform specific VEGF knock-out mice (Carmeliet et al., 1999; Stalmans et al., 2002). Mice expressing only VEGF165 are viable and healthy (Stalmans et al., 2002). These studies show the significance of VEGF165 as the principal effector of VEGF action. VEGF binds two related receptor tyrosine kinases (RTKs), VEGFR-1 and VEGFR-2 (Chen et al., 1997; Kolodkin et al., 1997). These VEGF receptors have seven immunoglobulin-like domains in the extracellular domain, a single trans-membrane region and a tyrosine kinase domain (Shibuya et al., 1990; Terman et al., 1991). VEGFR-1 (Flt-1) binds VEGF, VEGF-B and PLGF with high affinity (Shibuya et al., 1990), and VEGFR-2 (KDR or Flk-1) binds VEGF, VEGF-C and VEGF-D. However, recent studies show that VEGFR-2 is the major mediator of VEGF signals in endothelial cells (Gille et al., 2001; Meyer et al., 1999; Wise et al., 1999). VEGF induces the activation of various proteins such as PLC- $\gamma$ , PI-3 kinase, and the Src family in endothelial cells through VEGFR-2 (Eliceiri et al., 1999; Guo et al., 1995).

### The protein kinase (PKD) family

The protein kinase D (PKD) is made up of 3 isoforms (Fig. 1). Human and mouse PKD1, also known as protein kinase C $\mu$ , was identified by two different groups in 1994 (Johannes et al., 1994; Valverde et al., 1994) and isoforms PKD2 and PKD3 (PKC $\nu$ ) were discovered more recently (Hayashi et al., 1999; Rey et al., 2003). PKD1 is a serine/threonine kinase with 918-amino acids that is composed of two cysteine-rich, zinc finger-like regions and a pleckstrin homology (PH) domain in the N-terminal regulatory domain and a C-terminal catalytic domain (Johannes et al., 1994; Valverde et al., 1994), as illustrated in Fig. 1. Also, PKD2 and PKD3 have a similar structure and show a high homology to PKD1. The N-terminal regulatory regions of PKD inhibit the kinase activity of PKD and also control sublocalization of PKD (Rey and Rozengurt, 2001; Van Lint et al., 1995). The cysteine-rich domains play an important role in mediating PKD translocation to the plasma membrane and nucleus in cells stimulated with various stimuli and also suppresses the



**Fig. 2.** Schema for potential functions of PKD1 in VEGF-induced angiogenesis. VEGF-activated PKD1 phosphorylates class IIa HDACs (HDAC 5 and 7) and induces those cytoplasmic accumulations in endothelial cells. Eventually, VEGF-induced PKD1 pathway is involved in VEGF-induced gene expressions, EC migration, tube formation and microvessel sprouting.

kinase activity (Iglesias et al., 1998a). PKD also includes a PH domain, that has been determined to play an autoregulatory role (Iglesias and Rozengurt, 1998; Iglesias et al., 1998a). Accordingly, PKD with mutations in the PH domain are constitutively active (Iglesias et al., 1998a; Waldron et al., 1999), showing that the PH domain, like the cysteine-rich domains, is needed to maintain PKD in an inactive catalytic state.

The structural, enzymatic, and regulatory properties of PKD are different from the PKC family (Rozengurt et al., 2005; Valverde et al., 1994; Van Lint et al., 1995). PKD was initially divided to the AGC group (named for PKA, PKG, and PKC) (Mellor and Parker, 1998; Newton, 1997), but PKD is now classified as the calcium/calmodulin-dependent protein kinase (CAMK) group, separate from the AGC group (Hanks, 2003). This categorization is based on catalytic domain (Kunkel et al., 2007).

DAG triggers changes in localization, phosphorylation, and catalytic activation of PKD family. However, the PKD family is not only a target of diacylglycerol (DAG) but is also directly down-

stream of the PKC family. Thus, as a second mechanism, PKCs activate PKD by phosphorylation of Ser-744 and Ser-748 within the activation loop of the PKD catalytic domain (Zugaza et al., 1996; 1997), and this PKC-mediated phosphorylation plays an important role in PKD activation by various stimuli in intact cell systems (Iglesias et al., 1998b; Waldron and Rozengurt, 2003; Waldron et al., 2001). The activated PKD also translocates from the plasma membrane into the cytoplasm and nucleus (Rey and Rozengurt, 2001), and undergoes autophosphorylation at Ser916 (Matthews et al., 1999). PKD can be activated by a variety of stimuli including biologically active phorbol esters, growth factors, bombesin, endothelin, vasopressin, and T- and B-cell receptor agonists via PKC-dependent pathways (Lint et al., 2002; Rozengurt et al., 2005; Zugaza et al., 1997). PKD has been implicated in the regulation of a variety of cellular responses, including signal transduction, membrane trafficking, protein transport, and cell survival, migration, differentiation, and proliferation (Baron and Malhotra, 2002; Hausser et al., 2002; Jamora et al., 1999; Liljedahl et al., 2001; Rozengurt et al., 2005; Storz and Toker, 2003; Yeaman et al., 2004). Furthermore, PKD activation has been studied in many normal cell types, including fibroblasts (Chiu and Rozengurt, 2001a; Matthews et al., 1997; Zhukova et al., 2001), intestinal and kidney epithelial cells (Chiu and Rozengurt, 2001b; Chiu et al., 2002; Rey et al., 2001; 2004), smooth muscle cells (Abedi et al., 1998), cardiomyocytes (Haworth et al., 2000; 2004), and B and T lymphocytes (Matthews et al., 1999; 2000a; 2000b; Sidorenko et al., 1996). However, the regulation of PKD activation and its function in endothelial cells are very poorly studied.

### PKD1 functions in angiogenesis

Recently, functional activities of PKD family in the circulation system appear to mediate proliferation (Qin et al., 2006; Wong and Jin, 2005) and migration (Qin et al., 2006) in endothelial cells, hypertrophy in vascular smooth muscle cells (Xu et al., 2007), and activation in platelets (Stafford et al., 2003). In a recent report, PKD1 also mediates the VEGFR2-PLC $\gamma$ -PKC pathway to ERK1/2 activation and EC proliferation (Wong and Jin, 2005). Furthermore, disturbing PKD1 activity has been shown to block VEGF-stimulated angiogenesis in an *in vivo* model (Qin et al., 2006). A recent report also showed the physiological function of PKD2 in endothelial cells involved in angiogenesis (Hao et al., 2009). They found that PKD2 was an important PKD isoform mediating proliferation, migration, and *in vitro* angiogenesis in endothelial cells.

Although the cellular mechanism through which PKD1 mediates the pertinent process is unclear in many cases, several PKD1 downstream targets have been identified. Recent reports have revealed that class IIa histone deacetylase 5 (HDAC5) and 7 (HDAC7), an enzyme that induces chromatin modifications and acts as signal responsive repressors for control of gene expression (Bucks et al., 2006; Chang et al., 2006; McKinsey and Olson, 2005; Zhang et al., 2002), are direct downstream targets of PKD1 in endothelial cells (Ha et al., 2008a; 2008b). VEGF stimulates PKD-dependent phosphorylation of two serine 259/498 residues in HDAC5 in endothelial cells. Furthermore, VEGF induces HDAC5 translocation from the nuclei to the cytoplasm after VEGF stimulation and the PKD1-HDAC5 pathway is involved in VEGF-induced MEF2-dependent gene expression, NR4A1 expression, migration, and tube formation (Ha et al., 2008b). MEF2 family transcription factors have been implicated in blood vessel development and vascular integrity (Lin et al., 1998). Several MEF2-dependent genes, including NR4A1 and KLF2, have been identified (Chang et al., 2006; Youn and Liu, 2000).

In the recent studies, it has been shown that VEGF stimu-

lates PKD1-dependent HDAC7 phosphorylation at the sites of Ser178, Ser344, and Ser479 and cytoplasmic accumulation in endothelial cells, and that PKD1-HDAC7 pathway is involved in VEGF-induced angiogenic gene expression, including matrix metalloproteinases MT1-matrix metalloproteinase (MT1-MMP) and MMP10 expression, EC migration, tube formation, and microvessel sprouting (Ha et al., 2008a; Wang et al., 2008). It is increasingly apparent that PKD1 is a key player in the regulation of signaling transductions related to angiogenesis in endothelial cells.

### CONCLUSION

Investigation of the regulation and functions of PKD1 in endothelial cells is still in the beginning. Future studies using genetic engineered animal models should help us to further dissect the role of PKDs in VEGF signaling and angiogenesis *in vivo*. Nevertheless, the recent advances already suggest that PKD is responsive to important stimuli such as VEGF and may control physiological and pathological angiogenesis by mediating class IIa HDACs such as HDAC5 and HDAC7. In conclusion, these discoveries may implicate PKD in mediating angiogenesis by VEGF and may suggest PKD as a potential therapeutic target for angiogenesis in various diseases.

### ACKNOWLEDGMENTS

This work was partially supported by the American Heart Association Postdoctoral Fellowship (to C.H.H.), and Thomas R. Lee Award from the American Diabetes Association 1-06-CD-13, the Grant-In-Aid Award from the American Heart Association 0755916T and the National Institute of Health Grant HL-080611 (to Z.G.J.).

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