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ORIGINAL PAPER

Activation of diacylglycerol kinase α is required for VEGF-induced angiogenic signaling *in vitro*

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Vascular endothelial growth factor-A (VEGF-A) promotes angiogenesis by stimulating migration, proliferation and organization of endothelium, through the activation of signaling pathways involving Src tyrosine kinase. As we had previously shown that Src-mediated activation of diacylglycerol kinase-α (Dgk-α) is required for hepatocytes growth factor-stimulated cell migration, we asked whether Dgk-α is involved in the transduction of angiogenic signaling. In PAE-KDR cells, an endothelial-derived cell line expressing VEGFR-2, VEGF-A_{165}, stimulates the enzymatic activity of Dgk-α: activation is inhibited by R59949, an isoform-specific Dgk inhibitor, and is dependent on Src tyrosine kinase, with which Dgk-α forms a complex. Conversely in HUVEC, VEGF-A_{165}-induced activation of Dgk is only partially sensitive to R59949, suggesting that also other isoforms may be activated, albeit still dependent on Src tyrosine kinase. Specific inhibition of Dgk-α, obtained in both cells by R59949 and in PAE-KDR by expression of Dgk-α dominant-negative mutant, impairs VEGF-A_{165}-dependent chemotaxis, proliferation and *in vitro* angiogenesis. In addition, in HUVEC, specific downregulation of Dgk-α by siRNA impairs *in vitro* angiogenesis on matrigel, further suggesting the requirement for Dgk-α in angiogenic signaling in HUVEC. Thus, we propose that activation of Dgk-α generates a signal essential for both proliferative and migratory response to VEGF-A_{165}, suggesting that it may constitute a novel pharmacological target for angiogenesis control.
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Keywords: diacylglycerol kinase; VEGF; phosphatidic acid; Src; angiogenesis

Introduction

Tumor growth and metastasis to distant organs, as well as revascularization of ischemic tissues and female cycle are dependent on angiogenesis, the formation of new blood vessels by branching from pre-existing ones (Folkman, 1972; Carmeliet and Jain, 2000). Angiogenesis is promoted by angiogenic factors secreted by hypoxic tissues, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and hepatocytes growth factor (HGF) (Bussolino *et al*., 1992; Matsumoto and Claesson-Welsh, 2001). In particular, VEGF-A, whose receptors are mostly expressed on endothelial cells, plays a crucial role in the angiogenesis *in vivo* (Folkman, 1972; Carmeliet and Jain, 2000; Matsumoto and Claesson-Welsh, 2001). Because antiangiogenic therapy is currently believed to be a promising approach to cancer treatment (Folkman, 1972; Carmeliet and Jain, 2000; Matsumoto and Claesson-Welsh, 2001), the identification of new proteins involved in the biochemical mechanisms transducing angiogenic extracellular signals would provide novel biochemical targets for pharmacological intervention. VEGF-A stimulates angiogenesis by activating several signaling pathways in a spatially and timely coordinate manner, leading to endothelial cells migration, proliferation and organization in tubular structures (reviewed in Matsumoto and Claesson-Welsh, 2001). Among the numerous pathways activated, the generation of phosphatidylinositol(4,5)bis-phosphate ($PI(4,5)P_2$)-derived second messengers plays a crucial role in VEGF-A angiogenic signaling. Indeed, VEGF-A-induced angiogenic signaling requires both phosphorylation and hydrolysis of $PI(4,5)P_2$, mediated, respectively, by phosphatidylinositol 3-kinase (PI 3-kinase) and phospholipase C-γ (PLC-γ to generate phosphatidylinositol(3,4,5)tris-phosphate ($PI(3,4,5)P_3$) and diacylglycerol (DG). While $PI(3,4,5)P_3$ is required for activation of Akt, which mediates VEGF-A-induced survival signaling (Gerber *et al*., 1998), DG is required for activation of PKC-α, which mediates VEGF-A-induced proliferative and chemotactic signaling (Wellner *et al*., 1999; Matsumoto and Claesson-Welsh, 2001). Diacylglycerol kinase (Dgk) enzymes phosphorylate

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diacylglycerol to generate phosphatidic acid (PA), regulating in a reciprocal manner the level of two lipid second messengers (Topham and Prescott, 1999). Thus Dgk enzymes may act both as terminators of DG-mediated signaling and as activators of PA-mediated signals. Indeed, activation of Dgk enzymes has been reported to downregulate DG-regulated enzymes such as PKCs and Ras GTP releasing proteins (RasGRP), respectively, in platelets and T cells (De Chaffoy de Courcelles *et al*., 1989; Jones *et al*., 2002; Zhong *et al*., 2002). On the other hand, activation of Dgk-α has been reported to convey proliferative and motility signals, respectively, in IL-2-stimulated T lymphocytes and in HGF-stimulated endothelial cells (Flores *et al*., 1996; Cutrupi *et al*., 2000). In addition, generation of PA through phospholipase D (PLD)-mediated hydrolysis of phospholipid is required for vescicular trafficking, lamellipodia, cell migration and proliferation in different cellular systems (English, 1996; Honda *et al*., 1999; O'Luanaigh *et al*., 2002).

To date, nine distinct Dgk isoforms have been cloned in mammalians, encoding soluble proteins that reversibly associate to the membrane or to the nucleus (reviewed in Topham and Prescott, 1999). All isoforms share a highly conserved catalytic domain, preceded by at least two Zn fingers, homologous to the C1 domain of PKCs. Five different families have been defined according to the different N-terminal regulatory domains. Class I Dgk, comprising α-, β- and γ-isoforms, shares a common N-terminal autoinhibitory motif and a pair of EF-hand calcium-binding domains preceding the Zn fingers. Dgk-α is abundant in T lymphocytes, but is also expressed in endothelial and epithelial cells, fibroblasts and oligodendrocytes (Schaap *et al*. 1990; Cutrupi *et al*., 2000; data not shown). The biological functions and biochemical regulation of Dgk enzymes are currently under investigation (reviewed in van Blitterswijk and Houssa, 2000). We have previously shown that upon HGF stimulation of a porcine aortic cell line (PAE), Dgk-α is activated and associates in a complex with Src. In addition, we have also shown that Dgk-α is regulated by tyrosine phosphorylation and that its activation requires Src tyrosine kinase activity (Cutrupi *et al*., 2000). Specific inhibition of Dgk-α, either through expression of a dominant-negative mutant or by cell treatment with R59949, a pharmacological inhibitor, impairs HGF-induced chemotaxis of endothelial cells (Cutrupi *et al*., 2000) and IL-2-induced proliferation of T lymphocytes (Flores *et al*., 1996).

Thus, based on these data and on the absolute requirement for Src tyrosine kinase activity in VEGF-A-induced proliferation and motility of endothelial cells (Abu-Ghazaleh *et al*., 2001), we set to investigate the role of Dgk-α in VEGF-A signaling in PAE cells expressing VEGFR-2 (PAE-KDR) and in human umbilical vein endothelial cells (HUVEC). Herein we report that (i) VEGF-A_{165} stimulates Dgk-α activity in an Src-dependent manner and, at least in PAE-KDR, induces the formation of a Dgk-α/Src complex; (ii) both pharmacological inhibition of Dgk-α in both cells and expression of Dgk-α dominant-negative mutant in PAE-KDR impair VEGF-A_{165}-induced chemotaxis and proliferation, as well as *in vitro* formation of tubule structures in matrigel; (iii) specific downregulation of Dgk-α by siRNA in HUVEC impairs *in vitro* formation of tubule structures in matrigel. These are the first evidences indicating the involvement of Dgk-α in VEGF-A signal transduction, providing the first demonstration that Dgk-α activation is necessary for the VEGF-A-triggered angiogenic program. Furthermore, these data indicate Dgk-α as a promising novel pharmacological target for angiogenesis control.

Results

VEGF-A_{165} activates Dgk-α

Stimulation of VEGFR-2 tyrosine kinase activity mediates the activation of the angiogenetic response induced by VEGF-A in HUVEC and PAE-KDR cells (Waltenberger *et al*., 1994; Matsumoto and Claesson-Welsh, 2001). Thus, we have measured Dgk activity *in vitro* in cell homogenates obtained from either control or VEGF-A_{165}-stimulated PAE, PAE-KDR and HUVEC. Dgk activity was measured *in vitro* as phosphorylation of exogenous DG in the presence of radiolabeled ATP. The activity of homogenates obtained from PAE-KDR and HUVEC stimulated with VEGF-A_{165} was twofold higher than from untreated control cells (Figure 1a). The activation was sustained for at least 1 h (Figure 1b) and dependent on VEGF concentration (data not shown). On the other hand, Dgk activity from PAE cells, which do not express VEGFR-2 at significant

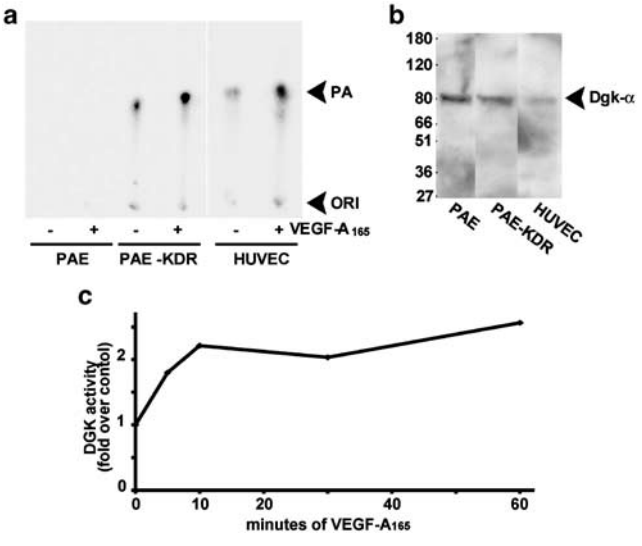

Figure 1 VEGF-A_{165} activates DGK in PAE-KDR and HUVEC. **(a)** Dgk activity assayed on whole-cell homogenates from either control or VEGF-A_{165} (10 ng/ml for 10 min)-stimulated quiescent PAE, PAE-KDR and HUVEC cells. **(b)** Dgk-α protein assayed by Western blot with anti-Dgk-α antibodies in whole-cell extracts from PAE, PAE-KDR and HUVEC. **(c)** Time course of Dgk activation in VEGF-A_{165}-stimulated (VEGF-A_{165} 50 ng/ml) PAE-KDR cells

levels, was not increased upon VEGF-A_{165} stimulation (Figure 1a). Intriguingly, even in the absence of exogenous VEGF-A_{165}, basal Dgk activity from PAE-KDR was much higher than from PAE cells, suggesting that the sole expression of VEGFR-2, putatively through an autocrine loop, is sufficient to generate a signal that upregulates Dgk activity. Indeed, PAE and PAE-KDR express VEGF, as measured by RT–PCR, and both basal ERK-1/2 and Src activities are upregulated in unstimulated PAE-KDR cells compared with PAE cells (data not shown).

However, these experiments, which clearly indicate that VEGF-A_{165} stimulates a Dgk activity, do not allow to establish which of the different Dgk isoforms are activated upon VEGF-A_{165} stimulation of these cells. Dgk-α is expressed at similar levels in PAE and PAE-KDR cells, as measured by Western blot with Dgk-α antibodies, while in HUVEC its expression is significantly lower (Figure 1c). Thus in order to verify whether Dgk-α is regulated by VEGF-A_{165}, we assayed *in vitro* the Dgk activity in anti-Dgk-α immunoprecipitates from lysates of PAE-KDR cells, either control or VEGF-A_{165} stimulated. Indeed, Dgk activity in anti-Dgk-α immunoprecipitates from VEGF-A_{165}-stimulated cells was higher than from unstimulated cells, while the amount of Dgk-α protein immunoprecipitated did not change (Figure 2a). VEGF-induced activation of Dgk-α was sustained for at least 60 min (Figure 2b), and was dependent on VEGF concentration (Figure 2c). Mock immunoprecipitates carried out in the absence of anti-Dgk-α antibodies did not contain either Dgk-α protein or activity.

In order to further characterize the Dgk activity stimulated by VEGF-A_{165} in PAE-KDR cells and HUVEC cells, we have verified whether it is inhibited *in vitro* by R59949, a class I Dgk-specific inhibitor with a strong preference for the α- and β-isoform rather than the γ-isoform (Kai *et al*., 1994), whose effects on tyrosine kinase signaling in PAE cells are reverted by overexpression of Dgk-α (Cutrupi *et al*., 2000). In endothelial cells, R59949 cell treatment did not affect both VEGFR-2 tyrosine phosphorylation (Figure 3a) and cell viability as measured by Trypan blue (Figure 3b). *In vitro*, 1 μM R59949 inhibited completely both basal and VEGF-induced Dgk activity assayed in PAE-KDR cell homogenates (Figure 3c). Conversely, R59949, even at 10 μM, inhibited only part of both basal and VEGF-stimulated Dgk activity assayed in HUVEC homogenates (Figure 3c), suggesting that in these cells VEGF-A_{165} may stimulate other Dgk isoforms also that are less sensitive to R59949.

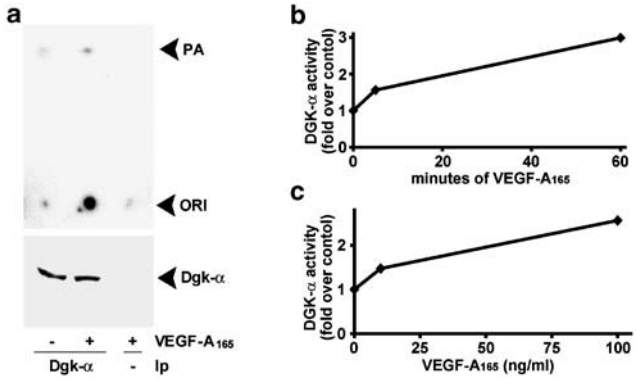

Figure 2 VEGF-A_{165} activates Dgk-α. **(a)** Dgk-α activity (upper panel) and protein (lower panel) assayed on anti-Dgk-α immunoprecipitates from lysates of either control or VEGF-A_{165} (10 ng/ml for 10 min)-stimulated PAE-KDR cells. Time course (**b**) and VEGF concentration dependence **(c)** of Dgk-α activation, assayed in anti-Dgk-α immunoprecipitates from PAE-KDR stimulated, respectively, with 50 ng/ml VEGF-A_{165} and upon 15 min stimulation

Expression of a dominant-negative mutant of Dgk-α impairs VEGF-A_{165}-induced in vitro angiogenesis

To further investigate the involvement of Dgk-α VEGF signaling, we expressed a catalytic inactive mutant of Dgk-α Dgk-α-K^-), which acts as dominant negative in PAE cells (Cutrupi *et al*., 2000). PAE-KDR cells were infected with PINCOS retrovirus containing myc-tagged Dgk-α-K^-, obtained as described previously (Cutrupi *et al*., 2000). The efficiency of infection, measured as green fluorescent protein (GFP) expression by FACS analysis, was about 70% (data not shown), while the expression of myc-tagged Dgk-α, either wt or K^-, was about two to threefold the expression of endogenous Dgk-α (data not shown). Thus the PAE-KDR-PINCOS cell lines, containing either empty vector, Dgk-α-wt or

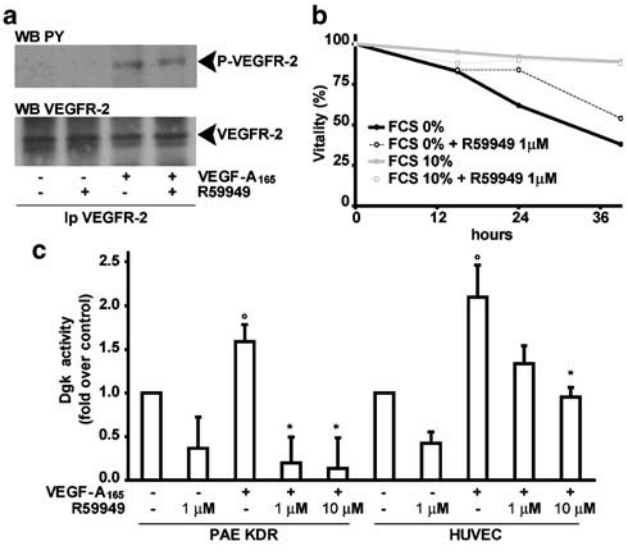

Figure 3 VEGF-A_{165}-stimulated Dgk is sensitive to R59949. (**a**) Antiphosphotyrosine (upper panel) and anti-VEGFR-2 (lower panel) Western blot of anti-VEGFR-2 immunoprecipitates from either unstimulated or VEGF-A_{165} (10 ng/ml for 10 min)-stimulated HUVEC, pretreated for 15 min with either R59949 1 μM or vehicle. **(b)** Cell viability, quantified by Trypan blue exclusion, of PAE-KDR cells maintained in the presence (10%) or absence of FCS and R59949 (1 μM) for the time indicated. **(c)** Dgk activity assayed in the presence of the indicated concentrations of R59949 on whole-cell homogenates from either control or VEGF-A_{165} (10 ng/ml for 10 min)-stimulated quiescent PAE-KDR and HUVEC cells (data are mean ± s.e.m. of five independent experiments normalized for control, °*t*-test *vs* control, **t*-test *vs* VEGF-A_{165}, $P<0.05$)

dominant-negative mutant, were assayed in a chemotaxis assay on a collagen I matrix. The expression of Dgk-α dominant-negative mutant in PAE-KDR significantly inhibited VEGF-A_{165}-induced cell migration (Figure 4a), while it did not affect cell migration induced by fetal calf serum (FCS) (data not shown). Conversely, the expression of Dgk-α-wt enhanced the migratory response of PAE-KDR cells to VEGF by about twofold, although the difference was not statistically significant (Figure 4a).

The same cell lines were also assayed for VEGF-A_{165}-induced DNA synthesis. The expression of Dgk-α dominant-negative mutant reduced VEGF-A_{165}-induced DNA synthesis of PAE-KDR, while the expression of wt Dgk-α did not affect DNA synthesis of these cells upon VEGF-A_{165} treatment (Figure 4b). Similar to the cell migration assay, the expression of Dgk-α dominant-negative mutant did not affect FCS-induced DNA synthesis of PAE-KDR cells (data not shown).

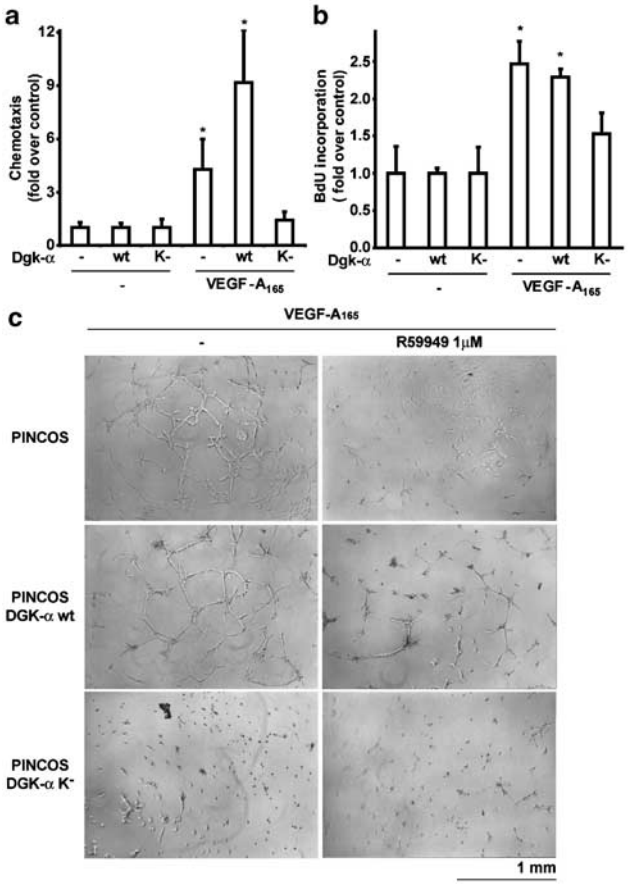

Figure 4 Expression of Dgk-α dominant negative inhibits VEGF-A_{165}-induced *in vitro* angiogenetic signaling. Quiescent PAE-KDR infected with either empty or Myc-Dgk-α-wt or Myc-Dgk-α-K^- virus were assayed for chemotaxis **(a)**, DNA synthesis **(b)** and network formation on matrigel **(c)** as follows. **(a)** Cells were plated in a modified Boyden chamber coated with collagen I and induced to migrate with 20 ng/ml of VEGF-A_{165} for 6 h. Data are shown as fold increase over control; each value is the mean ±s.e. of six points (**t*-test, $P<0.01\%$). **(b)** Cells were cultured in 96-well plates in the presence or absence of VEGF-A_{165} (10 ng/ml, absence of serum, 24 h). BrdU incorporation assay was carried out as described. Data are shown as fold increase over control; each value is the mean ±s.e. of eight points (**t*-test, $P<0.01\%$). **(c)** Cells were plated on growth factor-rich matrigel, and stimulated with VEGF-A_{165} (25 ng/ml) for 24 h, in the presence or absence of 1 μM R59949. Cells were photographed with contrast phase microscopy

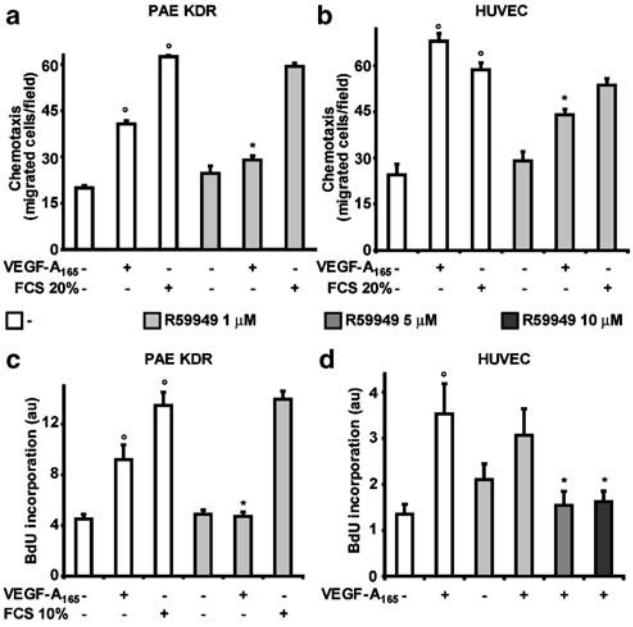

Figure 5 R59949 5 R59949 inhibits VEGF-A-induced cell motility and proliferation of PAE-KDR and HUVEC. Quiescent PAE-KDR **(a,c)** and HUVEC **(b,d)** were assayed for chemotaxis **(a,b)** and DNA synthesis **(c,d)** as follows: quiescent PAE-KDR **(a)** or HUVEC **(b)** were plated in a modified Boyden chamber coated with gelatin and induced to migrate in the presence of either 10 ng/ml **(a)** or 20 ng/ml **(b)** of VEGF-A_{165} or 20% FCS for 6 h, in the presence or absence of 1 μM R59949. Each value is the mean ±s.e. of triplicates (°*t*-test *vs* control, **t*-test *vs* VEGF-A_{165} stimulated, $P<0.01\%$). Quiescent PAE-KDR **(c)** or HUVEC **(d)** were cultured in 96-wells plates for either 24 h **(c)** or 48 h **(d)** with or without VEGF-A_{165} (**c**) 25 ng/ml in the absence of serum, (**d**) 10 ng/ml, 2% serum) or 10% FCS and in the presence or absence of R59949 at the indicated concentrations. BrdU incorporation was assayed as described. Each value is the mean ±s.e. of eight points (°*t*-test *vs* control, **t*-test *vs* VEGF-A_{165} stimulated, $P<0.01\%$)

To further investigate the involvement of Dgk-α in VEGF-A_{165} angiogenic signaling, we have verified whether the expression of Dgk dominant-negative mutant impairs the ability of endothelial cells to form tubular-like structures on matrigel, a mixture of laminin-rich basement membrane enriched in growth factors. In this assay, which is currently used to test *in vitro* pro- or antiangiogenic properties of molecules, endothelial cells are stimulated to migrate and to organize themselves in a chord structure network (Benelli and Albini, 1999). PAE-KDR cells infected with empty PINCOS vector or expressing Dgk-wt once plated on matrigel in the presence of VEGF-A_{165} (10 ng/ml) elongate and connect through chord-like structures to generate a complex network. However, PAE-KDR expressing Dgk-K^-, although still viable, fail to connect and to differentiate in chord-like structures, resulting in

a severe impairment of the formation of the characteristic network (Figure 4c).

Pharmacological inhibition of Dgk-α impairs in vitro VEGF-A_{165}-induced angiogenesis

Based on the sensitivity of Dgk-α to the inhibition by micromolar concentrations of R59949, we assayed its ability to inhibit VEGF-A_{165}-induced biological responses in both HUVEC and PAE-KDR cells. Indeed we had previously shown that R59949 inhibition of HGF-induced chemotaxis was completely reverted by overexpression of Dgk-α, providing a strong indication for the specificity of R59949-induced inhibition (Cutrupi *et al*., 2000).

In the presence of 1 μM R59949, VEGF-A_{165}-stimulated cell migration was inhibited by about 50 and 60%, respectively, in PAE-KDR and HUVEC, while basal cell motility of unstimulated cells was not significantly affected. However, in both PAE-KDR and HUVEC, R59949 did not inhibit cell migration induced by serum, suggesting that Dgk-α stands in the signaling pathway conveying signals specifically from the VEGFR-2 to the cell motility machinery (Figure 5a and b).

We then investigated the ability of R59949 to inhibit VEGF-A_{165}-induced DNA synthesis in both PAE-KDR and HUVEC. In serum-starved PAE-KDR, VEGF-A_{165} induced a twofold increase of DNA synthesis, which was completely abolished in the presence of 1 μM R59949 (Figure 5c). In HUVEC cultured in 2% FCS, VEGF-A_{165} stimulates DNA synthesis, which was inhibited by R59949 at concentrations equal or higher than 5 μM (Figure 5d). The reduced ability of R59949 to inhibit DNA synthesis in HUVEC may depend on serum sequestration of R59949 (De Chaffoy de Courcelles *et al*., 1989), although the involvement of other Dgk isoforms, less sensitive to the inhibitor (Jiang *et al*., 2000), could not be ruled out.

In addition, we have assayed the ability of R59949 to inhibit tubular-like structure formation on matrigel both by HUVEC and PAE-KDR-PINCOS. In the presence of 1 μM R59949, both PAE-KDR-PINCOS and HUVEC, although still viable, fail to connect and to differentiate in chord-like structures, resulting in a severe impairment of the formation of the characteristic complex network (Figures 4c, 6a and b). Cell viability in the presence of R59949 was verified by three distinct assays, Trypan blue, calcein-AM and MTT, reflecting three different cellular functions, respectively membrane impermeability, cytoplasmic esterase activity and mitochondrial respiratory chain activity (Poole *et al*., 1993; Baldanzi *et al*., 2002). R59949 treatment of HUVEC did not affect their viability as measured by calcein-AM and MTT (Figure 6a and b), while drug treatement of PAE-KDR cells did not affect cell viability as measured by Trypan blue and MTT (Figure3b and data not shown).

Interestingly, the specificity of R59949 in impairing angiogenic signaling through inhibition of Dgk-α is further suggested by the observation that in PAE-KDR overexpression of Dgk-wt reverts significantly the inhibition of chord-like formation on matrigel

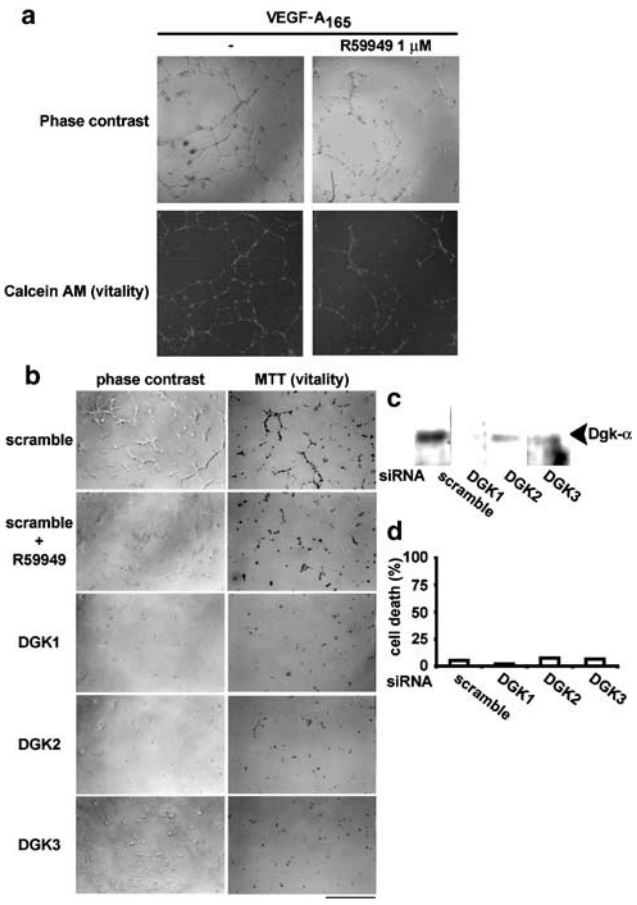

Figure 6 Downregulation of Dgk-α by RNA interference or R59949 treatment inhibits VEGF-A_{165}-induced *in vitro* angiogenesis in HUVEC. **(a)** HUVEC were plated on a growth factor rich matrigel and stimulated with VEGF-A_{165} (25 ng/ml) for 24 h in the presence or absence of 1 μM R59949. Cells were loaded with Calcein-AM fluorescent viability dye and photographed with contrast phase microscopy (upper panel) and green fluorescence (lower panel). **(b)** siRNA-transfected HUVEC were plated at 48 h following transfection on a growth factor rich matrigel for 24 h in the presence or absence of 1 μM R59949. Following image acquisition at contrast phase microscopy (left), cells were labeled with MTT vital dye and photographed with bright field microscopy (right). **(c)** siRNA-transfected HUVEC were homogenized, at 48 h from transfection, and their Dgk-α content was assayed by Western blot with anti-Dgk-α antibodies. **(d)** Cell death, quantified by Trypan blue exclusion assay, of siRNA-transfected HUVEC, 70 h following transfection

(Figure 4c). This finding is consistent with the reported ability of Dgk-α overexpression to revert the inhibition of HGF-induced chemotaxis exerted by R59949 (Cutrupi *et al*., 2000).

In addition, we have also verified whether R59949 impairs VEGF signaling through downregulation of VEGFR-2 tyrosine phosphorylation. However, VEGFR-2 tyrosine phosphorylation, as detected by antiphosphotyrosine Western blot of anti-VEGFR-2 immunoprecipitates, was not affected by cell treatment with 1 μM R59949 in VEGF-stimulated HUVEC (Figure 3a).

Downregulation of Dgk-α expression by RNA interference impairs HUVEC endothelial cell organization on matrigel

In order to provide further evidence that Dgk-α is involved in VEGF angiogenic signaling in HUVEC, we synthesized three single interfering RNAs for the Dgk-α transcript designed with Cenix algorithm, which did not overlap with any other known transcript from the human genome. Transfection of HUVEC cells with any of the three interfering RNAs lowers but does not abolish the expression of Dgk-α, as detected by Western blot on whole-cell lysates (Figure 6c). Transfection with the three interfering RNAs impairs the formation of chord-like structures on matrigel with efficacy similar to that obtained with 1 μM R59949 cell treatment. Conversely, transfection with a scrambled siRNA, which does not overlap with any known transcript, did not affect chord-like formation. In addition, downregulation of Dgk-α expression did not affect cell viability of HUVEC, as measured by Trypan blue exclusion (Figure 6d) and MTT (Figure 6b).

In summary, these data indicate that either inhibition of Dgk-α catalytic activity by both expression of dominant-negative mutant or cell treatment with a specific inhibitor, either downregulation of its expression by interfering RNA, severely impair the ability of endothelial cells to migrate, proliferate and organize as chord-like networks, providing further support to the claim that catalytic function of Dgk-α is required for the transduction of VEGF-A_{165} angiogenic signal.

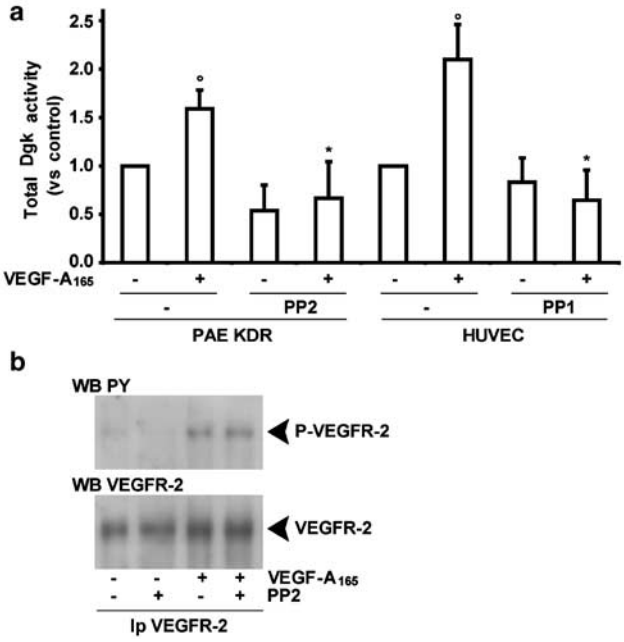

Figure 7 Src kinase activity is required for VEGF-A_{165}-induced activation of Dgk. **(a)** Dgk activity assayed in homogenates from either control or VEGF-A_{165} (10 ng/ml for 10 min)-stimulated quiescent PAE-KDR or HUVEC cells, pretreated for 15 min with either vehicle or with 5 μM PP2 or PP1 respectively (data are mean ± s.e.m. of five independent experiments normalized for control, °*t*-test *vs* control, **t*-test *vs* VEGF-A_{165}, $P<0.05$). **(b)** Antiphosphotyrosine (upper panel) and anti-VEGFR-2 (lower panel) Western blot of anti-VEGFR-2 immunoprecipitates obtained from cell lysates of either control or VEGF-A_{165} (10 ng/ml for 10 min)-stimulated quiescent HUVEC, pretreated with either vehicle or 5 μM PP2

VEGF-A_{165}-induced activation of Dgk-α is mediated by Src

Overall, these data demonstrate that VEGF-induced activation Dgk-α is required for VEGF angiogenic signaling in endothelial cells. We had previously shown that activation of Dgk-α by HGF is mediated by Src (Cutrupi *et al*., 2000), whose activation is also required for VEGF-A_{165}-induced cell motility, proliferation and increase of permeability (Abu-Ghazaleh *et al*., 2001; Eliceiri *et al*., 2002). Thus, we have explored the hypothesis that Dgk-α may stand as a downstream target of Src in VEGF-A signaling. Src tyrosine kinase activity was inhibited by pretreating both PAE-KDR and HUVEC, respectively, with 5 μM PP2 and PP1. At these concentrations, either PP1 or PP2 does not affect VEGFR-2 tyrosine kinase activity (Figure7b and data not shown) (Waltenberger *et al*., 1999; Abu-Ghazaleh *et al*., 2001). Inhibition of Src tyrosine kinase activity completely abrogated the stimulation of total Dgk activity induced by VEGF-A_{165}, demonstrating that Src mediates the activation of VEGF-A_{165}-induced stimulation of Dgk in both PAE-KDR and HUVEC (Figure 7a).

Then we investigated whether VEGF-A_{165} induces the formation of a Dgk-α/Src complex, by assaying Dgk activity co-purified with Src in anti-Src immunoprecipitates from either control or VEGF-A_{165}-stimulated PAE-KDR cells. Indeed, Dgk activity in anti-Src immunocomplexes from stimulated cells was higher than from control cells (Figure 8a, upper panel), while mock immunoprecipitates from VEGF-A_{165}-stimulated cells did not contain any Src protein and Dgk activity. No Dgk-α protein could be detected by Western blot in anti-Src immunoprecipitates, suggesting that the stoichiometry of Dgk-α association to Src is very low, below the level of sensitivity of the Dgk-α antibodies (data not shown). Indeed even upon HGF stimulation of PAE, only endogenous Dgk activity, but no endogenous Dgk-α protein, could be detected in a complex with Src (Cutrupi *et al*., 2000).

Thus in order to provide a more conclusive evidence for the formation of the complex between the two proteins, we have assayed the ability of myc-tagged Dgk-α to co-purify with Src in anti-Src immunoprecipitates. Myc-tagged Dgk-α was expressed in PAE-KDR cells by infection with PINCOS retrovirus containing myc-tagged Dgk-α, obtained as described previously (Cutrupi *et al*., 2000). Indeed in infected cells more myc-Dgk-α was detected by Western blot of anti-Src immunoprecipitates obtained from VEGF-A_{165}-stimulated rather than unstimulated cells (Figure 5b, upper panel). The amount of immunoprecipitated Src protein was not affected by VEGF-A_{165} stimulation, while no

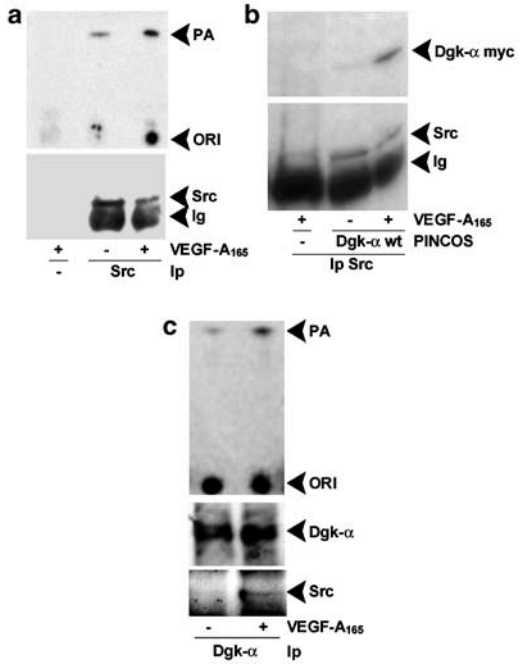

Figure 8 Dgk-α protein and activity are associated with Src in VEGF-A_{165}-stimulated cells. **(a)** Dgk enzymatic activity (upper panel) and Western blot of Src protein (lower panel) assayed in anti-Src immunoprecipitates obtained from lysates of either control or VEGF-A_{165} (50 ng/ml for 10 min)-stimulated quiescent PAE-KDR cells. (**b**) Myc-Dgk protein (upper panel) and Src protein (lower panel) assayed by Western blot, respectively, with anti-myc and anti-Src antibodies, of anti-Src immunoprecipitates obtained from lysates of VEGF-A_{165} (50 ng/ml for 10 min)-stimulated quiescent PAE-KDR infected with empty PINCOS vector or PINCOS/Myc-Dgk-α. (**c**) Dgk enzymatic activity (upper panel), Western blot of Dgk-α protein (central panel) and Western blot of Src protein (lower panel), assayed in anti-Src immunoprecipitates obtained from lysates of either control or VEGF-A_{165} (50 ng/ml for 10 min)-stimulated quiescent PAE-KDR cells

complex formation was observed in anti-Src immunoprecipitates from PAE-KDR cells infected with empty vector (Figure 8b, upper panel). In addition, we have been able the observe the formation of the Src/Dgk-α complex with endogenous proteins, by detecting Src protein in anti-Dgk-α immunoprecipitates from VEGF-A_{165}-stimulated but not unstimulated PAE-KDR cells (Figure 8c).

In summary, these data suggest that Src tyrosine kinase mediates the activation of Dgk by VEGF in both PAE-KDR and HUVEC. In addition, VEGF induces the formation of an Src/Dgk-α complex in PAE-KDR, while in the HUVEC the paucity of Dgk-α expression impairs to address the issue. Although we had previously shown that Dgk-α can be regulated by tyrosine phosphorylation, no tyrosine phosphorylation of Dgk-α could be detected upon VEGF-A_{165} stimulation, as well as upon HGF stimulation (Cutrupi *et al*., 2000).

No Dgk activity or protein, either endogenous or myc-tagged, was detectable in anti-VEGFR-2 immunoprecipitates (data not shown). Consistent with similar data obtained with HGF-R, these data suggest that Dgk-α does not form a stable complex with activated tyrosine kinase receptors (Cutrupi *et al*., 2000).

Discussion

Angiogenesis is a complex phenomenon required for embryonic development, wound healing, female cycle, solid tumor growth and revascularization of ischemic tissues (Matsumoto and Claesson-Welsh, 2001). Upon exposure to angiogenic factors, such as FGF, VEGF-A and HGF, endothelial cells lining blood vessels are activated in a spatially and timely coordinated manner to degrade the vessel basement membrane, invade the extracellular matrix according a chemotactic gradient, proliferate and differentiate to form a new lumen-containing vessel (Matsumoto and Claesson-Welsh, 2001; Gerhardt *et al*., 2003). Although the signaling pathways involved in the transduction of angiogenic stimuli have been extensively investigated, the complete picture of such signaling network is still missing.

We had previously shown that Dgk-α, which phosphorylates DG to PA, is activated by HGF through an Src-mediated mechanism, and is required for HGF-stimulated cell movement in a porcine aortic-derived cell line, raising the hypothesis that it may be involved in the transduction of the angiogenic signaling (Cutrupi *et al*., 2000). However, in order to provide more compelling evidence for the requirement of Dgk-α in the transduction of angiogenic signaling, we have investigated the role of Dgk-α in the signal transduction of VEGF-A, the major angiogenic factor *in vivo*, in both HUVEC primary endothelial cells and in PAE-KDR cells, an endothelial-derived cell line stably expressing VEGFR-2.

Herein we showed that VEGF-A_{165} stimulates Dgk activity in endothelial cells. In PAE-KDR cells, by assaying Dgk activity in anti-Dgk-α immunoprecipitates, we provided compelling evidence that Dgk-α isoform is activated by VEGF. In addition, most of the total Dgk activity assayed from PAE-KDR whole-cell homogenates is inhibited *in vitro* by R59949, which inhibits selectively class-I Dgk enzymes, that is, the α-, β- and γ- isoforms, while it does not affect the activity of other Dgk isoforms (Jiang *et al*. 2000), as well as the activity of other lipid and protein kinases (De Chaffoy de Courcelles *et al*., 1989; Cutrupi *et al*., 2000). The finding that overexpression of wt Dgk-α reverts the ability of R59949 to impair HGF-induced cell movement and network organization on matrigel, respectively, in PAE and PAE-KDR cells provides further support to the selectivity of R59949 for Dgk-α (Cutrupi *et al*., 2000; Figure 4c). Conversely, in HUVEC, which express lower levels of Dgk-α than PAE-KDR, VEGF-stimulated Dgk activity is only partially inhibited by R59949, suggesting that also R59949-resistant Dgk activities are activated by VEGF.

In order to investigate whether Dgk-α plays a role in VEGF angiogenic signaling, we have inhibited it by three independent strategies, that is, pharmacological inhibition with R59949 in both PAE-KDR and HUVEC, expression of a Dgk-α dominant-negative mutant in PAE-KDR cells and specific downregulation of Dgk-α expression by RNA interference in HUVEC. *In vitro*, the ability of endothelial cells to move toward a

chemotactic gradient, to proliferate and to organize in a network of chord-like structures on matrigel reflects their behavior during the angiogenic process *in vivo*.

In PAE-KDR cells, the inhibition by both expression of Dgk-α dominant-negative mutant, and cell treatment with 1 μM R59949 severely impairs VEGF-induced chemotaxis and DNA synthesis, as well as network formation on matrigel, without affecting cell viability. Thus, the data clearly demonstrate that in PAE-KDR cells Dgk-α is activated by VEGF and that its catalytic function is required for VEGF-induced angiogenic signaling *in vitro*.

In HUVEC, R59949, which inhibits part of the VEGF-stimulated Dgk activity, impairs VEGF-induced chemotaxis and DNA synthesis, as well as network organization on matrigel. The higher concentration of the drug required to impair DNA synthesis may further suggest that other R59949-resistant Dgk isoforms may be involved in the transduction of VEGF proliferative signaling in HUVEC. Alternatively, R59949 may be sequestered by the serum required to obtain HUVEC proliferation in cultures (De Chaffoy de Courcelles *et al*., 1989). However, more direct evidence that Dgk-α is required for *in vitro* angiogenesis in HUVEC primary endothelial cells comes from the demonstration that specific downregulation of Dgk-α expression by RNA interference totally impairs the ability of HUVEC to form networks on matrigel, without affecting cell viability. The same results were obtained with three different dsRNA, which target respectively two sequences at the N-terminal of the Dgk-α and a sequence next to the ATP-binding site. The three sequences do not feature any homology with other Dgk isoforms or other proteins.

In summary, these experiments strongly suggest that activation of Dgk-α is required for angiogenic signaling in both PAE and HUVEC endothelial cells, although the possibility that in HUVEC, which express a low level of the α-isoform, other Dgk isoform may also be involved in VEGF signaling should be taken into account.

Dgk-α does not contain any domain suggesting a direct interaction with tyrosine kinase receptors, and indeed does not associate with either VEGFR-2 or HGF-R (data not shown). However, a Dgk activity is upregulated upon expression of v-Src in fibroblasts (Sugimoto *et al*., 1984); we and others have recently shown that Src stimulates Dgk-α activity *in vitro* and mediates HGF- and IL-2-stimulated Dgk-α activation in intact cells (Cutrupi *et al*., 2000; Cipres *et al*., 2003). Here, we reported that in both PAE-KDR and HUVEC, the VEGF-induced stimulation of Dgk activity is blunted by cell treatment, respectively, with PP2 and PP1, two Src family-specific inhibitors that do not affect Dgk activity *in vitro* (data not shown). These data imply that even if other Dgk isoforms are also stimulated by VEGF in these cells, their activation is completely Src family kinase dependent. Indeed, very recent data indicate that Dgk-α associates with Src upon GnRH stimulation of pituitary cells (Davidson *et al*., 2004). In addition, in PAE-KDR cells, we were able to show that VEGF stimulation induces the formation of a complex between Dgk-α and Src, but not between Dgk-α and the activated receptor, as Dgk-α does not co-purify in antireceptor immunoprecipitates (data not shown). This may depend on the low stoichiometry of the Src/Dgk-α complex, that is, only a small fraction of receptor-associated Src forms a ternary complex with Dgk-α. Alternatively, receptor-activated Src may come off the complex with the receptor and may associate with Dgk-α. Indeed according to current models of its function, Src is recruited to the active receptor through its SH2 domain, switches to the active open conformation, allowing complex formation with its substrates, for instance p130Cas and FAK, their processive phosphorylation and establishing a more stable complex with them through its SH2 domain, excluding the receptor (Nakamoto *et al*., 1996; Thomas *et al*., 1998; Scott and Miller, 2000; Pellicena and Miller, 2001).

Although these data indicate Dgk-α as a putative substrate of Src tyrosine kinase activity, no tyrosine phosphorylation of Dgk-α could be detected upon VEGF-A stimulation of either PAE-KDR or HUVEC. However, tyrosine phosphorylation of Dgk-α was previously shown in PAE cells upon treatment with sodium pervanadate, and in COS cells upon coexpression of both Src and Dgk-α (Cutrupi *et al*., 2000). In both cases, tyrosine phosphorylation correlates with an increase of Dgk-α enzymatic activity. We speculate that either tyrosine phosphorylation of Dgk-α occurs at very low stoichiometry or is a transient event leading to the switch of Dgk-α to an active conformation. Alternatively, Src may induce tyrosine phosphorylation of a regulator of Dgk-α. Indeed recent findings indicate that $PI(3,4,5)P_3$, the lipid product of PI 3-kinase, binds and stimulates Dgk-α activity and that active Lck stimulates Dgk-α in a PI 3-kinase-dependent manner (Cipres *et al*., 2003). Therefore, it is likely that multiple mechanisms may participate in the regulation of Dgk-α. The identification of the molecular determinants of the physical and functional interactions between the two proteins will shed light on such mechanisms.

VEGF-A-induced angiogenesis, both *in vitro* and *in vivo*, requires Src function: Src is required for VEGF-A-induced chemotaxis and proliferation of endothelial cells *in vitro* (Abu-Ghazaleh *et al*., 2001), while genetic evidences *in vivo* indicate that deletion of either Src or Yes gene in mice impairs VEGF-dependent increase of endothelial permeability, that is, edema formation (Eliceiri *et al*., 2002). Furthermore, retroviral-mediated overexpression of Src dominant negative in a subcutaneous model of angiogenesis severely impairs neo-vascularization *in vivo* (Eliceiri *et al*., 1999). Thus, the demonstration that VEGF-induced stimulation of Dgk activity depends on Src activity, and that Dgk-α is required for VEGF-A-induced angiogenetic responses *in vitro* suggest that it may act as a crucial Src effector in VEGF angiogenic signaling in endothelial cells. In addition, at least in HUVEC, the involvement of other Src-regulated Dgk isoforms in VEGF signaling is not ruled out.

The signaling pathways downstream to Dgk-α have not been extensively investigated yet. Dgk enzymes phosphorylate DG to generate PA. Indeed VEGF-A stimulates PLC-γ-mediated hydrolysis of $PI(4,5)P_2$ to generate DG, which then leads to activation of PKC (Matsumoto and Claesson-Welsh, 2001). PKC-α and -β isoforms are required for VEGF-stimulated cell proliferation and migration *in vitro*, as well as for neovascularization *in vivo* (Wellner *et al*., 1999; Wang *et al*., 2002).

In some cell signaling system, activation of Dgk-α, which removes DG to generate PA, contributes to terminate DG-mediated signaling, while its inhibition results in the accumulation of DG, leading to sustained DG-mediated signaling. However, in endothelial cells, the increased activity of PKCα, which indeed is activated by VEGF-A in a DG-mediated manner, is expected to enhance rather than inhibit VEGF-A-induced angiogenic response (Wellner *et al*., 1999; Matsumoto and Claesson-Welsh, 2001; Wang *et al*., 2002). Conversely, the role of activation of Dgk-α in VEGF-A angiogenic signaling may consist in the generation of PA-mediated signals. The role of PA in VEGF-A signaling still remains to be investigated. *In vitro*, PA is a regulator of several signaling proteins, some of which are involved in VEGF-A signaling (English, 1996; Gomez-Cambronero and Keire, 1998; Topham and Prescott, 1999). For instance, VEGF-A activates PKCζ, which is positively regulated by PA and Src tyrosine phosphorylation (Limatola *et al*., 1994; Seibenhener *et al*., 1999). Similarly, VEGF-A activates Raf, which is regulated by PA *in vitro* and in intact cells (Andresen *et al*., 2002). Activation of both PKCζ and Raf is required for VEGF-induced angiogenic signaling (Matsumoto and Claesson-Welsh, 2001). Among other putative targets of PA *in vitro*, Rho-GDI and PI(4)P 5-kinase might play a role in VEGF-A signaling, respectively, by mediating Rac activation (Del Pozo *et al*., 2002; Zeng *et al*., 2002) and by participating in the dynamic recruitment of focal adhesion proteins required for cell movement (Jenkins *et al*., 1994; Di Paolo *et al*., 2002). In addition, both *in vitro* and in intact cells, PA also regulates activation of mTor, a serine kinase involved in the transduction of growth factor-stimulated proliferative signaling (Fang *et al*., 2001). However, the role of mTor in VEGF-A signaling has not been investigated. Alternatively, PA generated through activation of Dgk-α may be further metabolized to lysosphosphatidic acid (LPA), through a phospholipase A2. Then LPA, through an autocrine loop, may stimulate its receptor(s), edg.2 and -4, whose expression has been reported in endothelial cells (Panetti, 2002). Indeed, exogenously added LPA stimulates endothelial cell proliferation and cell matrix-adhesion dependent cell migration (Panetti, 2002). Interestingly, in prostate carcinoma, bombesin, a growth factor for these cells, has been shown to activate an LPA autocrine circuit, which is required for bombesin signaling (Xie *et al*., 2002). However, no evidence of growth factor-stimulated synthesis of LPA has been reported in endothelial cells.

Finally we provided evidence indicating that Dgk-α is an essential component of VEGF-A signal transduction, which is required to convey both chemotactic and proliferative signals downstream from Src. Because tumor cells produce a wide array of angiogenic factors, it has been suggested that successful strategies to block angiogenesis in cancer patients may repress the ability of endothelial cells to participate in the angiogenic process. As Dgk-α activity is required for angiogenesis and quite specific inhibitors are available, Dgk-α may represent a new and promising target for pharmaceutical control of angiogenesis.

Materials and methods

Cells culture, reagents and antibodies

Porcine aortic endothelial cell line (PAE), a cell line derived from porcine aortic endothelium, and PAE-KDR, a PAE-derived cell line stably expressing VEGFR-2, were a kind gift of Waltenberger (Waltenberger *et al*., 1994), and were cultured in Ham's F12 supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 50 μg/ml streptomycin (Life Technologies). Human endothelial cells from umbilical cord veins (HUVEC) were prepared and characterized as previously described, and were grown in M199 medium (BioWhittaker, Walkersville, MD, USA) supplemented with 20% FCS (Life Technologies), endothelial cell growth factor (100 μg/ml) (Sigma Chemical Co., St Louis, MO, USA) and porcine heparin (Sigma) (100 μg/ml). HUVEC were used at second passage and grown on a plastic surface coated with porcine gelatin (Sigma), otherwise specified. Recombinant $VEGF\text{-}A_{165}$ was purchased from R&D systems, and R59949, PP1 and PP2 were from Alexis. Anti-Src antibodies (N-16 for immunoprecipitation and SRC-2 for Western blotting) were from Santa Cruz Biotechnology, and 4G10 antiphosphotyrosine and 9E10 anti-myc tag antibodies were from UBI. Anti-Dgk-α antibodies were a mix of monoclonal obtained as described (Schaap *et al*., 1993). Anti-VEGFR-2 antibodies were from Santa Cruz Biotechnologies. ECL and secondary antibodies were from Perkin-Elmer. PINCOS, PINCOS/Myc-Dgk-α, PINCOS/Myc-Dgk-α-K^- and the PHOENIX retroviral system were previously described (Cutrupi *et al*., 2000). Unless specified, all other reagents were from Sigma-Aldrich.

Infection with retroviral vectors

Phoenix cells obtained by G Nolan (Swift *et al*., 1999) were transiently transfected by calcium phosphate (Cell Phect Transfection kit, Amersham-Pharmacia) with 20 μg PINCOS, PINCOS/Myc-Dgk-α or 20 μg PINCOS/Myc-Dgk-α-K^- in growth media containing chloroquin (25 μM). Cells were incubated for 8 h at 37°C (5% CO_2) and the precipitate was removed by washing with PBS. Then, cells were incubated with DMEM containing 10% FCS for 72 h; the retroviral supernatant was collected, the debris removed by 1500 *g* centrifugation, filtered by a 0.8 pore filter, and supplemented with Polybrene (8 μg/ml). Log-phase growing PAE-KDR cells were infected by addition of 5 ml retroviral supernatant. Following 16 h from infection, cells were placed into 10% FCS medium for 48 h and serum starved overnight in 0.5% FCS prior to further experimental manipulations. PINCOS-infected cells express both GFP and the protein of interest. Efficiency of infection was about 70% as measured by FACS analysis of GFP expression.

Preparation of cell lysate, homogenates, immunoprecipitation and Western blotting

Subconfluent PAE-KDR cultures were made quiescent by 16 h serum starvation. Subconfluent HUVEC cultures were made quiescent by incubating for 16 h in 5% FCS. Following cell stimulation, the cell lysates were prepared in buffer A (25 mM Hepes pH 8, 10% glycerol, 150 mM NaCl, 5 mM EDTA, 2 mM EGTA, 1 mM $ZnCl_2$, 50 mM ammonium molibdate, supplied before use with 1 mM sodium orthovanadate, 10 mM NaF, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 μg/ml pepstatin, 1 μg/ml soybean trypsin inhibitor) supplemented with 1% NP-40, as described previously (Cutrupi *et al*., 2000). Where indicated, cell extracts were prepared by collecting the cells with a rubber scraper in buffer A and homogenizing them in syringe–needle in buffer A. Protein concentration was determined by the BCA method (Pierce). Immunoprecipitation, SDS–PAGE and Western blots were performed as described previously (Cutrupi *et al*., 2000).

Dgk-α assay

Dgk-α was assayed in the immunoprecipitates essentially as described (Schaap *et al*., 1993): immunocomplexes were incubated for 5 min at 30°C with 1 mg/ml diolein (Fluka), 1 mM ATP, 30 μCi-[γ^{32}P]ATP (Amersham), 10 mM $MgCl_2$, 1 mM $ZnCl_2$, 1 mM EGTA in 25 mM Hepes pH 8, and the reaction stopped with 50 μl of 1 M HCl and 100 μl of chloroform : methanol 1 : 1. Lipids were then extracted, and separated by TLC in chloroform : methanol : water : 25% ammonium hydroxide (60 : 47 : 11 : 4). TLC was exposed on a autoradiographic film (Kodak Biomax), [^{32}P]PA was identified by co-migration with nonradioactive PA standards stained by incubation in an iodine chamber. PA spots were quantified using phosphor imager (Biorad). Dgk activity in homogenates (1–5 μl) was assayed as described above, except that ATP was 5 mM.

Cell migration assay

For migration on gelatin, endothelial cells were seeded (1×10^5 cells in 50 μl suspension) in the upper chamber of a modified Boyden chamber. The undersurface of PVDF filter (8 μm pores, Nucleopore) was coated with 0.1% gelatin. The lower chamber was filled with serum-free medium with or without VEGF-A_{165} and incubated at 37°C in air with 5% CO_2 for 6 h. Where indicated, 1 μM R59949 was added to the upper chamber with the cells. Filters were then removed, stained with Diff-Quik (Baxter Diagnostic AG) and the cells of five fields were counted at the inverted microscope with a high-power oil immersion objective (Zeiss). For migration on collagen I, endothelial cells were seeded (1×10^5 cells in 50 μl suspension) in the upper chamber of a chemotox chamber (Nuroprobe), coated with 20 ng/ml collagen I. The lower chamber was filled with serum-free medium with or without VEGF-A_{165} and incubated at 37°C in air with 5% CO_2 for 6 h. Filters were then removed, stained with crystal violet and the cells of five fields were counted at the inverted microscope (Zeiss axiovert).

Cell proliferation assay

PAE KDR cells were plated and after 6 h treated as indicated for 24 h. HUVEC cells were plated and after 24 h treated as indicated for additional 48 h. BrdU incorporation was measured with 'Cell Proliferation ELISA Biotrak System' from Amersham-Pharmacia as suggested by the manufacturer.

Matrigel morphogenetic assay

Matrigel (Collaborative Biomedical Products, Becton Dickinson, Milan, Italy; not purified from contaminant growth factors; 0.2 ml). was added to each well of a 48-well plate and incubated at 37°C for 30 min to allow gel formation. Endothelial cells (2×10^4/well) were plated onto Matrigel. After 48 h incubation in the presence or absence of the indicated treatments in 5% CO_2 humidified atmosphere at 37°C, the three-dimensional organization of the cells was examined under an inverted phase contrast photomicroscope (DM-IBM model; Leica Microsystems, Wetzlar, Germany) and then photographed. Not purified matrigel allows spontaneous *in vitro* angiogenesis (Marconcini *et al*., 1999).

RNA interference

HUVEC were transfected with siPORT lipid transfection agent (Ambion) as suggested by the manufacturer. Cell transfection procedures must be optimized for each HUVEC preparation using Silencer siRNA Transfection KIT (Ambion). siRNA used were chemically synthesized as double-strand RNA (Ambion). The sequence was designed using Cenix bioscience algorithm, which considers T_m, nucleotide content of the 3′ overhangs, nucleotide distribution over the length of the siRNA, and presence and location of mismatches to off target genes. A key step in the algorithm is a stringent analysis of each siRNA sequence to maximize the target specificity. Sequences were as follows: DGK1 sense GGUCA GUGAUGUCCUAAAGTT, antisense CUUUAGGACAU CACUGACCTT (target AAGGTCAGTGATGTCCTAAAG); DGK2 sense GGAUGGCGAGAUGGCUAAATT, antisense UUUAGCCAUCUCGCCAUCCTC (target AAGGATGGC GAGATGGCTAAA); and DGK3 sense GGAUUUAGA-GAUGAGUAAATT, antisense UUUACUCAUCUCUAAA UCCTT (target AAGGATTTAGAGATGAGTAAA). A GA PDH scramble siRNA was used as negative control (provided with Silencer siRNA Transfection KIT from Ambion).

Reproducibility and data analysis

All the experiments shown are representative of three or more independent experiments with similar results. In Figures 3c and 7a, data are mean ± s.e.m. of five independent experiments normalized for control.

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