

## Activation and translocation of PKC $\delta$ is necessary for VEGF-induced ERK activation through KDR in HEK293T cells

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### Abstract

VEGF-KDR/Flk-1 signal utilizes the phospholipase C- $\gamma$ -protein kinase C (PKC)-Raf-MEK-ERK pathway as the major signaling pathway to induce gene expression and cPLA2 phosphorylation. However, the spatio-temporal activation of a specific PKC isoform induced by VEGF-KDR signal has not been clarified. We used HEK293T (human embryonic kidney) cells expressing transiently KDR to examine the activation mechanism of PKC. PKC specific inhibitors and human PKC $\delta$  knock-down using siRNA method showed that PKC $\delta$  played an important role in VEGF-KDR-induced ERK activation. Myristoylated alanine-rich C-kinase substrate (MARCKS) translocates from the plasma membrane to the cytoplasm depending upon phosphorylation by PKC. Translocation of MARCKS-GFP induced by VEGF-KDR stimulus was blocked by rottlerin, a PKC $\delta$  specific inhibitor, or human PKC $\delta$  siRNA. VEGF-KDR stimulation did not induce ERK phosphorylation in human PKC $\delta$ -knockdown HEK293T cells, but co-expression of rat PKC $\delta$ -GFP recovered the ERK phosphorylation. Y311/332F mutant of rat PKC $\delta$ -GFP which cannot be activated by tyrosine-phosphorylation but activated by DAG recovered the ERK phosphorylation, while C1B-deletion mutant of rat PKC $\delta$ -GFP, which can be activated by tyrosine-phosphorylation but not by DAG, failed to recover the ERK phosphorylation in human PKC $\delta$ -knockdown HEK293T cell. These results indicate that PKC $\delta$  is involved in VEGF-KDR-induced ERK activation via C1B domain.

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**Keywords:** Vascular endothelial growth factor-A; KDR/Flk-1; HEK293T cells; Phospholipase C- $\gamma$ ; Protein kinase C; ERK; GFP; Myristoylated alanine-rich C kinase substrate; C1B domain

Recent studies have revealed that vascular endothelial growth factor-A (VEGF), also referred to as vascular permeability factor (VPF), is essential for many angiogenic processes in normal and abnormal states such as diabetic retinopathy, tumor vascularization, and rheumatic arthropathy [9,10,31]. VEGF exhibits two major activities, the capacity to stimulate vascular endothelial cell proliferation [3,8,25] and the ability to increase microvessel permeability to macromolecules [21,43]. In addition, VEGF stimulates migration [2],

protease production [39,54], cell survival [12], generation of nitric oxide and prostacyclin [30,53,55] in endothelial cells, and migration of peripheral blood monocytes [1,45]. The signaling mechanisms underlying these diverse process are still not well characterized.

VEGF binds two tyrosine kinase receptors, Flt-1 and KDR/Flk-1 (also known as VEGF receptor-1 and -2, respectively) with high affinity [5,29]. Flt-1 and KDR/Flk-1 belong to a receptor gene family distantly related to the Fms/Kit/platelet-derived growth factor receptor (PDGFR). In addition, they share seven immunoglobulin (Ig)-like domains in the extracellular domain and a long kinase insert in the middle of the kinase domain

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[28,46,52]. Since the mRNAs of the Flt-1 and KDR are specifically expressed in the vascular endothelial cells [6,17,18,40,57], they are considered to play a crucial role in the vascular system.

Unlike other representative growth factor receptor tyrosine kinases that mainly mediate Ras to activate Raf-MEK-ERK pathway, KDR/Flk-1 utilizes the phospholipase C- $\gamma$  (PLC- $\gamma$ )-protein kinase C (PKC)-Raf-MEK-ERK pathway but not Ras-mediated pathway [50,51]. In an agreement with this notion, Ras-GTP complex is below detectable levels after VEGF stimulation and dominant-negative Ras does not suppress the VEGF signaling. The precise mechanism of VEGF-KDR signaling to ERK phosphorylation is still controversial.

PKC plays a pivotal role in many signaling pathways, and the existence of multiple isoforms suggests that different isoforms have various functions. The PKC superfamily consists of at least 10 isoforms and is divided into three groups, classical PKC (cPKC;  $\alpha$ ,  $\beta$ I,  $\beta$ II,  $\gamma$ ), novel PKC (nPKC;  $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\theta$ ), and atypical PKC (aPKC;  $\zeta$ ,  $\iota/\lambda$ ), based on their structure and biochemical properties [32,33]. Each PKC isoform has differential sensitivity to activators and shows tissue-specific and cell-specific expression, suggesting that each isoform plays a specific role in various signal transduction pathways and in the regulation of numerous cellular processes [32–34]. Recently, we demonstrated using GFP-tagged PKC that various stimuli induce specific translocation and activation of each PKC isoform, and suggested that each isoform has specific translocation mechanism and function in response to various stimuli, respectively [19,36,41,48]. Several investigators have reported that a specific PKC isoform is involved in the VEGF signaling pathway. VEGF activated PKC $\beta$  in NIH3T3 cells overexpressing KDR [50], PKC $\alpha$  and PKC $\zeta$  or PKC $\delta$  is involved in VEGF-induced ERK activation in human umbilical vein endothelial cells [13,56].

In the present study, to clarify a PKC isoform involved in the signaling and to investigate its activation mechanism in VEGF-KDR-PLC $\gamma$ -PKC-Raf-ERK pathway, we examined the effect of a PKC specific inhibitor or siRNA on ERK phosphorylation induced by VEGF-KDR stimulation in HEK293T cells expressing KDR. We also investigated the spatial activation of PKC by VEGF-KDR stimulation. We demonstrated here that PKC $\delta$  mediates the cascade from VEGF-KDR to ERK and PKC $\delta$  translocation from the cytoplasm to the plasma membrane through its C1B domain is necessary for VEGF-induced ERK phosphorylation.

## Materials and methods

**Materials and chemicals.** Human VEGF-A was purchased from PeproTec EC. Polyclonal anti-ERK (#9102) and monoclonal

anti-phospho-ERK (Thr202/Tyr204) (#9106) antibodies were purchased from Cell Signaling Technology. Polyclonal anti-Flk-1 (sc-505), anti-PKC $\beta$ I (sc-209), anti-PKC-II (sc-210), anti-PKC $\gamma$  (sc-211), anti-PKC (sc-937), anti-PKC $\epsilon$  (sc-214), and anti-PKC $\eta$  (sc-215) antibodies were purchased from Santa Cruz Biotechnology. Monoclonal anti-PKC $\alpha$  antibody was purchased from Transduction Laboratories. Polyclonal anti-PKC $\zeta$  serum (#07-264) was purchased from Upstate. Other materials and chemicals were from commercial sources.

**Cell culture.** Human embryonic kidney cells (HEK293T) were cultured in Dulbecco's modified Eagle's medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO<sub>2</sub> at 37 °C. All media were supplemented with penicillin (100 U/ml) and streptomycin (100 mg/ml).

**Preparation of plasmids.** The expression plasmids bearing cDNA of KDR, PKC $\alpha$ , PKC $\beta$ I, PKC $\beta$ II, PKC $\gamma$ , PKC $\delta$ , PKC $\epsilon$ , PKC $\eta$ , PKC $\zeta$ , PKC $\delta$ -GFP, PKC $\delta$ -(Y311/322F)-mutant-GFP, PKC $\delta$ -mutant deleting C1B domain ( $\Delta$ C1B mutant)-GFP, and myristoylated alanine-rich protein kinase C substrate (MARCKS)-GFP were prepared as described previously [20,35–38,41,42].

**Transfection of plasmids into cultured cells.** Plasmids were prepared by purification protocol using CsCl density gradient. Transfection of plasmids was performed by lipofection using Polyfect Transfection Reagent (QIAGEN GmbH, Germany) or Lipofectamin 2000 (Invitrogen) according to the manufacturer's standard protocol. For co-expression of plasmids, the same amount of each plasmid was mixed and transfected. Experiments were 24–48 h after the transfection.

**Observation of translocation of MARCKS-GFP or PKC $\delta$ -GFP.** Six hours after transfection, cells were spread onto glass-bottomed culture dish (MatTek, Ashland, MA) and cultured for 18 h before observation. The culture medium was replaced with normal Hepes buffer composed of 5 mM Hepes, 135 mM NaCl, 5.4 mM KCl, 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, and 10 mM glucose, pH 7.3, at RT. Translocation of fusion proteins was triggered by a direct application of VEGF at a 10 times higher concentration into the buffer to obtain the final concentration at 25 ng/ml. The fluorescence of GFP was monitored under a confocal laser scanning fluorescence microscope (model LSM 510 invert, Carl Zeiss, Jena, Germany). GFP-fused protein was monitored at 488 nm argon excitation with a 510–535 nm-band pass barrier filter. Images were collected sequentially every 10 s. All experiments were performed at 37 °C.

**Knock down of endogenous PKC $\delta$  by siRNA.** Knock down of human PKC $\delta$  in HEK293T cells by using siRNA was performed as described previously [16]. Briefly, the siRNA sequences of human PKC $\delta$  siRNA corresponded to the coding region 603–621 relative to the first nucleotide to the start codon. Twenty-one-nucleotide RNAs with 3' overhangs of 2 deoxythymidine were chemically synthesized by Nihon Bio Service (Asaka, Japan). To generate a duplex of siRNA, 20  $\mu$ M sense and antisense single-strand RNAs were annealed by incubating the mixed siRNA in annealing buffer (30 mM Hepes-KOH, 100 mM potassium acetate, and 2 mM magnesium acetate) for 1 min at 90 °C followed by incubation for 1 h at 37 °C. Transfection of duplex siRNA was performed by using Lipofectamin 2000 (Invitrogen) according to the method described by Elbashir et al. [7]. Annealed siRNA was transfected at a final concentration of 250 nM into 90–100% confluent HEK293T cells by lipofection.

**ERK phosphorylation assay.** The transfected cells were serum-starved for 2 h and stimulated by final 25 ng/ml VEGF for 5 min. Then SDS-PAGE sample buffer was added to the cells to stop the signal transduction. Total cell lysates were collected with scrapper, heated to 95 °C for 5 min, fractionated by a 8% acrylamide SDS-PAGE gel, and immunoblotted with an antibody that specifically recognized ERK-1 and ERK-2 activated by phosphorylation at Thr202/Tyr204.

## Results

### Effects of selective PKC inhibitors on VEGF-KDR-induced ERK phosphorylation in transiently KDR-expressed HEK293T cells

VEGF induced (25 ng/ml) ERK phosphorylation in a time dependent manner in the HEK293T cells which transiently express KDR (Fig. 1), although ERK phosphorylation was not observed by the same treatment in wild HEK293T cells (data not shown). The phosphorylation reached a maximum between 3 and 5 min and the signal slightly decreased in intensity by 30 min after stimulation (Fig. 1). Using the HEK293T cells expressing KDR, we first examined whether ERK phosphorylation by VEGF depends on PKC or not. A broad PKC inhibitor, Gö6983, significantly reduced VEGF-induced ERK phosphorylation, indicating that PKC is involved in the ERK phosphorylation.

Western blotting using isozyme specific antibodies revealed that HEK293T cell mainly expressed PKC $\beta$ I,  $\beta$ II,  $\delta$ ,  $\epsilon$ , and  $\zeta$  (Fig. 2) in addition to faint PKC $\alpha$ . To determine which PKC isozyme is involved in the signaling, we investigated the effect of isozyme specific PKC inhibitors on VEGF-KDR-induced ERK phosphorylation. In addition to Gö6983, GF109203X, another broad PKC

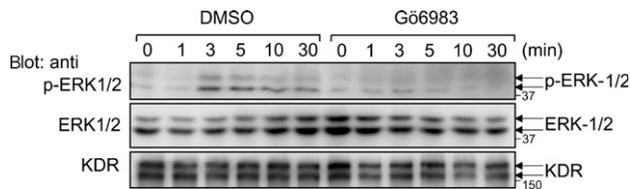


Fig. 1. PKC dependent ERK phosphorylation induced by VEGF in HEK293T cells expressing KDR. Forty-eight hours after the transfection with KDR, cells were pretreated without (DMSO) or with 2  $\mu$ M Gö6983 for 1 h and stimulated by 25 ng/ml VEGF for the indicated times. The signaling of the cells was stopped by adding SDS-sample buffer and the extracts were loaded to SDS-PAGE and transferred to a membrane. Phosphorylation of ERK1, 2 was detected with anti-phospho-Thr202/Tyr204 ERK antibody.

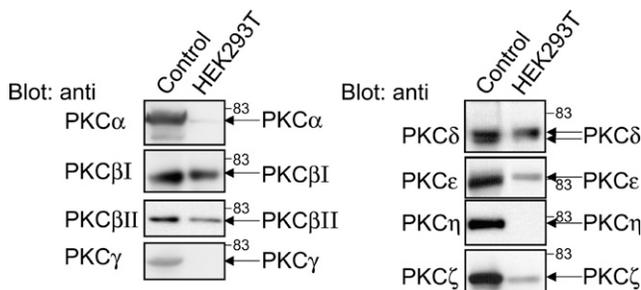


Fig. 2. Expression of multiple PKC isozymes in HEK293T cell. Total cell lysate (25 mg) extracted from HEK293T cells (HEK293T) was fractionated by SDS-PAGE and Western blot analyses were performed with anti-PKC isozyme specific antibodies. Cells expressing PKC $\alpha$ ,  $\beta$ I,  $\beta$ II,  $\gamma$ ,  $\delta$ ,  $\epsilon$ , and  $\zeta$  were used as a positive control (control).

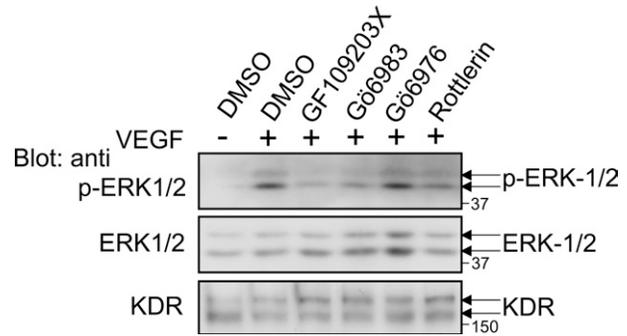


Fig. 3. Effects of PKC inhibitors on VEGF-induced ERK phosphorylation in HEK293T cells expressing KDR. HEK293T cells transfected with KDR were incubated for 1 h in absence (DMSO) or presence of 1  $\mu$ M GF109203X, 1  $\mu$ M Gö6983, 1  $\mu$ M Gö6976 or 5  $\mu$ M Rottlerin. Then these cells were treated with 25 ng/ml VEGF for 5 min. The signaling of the cells was stopped by adding SDS-sample buffer and the extracts were loaded to SDS-PAGE and transferred a membrane. Phosphorylation of ERK1, 2 was detected with anti-phospho-Thr202/Tyr204 ERK antibody. The results shown are representative of two experiments.

inhibitor, suppressed the ERK phosphorylation. On the other hand, Gö6976, a cPKC specific inhibitor, did not show any effect on ERK phosphorylation, while rottlerin, a PKC $\delta$  specific inhibitor, significantly suppressed the ERK phosphorylation. These data suggested that PKC $\delta$  plays a key role in VEGF-KDR-induced ERK phosphorylation among endogenous PKC isozymes (Fig. 3).

### Inhibition of VEGF-KDR-induced ERK phosphorylation by PKC $\delta$ siRNA

We have reported a successful knockdown of PKC $\delta$  in HEK293T cells using human PKC $\delta$ -specific siRNA [16]. To ascertain that PKC $\delta$  is involved in ERK phosphorylation in response to VEGF-KDR stimulation, we knocked down endogenous PKC $\delta$  in HEK293T cells using RNAi method. siRNA for human PKC $\delta$  significantly decreased PKC $\delta$  expression, while it had no effect on the expression of other PKC isozymes (Fig. 4A). Concomitantly siRNA targeting human PKC $\delta$  decreased VEGF-KDR-induced ERK phosphorylation in the HEK293T cell (Fig. 4B), but control siRNA with scrambled sequence failed.

### Translocation of MARCKS-GFP from the plasma membrane to the cytoplasm in response to VEGF-KDR stimulation depends on PKC

It is supposed that activation of PKC $\delta$  is necessary for ERK phosphorylation by VEGF treatment, although the targeting site of PKC $\delta$  is still unclear. It also remains unknown where PKC $\delta$  is activated and where the enzyme phosphorylates the substrate leading to ERK phosphorylation. Live imaging studies revealed

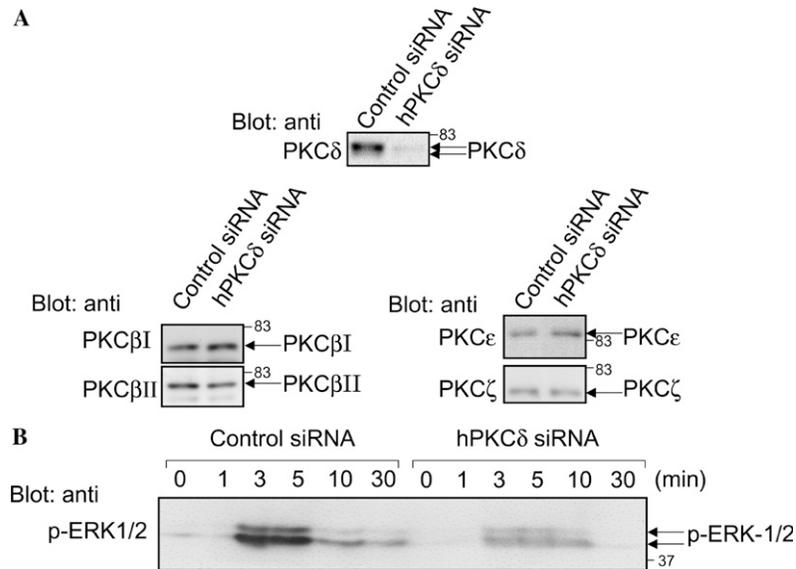


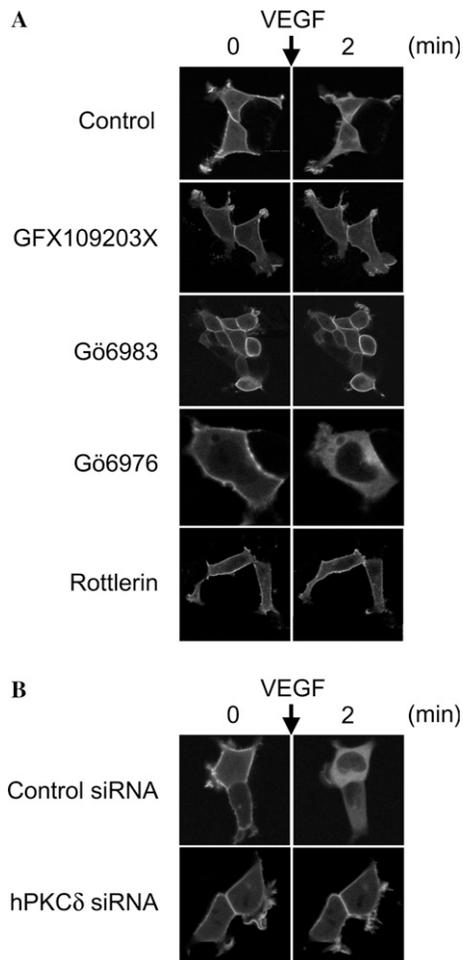
Fig. 4. Effect of endogenous PKC $\delta$  knockdown by siRNA on VEGF-induced ERK phosphorylation in transiently KDR-expressed HEK293T cell. HEK293T cells were co-transfected with KDR and either siRNA for human PKC $\delta$  (hPKC $\delta$  siRNA) or scrambled siRNA (random siRNA). (A) Forty-eight hours after the transfection, total cell lysate (25  $\mu$ g) were subjected to SDS-PAGE and Western blot analyses were performed with different PKC isoforms as indicated. (B) Forty-eight hours after the transfection, cells were stimulated by 25 ng/ml VEGF for the indicated times. Phosphorylation of ERK1, 2 was analyzed by Western blotting with anti-phospho-Thr202/Tyr204 ERK antibody.

that PKC $\delta$  is activated on the plasma membrane [36], in the cytoplasm [36], in the mitochondria [26], and at Golgi complex [19]. We examined where the active PKC $\delta$  is localized by using myristoylated alanine-rich C kinase substrate (MARCKS), as a marker of PKC activation. MARCKS, one of the physiological PKC substrates, cycles on and off plasma membranes by a mechanism termed the myristoyl-electrostatic switch. While at the membrane, MARCKS binds to and sequesters acidic phospholipids intervening basic effector domain. Activated PKC phosphorylates basic effector domain of MARCKS and then MARCKS translocates from the plasma membrane to the cytosol. Using GFP fused MARCKS (MARCKS-GFP), the translocation of MARCKS can be monitored in living cells by confocal laser scanning microscope [35]. It has enabled us to detect the activation of PKC on the plasma membrane in living cells by monitoring the location of MARCKS. As shown in Fig. 5, VEGF induced MARCKS-GFP translocation from the plasma membrane to the cytoplasm, suggesting that endogenous PKC was activated on the plasma membrane by VEGF-KDR stimulation (Fig. 5A). Pan PKC inhibitors including GF109203X and Gö6983 blocked the MARCKS-GFP translocation (Fig. 5A). Treatment of Gö6976 or BAPTA-AM (data not shown) had no effect on the MARCKS-GFP translocation, while inhibition of PKC $\delta$  with rottlerin blocked MARCKS translocation. These results strongly suggest that VEGF-KDR stimulation induces membrane translocation of PKC $\delta$  and its activation on the plasma membrane. In addition to the pharmacological

studies, we examined the translocation of MARCKS-GFP in HEK293T cells transfected with siRNA for human PKC $\delta$ . In PKC $\delta$ -knockdown HEK293T cells, VEGF-KDR stimulation failed to induce the translocation of MARCKS-GFP (Fig. 5B). These findings indicate that activation of PKC $\delta$  on the plasma membrane is indispensable for the ERK phosphorylation at downstream of VEGF-KDR signaling.

#### *C1B domain of PKC $\delta$ is necessary for VEGF-KDR induced ERK phosphorylation*

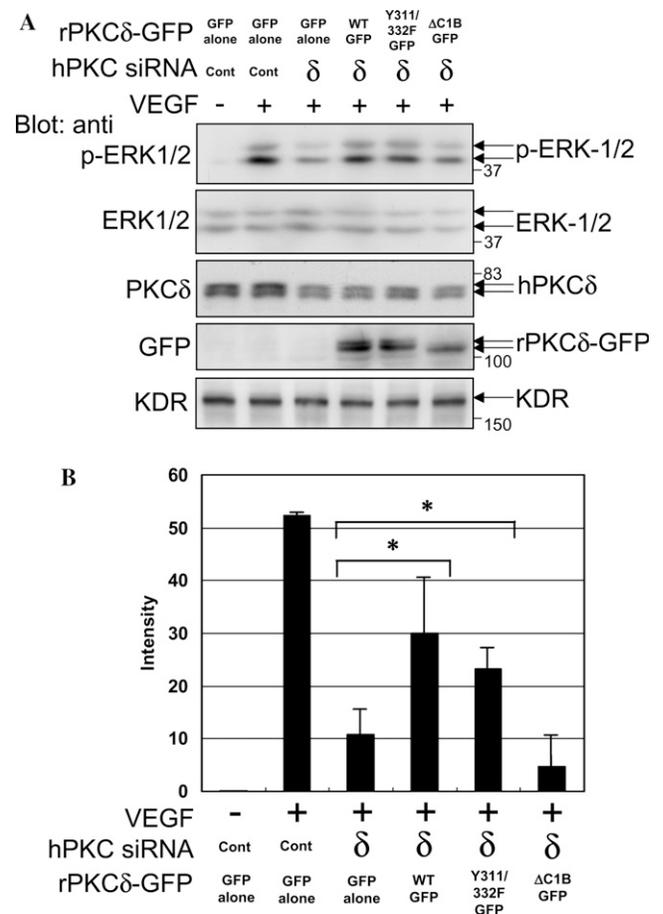
It is reported that PKC $\delta$  can be activated through several distinct processes [22]. Activation of PKC $\delta$  by diacylglycerol (DAG) binding to C1B domain is well established and DAG-independent activation of the PKC isozyme was recently shown [14,15,19,20,23,24,47,49,58]. Tyrosine phosphorylation of PKC $\delta$  after hydrogen peroxide or ceramide treatment results in the activation of the PKC isozyme without DAG binding to its C1B domain. We examined whether the knockdown of endogenous PKC $\delta$  can be compensated by the overexpression of wild or various PKC $\delta$  mutants to investigate how PKC $\delta$  is activated by VEGF/KDR stimulation. Overexpression of rat-derived wild PKC $\delta$  in HEK293T cells transfected with human PKC $\delta$  siRNA compensated VEGF-KDR induced ERK phosphorylation (Fig. 6), suggesting that siRNA for human PKC $\delta$  reduced the amount of endogenous PKC $\delta$  specifically but did not affect other signaling molecules. Phosphorylation of tyrosine 311(Y311) and 332(Y332)



**Fig. 5.** Inhibition of VEGF-induced MARCKS-GFP translocation by PKC inhibitor and siRNA. (A) HEK293T cells were co-transfected with KDR and MARCKS-GFP. Six hours after the transfection, the cells were seeded to a 35 mm glass-bottomed plate. Eighteen hours after the seeding, the cells were pretreated without (control) or with 1  $\mu$ M GF109203X, 1  $\mu$ M Gö6983, 1  $\mu$ M Gö6976, or 5  $\mu$ M Rottlerin for 1 h. Then the cells were treated with 25 ng/ml VEGF and translocation of MARCKS-GFP was analyzed by confocal laser scanning microscope. (B) HEK293T cells were co-transfected with KDR, MARCKS-GFP, and either scrambled siRNA (control siRNA) or siRNA for human PKC $\delta$  (hPKC $\delta$  siRNA). Six hours after the transfection, the cells were seeded to a 35 mm glass-bottomed plate. Nineteen hours after the seeding, the cells were treated with 25 ng/ml VEGF and translocation of MARCKS-GFP was analyzed by confocal laser scanning microscope. The results shown are representative of 12 experiments.

of PKC $\delta$  has been shown to be responsible for its DAG-independent activation [20,23,24]. Double mutant of Y311/332F (phenylalanine) which cannot be activated by tyrosine phosphorylation but can be activated by phorbol ester significantly compensated the ERK phosphorylation (Fig. 6). Rat PKC $\delta$  mutant lacking C1B domain ( $\delta$ C1B) failed to induce ERK phosphorylation (Fig. 6).

We also examined the translocation of these mutants in response to VEGF-KDR stimulation. Intense fluores-



**Fig. 6.** Recovery of ERK phosphorylation by rat PKC $\delta$ -GFP in human PKC $\delta$  siRNA treated HEK293T cell. HEK293T cells were co-transfected with KDR, siRNA for human PKC $\delta$ , and either one of GFP-tagged rat PKC (rPKC $\delta$ -GFP) or its mutants indicated or GFP alone. Forty-eight hours after the transfection, cells were treated without (–) or with 25 ng/ml VEGF (+) for 5 min. (A) The cell lysates were analyzed by Western blot analysis with the antibodies indicated. (B) The intensity of phosphorylation of ERK was analyzed by NIH image software. Results are presented as means  $\pm$  SD ( $n = 3$ ). \* $P < 0.05$  comparing ERK phosphorylation in the cells transfected with human PKC $\delta$  siRNA and GFP alone.

cence of PKC $\delta$  (wild type)-GFP was found throughout the cytoplasm and nucleoplasm of the HEK293T cell. Stimulation of VEGF-KDR induced a translocation of PKC $\delta$  (wild type)-GFP to plasma membrane. The translocation was observed within 2 min after the stimulation. Thereafter, PKC $\delta$  (wild type)-GFP re-translocated to the cytoplasm within 5 min (data not shown). PKC $\delta$  (Y311/332F mutant)-GFP also showed a translocation similar to PKC $\delta$  (wild type)-GFP in response to VEGF-KDR stimulation, while PKC $\delta$ ( $\delta$ C1B mutant)-GFP did not translocate from the cytoplasm to the plasma membrane in response to VEGF-KDR stimulation. (Fig. 7). These data indicated that C1B domain is responsible for the translocation of PKC $\delta$  by VEGF-KDR stimulation.

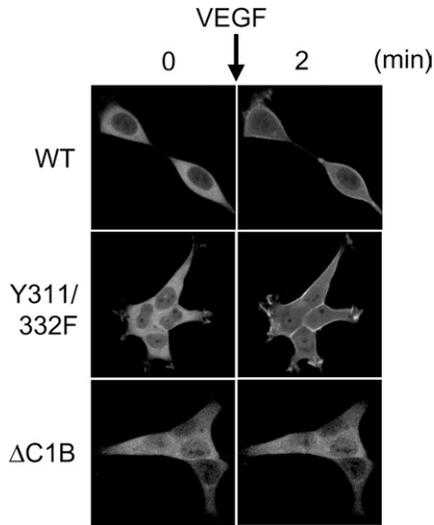


Fig. 7. Translocation of rat PKC $\delta$ -GFP or its mutants induced by VEGF-KDR stimulation. HEK293T cells were co-transfected with KDR and either one of GFP-tagged rat PKC or its mutants indicated. Six hours after the transfection, the cells were seeded to a 35 mm glass-bottomed plate. Eighteen hours after the seeding, the cells were treated with 25 ng/ml VEGF. Translocation of MARCKS-GFP is examined by confocal laser scanning microscope. The results shown are representative of six experiments.

## Discussion

In this report, we have investigated the mechanism of VEGF-KDR induced ERK activation shown by phosphorylation of ERK (Thr202/Tyr204) and identified that  $\delta$ -isoform of PKC is involved in this signaling. Furthermore, we have shown that VEGF-KDR-induced ERK activation requires the translocation of PKC $\delta$  from the cytoplasm to the plasma membrane and the activation of PKC $\delta$  at the plasma membrane via C1B domain.

Vascular endothelial cells express several kinds of VEGF receptors. Two structurally similar VEGF-receptors, Flt-1 and KDR/Flk-1, play independent and essential roles in vascular development. KDR-minus homozygous mice die on embryonic day 8.5–9.5 due to the lack of endothelial cell growth and blood vessel formation as well as an extremely poor hematopoiesis [44]. On the other hand, Flt-1-minus homozygous mice also die at the same stage; however, this is due to the overgrowth of endothelial cells and disorganization of blood vessels [11]. These results suggest that at least at the early stage of embryogenesis, KDR is essential for both proliferation and differentiation of endothelial cells, whereas Flt-1 is involved in the assembly of the vascular endothelium as a negative regulator of endothelial cell proliferation. Therefore to investigate VEGF-KDR specific signaling to ERK activation, we have used HEK293T cells transiently expressing KDR, since HEK293T cells do not express any endogenous VEGF receptors.

PKC activation has been shown to be mediated by PLC $\gamma$  activation through VEGF-KDR leading to ERK activation [50,51]. PLC $\gamma$  activation results in the production of DAG and Ca<sup>2+</sup> mobilization, suggesting that cPKC or nPKC would be involved in the signaling. However, it has been still controversial which isoform of PKC is activated by VEGF-KDR stimulation. Previous reports suggested PKC $\alpha$ ,  $\beta$ ,  $\delta$ , and  $\zeta$  as candidates responsible for key enzyme in VEGF-KDR-ERK pathway [13,42,50,56].

In our experiment, treatment of Gö6976, a cPKC specific inhibitor, had no effects on the ERK phosphorylation by VEGF-KDR signal, rottlerin inhibited the ERK phosphorylation (Fig. 3), supporting that PKC $\delta$  was involved between VEGF-KDR and Raf-MEK-ERK cascade. Although rottlerin has been used in many studies to implicate PKC $\delta$  in a variety of cellular events, it also inhibits a number of protein kinases including calmodulin-dependent protein kinase III, p38-regulated/activated kinase, and mitogen-activated protein kinase-activated protein kinase 2 [4,22]. Therefore to confirm the involvement of PKC $\delta$  in VEGF-KDR signaling, we performed the knock down of endogenous PKC $\delta$  using siRNA method in HEK293T cells. Knock down of human-derived endogenous PKC $\delta$  blocked VEGF-KDR-induced ERK phosphorylation (Fig. 4). Rat PKC $\delta$  co-transfected with siRNA of human PKC $\delta$  recovered VEGF-KDR-induced ERK phosphorylation (Fig. 6), but rat PKC $\delta$  kinase negative mutant did not (data not shown). These data indicate that PKC $\delta$  is indispensable for VEGF-KDR-induced ERK activation and that kinase activity of PKC $\delta$  is required to mediate VEGF-KDR signaling to induce ERK phosphorylation.

MARCKS was used for the spatio-temporal analysis of PKC activation. We have visualized the phosphorylation and translocation of MARCKS from the plasma membrane to the cytoplasm, when PKC was activated on the plasma membrane. In contrast, MARCK was not phosphorylated by PKC when PKC was activated in the cytosol [35]. In HEK293T cells transiently expressing KDR, VEGF stimulation induced the translocation of MARCKS-GFP from the plasma membrane to the cytoplasm. Pharmacological experiments using PKC inhibitors including rottlerin and siRNA treatment suggested that VEGF-KDR signal translocated and activated PKC $\delta$  on the plasma membrane and induced the phosphorylation of MARCKS. Although it is unknown why PKC $\delta$  is specifically activated by VEGF-KDR signal, it is possible that VEGF-KDR signal produces a lipid mediator specific for PKC $\delta$  activation or that receptor for activated C-kinase (RACK) for PKC $\delta$  is involved in this pathway.

Several activation mechanisms of PKC $\delta$  have been reported. It is activated by the binding with DAG or phorbol ester to C1 domain and by DAG-independent mechanisms such as tyrosine phosphorylation and

proteolytic reaction [22]. But it is still unknown how PKC $\delta$  is activated by VEGF-KDR signal. Previously, we have shown that ceramide induces the translocation of PKC $\delta$  to the Golgi complex and activates PKC $\delta$  via phosphorylation of Tyr311 and Tyr332 [19,20]. Unlike ceramide stimulation, unphosphorylatable PKC $\delta$  (Y311/332F mutant) could induce phosphorylation of ERK in response to VEGF, suggesting that tyrosine phosphorylation is not involved in VEGF-KDR induced PKC $\delta$  activation (Fig. 6). PKC $\delta$  consists of C2-like domain, pseudosubstrate-region, C1-domain including C1A and C1B, kinase domain, and several variable regions. Tandem C1 domains are not equivalent, DAG and phorbol ester mainly bind C1B domain of PKC $\delta$  [14,47,49]. Takahashi et al. reported that VEGF-KDR activates PLC $\gamma$  leading to ERK activation and that activated PLC $\gamma$  hydrolyzes PIP<sub>2</sub> to diacylglycerol (DAG) and IP<sub>3</sub>, inducing Ca<sup>2+</sup> influx. Therefore, it is predicted that PKC $\delta$  translocated from the cytoplasm to the plasma membrane, where this kinase is activated via DAG binding with C1B domain. In agreement with this hypothesis, rat PKC $\delta$  lacking the C1B domain could not recover VEGF-KDR-induced ERK activation in human PKC $\delta$  knocked-down HEK293T cells. Furthermore, PKC $\delta$  lacking C1B domain could not translocate from the cytoplasm to the plasma membrane by VEGF in HEK293T expressing KDR (Figs. 6 and 7).

VEGF-KDR signal has the important role in normal and pathological angiogenesis [9,10,31]. As the action induced by VEGF-KDR signal via ERK is necessary for angiogenesis, VEGF-KDR pathway has attracted great attention in the context of potential therapy for angiogenesis. Until now two categories of angiogenesis inhibitors have been clinically developed [27]. One is nucleotide- and peptide-based anti-VEGF reagents including Bevacizumab which is recombinant humanized anti-VEGF monoclonal antibody and Angiozyme which is a stabilized ribozyme directed against the pre-mRNA of the KDR gene. Another is KDR kinase chemical inhibitors including SU5416, SU6668, SU11248, ZD6474, and Vatalanib.

In this report we have revealed an important role of PKC $\delta$  and its activation mechanism in VEGF-KDR-induced ERK activation. These results suggested that in addition to drugs acting on VEGF or KDR, the blocker of PKC $\delta$  translocation by VEGF-KDR is also a candidate for pathological angiogenesis. However, the mechanism of PKC $\delta$  specific activation after VEGF treatment should be clarified.

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