

A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-A-dependent activation of PLC- γ and DNA synthesis in vascular endothelial cells

Tomoko Takahashi, Sachiko Yamaguchi, Kazuhiro Chida¹ and Masabumi Shibuya²

Department of Genetics, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108-8639 and ¹Laboratory of Cell Regulation, Department of Animal Resource Science/ Applied Biological Chemistry, University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan

²Corresponding author
e-mail: shibuya@ims.u-tokyo.ac.jp

KDR/Flk-1 tyrosine kinase, one of the two vascular endothelial growth factor (VEGF) receptors, induces mitogenesis and differentiation of vascular endothelial cells. To understand the mechanisms underlying the VEGF-A-induced growth signaling pathway, we constructed a series of human KDR mutants and examined their biological properties. An *in vitro* kinase assay and subsequent tryptic peptide mapping revealed that Y1175 and Y1214 are the two major VEGF-A-dependent autophosphorylation sites. Using an antibody highly specific to the phosphoY1175 region, we demonstrated that Y1175 is phosphorylated rapidly *in vivo* in primary endothelial cells. When the mutated KDRs were introduced into the endothelial cell lines by adenoviral vectors, only the Y1175F KDR, Tyr1175 to phenylalanine mutant, lost the ability to tyrosine phosphorylate phospholipase C- γ and, significantly, reduced MAP kinase phosphorylation and DNA synthesis in response to VEGF-A. Furthermore, primary endothelial cells microinjected with anti-phosphoY1175 antibody clearly decreased DNA synthesis compared with control cells. These findings strongly suggest that autophosphorylation of Y1175 on KDR is crucial for endothelial cell proliferation, and that this region is a new target for anti-angiogenic reagents.

Keywords: binding site/KDR/Flk-1/PLC- γ /tyrosine kinase receptor/vascular endothelial growth factor-A

Introduction

Recent studies have revealed that vascular endothelial growth factor-A (VEGF-A), also referred to as vascular permeability factor (VPF), is essential for many angiogenic processes in normal and abnormal states such as diabetic retinopathy and tumor vascularization (Folkman, 1990; Ferrara *et al.*, 1991; Mustonen and Alitalo, 1995; Shibuya, 1995). VEGF-A exhibits two major biological activities: one is the capacity to stimulate vascular endothelial cell proliferation (Connolly *et al.*, 1989; Ferrara *et al.*, 1989; Leung *et al.*, 1989) and the other is the ability to increase microvessel permeability to macromolecules (Senger *et al.*, 1983; Keck *et al.*, 1989).

VEGF-A binds two tyrosine kinase receptors, Flt-1 and KDR/Flk-1 (also known as VEGF receptor-1 and -2), with high affinity (de Vries *et al.*, 1992; Millauer *et al.*, 1993), and both 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 (Shibuya *et al.*, 1990; Matthews *et al.*, 1991; Terman *et al.*, 1992).

In spite of their similar structural features, recent studies conducted on the targeting of the two VEGF receptor genes demonstrated that they have independent and essential roles in vascular development. *KDR/flk-1*-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 (Shalaby *et al.*, 1995). On the other hand, *flt-1*-minus homozygous mice also die at the same stage; however, this is due rather to the overgrowth of endothelial cells and disorganization of blood vessels (Fong *et al.*, 1995). These results suggest that at least at the early stage of embryogenesis, KDR/Flk-1 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.

We have shown recently that, unlike other representative growth factor receptor tyrosine kinases, KDR/Flk-1 utilizes the phospholipase C- γ (PLC- γ)-protein kinase C (PKC)-Raf-mitogen-activated protein (MAP) kinase pathway as the major signaling pathway (Takahashi *et al.*, 1999). Ras is poorly activated, if at all, since the Ras-GTP complex is below detectable levels and dominant-negative Ras does not suppress the VEGF-A-dependent MAP kinase activation and DNA synthesis significantly.

In order to achieve a better understanding of the signal transduction mechanism of KDR/Flk-1, it is important to know which tyrosine residues on KDR/Flk-1 are autophosphorylated in response to VEGF-A, and what kind of biological signals are transduced from each autophosphorylation site. Recently, in an *Escherichia coli* expression system, tyrosine residues 951, 996, 1054 and 1059 on KDR/Flk-1 have been shown to be phosphorylated (Dougher-Vermazen *et al.*, 1994). However, Cunningham *et al.* (1997) reported that in the yeast system, tyrosine residues 801 and 1175, the putative binding sites for PLC- γ , were phosphorylated. In addition, Igarashi *et al.* (1998) reported that in the yeast two-hybrid system, one of the adaptor proteins binds KDR/Flk-1 through tyrosine residue 1175. However, it remains to be elucidated which tyrosine residues are phosphorylated in endothelial cells under physiological conditions, and which protein(s) tend to bind KDR/Flk-1 in response to VEGF-A *in vivo*.

In this study, using a variety of KDR/Flk-1 mutants introduced into either non-endothelial or endothelial cell lines and a phosphopeptide-specific antibody, we found that Y1175 is a major VEGF-A-dependent phosphorylation site on KDR/Flk-1, and that it is an essential binding site for PLC- γ via the SH2 domain. We also identified Y1214 as an additional autophosphorylation site of KDR/Flk-1. However, our results clearly indicate that Y1175, but not Y1214, plays a crucial role in the transduction of signals to the MAP kinase pathway and DNA synthesis in endothelial cells.

Results

Construction of mutants

In order to examine which tyrosine residues on KDR/Flk-1 are autophosphorylated in response to VEGF-A, we constructed a variety of deletion and/or point mutants of KDR/Flk-1. Subsequently they were expressed in a non-endothelial or an endothelial cell line, and the tryptic peptides analyzed in two-dimensional gels. We focused mainly on the C-terminal region downstream of the kinase domain, since representative receptor-type tyrosine kinases bear the autophosphorylation sites in this carboxyl region essential for signal transduction (Margolis *et al.*, 1989; Ronnstrand *et al.*, 1992; Valius *et al.*, 1993). Figure 1A summarizes the structural organization of the wild-type and mutant receptors analyzed in this study. Based on the amino acid sequences around tyrosine residues reported so far, Y1175, Y1214, Y1305 and Y1309 are well conserved in the C-terminal region of KDR/Flk-1 in various mammalian species (Matthews *et al.*, 1991; Terman *et al.*, 1991; Sarzani *et al.*, 1992) (Figure 1A).

We have shown recently that the PLC- γ -PKC pathway is crucial to the VEGF-A-dependent MAP kinase activation and DNA synthesis in primary endothelial cells (Takahashi *et al.*, 1999). Thus, we generated mutated Y1175 as well as Y801, putative docking sites for PLC- γ (Mohammadi *et al.*, 1991; Ronnstrand *et al.*, 1992; Rotin *et al.*, 1992; Obermeier *et al.*, 1993; Songyang *et al.*, 1993; Valius *et al.*, 1993; Middlemas *et al.*, 1994; Borrello *et al.*, 1996; Sawano *et al.*, 1997) (Figure 1A and B). In addition, we created a mutant Y1214, well matched to the consensus binding site for Grb2 (Songyang *et al.*, 1993), although we showed that the activation of the Grb2-Sos-Ras pathway is very poor in VEGF-A-stimulated endothelial cells (Seetharam *et al.*, 1995; Takahashi *et al.*, 1999).

Y1175 is one of the major autophosphorylation sites in the *in vitro* kinase assay

Initially, we examined the VEGF-A-dependent kinase activity of KDR/Flk-1 using an *in vitro* assay. Together with other investigators, we have reported previously that KDR/Flk-1 is highly phosphorylated on tyrosine residues in response to VEGF-A *in vivo* (Millauer *et al.*, 1993; Waltenberger *et al.*, 1994; Seetharam *et al.*, 1995; Takahashi and Shibuya, 1997; Takahashi *et al.*, 1999). Consistent with these data, the kinase activity *in vitro* was upregulated significantly in response to VEGF-A (Figure 2A). The phosphoamino acid analysis of KDR/Flk-1 revealed that the phosphorylation occurred only on tyrosine residues, not on serine or threonine residues (Figure 2B). Analysis of tryptic peptide mapping allowed

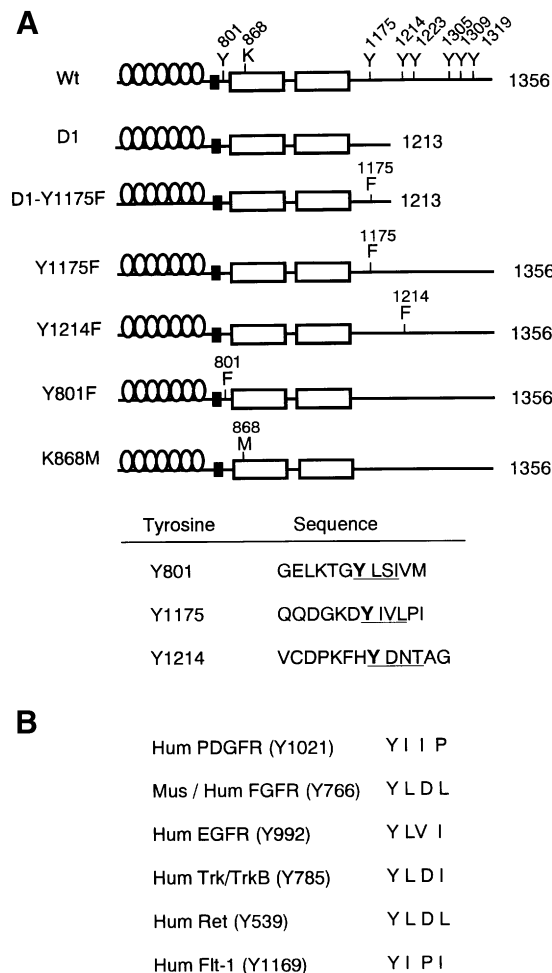


Fig. 1. Wild-type (Wt) and mutant receptors of KDR/Flk-1. (A) Schematic representation of Wt and mutant of KDR/Flk-1s. D1 lacks 143 amino acids from the C-terminal region of Wt. Y1175F, Y1214F and Y801F contain a Tyr→Phe mutation at positions 1175, 1214 and 801, respectively. K868M is a kinase-inactive receptor mutant containing a Lys→Met mutation at position 868. The last amino acids of each protein are numbered. Open circles in the extracellular domain of KDR/Flk-1 indicate immunoglobulin-like domains; the transmembrane-spanning domain and the tyrosine kinase domains are represented by closed and open boxes, respectively. Amino acid alignments around Y801, Y1175 and Y1214 of KDR/Flk-1 are shown below. The putative phosphorylated tyrosines are presented in bold. The tyrosine residue and three amino acids to the C-terminal side are underlined. (B) An alignment of amino acid sequences for the PLC- γ -binding sites in several growth factor receptors.

the detection of several phosphotyrosine-containing peptides of KDR/Flk-1 (Figure 2C). Essentially the same results were obtained from KDR/Flk-1 expressed in both NIH-3T3 and human umbilical vein endothelial (HUVE) cells (Figure 2A–C).

To identify the phosphorylated tyrosine residues, we compared the phosphopeptide maps of wild-type and several mutant KDR/Flk-1 molecules transiently expressed in 293 cells. The pattern of tryptic peptides of wild-type KDR/Flk-1 expressed in 293 cells was essentially the same as that expressed stably in NIH-3T3 cells (Figure 3A). The Y1175F and D1-Y1175F mutants lost one of the major spots, when compared with the patterns of wild-type and D1 mutant (Figure 3B–D). This indicates

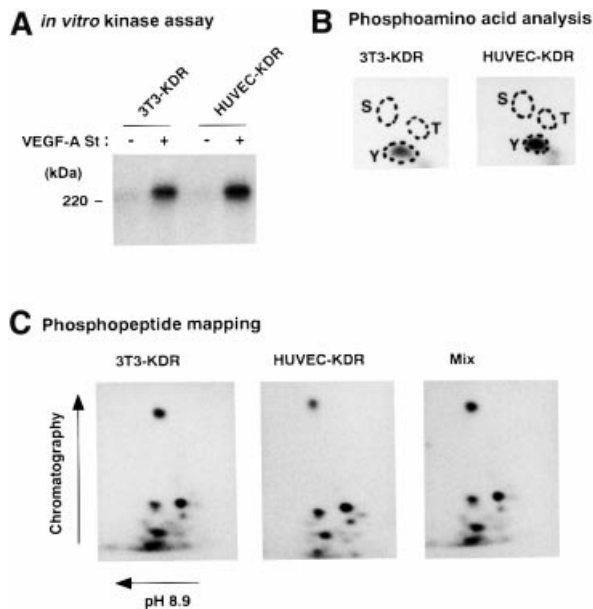


Fig. 2. The *in vitro* kinase assay, phosphoamino acid analysis and phosphopeptide mapping of KDR/Flk-1 overexpressed in NIH-3T3 and HUVE cells. (A) The *in vitro* kinase assay in KDR/Flk-1. The wild-type KDR/Flk-1 was immunoprecipitated from unstimulated or VEGF-A-stimulated NIH-3T3-KDR or HUVEC-KDR cells, and labeled in the *in vitro* kinase assay in the presence of [γ - 32 P]ATP. The radiolabeled KDR/Flk-1 was subjected to SDS-PAGE and autoradiography. (B) Phosphoamino acid analysis. The radiolabeled KDR/Flk-1 band was excised from the gel and subjected to trypsin digestion. The resulting phosphopeptides were then hydrolyzed and analyzed by two-dimensional chromatography (see Materials and methods). (C) Tryptic phosphopeptide mapping of KDR/Flk-1. The KDR/Flk-1 was labeled by the *in vitro* kinase assay and subjected to SDS-PAGE. The labeled KDR/Flk-1 band as shown in (A) was excised from the gel and subjected to trypsin digestion. The resultant phosphopeptides were resolved by electrophoresis at pH 8.9, followed by thin-layer chromatography (see Materials and methods).

that Y1175 is one of the major autophosphorylation sites of KDR/Flk-1. The map of the D1-Y1175F mutant retained only a few minor spots; thus, we suggest that most of the autophosphorylation sites of KDR/Flk-1 are located in its C-terminal region.

When the phosphopeptide map of the Y1214F mutant was compared with wild-type KDR/Flk-1, one of the major spots was completely absent in Y1214F KDR/Flk-1 (Figure 3E). Therefore, we conclude that Y1214 is another major autophosphorylation site of KDR/Flk-1. On the other hand, we could not identify Y801 as a major phosphorylation site in the series of our experiments (data not shown).

The involvement of Y1175 in signal transduction from VEGF in endothelial-derived cell lines

Since the Y1175-containing region has a possible binding motif for PLC- γ (Mohammadi *et al.*, 1991; Ronnstrand *et al.*, 1992; Rotin *et al.*, 1992; Obermeier *et al.*, 1993; Songyang *et al.*, 1993; Valius *et al.*, 1993; Middlemas *et al.*, 1994; Borrello *et al.*, 1996; Sawano *et al.*, 1997), we next examined the signal transduction from the Y1175F receptor in endothelial cells. We constructed recombinant adenovirus vectors containing wild-type (Ad-Wt) and various KDR/Flk-1 mutants as shown in Figure 1 and

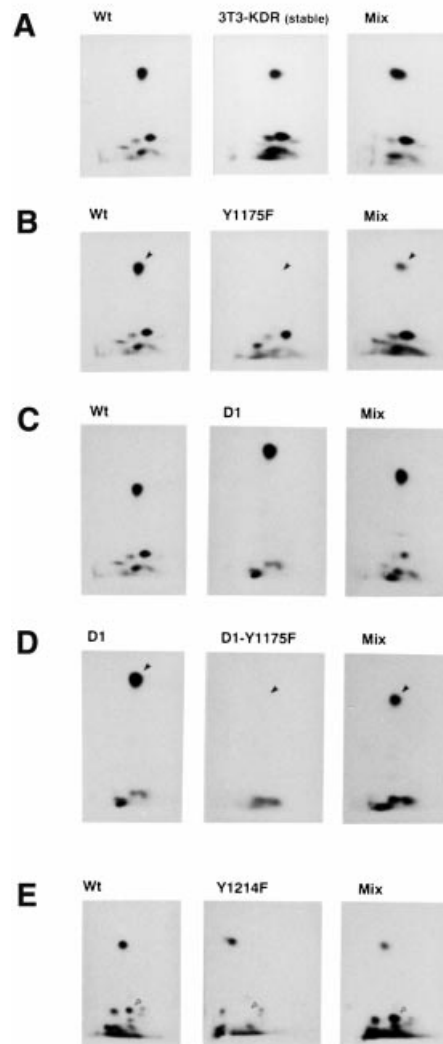


Fig. 3. Phosphopeptide mapping of KDR/Flk-1. Wild-type or mutant receptors were transiently expressed in 293 cells. NIH-3T3-KDR cells were used as the positive control. The KDR/Flk-1s were analyzed as indicated in the legend of Figure 2. The closed arrowheads (B and D) and the open arrowheads (E) indicate the phosphorylated peptide containing Y1175 and Y1214, respectively.

infected them into MSS31 cells. The MSS31 cell is an endothelial-derived cell line, which is characterized by the formation of a tubule-like structure in collagen gel, the uptake of acetylated low-density lipoprotein (Yanai *et al.*, 1991) and the expression of endothelial cell-related genes, such as *flt-1*, *KDR* and some *MMPs*, at RT-PCR levels (Namba *et al.*, 2000). When MSS31 cells were infected with various adenovirus vectors, KDR/Flk-1 was expressed (Figure 4A) and tyrosine phosphorylated efficiently in response to VEGF-A (Figure 4B) as in the case of NIH-3T3-KDR cells (Takahashi and Shibuya, 1997) or primary endothelial cells such as HUVE cells.

As shown in Figure 4C, the tyrosine-phosphorylated PLC- γ was undetectable in Y1175F mutant-expressing MSS31 cells. We previously reported that VEGF-A activates the MAP kinase pathway as an important signal transducer for cell proliferation in NIH-3T3-KDR cells (Takahashi and Shibuya, 1997) and in primary endothelial cells (Takahashi *et al.*, 1999). Thus, we next examined whether MAP kinase was activated in VEGF-A-stimulated

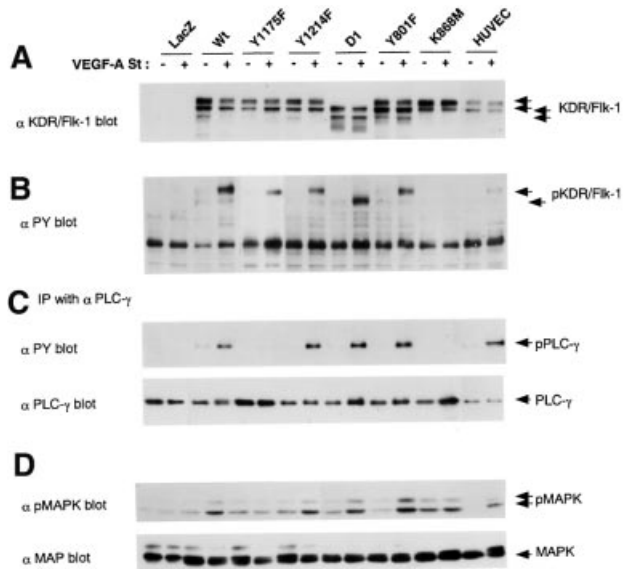


Fig. 4. Analysis of KDR/Flk-1 mutants expressed in the adenovirus vector expression system. MSS31 cells were infected with adenovirus vectors containing wild-type or mutant KDR/Flk-1 receptors. Two days later, the cells were first starved for 12 h in DMEM-0.1% FCS and subsequently were not stimulated or stimulated with VEGF-A (10 ng/ml). Total cell lysates expressing the wild-type or various mutants of KDR/Flk-1 were analyzed by western blotting using anti-KDR/Flk-1 (A) or anti-phosphotyrosine (B) antibody. (C) The cell lysates were immunoprecipitated with anti-PLC- γ antibodies and blotted with anti-phosphotyrosine or anti-PLC- γ antibodies. (D) The same total cell lysates were blotted with anti-phosphoMAP kinase or anti-MAP kinase antibody. HUVEC cells were used as the positive control.

MSS31 cells expressing various KDR/Flk-1 mutants. Consistent with the very low tyrosine phosphorylation of PLC- γ , VEGF-A-induced phosphorylation of MAP kinase was reduced significantly only in Y1175F mutant cells. The kinase-inactive mutant (K868M) did not show any autophosphorylation of the receptor, tyrosine phosphorylation of PLC- γ or MAP kinase phosphorylation (Figure 4B–D).

To confirm the involvement of Y1175 in signal transductions in another type of endothelial cell, we used cultured bovine aortic endothelial (BAE) cells in which endogenous VEGF receptors were downregulated. We found that VEGF-A could not induce tyrosine phosphorylation of PLC- γ (Figure 5C) or MAP kinase activation (Figure 5D) in the Y1175F mutant, in spite of significant expression and autophosphorylation of the receptor (Figure 5A and B). Thus, we conclude that Y1175 is involved in tyrosine phosphorylation of PLC- γ and MAP kinase activation in at least two independent endothelial cell systems.

The effects of Y1175F on DNA synthesis in cultured endothelial cells

Next, we examined the ability of Y1175F to initiate DNA synthesis in BAE cells. BAE cells were infected with adenovirus vectors containing LacZ, wild-type, Y1175F, Y1214F, Y801F or K868M KDR/Flk-1. Although the levels of these expressed KDR/Flk-1s were almost equivalent in western blot analysis (Figure 6A), Y1175F

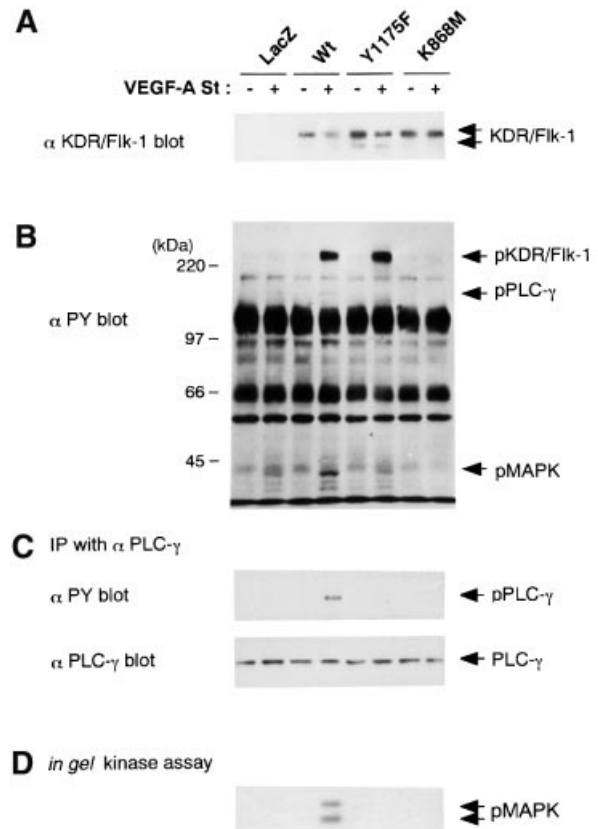


Fig. 5. Signal transduction from VEGF-A in BAE cells expressing LacZ, wild-type, Y1175F or K868M KDR/Flk-1. Intracellular signaling from various KDR/Flk-1s was analyzed as indicated in the legend of Figure 4, except for the in-gel kinase assay for MAP kinase (D) (see Materials and methods).

and K868M could not initiate DNA synthesis in response to VEGF-A (Figure 6B). Thus, we suggest that the autophosphorylation of Y1175 is essential for cell proliferation in VEGF-A signaling.

Y1175 is phosphorylated *in vivo* in VEGF-A-stimulated primary endothelial cells

To evaluate further the phosphorylation of Y1175 *in vivo*, we raised an antibody against the phosphorylated Y1175-containing peptide (anti-PY1175 Ab) via affinity purification. This anti-PY1175 Ab specifically recognized the VEGF-A-induced phosphorylation state of a variety of KDR/Flk-1 mutants expressed in MSS31 cells, except for the Y1175F mutant (Figure 7A). Moreover, it did not react with other tyrosine kinases that have been reported to bind PLC- γ , such as epidermal growth factor receptor (EGFR), PDGFR, fibroblast growth factor receptor (FGFR) and Flt-1 (Figure 7B). When HUVEC cells or rat sinusoidal endothelial (SE) cells were stimulated with VEGF-A, anti-PY1175 Ab clearly recognized the autophosphorylated KDR/Flk-1 of 230 kDa, indicating that Y1175 on KDR/Flk-1 is phosphorylated rapidly *in vivo* in primary endothelial cells (Figure 7C). Furthermore, plasma membrane and cytoplasm of HUVEC cells were stained transiently with the anti-PY1175 Ab after stimulation with VEGF-A, but not bFGF, PDGF (Figure 7D) or EGF (data not shown). These results suggest that anti-PY1175

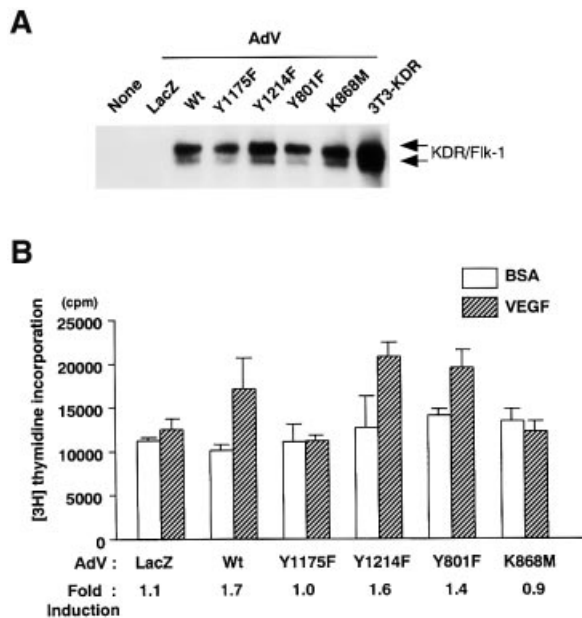


Fig. 6. Mutation of Y1175F in KDR/Flk-1 blocks VEGF-A-induced initiation of DNA synthesis. BAE cells were infected with adenovirus vectors containing wild-type or mutant receptors and subsequently starved for 48 h in DMEM–0.1% FCS. The total cell lysates were blotted with anti-KDR/Flk-1 antibody. NIH-3T3-KDR cells were used as the positive control (A). Alternatively, BAE cells were stimulated with BSA or VEGF-A (10 ng/ml). After incubation for 16 h, cells were pulse-labeled with [³H]thymidine (1 mCi/ml) for 4 h. Subsequently, they were harvested on glass filters and incorporated radioactivity was measured. Data represent the average of triplicate samples. Fold induction was calculated relative to the values for the cells stimulated by BSA in each mutant (B).

Ab is useful for the detection of activated KDR/Flk-1, not only in the western blotting but also in histological sections.

The C-terminal SH2 domain of PLC- γ associates with phosphorylated KDR/Flk-1 through Y1175

We have shown previously that KDR/Flk-1 associates with PLC- γ upon VEGF-A stimulation in NIH-3T3-KDR cells (Takahashi and Shibuya, 1997). We also confirmed the *in vivo* association of KDR/Flk-1 and PLC- γ through Y1175 in both BAE (Figure 8A) and MSS31 cells (data not shown).

To clarify the binding sites on PLC- γ with KDR/Flk-1, we tested the association of the SH2 domain in PLC- γ with phosphorylated KDR/Flk-1 in a pull-down assay using recombinant GST fusion protein. As shown in Figure 8B, the GST–PLC- γ SH2–SH2 domain, but not GST alone effectively associated with the 230 kDa protein of KDR/Flk-1. This long form of KDR/Flk-1 was shown to be expressed on the cell surface as a mature form and autophosphorylated on tyrosine in response to VEGF-A (Takahashi and Shibuya, 1997). Furthermore, we found that the C-terminal SH2 domain of PLC- γ has a much greater affinity for phosphorylated KDR/Flk-1 than the N-terminal domain. Other SH2 domains derived from Grb2 or phosphatidylinositol 3-kinase (PI3 kinase) did not bind to phosphorylated KDR/Flk-1 at detectable levels (Figure 8C).

To confirm that the association of C-terminal SH2 domain with KDR/Flk-1 is mediated by phosphoY1175 on KDR/Flk-1, we next assessed the ability of anti-PY1175 Ab or the PY1175 peptide to block the VEGF-A-induced association of KDR/Flk-1 with PLC- γ *in vitro* (Figure 8D and E). In the pull-down assay using the C-terminal SH2 domain of PLC- γ , anti-PY1175 Ab as well as the phosphopeptide completely blocked this association, whereas control antibody or non-phosphopeptide did not. These results strongly suggest that the C-terminal SH2 domain of PLC- γ is directly associated with the phosphorylated Y1175 on KDR/Flk-1.

Microinjection of PY1175 Ab specifically blocks VEGF-A-mediated DNA synthesis in endothelial cells

To assess the physiological role of phosphoY1175 in VEGF-A-stimulated DNA synthesis in primary endothelial cells, we microinjected the anti-PY1175 Ab into SE cells. These cells showed the uptake of 5-bromo-2'-deoxyuridine (BrdU) in a strictly VEGF-A-dependent manner (Figure 9A). In the cells injected with anti-PY1175 Ab, the uptake of BrdU was significantly decreased compared with the control rabbit IgG (Figure 9B and C). Thus, we conclude that phosphoY1175 is crucial for engaging VEGF-A-induced stimulation of DNA synthesis in primary endothelial cells.

Discussion

We previously reported that KDR/Flk-1 is highly phosphorylated on tyrosine residues and that one of the major target molecules of KDR/Flk-1 is PLC- γ , which binds to KDR/Flk-1 and is tyrosine phosphorylated in response to VEGF-A (Takahashi and Shibuya, 1997). In addition, we have demonstrated recently that the VEGF-A-induced activation of the Raf-1–MEK–MAP kinase pathway and the stimulation of DNA synthesis are mediated mainly by PKC (particularly PKC β) in primary SE cells (Takahashi *et al.*, 1999). Activation of PLC- γ is well known to generate the second messengers diacylglycerol and inositol trisphosphate from phosphatidylinositol 4,5-bisphosphate. Consequently, these messengers stimulate activation of PKC and Ca²⁺ mobilization, respectively (Nishizuka, 1992). Thus, these results support the possibility that the activation of PLC- γ and the subsequent stimulation of PKC are important for the mitogenic signaling in vascular endothelial cells.

Autophosphorylation of receptor-type tyrosine kinases (RTKs) is considered to be important for determination of the substrate specificity by recruiting limited substrates for the receptors (Fanti *et al.*, 1992). Numerous studies have been performed to identify specific phosphotyrosine residues in RTKs as the major or unique binding sites for each SH2 domain-containing protein(s). In most cases, the three amino acids on the C-terminal side of phosphotyrosine provide key recognition elements (Songyang *et al.*, 1993). In fact, specific docking sites for PLC- γ have been identified in PDGFR, EGFR and FGFR (Figure 1B) (Mohammadi *et al.*, 1991; Ronnstrand *et al.*, 1992; Rotin *et al.*, 1992; Obermeier *et al.*, 1993; Songyang *et al.*, 1993; Valius *et al.*, 1993; Middlemas *et al.*, 1994; Borrello *et al.*, 1996; Sawano *et al.*, 1997). The consensus sequences for

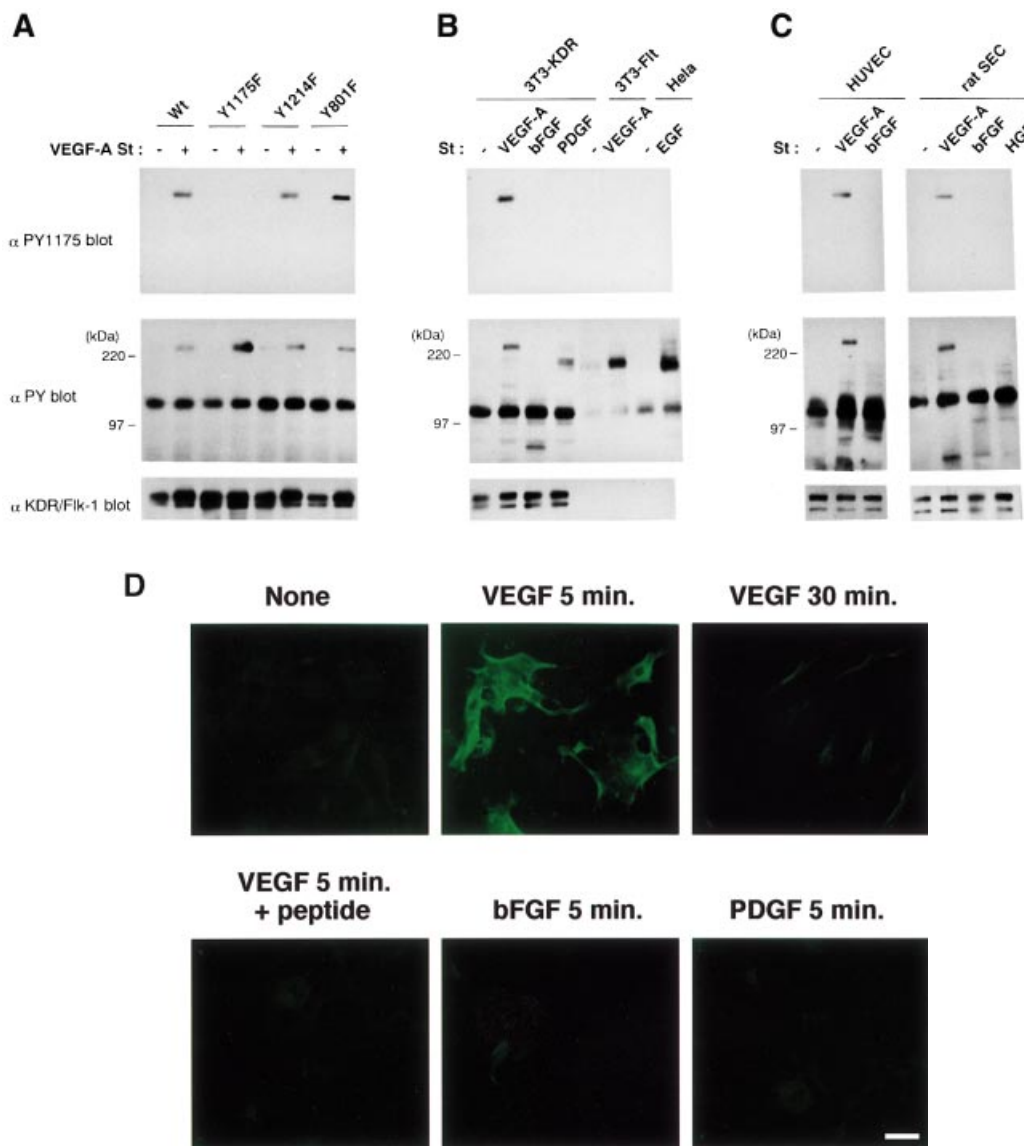


Fig. 7. *In vivo* Y1175 phosphorylation in VEGF-A-stimulated cells. MSS cells were infected with adenovirus vectors containing wild-type, Y1175F, Y1214F or Y801F. After 2 days, cells were stimulated with or without VEGF-A (A). NIH-3T3-KDR, NIH-3T3-Flt or HeLa cells were stimulated with VEGF-A, bFGF, PDGF or EGF as indicated (B). Primary endothelial cells, HUVE cells or rat SE cells were stimulated with VEGF-A, bFGF or HGF (C). Total cell lysates were blotted with anti-PY1175 (upper panel), anti-phosphotyrosine (middle panel) or anti-KDR/Flk-1 (lower panel) antibody. Histochemistry of HUVE cells using anti-PY1175. HUVE cells were starved and stimulated with VEGF-A, bFGF or PDGF for the indicated times. Subsequently, the cells were fixed and immunostained with anti-PY1175. Alternatively, PY1175 peptide was added as a competitor. The cells were examined with a fluorescent microscope (D). Bar, 50 μ m.

binding of the N- and C-terminal PLC- γ SH2 domains are thought to be PO₄-Y-(L, I, V)-(E, D)-(L, I, V) and PO₄-Y-(V, I)-(I, L)-(P, I, V), respectively (Songyang *et al.*, 1993). As for KDR/Flk-1, the C-terminal PLC- γ SH2 domain preferentially bound to the phosphorylated KDR/Flk-1 (Figure 8). The amino acid sequences around Y1175 (Y-I-V-L) and Y801 (Y-L-S-I) are partly matched to the binding sites of the C-terminal PLC- γ SH2 domains.

In this study, we have examined the autophosphorylation sites on KDR/Flk-1 and their functional roles in endothelial cell proliferation by using a variety of KDR mutants and two-dimensional peptide mapping. We were particularly interested in the analysis of Y801, Y1175 and Y1214 since the former two residues carry the surrounding peptide sequences well matched for PLC- γ -binding sites,

and the latter has a candidate sequence for a Grb2-binding site. Here we found that Y1175 and Y1214 are the major autophosphorylation sites on KDR/Flk-1 both *in vitro* and *in vivo*, but Y801 was not highly phosphorylated by KDR tyrosine kinase.

Using mutant KDR adenovirus vectors in cell culture and PLC- γ SH2-binding experiments *in vitro*, we demonstrated that the PY1175 site is the single major PLC- γ -binding site on KDR and plays a crucial role in the phosphorylation and activation of the PLC- γ -PKC-MAP kinase pathway for DNA synthesis in endothelial cells (Figure 6B). In general, this finding is quite surprising since representative receptor-type tyrosine kinases directly involved in cell growth usually utilize the Ras-mediated MAP kinase activation pathway, but poorly PKC-mediated

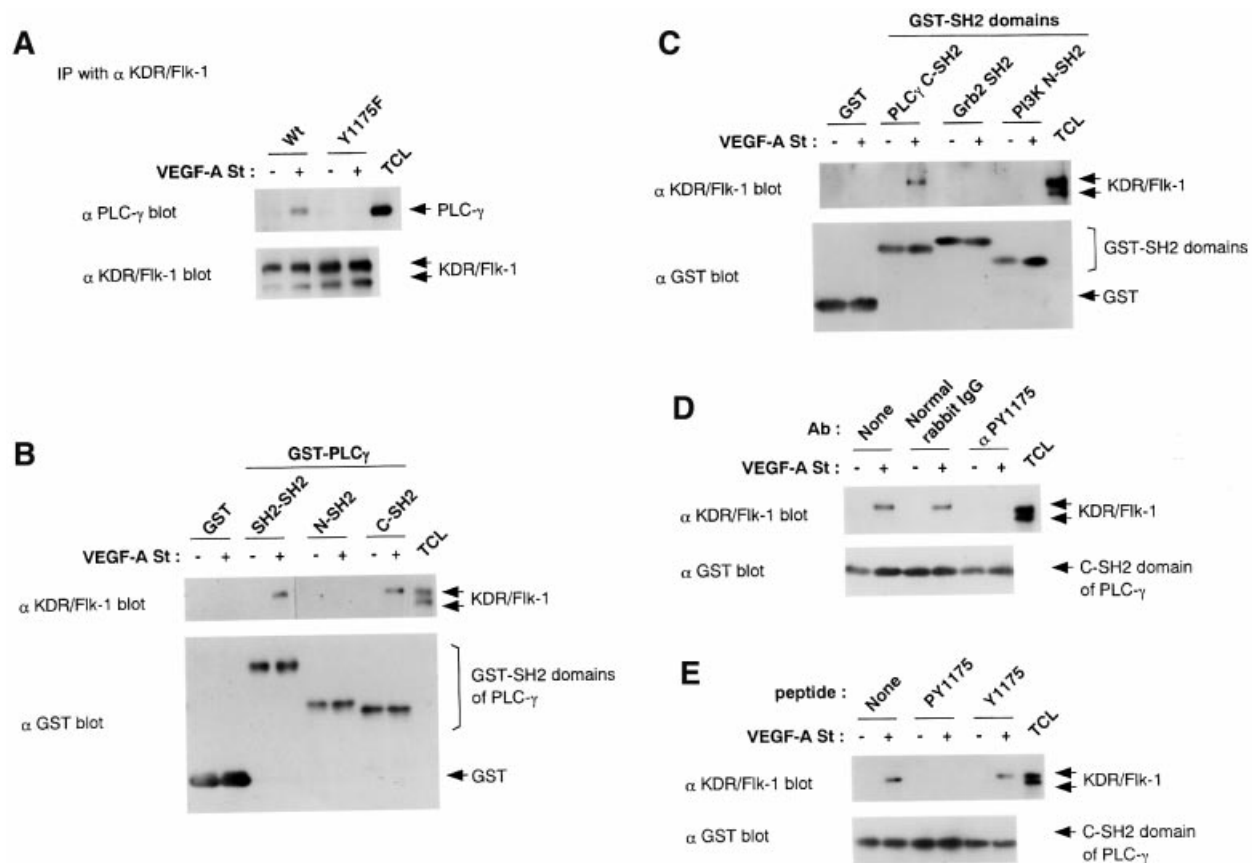


Fig. 8. The C-terminal SH2 domain of PLC- γ associates with KDR/Flk-1 through phosphorylated Y1175. (A) An *in vivo* association of KDR/Flk-1 with PLC- γ . BAE cells were infected with adenovirus vectors containing wild-type or Y1175F. After stimulation with VEGF-A (10 ng/ml), cell lysates were prepared and immunoprecipitated with anti-KDR/Flk-1 antibody. The samples were resolved by SDS-PAGE and immunoblotted with anti-PLC- γ antibodies or anti-KDR/Flk-1 as indicated. (B–E) *In vitro* association of KDR/Flk-1 with PLC- γ . NIH-3T3-KDR cell lysates unstimulated or stimulated with VEGF-A (10 ng/ml) were incubated with 1.5 μ g of GST, GST-PLC- γ SH2-SH2, GST-PLC- γ N-SH2 or GST-PLC- γ C-SH2 protein pre-bound to glutathione-Sepharose beads for 2 h at 4°C (B). The same experiments were carried out using GST, GST-PLC- γ C-SH2, GST-Grb2 SH2 or GST-PI3 kinase N-SH2 protein (C). The same cell lysates as in (A) were incubated with 1.5 μ g of GST-PLC- γ C-SH2 protein pre-bound to glutathione-Sepharose beads. During the incubation, antibody (D) or peptide (E) was added as a competitor. The beads were then washed three times with cold lysis buffer. The proteins bound to the beads or total cell lysate (TCL) were resolved by SDS-PAGE and immunoblotted with anti-KDR/Flk-1 or anti-GST antibody as shown.

pathway, for DNA synthesis. This observation, however, strongly supports our recent results that KDR/Flk-1 preferentially utilizes the PLC- γ -PKC-MAP kinase pathway for endothelial cell mitosis and that PKC inhibitors, but not PI3 kinase inhibitors nor dominant-negative Ras mutants, dramatically suppressed the VEGF-A-dependent DNA synthesis (Takahashi *et al.*, 1999).

Furthermore, by preparing an antiserum specific to the PY1175 region on KDR/Flk-1, we showed that the tertiary structure surrounding the phosphorylated Y1175 is clearly distinguishable from that of the autophosphorylation sites in EGFR, PDGFR, FGFR and Flt-1, which have been shown to bind and phosphorylate PLC- γ (Figure 7B). Based on this specificity, we were able to show a transient staining of primary endothelial cells after stimulation with VEGF-A (Figure 7D), and that microinjection of this antibody blocked VEGF-A-dependent DNA synthesis (Figure 9). These results strongly suggest that this antibody is useful in the detection of activated KDR/Flk-1 in pathological as well as physiological angiogenesis by histological staining, and that the region including the phosphorylated Y1175 is a good target for low molecular weight compound(s) with anti-angiogenic activity.

Recently, several compounds were reported to block the tyrosine kinase activity of KDR/Flk-1. However, such protein kinase inhibitors often cross-react with other protein kinases, which might generate severe side effects *in vivo*. Thus, as long as the structure surrounding PY1175 is highly specific and distinguishable from the PLC- γ -binding regions on other tyrosine kinase receptors, it appears reasonable to develop PY1175-blocking compounds for use alone or in combination with an KDR/Flk-1 tyrosine kinase inhibitor in anti-angiogenic therapy.

Y1214 was found to be another major phosphorylation site on KDR/Flk-1 (Figure 3E). Examination of the amino acid sequence containing Y1214 revealed a candidate for a Grb2-binding site (Songyang *et al.*, 1993). In our previous report, however, we showed that Grb2, an important adaptor molecule linking tyrosine kinase to Ras, was not associated with KDR/Flk-1 at detectable levels. In addition, VEGF-A-induced activation of MAP kinase and stimulation of DNA synthesis were not Ras dependent (Takahashi *et al.*, 1999). Consistent with this, we could not observe the association of Grb2 with phosphorylated KDR/Flk-1 in a pull-down assay using the Grb2 SH2 domain (Figure 8). These results suggest that Grb2 is not

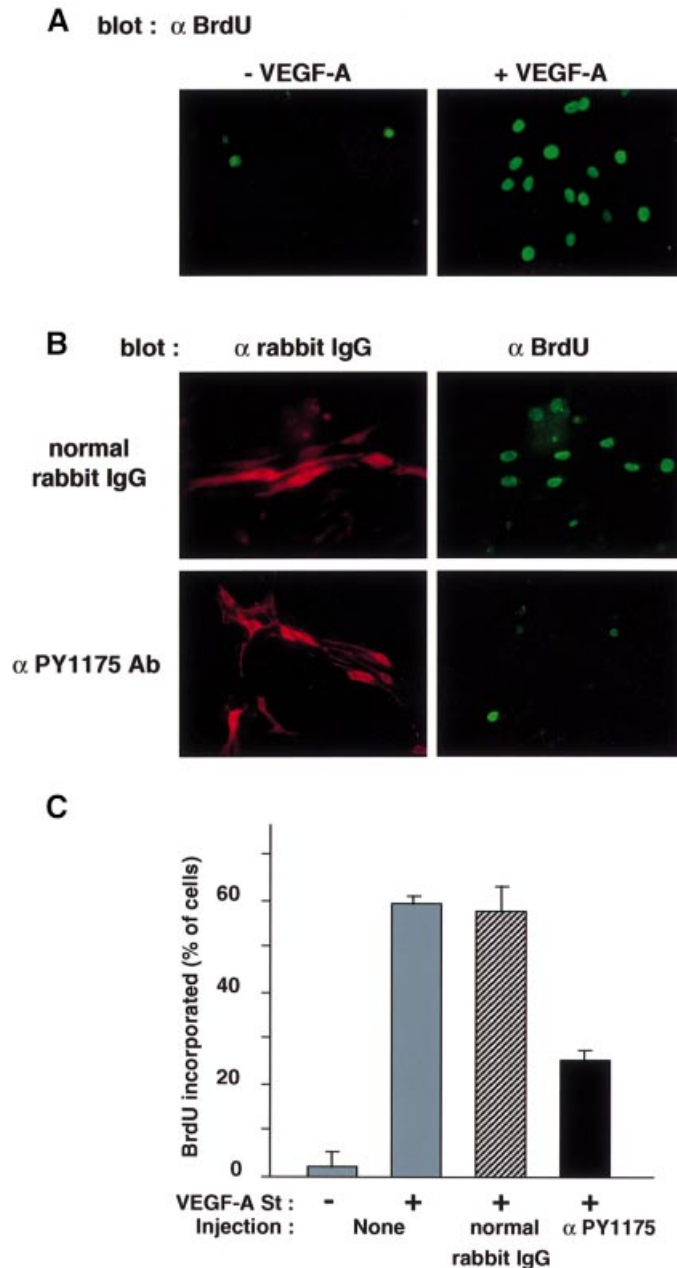


Fig. 9. Inhibition of VEGF-A-induced DNA synthesis in SE cells. SE cells were starved in modified HuMedia-EG2 medium without VEGF-A for 6–7 h. The cells were either not injected (A), injected with control normal rabbit IgG or injected with anti-PY1175 antibody (B). After 1–3 h, the injected cells were stimulated with VEGF-A and simultaneously labeled with BrdU for 20 h. The injected cells were identified by staining with FITC-labeled anti-rabbit antibody and BrdU-incorporated cells by staining with anti-BrdU antibody. Results were expressed as the percentage of stained cells exhibiting nuclear fluorescence (C).

greatly involved in the VEGF-A growth signaling pathway. In accordance with this, any reduction in the phosphorylation of MAP kinase in the Y1214F mutant was not observed in our experiments (Figure 4D). However, whether tyrosine phosphorylation of Y1214 is important for other signal pathways of VEGF-A in endothelial cells, such as the stimulation of chemotaxis (Waltenberger *et al.*, 1994), cell survival (Fujio, 1999) or the regulation of gene expression (Pepper *et al.*, 1991; Unemori *et al.*, 1992), remains to be elucidated.

In conclusion, autophosphorylation of the Y1175 residue on KDR/Flk-1 was found to create a high affinity interaction site with PLC- γ and to mediate cell prolifer-

ation signals via the MAP kinase pathway in vascular endothelial cells.

Materials and methods

Cell cultures, transfection and growth factors

NIH-3T3-KDR (Takahashi and Shibuya, 1997) and NIH-3T3-Flt cells (Seetharam *et al.*, 1995) were maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo), supplemented with 10% calf serum (CS), 2 mM L-glutamine, 40 μ g/ml kanamycin and 200 μ g/ml G418. The 293 cells and HeLa cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 40 μ g/ml kanamycin. For transient expression, 293 cells were transfected with plasmid DNAs using the standard phosphate calcium precipitation technique. After

2 days of culture, the cells were harvested for *in vitro* kinase assay. MSS31, which is an endothelial cell line derived from murine spleen stromal cells, was established and maintained as described elsewhere (Yanai *et al.*, 1991). BAE cells were maintained in M199 (Nissui) containing 20% FCS. Primary cultures of HUVE cells were purchased from Morinaga (Yokohama) and maintained in a complete medium containing 5% FBS and growth factors (Nissui) or HuMedia-EG2 medium (Kurabo, Osaka) according to the manufacturer's instructions. SE cells were isolated from rat liver as described before (Yamane *et al.*, 1994) and grown in HuMedia-EG2 medium supplemented with VEGF-A (10 ng/ml). Recombinant human VEGF-A (165 amino acid form) was purified by heparin column chromatography from the conditioned medium of Sf-9 insect cells expressing the VEGF-A gene (Cohen *et al.*, 1992). Recombinant human bFGF, PDGF B/B, EGF and HGF were obtained from Oncogene Research Products (Cambridge, MA), Boehringer Mannheim (Gmh, Germany), Toyobo Co. (Osaka) and R&D systems Inc., respectively.

Site-directed mutagenesis of KDR/Flk-1

Single point mutations were introduced using PCR-mediated mutagenesis. The mutagenic oligonucleotides were 5'-GATGGCAAAGAC-TTCATTGTTCTTCCC-3' (Y1175F), 5'-CCCAAATTCATTTCGAC-AACACAGCA-3' (Y1214F) and 5'-GACAGGCTTCTTGCCATCG-3' (Y801F), which replace the tyrosine residue at positions 1175, 1214 and 801 of KDR/Flk-1, respectively, with phenylalanine. The Y1175F and Y1214F PCR products were ligated into the *ApaI*-*PstI* site corresponding to nucleotide residues 3375–3668 (Terman *et al.*, 1991). The Y801F PCR product was ligated into the *SphI*-*BamHI* site corresponding to nucleotide residues 2213–2412. 5'-GTAGCAGTCATGAAATTGAAAG-3' (K868M) was used for the replacement of Lys868 with methionine and 5'-CTGTTCTAGACTAATGGAATTTGGGGTCCAC-3' (D1) for the change of Tyr1214 to the termination codon. These PCR products were ligated into the *BamHI*-*TaqI* site at nucleotide residues 2412–2631 and the *ApaI*-*XbaI* site at nucleotide residues 3375–4139, respectively. The point mutation Y1175F was also introduced into D1 (D1-Y1175F). All mutated nucleotide sequences were verified by sequencing.

Infection with recombinant adenovirus vectors

The recombinant adenovirus vectors encoding the wild-type or various mutants of KDR/Flk-1 were constructed by homologous recombination between the expression cosmid cassettes and the parental virus genome, according to the manufacturer's instructions (Takara, Japan). Cells were infected with the adenovirus vectors for 1 h at 37°C and used in various experiments 48 h after infection.

Antibodies

Rabbit polyclonal antisera to KDR/Flk-1 were generated against a synthetic peptide of the kinase insert region of human KDR/Flk-1 (residues 947–966). For blotting against rat KDR/Flk-1 in SE cells, the rabbit polyclonal antisera raised against another synthetic peptide (residues 1294–1316) were used. A monoclonal antibody specific for phosphotyrosine (PY-20) was obtained from ICN Biochemicals (Costa Mesa, CA). Mixed monoclonal antibodies to PLC- γ and rabbit polyclonal antibody to GST were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-p44/42 MAP kinase and anti-phosphoMAP kinase antibodies were purchased from New England Biolabs, Inc. (Beverly, MA) and Promega (Madison, WI), respectively. Secondary antibodies conjugated to horseradish peroxidase (HRP) were purchased from Amersham (Arlington Heights, IL).

The rabbit anti-phosphoY1175 polyclonal antibody was raised against a keyhole limpet hemocyanin (KLH)-conjugated phosphopeptide CDGKDY(PO₃)IVLPI (PY1175 peptide; amino acids 1171–1180), which was chemically synthesized (Peptide Institute, Osaka). The serum obtained was affinity purified by column chromatography using a HiTrap NHS-activated column (Amersham Pharmacia Biotech, Uppsala, Sweden) linked to the same phosphopeptide, followed by passage through a column linked to the corresponding unphosphorylated peptide CDGKDYIVLPI (Y1175 peptide). These peptides were also used for the *in vitro* binding assay.

Immunoblotting and immunoprecipitation

For *in vivo* phosphorylation, cells were starved for 6–7 h in the medium containing 0.1% CS or 0.1% FCS, and stimulated with or without 10 ng/ml VEGF-A for 5 min at 37°C. The preparation of cell lysates, immunoblotting and immunoprecipitation were carried out as described before (Takahashi and Shibuya, 1997).

Immunocytochemistry

HUVE cells were starved and stimulated with VEGF-A, bFGF, PDGF or EGF (50 ng/ml) for the indicated times. Cells were fixed in acetone/methanol for 2 min. Samples were then blocked in phosphate-buffered saline (PBS) containing 1% normal goat serum for 30 min, and immunostained with anti-PY1175 Ab for 2 h. Alternatively, PY1175 peptide (1 μ g/ml) was added as a competitor. The signals were visualized with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (1:100) (ICN Biochemicals) for 30 min.

In vitro kinase assay, phosphoamino acid analysis and phosphopeptide mapping

Subconfluent NIH-3T3-KDR, 293 or HUVE cells expressing wild-type or mutant receptors were incubated with or without VEGF-A (10 ng/ml) for 5 min at 37°C. Cells were lysed in 1% Triton X-100 buffer modified for kinase assay [50 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 5 mM EDTA, 2% aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM NaF, 10 mM Na₄P₂O₇ and 2 mM Na₃VO₄]. The cell extracts were immunoprecipitated with anti-KDR/Flk-1 serum and incubated with protein A–Sepharose beads for 1 h. The precipitates were then washed four times with modified 1% Triton X-100 buffer, and once with 50 mM HEPES pH 7.4. The kinase activity was assayed by incubation of the immunoprecipitates with 20 μ l of kinase buffer containing 50 mM HEPES pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 10 mM MgCl₂, 10 mM MnCl₂, 2% aprotinin, 1 mM PMSF, 50 mM NaF, 10 mM Na₄P₂O₇ and 2 mM Na₃VO₄ for 30 min at 30°C.

The radiolabeled KDR/Flk-1 was resolved by SDS–PAGE, excised from the gel and digested exhaustively with trypsin. For phosphoamino acid analysis, the samples were hydrolyzed in 500 μ l of 6 M HCl for 30 min and dried. The phosphoamino acids were then dissolved in electrophoresis buffer pH 1.9 containing phosphoamino acid standards, and resolved by two-dimensional electrophoresis (pH 1.9 followed by pH 3.5). For phosphopeptide mapping, the trypsin (Sigma Chemical Co., St Louis, MO)-digested phosphopeptides were resolved by thin-layer electrophoresis (pH 8.9), followed by ascending chromatography in a buffer containing isobutyric acid/pyridine/glacial acetic acid/water/butanol (65:5:3:29:2). Each plate was loaded with ~1000 c.p.m. sample or a mixture of two 500 c.p.m. samples.

In-gel kinase assay of MAP kinase

The separating gels were polymerized with 0.5 mg/ml of myelin basic protein (MBP; Sigma). Samples containing 15 μ g of protein were subjected to SDS–PAGE. Then the gels were denatured in 6 M guanidine–HCl, renatured and assayed for kinase activity as described previously (Takahashi and Shibuya, 1997).

Assay of DNA synthesis

BAE cells were grown in 24-well culture plates (2 \times 10⁴ cells/well). Next day, they were infected with adenovirus vectors containing wild-type or mutant receptors and then starved for 48 h in DMEM–0.1% FCS. At the end of the starvation period, the cells were stimulated with 10 ng/ml VEGF-A or bovine serum albumin (BSA) as negative control. After incubation for 16 h, cells were pulse-labeled with [³H]thymidine (1 μ Ci/ml) for 4 h. Finally, they were harvested on glass filters and incorporated radioactivity was measured. Data represent the average of triplicate samples.

In vitro binding assay with GST fusion proteins

NIH-3T3-KDR cell lysates stimulated with or without VEGF-A (10 ng/ml) were incubated with 1.5 μ g of GST, GST–PLC- γ SH2–SH2, GST–PLC- γ N-SH2, GST–PLC- γ C-SH2, GST–Grb2 SH2 or GST–PI3 kinase N-SH2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) pre-bound to glutathione–Sepharose beads (Amersham Pharmacia Biotech) for 2 h at 4°C. Alternatively, during the incubation, either 1 μ g of the antibody (normal rabbit IgG or anti-PY1175 Ab) or 10 μ g of peptide (PY1175 peptide or the corresponding Y1175 peptide) was added. The beads were then washed three times with cold lysis buffer. The proteins bound to the beads were eluted by boiling in SDS sample buffer, resolved by SDS–PAGE and immunoblotted with anti-KDR/Flk-1 or anti-GST antibody as indicated in Figure 8.

Microinjection

SE cells were plated on the collagen-coated glass coverslips and cultured for 2 days in modified HuMedia-EG2 medium supplemented with VEGF-A, in which basal medium was replaced by DMEM lacking thymidine. They were then starved for 6–7 h in the same medium without VEGF-A. Purified PY1175 Ab or normal rabbit IgG was microinjected

into the cytoplasm of each cell using an automatic Eppendorf microinjector 5246 and micromanipulator 5171. At 1–3 h after injection, the injected cells were stimulated with VEGF-A (50 ng/ml) and simultaneously labeled with 100 μ M BrdU (Sigma) for 20 h. Subsequently, the cells were fixed with 3.7% formaldehyde in PBS, permeabilized in methanol for 10 min and incubated in 2 M HCl for 10 min. They were then incubated with monoclonal anti-BrdU antibody (1:200) (Takara) in PBS containing 1% goat serum for 60 min and visualized by incubation with the mixture of FITC-conjugated anti-mouse IgG (1:200) and rhodamine-conjugated anti-rabbit IgG (1:100) (ICN Biochemicals) for 30 min. Data were means \pm SE of three independent experiments.

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