Utilization of Distinct Signaling Pathways by Receptors for Vascular Endothelial Cell Growth Factor and Other Mitogens in the Induction of Endothelial Cell Proliferation*

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This study was initiated to identify signaling proteins used by the receptors for vascular endothelial cell growth factor KDR/Flk1, and Flt1. Two-hybrid cloning and immunoprecipitation from human umbilical vein endothelial cells (HUVEC) showed that KDR binds to and promotes the tyrosine phosphorylation of phospholipase Cγ (PLCγ). Neither placental growth factor, which activates Flt1, epidermal growth factor (EGF), or fibroblast growth factor (FGF) induced tyrosine phosphorylation of PLCγ, indicating that KDR is uniquely important to PLCγ activation in HUVEC. By signaling through KDR, VEGF promoted the tyrosine phosphorylation of focal adhesion kinase, induced activation of Akt, protein kinase Cε (PKCe), mitogen-activated protein kinase (MAPK), and promoted thymidine incorporation into DNA. VEGF activates PLCγ, PKCe, and phosphatidylinositol 3-kinase independently of one another. MEK, PLCγ, and to a lesser extent PKC, are in the pathway through which KDR activates MAPK. PLCγ or PKC inhibitors did not affect FGF- or EGF-mediated MAPK activation. MAPK/ERK kinase inhibition diminished VEGF-, FGF-, and EGF-promoted thymidine incorporation into DNA. However, blockade of PKC diminished thymidine incorporation into DNA induced by VEGF but not FGF or EGF. Signaling through KDR/Flk1 activates signaling pathways not utilized by other mitogens to induce proliferation of HUVEC.

Angiogenesis is an important component of embryonic vascular development, wound healing, and organ regeneration, as well as pathological processes such as diabetic retinopathies, rheumatoid arthritis, and tumor growth (1, 2). A complex network of growth factors and cytokines regulates angiogenesis. Some factors, such as tumor necrosis factor, transforming growth factor, angiogenin, and prostaglandin E2, are believed to induce angiogenesis indirectly (1, 2). Other factors that play a role in blood vessel development, such as the acidic and basic fibroblast growth factors and platelet-derived growth factor, are mitogens for many cell types.

Vascular endothelial cell growth factor (VEGF)1 is unique, being an endothelial cell-specific mitogen that promotes many of the events necessary for angiogenesis (3–8). VEGF induces the proliferation and movement of endothelial cells, remodeling of the extracellular matrix, the formation of capillary tubes, and vascular leakage. VEGF is produced by normal and transformed cells (9, 10) and plays a significant role in the development of the cardiovascular system, in the physiology of normal vasculature, and in tumor-induced angiogenesis (11–16).

VEGF exerts its actions by binding to two cell surface receptors, KDR (the human homolog of Flk1) and Flt1 (17–21). Both receptors are structurally similar to members of the platelet-derived growth factor receptor family and consist of an extra-cellular domain composed of seven immunoglobulin-like motifs, a transmembrane domain, a juxtamembrane domain, a tyrosine kinase that is split by a kinase insert region, and a C-terminal tail (22). Gene targeting studies have shown that VEGF, Flk1, and Flt1 are essential for fetal angiogenesis. The loss of even a single allele for VEGF is lethal, suggesting the unique importance of this angiogenic factor (23). Mouse embryos null for either receptor die in utero between days 8.5 and 9.5; however, the phenotypes of the receptor knockout animals are distinct (24, 25). In Flk1 null mutant mice, endothelial and hematopoietic cell development are impaired (24), whereas in Flt1 null mutant mice there is apparent overgrowth of endothelial cells, and blood vessels are disorganized (25). The distinct phenotypes of the Flk1 and Flt1 knockout animals show that these receptors have different biological functions, which makes it likely that KDR/Flk1 and Flt1 utilize distinct signal transduction cascades to promote responses.

Flk1 is particularly abundant on the proliferating endothelial cells of vascular sprouts of embryonic and early postnatal brain; however, the level of Flk1 mRNA is dramatically reduced in adult brains in which endothelial cell proliferation has ceased (21). These observations and experiments using mutant forms of VEGF that selectively bind KDR/Flk1 or Flt1 (26) associate the former receptor with endothelial cell proliferation

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1 The abbreviations used are: VEGF, vascular endothelial cell growth factor; PIGF, placental growth factor; PLCγ, phospholipase Cγ; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; FAK, focal adhesion kinase; HUVEC, human umbilical vein endothelial cells; BAEC, bovine aortic endothelial cells; EGF, epidermal growth factor; FGF, fibroblast growth factor; GF 109203X, bisindolylmaleimide I; PD98059, 2‘-amino-3‘-methoxyflavone; PI 3-kinase, phosphatidylinositol 3-kinase; MEK, MAPK/ERK kinase.
and survival. The ability of VEGF and PI GF, a VEGF homolog that binds Flt1 but not KDR/Flk1, to induce chemotaxis and procoagulant activity associates these responses with signaling through Flt1 (27–29). These observations also support the conclusion that the functions of VEGF are segregated between two structurally related receptor tyrosine kinases.

Previously, we demonstrated that VEGF promotes the tyrosine phosphorylation of a group of signaling molecules that contain SH2 domains and associated this process with endothelial cell proliferation (30). However, at that time it was not possible to determine which signaling proteins bound to or were activated by KDR/Flk1 or Flt1. The present study identifies PLCγ, Akt, focal adhesion kinase (FAK), and PKCε as components of the signaling mechanism used by KDR to affect cellular responses. KDR also mediates activation of MAPK and promotes endothelial cell proliferation. Activation of PLCγ and PKCε by KDR occurred independently of one another and of PI 3-kinase. MAPK activation by VEGF was mediated by MEK, PLCγ, and PKCε but not by PI 3-kinase; however, only MEK was implicated in MAPK activation by EGFR or basic FGF. Finally, MAPK activity was important to the induction of endothelial cell proliferation induced by VEGF, EGFR, or FGF. However, PKC activity, acting through MAPK-dependent and MAPK-independent pathways was important to HUVEC proliferation induced by VEGF but not by FGF or EGFR. Thus, in the endothelium, VEGF utilizes multiple signaling pathways not accessed by other mitogens to elicit responses. This may explain the pre-eminent role of VEGF in angiogenesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant human VEGF and the cDNA for KDR were gifts from Genentech Inc. (South San Francisco, CA). PI GF was purchased from R & D Systems (Minneapolis, MN). EGFR and basic FGF were from Clonetics Inc. (San Diego, CA) and Life Technologies, Inc., respectively. S.U5416, a specific KDR antagonist, was a gift from Sugen Inc. (South San Francisco, CA). Horseradish peroxidase-conjugated monoclonal antiphosphotyrosine antibody (RC20), monoclonal antibodies to PLCγ, FAK, and antibodies to PKCα, PKCβ, and PKCε were from Transduction Laboratories (Lexington, KY). Anti-FRG was from Upstate Biotechnology (Lake Placid, NY). Yeast strains for two-hybrid screening, Y190 yeast cells were transformed with a HUVEC two-hybrid library, and pGAD10 for Matchmaker cDNA yeast two-hybrid library screening. Strains, Plasmids, and DNA Manipulation—Site-directed mutagenesis of KDR—Single point mutations in the cytoplasmic domain of KDR were achieved using the Quick Change Site-directed mutagenesis kit developed by Stratagene Inc. (La Jolla, CA). Using the pGBT-KDR-IC construct as a template for mutagenesis, three tyrosine residues (Tyr583, Tyr996, and Tyr1179) were mutated to phenylalanine, thereby generating mutants Y951F, Y996F, and Y1175F, respectively. The amino acid substitutions were verified by DNA sequencing.

**Immunoprecipitation and Western Blotting**—After treatments, HUVECs were washed twice with ice-cold phosphate buffered saline and lysed by incubation in 50 mM HEPES, pH 7.0, 150 mM NaCl, 1% glycerol, 1.2% Triton X-100, 1.5% MgCl2, 1 mM EDTA, 10 units/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate for 30 min at 4°C. Immunoprecipitations, SDS-polyacrylamide gel electrophoresis, and Western blotting were then conducted as described previously (30).

**Intercellular Distribution of PKC Isoforms**—The redistribution of PKC isoforms was assayed by modification of the procedure of Lu et al. (32). Briefly, HUVECs were grown to 80% confluence in endothelial cell growth medium. The day of the experiment, the cells were incubated in endothelial cell basal medium containing 0.1% bovine serum albumin for 2 h during which time they were treated with SU5416, U73122, or were left untreated (33°C) as described under "Cell Culture and Treatments" and then incubated in the absence or presence of VEGF. After the medium was aspirated, the cell monolayer was washed with ice-cold phosphate-buffered saline and then scraped into buffer A (20 mM Tris, pH 7.5, 80 mM β-glycerophosphate, 5 mM EDTA, 2 mM diithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 5 mM MgCl2 leupeptin), or lysate was prepared by repeated passage of the cells through a 29-gauge needle and centrifuged at 100,000g for 30 min at 4°C. The recovered supernatant represents the cytosolic cell fraction. The pellet was suspended in buffer A containing 1% Triton X-100 by vortexing for 10 min at 4°C. The recovered supernatant represents the cell membrane fraction. Thymidine Incorporation into DNA—HUVECs in endothelial cell growth medium were plated onto 96-well plates at a density of 5 × 103 cells/well. The next day the medium was changed to endothelial cell basal medium containing 0.5% fetal bovine serum and 0.1% bovine serum albumin (starvation medium). The cells were then pretreated with various inhibitors for 1 h and incubated with various mitogens for 30 h. [3H]Thymidine (1 μCi) was added to each well during the last 6 h of incubation. [3H]Thymidine-labeled DNA, which corresponds to de novo DNA synthesis, was then assayed by harvesting the cells onto Whatman GF/B paper filter mats using a Brandel Harvester 96. The filters were assayed for [3H] using a Beckman model LS6000IC liquid scintillation counter.

**RESULTS**

To identify proteins that bind the intracellular domain of KDR (KDR-IC), a KDR-IC/GAL4 DNA binding domain fusion was created and used as bait to screen a HUVEC cDNA library cloned into the GAL4 activation domain. A total of 5,260,000 colonies were screened. From among 20 His7′lacZ′ colonies thus isolated, 6 were true positives. DNA sequence analysis

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We considered it likely that activation and tyrosine phosphorylation of KDR promotes its interaction with one or both of the SH2 domains in PLCγ. Tyrosines corresponding to amino acids 951, 996, 1054, and 1059 in KDR are phosphorylated in bacteria (33) and are likely targets for phosphorylation in mammalian cells. Among these tyrosines, Tyr951 is unique to KDR and in the kinase insert domain, whereas the other three tyrosines are in KDR and Flt1. The conserved tyrosine 996 is also in the kinase insert domain, whereas Tyr1054 and Tyr1059 are in the catalytic domain. Tyr1175 in the KDR C-terminal domain corresponds to tyrosine Tyr1169 of Flt1, a binding site for PLCγ (34, 35). Given that Tyr951, Tyr996, and Tyr1175 are outside of the catalytic domain of KDR, these tyrosines were mutated to phenylalanine to yield KDRY951F, KDRY996F, and KDRY1175F. Testing of wild-type and KDR mutants for interaction with PLCγ in yeast two-hybrid system constructs of PLCγ with KDR-IC, suggesting that it may be a secondary contact site between PLCγ and KDR.

PLCy constructs illustrated in Fig. 1A were evaluated for their role in the KDR interaction. Individually or together, the N- and C-terminal SH2 domains of PLCγ interact strongly with wild-type KDR. This result suggests that either SH2 domain may mediate interaction with tyrosine 951 in KDR. The ability of either SH2 domain to interact weakly with tyrosine 1175 is consistent with the conclusion that this site also fosters the KDR/PLCγ interaction.

To determine whether KDR and PLCγ interact in vivo, KDR was immunoprecipitated from control and VEGF-treated HUVEC. As illustrated in Fig. 2, the proteins associate and VEGF stimulation increases the amount of interaction. Because increased tyrosine phosphorylation of PLCγ is associated with an increase of its enzymatic activity, we examined whether activation of KDR promotes the tyrosine phosphorylation of PLCγ. Thus, cells were incubated with vehicle (0.1% dimethyl sulfoxide) or SU5416, a specific antagonist that blocks the tyrosine phosphorylation and activation of KDR (36), and then with VEGF (Fig. 3A). Probing Western blots of KDR immunoprecipitate with an antibody to phosphotyrosine revealed that VEGF promoted the tyrosine phosphorylation of KDR and a second protein with mobility on SDS-polyacylamide gel electrophoresis identical to PLCγ; SU5416 antagonized the tyrosine phos-

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phorylation of both KDR and the protein corresponding to PLCγ. The definitive demonstration that KDR activation results in tyrosine phosphorylation of PLCγ is illustrated by the results shown in Fig. 3B. In this experiment, cells were stimulated with VEGF or PlGF. The former mitogen binds KDR and Flt1, whereas the latter only binds Flt1 (27–29). PLCγ was tyrosine phosphorylated in cells stimulated with VEGF, but not PlGF, showing that KDR is responsible for this process.

Treatment of cells with SU5416 to inactivate KDR followed by stimulation with VEGF or PlGF was used to identify additional signaling proteins that serve as substrates for KDR or Flt1. As shown in Fig. 4, VEGF, but not PlGF, promotes the tyrosine phosphorylation of FAK, and this process is abrogated by SU5416. A similar approach, used in combination with an antibody that only recognizes phosphorylated (activated) Akt on Western blots, showed that VEGF but not PlGF activates this enzyme, and this was inhibited by SU5416 (Fig. 5). Thus, FAK and Akt are activated by signaling through KDR.

We next determined whether common signaling proteins are activated by treatment of HUVEC with various mitogens. Thus, HUVEC were pretreated with SU5416 and then incubated with VEGF, EGF, or basic FGF. By probing Western blots of PLCγ immunoprecipitates with an antibody directed against phosphotyrosine, we observed that VEGF but not EGF or FGF promotes the tyrosine phosphorylation of PLCγ (Fig. 6A). The ability of SU5416 to abrogate VEGF stimulation of PLCγ tyrosine phosphorylation identifies KDR as the receptor responsible for this process.

Because PLCγ directly binds KDR, we considered it likely that other signaling molecules would not be required for its activation (tyrosine phosphorylation) by VEGF. This supposition was tested by treating HUVEC with wortmannin, an inhibitor of PI 3-kinase, or U73122 (Fig. 7B), an inhibitor of phospholipase C (39), or SU5416. In this experiment, cells were stimulated with VEGF or PlGF, or medium for 10 min. A Western blot of proteins that co-immunoprecipitated with PLCγ was probed with an antibody to phosphotyrosine (top) and reprobed with an antibody to PLCγ (bottom).

To determine whether VEGF-induced activation of PKCε is dependent on other signaling proteins, HUVEC were treated with U73122 (Fig. 7B), an inhibitor of phospholipase C (39), or wortmannin (Fig. 7C) and then with VEGF. Assay for translocation of PKCε showed that inhibition of PLC or PI 3-kinase did not abrogate the ability of VEGF to activate PKCε. Thus, activation of PLCγ and PKCε by VEGF occur independently of one another and of PI 3-kinase in HUVEC.

An experiment employing an antibody that exclusively recognizes dually phosphorylated (activated) MAPKs (ERK1 and ERK2) was conducted to determine whether VEGF activates these enzymes and, if so, which VEGF receptor was responsible for the response. VEGF activates ERK1 and ERK2, and this

Fig. 3. Tyrosine phosphorylation of PLCγ by signaling through KDR. A, HUVEC were incubated in the absence or presence of SU5416 before treatment with VEGF. A Western blot (WB) of proteins that co-immunoprecipitated with KDR was probed with an antibody to phosphotyrosine (PY, top) and reprobed with the antibody to KDR (bottom). C, control; SU, SU5416. B, HUVEC were incubated with VEGF, PlGF, or medium. A Western blot of proteins that co-immunoprecipitated with PLCγ was probed with an antibody to phosphotyrosine (top) and reprobed with an antibody to PLCγ (bottom).

Fig. 4. Phosphorylation of FAK by signaling through KDR. HUVEC were incubated in the absence or presence of SU5416 and then treated with VEGF, PlGF, or medium for 10 min. A Western blot of proteins that co-immunoprecipitated with FAK was probed with an antibody to phosphotyrosine (top) and reprobed with an antibody to FAK (bottom). IP, immunoprecipitation; WB, Western blot; C, control; SU, SU5416.

Fig. 5. Phosphorylation of Akt by signaling through KDR. HUVEC were incubated in the absence or presence of SU5416 and then treated with VEGF, PlGF, or medium for 30 min. A Western blot (WB) was probed with an antibody that recognizes phosphorylated (activated) Akt (top) or all Akt (bottom). C, control; SU, SU5416.
effect was blocked by SU5416, demonstrating that KDR facilitates MAPK activation (Fig. 8A). Consistent with this conclusion, PIGF was unable to elicit MAPK activation through Flt1. By using inhibitors, it was possible to identify upstream signaling molecules necessary for VEGF activation of MAPK in HUVEC. PD98059, a MEK inhibitor (40), but not wortmannin, abrogated activation of MAPK by VEGF (Fig. 8B). Additionally, U73122 (Fig. 8D), an inhibitor of PLCγ, and to a small degree GF 109203X (Fig. 8C), the inhibitor of PKC, blocked MAPK activation. Thus, MEK, PLCγ, and PKC but not PI 3-kinase play a role in the activation of MAPK by VEGF.

The ability of VEGF but not EGF or FGF to activate PLCγ indicated that these mitogens use different signaling pathways in HUVEC (Fig. 6A). For this reason the effects EGF and bFGF on MAPK activation were characterized. In HUVECs, EGF induced a more profound and FGF a less substantial activation of ERK1 and ERK2 than VEGF. Furthermore, these responses were unaffected by SU5416, an observation consistent with the ability of this reagent to specifically inhibit KDR but not other receptor tyrosine kinases (Fig. 9A). Whereas PD98059 inhibited MAPK activation by EGF or FGF in HUVEC, wortmannin and GF 109203X did not (Figs. 9B and C). Thus, the inability of EGF and FGF to act on PLCγ was not due to insensitivity of HUVEC to these mitogens or lack of mitogen activity. Rather, VEGF and the other mitogens differentially utilize various signaling proteins and transduction pathways in HUVEC.

Knowing that the mitogens act through distinct pathways in HUVEC, we determined how inhibition of these pathways affects de novo DNA synthesis, an index of cell proliferation. VEGF but not PIGF significantly promoted the incorporation of thymidine into DNA, and this response was blocked by SU5416 (Fig. 10A). These observations show that the VEGF/KDR signaling system promotes a proliferative response from endothelial cells. Reproducibly, SU5416 diminished thymidine incorporation into DNA in control and PIGF-treated HUVEC; this effect probably is the consequence of the production of low levels of VEGF by HUVEC (data not shown), which accounts for some constitutive phosphorylation of KDR in these cells, which was suppressed by SU5416 (Fig. 3).

A comparison of the effects of mitogens on HUVEC proliferation revealed that VEGF, EGF, and FGF induced 7-, 2-, and 16-fold increases in thymidine incorporation into DNA, respectively (Fig. 10B). However, the level of MAPK activation (Figs. 8 and 9) induced by these mitogens does not correlate with the extent to which they promote HUVEC proliferation (Fig. 10B). PD98059, the MEK inhibitor (40), diminished VEGF-, EGF-, and FGF-promoted thymidine incorporation into DNA by about 50%, indicating that MAPK plays a role in the mitogenic response induced by the growth factors. U73122, the phospholipase C inhibitor (39), was evaluated for its effects on mitogen-induced endothelial cell proliferation; at concentrations that inhibited PLCγ activity, it was cytotoxic to HUVEC during the time necessary to assay thymidine incorporation into DNA (data not shown). However, the demonstration that PLCγ plays a role in the activation of MAPK by VEGF (Fig. 8C) and that MAPK is important for the proliferative response of HUVEC to VEGF (Fig. 10B) implicates PLCγ in the proliferative response. GF 109203X, the PKC inhibitor (37), suppressed VEGF-in-
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FIG. 9. MAPK activation in response to mitogens. HUVEC were treated with medium, SU5416, wortmannin, PD98059, or GF 109203X (GFX) before stimulation with FGF or EGF. A Western blot (WB) of proteins from cell lysates was then probed with an antibody to activated (top) or total MAPKs (bottom).

Reduced thymidine incorporation into DNA by 70%, without significantly affecting the response of HUVEC to EGF or FGF. Interestingly, inhibition of PKC only modestly affected VEGF activation of MAPK but significantly blocked the proliferative response induced by this mitogen. These results suggest that VEGF-induced PKC activity affects endothelial cell proliferation through MAPK-dependent and MAPK-independent mechanisms and that PKC is important to the proliferative response induced by VEGF but not FGF or EGF in HUVEC.

DISCUSSION

In previous studies, we and others began identifying signal transduction pathways activated by VEGF. In BAEC, which express KDR/Flk1 and Fli1, VEGF promotes the tyrosine phosphorylation of a group of signal transduction mediators that contain SH2 domains (30). Treatment of these cells with genistein, a tyrosine kinase inhibitor, blocks phosphorylation of the cytoplasmic signaling proteins and attenuates VEGF-induced endothelial proliferation, thereby relating these processes (30). In HUVEC, VEGF promotes tyrosine phosphorylation of FAK and the focal adhesion associated protein paxillin, which may be associated with endothelial cell migration, and activates PI 3-kinase and MAPK (41). Although these studies begin to provide a basis for understanding VEGF action, it is important to identify which signaling pathways are utilized by either or both of the VEGF receptors.

One approach used to address this issue has been to transfect KDR/Flk1 or Flt1 into cells that do not ordinarily express these receptors and then test the ability of VEGF to elicit responses. Stimulation of porcine aortic endothelial cells overexpressing KDR/Flk1 with VEGF resulted in association of the receptor with Shc, Grb2, Nck, two protein-tyrosine phosphatases, SHP-1 and SHP-2, MAPK activation, changes of cell morphology, actin reorganization, membrane ruffling, chemotaxis, and a proliferative response; none of these events were detected in cells transfected with Fli1 (42). However, although PI 3-kinase and PLCγ are responsive to VEGF in BAEC (30), these putative mediators of VEGF functions were not activated in porcine aortic endothelial cells overexpressing KDR/Flk1 or Fli1 (43). In NIH3T3 cells overexpressing KDR/Flk1, VEGF did induce the tyrosine phosphorylation of PLCγ, MAPK activation, and a weak mitogenic response (44); however, activation of MAPK and PLCγ phosphorylation were also observed in NIH3T3 cells transfected with Fli1 (34, 45). As an alternative to receptor overexpression, immortalized endothelial cells have been used as a model system. Here, Affi-Gel-immobilized Fli1 precipitated PLCγ, SHP-2, Grb2, Crk, and Nck from cell lysates (46). Thus, when overexpressed or studied in transformed cells, KDR/Flk1 and Fli1 appear to associate with a similar array of cytoplasmic signaling proteins.

Proteins that bind the cytoplasmic domains of the VEGF receptors have been identified in yeast using the two-hybrid system. A fusion of the intracellular domain of Fli1 with the GAL4-DB interacted with fusions of PI 3-kinase, Nck, SHP-2, and PLCγ with the GAL4-AD (47, 48). In Fli1, tyrosine 794 in the juxtamembrane region, as well as tyrosine 1169, in the C-terminal tail, interacted with the N-terminal SH2 domain of PLCγ (35). The amino acids downstream of these tyrosines are LSI and IPI, respectively, in which leucine/isoleucine at position +1 and +3 conform to a consensus binding sequence for the N-terminal SH2 domain of PLCγ (35). The analogous amino acids in KDR/Flk1, tyrosine 801, upstream of LSI, and tyrosine 1175, upstream of IVL, weakly interact with PLCγ (35). In the present study, the intracellular domain of Flt1/Flk1 was used to screen a HUVEC two-hybrid library, leading to the identification of PLCγ as a KDR/Flk1-binding protein. Stronger interaction was detected with tyrosine 951, in the kinase insert domain of KDR/Flk1, although weaker interaction with tyrosine 1175 also occurred. Although tyrosine 1175 in KDR/Flk1 is followed by an IVL consensus binding site for PLCγ, the stronger interacting site at tyrosine 951 is flanked by VGA. This amino acid sequence does not correspond to a typical binding site for the N-terminal SH2 domain of PLCγ or the C-terminal SH2 domain, which recognizes a Y(L/I)I motif, with the proline in the +3 position essential for binding. Thus, our observations suggest that the primary binding site for PLCγ in KDR/Flk1 is novel in that interaction occurs in the kinase insert domain and the amino acid sequence of the recognition site is unique.

We used HUVEC, a physiologically relevant target cell, for studies of VEGF signaling and action. This choice obviated the necessity for transfections and the possibility that receptor overexpression would promote nonspecific or nonphysiologically relevant interactions of KDR/Flk1 and Fli1 with signaling proteins. Our strategy was to stimulate HUVEC with VEGF, which binds to and activates both VEGF receptors, or PIGF, which binds to and activates only Fli1 (27–29), as a first approach toward identifying which signaling proteins are utilized by either VEGF receptor. Additionally, to refine the distinction between KDR/Flk1 and Fli1, experiments were conducted with HUVEC incubated in the absence or presence of SU5416, a specific inhibitor of the KDR/Flk1 receptor tyrosine kinase. Finally, because VEGF is uniquely important to the endothelium, we compared the mechanism through which it induces proliferation to that of two less tissue-specific mitogens, basic FGF and EGF.

Our identification of PLCγ as a protein that binds KDR by two-hybrid cloning was validated by co-immunoprecipitation of the proteins from HUVEC. The ability of VEGF, but not PIGF, to promote the tyrosine phosphorylation of PLCγ, and the ability of SU5416 to impair this process, identifies this protein as a substrate for KDR but not Fli1. Using this same approach, we found that FAK is a downstream target for KDR and that the
Akt serine-threonine kinase, ERK1 and ERK2, and endothelial cell proliferation are induced by signaling through KDR. Our observations related to Akt are consistent with those of Gerber et al. (50), who also showed that Akt is activated by VEGF and downstream of KDR/Flk1. Akt and MAPK are components of signaling pathways that promote cell survival (50, 51), suggesting that by activating multiple signaling pathways, KDR/Flk1 is important to endothelial cell growth and viability as well.

In BAEC, VEGF sequentially activates PLC-γ and a downstream target, PKCβ, and this pathway appears to be a component of the mechanism through which VEGF elicits mitogenic responses (38). In contrast with observations made in BAEC, Wellner et al. (52) associated activation of PKC-α and PKC-ζ with the proliferative response induced by VEGF in HUVEC. We were unable to detect expression of PKCβ in HUVEC and found that by acting through KDR, VEGF induced membrane translocation of PKC-ε but not PKC-α or PKC-ζ. We conclude that activation of PKC isoforms by VEGF is species-specific, likely to be sensitive to differences in how primary cells are isolated and cultured, and likely to be sensitive to variances in experimental procedures. Nevertheless, PKC is added to the group of signaling proteins activated by KDR.

Our inability to identify a signaling cascade downstream of Flt1 does not suggest that this receptor is nonfunctional. Flt1 induces tissue factor expression in macrophages and endothelial cells (29), and mice in which this gene has been knocked out die in utero (25). The ability of the extracellular domain of Flt1 to promote normal vascular development in the fetus (53) and the much weaker enzymatic activity of Flt1 relative to KDR/Flk1 (43) may suggest that this receptor works through novel mechanisms.

VEGF is unique in its ability to promote so many events necessary for angiogenesis, and its activity is associated with the progression of important pathologies, including cancer (2). Neutralization of VEGF or inhibition of KDR/Flk1 blocks the growth and spread of cancers in animals (36, 54, 55). These observations suggest that VEGF, acting through KDR, may induce tissue factor expression in macrophages and endothelial cells (29), and mice in which this gene has been knocked out die in utero (25). The ability of the extracellular domain of Flt1 to promote normal vascular development in the fetus (53) and the much weaker enzymatic activity of Flt1 relative to KDR/Flk1 (43) may suggest that this receptor works through novel mechanisms.

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![Fig. 10. Proliferative response of HUVEC to mitogens.](http://www.jbc.org/content-pdf/272/18/5102.full.pdf)
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Utilization of Distinct Signaling Pathways by Receptors for Vascular Endothelial Cell Growth Factor and Other Mitogens in the Induction of Endothelial Cell Proliferation

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