THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 269, No. 43, Issue of October 28, pp. 26988–26995, 1994
Printed in U.S.A.

Different Signal Transduction Properties of KDR and Flt1, Two Receptors for Vascular Endothelial Growth Factor*

(Received for publication, January 31, 1994, and in revised form, July 15, 1994)

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Vascular endothelial growth factor (VEGF) is a homodimeric peptide growth factor which binds to two structurally related tyrosine kinase receptors denoted Flt1 and KDR. In order to compare the signal transduction via these two receptors, the human Flt1 and KDR proteins were stably expressed in porcine aortic endothelial cells. Binding analyses using ^{125}I-VEGF revealed K_d values of 16 pM for Flt1 and 760 pM for KDR. Cultured human umbilical vein endothelial (HUVE) cells were found to express two distinct populations of binding sites with affinities similar to those for Flt1 and KDR, respectively. The KDR expressing cells showed striking changes in cell morphology, actin reorganization and membrane ruffling, chemotaxis and mitogenicity upon VEGF stimulation, whereas Flt1 expressing cells lacked such responses.

KDR was found to undergo ligand-induced autophosphorylation in intact cells, and both Flt1 and KDR were phosphorylated *in vitro* in response to VEGF, however, KDR much more efficiently than Flt1. Neither the receptor-associated activity of phosphatidylinositol 3′-kinase nor tyrosine phosphorylation of phospholipase C-γ were affected by stimulation of Flt1 or KDR expressing cells, and phosphorylation of GTPase activating protein was only slightly increased. Members of the Src family such as Fyn and Yes showed an increased level of phosphorylation upon VEGF stimulation of cells expressing Flt1 but not in cells expressing KDR.

The maximal responses in KDR expressing porcine aortic endothelial cells were obtained at higher VEGF concentrations as compared to HUVE cells, *i.e.* in the presence of Flt1. This difference could possibly be explained by the formation of heterodimeric complexes between KDR and Flt1, or other molecules, in HUVE cells.

The endothelium represents a highly differentiated cell type, which has specific requirements based on its anatomical localization. It plays a central role during early stages of development and is involved in the structural and functional regulation of hemostasis and tissue perfusion including the regulation of the vascular tone and vascular permeability (1) as well as angiogenesis (2). It furthermore plays a role in various disease processes such as atherosclerosis (3), the growth of solid tumors, as well as metastasis (4). A wide range of different factors has been shown to regulate the various endothelial functions such as vasconstrictors (*e.g.* angiotensin and endothelin), vasodilators (*e.g.* endothelial-derived relaxing factors), pro- and anticoagulant factors, adhesion molecules, as well as various growth inhibitors, growth factors and cytokines (*e.g.* transforming growth factor-β, and platelet-derived growth factor (PDGF)),[1] and their imbalanced function and/or expression has been implicated to result in "endothelial dysfunction."

The recently identified and characterized vascular endothelial growth factor (VEGF) (5), also described as vascular permeability factor (6) or vasculotropin (7), is a 46-kDa homodimeric protein that was found to specifically bind to endothelial cells, to stimulate endothelial cell growth *in vitro* (8) and angiogenesis *in vivo* (9). It has been implicated to be involved in several endothelial-specific functions including endothelial proliferation and angiogenesis (8, 10), the regulation of vascular permeability (8), and thrombogenicity (11). Moreover, VEGF is induced by hypoxia (12, 13) and may therefore be regarded as an important regulator of the endothelium.

Recently, two receptor-tyrosine-kinases have been shown to specifically bind VEGF, namely the *fms*-like tyrosine kinase Flt1 (14) and KDR (15), the mouse homologue of which is called Flk-1 (16–18) or NYK/FLK-1 (19), and the rat homologue TKr-III (20). Both receptors contain large insert sequences in their intracellular kinase domains and seven immunoglobulin-like domains in the extracellular NH_2-terminals. Both have been shown to be almost exclusively expressed in endothelial cells (18, 21) and therefore represent targets for transducing signals upon VEGF stimulation of the endothelium.

In order to elucidate the functional role of the two VEGF receptors in the transduction of the different effects of VEGF in endothelial cells, we have expressed the two receptors separately in porcine aortic endothelial (PAE) cells which lack endogenous VEGF receptors. In this paper we report that Flt1 and KDR transduce different cellular effects and compare some of the initial signal transduction events.

MATERIALS AND METHODS

Establishment of Stably Transfected Cell Lines—A full-length *Flt1* cDNA (clone 3–7) (22) was cloned into the cytomegalovirus-based eu-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. This work was supported in part by Grant Wa734/1–1 from Deutsche Forschungsgemeinschaft and a grant from Fritz-Thyssen-Stiftung (to J. W.).

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[1] The abbreviations used are: PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; PAE cells, porcine aortic endothelial cells; HUVE cells, human umbilical vein endothelial cells; PBS, phosphate-buffered saline; FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis; PI3-K, phosphatidylinositol 3′-kinase; PLC-γ, phospholipase C-γ; PDGFR, platelet-derived growth factor receptor; GAP, GTPase activating protein.

karyotic expression vector pcDNAI/Neo (Invitrogen). This construct was transfected into PAE cells (23) using electroporation as described before (24). In a similar way, *KDR* cDNA ligated into a modified pcDNAI (Invitrogen) expression vector (15) was transfected into PAE cells together with pVV3 (24), a plasmid containing a neomycin resistance gene. PAE cells were cultured in Ham's F-12 (Life Technologies, Inc. or Biochrom) supplemented with penicillin/streptomycin (Life Technologies, Inc.), 10% fetal calf serum (Life Technologies, Inc.), and 0.4 mg/ml of G418 sulfate (Geneticin®, Life Technologies, Inc.). Clones were picked after 2 weeks and selected based on their ligand binding abilities, *i.e.* clones 7 and 13 of PAE/Flt1 and clones 11 and 23 of PAE/KDR.

Antisera—Polyclonal rabbit antisera against Flt1 and KDR were raised as described previously (25) against synthetic peptides and named according to the sequence of the three NH_2-terminal amino acids: EPK against amino acid residues 973–993 in the kinase insert region of Flt1 (22), VSE against amino acid residues 1300–1319, P1–3 against the COOH-terminal region of Flt1 (26), and NEF against the 25 amino acid residues of the rat TKr-III sequence (20) that correspond to amino acid residues 935–959 in the human KDR (15). Some of the peptide antisera were loaded on affinity columns containing the corresponding synthetic peptides coupled to CH-Sepharose 4B (Pharmacia LKB), and the bound antibodies were eluted at pH 3, neutralized, and dialyzed against PBS containing 10% glycerol. The polyclonal rabbit antiserum R3 was used for immunoprecipitation of the PDGF β-receptor as described before (27). The monoclonal antibody PY20 (Upstate Biotechnology Incorporated, UBI) was used for detection of phosphotyrosine, and as a secondary antibody, peroxidase-conjugated rabbit anti-mouse Ig (Dakopatts); alternatively the horseradish peroxidase-conjugated phosphotyrosine antibody RC20H (Transduction Laboratories) was used. For detection of PLC-γ, the monoclonal antibody PLC-γ1 (Transduction Laboratories) was applied.

Affinity-purified rabbit antibodies recognizing the carboxyl-terminal sequence of $pp60^{c\text{-}src}$ (Src), $p59^{fyn}$ (Fyn), and $pp62^{c\text{-}yes}$ (Yes) (αcst.1) as well as antisera directed against Fyn (αfyn.2) or Yes (αyes.6) were gifts of S. Courtneidge (28). An antiserum (α GAP) against GTPase activating protein was obtained from J. Downward, Imperial Cancer Research Fund, London, and an antiserum recognizing the p85 subunit of phosphatidylinositol 3′-kinase (PI3-K) was obtained from M. Waterfield, Ludwig Institute for Cancer Research, London.

Growth Factors—Recombinant VEGF expressed in the baculovirus system (VEGF cDNA encoding the 165 amino acid form of VEGF) was a gift of D. Gospodarowicz, Chiron Corp., Emeryville, CA. PDGF-BB was recombinant protein expressed in the yeast strain *Saccharomyces cerevisiae* (29).

Receptor-binding Analysis—Binding experiments were performed as described in detail previously (30) on either transfected PAE or HUVE cells grown to confluence in 24-well tissue culture plates under the culture conditions described above and using iodinated VEGF (1–2 ng/ml) and different concentrations of unlabeled VEGF. Iodination of VEGF was performed using the chloramine-T method (31) to specific activities of 2.5×10^5 to 3.8×10^5 counts/min/ng ^{125}I-VEGF. The data were subjected to Scatchard analysis (32) as described before (30).

Mitogenicity Assay—Cells were seeded sparsely in 12-well culture dishes. Two days later, the medium was changed to serum-free Ham's F-12 medium supplemented with 0.1 mg/ml bovine serum albumin. After an additional 24 h of incubation, the medium was renewed, and after a total of 40 h of serum-free incubation, the cells received VEGF (0–100 ng/ml) or 5% FCS for another 24 h. During the final 2 h of stimulation, 0.25 μCi of [^{3}H]thymidine/ml (Amersham) was present. High molecular weight ^{3}H radioactivity was then precipitated with 5% trichloroacetic acid at 4 °C for 20 min. After washing twice with ice-cold H_2O, ^{3}H radioactivity was solubilized in 1 M NaOH (400 μl/well) for 20 min at room temperature, acidified by the addition of 2 M HCl (400 μl/well), and quantitated by liquid scintillation counting.

Actin Reorganization Assay and Changes in Cell Morphology—The assay was performed as described previously (33). In brief, cells were seeded sparsely onto microscope glass coverslips placed within 6-well culture plates and allowed to grow for 2 days with one change of the medium. VEGF (100 ng/ml) was added, and the cells were incubated for 2–20 min at 37 °C. The cells were fixed in freshly prepared 4% paraformaldehyde in PBS at room temperature for 30 min, followed by acetone fixation at 4 °C for 5 min. After three washes in PBS, rhodamine-conjugated phalloidin was applied in a humidified chamber at room temperature for about 60 min. After rinsing the coverslips in PBS and distilled H_2O, they were mounted onto object glasses using Fluoromount C® (Southern Biotechnology). The slides were viewed with a Wild-Leitz epifluorescence microscope, and photomicrographs were taken using Kodak T-MAX film.

Chemotaxis Assay—The motility response of cells was assayed by means of the leading front technique using a modified Boyden chamber and 150-μm collagen-coated nitrocellulose filters, as described before (34–36). For each set of experiments, the migration of the respective cell-clone in Ham's F-12 medium with 10% FCS and with the same medium below the filter (chemokinesis) served as a control and is referred to as 100% migration. All experiments were performed in duplicate for every concentration of VEGF, and several independent cell clones expressing KDR or Flt1 were analyzed.

Immunoprecipitation and in Vitro Immune Complex Kinase Assay—Subconfluent or confluent cells were preincubated for 5 min at 37 °C with 100 μM Na_3VO_4 to inhibit phosphatase activity. They were stimulated for 8 min at 37 °C, or for 1 h at 4 °C followed by 8 min at 37 °C, with 1–100 ng/ml VEGF or with 50–100 ng/ml PDGF-BB. After a wash with ice-cold PBS containing 100 μM Na_3VO_4, cells were solubilized in a lysis buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1% CHAPS (Sigma), 10 mM EDTA, 10% glycerol, 100 μM Na_3VO_4, 1% Trasylol (Bayer), 1 mM phenylmethylsulfonyl fluoride). The cell lysates were centrifuged at $10,000 \times g$ for 20 min, and the supernatants were precleared for 1 h at 4 °C using 20 μl of Protein A-Sepharose CL 4B. The supernatant was used for immunoprecipitation as described before (37) with receptor-specific antisera, antisera reactive with GAP, PLC-γ, or with members of the Src family. Alternatively, the monoclonal antibody PY20 recognizing phosphotyrosine was used. Immunoprecipitates immobilized on Protein A-Sepharose CL 4B were used for immune complex kinase assays by incubation in 40 μl of 50 mM HEPES, pH 7.4, 10 mM $MnCl_2$, 1 mM dithiothreitol, and 5 μCi of [γ-^{32}P]ATP (Amersham) for 7 min at room temperature. The samples were analyzed by SDS-PAGE using 5–10% polyacrylamide. The gels were incubated for 30 min in 10% acetic acid, 40% methanol, followed by a 30-min incubation in 2.5% glutaraldehyde (38) in order to cross-link proteins to the gel, and further treated for 1 h at 55 °C in 1 M KOH to remove serine-bound phosphate (39) before gel drying and exposure to Hyperfilm MP (Amersham) was performed.

Phosphorylation in Intact Cells and Immunoblotting—Cell culture and immunoprecipitation was performed essentially as described above. Confluent cells (75 cm^2) were preincubated with 100 μM Na_3VO_4 for 5 min, stimulated with 50 ng/ml of VEGF for 60 min at 4 °C followed by 8 min at 37 °C. Washing with PBS containing 100 μM Na_3VO_4 was followed by solubilization in Nonidet P-40 lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40 (Nonidet P-40, Sigma), 10 mM EDTA, 10% glycerol, 100 μM Na_3VO_4, 1% Trasylol, 1 mM phenylmethylsulfonyl fluoride, 1 mM zinc acetate). Lysates were used for immunoprecipitation, and the samples were subjected to SDS-PAGE and blotted onto a nitrocellulose membrane (Hybond C extra®, Amersham). Phosphorylated proteins were detected by immunoblotting as described before (37), using PY20, and as a secondary antibody, peroxidase-conjugated rabbit anti-mouse Ig (Dakopatts), or alternatively the horseradish peroxidase-conjugated phosphotyrosine antibody RC20H was used. A chemoluminescence-based detection system (ECL®; Amersham) was used for detection of immunoreactivity.

Phosphatidylinositol 3′-Kinase Assay—Confluent cells, grown in Ham's F-12 containing 10% fetal calf serum, were stimulated with 50 ng/ml of either VEGF or PDGF-BB in the presence of 100 μM Na_3VO_4 for 8 min at 37 °C, washed once with ice-cold PBS containing 100 μM Na_3VO_4, and lysed in Nonidet P-40 lysis buffer (see above) on ice. The lysate was centrifuged at $10,000 \times g$ for 20 min at 4 °C, and the supernatant was incubated with a receptor-specific antiserum or an antiserum recognizing the regulatory subunit p85 (5α) for 2 h on ice. Protein A-Sepharose CL 4B (50 μl of a slurry containing 50% beads in PBS) was added, and the incubation was prolonged for another 30 min. Analysis of receptor-mediated stimulation of PI3-K was essentially performed as described earlier (40). In brief, the Sepharose beads were collected and washed several times to remove nonspecific phosphatidylinositol 4′-kinase activity. Phosphatidylinositol was used as a substrate for PI3-K *in vitro*. The phospholipids were extracted with chloroform, separated by thin layer chromatography, and detected by autoradiography.

RESULTS

PAE/Flt1 and PAE/KDR Cells Express High Affinity Binding Sites for VEGF: Comparison with HUVE Cells—In order to study the properties of the endothelial cell-specific receptors Flt1 and KDR, porcine aortic endothelial cells were chosen as recipient cells for the expression of these receptors. Receptor binding analyses did not show any significant VEGF binding on untransfected PAE cells (Fig. 1*c*), whereas high affinity binding

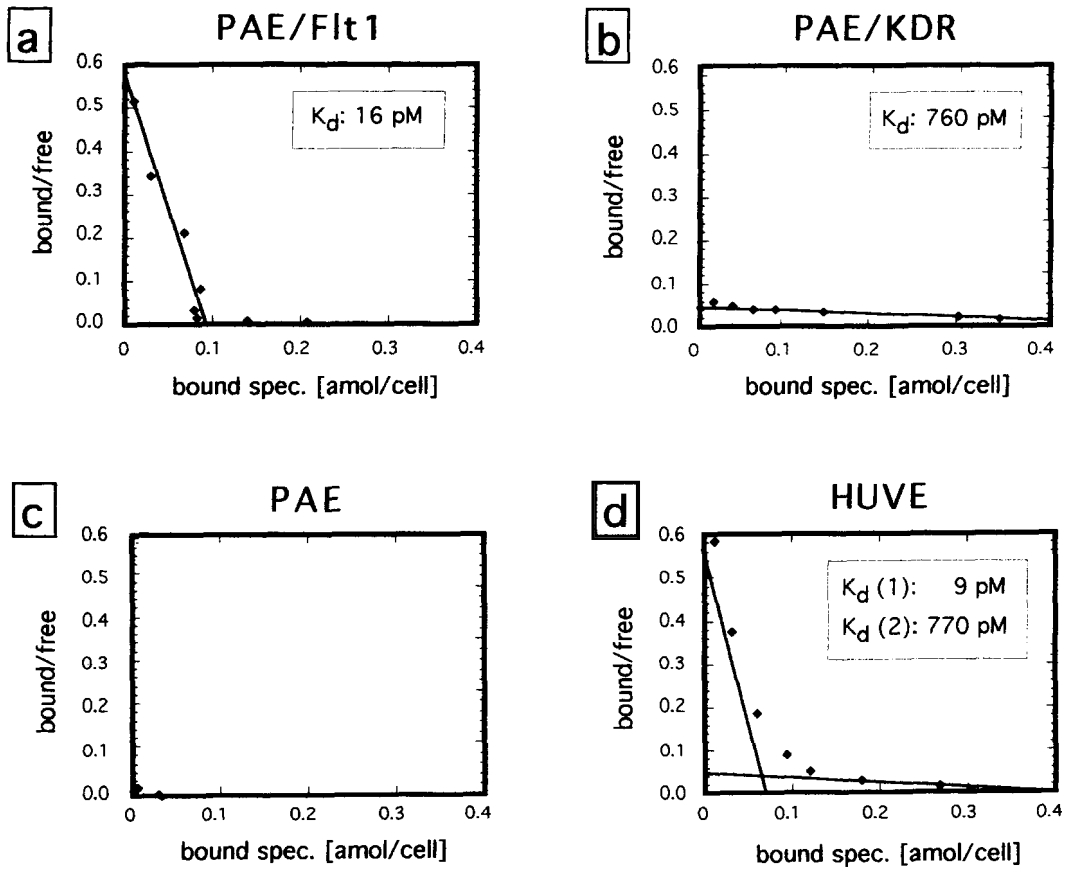

FIG. 1. **Scatchard analyses of receptor binding of VEGF to PAE/Flt1 cells (*a*), PAE/KDR cells (*b*), untransfected PAE cells (*c*), as well as to HUVE cells (*d*), are shown.** All data points represent the mean of triplicates, and similar results were obtained in at least two different experiments.

sites were found on cell lines expressing Flt1 or KDR (*PAE/Flt1* and *PAE/KDR* cells, respectively). The affinities for the two receptors differed significantly, with K_d values of about 16 pM for Flt1 (50,000 receptors/cell; Fig. 1*a*) and 760 pM for KDR (150,000 receptors/cell; Fig. 1*b*). On HUVE cells two distinct VEGF receptor populations were identified (Fig. 1*d*) with K_d values of 9 and 770 pM, *i.e.* comparable to those determined for Flt1 and KDR.

Different Abilities of Flt1 and KDR to Transduce Ligand-dependent Changes in Cell Shape, Membrane Ruffling, Chemotaxis, and Mitogenicity—The changes in cellular shape upon ligand stimulation were determined by fixation and staining with rhodamine-conjugated phalloidin in order to visualize cytoskeletal structures. The addition of 100 ng/ml VEGF to PAE/KDR cells resulted in the formation of cytoplasmic protrusions and edge ruffles after 5 min of stimulation (Fig. 2). The formation of pili and major changes in the cell shape could be seen after 15 min of ligand stimulation, the edge ruffles still being partly present. Changes of very similar morphology and kinetics appeared in HUVE cells, when treated in the same way (Fig. 2). Under identical conditions, the Flt1 expressing PAE cells and untransfected PAE cells did not respond in changes of cellular shape or cytoskeletal reorganization.

Chemotaxis of PAE cells expressing KDR or Flt1 was examined using a modified Boyden chamber technique. The migration distance of the leading front of cells into a 150-μm nitrocellulose filter, toward increasing concentrations of VEGF, was scored. Calf serum at 10% was present in both the upper and lower chamber, in order to avoid scoring for random migration (chemokinesis). As seen in Fig. 3, an efficient chemotaxis of KDR expressing cells was achieved using concentrations of 10–50 ng/ml of VEGF. Several different PAE cell clones expressing Flt1 were examined, and all failed to mediate an appreciable chemotactic response at concentrations of VEGF between 0.1 and 100 ng/ml. Untransfected PAE cells also failed to migrate toward VEGF.

The abilities of KDR and Flt1 to transduce mitogenic signals were investigated using a [^{3}H]thymidine incorporation assay. Whereas untransfected PAE cells and PAE/Flt1 cells failed to increase the incorporation of [^{3}H]thymidine upon stimulation with VEGF, the PAE/KDR cells responded in a dose-dependent fashion to concentrations of 1–10 ng/ml VEGF (Fig. 4). At a concentration of 30 ng/ml, the majority of cells had detached from the culture dish after 24 h of incubation allowing essentially no measurable incorporation and precipitation of [^{3}H]thymidine. Additionally, HUVE cells responded to VEGF and showed maximal response at 1 ng/ml, *i.e.* a significantly lower concentration than that giving maximal stimulation of PAE/KDR cells.

Flt1 and KDR: Distinct Patterns of Phosphorylation and Association with Cellular Proteins—Stimulation of intact PAE/KDR cells with VEGF (50 ng/ml) induces phosphorylation of the 205-kDa receptor on tyrosine residues, as assessed by immunoprecipitation using the KDR-specific NEF antiserum and subsequent immunoblotting using the anti-phosphotyrosine antibody PY20 (Fig. 5). The PDGF β-receptor expressed in PAE cells (41) was tyrosine phosphorylated in a similar way, when stimulated with 50 ng/ml of PDGF-BB. Using a similar assay no VEGF-induced tyrosine-phosphorylation of Flt1 was detectable in PAE/Flt1 cells when precipitation was performed using the EPK antiserum; it should be noted though that this antiserum has a lower titer than the NEF antiserum. The Flt1

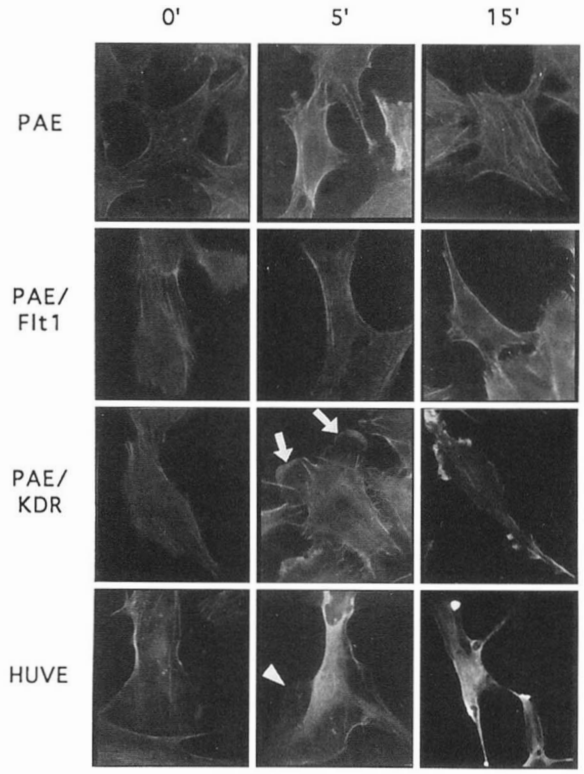

FIG. 2. **Actin reorganization upon stimulation with VEGF.** Sparsely seeded untransfected (*PAE*) and transfected PAE cells (*PAE/Flt1*, *PAE/KDR*) as well as HUVE cells (*HUVE*), were stimulated with 100 ng/ml VEGF for 5 or 15 min, fixed with paraformaldehyde, permeabilized with acetone, and stained with rhodamine-phalloidin for visualization of the actin filaments. After 5 min of stimulation, membrane edge ruffles ("leading lamellae") can be observed in PAE/KDR cells (→) as well as in HUVE cells (▶). Photographs were taken using a Leitz-Wild microscope and Kodak TMY film.

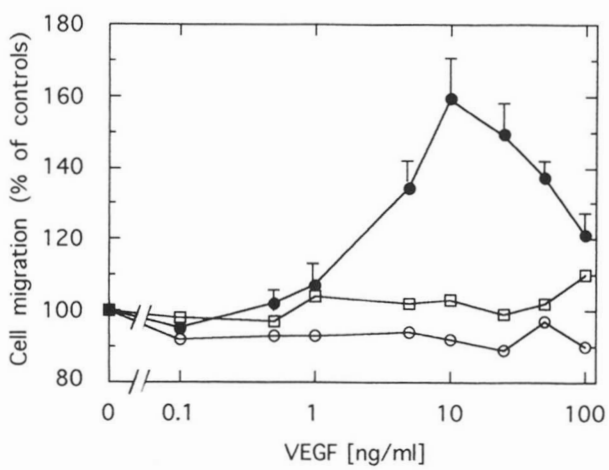

FIG. 3. **Chemotactic response of PAE/KDR cells (●), PAE/Flt1 cells (□), and untransfected PAE cells (○) to VEGF.** Cells were suspended in Ham's F-12 medium supplemented with 10% FCS and incubated for 6 h at 37 °C in a modified Boyden chamber containing a 150-μm nitrocellulose filter membrane. Data points are mean ± S.E. of three experiments performed in duplicate for PAE/KDR cells. For PAE/Flt1 and untransfected PAE cells, data represent the mean of duplicates.

protein, however, can be demonstrated as a 205-kDa protein in PAE/Flt1 cells, when Flt1 is enriched by EPK-specific immunoprecipitation followed by Western blot analysis with another antiserum against Flt1 (P1–3; data not shown).

Using an *in vitro* phosphorylation assay, VEGF-inducible tyrosine phosphorylation of Flt1 could be demonstrated; after

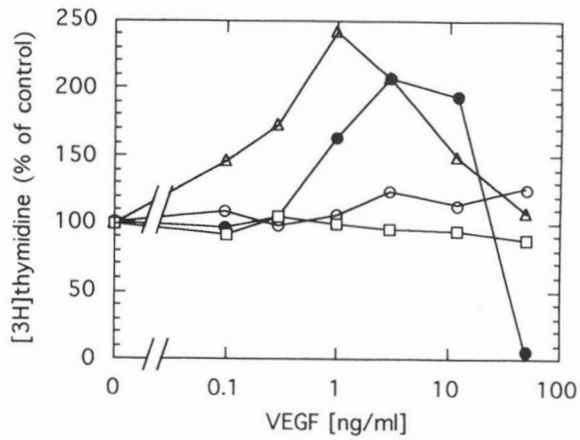

FIG. 4. **Incorporation of [^{3}H]thymidine.** PAE/KDR cells (●), PAE/Flt1 cells (□), untransfected PAE cells (○), and HUVE cells (Δ) were grown in 12-well tissue culture plates to subconfluence in the presence of 10% FCS, serum starved for 40 h, and consecutively stimulated with VEGF (up to 50 ng/ml) for 24 h. Incorporation of [^{3}H]thymidine as compared with the unstimulated control was estimated. Data represent the mean of triplicates, and similar results were obtained in at least two different experiments.

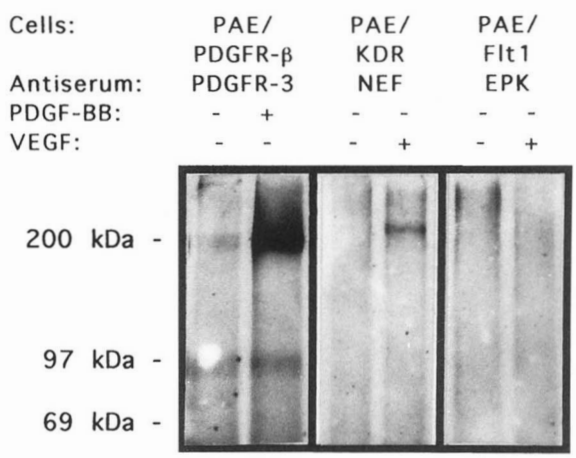

FIG. 5. **Phosphorylation of PDGFR-β, KDR, and Flt1 in intact cells.** Confluent monolayers of PAE/PDGFR-β were stimulated with PDGF-BB, and PAE/KDR and PAE/Flt1 cells were stimulated with VEGF for 60 min at 4 °C, followed by 8 min at 37 °C. Lysates were immunoprecipitated using the PDGFR-β-specific antiserum PDGFR-3, or KDR- and Flt1-receptor-specific antisera (NEF and EPK, respectively), separated by SDS-PAGE, and blotted onto a nitrocellulose membrane. Phosphotyrosine-containing proteins were visualized by incubation with an anti-phosphotyrosine antibody (PY20) and a chemoluminescence-based detection system.

immunoprecipitation from PAE/Flt1 cells using the EPK antiserum or PY20, followed by incubation of the immunoprecipitate with [γ-^{32}P]ATP, a phosphorylated 205-kDa component was seen (Fig. 6*b*). Using a similar assay and another Flt1-specific antiserum, VSE, a 205-kDa component was phosphorylated in HUVE cells after stimulation with VEGF (Fig. 6*c*). However, a much stronger phosphorylation was seen of the KDR protein in VEGF-stimulated HUVE cells precipitated with NEF (Fig. 7), as well as in PAE/KDR cells after immunoprecipitation with NEF (Figs. 7 and 6*a*) or with PY20 (Fig. 6*a*). In conclusion, both Flt1 and KDR are tyrosine phosphorylated in response to VEGF in transfected PAE cells and in HUVE cells, but KDR appears to undergo autophosphorylation much more efficiently.

A number of proteins were coprecipitated and phosphorylated together with the tyrosine kinase receptors Flt1 and KDR in the *in vitro* kinase assay (Fig. 6, *a-c*). The sets of associated phosphoproteins differed substantially between Flt1 and KDR and were also different from those associated with the PDGF

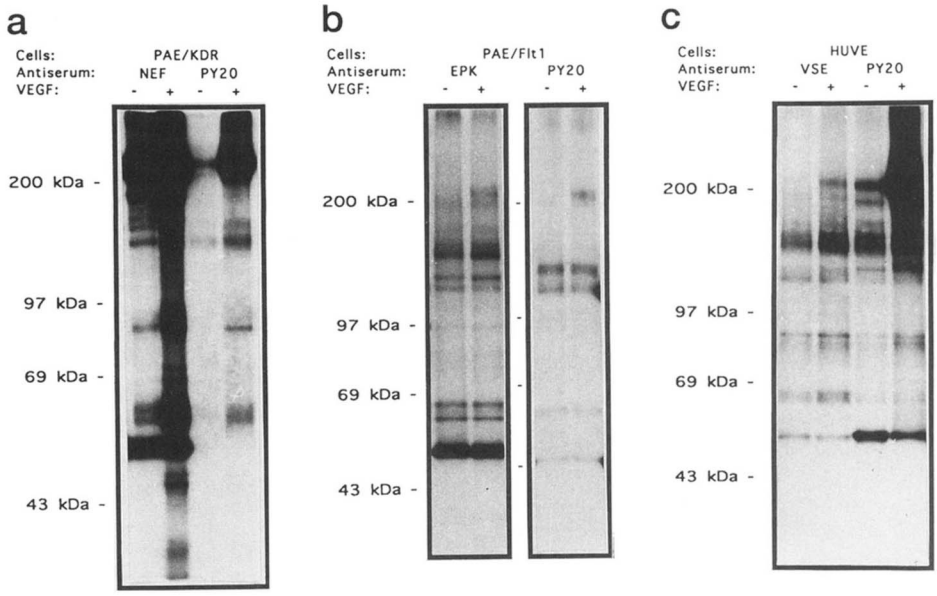

FIG. 6. ***In vitro* phosphorylation/substrate association of VEGF receptors.** *a*, KDR was immunoprecipitated from PAE/KDR cells stimulated or not with VEGF, using either the receptor-specific polyclonal antiserum NEF or the monoclonal anti-phosphotyrosine antibody PY20. The immunoprecipitates were subjected to an *in vitro* kinase assay and analyzed by SDS-PAGE, followed by KOH treatment of the gel, and autoradiography (Hyperfilm MP, Amersham) for detection of phosphoproteins. Similar analyses were done on EPK and PY20 immunoprecipitates of Flt1 from PAE/Flt1 cells (*b*), and on VSE immunoprecipitates of Flt1 from HUVE cells as well as on PY20 immunoprecipitates of HUVE cells (*c*).

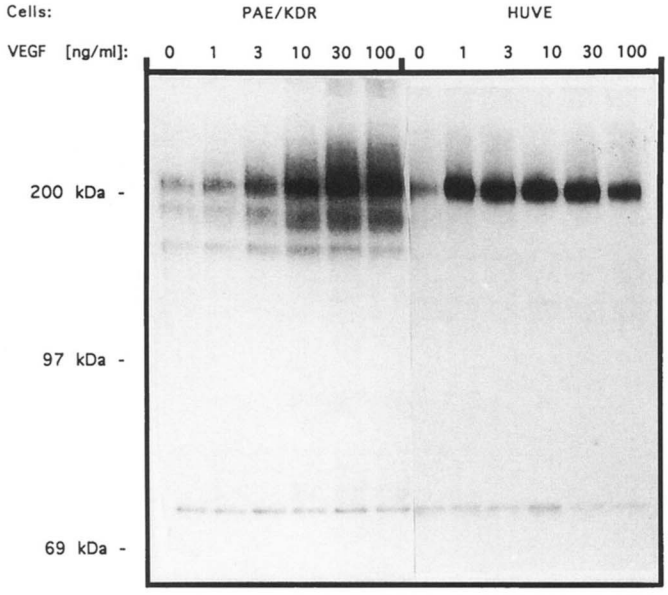

FIG. 7. ***In vitro* phosphorylation of KDR in PAE/KDR and in HUVE cells.** Cell monolayers were stimulated with different concentrations of VEGF and lysed. KDR-specific immunoprecipitation using the NEF antiserum was followed by an *in vitro* kinase assay; samples were analyzed by SDS-PAGE, followed by KOH treatment of the gel, and autoradiography on FUJI RX film.

β-receptor in the same cellular background (data not shown). The most prominent phosphoproteins that were coprecipitated with KDR migrated at sizes corresponding to 36, 46, 52, 60, 85, and 140 kDa (Fig. 6*a*), and a phosphoprotein that associated with Flt1 and increased after VEGF stimulation migrated at about 140 kDa (Fig. 6, *b* and *c*).

The dose-response of VEGF-induced phosphorylation of KDR was assessed in both PAE/KDR and HUVE cells using an *in vitro* kinase assay (Fig. 7). The maximal response was higher in PAE/KDR cells, which may at least in part be explained by the higher receptor number/cell and the higher cell density in the monolayer. The major difference, however, was the dose dependence in *in vitro* phosphorylation of KDR; whereas the maximum in PAE/KDR cells was reached at about 30 ng/ml VEGF, the maximum was shifted to 1 ng/ml or below in HUVE cells, which express in addition to KDR also Flt1.

Signal Transduction of Flt1 and KDR: Association of Signal Transduction Molecules—Some of the known signal transduction molecules that associate with tyrosine kinase receptors were tested with respect to association with and stimulation by Flt1 and KDR. Analyses of immunoprecipitates from PAE/Flt1 and PAE/KDR cells stimulated or not with VEGF did not reveal any PI3-K activity associated with neither of the two VEGF receptors (Fig. 8); the same results were obtained independent of whether receptor-specific antisera, or the antiserum 5α, directed against the regulatory subunit (p85) of PI3-K was used for immunoprecipitation prior to the *in vitro* kinase reaction (data not shown). In contrast, PI3-K activity was found associated with activated PDGF β-receptors, consistent with previous findings (42–44) (Fig. 8). Moreover, unlike in the case of the PDGF β-receptor no tyrosine phosphorylation of PLC-γ was found to be induced by Flt1 or KDR receptors (data not shown). In contrast, both Flt1 and KDR showed some ligand-dependent association of Src family members (Fig. 9), as visualized by immunoprecipitation with antibodies against Src family members, namely $p59^{fyn}$ (Fyn) and $pp62^{c\text{-}yes}$ (Yes), followed by *in vitro* kinase assay; the association was, however, much weaker than that to the PDGF β-receptor (Fig. 9*B*). Whereas VEGF stimulation of Flt1 expressing cells resulted in a 2.4-fold increase of Fyn phosphorylation and a 2.1-fold increase in the phosphorylation of Yes (Fig. 9*A*), the level of phosphorylation decreased for both Src family members after stimulation of PAE/KDR cells. Furthermore, there was a weak VEGF-inducible stimulation of tyrosine phosphorylation of GAP, but no significant association of GAP with KDR or Flt1 (Fig. 10).

DISCUSSION

We show in the present paper that the two VEGF receptors Flt1 and KDR have different affinities for VEGF. Both Flt1 and KDR can be phosphorylated on tyrosine residues, but show different patterns of potential intracellular substrates in the *in vitro* immune complex kinase assay, and they also mediate different cellular responses.

The transfected cell lines PAE/Flt1 and PAE/KDR express functional VEGF receptors, assessed both by ligand binding and ligand-dependent phosphorylation in the *in vitro* kinase assay. In HUVE cells, which express both Flt1 and KDR receptors (our data and Ref. 45), ligand-induced phosphorylation could be seen in a similar way. The extent of detectable phosphorylation, both at base line and upon VEGF stimulation, was

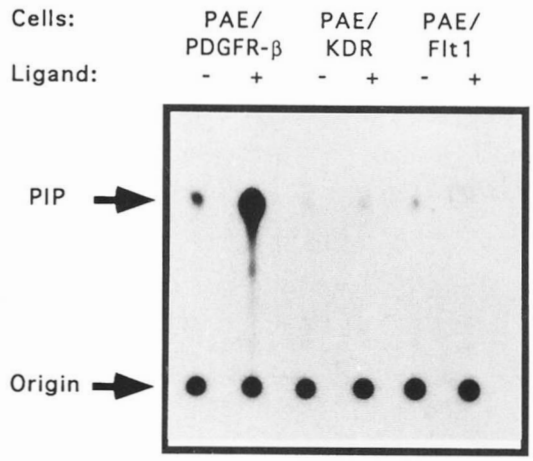

FIG. 8. **PI3-K reaction products separated by thin layer chromatography.** PAE cells expressing either PDGFR-β, KDR or Flt1 were incubated with the corresponding ligand (+) or carrier (–); cell lysates were immunoprecipitated using receptor-specific antisera. Immune complexes were subjected to PI3-K assay, and the PI3-K reaction products were separated by thin layer chromatography and detected by fluorography. The positions of the origin and the phosphatidylinositol phosphate (*PIP*) are indicated.

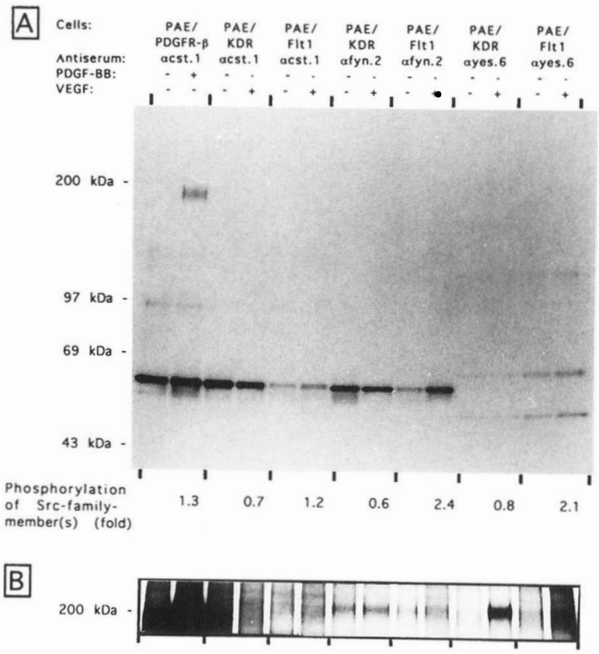

FIG. 9. **Assay of the phosphorylation of Src family members.** *A*, VEGF-dependent *in vitro* phosphorylation of Src family members in transfected porcine aortic endothelial cells expressing the PDGFR-β (*PAE/PDGFR-β*), KDR (*PAE/KDR*), or Flt1 (*PAE/Flt1*). Following immunoprecipitation using different antisera against Src family members, samples were subjected to *in vitro* kinase assay, separated by SDS-PAGE, and detected by autoradiography. Changes in the extent of phosphorylation were quantified on a phosphor-storage based detection system (PhosphorImager®, Molecular Dynamics) and are given below the figure of the gel. Moreover, in the case of the cst.1 antiserum, a clear ligand-induced association of Src family members and the PDGFR-β can be seen. *B*, a longer exposure of the same gel (2 weeks at –70 °C) demonstrates the association of KDR and Flt1 with Src family members and the changes induced by ligand stimulation.

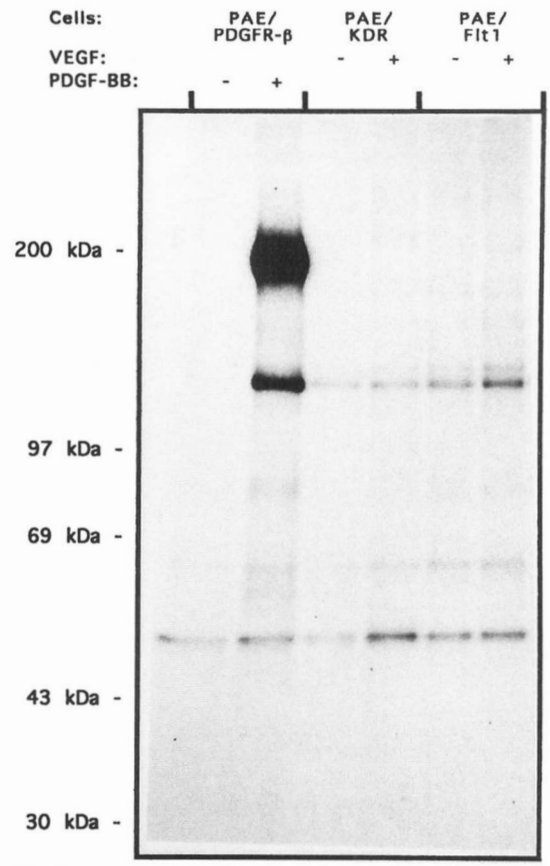

FIG. 10. **Assay of GAP phosphorylation.** Transfected PAE cells expressing the PDGFR-β, KDR, or Flt1 were stimulated with 50 ng/ml of their specific ligand. Cells were lysed and subjected to immunoprecipitation using a GAP-specific antiserum and *in vitro* kinase assay; samples were separated by SDS-PAGE and analyzed by autoradiography. Stimulation of GAP phosphorylation can be seen in all three cases, whereas ligand-dependent association with GAP only occurs in the PDGFR-β expressing cells.

severalfold higher in PAE/KDR cells as compared with PAE/Flt1 cells and was somewhat weaker than in PDGF-BB-stimulated PAE/PDGFR-β cells expressing a similar number of receptors (46). This may be due to a difference in the intrinsic properties of the kinases or to different susceptibilities to dephosphorylation by phosphotyrosine phosphatases. It may also in part reflect the lower number of Flt1 receptors/cell or may be attributed to a different sensitivity of the antisera used in this study. The NEF antiserum recognizing KDR has proven to be a sensitive as well as specific tool in immunoprecipitation, immunoblotting,[2] as well as in immunohistochemistry (47). On the other hand, the antisera EPK and VSE recognized Flt1 in PAE/Flt1 cells as well as in HUVE cells using the *in vitro* kinase assay. However, their sensitivity was lower as compared with NEF, and neither of the antisera were suitable for immunoprecipitation of detectable amounts of metabolically labeled Flt1 protein in these two cell lines. The reason for the difficulty in obtaining good polyclonal antisera against Flt1 is unclear.

Tyrosine phosphorylation of KDR can be shown by different methods including phosphorylation in the intact cell, whereas tyrosine phosphorylation of Flt1 could only be demonstrated in the *in vitro* immunocomplex kinase assay. This does not formally exclude the possibility that another kinase was coprecipitated and mediated phosphorylation of Flt1.

In our system KDR transduced signals for mitogenicity, chemotaxis, actin reorganization, and changes in gross morphology of the cell, whereas Flt1 did not transduce any of these effects despite the fact that it bound VEGF with higher affinity than KDR. Negative results may have trivial explanations, *e.g.* a mutation in the cDNA used for transfection. It is, however, notable that also another group has been unable to demonstrate mitogenic signaling using an independently isolated *Flt1* cDNA transfected into NIH3T3 cells.[3] The reason for the dif-

[2] J. Waltenberger, unpublished data.
[3] T. P. Quinn and L. T. Williams, personal communication.

ferences in the effects mediated by KDR and Flt1 is not known. It is possible that PAE cells, a stable cell line that had been in culture for years (23), lack the particular signal transduction molecules necessary for the transduction of Flt1 signals, although PAE cells are of endothelial origin and display several endothelial characteristics such as the expression of factor VIII,[4] the expression of endoglin, and a cobblestone morphology (23). It is also possible that Flt1 is involved in other not yet analyzed pathways. Similar to our results obtained for Flt1, the product of the *met* proto-oncogene was found not to mediate mitogenesis when expressed in NIH 3T3 fibroblasts and stimulated with its specific ligand (48). Nonetheless, activation of Met induced blood vessel formation *in vivo* (49). By analogy, it is possible that Flt1 stimulates other endothelial functions than mitogenicity. An alternative possibility to explain the lack of effects of Flt1 *per se* would be to postulate that Flt1 is functionally active only when present in a heterodimer with KDR or another molecule, similarly as for HER2, which can only be activated by heregulin in the presence of HER4 (50). This could also explain the different dose-response behavior of KDR phosphorylation in PAE/KDR and HUVE cells (Fig. 8). Heterodimerization (cooperativity) of the two VEGF receptors may shift the maximum of the dose-response curve to lower values in the case of HUVE cells, where both Flt1 and KDR are expressed: mitogenicity and *in vitro* phosphorylation occurred at a 10-fold lower concentration of VEGF in HUVE cells as compared with PAE/KDR cells. The concept of heterodimerization of receptor tyrosine kinases is well established and has been demonstrated for the structurally similar PDGFR-α and PDGFR-β which form hetero- or homodimers, depending on the stimulating PDGF isoform (51, 52). It is an interesting perspective that the four different VEGF isoforms known (53) would differ in their abilities to induce heterodimers *versus* homodimers. The recent discovery of Flt1 processing into truncated receptors and soluble Flt1 proteins (54) represents an alternative and intriguing possibility to explain the lack of cellular responses (*e.g.* mitogenesis), when PAE/Flt1 cells (Fig. 4) or NIH3T3/Flt1 cells[3] are stimulated with VEGF. This finding, however, does not rule out a potential role of Flt1 for intracellular signaling. In our system there was no indication for processing of Flt1.

The receptors for VEGF do not appear to associate with PI3-K, a signal transduction molecule which binds to many other receptor tyrosine kinases such as the receptors for PDGF (55, 56) or the stem cell factor (*c-Kit*) (57). PI3-K binds to the consensus sequence phosphorylated Tyr-Xaa-Xaa-Met (58). KDR and Flt1 contain only one such motif each; in both receptors they are located inside the kinase domains and are therefore unlikely to serve as autophosphorylation sites mediating binding to PI3-K or other signal transduction molecules. The PDGFR-β has been shown to mediate the signal for chemotaxis via stimulation of PI3-K (44, 59), which could be abolished by mutations of the PI3-K-binding site in the kinase insert of the receptor. KDR-induced chemotaxis is apparently mediated without a direct interaction between PI3-K and the receptor, since the receptor-bound PI3-K activity is not increased after ligand binding; however, the possibility that PI3-K activity is activated downstream in the signal transduction pathways originating from activated KDR, *e.g.* via activation of Src family kinases (60), has not been excluded. The effects of KDR and Flt1 on binding and activation of members of the Src family of tyrosine kinases are interesting. Src-like molecules have been implicated in mitogenic signaling via PDGF receptors (61), but their exact roles in signal transduction remain to be elucidated.

[4] E. Landgren, personal communication.

The expression of Flt1 has been shown to be highly specific for the endothelium (21). Similarly, KDR (Flk-1) expression is largely restricted to capillaries and the endothelial layers of blood vessels (17–19), but is also present in fetal liver cells (16), the source of its original isolation. However, there is recent evidence for the expression of KDR in pancreatic duct cells (47), demonstrating that KDR is not exclusively expressed in the endothelium.

The significance of the regulation of endothelial function by receptor tyrosine kinases is unclear. By now, several more or less endothelial-specific receptor tyrosine kinases have been described, namely the two VEGF receptors Flt1 and KDR and the receptors Tek (62, 63), Tie-1 (64, 65), and Tie-2 (65) for which the ligands remain to be identified. Both KDR (17–19) and Flt1 (21) are developmentally regulated, and an important role in endothelial cell differentiation is assumed. The present study demonstrates cellular responses and early events in the signal transduction via VEGF receptors. KDR was found to mediate essential functions such as chemotaxis, mitogenesis, and cytoskeletal reorganizations, whereas the functional significance of the Flt1 receptor remains to be determined. This provides a basis for further characterization of the molecular events involved in VEGF stimulation of normal endothelial cells, notably with respect to the heterogeneity of the endothelium (66) as well as with respect to endothelial dysfunction and related cardiovascular diseases, vascular remodeling (67), and therapeutic angiogenesis (9).

Acknowledgments—We wish to thank Seijiro Mori for help with calculations for the binding analysis, Stefan Wennström for valuable discussions concerning the PI3-K assay, Mitsuyashu Kato for help with establishing the HUVE cell culture, Ulla Engström for peptide synthesis, Mathilda Johnell for help with chemotaxis assays, as well as Lata Seetharam and Yoshiro Maru for preparation of the Flt-1 antibody P1–3. KDR cDNA was kindly provided by Bruce I. Terman, Pearl River, NY, and recombinant VEGF was generously provided by Denis Gospodarowicz, Emeryville, CA. The antisera recognizing Src family kinases were a generous gift from Sara Courtneidge, Heidelberg, Germany, and the antisera against GAP and p85 were kindly provided by Julian Downward and Mike Waterfield, respectively, both from London, Great Britain.

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