THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 9, Issue of February 26, pp. 5612–5619, 1999
Printed in U.S.A.

Vascular Endothelial Growth Factor (VEGF) Receptor II-derived Peptides Inhibit VEGF*

(Received for publication, September 2, 1998, and in revised form, November 25, 1998)

Christine Piossek‡, Jens Schneider-Mergener§, Michael Schirner¶, Evangelia Vakalopoulou¶, Lothar Germeroth‡‖, and Karl-Heinz Thierauch¶**

From ‡JERINI BIO TOOLS GMBH, Rudower Chaussee 5, 12489 Berlin, Germany, the §Institut für Medizinische Immunologie, Universitätsklinikum Charite, Humboldt-Universität zu Berlin, Schumannstraße 20-21, 10098 Berlin, Germany, and ¶SCHERING AG Research Laboratories, Müllerstraße 170–178, 13342 Berlin, Germany

Vascular endothelial growth factor (VEGF) directly stimulates endothelial cell proliferation and migration via tyrosine kinase receptors of the split kinase domain family. It mediates vascular growth and angiogenesis in the embryo but also in the adult in a variety of physiological and pathological conditions. The potential binding site of VEGF with its receptor was identified using cellulose-bound overlapping peptides of the extracytosolic part of the human vascular endothelial growth factor receptor II (VEGFR II). Thus, a peptide originating from the third globular domain of the VEGFR II comprising residues ^{247}RTELNVGIDFNWEYP^{261} was revealed as contiguous sequence stretch, which bound ^{125}I-VEGF$_{165}$. A systematic replacement with L-amino acids within the peptide representing the putative VEGF-binding site on VEGFR II indicates Asp^{255} as the hydrophilic key residue for binding. The dimerized peptide (RTELNVGIDFNWEYPAS)$_2$K inhibits VEGF$_{165}$ binding with an IC$_{50}$ of 0.5 μM on extracellular VEGFR II fragments and 30 μM on human umbilical vein cells. VEGF$_{165}$-stimulated autophosphorylation of VEGFR II as well as proliferation and migration of microvascular endothelial cells was inhibited by the monomeric peptide RTELNVGIDFNWEYPASK at a half-maximal concentration of 3–10, 0.1, and 0.1 μM, respectively. We conclude that transduction of the VEGF$_{165}$ signal can be interrupted with a peptide derived from the third Ig-like domain of VEGFR II by blockade of VEGF$_{165}$ binding to its receptor.

Angiogenesis, the formation of new blood vessels sprouting from existing ones, plays an essential role in fetal and adult life. It is important for proliferative processes in the reproductive tract, tissue regeneration, and wound healing as well as for pathological conditions such as solid tumor growth, rheumatoid arthritis, and retinopathies (1, 2).

Several putative angiogenic factors have been identified. Many of these factors possess only very little or no direct mitogenicity on vascular endothelial cells (3). Vascular endothelial growth factor (VEGF)[1] in contrast is a potent endothelial cell-specific mitogen *in vitro* (4–6) enhancing vascular permeability and stimulating angiogenesis *in vivo* (7, 8). Its importance has been demonstrated by blockade of tumor growth by neutralizing anti-VEGF monoclonal antibodies (9) and by experiments where tumor growth was blocked by evoking the expression of kinase truncated VEGFR II on cells in the vicinity of growing tumors (10).

VEGF expression is induced by hypoxia in a variety of differentiated cells (11, 12) whereas the two corresponding VEGF receptors denoted fms-like tyrosine kinase (FLT-1, VEGFR I) and kinase insert domain-containing receptor (KDR, VEGFR II), appear to be expressed exclusively by endothelial and hematopoietic cells (13–16). VEGF is encoded by a single gene yielding four isoforms containing 121, 165, 189, and 206 amino acids due to alternative splicing (17, 18). Isoforms VEGF$_{189}$ and VEGF$_{206}$ remain bound to the extracellular matrix. Isoforms VEGF$_{121}$ and VEGF$_{165}$ are secreted (19). The most abundantly expressed VEGF isoform is the homodimeric VEGF$_{165}$ with an apparent molecular weight of 43,000. VEGF is a member of the cysteine knot family of growth factors and thus structurally related to the platelet-derived growth factor (PDGF) and transforming growth factor β (TGFβ) (20, 21). It contains a heparin-binding site and glycosylation sites, which appear not to be involved in binding (22, 23). Recently three VEGF homologues, placental-like growth factor (PlGF), VEGF-B, and VEGF-C, have been identified (24–26). Placental-like growth factor originally found in placenta is binding to VEGFR I but not to VEGFR II (26). VEGF-B heterodimerizes with VEGF$_{165}$ *in vitro* and is particularly expressed in muscle. VEGF-C is binding to the FLT-4 receptor (VEGFR III), which is mainly expressed in the lymphatic system, and after complete processing it also binds to VEGFR II (25). Formation of heterodimers among the various VEGF homologues in addition may govern the diverse physiological functions of VEGF (27–29).

The two VEGF receptors I and II with apparent molecular weights of about 220,000 consist of seven immunoglobulin-like extracellular domains, one transmembrane stretch, and intracellular split tyrosine kinase domains. These structural elements relate the VEGF receptors closely to the platelet-derived growth factor α/β receptors having five extracellular immunoglobin-like domains and pertaining to the class III tyrosine kinase receptors (30–32).

The VEGFR I and II bind most likely to different VEGF epitopes dominated by basic and acidic residues, respectively, which appear to be essential for ligand-receptor interaction (33). This is further indicated by the finding that placental-like

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‖ To whom correspondence may be addressed. Tel.: 49-30-6392-6392; Fax: 49-30-6392-6395; E-mail: biotools@jerini.de.

** To whom correspondence may be addressed. Tel.: 49-30-4681-5798; Fax: 49-30-4681-8069; E-mail: karlheinz.thierauch@schering.de.

[1] The abbreviations used are: VEGF, vascular endothelial growth factor/vascular permeability factor; VEGFR II, VEGF receptor II or KDR (kinase domain insert receptor); VEGFR I, VEGF receptor I or Flt-1 (fms like tyrosine kinase); FLT-4, VEGF receptor III or VEGFR III; HUVEC, human umbilical vein cell; MVEC, microvascular endothelial cell; sVEGFR II, soluble VEGF receptor II; c-kit, stem cell growth factor receptor; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

growth factor competes for VEGF binding at VEGFR I but not at VEGFR II (34). When deletion mutants of VEGFR I were constructed lacking extracellular Ig-like domains only the deletion of domain 2 abolished VEGF binding completely. Binding was restored by substituting with domain 2 of VEGFR II. However, the specificity had changed and the placental-like growth factor did not displace VEGF any longer (35). Truncation studies of both VEGF receptors, VEGFR I (34) and VEGFR II (36), have mapped their VEGF-binding site to the first three N-terminal globular domains. Concomittant with the recognition of different binding sites, the VEGFR I and II appear to exhibit different functions in angiogenesis. While VEGFR II is mediating the stimulation of endothelial cell proliferation during angiogenesis and vasculogenesis (13), the VEGFR I seems to be involved in the regulation of the assembly of the vascular endothelium, which was demonstrated by studies with transgenic knockout mice (37). VEGFR I-deficient embryos die at day 10.5 of gestation. Although angioblasts are formed the assembly into functional blood vessels is impaired. Similarly died homocygotic VEGFR II deficient embryos at day 8.5 with no vessels forming and defects in angioplastic and hematopoietic lineages (38).

The molecular basis of the VEGF-VEGFR II interaction is poorly understood. But recently the high resolution crystal structures of VEGF (21, 39), VEGF complexed with domain 2 of the VEGFR I and VEGF mutation data became available (21, 33). This VEGF mutation studies demonstrated that high affinity binding of VEGF to VEGFR II is dependent on the presence of three locally vicinal isoleucins (Ile^{43}, Ile^{46}, and Ile^{83}) and positively charged amino acids Arg^{82}, Lys^{84}, and His^{86} in VEGF. In addition, Asn^{63}, Glu^{64}, and Glu^{67} are important especially for VEGFR II binding.

The aim of our investigations was to contribute to the topology of VEGF-VEGFR II interaction and to use that knowledge to design novel VEGF inhibitors. In the present study we report the mapping and characterization of a putative $VEGF_{165}$/VEGFR II contact site using cellulose-bound peptide libraries derived from the VEGFR II sequence. The resulting peptide was characterized with respect to $VEGF_{165}$ binding, inhibition of VEGFR II autophosphorylation, as well as growth and migration of microvascular endothelial cells.

EXPERIMENTAL PROCEDURES

Synthesis of Peptides and Cellulose-bound Peptide Libraries—Cellulose-bound and cleavable sets of peptides (PepSpots™ and Cleavable PepSpots™, Jerini Bio Tools GmbH, Berlin, Germany) were automatically prepared according to standard spot synthesis protocols (40) using a spot synthesizer (Abimed GmbH, Langenfeld, Germany) as described previously (41). Cleavable peptides were released as amides from the cellulose support using ammonia vapor (42). Subsequently, filter discs with adsorbed peptides were punched out into microtiter plates. After desorption from the cellulose matrix with buffer the peptide solution was directly used in the test system. For generation of the sequence files, the in house developed software DIGEN was applied. Soluble peptides in milligram quantities were synthesized as amides on a multiple peptide synthesizer AMS 422 (Abimed) according to the standard Fmoc machine protocol (43) using TentaGel S RAM resin (50 mmol/g; Rapp Polymere, Tübingen, Germany) and PyBOP activation. All peptides were analyzed by high performance liquid chromatography on a Vydac C18 column using a linear gradient 5–60% acetonitrile/water (0.05% trifluoroacetic acid) for 20 min at 1.2 ml/min flow rate (detection at 214 nm) and by laser desorption time-of-flight mass spectrometry using α-cyano-4-hydroxycinnamic acid as matrix (LaserTec fOCUS mass spectrometer; VESTEC/Perseptive Biosystems, Wiesbaden-Nordenstadt, Germany).

Solid Phase Binding Assay—Peptide libraries bound covalently to cellulose membranes were incubated in Tris-buffered saline (50 mM Tris, pH 7.5, 150 mM NaCl) containing 0.05% Tween 20 (TBS/Tween 20). After two changes of buffer the paper was blocked by incubation for 60 min in TBS/Tween 20 containing 5% milk powder at 4° C. To this solution ^{125}I-$VEGF_{165}$ was added to a concentration of 0.1 μC/ml. The incubation was continued for 60 min before the cellulose libraries were washed three times with TBS/Tween 20. Overnight the cellulose sheets were placed together with an autoradiography film (Reflection NEF 496, DuPont, Bad Homburg, Germany) and an intensifying screen NEF 491 (DuPont) before the film was developed. Binding peptides were detected as black dots.

Human Umbilical Vein Endothelial Cell Culture—Human umbilical cords were canulated with a conical device, which was fixed by a thread, and flushed with sterile phosphate-buffered saline (PBS) containing calcium, magnesium (Biochrom KG, Berlin, Germany), penicillin (500 units/ml), and streptomycin (1000 μg/ml) to remove blood cells. The veins were then filled with PBS containing chymotrypsin (0.1% w/v) and incubated at room temperature for 25 min. After slight manual massage of the umbilical cords the cell suspension was brought into a tube containing 2 ml of fetal calf serum. After dilution with buffer, cells were collected by centrifugation, washed once in buffer, and seeded into two 25-cm^2 flasks (coated with 10 μg/ml Collagen) per cord in medium 199 (M199) containing 10% fetal calf serum, 10% human serum, glutamin, or glutamax (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), ascorbic acid (1.27 mM), pyruvic acid (1 mM), and 1% nonessential amino acids (Biochrom), 6 μg/ml endothelial growth factor from bovine brain (Sigma), and 7.5 μg/ml heparin (Sigma). After 2–3 h of plating we washed the adherent cells twice with PBS and cells were cultured under standard conditions in medium described above. Cells were passaged by trypsin digestion (0.02% trypsin, 0.01% EDTA in phosphate-buffered saline without bivalent ions) into microtiter plates at a density of 1.6×10^4 cells/well and cultured from 3 to 7 days.

Preparation of Microvascular Endothelial Cells from Human Foreskin—Preparation of magnetic beads according to Jackson *et al.* (Ref. 44, 10 million Dynabeads (anti-mouse Ig coated by means of an DNA linker)) were washed with PBS/bovine serum albumin (BSA) three times using the magnetic device. Anti-CD31 antibody (Immunotech, Westbrook, ME) was added at a concentration of 3.5 μg/10 million beads and shaken for 30 min. The suspension was washed five times with PBS/BSA and stored in 1.25 ml of 0.01% azide containing PBS/BSA at 4° C. Beads were washed directly before use to remove azide.

Human Foreskin was washed with PBS containing penicillin (1000 units/ml) and streptomycin (1000 μg/ml) to remove blood. The skin was cut in 1 ml of PBS into 2 × 2-mm pieces and left for 60 min in 0.3% trypsin/EDTA in PBS in the incubator at 37° C. Trypsin was removed and the skin fragments were incubated for 4 h with dispase grade II (2.4 units/ml; Boehringer Mannheim GmbH, Mannheim, Germany) at 37° C. The dermis of the skin was scraped with a spatula and the resulting cell suspension was diluted in PBS and centrifuged at 200 × *g* for 5 min. The pellet was resuspended in complete medium and filtered over a 100-μm net. The cell suspension was distributed in one or two culture wells of 10-cm^2 area. After 16 h in the incubator adherent cells were washed several times with PBS and culture was continued until 50% confluence is observed. Cell mixtures originating from 10 cm^2 surface were incubated at 4° C with 300,000 washed beads of the preparation for 15 min and then washed cautiously five times with cold PBS/BSA. To the pellet 50 μl of M199 containing 1% fetal calf serum and 200 units of DNase was added and incubation was continued for 15 min at 37° C. Then the cells were plated into a collagen-coated tissue culture flask in the same full medium as HUVEC.

Receptor Binding Assay—$VEGF_{165}$ binding to soluble extracellular fragment of VEGFR II (sVEGFR II) using microtiter plates (Maxisorb, Nunc, Roskilde, Denmark) shaken for 30 min with 50 μl of sVEGFR II solution in PBS (Biochrom), in concentrations of about 0.4 μg depending on the preparation. Then 200 μl of 4% BSA in PBS was added for another 30 min. The solution was removed and the plates were washed with 0.1% BSA in PBS. 10 μl of compounds in PBS, 0.1% BSA were added and 40 μl of ^{125}I-$VEGF_{165}$ (approximately 15,000 cpm or 300–600 pM) was added in PBS, 0.1% BSA. The incubation mixture was shaken for 60 min and radioactivity was removed. After three washes with 0.1% BSA in PBS, 100 μl of 0.5% sodium dodecyl sulfate (SDS) was added and the plate was shaken for 30 min. 80 μl of the solution was utilized for γ-counting.

$VEGF_{165}$ Binding to HUVEC—300,000 HUVECs were seeded per well (24-well plates) and cells were cultured for 2 days in complete medium. Cells were washed with PBS containing calcium and magnesium chloride (Biochrom). At 4° C 100 μl of medium M199 without additives but containing 0.1% BSA was added followed by 10 μl of peptide solution, that had been dissolved in dimethyl sulfoxide and diluted appropriately in advance, and 40 μl of ^{125}I-$VEGF_{165}$. After 2 h of incubation at 4° C the medium was aspirated and washed three times with 500 μl of PBS. The cells were lysed with 100 μl of 0.5% SDS solution by vigorous shaking. The solution was transferred to a count-

ing vial and γ-radiation was measured. The result is given as the concentration that reduces specific binding to 50%.

VEGFR II Autophosphorylation Assay—100,000 MVECs were plated into a 12-well plate. After 48 h the cells were put on ice and fresh medium containing the solvent control, $VEGF_{165}$ or $VEGF_{165}$ plus peptides in ice-cold medium M199 with 0.1% BSA was added. Incubation was continued for 1 h. The medium was removed and ice-cold RIPA buffer was added (50 mM HEPES, pH 7.2, 10 mM EDTA, 0.1% SDS, 1% Nonidet P-40, 0.5% deoxycholate, 50 mM sodium pyrophosphate, 100 mM sodium fluoride, 2 mM orthovanadate, 1 mM zinc acetate, 1.25 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin). The DNA was removed by filtering through a Millipore filter (0.65 μm). 50 μl of wheat germ agglutinin-Sepharose was added to the filtrate and the mixture was incubated under rolling for 1 h at 4° C. Sepharose was separated by centrifugation, the supernatant was discarded and 25 μl of twice concentrated SDS-electrophoresis buffer according to Laemmli was added and boiled for 5 min. Proteins were separated on a 6% SDS gel according to size. The proteins were transferred to a polyvinylidene difluoride membrane by semidry electroblotting. The membrane was blocked by 0.25% Tween 20 in PBS containing magnesium and calcium chloride. After three washes for 15 min with 0.05% Tween 20 in the same buffer, horseradish peroxidase-conjugated anti-phosphotyrosine monoclonal antibody (PY-20, Transduction Laboratories, Inc., Lexington, Ky) was added at a final concentration of 250 ng/ml. After three washes for 15 min each, the blot was developed with an enhanced chemiluminescence system (Amersham, Braunschweig, Germany). Alternatively the blot was developed for control purposes with a poly- or monoclonal antibody against VEGFR II (Dr. Towbin, Basel; Dr. Martiny-Baron, Freiburg, Germany).

c-kit Autophosphorylation Assay—TF-1 cells (CRL-2003, ATCC, Manassas, VA) that had been cultured in the presence of 2 ng/ml granulocyte macrophage-colony stimulating factor were centrifuged. Three million cells in 50 μl of PBS containing 0.1% BSA were transferred into wells of a C96 white microtiter plates (Maxisorb, Nunc) that had been coated previously with 1 μg/ml anti-c-kit = CD 117 antibody (Research Diagnostics Inc., Flanders, NJ) overnight at pH 9.6 and then blocked with BSA. 10 μl of inhibitor solution was added 5 min before stem cell factor (40 μl, 1 nM) was added. Incubation time was 60 min at 4° C. Cells were lysed in the original plate with 50 μl of lysis buffer (150 mM HEPES pH 7.1, 450 mM sodium chloride, 3 mM magnesium chloride, 30 mM sodium diphosphate ($Na_4P_2O_7$), 300 mM sodium flouride, 3 mM sodium vanadate (Na_3VO_4), 30% glycerol, 4.5% Triton X-100, 3 tablets/50 ml of protease inhibitor mixture (B#1836145, Boehringer Mannheim GmbH). After washing several times (50 mM Tris pH 7.4, 150 mM NaCl, Tween 0.1%), the wells were incubated with 1:20,000-fold diluted anti-phosphotyrosine antibody horseradish peroxidase-conjugated (4G10, Upstate Ltd., Lake Placid, NY) and after repeated washings developed using chemiluminescence substrate (Boehringer Mannheim GmbH), according to the manufacturer.

Endothelial Cell Migration—Endothelial cells were passaged and maintained overnight in medium 199, 10% fetal calf serum, 10% human serum containing glutamin, penicillin, and streptomycin but no growth factors. The next day cells were detached from the culture plates with trypsin. Ten thousand cells in 100 μl of M199 containing 2% human serum and glutamin were given into a culture well insert with a porous filter bottom that had been washed with PBS and coated with collagen (10 μg/ml) previously. In the lower chamber are 600 μl of medium (M199 + 2% human serum). After 2 h at standard culture conditions $VEGF_{165}$ (250 pM final concentration) and 10 μl of the inhibitors were added. The medium was mixed by cautious swirling and cells were incubated for an additional 18 h. The inserts were rinsed with PBS and stained by immersion into a PBS solution containing 1% rose bengal in 30% of ethanol. Excess rose bengal was removed by washing with PBS. Cells from the upper side of the insert were removed by cotton swaps. For quantification randomized inserts were evaluated under the microscope counting three field each of three independent experiments. Quantitative image scan was performed with Densitometer SI (Molecular Dynamics, Sunnyvale, CA).

Proliferation of Microvascular Endothelial Cells—50,000 cells were plated into a 24-well plate in full medium based on M199. After 2 h the medium was changed to 3% human serum. The next morning various amounts of $VEGF_{165}$ and inhibitors were added. After 3 days in culture the cells were washed and 1% rose bengal in 30% ethanol was added. After 5 min the dye solution was removed, and the cells were washed three times thoroughly with PBS and lysed with 0.1% sodium dodecyl sulfate in PBS. Optical density of the resulting solutions was determined at 560 nm.

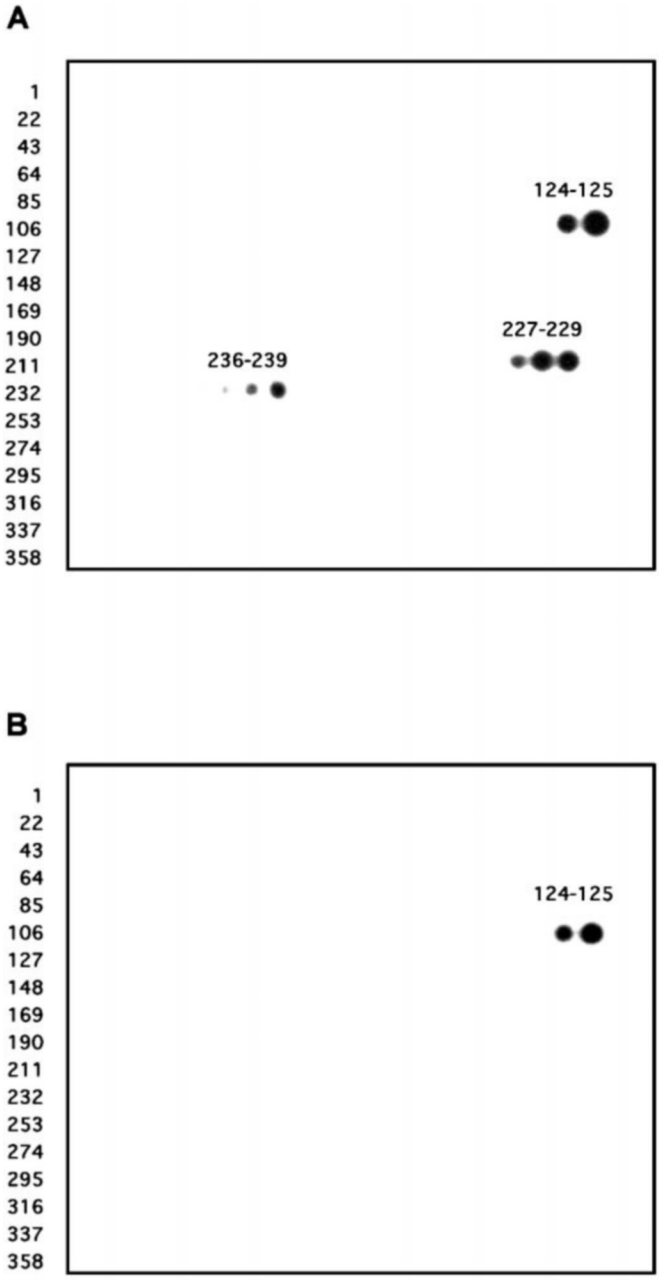

FIG. 1. **Autoradiographs of the cellulose-bound VEGFR II-derived peptides probed with ^{125}I-$VEGF_{165}$.** The primary sequence of VEGFR II is fragmented in 376 13-mer peptides overlapping 11 amino acids. *A*, original sequence of VEGFR II. The signals represent the following sequences: spot 124, RTELNVGIDFNWE; spot 125, ELNVGIDFNWEYP; spot 227, HIHWYWQLEEECA; spot 228, HWYWQLEEECANE; spot 229, YWQLEEECANEPS; spot 236, AVSVTNPYPCEEW; spot 237, SVTNPYPCEEWRS; spot 238, TNPYPCEEWRSVE; spot 239, PYPCEEWRSVEDF. *B*, the cysteine residues of the original sequence are replaced by serine residues. The signals represent the following sequences: spot 124, RTELNVGIDFNWE; spot 125, ELNVGIDFNWEYP.

RESULTS

Mapping of the Potential VEGF/VEGFR II Contact Site—Two scans of overlapping peptides (13-mers, shifted by 2 amino acids) derived from the entire soluble VEGFR II (sVEGFR II) sequence were independently synthesized (Fig. 1). In the second scan (Fig. 1*B*) all cysteine residues in the peptides were substituted by serine to prevent unspecific disulfide bridging of the target molecule (^{125}I-$VEGF_{165}$). Incubation of both scans with radiolabeled ^{125}I-$VEGF_{165}$ was performed utilizing only short washing times in order to permit kinetically rapid bind-

ing processes. The peptide scan with cysteine containing peptides probed with soluble ^{125}I-VEGF$_{165}$ showed eight strong spots (Fig. 1*A*), whereas the peptide scan with the serine substitutions (Fig. 1*B*) displayed only two spots (124 and 125) corresponding to the peptide sequences ^{247}RTELNVGIDFNWE^{259} and ^{249}ELNVGIDFNWEYP^{261} of VEGFR II. The activity bound to spots 124 and 125 could be easily stripped with routinely used buffers (Tween 20, Tris-buffered saline, data not shown). In contrast, the activity displayed on spots 227–229 and 236–239 (Fig. 1*A*), where all corresponding peptides contain a cystein residue, could only be removed under reducing conditions indicating nonspecific disulfide bridging of the ligand with the matrix-bound peptides. The specific signals of the spots 124 and 125, which thus were reproduced in two independent experiments (Fig. 1), represent an amino acid sequence located in the third globular domain of the extracellular part of the VEGFR II.

A complete L-substitution analysis of the peptide ^{249}ELNVGIDFNWEYP^{261} (Fig. 1, *A* and *B*), spot 125, which exhibits the stronger signal in the peptide scan in comparison to spot 124) in which all residues were replaced by all other 19 L-amino acids (Fig. 2) was performed to determine the residues involved in binding of VEGF$_{165}$. The results indicate that Asp^{255} is the essential residue for peptide binding since no other amino acid substitution is allowed at this position. Glu^{249} can only be replaced by Asp and Glu with comparable signal intensity favoring a negatively charged amino acid residue at position 249. Important hydrophobic residues are located in positions Leu^{250}, Val^{252}, Ile^{254}, Phe^{256}, and Trp^{258}. These five hydrophobic residues can only be exchanged by physicochemically similar amino acids comprising aliphatic and aromatic residues. Interestingly, amino acids Val^{252} and Ile^{254} can be replaced by Phe but not by Tyr suggesting that the hydroxyl groups of Tyr are unfavorable for binding. Ser and Thr can be substituted for Asn^{251} with equal signal intensity favoring H-bonding of that amino acid residue, which appears also to be

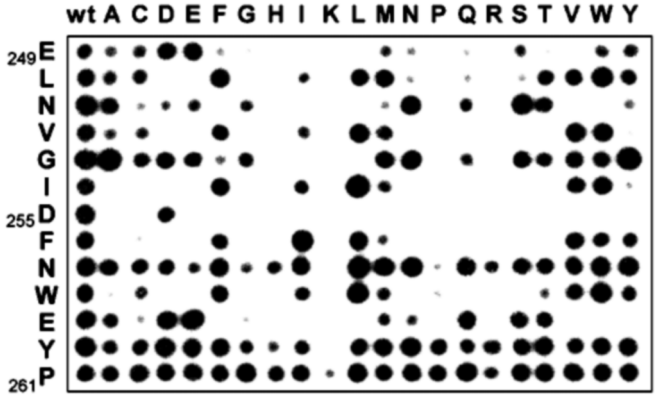

FIG. 2. **Autoradiograph of the cellulose-bound L-substitutional analysis of VEGFR II$_{249–261}$-peptide probed with ^{125}I-VEGF$_{165}$.** Every amino acid in the wild type peptide (*wt, left column*) is exchanged against the 20 L-amino acids (rows) resulting in a complete set of the possible point substitutions.

the type of interaction at position Glu^{259} since charged amino acids can be replaced with comparable intensity by potential H-bond donors (Gln, Ser, and Thr). Positively charged amino acids (Lys, Arg, and His) are strongly disfavored for substitution since only C-terminal positioned amino acids (Tyr^{260}, Pro^{261}, and Asn^{257}) can be replaced by Arg and His without eliminating VEGF$_{165}$ binding. In addition, substitution of Pro is only allowed at the C-terminal position suggesting that the secondary structure inducing Pro interferes with the binding conformation of the peptide. A similar situation is found for Gly, which also can only be exchanged with comparable signal intensities for the C-terminal amino acids and additionally for Gly^{253}.

Inhibition of Peptide Binding to VEGFR II—The peptides obtained from this analysis (Table I) were synthesized according to standard methods (44). The resulting purity of the peptides was >95%, which was analyzed by high performance liquid chromatography and mass spectrometry. To overcome solubility problems the peptides were prolongated with hydrophilic amino acids at the C termini according to the primary sequence of VEGFR II. The elongated peptides exhibit in the solid-phase VEGF binding assay similar signal intensities (data not shown) as compared with the peptides obtained in the initial peptide scan (Fig. 1). The peptide representing the potential VEGFR II-binding site was synthesized as monomer and dimer. The dimeric peptides were synthesized as branched peptides with one common C-terminal lysine.

To find out whether the peptides bind to VEGF$_{165}$ at a site relevant for the interaction of VEGF$_{165}$ with its receptor the peptides were studied as competitors in a binding test utilizing either the extracellular part of a VEGFR II bound to microtiter plates or endothelial cells as receptor source and ^{125}I-VEGF$_{165}$ as ligand. In Fig. 3 the competition of peptide RTELNVGIDFNWEYPASK with ^{125}I-VEGF$_{165}$ is compared with that of VEGF$_{165}$. Unlabeled VEGF$_{165}$ reduces binding of ^{125}I-VEGF$_{165}$ with an IC$_{50}$ of about 1 nM from its receptor. Peptides RTELNVGIDFNWEYPASK (Je-7) and (RTELNVGIDFNWEYPAS)$_2$K (Je-11) inhibit binding of ^{125}I-VEGF$_{165}$ at 10 and 0.5 μM, respectively.

Inhibition of VEGF$_{165}$ Signal Transduction—For functional testing MVECs were incubated with VEGF$_{165}$, or VEGF$_{165}$ in the presence of peptides. Receptor autophosphorylation of lysed cells was quantified in a Western blot using wheat germ agglutinin-Sepharose for precipitation and an anti-phosphotyrosine monoclonal antibody coupled to horseradish peroxidase for the detection. With increasing concentrations of VEGF$_{165}$ a band at 200 kDa stained increasingly at an IC$_{50}$ of 75 pM (Fig. 4). In control blots polyclonal anti-VEGFR II antibody was used for identification of the receptor band (Dr. Towbin, Basel, Switzerland). Compared with VEGF$_{165}$ addition alone the intensity of this phosphotyrosine containing band was suppressed in the presence of the peptide with an IC$_{50}$ 3–10 and 0.3–1 μM for peptide monomer (Je-7) and dimer (Je-11), respectively, thus providing evidence of the antagonistic effect (Fig. 5). The peptides alone did not activate receptor phosphorylation nor did

TABLE I
Summary of results
Competition of peptides RTELNVGIDFNWEYPASK (Je-7) and (RTELNVGIDFNWEYPAS)$_2$K (Je-11) in different assays.

Peptide	IC$_{50}$ (μM)				
	Competition with VEGFR-II	Competition with HUVEC	Receptor phosphorylation assay	Migration assay	Proliferation assay
RTELNVGIDFNWEYPASK (Je-7)	10	ND	3–10	0.1	0.1
(RTELNVGIDFNWEYPAS)$_2$K (Je-11)	0.5	30	0.3–1	0.3	0.5
VEGF$_{165}$	0.001	0.0006	Stimulation 0.000075	Stimulation 0.00025	Stimulation 0.00025

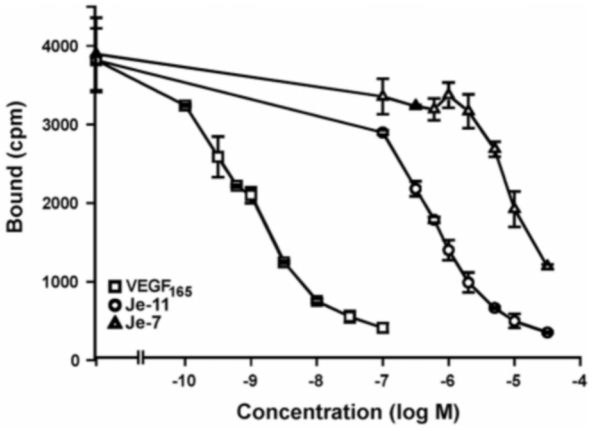

FIG. 3. **Binding competition of the peptides and VEGF for the VEGFR II.** Competition curves for the monomeric peptide RTELNVGIDFNWEYPASK (Je-7), the dimeric peptide $(RTELNVGIDFNWEYPAS)_2K$ (Je-11), and $VEGF_{165}$ were obtained according to "Experimental Procedures" by adding increasing concentrations of peptides and $VEGF_{165}$ to microtiter plates, coated previously with soluble VEGFR II preparations before adding ^{125}I-$VEGF_{165}$.

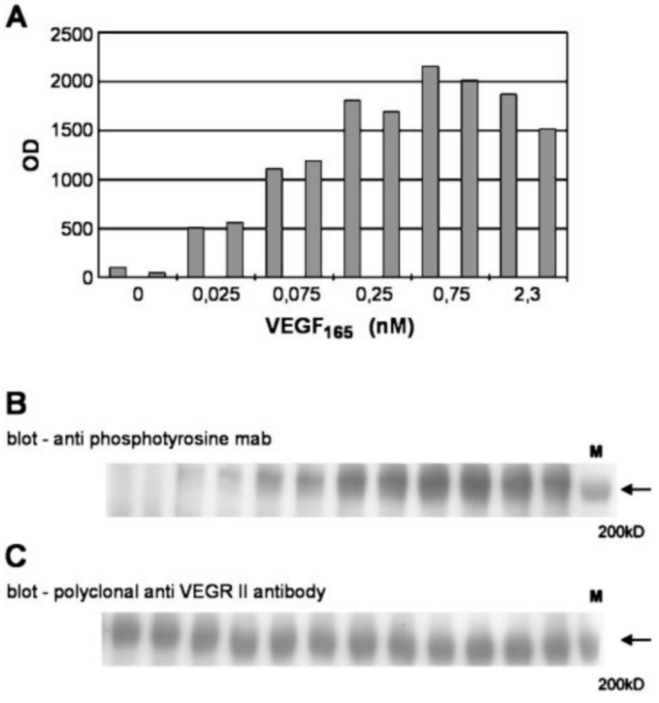

FIG. 4. **Autophosphorylation of VEGFR II in MVECs induced by $VEGF_{165}$.** Cells were treated as described under "Experimental Procedures." The experiments were performed as duplicates of each concentration. *A*, quantitative densitometric scan of the Western blot of lysed cells after stimulation with $VEGF_{165}$. *B*, 200-kDa band of the Western blot stained with an anti-phosphotyrosine antibody. *C*, as a control the stripped membrane was reprobed with a polyclonal antibody against VEGFR II (obtained from Dr. Towbin).

they have any effect on the morphology of the cells (data not shown).

To determine the specificity of the peptides inhibiting autophosphorylation of VEGFR II we performed control experiments with TF-1 cells expressing the c-kit receptor. C-kit is a member of the receptor tyrosine kinases class III comprising split kinase domains comparable to VEGFR II differing only in the number of extracellular Ig loops. VEGFR II possesses seven Ig loops whereas c-kit consists of five Ig loops thus belonging to the platelet-derived growth factor-receptor family. Using stem cell factor, the endogenous c-kit ligand, we found a concentration-dependent increase in receptor autophosphorylation. The autophosphorylation was blocked by the kinase inhibitor staurosporine, but not by peptides Je-7 and Je-11 (Table II).

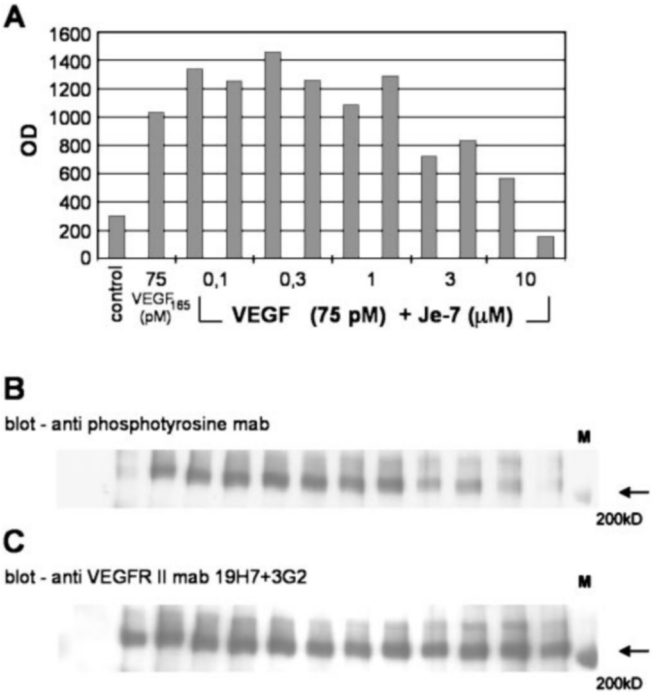

FIG. 5. **Inhibition of $VEGF_{165}$-stimulated VEGFR II autophosphorylation in MVECs.** Cells were incubated with 75 pM $VEGF_{165}$ and increasing concentrations of peptide RTELNVGIDFNWEYPASK (Je-7). Each concentration was tested twice. The preparation of the blots was performed as described under "Experimental Procedures" (*right lane* marker protein myosin). *A*, quantitative densitometric scan of the Western blot of lysed cells after stimulation with VEGF and competition with Je-7. *B*, 200-kDa band of the Western blot stained with an anti-phosphotyrosine antibody. *C*, as a control the stripped membrane was reprobed with a polyclonal antibody against VEGFR II (obtained from Dr. Towbin).

Inhibition of Endothelial Cell Activity—To further substantiate the antagonistic activity of the peptides we studied the migration of microvascular endothelial cells through nucleopore filters. The number of cells migrating through the filters to the lower compartment within 18 h can be increased in the presence of 250 pM $VEGF_{165}$ by a factor of 2–4 depending on the cell preparation. If in addition increasing concentrations of the antagonistic peptide are added the number of transmigrating cells is decreased (Fig. 6). Migration of MVECs was inhibited at half-maximal concentrations of about 0.1 and 0.3 μM by RTELNVGIDFNWEYPASK (Je-7) and $(RTELNVGIDFNWEYPAS)_2K$ (Je-11), respectively.

$VEGF_{165}$ stimulates MVEC proliferation in a concentration-dependent manner with an EC_{50} of 100 pM (Fig. 7). In the presence of peptides Je-7 and Je-11, the cell number assayed as described was reduced concentration dependently with an IC_{50} of 0.1 and 0.5 μM, respectively (Fig. 8). The peptides did not inhibit cell proliferation in the absence of $VEGF_{165}$, showing the lack of toxicity of the agents for basal cell growth.

DISCUSSION

VEGFR II and VEGF dimers are relatively large proteins that interact in a 2:1 stoichiometry at the extracellular part of the molecules. The cytoplasmic tails of the dimerizing receptors are autophosphorylated subsequently.

To get more information on the size of the interacting surface on both molecules we have mapped a potential contact site of $VEGF_{165}$/VEGFR II using cellulose-bound overlapping peptides, which were already successfully applied for mapping

TABLE II
C-kit autophosphorylation in TF-1 cells

The peptides Je-7 and Je-11 as well as the tyrosine kinase inhibitor staurosporine were incubated with TF-1 cells in the presence of stem cell factor. Stem cell factor concentration used in the receptor autophosphorylation assay was determined using increasing concentrations of the ligand.

	Receptor autophosphorylation assay, EC_{50}	Receptor autophosphorylation assay, IC_{50} (at SCF-concentration 0.6 nM)
Stem cell factor	0.3 nM	
Staurosporine		200 nM
RTELNVGIDFNWEYPASK (Je-7)		No inhibition at 10 μM
$(RTELNVGIDFNWEYPAS)_2K$ (Je-11)		No inhibition at 10 μM

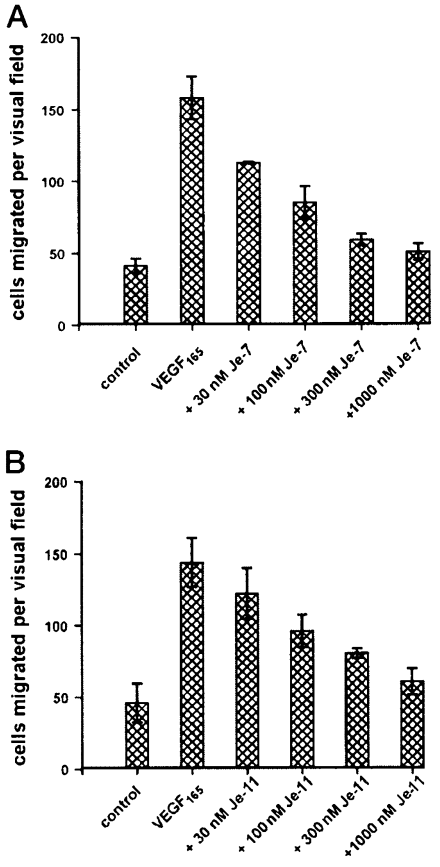

FIG. 6. **Migration assay with MVEC through micropore filters.** Inhibition of $VEGF_{165}$-stimulated migration of MVECs with increasing concentrations of peptides. Three independent filters, three visual fields each. *A*, peptide Je-7 (RTELNVGIDFNWEYPASK). *B*, peptide Je-11 ($(RTELNVGIDFNWEYPAS)_2K$).

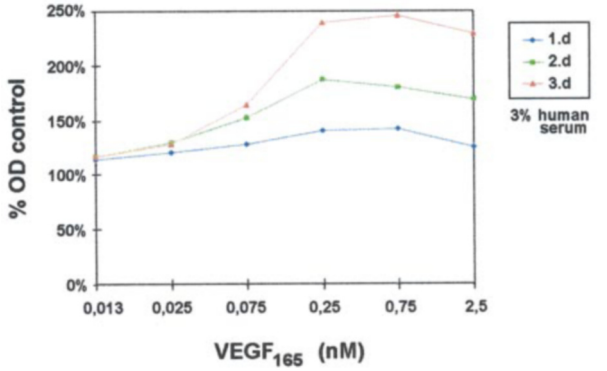

FIG. 7. **Proliferation assay with MVEC.** MVEC were cultured overnight in a serum-reduced medium before increasing concentrations of $VEGF_{165}$ were added. After 1 (*blue curve*), 2 (*green curve*), or 3 (*red curve*) days in culture, cell mass was determined by colorimetric stain.

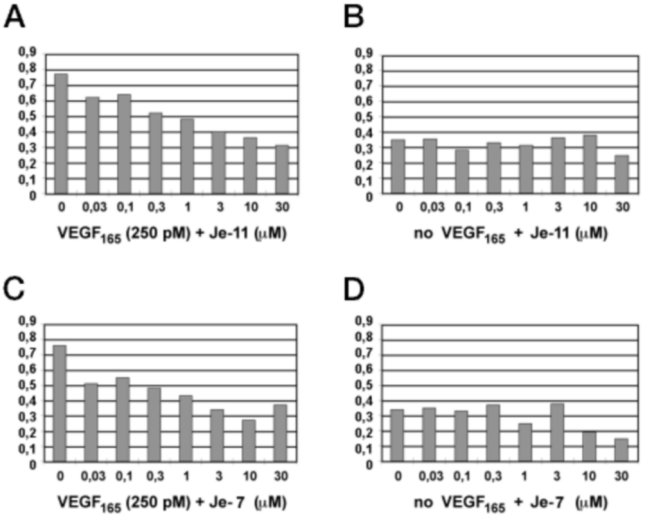

FIG. 8. **Peptide inhibition of $VEGF_{165}$-stimulated MVEC proliferation.** *A* and *C*, inhibitory peptides were present in increasing concentrations. *B* and *D*, for control of peptide toxicity the cells were cultured with peptides in the absence of $VEGF_{165}$.

different types of protein-protein interactions (45–48). The mapped peptides were further characterized by a complete L-substitutional analysis which reveals binding information on a resolution of one amino acid side chain. A special feature of the peptide/$VEGF_{165}$ contact site which may reflect partly the VEGFR II-VEGF-binding site is that only negatively charged and hydrophobic amino acids contribute to binding.

The VEGF crystal structure and mutation analysis (21) reveal a hydrophobic groove in the VEGF surface which is accompanied by two nearby hydrogen bond forming amino acids (Asp^{63}, Glu^{64}, and Glu^{67}) or basic residues Arg^{82}, Lys^{84}, and His^{86}, the basic amino acids being relevant for VEGFR II binding, the acidic ones for VEGFR I binding (21, 33).

As indicated through binding experiments using shortened extracellular domains of the VEGFR I by Davis-Smyth *et al.* (35) and Barleon *et al.* (34) only extracellular Ig-like domains 1–3 appear to be necessary for binding. These findings are in line with the presumed VEGF contact site on the third globular domain of VEGFR II. Recently it was shown that domains 2 and 3 of VEGFR I and VEGFR II were sufficient to bind VEGF with comparable affinities to the wild type receptors (39). If only the Ig-like domain 2 of both receptors was tested for binding, a 60-fold decrease of binding affinity was observed for the VEGFR I, whereas the binding of VEGFR II-domain 2 was reduced by a factor of 1000. This clearly indicates that the Ig-like domain 3 of the VEGFR II contributes significantly to the binding of VEGF, which is also strongly supported by our results since we only detect a contiguous peptide sequence on the third Ig-like domain of VEGFR II and not in the second domain. High resolution data obtained from the crystal structure analysis of $VEGF_{8-109}$ complexed with the second Ig-like domain of VEGFR I (FLT-1) (39) reveal similar VEGF-binding sites for the VEGFR I and VEGFR II. Five of seven residues of VEGF reported to be important for VEGFR II interaction are located in the interface of the VEGF/VEGFR I-domain 2 complex (33, 39). Interestingly, the residual 2 VEGF amino acids described to be involved in VEGFR II binding (21, 33, 49) form a groove adjacent to the C-terminal segment of VEGFR I-domain 2 suggesting that this groove is a potential binding site of

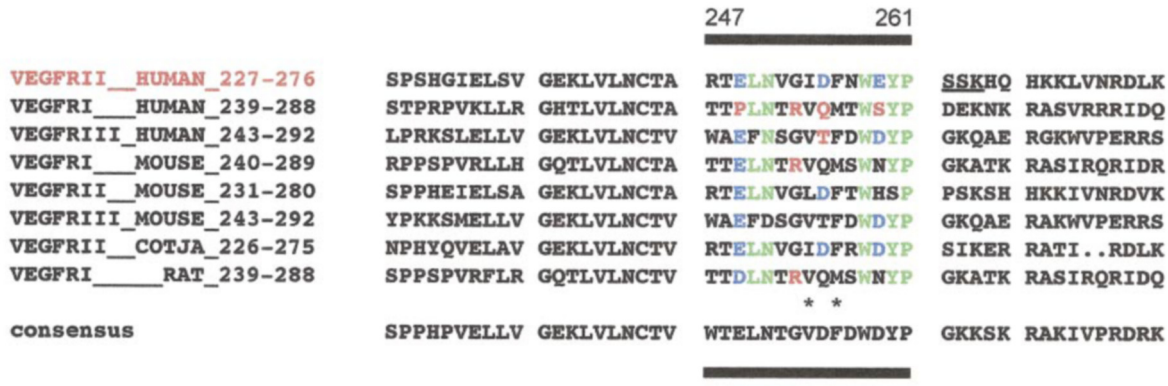

FIG. 9. **Sequence comparison of different VEGF receptors.** The *bar* and the *asterisks* indicate the mapped VEGFR II-derived peptide sequence and physicochemically similar amino acids, respectively. The *underlined* residues, *SSK,* were used to improve the solubility of the VEGFR II-derived peptides. Conserved amino acids are shown in *green*, the negatively charged amino acids are stained in *blue*. Amino acid residues differing significantly from the sequence VEGFR $II_{247–261}$ are colored in *red*.

domain 3 of the native complex (39). It is tempting to speculate that the discussed groove is the binding site for the identified peptide VEGFR $II_{247–261}$ located on the third Ig-like domain. This would partly explain why we could only map a peptide binding $VEGF_{165}$ with good affinity on the third domain and not on the second domain of VEGFR II; because for peptide binding, a binding groove is much more favorable than a flat discontinuous surface as demonstrated for the interface of VEGF and VEGFR I-domain 2. This discussed mode of binding would also be in agreement with the low solubility observed for the initially synthesized hydrophobic peptides and the importance of 5 hydrophobic residues in the VEGF-peptide interaction as deduced from the substitutional analysis (Fig. 2). Although the data strongly suggest a direct binding of the mapped peptide to the VEGFR II-binding site of VEGF, we cannot rule out an allosteric mode of action or an interference with VEGF dimerization, which causes the inhibitory effect of the peptide.

A sequence comparison of the mapped VEGFR II-derived epitope with the corresponding sequences of VEGFR I and VEGFR III of different species (Fig. 9) reveal significant differences among the VEGF receptors. No negative charge is present in the compared sequence of the human VEGFR I. Moreover, the VEGFR I contains an Arg and a Met at positions 253 and 256 (human VEGFR II numbering) instead of Gly and Phe in all other VEGF receptors. Both residues specific for VEGFR I would abolish binding of the VEGFR II-derived peptide (Fig. 2). Asp^{255}, the key residue for peptide binding (Fig. 2), is the only residue specific for VEGFR II in different species suggesting that it might also play a role in VEGFR II binding. Besides two strictly conserved residues (Trp^{258} and Pro^{261}) the potential VEGFR II-binding site comprise several residues differing strongly from the VEGFR I which may contribute to the different binding modes of the two major VEGF receptors.

The identified contiguous peptide from the sequence of Ig-like domain 3 binds to $VEGF_{165}$ and competes with VEGFR II for $VEGF_{165}$ binding. Utilizing this peptide in a monomeric and dimeric form, the signal transduction pathway of $VEGF_{165}$ in endothelial cells could be interrupted in cellular test systems measuring receptor autophosphorylation, proliferation, and migration. The inhibition of the VEGF/VEGF-receptor function is specific for VEGF signaling since the ligand-receptor interaction of stem cell factor/c-kit, belonging together with VEGFR II to the same tyrosine kinase receptor class III, is not affected.

The concentrations observed for inhibition of VEGF are slightly different for the different assays which might be due to the different duration, variation in protein content of the media, and sensitivity of the experiments (1 h at 4° C for receptor autophosphorylation, 18 h at 37° C for migration, and 3 days at 37° C in the proliferation assays). Especially the duration of the experiment could have an influence on the effective concentration of the peptides showing a limited solubility. This is in line with the fact that the monomeric peptide (Je-7) possesses a lower solubility compared with the dimeric peptide (Je-11), which does not show relevant differences in the IC_{50} values in the endothelial cell migration assay and proliferation assay in contrast to the competition with sVEGFR and the receptor autophosphorylation assay. In addition, the different sensitivity of the respective assays might contribute to the different IC_{50} values. Experiments with other tested compounds indicate that the migration and proliferation seem to be more sensitive assays compared with the binding and phosphorylation assays.

The binding constant of the monomeric peptide to $VEGF_{165}$ is considerably lower than the VEGF/VEGFR II binding constant which might also be due to an avidity effect of the homodimeric $VEGF_{165}$, which possesses two identical receptor-binding sites. This is supported by the fact that the dimeric peptide has an order of magnitude higher binding constant than the monomeric peptide. We assume that the binding between the peptide dimer and VEGF occurs in a 1:2 stoichiometry since the length of the peptide length does not allow for the simultaneous occupation of the two VEGF-binding sites, which are most likely located on the poles of the oval VEGF molecule (21, 39). Our findings open the opportunity to search for non-peptidic small molecules that block the interaction of VEGF with VEGFR II for possible use for pharmaceutical purposes.

Acknowledgments—We thank Dr. Towbin and Dr. Reusch for the VEGFR II antibodies. G. Fenten and M. Seeman for expert technical help, Dr. Martiny-Baron for baculovirus, expressing VEGFR II protein, and VEGF, Dr. Holger Wenschuh for peptide synthesis, and Dr. Ulrich Reineke for critical reading of the manuscript.

Note Added in Proof—The importance of the third Ig-like domain of VEGFRII in VEGF binding is supported by studies of Shinkai, A., Ito, M., Anazawa, H., Yamaguchi, S., Shitara, K., and Shibuya, M. (1998) *J. Biol. Chem.* **273,** 31283–31288, published after submission of this manuscript.

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