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 celluose-bound overlapping peptides of the extracytoplasmic 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 \(^{247}\text{RTELNVGIDFNWEYP}\)\(^{261}\) was revealed as contiguous sequence stretch, which bound \(^{125}\text{I}-\text{VEGF}_{165}\). 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 \((\text{RTELNVGIDFNWEYP})_2\) \(^K\) inhibits VEGF\(^{165}\) binding with an \(IC_{50}\) of 0.5 \(\mu M\) on extracellular VEGFR II fragments and 30 \(\mu M\) 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 \((\text{RTELNVGIDFNWEYP}_K)_2\) at a half-maximal concentration of 3–10, 0.1, and 0.1 \(\mu M\), 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)\(^3\) 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-I, 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 \(\beta\) (TGF\(\beta\)) (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 (PIGF), 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 \(\alpha/\beta\) receptors having five extracellular immunoglobulin-like domains and pertaining to the class III tyrosine kinase receptors (30–32).

The VEGFRI 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 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.

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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. Concomitant 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 homoygotic 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 (Ile43, Ile46, and Ile83) and positively charged amino acids Arg82, Lys84, and His86 in VEGFR II autophosphorylation, as well as growth and mitogenic binding of VEGF to VEGFR II. This VEGF mutation studies demonstrated that high affinity binding of VEGF to VEGFR II is dependent on the presence of three locally vicinal isoleucins (Ile43, Ile46, and Ile83) and positively charged amino acids Arg82, Lys84, and His86 in VEGF. In addition, Asn63, Glu64, and Glu67 are important for VEGFR II-derived Peptides Inhibit VEGF...
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, VEGF165 or VEGF165 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 phenethylsulfonyl 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 5% bovine serum 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 96 white microtiter plates (Maxisorb, Nunclon) that had been coated 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 μg/ml) 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 (Na₂P₂O₇), 300 mM sodium fluoride, 3 mM sodim pyrophosphate, 0.25% Nonidet P-40, 0.5% deoxycholate, 50 mM sodium pyrophosphate, 100 mM sodium fluoride, 2 mM orthovanadate, 1 mM zinc acetate, 1.25 mM phenethylsulfonyl fluoride, 10 mg/ml aprotinin). The DNA was removed by filtering through a Millipore filter (0.65 μm). In the lower chamber are 600 μl of M199 containing magnesium and calcium chloride. The cells were washed and 1% rose bengal in 30% ethanol was added. 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 VEGF165 were added and incubated. 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.

**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 VEGFR II (250 μl 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% 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).

**VEGFR II-derived Peptides Inhibit VEGF**

Fig. 1. Autoradiographs of the cellulose-bound VEGFR II-derived peptides probed with 125I-VEGF165. The primary sequence of VEGFR II is fragmented in 378 13-mer peptides overlapping 11 amino acids. A, original sequence of VEGFR II. The signals represent the following sequences: spot 124, RTELNVGIDFNW; spot 125, ELNVGIDFNWEP; spot 227, HIHWYWQLEEC; spot 228, HWYWQLEEECANP; spot 229, YWQLEEECANP; spot 236, AVSVTNPYPC-EEW; spot 237, SVTNYPYPCEDW; spot 238, TNPYPCEDWBSVE; spot 239, PYPCEEDWBSVEF. B, the cysteine residues of the original sequence are replaced by serine residues. The signals represent the following sequences: spot 124, RTNLNVGIDFNWE; spot 125, ELNVGIDFNWEPY.

**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 (cVEGFR II) sequence were independently synthesized (Fig. 1). In the second scan (Fig. 1B) all cysteine residues in the peptides were substituted by serine to prevent unspecific disulfide bridging of the target molecule [125I-VEGF165]. Incubation of both scans with radiolabeled [125I-VEGF165] 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-VEGFr165 showed eight strong spots (Fig. 1A), whereas the peptide scan with the serine substitutions (Fig. 1B) displayed only two spots (124 and 125) corresponding to the peptide sequences $^{247}$RTELNVGIDFNWE$^{259}$ and $^{249}$ELNVGIDFNWEP$^{261}$ 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. 1A), 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 $^{249}$ELNVGIDFNWE$^{261}$ (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 VEGF165. 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$^{256}$, 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 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 VEGF165 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 prolonged 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 VEGF165 at a site relevant for the interaction of VEGF165 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-VEGFr165 as ligand. In Fig. 3 the competition of peptide RTELNVGIDFNWEYPASK with $^{125}$I-VEGFr165 is compared with that of VEGF165. Unlabeled VEGF165 reduces binding of $^{125}$I-VEGFr165 with an IC$_{50}$ of about 75 pM (Fig. 4). In control blots polyclonal anti-VEGFR II antibody was used for the detection. With increasing concentrations of VEGF165 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 VEGF165 addition alone the intensity of this phosphotyrosine containing band was suppressed in the presence of the peptide with an IC$_{50}$ of 3–10 and 0.3–1 $\mu$m 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**

| Peptide                        | 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)K (Je-11)   | 0.5                       | 30                     | Stimulation                   | 0.3–1          | 0.5                 |
| VEGFr165                       | 0.001                     | 0.0006                 | Stimulation                   | 0.000075       | 0.00025             |

**Fig. 2. Autoradiograph of the cellulose-bound L-substitutional analysis of VEGFR II-derived peptide probed with $^{125}$I-VEGFr165.**

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.
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).

**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 VEGF165/VEGFR II using cellulose-bound overlapping peptides, which were already successfully applied for mapping VEGF.
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/VEGF165 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 (Asp63, Glu64, and Glu67) or basic residues Arg82, Lys84, and His86, 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 VEGF8–109 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 VEGF.

### TABLE II

| Receptor autophosphorylation assay, IC_{50} (at SCF-concentration 0.6 nM) |
|---------------------------------------------------------------|
| **Stem cell factor**                                         | 0.3 nM |
| **Staurosporine**                                           | 200 nM |
| RTELNVGIDFNWEYPASK                                         | No inhibition at 10 μM |
| (RTELNVGIDFNWEYPAS)_{2}K                                     | 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)_{2}K).

**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}.
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 I\textsubscript{II}-derived located on the third Ig-like domain. This would partly explain why we could only map a peptide binding VEGF\textsubscript{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\textsuperscript{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\textsuperscript{258} and Pro\textsuperscript{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\textsubscript{165} and competes with VEGFR II for VEGF\textsubscript{165} binding. Utilizing this peptide in a monomeric and dimeric form, the signal transduction pathway of VEGF\textsubscript{165} in endothelial cells could be interrupted in cellular test systems measuring receptor autophosphorylation, proliferation, and migration. The inhibition of the VEGF/VEGFR autophosphorylation 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 medium, 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\textsubscript{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\textsubscript{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\textsubscript{165} is considerably lower than the VEGF/VEGFR II binding constant which might also be due to an avidity effect of the homodimeric VEGF\textsubscript{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.

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