Vascular endothelial growth factors (VEGFs) and their receptors play key roles in angiogenesis and lymphangiogenesis. VEGF activates VEGF receptor-1 (VEGFR-1) and VEGFR-2, whereas VEGF-C activates VEGFR-2 and VEGFR-3. We have created a library of VEGF/VEGF-C mosaic molecules that contains factors with novel receptor binding profiles, notably proteins binding to all three VEGF receptors ("super-VEGFs"). The analyzed super-VEGFs show both angiogenic and lymphangiogenic effects in vivo, although weaker than the parental molecules. The composition of the VEGFR-3 binding molecules and scanning mutagenesis revealed determinants of receptor binding and specificity. VEGF-2 and VEGFR-3 showed striking differences in their requirements for VEGF-C binding; extracellular domain 2 of VEGF-2 was sufficient, whereas in VEGFR-3, both domains 1 and 2 were necessary.

The vascular endothelial growth factors (VEGFs)² are key molecules in the development and growth of blood and lymphatic vessels (1–3). Each VEGF is characterized by an individual receptor binding profile, which determines its scope of biological effects. For example, VEGF binds both VEGFR-1 and VEGFR-2 and is predominantly angiogenic, whereas VEGF-C binds VEGFR-2 and VEGFR-3 and promotes mainly lymphangiogenesis. The co-crystallization of placenta growth factor (PIGF) and VEGF with domain 2 of VEGFR-1 (4, 5) shows that a virtually identical set of residues in PIGF and VEGF form very similar receptor binding interfaces (5–7).

VEGF amino acid residues important for binding to VEGFR-2 have been identified by mutagenesis studies (7–9). However, it is unclear how receptor specificity is encoded within the VEGF family of growth factors. Furthermore, there are no structural data regarding VEGF-C interactions, we created a library of mosaic molecules from VEGF and VEGF-C. Additional to the evaluation of individual residues based on the loss of ligand affinity upon mutation, we grafted VEGF-C segments into VEGF to gain VEGFR-3 binding. Because such DNA shuffling did not resolve the interaction determinants at the individual residue level, we scanned the regions that conferred VEGFR-3 binding by alanine mutagenesis to identify the residues that are important for specific receptor interaction. Finally, we showed in vivo that the introduction of additional receptor affinities into VEGF or VEGF-C modifies their biological effects, creating dual angiogenic/lymphangiogenic properties, which are somewhat weaker than the angiogenic and lymphangiogenic effects of VEGF and VEGF-C, respectively.

**EXPERIMENTAL PROCEDURES**

Creation and Screening of the VEGF/VEGF-C Mosaic Library—We maximized the identity of nucleotide sequences coding for the VEGF homology domain of VEGF and VEGF-C by silent mutagenesis (see the supplemental data). Two sets of nine DNA fragments coding for segments of VEGF and VEGF-C, respectively, were synthesized. The overlaps of these fragments corresponded to stretches of 100% nucleotide sequence identity between VEGF and VEGF-C. Two or three adjacent fragments were simultaneously ligated into a modified pKO Scrambler V912 vector (Stratagene, La Jolla, CA). Thus, four different vector sets were obtained (Fig. 1B). Two vector sets were then combined into 32 different vectors coding for the N-terminal halves of the mosaic molecules. Similarly, the two other vector sets were combined into 16 different vectors coding for the C-terminal halves. The final ligation created 512 different vectors. The expression plasmid pMosaic that hosted the library was constructed using sequences from pSecTagA and pLCzA-A (both from Invitrogen), site-directed mutagenesis, and synthetic linkers. pMosaic contained a cytomegalovirus promoter, the immunoglobulin leader peptide for secretion, a C-terminal Myc epitope, and a hexahistidine tag for detection. The assembly of the library is schematically shown in Fig. 1B, and details can be found as supplemental data. All 512 constructs were separately transfected into 293T cells. After metabolic labeling with [³⁵S]methionine and [³⁵S]cysteine for 24 h, the conditioned medium was immunoprecipitated with anti-pentahistidine antibody (Qiagen, Valencia, CA) and VEGF extracellular domain/immunoglobulin G Fc (VEGF/IgGFc) fusion proteins. For the latter, the
VEGF/VEGF-C Mosaic Molecules

medium was supplemented with bovine serum albumin and heparin to
final concentrations of 1% and 0.1 μg/ml. The protein A-bound fraction
was analyzed in 15% SDS-polyacrylamide gels.

Construction of VEGFR/igGFc Expression Vectors—To construct the
VEGFR-2/igGFc expression plasmid, the first three Ig homology
domains of the extracellular part of VEGFR-2 were amplified by PCR
using primers 5'-GGGATCCCTTCAGTTGTTTCTCTGAC-3' and
3'-CCAGTCACCCTGCTCCGGAAGCTTCTGGAGAG-3' and cloned into the Signal pII plus vector (Novagen, Madison, WI). The
resulting plasmid was cut with BamHI and KpnI, treated with
T4 polymerase, and ligated to itself. The expression plasmids for VEG-
FR-2/igGFc and VEGFR-3/igGFc (12) were transiently transfected into
293T cells, and the conditioned medium was used for the precipitation
of growth factors. VEGFR-1/igGFc was expressed in S2 cells and
purified as previously described (12). The deletion series of the linker
between VEGFR-3 domains 2 and 3 was created by PCR using the ex-
pression plasmid VEGFR-3/igGFc as template, T7 as forward primer,
and the following reverse primers: 5'-CTGGATCCATCCGCGAGCTGTT-
TGCTCGT-3', 5'-TACAGGATCCCTCGGTGTTGACCCAG-3', 5'-
TCAGGATCCGCGAGCTCAGGGAGAAGG-3', and 5'-TACAGGATCC-
CGGAAGGGGTGGATAAGG-3'.

The Ig homology domain 1 was deleted from the expression plasmid
VEGFR-3/igGFc by site-directed mutagenesis using primers 5'-CTGG-
ATACTCAGGAGCTCAGGGAGAAGG-3' and 5'-AGCTGCTGTTAGGGGAGA-
AGGATCCGCGAGCTCAGGGAGAAGG-3' and excision of the BamHI
fragment from the resulting plasmid. The plasmid coding for
VEGFR-3/igGFc was then cloned into the plasmid encoding only VEGFR-3 domain 2. The sequences encoding the four different versions of domain 2 of VEGFR-2 were amplified using forward primer 5'-AGCGATAGCTGATTCAAGAGATTACAGAATTC-
C-3' and reverse primers 5'-TACAGGATCCGCTCAGGGAGAAGG-3',
5'-AGCTGCTGTTAGGGGAGAAGGATCCGCGAGCTCAGGGAGAAGG-
AGGATCCGCGAGCTCAGGGAGAAGG-3', and 5'-TACAG-
GATCCGCGAGCTCAGGGAGAAGG-3'.

Protein Production, Purification, and Gel Filtration—Ten selected
mosaic VEGFs were produced in insect cells using the Bac-to-Bac sys-
tem. Sf9 and High Five cells were maintained in S90011 (all Invitrogen)
according to the instructions of the supplier. First, the coding sequences
for the melittin signal peptide and a hexahistidine tag were inserted into
the transfer vector pFASTBAC1 as described previously (20), and sub-
sequently, the coding sequences of the VEGF/VEGF-C mosaic mole-
ecules were subcloned into this construct. Details about the growth fac-
tors and control proteins are listed in the supplemental data. To
produce the proteins for the VEGFR-1 bioassay, the conditioned
medium of the baculovirus-infected High Five cells was concentrated
50-fold using Centricon C-10 spin columns (Millipore, Bedford,
MA) diluted into Dulbecco's modified Eagle's medium, and the concentra-
tion of the mosaic molecules was determined by densitometry.

To purify protein for the chorioallantoic membrane (CAM) assay,
conditioned medium of High Five cells was harvested 72 h after infec-
tion and dialyzed against 30 mM sodium phosphate and 400 mM sodium
chloride, pH 6. The pH was adjusted to 8.0, and Ni2+-nitrilotriacetic
acid Superflow resin (Qiagen) was added. The samples were agitated for
12 h at +4 °C. The resin was then collected and applied to the chromo-
tography columns. The columns were washed with 30 mM sodium
phosphate, 400 mM sodium chloride, 600 mM glycerol, and 20 mM imid-
azole at pH 8.0, and the bound proteins were eluted with imidazole,
dialyzed against 0.1% trifluoroacetic acid, and sterilized using Mil-
lex-GV filters (Millipore). The proteins were checked in silver-stained
15% polyacrylamide gels and quantified using the BCA protein assay kit
(Pierce).

Coding sequences for the alanine scan mutants used in the surface
plasmon resonance analysis and the VEGFR-2/VEGFR-3 bioassays
were generated by site-directed mutagenesis using megaprime-based
PCR mutagenesis or the QuikChange method (Stratagene). Coding
sequences were then subcloned into the pMT-BiP/V5HisC vector
(Invitrogen) for expression in insect cells. Protein from 50 ml of HyQ Sf
insect medium (Hyclone, Logan, UT) conditioned for 5 days by stably
expressing S2 cells was purified by Ni2+-nitrilotriacetic acid affinity
chromatography as described above followed by buffer exchange
against standard Bicore running buffer using a HR10/10 Fast Desalting
column on an Äkta Explorer (GE Healthcare, Chalfont St. Giles, UK).

Surface Plasmon Resonance Analysis—Binding of VEGF-C and its
mutants to VEGFR-1, -2, and -3 were analyzed with surface plasmon
resonance in the Biacore 2000™ biosensor (Uppsala, Sweden). Flow
cells of a CM5 biosensor chip were covalently coated with VEGF/
igGFc fusion proteins via standard amine coupling. The binding of the
alanine scan mutants was then analyzed in the standard Bicore running
buffer (10 mM Hepes, pH 7.4, 150 mM sodium chloride, 3 mM EDTA,
0.005% surfactant P-20) following the instructions of the manufacturer.
The kinetics of the VEGF-C interaction with VEGFR-2 and -3 were
determined at varying concentrations of VEGF-C (20–300 nM) over a
surface to which 1500 resonance units of the respective receptor had
been coupled. The VEGF-2 and -3-immobilized flow cells were regen-
erated after every injection with a pulse of 100 or 20 mM hydrochloric
acid, respectively. The coupling level used in the screening experiments
of the interactions was chosen to be 6000 resonance units for VEGFR-2,
and the concentration of VEGF-C mutants was 1 μM. The coupling level
of the more reusable VEGFR-3 surface was kept at 1500 resonance units,
and the concentration for screening of mutants was chosen to be 100
nM. Replicative screening experiments were done on freshly coupled
surfaces. The coupling levels of the receptors and the concentrations
of the mutants varied between the experiments. The contact time of
VEGF-C and its mutants was 5 min and the flow rate 20 μl/min.

The data were evaluated by first subtracting the sensogram obtained from
the empty control flow cell from the sensograms of the flow cells con-
taining VEGFR-2 or -3. The obtained curves were fitted to the natural
logarithmic 1:1 Langmuir binding model of the BiaEvaluation 3.1 soft-
ware package (Biacore) to obtain the relative binding levels and the
relative dissociation constants of the mutated VEGF-C proteins.

Downloaded from www.jbc.org at FINELIB - Helsinki Univ on August 10, 2006
Gel filtration was performed on a Superdex 75 10/300 GL column (GE Healthcare) calibrated with a gel filtration low molecular weight calibration kit (GE Healthcare) using phosphate-buffered saline as eluent, a flow rate of 0.75 ml/min, and a sample volume of 200 µl. Analysis was performed using the Unicorn 4.1 software package (GE Healthcare).

CAM Assay—The CAM assay was carried out as described previously (21). Disks punched from Thermanox coverslips (Nunc) with the sterile salt- and carrier-free protein were applied to day 13 CAM, and after 3 days, the specimens were either fixed in 4% paraformaldehyde or 0.5% ZnCl and embedded into paraffin or embedded into TissueTek and subsequently frozen. Specimens were photographed in an Olympus SZX9 stereomicroscope. Carrier disks alone and human serum albumin were used as negative controls. Quantitation of angiogenesis in the transparent CAM was based on the optical density of the digitized microphotographs using computer-assisted image analysis (22). Mean relative optical density was measured from vessels with a diameter <0.1 mm using the program ImageJ (23).

RESULTS

Mosaic VEGFs with Novel Receptor Binding Profiles—Based on eight hot spots of sequence homology at the nucleotide level (supplemental data), we fragmented the VEGF homology domains of VEGF and VEGF-C into nine subunits and swapped the homologous fragments in all combinations (Fig. 1). All clones were transiently expressed in 293T cells, and the factors were precipitated from the conditioned medium of metabolically labeled cells using soluble VEGFR/IGF fusion proteins. To our surprise, the library contained molecules with all possible recep-
VEGFR-1 or -2 binding.

The presence of VEGF-C-derived fragments 4, 5, and 8 did not inhibit VEGFR-3 binding alone are sufficient to introduce VEGFR-3 binding into VEGF. Binding is exemplified in mosaic molecule 14–9, where these two fragments derived from VEGF-C could bind VEGFR-3. However, a bias toward VEGF-C-derived fragments was observed in the presumed bottom face of the molecule (fragments 4, 5, and 8). A predominantly VEGF-derived bottom face of the molecule was observed in the presumed bottom face of the molecule (fragments 4, 5, and 8).

Composition of the Mosaic Molecules—The apparent molecular masses of the dominant polypeptide species (19 or 21 kDa) were 4 or 6 kDa higher than predicted from the amino acid sequence. A comparison of the mobilities of the recombinant proteins shown in Fig. 2A and the schematic structures shown in Fig. 2E indicate that the reason for the size difference is the utilization of the N-linked glycosylation sites in fragments 7 (both VEGF and VEGF-C) and 9 (VEGF-C). Enzymatic deglycosylation and analysis of glycosylation-deficient mutants of VEGF-C confirmed that VEGF-C indeed uses both potential N-glycosylation sites (supplemental data). The compositions, apparent molecular masses, and receptor binding profiles of 10 mosaic molecules that were selected for further analysis are described in Fig. 2E.

The pattern of receptor interaction correlated with the composition of the mosaic molecules. VEGF-derived fragments 2 and 7 were required for VEGFR-1 binding, as none of the molecules with these fragments derived from VEGF-C could bind VEGFR-1. However, many mosaic molecules containing VEGF-derived fragments 2 and 7 bound efficiently to VEGFR-2 and -3; thus, these fragments were classified as "promiscuous." Also, fragment 3 of VEGF favored VEGFR-1 binding. Although required, fragments 2, 3, and 7 alone were not sufficient for VEGFR-1 binding.

VEGFR-3 Specificity and Binding Determinants—Unlike the binding to VEGFR-1, which depended strictly on VEGF-derived fragments 2 and 7, no single fragment of VEGF-C was absolutely required for binding to VEGFR-3. However, a bias toward VEGF-C-derived fragments was observed in the presumed bottom face of the molecule (fragments 4, 5, and 8). A predominantly VEGF-derived bottom face of the molecule precluded VEGFR-3 binding. Of the 67 VEGFR-3 binding molecules, 52, 56, and 45 had VEGF-C-derived fragments 4, 5, and 8, respectively. Although required, fragments 2, 3, and 7 alone were not sufficient for VEGFR-1 binding.

TABLE 1

Receptor binding frequencies among mosaic VEGFs

<table>
<thead>
<tr>
<th>Receptor binding profile</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFR-1 and VEGFR-2</td>
<td>26</td>
</tr>
<tr>
<td>VEGFR-2 and VEGFR-3</td>
<td>28</td>
</tr>
<tr>
<td>VEGFR-1</td>
<td>25</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>25</td>
</tr>
<tr>
<td>VEGFR-3</td>
<td>26</td>
</tr>
<tr>
<td>VEGFR-1 and VEGFR-3</td>
<td>3</td>
</tr>
<tr>
<td>All three VEGF receptors</td>
<td>10</td>
</tr>
<tr>
<td>None</td>
<td>255</td>
</tr>
<tr>
<td>Not expressed or not secreted</td>
<td>114</td>
</tr>
<tr>
<td>Total</td>
<td>512</td>
</tr>
</tbody>
</table>

binding was compromised, with VEGFR-3 binding remaining largely unaffected. To determine the cause of this difference, we used gel filtration to check the dimerization status of these mutants. For the Cys156→Ala mutant, the molar ratio of monomeric to dimeric molecules was 4.6 to 1. However, this can only partially explain the difference, because the Cys156→Ser mutant also showed a significant monomeric component with a molar ratio of 2 to 1. In fragments 4 and 8, the highest increase in the apparent $K_d$ for VEGFR-3 binding was observed upon mutation of Phe151 and Pro198 residues, respectively.

We confirmed that the increase of apparent $K_a$ and the reduction in total binding translated into reduced signaling using a cell growth/survival bioassay in Ba/F3 cells expressing a chimeric VEGFR-3/EC/Epo ligand binding nor survival signaling in the Ba/F3 assay were significantly affected.

The Minimal VEGF-C Binding Fragment of VEGFR-3 and VEGFR-2—The extent of the extracellular domain necessary for VEGF binding differs between VEGFR-1 and VEGFR-2. The second extracellular Ig homology domain of VEGFR-1 alone is sufficient for VEGF binding, whereas VEGFR-2 also requires the presence of the third domain. Moreover, the exact amount of linker between domains 2 and 3 of VEGFR-1 determines whether a specific domain

FIGURE 2. The segment composition, molecular masses, and receptor binding properties of 10 mosaic molecules. A-D, conditioned medium from transfected and metabolically labeled 293T cells was precipitated with the indicated VEGFR/IgGFc fusion proteins or the anti-histidine tag antibody. Note that VEGFR-1/IgGFc and VEGFR-2/IgGFc precipitate also endogenous VEGF (in the Mock lanes of B and C). E, red segments, VEGF sequence; green segments, VEGF-C sequence; white segments, stretches of identity used in the construction of the library. The arrows indicate fragments containing receptor specificity determinants. Blue dots mark the glycosylation sites. Relative affinities are given as $E_c^p$ parent molecule/$E_c^m$ mosaic molecule. VEGF-2 affinities relative to both VEGF109 (left) and VEGF-C109 (right) were determined; numbers >1 indicate reduced binding affinity. A minus sign indicates no detectable interaction. The supplemental data provide a complete list of all 512 mosaic molecules and their receptor binding profiles.

$K_d$, not quantified due to low signal level; n.q., not quantified due to low signal level; Ab, antibody.

$K_a$, not quantified due to low signal level; n.q., not quantified due to low signal level; Ab, antibody.

$K_a$, not quantified due to low signal level; n.q., not quantified due to low signal level; Ab, antibody.
only construct can bind VEGF (4). To determine how much of the linker between the corresponding domains of VEGFR-2 and -3 is necessary for VEGF-C binding, we created a series of successive receptor deletion mutants. VEGF-C bound efficiently to soluble fusion proteins containing domains 1 and 2 of VEGFR-3 plus the linker between domains 2 and 3 up to Gly226 (supplemental data). When three additional residues were deleted, the fusion protein failed to be expressed. Thus, the minimal VEGF-C binding fragment of VEGFR-3 appears to extend C-terminally approximately to the same boundary as the minimal VEGF binding fragment of VEGFR-1. Although domain 2 of VEGFR-1 is sufficient for VEGF binding (4), no binding of VEGF-C occurred to VEGFR-3/IgGFc fusion proteins consisting of only domain 2 (including various lengths of the linker) or domains 2 and 3 (supplemental data). In contrast, VEGF-C bound efficiently to the second domain of VEGFR-2 consisting of residues 118–220; constructs containing an additional 6 or 12 C-terminal residues showed reduced interaction (supplemental data).

Mosaic VEGFs Induce VEGFR-2 and -3 Phosphorylation—To confirm the binding data, we assayed the dimerization-induced autophosphorylation of native VEGF receptors. For the phosphorylation assay, we chose a growth factor concentration that would saturate the response to VEGF and VEGF-C but allow the detection of differences in less active molecules. The mosaic factors 12–11, 84–11, and 12–14 were almost as potent as VEGF-C in inducing the phosphorylation of VEGFR-3 (Fig. 4A). Many of the mosaic molecules, however, induced a weaker VEGFR-2 phosphorylation than VEGF or VEGF-C (Fig. 4A). The receptor phosphorylation data mirrored the receptor binding data, with the exception of molecule 12–14. This mosaic factor induced VEGFR-2 phosphorylation but did not precipitate with the soluble VEGFR-2/IgGFc protein for reasons yet to be determined. As VEGFR-1 activation cannot be measured in terms of receptor phosphorylation (25, 26), we used a bioassay to demonstrate biological activity (Fig. 4B).

Mosaic VEGFs Are Angiogenic and Lymphangiogenic—To simultaneously analyze the angiogenic and lymphangiogenic potential of the mosaic molecules in vivo, the purified proteins were applied to differentiated chick CAM (Fig. 5) (21). After incubation for 3 days, the blood vessels of the CAM were identified by staining for αSMA and von Willebrand factor, whereas the transcription factor Prox-1 was stained to identify the lymphatic vessels (Fig. 6A).

The mosaic VEGFs that bound to VEGFR-2 gave an angiogenic response in the CAM (Fig. 5). Similarly, a lymphangiogenic response was obtained with all tested mosaic VEGFs that showed significant binding to VEGFR-3. However, the effects of the mosaic VEGFs on the CAM were weaker than those of either parental molecule; to achieve comparable effects, 5–10-fold more protein had to be applied. The angiogenic control protein VEGF109 and the lymphangiogenic control protein VEGF-C109, which comprised exactly the same minimal receptor binding domain as the mosaic molecules, also showed reduced potency when compared with native VEGF165 and mature VEGF-C, respectively. Of the two super-VEGFs tested, 12–11 was mostly angiogenic, with only a minor lymphangiogenic effect (Fig. 5B), and 84–11 was mostly lymphangiogenic, with only minor angiogenic activity (Fig. 5B).

Because the effects of the monospecific mosaic VEGFs were moderately weak in the CAM assay, we expressed and assayed for comparison

![Graph](image-url)
VEGF family members VEGF-B186 (27), VEGF-E (28, 29), and the Cys156→Ser mutant of VEGF-C (10). VEGF-E and the mosaic factor 53-3, which bind only VEGFR-2 were equally angiogenic on the CAM, but weaker than the factors that bound to both VEGFR-1 and -2 (data not shown). Compared with VEGF-C, the Cys156→Ser mutant of VEGF-C induced a somewhat weaker lymphangiogenic response. VEGF-B186 was without effect in the CAM.

**DISCUSSION**

Three ligands and two ligand-receptor complexes have been crystallized in the VEGF/VEGFR family, yet the mechanisms of receptor specificity determination are not clear. To pinpoint the determinants of receptor specificity in VEGF-C versus VEGF, we shuffled their coding.

**FIGURE 5.** Angiogenic effects of the mosaic molecules in the chick CAM. Different amounts of the recombinant mosaic proteins were dried on coverslips and applied to the 13-day CAM for 3 days. Note that the area of highest growth factor concentration and the strongest angiogenic response forms an uneven circle near the rim. The lymphatic vessels are inconspicuous. Occasionally, however, the huge lymphatic sinuses observed after the application of excess amounts of VEGF-C were filled with blood (data not shown).

* and ** denote p values of <0.05 and <0.01, respectively. HSA, human serum albumin.
sequences. Classical DNA shuffling was not feasible, as the identity between VEGF and VEGF-C is concentrated within the cystine knot; therefore, this method would have caused a strong crossover bias (30). A non-random approach resulted in a library size that allowed exhaustive profiling of the receptor binding pattern of each individual clone. By combining this data with scanning mutagenesis and analysis of receptor deletion mutants, we were able to resolve the VEGF-C binding determinants at three different levels: 1) at the protein domain level of the receptor, 2) at the secondary structure level of the ligand, and 3) at the amino acid residue level of the ligand (summarized in Fig. 7).

Loops 1 and 3 of VEGF-C Contain the VEGFR-3 Specificity Determinants—Large parts of VEGF and VEGF-C were compatible with binding to all receptors. Based on the receptor binding profiles of 398 mosaic molecules, we identified three determinants of VEGF-3 receptor specificity, two of which are localized in the two loop regions of the cystine knot. VEGF-C-derived fragments 4 and 8 (corresponding to loops 1 and 3) could confer VEGFR-3 binding to VEGF. In similar experiments, VEGF-E-derived loops 1 and 3 conferred VEGFR-2 binding to PIGF, and VEGF-A-derived loops 1 and 3 could functionally replace their counterparts in VEGF-E (31). Such functional replacements required that both loops be derived from the same parent molecule. In contrast to this, the same VEGF-C-derived loops could not functionally replace their VEGF counterparts for VEGFR-2 binding. Together with the fact that VEGF-C binding to VEGFR-2 does not require the presence of the third receptor domain, we conclude that VEGF-C binds VEGFR-2 in a fashion substantially different from VEGF or VEGF-E.

The mutation of seven residues within fragments 4 and 8 into alanine residues resulted in a moderate increase of the $K_d$ for VEGFR-3 between 2 and 8-fold. A more pronounced effect on VEGFR-3 binding was observed for only three mutants, Phe$^{151}$, Cys$^{156}$, and Pro$^{198}$. This suggests the presence of a large binding interface with only few prominent key residues. The homologous residues of Phe$^{151}$ and Pro$^{198}$ in the VEGF- and PIGF-VEGFR-1 complexes directly contact the receptor (4, 5), whereas the homologous cysteine residue is crucial for structural integrity and biological activity (32, 33).

Domain 2 of VEGF-2 Is Sufficient for VEGF-C Binding, whereas in VEGFR-3, Both Domains 1 and 2 Are Necessary—The bottom regions of VEGF and PIGF may contact the third extracellular Ig homology domain of VEGF receptors, and they have been implicated in receptor binding and specificity (4, 5). Surprisingly, however, the third domain of VEGF-3 was not required for VEGF-C binding, and instead, the first domain appeared necessary. Given that all VEGFs bind their receptors in a highly similar fashion, it appears unlikely that VEGF-C would interact directly with this domain of VEGFR-3. It has been proposed that domain 1 of VEGF-1 provides a shield for the hydrophobic surface of domain 2 in the absence of ligand (4). That the first extracellular domain of VEGF-3 could accomplish such function is supported by the fact that replacement of the first domain of VEGFR-3 by the corresponding domains of platelet-derived growth factor receptor $\alpha$ or colony stimulation factor-1 receptor resulted in the loss of protein secretion, indicating impaired protein folding.

The Role of Subunit Flexibility for Multiple Receptor Binding—In fragment 5, which comprises much of the presumed dimerization interface, 7 of 11 amino acid residues are conserved between VEGF and VEGF-C, most likely because of the constraints imposed by the cystine knot. VEGF-C-derived fragment 5 correlated strongly with VEGFR-3 binding but not with VEGFR-1 binding. In 56 of 67 VEGF-3 binding mosaic factors, this segment was derived from VEGF-C. The alanine scan of this fragment indicated cysteine 156 as the major important residue within fragment 5. Mutation of this cysteine residue into a serine residue abolished only VEGF-2 binding (10), whereas mutation to alanine resulted in the loss of binding to both VEGFR-2 and VEGFR-3. For the Cys$^{156}$→Ala mutant, the ratio of monomeric to dimeric molecules was 4.6 to 1, a similar value as reported for the corresponding Cys→Ser mutation in VEGF, which also abolishes most of the biological activity.

2 M. Jeltsch, and K. Alitalo, unpublished data.
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FIGURE 7. Schematic summary of receptor binding requirements of VEGF versus VEGF-C. A, ligand binding domains of VEGF receptors. Domain 2 of VEGFR-1 is sufficient for VEGF binding, whereas in VEGFR-2, both domains 2 and 3 are necessary (4). Domain 2 of VEGFR-2 is sufficient for VEGF-C binding, but in VEGFR-3, both domains 1 and 2 are required. The minimal ligand binding domains are shown in yellow. B, structural elements for receptor binding of VEGF and VEGF-C shown on a model of VEGF. The VEGF-C regions required and sufficient for VEGF-C binding but incompatible with VEGFR-1 binding are shown in green. VEGF regions required but not sufficient for VEGFR-1 binding and compatible with VEGFR-3 binding are shown in red. The promiscuous part of the receptor binding interface is shown in blue, and the van der Waals surface of receptor domain 2 is rendered in yellow. C, importance of individual amino acid residues from the specificity-determining regions of VEGF-C for VEGFR-2 and VEGFR-3 binding. Indicated are all residues resulting in a minimal ligand binding domain. Red represents the N-terminal helix of VEGF and the corresponding part of VEGF-C, and blue represents binding determinants compatible with all VEGF receptors.

Because the Cys^{156}_{Ser} mutant also showed a significant monomeric component with a ratio of 2:1, other factors are likely to contribute to the loss of activity of the Cys^{156}_{Ala} mutation.

Both growth factor ligands of VEGFR-3 are non-covalent dimers (34, 35). Thus, fragment 5 might be involved in rendering the groove between the two subunits of VEGF-C more accessible and/or more flexible than in VEGF, thereby enabling VEGF-C binding. However, the non-covalent mode of dimerization does not seem to be directly responsible for the higher accessibility, as several VEGF-3 binding mosaic molecules were covalent dimers (supplemental data).

The flexibility that allows variable exposure of the bottom groove of VEGF has been implicated in its ability to bind to different receptors (36). However, only the structures of VEGF-VEGFR-2 or VEGF-C-VEGFR-2 and VEGF-C-VEGFR-3 complexes can tell whether the subunit and loop flexibility of VEGFs participate in the receptor specificity determination. The absolute requirement for VEGF-derived fragments 2 and 7 for VEGF-1 binding and the existence of super-VEGFs support the view that receptor discrimination requires specific structural features separate from an overlapping receptor binding core interface, which might not allow receptor discrimination because of its high conservation. Such structures would include the N-terminal helices of PIGF/VEGF and specific features of the VEGF-C bottom face and would be complemented by specificity-determining structures of the receptors. Because VEGF-derived fragments 2 and 7 did not confer VEGFR-1 binding to VEGF-C (not even in combination with fragment 3), such specificity-determining structures might be accessory and not responsible for the majority of free energy change.

The Specificity-determining Regions of VEGF and VEGF-C Do Not Overlap—Because the specificity-determining structures did not overlap, we were able to create VEGF/VEGF-C mosaic molecules that interact with all three VEGF receptors. We were interested in seeing how such changes in the receptor interaction patterns would translate to biological function. In the CAM assay, which is well suited for the simultaneous evaluation of both the angiogenic and lymphangiogenic potential of growth factors (21, 37, 38), the biological responses toward the VEGF/VEGF-C mosaics were attenuated when compared with equal amounts of the parental molecules. This is not surprising given the library size and the fact that no optimization was performed on the molecules. The relative affinities toward VEGF-2 and VEGF-3 were always weaker than those of the parental molecules. Yet, the relative affinity toward VEGF-1 as well as the potency in the Ba/F3 bioassay appeared unaffected or even slightly increased for some VEGF-1 binding molecules. This is likely because of the function of the N-terminal helix for VEGF-1 binding. It carries several VEGF-1 binding determinants, whereas it is not an integral part of the cystine knot and the associated loops. Therefore, changes in the fine structure of the cystine knot with its loops (which likely comprise more of the binding determinants for VEGF-2 and VEGF-3) will have less impact on VEGF-1 binding than on VEGF-2 and -3 binding. The weaker biological activity is furthermore explained by the fact that the mosaic molecules contained only the receptor binding domain and no accessory domains. A similar difference in the biological potency between VEGF^{121}_{165} and VEGF^{121}_{165} has been attributed to the lack of the C-terminal domain in VEGF^{121}_{165}, which mediates the interaction with neuropilin (39, 40).
The lack of strong lymphangiogenic activity of mosaic 12–11 was unexpected, considering its ability to induce phosphorylation of VEGFR-3. Similarly unexpected was the weak angiogenic response of 84–11, despite its higher potency in activating VEGFR-2 when compared with 12–11, with the latter producing a strong angiogenic response. Whether this difference can be explained by their differential affinity for and activation of VEGFR-1 remains unclear, which highlights our incomplete understanding of the dual decoy versus signaling functions of VEGFR-1 in angiogenesis (25, 41, 42). For a few mutants, the changes in affinity, ligand binding, and biological response appeared at least partially uncoupled, something that has been previously described for the ErbB receptor family and its viral ligands (43). A similar mechanism of low level but sustained receptor activation could explain why the Pro198→Ala mutant performed well in the bioassay despite its high KC.

Angiogenesis or the lack thereof are key events in the development and progression of major pathological conditions, such as tumor growth or ischemic heart disease. Consequently, VEGF-mediated signaling is a major target for therapeutic intervention (44). Recently, it has become clear that VEGF-C also plays a key role in several diseases. Insufficient signaling via VEGFR-3 can be the cause of hereditary lymphedema type 1 (45), and VEGF-C can promote tumor dissemination via its lymphangiogenic effect (46–49). The mosaic molecules we have described provide a platform for applications that require the fine-tuning of proangiogenic or prolymphangiogenic signaling, whereas knowledge of the receptor interactions of VEGF-C can be utilized to design inhibitors of VEGF-C signaling.

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