Mapping of the Sites for Ligand Binding and Receptor Dimerization at the Extracellular Domain of the Vascular Endothelial Growth Factor Receptor FLT-1*  

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The vascular endothelial growth factor (VEGF) receptor FLT-1 has been shown to be involved in vasculogenesis and angiogenesis. The receptor is characterized by seven Ig-like loops within the extracellular domain. Upon VEGF binding FLT-1 becomes phosphorylated, which has been thought to be preceded by receptor dimerization. To further investigate high affinity binding of VEGF to FLT-1 and ligand-induced receptor dimerization, we expressed in Sf9 cells the entire extracellular domain comprising all seven Ig-like loops: sFLT-1(7) and several truncated mutants consisting of loop one, one and two, one to three, one to four, and one to five. The corresponding proteins, named sFLT-1(1), (2), (3), (4), (5), and (7) were purified. Only mutants sFLT-1(3) to (7) were able to bind 125I-VEGF with high affinity. No binding of VEGF was observed with sFLT-1(1) and sFLT-1(2), indicating that the first three Ig-like loops are involved in high affinity binding of VEGF. The binding of VEGF to sFLT-1(3) could be competed with placenta growth factor (PlGF), a VEGF-related ligand, suggesting that high affinity binding of VEGF and PlGF is mediated by the same or closely related contact sites on sFLT-1. Deglycosylation of the sFLT-1(3), (4), (5), and (7) did not abolish VEGF binding. Furthermore, unglycosylated sFLT-1(3), expressed in Escherichia coli, was able to bind VEGF with similar affinity as sFLT-1(3) or sFLT-1(7), both expressed in Sf9 cells. This indicates that receptor glycosylation is not essential for high affinity binding. Dimerization of the extracellular domains of FLT-1 upon addition of VEGF was detected with all mutants containing the Ig-like loop four. Although sFLT-1(3) was able to bind VEGF, dimerization of this mutant was inefficient, indicating that sites on Ig-like loop four are essential to stabilize receptor dimers.

The vascular endothelial growth factor (VEGF), a potent mitogen for endothelial cells, is an important angiogenic factor also involved in the differentiation of endothelial cells and the development of the vascular system (1, 2). It has been shown to be implicated in human diseases such as diabetic retinopathy, rheumatic arthritis, and cancer (3). VEGF in particular appears to be the most important angiogenic factor of many solid tumors, promoting vascularization and formation of metastases (4).

Four different VEGF isoforms have been described so far, all encoded by a single gene: VEGF121, VEGF165, VEGF189, and VEGF206 (5). All different isoforms are secreted dimeric proteins, sharing similarities with platelet-derived growth factor (PDGF) and belong to the family of growth factors containing a cysteine knot motif (6).

Two receptor tyrosine kinases, FLT-1 (7, 8) and KDR/FLK-1 (9, 10), have been identified, which bind VEGF with high affinity. Both receptors belong to the type III tyrosine kinases and are characterized by seven Ig-like loops within their extracellular domain and a split kinase domain within the cytoplasmic moiety (11). The Ig-like loop motif is a common feature of extracellular domains of membrane-anchored proteins. Members of the immunoglobulin superfamily are often involved in cell surface recognition (12). Both VEGF receptors contain several putative N-glycosylation sites and the apparent molecular weights of the mature proteins suggest that both receptors are extensively glycosylated (8, 10).

The activation of growth factor receptors in general is preceded by the formation of receptor dimers and subsequent receptor phosphorylation. The resulting phosphorytrosine residues are docking sites for signal coupling components such as SH-2 proteins (13). The molecular structures that are responsible for ligand/receptor interaction and ligand-induced dimerization are poorly understood for most receptors. Since the dimeric structure of VEGF is a prerequisite of receptor activation, it can be speculated that one VEGF molecule bridges two receptors via two similar recognition sites, as has been suggested for PDGF (14). Characterization of VEGF binding to its receptors by mutational analysis of the ligand supports the assumption that VEGF has two contact sites for its receptors (15).

Nothing is known about which part of the receptor is involved in ligand binding and which part of the receptor's extracellular domain is involved in VEGF-dependent dimerization. To investigate these questions, we generated several soluble mutants of the extracellular domain of FLT-1, each consisting of a different stretch of Ig-like loops. The data presented here suggest that the recognition site for VEGF is located on the first three Ig-like loops, whereas dimerization is stabilized due to an additional domain located on Ig-like loop four. Glycosylation was found not to be a prerequisite of high affinity binding of VEGF to FLT-1.
EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—SF9 cells were cultured routinely in 1-liter spinner flasks (Technomara, FRG) in ExCell 400 (JRH Bioscience, UK) without any further supplements. Human umbilical vein endothelial cells (HUVECs) were obtained from PromoCell (FRG) and cultured according to the provided protocol. VEGF_165 was expressed in Escherichia coli and purified as described previously (16). VEGF_165 was induced to a specific activity of 100,000 rcp/mg by using the chloramphenicol T method (17) (Immundiagnostik, FRG). PlGF_152 was expressed with the baculovirus/insect cell system as described previously (18). N-Glycosidase F and disuccinimidyl suberate (DSS) were obtained from Boehringer Mannheim (FRG), N-hydroxysuccinimidyl-biotin was from Pierce (FRG).

Expression and Purification of Soluble FLT-1 Receptor Mutants—Soluble FLT-1 (sFLT-1) fragments were cloned by polymerase chain reaction from the full-length cDNA clone 3–7 (7) using the upstream primer: 5'-GGAGTTTCCGCTCCATTTGTCAGC-3' and downstream primer: 5'-GGATCCCGCGCTCACCATGGTCAGC-3', containing an EcoRI site and various downstream primers, which all contain an NcoI site. The PCR products were isolated from agarose gel with the Quiax DNA gel extraction kit (Quiagen, FRG) and purified with the Qiaprep Spin Miniprep kit (Qiagen, FRG). The cDNA was cloned into the expression vector His-pET (16). Thus the expression plasmid was purified with the Quiax DNA gel extraction kit (Quiagen, FRG) and subcloned into the baculovirus transfer vector pVL1392 as EcoRi/BamHI fragments. Plasmids containing the cDNA were isolated from transfected bacteria and then used for transfection into SF9 cells along with wild-type baculovirus DNA. Recombinant baculoviruses were obtained using the BaculoGold™ transfection kit following standard protocol (Promega, Madison, WI). SF9 cell protein expression was induced at a density of 2×10⁶ cells/ml with 2 volumes of buffer D (6M urea, 0.5M cystamine, 0.1M trichloroacetic acid, 0.05% sodium deoxycholate and 1% Nonidet P-40) and recentrifuged. The pellet was resuspended in 25 ml of buffer A supplemented with 0.4 M NaCl for sFLT-1(3), or 0.6 M NaCl for sFLT-1(4), or 10 ml of 0.4 M NaCl for sFLT-1(5) and at 2407 g for 17 min, respectively. The polymerase chain reaction products were purified with the Qiagen kit (Quiagen, FRG) and concentrated with the Qiaprep Spin Miniprep kit (Qiagen, FRG). For bacterial expression, the plasmid construct was transfected into E. coli strain BL21 (DE3) carrying an inducible T7 RNA polymerase gene (19). Bacterial cultures of 250 ml of LB medium (pH 7.4) with 200 μM IPTG were grown in shaking flasks at 37 °C. The mixture was incubated at 95 °C for 2 min. Then 10 μl of 2× reaction buffer were added (100 mM sodium phosphate, pH 7.2, 20 mM EDTA, 1% Nonidet P-40) and again incubated at 95 °C for 2 min. After cooling down, N-glycosidase F (0.2 unit/assay) was added, and the mixture was incubated at 37 °C overnight with 4× SDS sample buffer, and proteins were resolved by SDS-PAGE.

Antibodies and Western Blot Analysis—Approximately 50 μg of sFLT-1(7)-145 dissolved in PBS and emulsified with Freund’s complete adjuvant were injected both intraperitoneally and subcutaneously into Lou/C rats and again as a booster 8 weeks after the first immunization. Purified Plgf_152 was expressed in E. coli and purified as described previously (20). Supernatants were screened for anti-sFLT-1 antibodies using a solid phase enzyme-linked immunosorbent assay. The mAb 7A6 was able to detect the various sFLT-1 mutant proteins and was characterized as a rat IgG2a. For Western analysis, aliquots (20 μl) of the elution fractions of the heparin-Sepharose columns were resuspended in SDS sample buffer and resolved by SDS-PAGE. The gels were electroblotted onto a polyvinylidine difluoride membrane, and blots were blocked for 30 min with 5% milk powder in TBST (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) and incubated for 1 h in the same buffer with 1 μg/ml mAb 7A6. The blots were washed twice in water and TBST and incubated for 45 min in TBST containing a 5000-fold diluted peroxidase-conjugated goat-anti-rat IgG (Jackson Immunoresearch Labs., Inc., West Grove, PA). The blots were washed as described before, and peroxidase-coupled antibodies were visualized using the ECL chemiluminescent Western blotting detection system (Amersham, FRG).

Ligand Blotting—For ligand blotting, 0.5 μg of purified sFLT-1 mutant proteins was mixed with nonreducing SDS sample buffer, and SDS-PAGE was performed. After blotting the gel onto a polyvinylidene difluoride membrane and the mixture was incubated at 37 °C overnight, the reaction was stopped and the blot was washed twice in water and TBST and incubated for 45 min in TBST containing a 5000-fold diluted peroxidase-conjugated goat-anti-rat IgG. The blots were washed twice as described before, and peroxidase-coupled antibodies were visualized using the ECL chemiluminescent Western blotting detection system (Amersham, FRG).

Bacterial Expression and Purification of sFLT-1(3)—A 0.9-kilobase pair NcoI/BamHI fragment encoding amino acids 31–338 of human FLT-1 was generated by polymerase chain reaction and ligated into the bacterial expression vector His-pEL (16). Thus the expression plasmid encoded sFLT-1 amino acids 31–338 fused to an N-terminal 6× His-tag and amino acids Met and Glu, which were derived from the bacterial NcoI site. For bacterial expression, the plasmid construct was transformed into E. coli strain BL21 (DE3) carrying an inducible T7 RNA polymerase gene (19). Bacterial cultures of 250 ml of LB medium containing 100 μg/ml ampicillin were grown in shaking flasks at 37 °C to an A₆₀₀ of 0.9. Isopropyl-b-D-thiogalactoside was added to a final concentration of 0.5 mM, and the culture was grown for 4 h at 37 °C. Cells were harvested and washed, and the pellet was frozen at −80 °C. Upon use, cells were thawed at 37 °C, resuspended in 25 ml of buffer A (50 mM Tris-HCl, 10 mM 2-mercaptoethanol, 2 mM EDTA, 5% (v/v) glycerol, 0.2 mM EDTA, 0.01% DMSO, pH 8.0) and incubated for 30 min at 22 °C. The suspension was sheared by five high-speed treatments of 20 s in an Ultra-Turrax dispersing apparatus and incubated for 10 min at 22 °C. The mixture was cooled on ice and sonicated six times for 15 s with the microtip of a Bronson Sonifier 250. After the addition of sodium deoxycholate and Nonidet P-40 to a final concentration of 0.05% and 1% (w/v), respectively, the mixture was incubated for 10 min at 4 °C and then centrifuged at 10,000 rpm for 30 min at 4 °C. The pellet was resuspended in 25 ml of buffer A supplemented with 0.05% sodium deoxycholate and 1% Nonidet P-40 and recentrifuged. The inclusion body pellet was solubilized in 25 ml of buffer B (6 M guanidine HCl, 0.15 M NaCl, 0.1 mM dithiothreitol, 50 mM NaN₃PO₄ buffer at pH 6.5). The solubilized protein was dialyzed twice against 250 ml of buffer C (6 M urea, 0.1 mM dithiothreitol, 50 mM MES at pH 5.5) at 4 °C, and the volume of the dialysate was reduced 20-fold by ultrafiltration (Millipore Ultrafree-15). The concentrated protein solution was mixed with solution of buffer D (0.1 M sodium phosphate, 20 mM Hepes at pH 7.4) and incubated for 4 h at 4 °C with gentle agitation. For refolding, the solution was sequentially diluted to final 0.3 M urea by the addition of PBS, 50 mM glycine. The refolded sFLT-1(3) protein was concentrated by ultrafiltration and referred to as sFLT-1(3)₀.₅.₇.

Deglycosylation of Proteins—For deglycosylation of recombinant sFLT-1 mutants, N-glycosidase F was used. SDS (final concentration, 0.3%) was added to sFLT-1 proteins (250–500 ng in 10 μl total volume), and the mixture was incubated at 95 °C for 2 min. Then 10 μl of 2× reaction buffer were added (100 mM sodium phosphate, pH 7.2, 20 mM EDTA, 1% Nonidet P-40) and again incubated at 95 °C for 2 min. After cooling down, N-glycosidase F (0.2 unit/assay) was added, and the mixture was incubated at 37 °C overnight with 4× SDS sample buffer, and proteins were resolved by SDS-PAGE.

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RESULTS

Recombinant Expression of sFLT-1 Mutants—To express the soluble extracellular domain of the VEGF receptor FLT-1 and its truncated mutants, constructs were made by introducing stop codons at various sites of the flt-1 cDNA as shown in Fig. 1. The resulting cDNAs were cloned into the baculovirus transfer vector pVL1392, and all proteins were expressed in Sf9 insect cells. Since all constructs contain the N-terminal leader sequence but lack the transmembrane domain, the expressed proteins were expected to be secreted from the infected Sf9 cells. To enable detection of the expressed proteins, monoclonal antibodies had been raised against the extracellular domain of FLT-1. The monoclonal antibody 7A6 was found to detect all sFLT-1 mutant proteins, was expressed and secreted (Fig. 2). Western blot analysis of partially purified sFLT-1 proteins from conditioned media of infected Sf9 cells confirmed that all FLT-1 mutants were expressed and secreted (Fig. 2). The apparent molecular masses were estimated from SDS-PAGE to be 16 kDa for sFLT-1(1), 28 kDa for sFLT-1(2), 45 kDa for sFLT-1(3), 57 kDa for sFLT-1(4), 72 kDa for sFLT-1(5), and 105 kDa for sFLT-1(7). sFLT-1(1) and sFLT-1(2) matched more or less the calculated molecular masses deduced from the amino acid sequence, whereas the estimated size of sFLT-1(3) to sFLT-1(7) was increased by about 10–20 kDa as compared with the calculated molecular mass. This is most likely due to N-linked sugars since the extracellular domain contains 12 putative glycosylation sites (see Fig. 1).

Binding of VEGF and PlGF to sFLT-1 Mutants—To investigate whether the expressed sFLT-1 mutant proteins are able to bind VEGF, we used a solid phase binding assay on microtiter plates. Specific binding of VEGF could be observed to all sFLT-1 mutants containing the first three N-terminal Ig-like loops: sFLT-1(1), (2), (3), (4), (5), and (7). No specific VEGF binding could be obtained with sFLT-1(1) and sFLT-1(2) containing the first or the first and the second Ig-like loops, respectively (Fig. 3, inset). In a competition experiment, using increasing amounts of unlabeled VEGF, comparable high affinity binding of VEGF to all sFLT-1 mutants containing the first three Ig-like loop could be demonstrated (Fig. 3). To make sure that lack of ligand binding of the two shorter mutants sFLT-1(1) and sFLT-1(2) was not due to inefficient immobilization to the microplates, we performed a cellular binding assay with HUVECs that express both VEGF receptors KDR and FLT-1 (21). All sFLT-1 mutants containing the first three Ig-like loops were able to compete efficiently with VEGF binding to HUVECs, the mutants sFLT-1(1) and (2), which failed to bind VEGF in the solid phase assay, also failed to compete with VEGF binding to endothelial cells (Fig. 4). The slight decrease of total binding observed with the sFLT-1(1) and sFLT-1(2) preparations is statistically not significant.

PlGF, similar to VEGF, is a disulfide-bridged homodimer and shares about 30% identity with VEGF (18, 22). PlGF has been found to bind to FLT-1 and to displace VEGF from this
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To test whether this is also true for the soluble extracellular domain of FLT-1, we performed the solid phase binding assay (Fig. 5). PIGF152 competed with VEGF165 for binding to sFLT-1(3) and (7). No difference could be detected between the full-length extracellular domain and sFLT-1(3), the shortest sFLT-1 mutant still exhibiting VEGF binding. From this we conclude that PIGF and VEGF share similar contact sites on the receptor.

Inhibition of VEGF-mediated DNA Synthesis by sFLT-1 Mutants—Since HUVECs proliferate in response to VEGF, we investigated the ability of the sFLT-1 mutants to antagonize VEGF-mediated incorporation of [3H]thymidine into HUVECs (Fig. 6). A dose-dependent inhibition of VEGF-stimulated DNA synthesis could be observed with sFLT-1(3), (4), (5), and (7), confirming the results from the ligand binding studies. Addition of sFLT-1(1) and sFLT-1(2) had only a minor VEGF-antagonizing effect. From these experiments we conclude that the presence of Ig-like loop three is a prerequisite for high affinity binding of VEGF to its receptor.

Effect of Glycosylation on VEGF Binding—The presence of putative N-glycosylation sites (Fig. 1) and the obvious differences between the apparent and calculated molecular weights of the sFLT-1 mutants (3) to (7) suggest that those sFLT-1 mutants, which are able to bind VEGF, are released from Sf9 cells as glycosylated proteins. To investigate whether glycosylation has any influence on VEGF binding, we incubated the recombinant sFLT-1 mutants with N-glycosidase F. The N-glycosidase F-treated proteins and appropriate controls were subjected either to Western blot analysis or ligand blotting (Fig. 7). The Western blot analysis revealed a significant decrease of the apparent molecular weight due to the glycosidase treatment, demonstrating that the polypeptides contain N-linked sugar residues (Fig. 7A). Incubation with radiolabeled 125I-VEGF identified both the glycosylated and deglycosylated polypeptides as VEGF binding proteins (Fig. 7B). In several preparations we detected high molecular weight complexes in the absence of VEGF with the mAb 7A6. The appearance of these complexes varied in individual preparations of different sFLT-1 mutants. An example is shown for sFLT-1(5) in Fig. 7, A and B (arrowheads). The reason for this complex formation is unclear. Since this effect was most obvious on nonreducing SDS gels, we speculate that the additional bands might be due to incorrect formation of disulfide bridges during the recombinant protein expression and partial proteolytic degradation.

To confirm that VEGF binding does not depend on glycosylation, we used a different experimental approach, expressing the sFLT-1 sequence corresponding to amino acid Asp31 to receptor (23–25). To test whether this is also true for the soluble extracellular domain of FLT-1, we performed the solid phase binding assay (Fig. 5). PIGF152 competed with VEGF165 for binding to sFLT-1(3) and (7). No difference could be detected between the full-length extracellular domain and sFLT-1(3), the shortest sFLT-1 mutant still exhibiting VEGF binding. From this we conclude that PIGF and VEGF share similar contact sites on the receptor.

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DISCUSSION

We expressed different truncated mutants of the extracellular domain of the VEGF receptor FLT-1 to get further insight into the structure/function relationship for VEGF binding and receptor dimerization. The baculovirus/insect cell expression system has already been used to express a soluble extracellular domain of FLT-1, consisting of the Ig-like loops one to six, which retained the full capacity of VEGF binding (26). We used the same expression system to express different sFLT-1 mutants that were constructed by introducing artificial stop codons into appropriate positions, resulting in cDNAs for Ig-like loop one, Ig-like loop one and two, Ig-like loop one to three, Ig-like loop one to four, Ig-like loop one to five, and Ig-like loop one to seven. Monoclonal antibodies were raised against the extracellular domain of FLT-1 to confirm protein expression and to follow protein purification. mAb 7A6 was able to detect all different constructs, indicating that its binding epitope is located within the first N-terminal Ig-like loop (Fig. 2).

To investigate binding of VEGF to the various sFLT-1 mutants, we used either a solid phase binding assay or an endothelial cell-based receptor binding assay. High affinity binding of VEGF to sFLT-1(3), (4), (5), and (7) could be observed with the solid phase binding assay. The same sFLT-1 mutants were also able to compete with VEGF receptors on HUVECs for VEGF binding. This is in agreement with the results obtained with the solid phase binding assay (compare Figs. 3 and 4). No binding of VEGF to sFLT-1(1) or (2) containing only the first, or the first and second N-terminal Ig-like loops, respectively, could be detected in the solid phase assay, and only a very weak competition, if any at all, was observed in cell-based binding assay (Figs. 3 and 4). Furthermore, VEGF-stimulated DNA synthesis in HUVECs was inhibited in a dose-dependent fashion by all sFLT-1 mutants except sFLT-1(1) and (2). This confirms our conclusion that the contact site(s) of VEGF is(are) located within the first three N-terminal Ig-like loops of sFLT-1.

We further hypothesize that especially Ig-like loop three contributes to VEGF binding, since the deletion of this loop completely abolishes VEGF binding. Similar results have been reported by others for c-Kit and the PDGF receptor, two receptors related to the VEGF receptors and consisting of five Ig-like loops in their extracellular domain. In each case ligand binding could be mapped to the first three Ig-like loops (27–29). Further mutational analysis for the PDGF receptor type α revealed that high affinity binding of PDGF is mediated by Ig-like loops two and three and that loop one defines specificity between PDGF AA and PDGF BB (27).

PIGF, a growth factor related to VEGF, has been described to
bind to FLT-1 (23–25), and heterodimers of VEGF/PlGF have been found to activate VEGF receptors (30). Competition of PlGF with VEGF binding to FLT-1 has been found by others, suggesting that PlGF and VEGF share similar receptor recognition sites (23–25). We confirmed these results by testing the influence of PlGF on VEGF binding to sFLT-1 proteins in the solid phase binding assay (Fig. 5). PlGF was able to displace VEGF from both the entire extracellular domain sFLT-1(7) and sFLT-1(3), the shortest deletion mutant which retained VEGF binding.

The presence of potential N-glycosylation sites within the extracellular domain of FLT-1 and the difference between the apparent molecular weights obtained from SDS-PAGE and the calculated molecular weight, both suggest that sFLT-1 and larger sFLT-1 mutants were secreted as glycosylated proteins from infected Sf9 cells (Figs. 1 and 2). We therefore addressed the question, whether glycosylation of FLT-1 participates in the recognition of VEGF. Two lines of evidence demonstrate that glycosylation is not a prerequisite for ligand binding. First, enzymatic deglycosylation of sFLT-1 mutants, using N-glycosidase F, did not abolish VEGF binding (Fig. 7), and second, sFLT-1(3) expressed in E. coli retained the ability to bind VEGF (Fig. 8).

It has been shown, for a variety of receptor tyrosine kinases, that receptor activation is preceded by receptor dimerization (for review, see Heldin (13)). The ability of recombinant-purified extracellular receptor domains to dimerize in the presence of the appropriate ligand has already been described for the PDGF receptor α and β (14). To investigate whether the soluble extracellular domain of FLT-1 can dimerize and whether dimerization is VEGF-dependent, we performed chemical cross-linking experiments of sFLT-1 and VEGF complexes. Western analysis identifying sFLT-1 mutants with the monoclonal antibody 7A6 or the use of radiolabeled VEGF revealed that soluble receptor mutants sFLT-1(4) and (5) are able to form dimers in the presence of VEGF, whereas sFLT-1(3) associated predominantly in its monomeric form with VEGF (Fig. 9). This leads us to the conclusion that Ig-like loop four is of particular importance for the formation of VEGF-mediated receptor dimerization. Similar observations have been reported for c-Kit, a receptor consisting of five Ig-like loops on the extracellular part. Ig-like loop four of c-Kit has been identified as an intrinsic ligand-dependent dimerization site. Receptor mutants of c-Kit lacking Ig-like loop four retained their capacity of ligand binding but no longer formed receptor dimers (31). No significant differences have been observed with respect to the affinity for VEGF binding to all sFLT-1 mutants (Figs. 3 and 4). Thus it is unlikely that Ig-like loop four has a strong impact on ligand recognition. However, it might be responsible for a direct interaction of two sFLT-1 monomers. Since efficient dimerization requires the presence of VEGF, presentation of the active dimerization site seems to be the consequence of a VEGF-dependent conformational change. Unlike the other Ig-like loops, loop four lacks the disulfide bridge, which is thought to stabilize the two β sheets of the Ig-like loop structure. Thus Ig-like loop four might gain additional flexibility. However, participation of other domains involved in formation of receptor dimers cannot be excluded.

The results presented here clearly suggest that VEGF binding can be attributed to the first three N-terminal Ig-like loops.
and that Ig-like loop four is involved in the formation of receptor dimers.

Addendum—While this work was under review, the participation of the first three Ig-like loops of FLT-1 in VEGF binding has been confirmed by Davies-Smyth et al. (32).

REFERENCES
1. Carmeliet, P., Ferreira, V., Breier, G., Pollefy, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenheuvel, A., Harpal, K., Eberhardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W., and Nagy, A. (1996) Nature 380, 435–439
2. Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., Powell-Braxton, L., Hillan, K. J., and Moore, M. W. (1996) Nature 380, 439–442
3. Folkman, J. (1995) Nat. Med. 1, 27–31
4. Martiny-Baron, G., and Marme, D. (1995) Curr. Opin. Biotechnol. 6, 675–680
5. Ferrara, N. (1995) Breast Cancer Res. Treat. 36, 127–137
6. McDonald, N. Q., and Hendrickson, W. A. (1993) Cell 73, 421–424
7. Shibuya, M., Yamaguchi, S., Yamane, A., Ikeda, T., Tojo, A., Matsushime, H., and Sato, M. (1990) Oncogene 5, 519–524
8. de Vries, C., Rccobedo, J. A., Ueno, H., Houck, K., Ferrara, N., and Williams, L. T. (1992) Science 255, 989–991
9. Terman, B. I., Dougher-Vermazen, M., Carrion, M. E., Dimitrov, D., Armellino, D. C., Gospodarowicz, D., and Bohlen, P. (1992) Biochem. Biophys. Res. Commun. 187, 1579–1586
10. Millauer, B., Wizigmann-Voos, S., Schnurich, H., Martinez, R., Moller, N. P. H., Risau, W., and Ulbrich, A. (1993) Cell 72, 835–846
11. Fantl, W. J., Johnson, D. E., and Williams, L. T. (1993) Annu. Rev. Biochem. 62, 453–481
12. Williams, A. F., and Barlay, A. N. (1988) Annu. Rev. Immunol. 6, 381–405
13. Heldin, C.-H. (1995) Cell 80, 213–223
14. Herren, B., Rooney, B., Weyer, K. A., Iberg, N., Schmid, G., and Pech, M. (1993) J. Biol. Chem. 268, 15088–15095
15. Keyt, B. A., Nguyen, H. V., Berleas, L. T., Duarte, C. M., Park, J., Chen, H., and Ferrara, N. (1996) J. Biol. Chem. 271, 5638–5646
16. Siemeister, G., Schnurr, B., Mohrs, K., Schachttele, C., Marmé, D., and Martiny-Baron, G. (1996) Biochem. Biophys. Res. Commun. 222, 249–255
17. Hunter, W., and Greenwood, F. C. (1962) Nature 194, 495–496
18. Hauser, S., and Weich, H. A. (1993) Growth Factors 9, 259–268
19. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorf, J. W. (1990) Methods Enzymol. 185, 60–89
20. Kremmer, E., Kranz, B. R., Hille, A., Klein, K., Eulitz, M., Hoffmann-Fezer, G., Feiden, W., Hermann, K., Delecluse, H. J., Delso, G., Borkammm, G. W., Mueller-Lantzsch, N., and Grasser, F. A. (1995) Virology 206, 336–342
21. Barleon, B., Hauser, S., Schöllmann, C., Weindel, K., Marmé, D., Yayon, A., and Weich, H. A. (1994) J. Cell. Biochem. 54, 56–66
22. Maglione, D., Guerriero, V., Viggio, G., Delli-Bovi, P., and Persico, M. G. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8967–8971
23. Kendall, R. L., Wang, G., DiSalvo, J., and Thomas, K. A. (1994) Biochem. Biophys. Res. Commun. 201, 326–330
24. Park, J. E., Chen, H. H., Winer, J., Houck, K. A., and Ferrara, N. (1994) J. Biol. Chem. 269, 29646–29654
25. Sawano, A., Takahashi, T., Yamaguchi, S., Aonuma, M., and Shibuya, M. (1996) Cell Growth Differ. 7, 213–221
26. Kendall, R. L., and Thomas, K. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10705–10709
27. Mahadevan, D., Yu, J.-C., Saldanha, J. W., Thanki, N., McPhie, P., Uren, A., LaRochele, W. J., and Heidaran, M. A. (1995) J. Biol. Chem. 270, 27593–27600
28. Blechman, J. M., Levi, S., Brizzi, M. F., Leitner, O., Pegoraro, L., Givol, D., and Yarden, Y. (1993) J. Biol. Chem. 268, 4399–4406
29. Levin, S., Blechman, J., Nishikawa, S.-I., Givol, D., and Yarden, Y. (1993) Mol. Cell Biol. 13, 2224–2234
30. Cao, Y., Chen, H., Zhou, L., Chiang, M.-K., Anad-Apte, B., Weatherbee, J. A., Williams, A., Fang, P., Flanagan, J. G., and Tsang, M. L.-S. (1996) J. Biol. Chem. 271, 3154–3162
31. Blechman, J. M., Levy, S., Barg, J., Eisenstein, M., Vaks, B., Vogel, Z., Givol, D., and Yarden, Y. (1995) Cell 80, 103–113
32. Davies-Smyth, T., Chen, H., Park, J., Presta, L. G., and Ferrara, N. (1996) EMBO J. 15, 4919–4927
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