Placental growth factor (PIGF) is a member of the vascular endothelial growth factor (VEGF) family and plays an important role in pathological angiogenic events. PIGF exerts its biological activities through binding to VEGFR1, a receptor tyrosine kinase that consists of seven immunoglobulin-like domains in its extracellular portion. Here we report the crystal structure of PIGF bound to the second immunoglobulin-like domain of VEGFR1 at 2.5 Å resolution and compare the complex to the closely related structure of VEGF bound to the same receptor domain. The two growth factors, PIGF and VEGF, share a sequence identity of ~50%. Despite this moderate sequence conservation, they bind to the same binding interface of VEGFR1 in a very similar fashion, suggesting that both growth factors could induce very similar if not identical signaling events.

Angiogenesis, the process of new blood vessel formation, is a complex process that involves a number of different growth factors. It is essential for a variety of physiologically important events such as embryogenesis, wound healing, and tissue repair but is also critical in a number of diseases such as tumor progression, psoriasis, rheumatoid arthritis, and diabetic retinopathy (1, 2). Consequently, the molecules that induce or mediate angiogenic events are potentially important targets for the treatment of these diseases. VEGF-A (or VEGF), the most important inducer of angiogenesis, is also the founding member of the family of vascular endothelial growth factors (3). This family of structurally and functionally related growth factors includes VEGF-A (VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PIGF). The members of the VEGF family are responsible for an array of angiogenic, vasculogenic, and lymphangiogenic processes and mediate their function mainly through differential binding to the three homologous receptor tyrosine kinases VEGFR1 (also called flt-1), VEGFR2 (or KDR), and VEGFR3. These receptors are a subclass of the platelet-derived growth factor (PDGF) receptor family, but whereas the extracellular domain of the PDGF receptors have five Ig-like domains, the ectodomains of the VEGFRs contain a total of seven Ig-like domains. The extracellular portion of all receptor tyrosine kinases is connected to the intracellular tyrosine kinase domain via a single transmembrane helix.

VEGF, the most potent angiogenic factor of the VEGF family, signals through binding to VEGFR1 and VEGFR2. A number of studies have identified VEGFR2 as the key signaling receptor mediating the proliferative effects of VEGF (1, 3). The role of VEGFR1, the only tyrosine kinase receptor for PIGF, is still less well understood. Mice devoid of VEGFR1 develop endothelial cells but die because of severe disorganization of the vascular system (4). In contrast, mice that express only a tyrosine kinase-deficient variant of the receptor are viable and do not display impaired embryogenesis (5). PIGF is specific for VEGFR1, and studies of PIGF isoforms showed that it has only limited mitogenic activity but that it augments and potentiates the activity of low concentrations of VEGF in vitro and in vivo (6). Therefore it has been suggested that VEGFR1 acts as a nonsignaling reservoir for VEGF during development or at least, under certain conditions, as a decoy receptor (7). Recent studies showed that VEGFR1 is expressed not only on vascular endothelial cells but is also present on the surface of pluripotent stem cells. PIGF is specific for VEGFR1 and can reconstitute hematopoiesis by recruiting these stem cells from the bone marrow (8). It was further shown that selective activation of VEGFR1 induces proliferation of hepatocytes rather than endothelial cells (9). Interestingly, PIGF is not required for embryonic angiogenesis, as mice that lack PIGF are healthy and unaffected by the mutation (10). However, PIGF-deficient mice display attenuated responses to VEGF in pathological angiogenesis (11), and PIGF is necessary for angiogenic events in adult tissues during ischemia, inflammation, wound healing, and cancer (10). Antibodies against VEGFR1 suppress neovascularization in tumors and ischemic retina, angiogenesis, and inflammatory joint destruction in autoimmune arthritis (12). These findings make PIGF and its receptor, VEGFR1, potentially attractive targets for modulation of inflammation and angiogenesis.

All members of the VEGF family are secreted dimeric glycoproteins. As a result of alternative splicing events, PIGF and VEGF appear in a number of isoforms. So far three isoforms of mature human PIGF have been reported (13, 14). The shortest isoform of PIGF, PIGF-1, comprises 131 residues in its mature form and, like the corresponding isoform VEGF121 of VEGF, entails the receptor-binding domain. PIGF-2 has an insertion of...
21 residues near its C terminus and like the longer VEGF-isoforms is able to bind heparin. PlGF-3 has an insertion of 72 amino acids near the C terminus of PlGF-1 but, unlike PlGF-2, does not bind to heparin (14).

Although the structures of the VEGF (15) and PlGF (16) receptor-binding domains in their free forms have been reported previously, only the complex between VEGF and VEGFR1 has been available until now (17). Here we describe the crystal structure of the minimal ligand binding fragment of VEGF co-complexed with a short ligand binding domain of PlGF and compare it with the complex between VEGFR1-d2 and VEGF. The structure and comparison of both complexes should shed light on the differences in the downstream effects of VEGFR1 activation via PlGF and VEGF (18).

**EXPERIMENTAL PROCEDURES**

Expression, Refolding, and Purification—VEGFR constructs were expressed, purified, and tested for functional integrity as described (17, 19). VEGFR-d1–7 and -d23 were expressed in mammalian cell by transient transfection (19), and VEGFR-d2 was expressed in *Escherichia coli* and refolded as reported (17). A construct comprising residues 19–116 of PlGF-2, corresponding to the receptor-binding domain, was expressed as insoluble protein in *E. coli*. Inclusion bodies were isolated by passing homogenized cells in 20 mM Tris-HCl (pH 7.5) through a French pressure cell and centrifuging the homogenate for 15 min at 4000 × g. The inclusion bodies were resuspended and the centrifugation repeated. The pellet, consisting primarily of PlGF, was dissolved in 6 M urea, 20 mM Tris-HCl, pH 7.5, and stirred for 1 h in 20 mM dithiothreitol. The protein solution was diluted to a concentration of 0.75 mg/ml in 0.02% NaN3 and 0.1 M bis-Tris propane (BTP), pH 6.5, was mixed with temperature using the hanging drop method. Crystallization buffer containing 0.15 M NaCl, 10 mM imidazole (pH 6.5), and 3% polyethylene glycol 8000 in liquid nitrogen.

**TABLE I**

| Binding affinities | IC50 (nM) |
|-------------------|----------|
| VEGFR1-d2         | 3.0      |
| VEGFR1-d2-3       | 1.3      |
| VEGFR1-d1-7       | 0.024    |
| VEGFR1-19-116     | 275      |

Affinity of the PlGF Binding Domain to VEGFR1—Based on sequence alignments and the crystal structure of VEGF in complex with VEGFR1-d2 (17) a construct that included only the minimal receptor-binding domain of PlGF was designed. The resulting protein, PlGF19-116, consists of residues 19–116. VEGFR1 consists of seven immunoglobulin-like domains in its extracellular portion and binds to PlGF with sub-nanomolar affinity (6).

To establish the minimal ligand binding domain toward PlGF, we tested three constructs of VEGFR1 containing the entire ectodomain (VEGFR1-d1–7), domains 2 and 3 (VEGFR1-d2–3), and domain 2 only (VEGFR1-d2) in Biacore competition assays. In these assays, the longest construct, containing the entire ectodomain of the receptor, binds PlGF19-116 with an IC50 of 0.5 nM. The affinity of PlGF19-116 toward the VEGFR1-d2 construct is only about 2-fold weaker, whereas VEGFR1-d2, the shortest construct tested, bound to the ligand with an IC50 of about 270 nM and therefore binds PlGF19-116 more than 500-fold weaker than the full-length receptor (Table I).

Quality of the Model—The crystal structure of the VEGF-VEGFR1-d2 complex was determined at 2.5 Å resolution. The asymmetric unit contains one full complex comprising two molecules of VEGFR1-d2 bound to a PlGF homodimer, one molecule of BTP, and 161 solvent molecules. The model of PlGF comprises residues 22–115 for one molecule and residues 21–115 for the second (residue numbers refer to the sequence of the mature protein and differ by 1 compared with the numbering used by Iyer et al. (16)). Both copies of VEGFR1-d2 contain residues 133–224. The structure was refined to an R-value of 19.4% (Rfree 26.0%) using all reflections between 20 and 2.45 Å resolution (Table II). Of the 323 non-glycine and non-proline residues, 89.2% have their main chain torsion angles in the “most-favorable” and 10.5% in the “additionally allowed” regions of the Ramachandran plot (25). A single residue lies in the “generously allowed” region.

Overall Structure of PlGF and VEGFR1—The overall architecture of PlGF in complex with VEGFR1-d2 strongly resembles the complex between VEGF and same receptor fragment. The center of the complex is formed by the PlGF homodimer. Both receptor fragments are bound to the distant poles of this dimer giving the entire complex the dimensions 85 × 40 × 35 Å (Fig. 1).
The overall structure of the receptor-binding fragment of PlGF in complex with VEGFR1-d2 is very similar to previously reported structures of PlGF or VEGF (15, 16, 17). Like all members of the cystine knot family of growth factors, the PlGF monomer has an elongated shape with two pairs of twisted, antiparallel, two-stranded $\beta$-sheets in its central portion (strands A, B, C, and D) and the characteristic cystine knot motif, as well as the N and C termini on one end of the molecule (26). The cystine knot is formed by three disulfide bridges. In this motif, two disulfide bridges together with the protein backbone form a closed ring that is penetrated by the third disulfide bridge. Like the related VEGF, PlGF has an N-terminal helix (a1). The protein segment connecting strands A and B includes residues 43–58 and contains a single turn of $\alpha$-helix (a2) as well as a short additional $\beta$-strand (A') that forms hydrogen bonds with strand C. The hydrogen bonding pattern between strand C and D is interrupted so that strand D is broken into two shorter $\beta$-strands, named strands D and D'. The BC loop and the CD loops are both relatively short and span residues 65–74 and 91–97, respectively (Fig. 1).

In the biologically active dimer, two PlGF monomers are assembled in an antiparallel manner and are covalently connected through the formation of two disulfide bonds between residues Cys$^{59}$ and Cys$^{65}$. This homodimer, which has a rather unusual shape, can be described as a curved sheet that is about 70 Å long and 35 Å wide but less than 15 Å thick in its central portion (Fig. 1). A total of 2700 Å$^2$ is buried in the interface between the two PlGF monomers. A large portion of this interface is formed by the N-terminal helices of PlGF, which pack on top of the respective other dimer within the complex and thus stabilize the dimeric assembly.

The overall architecture of the complex between ligand and receptor is dictated by the internal 2-fold symmetry of the PlGF dimer. The two receptor fragments of VEGFR1 bind on the opposing edges of the PlGF dimer with the closest distance between any two atoms between both receptor fragments of about 30 Å. VEGFR1-d2 is a member of the I-set of Ig-like domains (17). Generally, Ig-like domains consist of about 100 residues, which form two $\beta$-sheets that fold against each other to form a $\beta$-sandwich. In VEGFR1-d2 one $\beta$-sheet is formed by strands A', G, F, C, and C' and the other contains strands B, E, and D. One disulfide bond forms part of the hydrophobic core and connects strands B and F, thus stabilizing the fold of the domain.

The two halves of the complex are almost identical and superimposing the entire complex onto itself according to the internal 2-fold symmetry results in an r.m.s.d. of 0.75 Å for 372 Ca positions with the largest deviations occurring in regions that are also flexible in the unbound VEGFR1-d2 (27).

**Interface**—The total surface area buried in each of the interfaces between PlGF and VEGFR1-d2 amounts to $\sim$1650 Å$^2$. Both PlGF monomers participate in the binding of each receptor with one monomer contributing about 70% and the other 30% of the buried surfaces. Each receptor molecule is in contact with five segments of the PlGF dimer. Three of these, including the N-terminal helix of PlGF (residues 24–33), the loop connecting strands B and C, and the C-terminal residues 110–114, stem from one PlGF monomer; the other two segments, including residues of the AB loop (residues 54–56) and the CD loop (residues 87–99), stem from the other PlGF monomer.

On the VEGFR1-d2 side, residues from four distinct seg-
ments of amino acids form the interface. They include residues 140–147 from the N-terminal strand A, residues 171–175 from strand C and the loop connecting to strand C', residues 199–204 from strand F and the short helix following it, as well as residues 217–224 from strand G (Fig. 1). The C terminus of the last VEGFR1-d2 residue with defined electron density, Arg224, projects toward a groove formed by the AB loop of one PlGF monomer and the CD loop of the second monomer suggesting that the linker connecting VEGFR1-d2 to the third domain of the intact ectodomain might also contribute to the formation of the complex.

The interface is largely of hydrophobic nature with no apparent “knob-into-hole” interactions. Hydrophobic residues constitute ~50% of the interface, with nine leucine and isoleucine residues being responsible for almost one-third of the buried surface. There are only three direct polar interactions; one hydrogen bond is formed between the side chain of PlGF residue Gln26 and the main chain carbonyl of Glu141 of VEGFR1-d2, and two charged interactions occur between Asp71 of PlGF and Arg224 of VEGFR1-d2. In addition several hydrogen bonds are mediated by a bound BTP molecule and a number of water molecules.

**bis-Tris Propane Bound in the Interface**—In addition to one full complex between PlGF and VEGFR1-d2, the asymmetric unit contains a single molecule of BTP. The buffer molecule is near the interface between PlGF and VEGFR1-d2 and located on a 2-fold noncrystallographic symmetry (NCS) axis, with the NCS axis going through the central atom of the propane unit. Thus both of the tris(hydroxymethyl)methylamino head groups form nearly identical interactions with the proteins. Each head group is involved in hydrogen bond formation to Glu141 of the receptor domain and to Gln26 and Glu201 of VEGF (Fig. 2). Interestingly, the BTP molecule is therefore in contact with both PlGF and both VEGFR1-d2 molecules of the asymmetric unit, thus presumably greatly stabilizing the packing arrangement in these crystals (Fig. 2).

**FIG. 3.** *Superposition of free (blue) and receptor-bound (wheat and orange) PlGF.* A, ribbon representation in orientation similar to that shown in Fig. 1B. Note the large movement of the CD and AB loops between both structures. B, stereo view of the AB region, an area in which the two structures are dramatically different.

**FIG. 4.** *Superposition of PlGF–VEGFR1-d2 and VEGF–VEGFR1-d2 complexes.* The color code of PlGF–VEGFR1-d2 is the same as described in the legend for Fig. 1; VEGF–VEGFR1-d2 is shown in blue. A, ribbon diagram with orientation similar to that in Fig. 1B. B, “open book” view of the VEGFR1-d2 (left) and the growth factors (right) shown as tubes, with selected side chains shown as sticks.

**DISCUSSION**

**Structure of Bound Versus Unbound PlGF**—Because of the dimeric nature of the PlGF complex, there are two ways to superimpose the PlGF dimer in its free form (Protein Data Bank code 1FZV) and the PlGF in complex with VEGFR1-d2 on top of each other. In either case, the molecules have an r.m.s.d. of ~2.1 Å for a total of 188 Ca atoms (residues 22–115). Even the superposition of a single PlGF monomer in its unbound form onto a PlGF monomer in complex with VEGFR1-d2 re-
sults into a rather large r.m.s.d. of 1.6 Å for 94 atoms. This deviation is the result of a number of changes. First, the overall shape of the PlGF dimer is slightly altered when bound to the receptor (Fig. 3A). The CD loops form the far end of the sheet-like PlGF dimer. When bound to the receptor, they have moved away from the center by ~4 Å, which results in a more open appearance of the PlGF dimer. Second, and more dramatic, is the conformational change of the peptide segment connecting the short helix α2 with strand B (residues 48–54). The helical turn α2 in the region is present in both structures, but the following loop has completely rearranged (Fig. 3B). However, this peptide segment is not in contact with the bound receptor, and the changes are most likely the result of different crystal packing environments and influenced by a bound 2-methyl-2,4-pentanediol (MPD) molecule that packs against the aromatic ring of Tyr47 in the crystal structure of free PlGF. Because the largest conformational change between PlGF bound versus unbound is likely to be induced by weak crystal packing forces, it can be expected that, as previously seen for VEGF, receptor binding does not involve major conformational changes in the ligand.

Comparison of VEGFR1-d2 Binding to PlGF and to VEGF—A comparison of PlGF bound to VEGFR1-d2 with the high resolution structure of VEGF (Protein Data Bank code 1FLT) bound to the same receptor domain allows for the determination of the most important specificity determinants of both growth factors toward VEGFR1. For the following comparison of the interfaces, only residues that are well defined in all structures are discussed. The set of residues to be considered includes residues 133–224 of VEGFR1-d2, 14–107 of VEGF, and the equivalent residues of PlGF, 22–115.

As Autiero et al. (18) have shown, only about half of the VEGF residues that participate in the interface with VEGFR1-d2 are conserved between PlGF and VEGF. Nevertheless, PlGF and VEGF bind to VEGFR1-d2 in a strikingly similar way. Superposition of both dimers in their receptor-bound state results in an r.m.s.d. of 1.5 Å; thus PlGF and VEGF are more similar to each other in their bound states than receptor-bound PlGF is to free PlGF. Similarly, superposition of VEGFR1-d2 from both complexes results into r.m.s.d.s of 0.6–0.7 Å, indicating that there is no induced-fit mechanism required for binding of the receptor domain to either growth factor. Clearly, both complexes are very similar. Moreover, despite the low sequence conservation within of the contacting residues, the interfaces in both complexes are virtually identical; every VEGFR1-d2 residue that contributes more than 10 Å² of buried surface to the interface with VEGF is also involved in PlGF, binding and an almost identical set of residues on PlGF and VEGF contributes to binding to the receptor domain.

The Differences between PlGF and VEGF in the Core of the Interface—Only five of the nine residues that form the core of the interface with VEGFR1-d2 are conserved between human PlGF and human VEGF (Figs. 4 and 5). One of the differences is the exchange of Leu86 in PlGF with Met81 in VEGF. The side chains of both residues, however, occupy the same space in both complexes and have very similar interactions with the receptor. The other three sequence differences are more interesting; most noticeable is the exchange of Trp29 in PlGF against the smaller Tyr21 in VEGF (Fig. 5). This opens up additional space in the interface, which is filled by the exchange of the small Ser66 to the larger Lys48 and Gly70 to Asn62. As a result, the binding surface presented to the receptor is almost unchanged despite the low sequence conservation, and VEGFR1-d2 can bind both ligands without any significant conformational changes.

Interestingly, the three residues compensating each other in their space requirement are almost entirely conserved among the four PlGF sequences on the one hand and among the 10 mammalian VEGF sequences on the other. (Only the Gly70–Asn62 has two exceptions in the sequences deposited in the Swiss Protein data base.) Thus it seems likely that these residues have a biologically important role, for example as specificity determinants or to modulate the binding properties of PlGF/VEGF heterodimers (see below).

Specificity—VEGF is capable of binding VEGFR1 and VEGFR2 with high affinity, but PlGF selectively binds to
VEGF1. It is intriguing but difficult to understand how specificity between these growth factors and their receptors is accomplished. Domain 2 of VEGFR1 and VEGFR2 share a sequence identity of about 32%. This degree of sequence conservation clearly indicates that the overall fold of both domains is conserved, and using the available structures of VEGFR1-d2, it is possible to create a model of VEGFR2-d2 with fairly high confidence. Based on the sequence similarity among the receptors and the various growth factors, it is expected that the overall architecture be retained in all complexes between VEGF family members and VEGFR1 and VEGFR2. However, only two of the 19 VEGFR1 residues involved in VEGF or Pigf binding are conserved in VEGFR2. This low level of sequence conservation in the binding interface prevents any model from providing reliable insights about the residues responsible for the discrimination between VEGF and Pigf.

In addition, domain 2 is unlikely to be the only specificity-determining domain of the VEGF system. Based on the crystal structures between Pigf or VEGF in complex with VEGFR1-d2 and alanine scanning experiments on VEGF, it is apparent that domain 3 is also involved in ligand binding (17).

The role of domain 3 seems to be crucial in the VEGFR2 system. VEGFR2-d1–d2 binds to VEGF with about 1000-fold lower affinity than a construct that entails domains 1–3 of that receptor (19). The same domain of VEGFR1 seems not to be as pivotal for VEGF binding, as the affinity toward VEGF is only affected by a factor of 50 when compared with the affinity of the full-length receptor. Interestingly, the presence of VEGFR1-d3 has a much greater effect on the binding of Pigf, which binds about 500-fold more weakly to VEGFR1-d2 than to the full-length receptor (Table I). This strongly suggests that domain 3 of the VEGFRs plays an important part in determining their specificities.

Pigf/VEGF Heterodimers—The sequence identity between Pigf and VEGF is 52%. Although the sequence conservation in the receptor-binding site is not higher than the overall sequence identity, the residues that are involved in forming the interface between the two monomers in the growth factor homodimers do show a higher degree of sequence conservation: About 65% of residues involved in the dimer interface are conserved between Pigf and VEGF (Fig. 4). Therefore the sequence conservation within the Pigf family (67% between human and rat Pigf) and the sequence conservation of residues that form the interface within each growth factor homodimer are approximately the same, and indeed it has been shown that Pigf and VEGF are able to form naturally occurring heterodimers (28).

These Pigf/VEGF heterodimers are asymmetric and have two different receptor-binding sites (Fig. 6). It is conceivable that one of those interfaces binds preferentially or exclusively to VEGFR1, whereas the other does so to VEGFR2. As mentioned above, there are three residues that are almost entirely conserved in the core of the receptor binding interface as one type in all VEGFs and as another in the Pigfs. In each receptor-binding site these residues come from different monomers. Thus in Pigf/VEGF heterodimers there will be one tightly packed interface with the large side chains of Trp29 (Pigf) as well as Lys40 and Asn23 (VEGF) close to each other and one rather loosely packed interface containing the smaller side chains of Tyr14 (VEGF), Ser17, and Gly19 (Pigf). In these asymmetric dimers, the tightly packed interface may have a bulge, whereas the loosely packed interface could contain a crevice (Fig. 6). This will have differential effects toward binding to VEGFR1 or VEGFR2. As of yet, the consequences of the asymmetry in the Pigf/VEGF heterodimer assembly are unclear, and it is not possible to predict which of these binding interfaces is able to bind to which receptor.

Currently, the biological function of the Pigf/VEGF heterodimer is controversial. DiSalvo et al. (28) report that it has nearly the same mitogenic activity as VEGF homodimers but Eriksson et al. (29) find that the same heterodimer is functionally inactive. Yet again, a recent report by Autiero et al. (18) suggests that the heterodimer may lead to heterodimerization of VEGFR1 and VEGFR2 and to enhanced angiogenic responses.

Implications—Pigf and VEGF bind VEGFR1 in a surprisingly similar way. It was noted that not only about half of the residues in the receptor binding interface are conserved between VEGF and Pigf. Based on this and other evidence, Autiero et al. (18) suggested that Pigf and VEGF might be able to activate VEGFR1 in distinct ways. A comparison of the structure presented here and the structure of the complex between VEGF and VEGFR1-d2 disputes this idea. The comparison not only shows that the overall orientation of Pigf and VEGF toward the receptor is virtually identical but also emphasizes the concurrence of details in the binding interfaces in both complexes. Despite the low level of sequence conservation the presented interfaces are surprisingly similar, and thus the crystal structures suggest that Pigf and VEGF activate VEGFR1 in the same way.

The activation of distinct pathways through binding of VEGF or Pigf to VEGFR1 may however be the result of complex formation with other co-receptors such as neuropilin or heparin (30). More studies of the structure and function of these co-receptors are needed to achieve a full understanding of how Pigf and VEGF exert all of their functions.

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The Crystal Structure of Placental Growth Factor in Complex with Domain 2 of Vascular Endothelial Growth Factor Receptor-1
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