Identification of Placenta Growth Factor Determinants for Binding and Activation of Flt-1 Receptor*

Michela Erri1, Teresa Riccioni2, Shalini Iyer3, Claudio Pisano4, K. Ravi Acharya4, M. Graziella Persico5, and Sandro De Falco2 2 2

From the 1 Institute of Genetics and Biophysics "Adriano Buzzati-Traverso," Consiglio Nazionale delle Ricerche, 80131 Naples, Italy, 2 Research and Development, Sigma-Tau SpA, Industrie Farmaceutiche Riunite, 00040 Pomezia (Rome), Italy, and the 3 Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, United Kingdom

Placenta growth factor (PIGF) belongs to the vascular endothelial growth factor (VEGF) family and represents a key regulator of angiogenic events in pathological conditions. PIGF exerts its biological function through the binding and activation of the seven immunoglobulin-like domain receptor Flt-1, also known as VEGFR-1. Here, we report the first detailed mutagenesis studies that provide a basis for understanding molecular recognition between PIGF-1 and Flt-1, highlighting some of the residues that are critical for receptor recognition. Mutagenesis analysis, performed on the basis of a structural model of interaction between PIGF and the minimal binding domain of Flt-1, has led to the identification of several PIGF-1 residues involved in Flt-1 recognition. The two negatively charged residues, Asp-72 and Glu-73, located in the β3–β4 loop, are critical for Flt-1 binding. Other mutations, which bring about a significant decrease in PIGF binding activity, are Gln-27, located in the N-terminal α-helix, and Pro-98 and Tyr-100 on the β6 strand. The mutation of one of the two glycosylated residues of PIGF, Asn-84, generates a PIGF variant with reduced binding activity. This indicates that, unlike in VEGF, glycosylation plays an important role in Flt-1 binding. The double mutation of residues Asp-72 and Glu-73 generates a PIGF variant unable to bind and activate the receptor molecules on the cell surface. This variant failed to induce in vitro capillary-like tube formation of primary endothelial cells or neo-angiogenesis in an in vivo chorioallantoic membrane assay.

Placenta growth factor (PIGF) 1, 2, a member of the vascular endothelial growth factor (VEGF) family (3, 4), is one of the key regulators of pathological angiogenesis as demonstrated by gene inactivation studies (5). Human PIGF is encoded by a single gene, but alternative splicing of mature mRNA (6–8) gives rise to three known isoforms of varying length: PIGF-1 (PIGF131), PIGF-2 (PIGF152), and PIGF-3 (PIGF203). Apart from the differences in the number of amino acids, these isoforms also differ in their ability to bind heparin. The presence of a highly basic 21-amino acid insert at the C-terminal end of the protein confers upon PIGF-2 the ability to bind heparin molecules. Recently, however, a novel variant of PIGF, termed PIGF-4, was reported (9). This splice variant is believed to have the same sequence as PIGF-3 plus the heparin binding domain previously thought to be present only in PIGF-2. PIGF is secreted as a glycosylated homodimer. The most characteristic feature of PIGF is the presence of a cysteine-knot motif, consisting of an eight-residue ring formed by three intrachain and one interchain disulfide bond.

The VEGF-A homodimer exerts its biological activities through activation of two distinct tyrosine kinase receptors: fms-like tyrosine kinase receptor-1 (Flt-1; also known as VEGFR-1) and the kinase domain-containing receptor/fetal liver kinase receptor (KDR/Flk-1; also known as VEGFR-2) (10). VEGF-A is central to several physiological as well as pathological conditions (11, 12). The PIGF homodimer, on the other hand, binds and induces auto-phosphorylation of only Flt-1 (13). Conversely, the knockout of PIGF does not affect development, reproduction, or normal postnatal life but impairs angiogenesis and arteriogenesis during pathological conditions such as tumor growth, ischemic conditions, and wound healing (5, 14, 15). Synergistic cooperation between PIGF and VEGF in pathological conditions is specific (5). Up-regulation of PIGF by endothelial cells leads to displacement of VEGF from VEGFR-1. As a result, increased amounts of VEGF are available to bind to the mitogenic response-inducing receptor, VEGFR-2. Administration of recombinant PIGF amplifies VEGF-driven angiogenesis in vivo, stimulating the formation of mature and durable vessels in the ischemic heart and enlarging collateral arterioles in the ischemic limb with marked perfusional and functional improvement (16), without resulting in side effects such as edema and hypotension, usually observed after administration of recombinant VEGF-A (17). Furthermore, the blocking of Flt-1 activity with a neutralizing monoclonal antibody suppresses neovascularization in tumors and ischemic retina as well as in angiogenesis and inflammatory joint destruction in autoimmune arthritis (16). Altogether, these data clearly indicate that the PIGF/Flt-1 pathway plays a central role in pathological angiogenesis (5, 18).

The complexity of the biochemical relation between PIGF, VEGF-A, and the two receptors Flt-1 and Flk-1 has been explained recently, to a certain extent. Flk-1 is trans-phosphoryl-
PlGF and VEGF-A activate Flt-1 in a different manner because VEGF-A and PlGF-1 (24). Recently, the three-dimensional turn is reflected in the remarkable topological identity between molecules share 42% amino acid sequence identity, which in the preparation of the variant with the double mutation, primers carry nucleotide modification (QuikChange mutagenesis kit, Stratagene). For primers were designed to use the same PCR conditions: 95 °C 30 s, 1 cycle; 95 °C 30 s, 55 °C 1 min, 72 °C 12 min, 16 cycles. Then the methylated template DNA was digested with 10 units of DpnI for 60 min at 37 °C. The amplified DNA was purified and used to transform competent bacteria. In all cases, the plasmids were sequenced in both directions by the dideoxynucleotide method using SP6 and T7 standard primers. The following 10 PlGF-1 single residues were mutated to Ala: Asn-16, Pro-25, Glu-27, Cys-60, Asp-72, Glu-73, Asn-74, Asn-84, Pro-98, and Tyr-100. The eleventh PlGF-1 variant to be generated was the double mutant D72A/E73A.

**Transient Transfection and Selection of Stable Cell Lines**—Transient transfections were performed in the HEK 293T cell line with either pCHPlGF-1 vector or the plasmids carrying the PlGF-1 variants using calcium phosphate precipitation technique. DNA precipitate was used to transfect 293-hFlt-1 cell line stably transfected with the empty vector was also established. The cell line 293-h-Flt-1 was selected for the maximal amount of hFlt-1 expression as determined by Western blot analysis.

**Western Blot Analysis**—Aliquots of conditioned cell media obtained from transient transfections, were precipitated overnight at 20 °C with two volumes of chilled acetone and then centrifuged at 4 °C for 30 min at 3,000 × g. The pellets were suspended in SDS loading buffer, loaded onto a nonreducing 12% SDS-PAGE, and following electrophoresis, blotted onto polyvinylidene difluoride membranes. The polyvinylidene difluoride filters were first blocked for 1 h at room temperature (RT) with 5% dried milk in TBS (25 mM Tris pH 8, 150 mM NaCl, 2.5 mM KCl) and then incubated for 1 h at RT with 100 ng/ml PlGF polyclonal antibody diluted in the blocking buffer at 1 μg/ml. After three washings of 10 min each with TBS, the filter was incubated for 1 h at RT with a goat anti-rabbit antibody conjugated with HRP and diluted 1:10,000 in blocking buffer. For detection, the chemiluminescent ECL reagent was used according to the manufacturer’s instructions.

**ELISAs**—For quantitative determination of PlGF-1 variants, anti-human PlGF polyclonal antibody, diluted at 1 μg/ml in PBS, pH 7.5, was used to coat a 96-well plate, 100 μl/well, overnight at 4 °C. The wells were washed five times with PBS containing 0.004% Tween 20 (PBT), and the aspecific sites of wells, aliquots of conditioned media were diluted in PBE containing 0.1% bovine serum albumin, 5 μl. The conditioned media from HEK-293T transfected with PlGF-1 variants were diluted in PBE containing 0.1% bovine serum albumin, 5 μl EDTA, 0.004% Tween 20 and incubated overnight at 4 °C. Wells were washed again, and biotinylated anti-human PlGF polyclonal antibody, diluted in PBT at 500 ng/ml, was added to the wells and incubated for 1 h at 37 °C followed by 1 h at RT. Wells were washed and incubated with a solution containing a preformed avidin and biotinylated HRP macromolecular complex (Vectorstain elite ABC kit) for 1 h at RT. After the last wash, 100 μl of HRP substrate composed of 1 mg/ml of ortho-phenylenediamine in 50 mM citrate phosphate buffer, pH 5, 0.006% H₂O₂ was added and incubated for 15 min. The reaction was stopped with 30 μl/well of 4 N H₂SO₄, and the absorbance was measured at 490 nm on a microplate reader (Bio-Rad BenchMark). PlGF-1 variant concentrations were determined by interpolation of the standard curve using linear regression analysis.

To determine the binding activity of PlGF-1 variants to Flt-1, a 96-well plate was coated with a soluble form of human Flt-1 (Flt-1/Fc chimera) at 0.5 μg/ml in PBS, pH 7.5, 100 μl/well, overnight at RT. The plate was washed five times with PBT, and after the blocking of the aspecific sites of wells, aliquots of conditioned media were diluted in PBT and added at concentrations ranging from 1 to 100 ng/ml. The binding reaction was performed for 1 h at 37 °C and 1 h at RT. Wells were washed again with PBT and incubated with biotinylated anti-human PlGF polyclonal antibody, 300 ng/ml in PBE, for 1 h at 37 °C and 1 h at RT. The detection was performed as described above. All of the ELISAs were analyzed by means of three independent experiments. **Flt-1 Phosphorylation Analysis**—Subconfluent 293-h-Flt-1 cells were
starved overnight in serum free medium. The medium was removed, and the cell monolayer was preincubated for 5 min with 100 μM Na2VO4 to inhibit endogenous phosphatase activity. Subsequently, cells were incubated for 10 min at 37 °C with wt PlGF-1 or with the PlGF variants Q27A, D72A, or D72A/E73A at concentrations ranging from 10 to 50 ng/ml. Conditioned medium from 293-pcDNA3 cells was used as control. After stimulation, cells were first washed with pre-chilled 100 μM Na2VO4 and then lysed in a buffer containing 20 mM Tris-HCl, pH 8.5, 5 mM EDTA, 150 mM NaCl, 1% Triton-X100, 10% glycerol, 10 mM zinc acetate, 100 μM Na2VO4 and a mixture of protease inhibitors for 1 h at 4 °C. The samples were centrifuged at 12,000 × g for 15 min, and the supernatants were recovered and stored at −80 °C. The protein concentration was determined by the Bradford method (Bio-Rad reagent). To confirm the specificity of PlGF-1 activity, a neutralizing antibody, and a goat anti-mouse HRP-conjugated antibody, diluted 1:10,000 in blocking buffer, was added to 1 mg of protein extract and incubated overnight at 4 °C. Subsequently, the resin was recovered by centrifugation, washed once with complete lysis buffer, twice with lysis buffer without detergent, and finally with Tris-buffered saline. The pellets were loaded on a reducing 8.5% SDS-PAGE. Proteins were detected by Western blot as described above.

For the phosphotyrosine detection, mouse antibody against phosphotyrosine (anti-pY), diluted 1:1000 in blocking buffer, was used as primary antibody, and a goat anti-mouse HRP-conjugated antibody, diluted 1:10,000 in blocking buffer, was used as secondary antibody. The same filter was stripped with 0.2 N NaOH in Tris-buffered saline for 5 min at RT and used for Flt-1 detection obtained using a goat anti-human Flt-1 antibody diluted at 800 ng/ml in blocking buffer. Donkey anti-goat HRP-conjugated antibody, diluted 1:10,000 in blocking buffer, was used to detect the primary antibody.

For phospho-Erk and Erk-1–2 detection, mouse anti-pErk-1 and goat anti-Erk, were used according to the manufacturer’s instructions.

Densitometry analyses to quantify the degree of Flt-1 and Erk phosphorylation were performed using ImageQuant version 5.2 software (Amersham Biosciences).

Capillary-like Tube Formation Assay—A 24-well plate was coated with Matrigel (250 μg/well) for 30 min at 37 °C. HUVECs (8 × 104 cells/well) were seeded in 500 μl of EBM-2 or EBM-2 in the presence of either wt PlGF-1 or PlGF-1 variants at concentrations ranging from 100 to 300 ng/ml. EGM-2 and EBM-2 were used as positive and control, respectively. After 6 h of incubation, capillary-like tube formation was examined under an inverted phase microscope. Cells were fixed with PBS containing 0.2% glutaraldehyde, 1% paraformaldehyde and photographed. To confirm the specificity of PlGF-1 activity, a neutralizing monoclonal antibody (2.5 μg/ml) was preincubated with the conditioned media containing either wt PlGF-1 or D72A/E73A-PlGF-1 variant overnight at 4 °C. The assays were performed in triplicate.

Chicken Embryo Chorioallantoic Membrane (CAM) Assay—Fertilized chicken eggs were incubated under conditions of constant humidity at 37 °C. On the fourth day of incubation, a window was opened in the egg shell after removal of 3 ml of albumen so that the developing CAM was kept detached from the shell. The window was sealed with a piece of glass, and the eggs were incubated at 37 °C. On the ninth day, pellets of 3 × 104 cells of stable clones over-expressing wt PlGF-1, the PlGF-1 variants Q27A, D72A, or D72A/E73A, or the stable clone transfected with the empty vector were applied on the chorioallantoic membrane of 10 eggs for each sample. The allantoic vessels around the cell pellets were counted under a stereomicroscope and photographed with a digital camera at the time of cell implants and 72 h later.

RESULTS

Model-based Selection of PlGF-1 Residues Involved in Flt-1 Recognition—The structure-based sequence alignment of PlGF and VEGF is shown in Fig. 1A. A structural model of PlGF-1/Flt-112 interaction was built based on the three-dimensional structure of PlGF-1 (25) and the crystal structure of VEGF-A in complex with domain 2 of Flt-1 (26), the minimal domain required for binding. The model predicted a similar type of interaction as that observed for VEGF-A in that the binding is essentially driven by hydrophobic interactions. Each pole of the PlGF-1 homodimer contacts one Flt-1D2 molecule, and residues from both monomers are involved in the recognition of each receptor domain. Based on this model, seven residues were chosen and mutated to alanine. Five residues, Pro-25, Gln-27, Asp-72, Glu-73, Asn-74, are located in one monomer, and the other two residues, Pro-98 and Tyr-100, are in the other monomer (Fig. 1B). The residue Asp-72 (corresponding to Asp-63 of VEGF-A), located in the β3–β4 loop, is the only residue predicted to make a possible hydrogen-bonding interaction with Flt-112 domain. The residue Asn-74 again located in the β3–β4 loop, the residue Gln-27 located in the N-terminal α-helix, and the residue Tyr-100 located in strand β6 were chosen because the model predicted they would make both hydrogen-bonding and van der Waals contacts with the residues implicated in Flt-1 binding, whereas Gln-73, located in the β3–β4 loop, seems to be involved only in van der Waals interactions. The two residues Pro-25 and Pro-98, located at the beginning of the N-terminal helix and strand β6, respectively, appear to be part of the interface. The residue Cys-60, involved in interchain disulfide bond formation, was mutated to verify whether the monomeric form of PlGF-1 is expressed and has the ability to bind Flt-1. In addition to the above mutations, the double mutation D72A/E73A was modeled in the crystal structures of both native PlGF-1 and PlGF-1 in complex with Flt-112 (25) to look for possible conformational changes. Finally, the two putative glycosylation sites, Asn-16 and Asn-84, were also mutated to investigate the relationship between glycosylation and PlGF-1 activity.

Generation of PlGF Variants—All of the PlGF variants were generated using a PCR-based protocol and were transiently transfected in HEK 293T cells (Fig. 2). Wild type PlGF-1 and all recombinant PlGF-1 variants except N16A, N84A, and C60A showed a major band of expected molecular mass of ~46 kDa. The two glycosylation site mutants, N16A and N84A, displayed a higher mobility, indicating that both residues are post-translationally modified. The C60A mutation determined the expression and secretion of the monomeric form of PlGF-1 with the expected molecular mass (~23 kDa). In some lanes, in addition to the 46-kDa band, the antibody recognized a band with mobility corresponding to the monomeric form because of over-expression by transient transfection.

Binding of PlGF Variants to the Flt-1 Receptor—To perform the binding of PlGF variants with the Flt-1 receptor, first the concentration of recombinant proteins in the conditioned media was determined using a sandwich ELISA. The concentration was calculated from a standard curve as reference, constructed using recombinant human PlGF. The mutants were expressed with a variable yield, and their concentrations ranged from 25 to 200 ng/ml. The binding experiments were achieved using ELISA. A fixed concentration of soluble Flt-1 receptor fused to an Fc fragment was used to coat the plate, and variable amounts of PlGF variants or wt PlGF, ranging from 0 to 16 μg/ml, were added to perform a linear binding (Fig. 3A). The interaction was evaluated using a biotinylated polyclonal antibody against hPlGF. The N16A and N74A variants showed a binding comparable with that of the wild type protein; the P25A, E73A, and Y100A variants showed a reduction of about 25% of binding, whereas Q27A, D72A, N84A, and P98A variants displayed a reduction of binding activity around 50%. The C60A variant was unable to interact with immobilized Flt-1 as expected, since residues from both the monomers of the active PlGF-1 dimer are necessary for Flt-1 recognition. Interestingly, the double mutant D72A/E73A-PlGF-1 showed a binding activity very close to that of the C60A mutant. The percentage of binding of PlGF-1 variants in comparison with the wt PlGF at a concentration of 8 ng/ml is reported in Fig. 3B.

To confirm the observations of the linear binding assay, some of the PlGF-1 variants and the wild type protein were assayed at saturating concentrations (Fig. 4A). The linear binding assay, performed with a protein concentration up to 100 ng/ml, confirmed that the variant E73A showed a reduction in binding.
of about 25%; the two variants, Q27A and D72A, displayed a binding activity close to 50% with respect to wt PlGF-1, and D72A/E73A-PlGF-1 gave a binding activity close to zero (Fig. 4B). These results suggest that the observations derived from the modeling studies are correct. Except for N16A and N74A residues, all mutations involved residues that contribute toward the binding of Flt-1. It is of particular relevance that the double mutation of the contiguous residues D72A and E73A generated
a variant that severely compromised the binding of PlGF to Flt-1.

**The Double Mutant D72A/E73A-PlGF-1 Failed to Activate Flt-1**—The side-by-side, head-to-tail orientation of the PlGF-1 monomers places the receptor-binding interface at each pole of the PlGF-1 dimer. This structure facilitates receptor dimerization and, consequently, auto-phosphorylation of Flt-1, which is essential for initiating the signal transduction cascade (13). The *in vitro* data shown above indicate that the D72A/E73A-PlGF-1 mutant is unable to bind the soluble recombinant form of Flt-1, whereas the two single mutants, Q27A and D72A, show a 50% reduction of binding activity. To investigate whether this experimental evidence correlated with the inability of the double mutant to activate Flt-1 on cell surface, the stable cell line 293-Flt-1 was exposed to the wt PlGF-1 and the single or double mutant. We evaluated how these mutations affected Flt-1 phosphorylation by means of immunoprecipitation and Western blot assays. When starved 293-Flt-1 cells were incubated with 10 ng/ml PlGF-1 for 10 min (Fig. 5A), a clear induction of Flt-1 phosphorylation with respect to the control (Fig. 5A, lane 1) was revealed using anti-phosphotyrosine antibody. Stimulation of 293-Flt-1 cells by the two single mutants Q27A-PlGF-1 and D72A-PlGF-1 (at the same concentration) resulted in Flt-1 phosphorylation at the level of 42.5 and 59.3%, respectively, when compared with that induced by wt PlGF-1 (Fig. 5A, lanes 4 and 5). When 293-Flt-1 cells were incubated with D72A/E73A-PlGF-1, no phosphorylation was detected (Fig. 5A, lane 3). These results were confirmed by the study of the activation status of the signaling proteins triggered by the receptor. Flt-1 activation determines the activation of the mitogen-activated protein kinase pathway for signal transduction (30, 31). We analyzed the activation of the extracellular signal-regulated kinase (Erk) protein in 293-Flt-1 cells after exposure to the wt or the variants of PlGF-1. To detect the phosphorylation of Erk, total cellular proteins were analyzed by Western blot using the monoclonal anti-pErk antibody and, subsequently, the anti-Erk-1 antibody for normalization. The incubation of 293-Flt-1 cells with the wild type PlGF-1 indicated phosphorylation of Erk, whereas incubation with the double mutant D72A/E73A-PlGF-1 resulted in a non-activation status of Erk, comparable with the negative control (Fig. 5B). Again, when the 293-Flt-1 cells were incubated with the two single mutants Q27A-PlGF-1 and D72A-PlGF-1, a partial activation of Erk (as compared with Erk activation by wt PlGF-1) of ~40% was detected (Fig. 5B). Taken together, these findings suggest that the residues Glu-27, Asp-72, and Glu-73 play an important role in binding and activating Flt-1. The double mutation of the residue Asp-72 and Glu-73 generated an inactive PlGF-1 variant that failed to activate the Flt-1 signaling pathway.

**Effect of D72A/E73A-PlGF-1 on Capillary-like Tube Formation**—Cell proliferation, migration, and differentiation are processes integral to the formation of capillary-like tube structures. We examined the effect of the D72A/E73A mutation on the activity of PlGF-1 on tube formation using an *in vitro* Matrigel assay performed with human primary endothelial cells. HUVECs were plated on Matrigel and stimulated with 100 ng/ml proteins diluted in growth factor-free EBM-2 for 6 h. As a negative control, cells were incubated in EBM-2, and for positive control HUVECs were incubated in complete EGM-2 normally utilized for their *in vitro* growth. HUVECs plated on Matrigel and stimulated with 100 ng/ml hPlGF-1 formed capillary-like networks to an extent similar to that observed for the positive control (Fig. 6, B and C), whereas stimulation with the double mutant D72A/E73A-PlGF-1 was ineffective (Fig. 6E). Increasing the concentration of the double mutant proved to be just as ineffective (data not shown). The specificity of PlGF-1 stimulation was confirmed by preincubating the wt PlGF or the D72A/E73A-PlGF-1 with a neutralizing mAb against mPlGF, at a concentration of 2.5 μg/ml. The binding of PlGF-1 to Flt-1 was completely inhibited by the neutralizing mAb as observed from the lack of response of the endothelial cells to induce capillary-like tube formation (Fig. 6, D and F), comparable with the negative control (Fig. 6A). These data indicate that the PlGF-1/Flt-1 pathway is sufficient to induce the *in vitro* organization of endothelial cells into capillary-like structures. Furthermore, the double mutant D72A/E73A-PlGF-1 was also unable to stimulate formation of capillary-like structures, similar to the inhibitory effect exerted by the neutralizing mAb on the binding of PlGF-1 to Flt-1 and the subsequent activation of the receptor.

**Mutants Q27A, D72A, and D72A/E73A Were Unable to Induce In Vivo Angiogenesis**—The angiogenic ability of D72A/E73A-PlGF-1 was also investigated *in vivo* by means of a CAM assay. The assay was performed using the stable cell lines 293-PlGF-1, 293-Q27A-PlGF-1, 293-D72A-PlGF-1, and 293-D72A/E73A-PlGF-1 over-expressing recombinant proteins at similar concentrations (30–40 ng/ml) as determined by quantitative ELISA. HEK-293T cells stably transfected with pCDNA3 empty vector were used as a control. A pellet of 3 × 10⁶ cells was implanted on the embryonic chorioallantoic membrane at day 9 of incubation. After 72 h of implantation, only 293-PlGF-1 cells (Fig. 7B) were surrounded by an increased number of allantoic vessels that developed radially toward the implant, compared with 293-pCDNA3 cells (Fig. 7A), whereas no increased vascular reaction was detectable around the 293-
Q27A-PlGF-1 (Fig. 7C), 293-D72A-PlGF-1 (Fig. 7D), and 293-D72A/E73A-PlGF-1 pellets (Fig. 7E).

Measurement of the angiogenic response, performed at day 12 of incubation, revealed that the microvessel density induced by wt PlGF-1 is comparable with that reported for VEGF-A (32). However, the double mutant D72A/E73A, as well as the two single mutants Q27A-PlGF-1 and D72A-PlGF-1, could not induce significant angiogenic response (Fig. 7F) despite their ability to partially activate Flt-1 receptor.

DISCUSSION

In the present study we analyzed the role of various PlGF-1 residues by means of an alanine-screening approach based on the structural model of the PlGF/Flt-1p32 three-dimensional complex (25). The receptor binding activity of the different PlGF-1 variants indicates that, as seen with VEGF-A (27), the β3-β4 loop plays a crucial role in Flt-1 recognition; in particular, two residues of this loop, Asp-72 and Glu-73, are essential for interaction with the receptor. The Asp-72 residue, the only...
amino acid able to make direct polar interactions with Flt-1 D2 according to the hypothetical model, when mutated to alanine showed only about 50% receptor binding activity compared with wild type PlGF-1 (Figs. 3 and 4). Interestingly, a single mutation of the corresponding VEGF residue, Asp-63, did not give a significant decrease in receptor binding (27) indicating that perhaps Asp-72 has a more important role in Flt-1 recognition in the case of PlGF-1. Recent data from the three-dimensional crystal structure of PlGF/Flt-1 D2 seem to agree with our data. The authors report that Asp-72 of PlGF-1 does indeed make two polar interactions with Arg-224 of Flt-1 D2 (28) as hypothesized previously (25).

The single mutation of Glu-73 showed a moderate reduction of PlGF-1 binding activity (about 25%) (Figs. 3 and 4), whereas the mutation of the corresponding residue of VEGF-A (Glu-64) again did not show a significant change in receptor recognition (27). This residue, as the homologue in VEGF, is exposed to the wide groove between the monomers and appears to be involved in the interaction with the third Ig-like domain of the receptor (25, 26). Previous data have indicated that domain 3 seems more critical for PlGF than for VEGF binding to the receptor. Alanine mutations of residues 279–283 of Flt-1 domain 3, adjacent to domain 2, resulted in a 30% reduction in bound VEGF, whereas PlGF appeared to be more sensitive to these changes, because the amount of ligand bound decreased by 60% (33). It has been recently shown that as for VEGF (26), Flt-1 domains 2 and 3 are necessary and sufficient for the binding of PlGF with near native affinity. However, the deletion of domain 3 causes only a 50-fold decrease in VEGF binding, whereas the effect on PlGF is more consistent, resulting in about a 500-fold reduction of binding of PlGF to the domain 2 (28). Thus, the mutation of residues involved in Flt-1 D2 interaction is more critical for PlGF than for VEGF binding.

Mutation of the two contiguous residues Asp-72 and Glu-73
to alanines resulted in the generation of a PlGF-1 variant in which binding activity was severely impaired. The mutant D72A/E73A-PlGF-1 was unable to bind Flt-1 even when saturating concentrations were used in the receptor-binding assay (Figs. 3 and 4). In VEGF-A a comparable impairment of Flt-1 recognition is obtained only when a triple mutant involving the three negatively charged residues Asp-63, Glu-64, and Glu-67 is generated (27). In PlGF-1, only the first two negatively charged amino acids are conserved (Asp-72 and Glu-73), because the third one is replaced by the positively charged amino acid His-76 (Fig. 1A). Thus, the combined mutation of the Asp-72, which makes polar interactions, and the contiguous residue Glu-73, potentially involved in Flt-1 domain 3 contact, is sufficient to generate a PlGF-1 variant that is unable to interact with Flt-1.

Although point mutations can dramatically influence both the stability and function of a protein, large or gross structural changes are rarely observed when such single/double mutations are generated. Usually the mutated residues themselves undergo conformational adaptations. The double mutation D72A/E73A is located in the flexible, solvent-exposed β3-β4 loop that forms an important part of the receptor-binding surface on PlGF-1. These mutations were modeled both in the native structure of PlGF-1 and the structure in complex with Flt-1D2 (25) and were subjected to energy minimization in order to remove steric clashes. Superposition of these structures did not reveal any significant rearrangement of the structure in and around the mutation site. The secondary structural core of the molecule remained unperturbed. The loop containing the double mutation in the modeled mutant complex also did not show much divergence when compared with the loop in the native PlGF-1 structure (root mean square deviation of 1.1 Å for the same backbone atoms when the loop in the structure of the PlGF-1/Flt-1D2 complex is superimposed onto that of the native PlGF-1 structure. These observations indicate that the ineffectiveness of the double mutant in all of the receptor-binding assays is not because of gross conformational change(s).

Our studies indicate that another structural element of

**Fig. 5. Analysis of Flt-1 activation induced by wt PlGF-1 and PlGF-1 variants.** Starved 293-hFlt1 cells were stimulated with PlGF-1 and Q27A-PlGF-1, D72A-PlGF-1, and D72A/E73A PlGF-1 variants at a concentration of 10 ng/ml for 10 min. A, 1 mg of cell lysate was immunoprecipitated (IP) with anti-Flt1 antibodies and analyzed by Western blot, probed first with anti-phosphotyrosine antibodies (Anti p-Tyr), and subsequently normalized with anti-Flt1 antibodies (Anti Flt-1). B, 100 μg of the cell protein extracts was analyzed by Western blot (WB) to reveal the phosphorylation state of the signaling protein, Erk. The blots were first immunodetected with anti-phospho-Erk antibodies (Anti p-Erk) and subsequently with anti-Erk1 antibodies (Anti Erk1). The values of densitometry analyses performed using ImageQuant 5.2 software are reported. The values of 0 and 100 have been assigned arbitrarily to non-induced and wt PlGF-1-induced samples, respectively.

|                | WT PlGF-1 | D72AE73A-PlGF-1 | D72A-PlGF-1 | Q27A-PlGF1 |
|----------------|-----------|-----------------|-------------|------------|
|                |           |                 |             |            |
| pFlt-1/Flt-1 % | 0         | 100             | 59.3        | 42.5       |
| Anti p-Tyr     | [Image]   |                 |             |            |
| 200 kDa        |           |                 |             |            |
| Anti Flt-1     | [Image]   |                 |             |            |
| 200 kDa        |           |                 |             |            |
| Anti p-Erk     | [Image]   |                 |             |            |
| 43 kDa         |           |                 |             |            |
| Anti Erk1-2    | [Image]   |                 |             |            |
| 43 kDa         |           |                 |             |            |
| pErk/Erk1-2 %  | 0         | 100             | 37.9        | 43.9       |
PIGF-1 that is critical for receptor binding is the α1-helix at the N-terminal end. We muta ted the first residue of the N-terminal helix, Pro-25, and residue Gln-27, which, based on the structural model of PIGF-1/Flt-1, appears to be involved in both polar and van der Waals interactions with Flt-1p22. The variant P25A-PIGF-1 showed a limited decrease of binding affinity (about 25%), whereas the Q27A-PIGF-1 variant showed a reduction of about 50% in the binding activity. The mutation data for the corresponding VEGF-A residue Met-18 or for any residue from the N-terminal α1-helix are not available, except for Arg-23, which did not show a significant reduction in affinity for Flt-1 (27). Thus, our results with the Q27A-PIGF-1 variant indicate a different role for this residue and probably for the N-terminal α1-helix in Flt-1 recognition. The recent data from the PIGF/Flt-1p22 crystal structure (28) have confirmed the important role of Gln-27 in Flt-1 recognition and of the α1-helix in stabilizing the PIGF-1 dimer. The residue Gln-27 is the other PIGF-1 residue in addition to Asp-72 that is able to make polar interactions with Flt-1p22. The N-terminal helix has a fundamental role in the stabilization of the PIGF-1 dimer, because each helix packs on top of the other monomer.

Among the mutated residues, two other PIGF-1 determinants, both located on the other monomer of PIGF dimer, resulted in reduction of binding activity. The mutation of Tyr-100, which in the PIGF/Flt-1p22 model is predicted to make both polar and van der Waals interactions, showed a reduction of about 25% in binding activity, whereas the mutation of Pro-98, which appears to be part of the interface and is localized at the beginning of the β6 strand, showed about 50% decrease in binding activity. These results indicate that residues from the other monomer also contribute significantly to the affinity of PIGF-1 interaction with Flt-1. This finding further confirms that PIGF-1 is biologically active as a dimer, an aspect corroborated by the data obtained with the C60A-PIGF-1 variant. This mutation prevents the formation of the interchain disulfide bridge between the two PIGF-1 monomers and generates, instead, the monomeric form. The monomer was expressed and was sufficiently stable to be secreted by the cells (Fig. 2) but was unable to bind the receptor in vitro (Fig. 3).

We also investigated whether glycosylation plays any role in the binding of PIGF-1 to Flt-1. PIGF-1 is glycosylated at two positions, Asn-16 and Asn-84 (Fig. 2). The mutation N16A did not modify the receptor binding activity, whereas the mutation N84A, located in the β6 strand, which, according to the model of PIGF-1/Flt1p22 complex, takes part in the interaction, caused a decrease in the binding activity by about 50%. Conversely, the mutation of the corresponding residue in VEGF-A, Asn-75, did not show any reduction in binding affinity to the receptor (27). Thus, glycosylation seems to play a different role for PIGF-1 and VEGF-A in Flt-1 recognition.

With these mutation studies, we have demonstrated that the double mutation D72A/E73A is sufficient to prevent the binding of PIGF-1 to Flt-1 in vitro, whereas the single mutants Q27A-PIGF-1 and D72A-PIGF-1 show a 50% reduction of binding activity. The activation of the receptor is determined by the ability of PIGF-1 to induce receptor dimerization followed by auto-phosphorylation of Flt-1.

To confirm the results obtained in the ELISA-based tests, the ability of the variants Q27A-PIGF-1, D72A-PIGF-1, and D72A/E73A-PIGF-1 to induce phosphorylation of Flt-1 on the cell surface was investigated. The D72A/E73A-PIGF-1 mutant was unable to induce phosphorylation of Flt-1, whereas both of the single mutants gave an intermediate activation of Flt-1 receptor (Fig. 5A). As a consequence, the double mutant failed to activate the signaling proteins normally triggered by Flt-1 binding, whereas the partial activation of Flt-1 receptor by the single mutants resulted in a partial activation of the signaling proteins (Fig. 5B).

The inability of D72A/E73A-PIGF-1 variant to activate Flt-1 was further confirmed by two of the most common angiogenic assays, the in vitro capillary-like tube formation and the in vivo CAM assay. The double mutant was unable to induce HUVECs to organize into complex tubular structures as stimulated by wt PIGF-1. The specificity of this process was confirmed by inhibition of PIGF-1 binding to Flt-1 by means of a neutralizing monoclonal antibody (Fig. 6). Similarly, cells over-expressing the D72A/E73A-PIGF-1 variant were unable to stimulate in vivo neovascularization in CAM assay (Fig. 7E), whereas, as previously reported (34), wt PIGF-1 induces a significant angiogenic response (Fig. 7B). Interestingly, the cells over-expressing the single mutants Q27A-PIGF-1 and D72A-PIGF-1 failed to stimulate neovascularization in CAM assay (Fig. 7, C and D). In the same manner, both of the single mutants, despite their ability to induce a partial activation of Flt-1 receptor, were not able to induce capillary-like tube formation even when used at higher concentration (data not shown). These mutations generate PIGF variants that are unable to stimulate in vitro angiogenesis. These results have confirmed the ability of PIGF-1 to induce in vitro and in vivo angiogenesis and that the two residues, Asp-72 and Gln-73, of the β3-β4 loop, as well as residue Gln-27, located in the N-terminal α-helix, play a crucial role in PIGF-1-mediated angiogenic response.

The data obtained in this study have provided key information on the molecular basis of PIGF-1/Flt-1 interaction that, together with the recent crystal structure of PIGF/Flt-1p22 (28), becomes essential from a therapeutic perspective. Until now, efforts to stimulate or inhibit vessel growth therapeutically have been focused primarily on VEGF-A and its receptor Flk-1 (35), but the recent developments and insights are shifting this focus on PIGF and Flt-1. PIGF is able to stimulate neo-angiogenesis at levels comparable with VEGF-A without triggering the undesired side effects, such as vascular leakage and edema.
observed after administration of VEGF-A (17, 36). At the same time, a neutralizing monoclonal antibody against Flt-1 suppresses the growth of new vessels as well as angiogenesis-mediated inflammatory joint destruction in autoimmune arthritis and prevents the growth and rupture of atherosclerotic plaques (16). These results clearly indicate that PlGF and its receptor, Flt-1, represent potential therapeutic candidates for angiogenesis and inflammation (2, 18). The molecular details of PlGF/Flt-1 interaction now available could be utilized to design or to identify molecules that are able to modulate the pathway activated by the PlGF/Flt-1 interaction.

Furthermore, the D72A/E73A-PlGF-1 variant could represent a tool to investigate the role of PlGF/VEGF heterodimer. The function of the PlGF/VEGF heterodimer still remains controversial. Recently, Eriksson et al. (22) have reported that the PlGF/VEGF heterodimer is functionally inactive, whereas Autiero et al. (19) suggest that the heterodimer induces the heterodimerization of Flt-1 and Flk-1 receptors and induces an angiogenic response comparable with that of VEGF-A homodimer.

It could be hypothesized that the D72A/E73A-PIGF-VEGF heterodimer is unable to exert its biological function because of its inability to interact with the receptor on one end of the heterodimer. This heterodimer is probably unable to serve as a molecular bridge that brings about receptor dimerization. In this perspective, a knock-in approach with the substitution of wild type PIGF with the D72A/E73A variant will generate a mouse expressing an inactive form of PIGF homodimer (comparable with the knock-out PIGF mice) but also an inactive form of PIGF/VEGF heterodimer (not present in the knock-out PIGF mice). The verification of correct embryonic development and of the response after angiogenic stimulus in the adult will help in the understanding of the role of the PIGF/VEGF heterodimer under physiological and pathological conditions.

Acknowledgments—We thank Dr. P. Carmeliet for the monoclonal antibody anti-mPIGF. We also thank Maria De Mol for suggestions about the ELISAs, Salvatore Ponticelli for technical assistance, and Maria D’Agostino for typing the manuscript.

REFERENCES
1. Maglione, D., Guerriero, V., Viglietto, G., Delli-Bovi, P., and Persico, M. G. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9267–9271
2. De Falco, S., Gigante, B., and Persico, M. G. (2002) Trends Cardiovasc. Med. 12, 241–246
3. Ferrara, N., Gerber, H. P., and LeCouter, J. (2003) Nat. Med. 9, 669–676
4. Yancopoulos, G. D., Davis, S., Gale, N. W., Rudge, J. S., Wiegand, S. J., and Holash, J. (2000) Nature 407, 242–248
5. Carmeliet, P., Moons, L., Luttun, A., Vincenti, V., Campernelle, V., De Mol, M., Wu, Y., Bonu, F., Devy, L., Beck, H., Scholz, D., Acker, T., DiPalma, T., Dewerehin, M., Noel, A., Stalmans, I., Barra, A., Blacher, S., Vanden
PlGF Variants and Receptor Recognition

43939

dreesche, T., Ponten, A., Eriksson, U., Plate, K. H., Foidart, J. M., Schaper, W., Charnock-Jones, D. S., Hicklin, D. J., Herbert, J. M., Collen, D., and Persico, M. G. (2001) Nat. Med. 7, 575–583

6. Maglione, D., Guerrieri, V., Viglietto, G., Ferraro, M. G., Aprilekova, O., Alitaleo, K., Del Vecchio, S., Lei, K. J., Chou, J. Y., and Persico, M. G. (1993) Oncogene 8, 925–931

7. Hauser, S., and Weich, H. A. (1993) Growth Factors 9, 259–268

8. Cao, Y., Ji, W. R., Qi, P., and Rosin, A. (1997) Biochem. Biophys. Res. Commun. 235, 493–498

9. Yang, W., Ahn, H., Hinrichs, M., Torry, R. J., and Torry, D. S. (2003) J. Reprod. Immunol. 60, 53–60

10. Sato, Y., Kanno, S., Oda, N., Abe, M., Itu, M., Shitara, K., and Shibuya, M. (2000) Ann. N. Y. Acad. Sci. 902, 201–205, 205–207

11. 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

12. Carmeliet, P., Ng, Y. S., Nuyens, D., Theilmeier, G., Brusselmans, K., Cornelissen, I., Eller, E., Kakkar, V. V., Stalmans, I., Mattot, V., Perriard, J. C., Dewerchin, M., Flament, W., Nagy, A., Lupu, F., Moons, L., Collen, D., D’Amore, P. A., and Shima, D. T. (1999) Nat. Med. 5, 495–502

13. Park, J. E., Chen, H. H., Winer, J., Houck, K. A., and Ferrara, N. (1994) J. Biol. Chem. 269, 25646–25654

14. Pipp, F., Heil, M., Isbrucker, K., Ziegelschloeffler, T., Martin, S., van den Heuvel, J., Weich, H., Fernandez, B., Golomb, G., Carmeliet, P., Schaper, W., and Claus, M. (2003) Circ. Res. 92, 378–385

15. Luttun, A., Brusselmans, K., Fukao, H., Tjwa, M., Ueshima, S., Herbert, J. M., Matsuo, O., Collen, D., Carmeliet, P., and Moons, L. (2002) Biochem. Biophys. Res. Commun. 295, 428–434

16. Luttun, A., Tjwa, M., Moons, L., Wu, Y., Angelillo-Scherrer, A., Lia, F., Nagy, J. A., Hooper, A., Priller, J., De Klerk, B., Compernelle, V., Daci, E., Buchen, P., Dewerchin, M., Herbert, J. M., Fava, R., Mattijs, P., Carmeliet, G., Collen, D., Dvorak, H. F., Hicklin, D. J., and Carmeliet, P. (2002) Nat. Med. 8, 831–840

17. Thurston, G., Rudge, J. S., Ioffe, E., Zhou, H., Ross, L., Croll, S. D., Glazer, N., Hladik, J., McDonald, D. M., and Yancopoulos, G. D. (2000) Nat. Med. 6, 460–463

18. Luttun, A., Tjwa, M., and Carmeliet, P. (2002) Ann. N. Y. Acad. Sci. 979, 80–93

19. Autiero, M., Waltenberger, J., Communi, D., Kranz, A., Moons, L., Lambrecht, D., Kroll, J., Plaisance, S., De Mol, M., Bon, F., Kilche, S., Fellrich, G., Ballmer-Hofmann, K., Maglione, D., Mayr-Beyle, U., Dewerchin, M., Dombrowski, S., Stanimirovic, D., Van Hummelen, P., Dehio, C., Hicklin, D. J., Persico, G., Herbert, J. M., Shibuya, M., Collen, D., Conway, E. M., and Carmeliet, P. (2003) Nat. Med. 9, 936–943

20. DiSalvo, J., Bayne, M. L., Conn, G., Kwek, P. W., Trivedi, P. G., Soderman, D. D., Palisi, T. M., Sullivan, K. A., and Thomas, K. A. (1995) J. Biol. Chem. 270, 7717–7723

21. Cao, Y., Chen, H., Zhou, L., Chiang, M. K., Anand-Apte, B., Weatherbee, J. A., Wang, Y., Fang, F., Flanagan, J. G., and Tsang, M. L. (1996) J. Biol. Chem. 271, 3154–3162

22. Eriksson, A., Cao, R., Pawliuk, R., Berg, S. M., Tsang, M., Zhou, D., Fleet, C., Triatsaris, K., Dissing, S., Leboulch, P., and Cao, Y. (2002) Cancer Cell 1, 99–108

23. Cao, Y., Linden, P., Shimada, D., Browne, F., and Folkman, J. (1996) J. Clin. Investig. 98, 2507–2511

24. Muller, Y. A., Christinger, H. W., Heyt, B. A., and de Vos, A. M. (1997) Structure 5, 1325–1338

25. Iyer, S., Leonidas, D. D., Swaminathan, G. J., Maglione, D., Battisti, M., Tucci, M., Persico, M. G., and Acharya, K. R. (2001) J. Biol. Chem. 276, 12153–12161

26. Wiesmann, C., Fuh, G., Christinger, H. W., Eigenbrot, C., Wells, J. A., and de Vos, A. M. (1997) Cell 91, 685–704

27. Krey, B. A., Nguyen, H. V., Berleau, L. T., Duarte, C. M., Park, J., Chen, H., and Ferrara, N. (1996) J. Biol. Chem. 271, 5638–5646

28. Christinger, H. W., Fuh, G., De Vos, A. M., and Wiersmann, C. (2004) J. Biol. Chem. 279, 10382–10388

29. Maglione, D., Guerrieri, V., Rambaldi, M., Rosso, G., and Persico, M. G. (1993) Growth Factors 8, 141–152

30. Desai, J., Holt-Shore, V., Torry, R. J., Caudle, M. R., and Torry, D. S. (1999) Biol. Reprod. 60, 887–892

31. Solvaraz, S. K., Giri, R. K., Perelman, N., Johnson, C., Malik, P., and Kalra, V. K. (2003) Blood 102, 1515–1524

32. Ribatti, D., Nico, B., Morbidelli, L., Donnini, S., Ziche, M., Vacca, A., Roncali, L., and Presta, M. (2001) J. Vasc. Res. 389, 389–397

33. Davis-Smyth, T., Presta, M. G., and Ferrara, N. (1998) J. Biol. Chem. 273, 3216–3222

34. Ziche, M., Maglione, D., Ribatti, D., Morbidelli, L., Lago, C. T., Battisti, M., Fasolato, I., Barra, A., Tucci, M., Parise, G., Vincenti, V., Granger, H. J., Viglietto, G., and Persico, M. G. (1997) Lab. Investig. 76, 517–531

35. Imer, J. M., Vale, P. R., Symes, J. F., and Lossorso, D. W. (2001) Circ. Res. 89, 409–413

36. Epstein, S. E., Kornowski, R., Fuchs, S., and Dvorak, H. F. (2001) Circulation 104, 115–119