β-Hairpin Peptide That Targets Vascular Endothelial Growth Factor (VEGF) Receptors

DESIGN, NMR CHARACTERIZATION, AND BIOLOGICAL ACTIVITY* SS

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VEGF receptors have been the target of intense research aimed to develop molecules able to inhibit or stimulate angiogenesis. Based on the x-ray structure of the complex placental growth factor-VEGF receptor 1D2, we designed a VEGF receptor-binding peptide reproducing the placental growth factor β-hairpin region Gln87–Val100 that is involved in receptor recognition. A conformational analysis showed that the designed peptide adopts the expected fold in pure water. Moreover, a combination of NMR interaction analysis and cell binding studies were used to demonstrate that the peptide targets VEGF receptors. The VEGF receptor 1D2-interacting residues were characterized at the molecular level, and they correspond to the residues recognizing the placental growth factor sequence Gln87–Val100. Finally, the peptide biological activity was characterized in vitro and in vivo, and it showed a VEGF-like behavior. Indeed, the peptide activated VEGF-dependent intracellular pathways, induced endothelial cell proliferation and rescue from apoptosis, and promoted angiogenesis in vivo. This compound is one of the few peptides known with proangiogenic activity, which makes it a candidate for the development of a novel peptide-based drug for medical applications in therapeutic angiogenesis.

Angiogenesis is a fundamental physiological process involving the formation of a blood vessel from a preexisting one. It is particularly active during embryogenesis, whereas it is mainly quiescent during the adult life except for specific physiological conditions and pathological states. Angiogenesis is tightly regulated by several angiogenic factors, especially growth factors such as the vascular endothelial growth factor (VEGF). VEGF is a cytokine affecting proliferation, migration, and survival of endothelial cells (ECs) 1 (1), and it is involved in pathological angiogenesis (2). VEGF is a homodimeric disulfide-bound glycoprotein belonging to the cysteine knot growth factor family. It is encoded by a single gene, which is expressed in several isoforms due to different splicing events; VEGF165, the most abundant isoform, binds to heparin with high affinity (3). The biological function of VEGF is mediated by two tyrosine kinase transmembrane receptors: VEGF receptor 1 (VEGFR1/Fms-related tyrosine kinase 1) and VEGF receptor 2 (VEGFR2/kinase insert domain receptor). They are composed of an intracellular kinase domain, a short transmembrane region, and an extracellular portion constituting seven immunoglobulin-like domains. Receptors dimerize upon ligand binding, and the dimer formation triggers autophosphorylation of the cytosolic kinase domains, which turn on the intracellular signaling, ending in endothelial cell activation (4). VEGF possesses several homologs, which differ in the ability to recognize membrane receptors and in their biological activity. In particular, VEGF strongly binds to both receptors, whereas placental growth factor (PIGF) (5), which is receiving much attention because of its role in pathological angiogenesis (6, 7), only interacts with VEGFR1.

Much structural information on the natural ligands and their receptors has been reported. In particular, the x-ray structures of the complex between VEGF or PIGF and the second extracellular domain of VEGF receptor 1 (VEGFR1D2) have been described (8, 9). The recognition ligand interface is divided between both monomers and is distributed over a discontinuous surface that includes residues from the N-terminal helix, the loop connecting strands β3 and β4, and the β-hairpin region composed of strands β5 and β6 together with the connecting turn (8, 9).

Here we report the design, conformational analysis in aqueous solution, receptor interaction studies by NMR, and biological properties of a 17-mer β-hairpin peptide, HPLW, based on the β-hairpin sequence 87–100 of PIGF. Interestingly, HPLW adopts the expected fold in pure water, targets VEGF receptors, and shows in vitro and in vivo a VEGF-like activity. It is one of the few peptides with proangiogenic activity so far reported.
This peptide could find application in the area of therapeutic angiogenesis.

**EXPERIMENTAL PROCEDURES**

**Materials and Methods**

N,N-Dimethylformamide and N,N-diisopropylethylamine (DIPEA) were from Romil (Cambridge, UK), fluorenymethyl-oxycarboxy (Fmoc)-amino acids, and coupling reagents were purchased from Merck. All other reagents were from Sigma-Aldrich. Human umbilical vein endothelial cells (HUVECs) were purchased from Promocell (Heidelberg, Germany). All experiments were performed on low passage cell cultures. Cells were grown in EGM-2 ( endothelial growth medium) from Cambrex (Carlsbad, CA) at 37 °C and in 5% CO2. Experiments were performed on low passage cell cultures. Cells were purchased from Promocell (Heidelberg, Germany). All reagents were from Sigma-Aldrich. Human umbilical vein endothelial cells (HUVECs) were grown in EGM-2 (endothelial growth medium) from Cambrex (Carlsbad, CA) at 37 °C and in 5% CO2. Human recombinant VEGF165 was obtained from R&D Systems (Minneapolis, MN). VEGFR2D1–D6 was obtained from VEGFR1, and phycocerythrin-conjugated integrin 81 integrin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies against phospho-ERK1/2 (Thr202/Tyr204), phospho-AKT (Ser 473), AKT, VEGFR2, phospho-VEGFR2 (Tyr951), and phospho-RB (Ser780) were purchased from Cell Signaling Technology, Inc. (Danvers, MA).

**Animals**

Female CD1 mice (20–25 g) were supplied by Charles River and kept in temperature- and humidity-controlled rooms (22 °C and 50%, respectively) with lights on from 07:00 to 19:00 h with water and food available ad libitum. All procedures were carried out in accordance with the Italian law (Legislative Decree Number 116, January 27, 1992), which acknowledges the European Directive 86/609/EEC, and were fully compliant with GlaxoSmithKline policy on the care and use of laboratory animals and codes of practice. Furthermore, all efforts were made to minimize the number of animals used and their suffering.

**Peptide Synthesis**

Peptide HPLW and its scrambled analog (scHPLW; NH2-DQTRILWYSWRGLSFPGK-OH) were synthesized on a solid phase by standard Fmoc chemistry. The synthesis was carried out on the Tentagel HL-PHB resin (Fluka) (loading 0.35 mmol/g) with 30% piperidine in N,N-dimethylformamide, and coupling reactions were carried out with a 10-fold excess of amino acid and 9.9 eq of 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylethylene hexafluoraphosphoramide, 9.9 eq of 1-hydroxybenzotriazole, and 20 eq of DIPEA in N,N-dimethylformamide. Each coupling was carried out for 60 min followed by a capping step (20 min) performed with a solution of 2 M acetic anhydride, 0.06 M 1-hydroxybenzotriazole, and 0.55 M DIPEA in N-methylpyrrolidone. The peptides were cleaved off the resin and deprotected using a mixture of TFA/H2O/triisopropylsilane (95:2.5:2.5, v/v/v) for 3 h at room temperature. The resins were finally filtered, and the peptides were precipitated using cold diethyl ether. Peptides were labeled with fluorescein on a solid phase after selective deprotection of the methyltrityl side chain-protecting group of the N-terminal Lys with 1% TFA and 5% triisopropylsilane in dichloromethane. The Lys side chain amine group was incubated after a neutralization step (1.2 M DIPEA) with 3 eq of 6-[fluorescein-5(6)-carboxamido]hexanoic acid (Fluka) in the presence of 3 eq of N,N,N’,N’-tetramethyl-O-(7-azabenzotriazol-1-yl)uronium hexafluorophosphate (Advanced Biotech Italia) and 6 eq of DIPEA in N,N-dimethylformamide overnight at room temperature.

All crude products were purified by preparative reverse phase HPLC using a C12 column (Jupiter Proteo, 25 × 2.2 cm, 90 Å, 10 μm; Phenomenex). Peptide analysis was performed on an LC-MS instrument (LCQ DECA XP, ThermoFinnigan) equipped with diode array detector combined with an electrospray ion source and an ion trap mass analyzer using a C18 column (Jupiter, 50 × 2 mm, 5 μm, 300 Å; Phenomenex) and a linear gradient of H2O (0.1% TFA) and CH3CN (0.1% TFA) from 5 to 70% CH3CN in 30 min. All peptides showed a purity above 95% based on the chromatographic peak area revealed at 210 nm: HPLW MS m/z calc, 2168.5 atomic mass units; found, 2167.9; scHPLW MS m/z calc, 2168.5 atomic mass units; found, 2168.4; fluorescein-HPLW MS m/z calc, 2640.0 atomic mass units; found, 2638.9; fluorescein-scHPLW MS m/z calc, 2640.0 atomic mass units; found, 2639.2 atomic mass units.

**VEGFR1D2 Preparation**

The 15N-labeled protein was prepared according to the procedure reported in Di Stasi et al. (10). Briefly, 15N-labeled recombinant His-tagged VEGFR1D2 was expressed in Escherichia coli BL21-CodonPlus (DE3)-RIL cells. The protein was solubilized from inclusion bodies in 50 mM Tris-HCl, 10 mM imidazole, and 8 M urea, pH 8; refolded by equilibrating the nickel-nitriol triacetic acid resin in 50 mM Tris-HCl, 10 mM imidazole, and 300 mM NaCl, pH 8 with decreasing concentrations of urea; and then eluted by increasing the imidazole concentration from 100 to 300 mM. After tag cleavage with tobacco etch virus protease, the protein was concentrated and purified to homogeneity by size exclusion chromatography using an S75 column (GE Healthcare) equilibrated in 50 mM Tris-HCl and 250 mM NaCl, pH7. Finally, the protein was concentrated to 0.9 mM by using an Amicon Ultra system (Millipore).

**NMR Spectroscopy**

NMR samples were prepared in either a 90% H2O and 10% 2H2O mixture or 99.9% 1H2O. The peptide concentration was 1.0 mM as measured by reading the absorbance at 280 nm and
using a molar extinction coefficient of 13,980 M$^{-1}$ cm$^{-1}$. NMR data were collected on a 600-MHz Varian INOVA spectrometer equipped with a cold probe. Data for homonuclear two-dimensional total correlation spectroscopy (70-ms mixing time) and two-dimensional NOESY (250-ms mixing time) spectra were recorded by using presaturation of the water signal and the time-proportional phase incrementation mode. Double quantum-filtered COSY was performed with 4096 data points in the F2 dimension and 500 increments with 64 scans to obtain a suitable resolution to measure 31H, 1H$\alpha$ coupling constants. All spectra were processed with the software SPARKY (11) and analyzed with Neasy (12), a tool of computer-aided resonance assignment (CARA) software.

Starting structures were calculated using CYANA 2.1 (13) initiating from 100 random conformers. The 20 structures with the lowest CYANA target function were analyzed with MOLMOL (14) and PyMOL and further refined using SWISS-PdbViewer (15). A total of 600 steps of steepest descent minimization using the Gromos 96 force field were applied to each of the 20 structures over three steps. The diffusion-ordered NMR spectroscopy (16) was performed using the pulsed gradient spin-echo NMR method. The following relationship exists between the translational self-diffusion parameter $D$ and the NMR parameters: $I/\eta_0$ = $\exp(D\gamma^2\delta^2G^2(\Delta - \delta/3))$ where $I$ is the measured peak intensity of a particular group of resonances, $I_0$ is the maximum peak intensity of the same group of resonances at the smaller gradient strength, $D$ is the translational self-diffusion constant (in m$^2$ s$^{-1}$), $\gamma$ is the gyromagnetic ratio of a proton $(2.675 \times 10^8$ radians G$^{-1}$ s$^{-1}$), $\delta$ is the duration (in seconds) of the gradient, $G$ is the strength of the gradient (in G cm$^{-1}$), and $\Delta$ is the time (in seconds) between the two gradients. Experiments were acquired by using the pulsed field gradient-longitudinal eddy current delay pulse sequence with a postgradient eddy current relaxation of 5 ms. Each experiment was averaged over 128 scans, and the number of points was 16,000. The strength of the gradient pulses was varied from 2% of the total power of the gradient coil to 95%, and their shape was a sine function. The duration of the gradient was varied between 3.0 and 2.0 ms, and the time between both gradients was changed between 100 and 150 ms.

The equilibrium constant ($K_{eq}$) for folding was estimated assuming a two-state model in which the folded and unfolded states are in rapid equilibrium producing time-averaged chemical shifts. The r.m.s. deviation in H$\alpha$ chemical shifts from random coil values taken over all residues was used to estimate hairpin populations. The 0% folded limit can be equated with the established random coil chemical shift (RMS$\Delta$H$\alpha$ = 0), whereas the 100% folded limit was determined from the 50% methanol data at 277 K (RMS$\Delta$H$\alpha$ $\alpha$ limits). Thus, $K_{eq}$ = [folded]/[unfolded] where $K_{eq}$ = $1(1 - (\text{RMS}\Delta H_{\alpha}/\text{RMS}\Delta H_{\alpha} \text{ limits}))$ (17).

To map the binding site of HPLW on VEGFR1$_{D2}$, 15N uniformly labeled VEGFR1$_{D2}$ (200 mm) in 50 mM Tris and 100 mm NaCl at pH 7 was titrated with increasing amounts of unlabeled peptide (0.05, 0.10, 0.15, 0.20, 0.40, 0.60, and 1.0 mm). The per residue chemical shift perturbation upon binding weighted average chemical shift differences, $\delta$H$_{\alpha}$, were calculated for the amide $^{15}$N and $^1$H resonances using the following equation:

$$\delta H_{\alpha} = ((\Delta H)^2 + (0.17 \times \Delta^{15}N)^2)^{1/2}$$

where $\Delta H$ and $\Delta N$ are the differences between free and bound chemical shifts. The dissociation constants ($K_{eq}$) were estimated from the changes in both $^1$H and $^{15}$N chemical shifts as observed in the two-dimensional $^1$H, $^{15}$N heteronuclear single quantum correlation spectra of VEGFR1$_{D2}$ upon binding to HPLW peptide. The chemical shift changes for each residue measured at various protein/peptide ratios were plotted versus ligand concentration, and the results were fitted by nonlinear regression according to the equation (18)

$$\Delta \delta = \Delta \delta_{\text{max}}(K_p + [P] + [L] - ((K_p + [P] + [L]^2) - 4[P][L])^{1/2}/2[P])$$

Flow Cytometry

VEGFR Expression Determination—HUVECs (10$^6$) and WS1 cells (10$^6$) were resuspended in complete medium and incubated for 1 h at 4 °C. Then cells were blocked with 10% normal goat serum in 1× PBS (Jackson ImmunoResearch Laboratories) for 20 min on ice and incubated with VEGFR1 and VEGFR2 polyclonal antibodies in PBS containing 2% normal goat serum for 60 min at RT. Rabbit IgG was used as a negative control. After washing with PBS, cells were stained with rabbit anti-DyLight 488 conjugate (1:500) for 60 min on ice. After washing with PBS, the cells were resuspended in PBS and analyzed with a FACScan (BD Biosciences) flow cytometer.

Peptide Binding Experiments—HUVECs (3 × 10$^5$) were incubated with different concentration of fluorescein-conjugated peptides and the other indicated reagents (VEGF$_{165}$, VEGFR1$_{D2}$ or VEGFR2$_{D1-D6}$) for 30 min at 4 °C in the dark. After washing with PBS, the cells were resuspended in PBS and analyzed with a FACScan (BD Biosciences) flow cytometer.

Immunoprecipitation

HUVECs were plated in 100-mm dishes at 1 × 10$^5$ cells/cm$^2$. On the next day, cells were incubated in starvation medium (EBM-2 (endothelial cell basal medium), 0.1% heparin, and 0.1% BSA) alone or in the presence of VEGF$_{165}$ (25 ng/ml) and with HPLW (100 ng/ml) or scrambled HPLW (100 ng/ml) for 15 min at 37 °C. After treatment, cells were harvested and lysed with TNN buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, and 0.1% Triton X-100). 500 µg of proteins were used for the immunoprecipitation assay using VEGFR2 polyclonal antibody (Cell Signaling Technology, Inc.) at a 1:100 dilution with incubation at 4 °C overnight on a tube rotator. 30 µl of protein A-Sepharose (Amersham Biosciences) were then added, and after incubation for 1 h at 4 °C, the immunocomplexes were precipitated and washed five times with TNN buffer. Proteins obtained from immunoprecipitations were loaded for SDS-PAGE and blotted on a nitrocellulose membrane.
Western Blotting
HUVECs were treated in starvation medium with HPLW (25–100 ng/ml) and scHPLW (100 ng/ml) peptides and VEGF_{165} (25 ng/ml) for 15 and 30 min at 37 °C. After treatment, whole cell lysates were obtained by using radioimmuno precipitation assay lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM EGTA, and 0.05% SDS) supplemented with complete protease inhibitors and phosphate inhibitors (Pierce). Proteins (25 μg) were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane (Millipore) by semidy electromblotting (Transblot, Bio-Rad). Primary antibodies were incubated in Tris-buffered saline-Tween 20 containing 5% BSA or 5% nonfat dry milk overnight at 4 °C. Immunoreactivity was detected by sequential incubation with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) and standard chemiluminescence (Pierce).

Analysis of Caspase-3 Activity
Determination of caspase-3 activity was performed by a fluorometric assay based on the proteolytic cleavage of the 7-amido-4-methylcoumarin (AMC)-derived substrate (Ac-DEVD-AMC, Sigma-Aldrich), which yields a fluorescent product. HUVECs were treated in starvation medium with HPLW (1, 2.5, 5, 10, 25, 50, and 100 ng/ml) or scHPLW (100 ng/ml) peptide for 8 h at 37 °C. VEGF_{165} (25 ng/ml) was used as a positive control. After 8 h, cells were processed with 150 ml of caspase-3 reaction buffer (50 mM HEPES, pH 7.5, 0.1 mM EDTA, 0.1% Nonidet P-40, 0.1% CHAPS, and 1 mM DTT), and 20 mg of lysates were incubated in 96-well plates with 20 mM Ac-DEVD-AMC at 37 °C for 3 h. Samples were analyzed using a microplate reader (L5.5 luminescence spectrometer, PerkinElmer Life Sciences) (excitation, 360 nm; emission, 440 nm).

Cell Proliferation Assay
HUVECs were plated at a density of 1200 cells/well in 96-well poly-D-lysine-coated microplates (BD Biosciences). After 24-h incubation in starvation medium, cells were treated with VEGF_{165} (25 ng/ml), HPLW peptide (10–100 ng/ml), or scHPLW peptide (100 ng/ml). Cell proliferation was determined by using the CyQUANT NF cell proliferation assay kit (Molecular Probes) at 24, 48, and 72 h after treatment. Briefly, medium was removed, and cells were incubated with CyQUANT NF reagent for 1 h at 37 °C according to the manufacturer’s instructions. Plates were then analyzed by using a microplate reader (L5.5 luminescence spectrometer, PerkinElmer Life Sciences) (excitation, 485 nm; emission, 520 nm).

In Vivo Angiogenesis Assay
In vivo angiogenesis was assayed by using a directed in vivo angiogenesis assay (DIVAA) (Cultrex, Trevigen). Sterile silicone cylinders closed at one end, angioreactors, were filled with 20 ml of basement membrane extract premixed with or without angiogenesis factors (VEGF and FGF) to obtain positive and negative controls, respectively. Furthermore, HPLW (5, 10, and 100 ng/ml) or scHPLW (100 ng/ml) peptide was added to angioreactors, which were incubated at 37 °C for 1 h to allow gel formation before subcutaneous implantation into the dorsal flank of CD1 mice. Vessel formation evaluation was performed after 21 days. Matrigel was removed from the angioreactors and digested in 300 μl of CellSperse solution for 1 h at 37 °C. After digestion, the incubation mixture was cleared by centrifugation at 800 rpm. Cell pellets were resuspended in 500 ml of Dulbecco’s modified Eagle’s medium (DMEM) and 10% FBS and plated on coverslips in 24-well plates for 16 h at 37 °C in 5% CO₂. Cells were fixed with a 3.7% formaldehyde solution for 30 min at RT and quenched by incubation with 0.1 mM glycine for 5 min. Subsequently, cells were incubated with FITC-lectin reagent (in the kit) and then observed with a fluorescence microscope (Zeiss).

Statistical Analysis
Statistical analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software). Results are reported as the mean with S.D. of at least three experiments run in three to five replicates. Comparisons between control and treated samples were made with a paired Student’s t test.

RESULTS

Peptide Engineering—Based on the x-ray structure of the PlGF-VEGFR1D2 complex (9), we designed and synthesized a peptide reproducing the receptor binding region of PlGF spanning the amino acid sequence Gln^{87}–Val^{100}. This region contains five (Gln^{87}, Leu^{89}, Ile^{91}, Pro^{97}, and Tyr^{99}) of 21 residues situated at less than 4.5 Å from the receptor, and it assumes in the natural protein a β-hairpin conformation. The design strategy we adopted was to keep fixed the three-dimensional arrangement of the residues interacting with the receptor, stabilizing the β-hairpin conformation with the lowest number of mutations. All five interacting residues face the same side of the hairpin and make hydrophobic interactions with the receptor. The β-hairpin conformation was stabilized by introducing aromatic amino acids in opposite strands to create a hydrophobic cluster (19). To keep the natural loop conformation, amino acid substitutions in the loop were avoided. Lys^{90}, Ser^{98}, and Val^{100} (PlGF numbering) were replaced by Trp^{5}, Trp^{13}, and Tyr^{15} (peptide numbering), respectively (Fig. 1A). Trp^{5} and Trp^{13} will make a cross-strand interaction like Leu^{2} and Tyr^{15}, which will complete the hydrophobic cluster. At the N terminus a lysine residue was inserted to allow further peptide derivatization, whereas the Glu^{101} was replaced with a threonine residue. Finally, a Ser residue was added to the C terminus to make the two strands the same length. Serine and threonine residues were chosen because of their intrinsic preference for a β conformation and their hydrophilic character. The amino acid sequences of the designed peptide HPLW and the corresponding PlGF-β-hairpin region are reported in Fig. 1A.

Conformational Analysis in Solution—HPLW conformation in solution was analyzed using NMR techniques at a concentration of 1 mM and pH of 6.8; under these conditions, the peptide showed a monomeric aggregation state as determined by diffusion-ordered spectroscopy. In particular, diffusion-ordered spectroscopy measurements (supplemental Fig. S1) provided a diffusion coefficient ($D_{1}$) value of $1.95 \times 10^{-10}$ m$^2$ s$^{-1}$, which is
VEGF Receptor-binding β-Hairpin Peptide

A

PLGF

HPLW

87

QLLKIRSGDRPSYVE

KOLLWIRSGDRPWYYS

101

B

C

D

FIGURE 1. Solution structure of HPLW peptide. A, amino acid sequences of the natural PLGF β-hairpin spanning amino acids 87–101 and the designed peptide HPLW. B, superposition of the backbone atoms (residues 3–15) of the 20 lowest CYANA target function structures calculated for the HPLW peptide. C, backbone superposition of the HPLW representative structure (blue) and sequence 88–99 of PLGF (yellow). D, backbone superposition of the HPLW representative structure (blue) and sequence 80–91 of VEGF (red).

TABLE 1

| NMR structural statistics of HPLW peptide | r.m.s.d., root mean square deviation. |
|------------------------------------------|--------------------------------------|
| Quantity                                 | Value                                |
| NOE upper distance limit                 | 191                                  |
| Intraresidue                             | 105                                  |
| Short distance                           | 47                                   |
| Medium/long distance                     | 39                                   |
| Dihedral angle constraints               | 81                                   |
| Residual target function (Å)             | 0.16 ± 0.02                          |
| Residual NOE violations                   | Number >0.1 Å                         | ±1
greater than 2 Å                                                              |
| Maximum (Å)                              | 0.15 ± 0.02                          |
| Residual angle violations                 | Number >2.0°                          | 0 ± 0                           |
| Maximum (°)                              | 0                                    |
| Amber energies (kJ/mol)                  | Total                                | −386 ± 20                       |
|                                          | van der Waals                        | −111 ± 15                       |
|                                          | Electrostatic                        | −208 ± 18                       |
| Correlation coefficients (Å)             | Total                                | 0.24 ± 0.20                     |
|                                          | Maximum (Å)                          | 1.03 ± 0.31                     |

Overall, the NMR analysis suggests that the HPLW folds into a well-defined β-hairpin conformation and shows good structural features in agreement with what reported for other monomeric peptides in the same conditions (20).

The sequence-specific resonance assignment of the peptide HPLW was accomplished using a combination of double quantum-filtered COSY, total correlation spectroscopy, and NOESY experiments (21). The complete 1H resonance assignment is listed in supplemental Table S1. The analysis of HPLW HA chemical shifts revealed significant deviations from random coil values (ΔδHα) (22). As shown in supplemental Fig. S2A, the chemical shift index analysis of the ΔδHα indicated the presence of two β-strands consisting of residues 3–6 and 11–14 separated by a turn made by residues 7–10. This folded structure was confirmed by the pattern of sequential and long range NOEs consistent with the antiparallel alignment of two β-strands (supplemental Fig. S2B). In particular, a number of intense interstrand Hα-Hα (between residues Gln2 and Thr16, Leu4 and Tyr14, and Ile6 and Pro12) and HN-HN (between residues Leu3 and Tyr15, Trp5 and Trp13, and Arg7 and Asp10) interactions were detected and support the presence of a two-stranded β-sheet. Furthermore, several short range (δαN(i,i+2)) NOEs between residues Arg7 and Gly9 and Ser8 and Asp10 are diagnostic of the involvement of these residues in a β-turn.

For structure refinement, the final input file for the CYANA structure calculation software contained 191 meaningful distance constraints (105 intraresidue, 47 sequential NOEs, and 39 medium and long range NOEs) and 81 angle constraints. NMR structural statistics are shown in Table 1. The backbone superposition of the best 20 CYANA conformers (reported in Fig. 1B) shows that the NMR solution structure of the HPLW is well defined, satisfying the experimental distance restraints. The r.m.s. deviation values of the backbone and of all heavy atoms from residues 3 to 15 are 0.24 and 1.02 Å, respectively. The HPLW structure consists of two antiparallel β-strands connected by a type I β-turn. This turn is stabilized by a hydrogen bond between the backbone carbonyl oxygen of Arg7 and the amide hydrogen of Asp10, whereas the β-hairpin conformation is further stabilized by four interstrand hydrogen bonds created by the amide protons of residues Leu4, Trp5, Trp13, and Tyr15 interacting with the carbonyl group of residues Tyr15, Trp13, Trp5, and Leu4, respectively (supplemental Fig. S2C). The presence of these H-bonds was further experimentally confirmed by the measured amide proton temperature coefficients (supplemental Fig. S2D). The β-hairpin conformation is also stabilized by several short distance interactions between non- vicinal peptide residues that are brought close in space because of the folding of the peptide; in particular, several NOE contacts between the hydrophobic side chain of the residues Leu4, Leu4, Trp5, Trp13, Tyr14, and Tyr15 were identified, suggesting the presence of a hydrophobic cluster that contributes to stabilizing the β-hairpin conformation. Finally, the chemical shifts of the two Trp aromatic rings merit consideration because they could provide information about their relative orientation (23). In HPLW, the chemical shifts of the Hα and Hδ protons of the two tryptophans are upfield-shifted, suggesting that the Trp indole rings are packed in a face to edge arrangement, according to NOE data and as found in the calculated structures (supplemental Fig. S3). To measure the folded population, r.m.s. deviations of Hα chemical shifts (RMSDδHα) from random coil values were used as a quantitative measure of percent folded. The RMSDδHα of the fully unfolded states was set to zero, whereas data for HPLW in 50% methanol at 277 K were used as a reasonable estimate for the fully folded state in agreement with a number of other model β-hairpin peptides (17, 24). Based on RMSDδHα values, the designed peptide folds to a significant degree (∼70%) in water at 298 K.

Overall, the NMR analysis suggests that the HPLW folds into a well-defined β-hairpin conformation and shows good struc-
tural similarity with PIGF 88–99 (r.m.s. deviation, 1.02 Å; calculated on backbone atoms from residues 3 to 15) and VEGF 80–91 (r.m.s. deviation, 1.16 Å) sequences (Fig. 1, C and D, respectively). Furthermore, the five interacting residues are displayed on the same side of the β-hairpin as required for optimal interaction with the receptor (supplemental Fig. S4).

Receptor Binding Interaction by NMR—NMR titration experiments were carried out to study the interaction between HPLW peptide and VEGFR1D2 protein (10, 25) and to estimate the dissociation constant ($K_d$) of the complex. Upon progressive additions of HPLW to the free 15N uniformly labeled VEGFR1D2, continuous $^1$H and $^{15}$N chemical shift perturbations of several signals of VEGFR1D2 were detected in the two-dimensional $[^1$H, $^{15}$N] heteronuclear single quantum correlation spectra (supplemental Fig. S5). VEGFR1D2 showing the most significant changes of $^1$H and $^{15}$N chemical shifts upon formation of the complex was normalized (Fig. 2A) (26) and mapped onto the NMR solution structure of VEGFR1D2 (Protein Data Bank code 1QSV). These residues are located in strand A’ (Gln144, Ile145, Ile146, His147, and Met148), in strand G (Leu221, Thr222, His223, Arg224, Glu225, and Thr226), and in the N-terminal region of the protein encompassing residues Ser149, Glu141, and Ile142 (Fig. 2B). These data indicate that the binding surface recognized by the peptide is very similar to the site interacting with residues 87–99 of PIGF (9). The $K_d$ for the HPLW-VEGFR1D2 complex (1:1 binding stoichiometry) was estimated to be in the micromolar range (32 ± 2 μM) after analysis of the NMR titration curves of several VEGFR1D2-interacting residues (Fig. 2C).

Receptor Binding Studies on HUVECs—The ability of HPLW peptide to bind to cell membrane VEGF receptors was checked by a flow cytometry analysis on ECs. HUVECs were incubated with an increasing concentration of fluorescein-conjugated peptides. Untreated cells or cells incubated with scHPLW showed a lower fluorescence in comparison with cells incubated with HPLW peptide, suggesting a specific binding to the cell membrane in a dose-dependent manner (Fig. 3A). A small amount of unspecific binding was observed with a high concentration of the control peptide probably because of its high hydrophobicity. Next, we verified whether VEGF receptors were implicated in peptide binding to ECs. A competition experiment was performed in which HUVECs were incubated with 500 nM fluorescein-HPLW peptide and increasing VEGF concentrations (from 0.05 to 500 nM). The analysis of flow cytometry data showed a decrease of HPLW fluorescent signal when the VEGF concentration was increased up to 500 nM (Fig. 3B). No variation in fluorescence intensity was observed in an experiment in which the fluorescein-scHPLW competed with VEGF, suggesting that the unspecific binding on ECs was not mediated by VEGF receptors (supplemental Fig. S6A). Moreover, we also verified whether in the experimental condition used for the competition experiments VEGF binding was specific to the receptors. In fact, no variation of fluorescence intensity was observed when VEGF was added to cells treated with phycocerythrin-conjugated β1 integrin antibody (anti-phycocerythrin-β1 integrin) because of its failure to interact with integrins present on the EC surface (supplemental Fig. S6B). WS1 human fibroblast cells were used to verify the binding of peptide HPLW to cells not expressing VEGF receptors. The absence of VEGFRs was confirmed by Western blot and flow cytometry analyses (supplemental Fig. S7). Incubation of WS1 cells with fluorescein-HPLW peptide (500 nM) did not affect cell fluorescence (supplemental Fig. S8), indicating that the peptide did not bind cells without VEGFRs. Moreover, to confirm the binding specificity of peptide HPLW to VEGF receptors, a binding competition experiment was performed in which cells were incubated with HPLW and an increasing concentration of soluble VEGF (Fig. 3C) or VEGFR2 (supplemental Fig. S6D). The presence of the receptors clearly shifted the fluorescent signals toward the untreated control, indicating that peptide is no longer binding to ECs but is quenched by the soluble VEGF receptors. Soluble receptor binding was specific for the peptide as VEGFR1D2 or VEGFR2D1–D6 did not alter the fluorescence intensity observed when cells were treated with anti-phycocerythrin-β1 integrin (supplemental Fig. S9). These results suggest that HPLW is a VEGF receptor-binding peptide sharing part of the VEGF binding site on both receptors.

EC Rescue from Apoptosis—To preliminarily evaluate the biological behavior of the peptide HPLW, we performed a caspase-3 fluorometric assay. VEGF is a survival factor for ECs. Serum-deprived HUVECs undergo apoptosis, which is monitored by the increase of caspase-3 activity, and are rescued in the presence of VEGF (27). HUVECs were treated in serum-deprived conditions with HPLW peptide (100 ng/ml) in the absence or presence of VEGF (25 ng/ml). In the absence of VEGF, HPLW peptide significantly reduced caspase-3 activity (65%; $p = 0.002$) with respect to starvation analogously to the
effect elicited by VEGF. When ECs were incubated with peptide and VEGF, no inhibition of VEGF biological activity was observed (Fig. 4A). To confirm the VEGF-like prosurvival activity of HPLW peptide, a dose dependence experiment was performed. Caspase-3 activity was measured in serum-deprived HUVECs treated with HPLW peptide at a concentration range of 1–100 ng/ml. Fig. 4B shows that HPLW was able to significantly reduce caspase-3 activity in a dose-dependent manner, and notably, a 20% recovery from serum deprivation-induced apoptosis was observed at a concentration of 5 ng/ml. The peptide scHPLW at a concentration of 100 ng/ml was ineffective in reducing caspase-3 activity; in comparison, HPLW at the same concentration completely inhibited caspase-3 activity ($p \leq 0.0007$) similarly to VEGF. We evaluated the effect of soluble VEGFRs on the prosurvival activity of peptide HPLW. In particular, they should compete with cell membrane receptors for
peptide binding, reducing the HPLW effective concentration in solution and indeed its prosurvival effect. In the presence of 50 nM HPLW, increasing concentrations of VEGFR1D2 or VEGFR2D1–D6 increased caspase-3 activity in a dose-dependent manner (Fig. 4C). When peptide scHPLW was used, no variation was observed (Fig. 4C).

**Effect of HPLW Peptide on Cell Proliferation**—In addition to rescuing endothelial cells from serum starvation conditions, VEGF is also a potent activator of EC proliferation. This effect is principally mediated by the activation of the MAPK pathway. HUVECs were treated in serum deprivation conditions with HPLW (10, 25, 50, and 100 ng/ml) or scHPLW (100 ng/ml) (Fig. 5A). After 24, 48, and 72 h of treatment, cell proliferation was measured using a CyQUANT NF cell proliferation assay kit. HPLW peptide was able to induce in a dose-dependent manner EC proliferation in a manner similar to VEGF165 (25 ng/ml). Indeed, after 24 h, with respect to untreated cells, VEGF165 increased cellular proliferation by $45 \pm 14\%$ ($p = 0.007$), whereas HPLW (100 ng/ml) increased cellular proliferation by $46 \pm 8\%$; a $48 \pm 7\%$ increase was obtained in respect to scHPLW-treated cells. VEGF165 treatment at 48 h increased cellular proliferation by $59 \pm 6\%$ in respect to untreated cells ($p = 0.00068$), whereas HPLW treatment (100 ng/ml) increased cellular proliferation by $71 \pm 8\%$ and $77\%$ compared with untreated cells ($p = 0.00068$) and scHPLW-treated cells ($p = 0.00031$), respectively. Similar results were obtained after 72 h of treatment. Considerable effects were also observed with HPLW treatment at concentrations of 25 and 50 ng/ml at 48 and 72 h (Fig. 5A). Moreover, Western blot analysis of whole cells proteins obtained after 24 h of treatment confirmed ERK1/2 activation in cells treated with HPLW peptide and VEGF165. As a marker of cell proliferation, the RB phosphorylation status was also checked in the same experimental conditions. RB protein in fact regulates proliferation by controlling cell cycle progression through the restriction point within the G1 and S phases. HPLW and VEGF165, but not scHPLW, enhanced RB phosphorylation, thus indicating cell cycle progression from G1 to S phase (Fig. 5B). The proliferative effect of peptide HPLW was abolished when HUVECs were incubated with soluble VEGFRs. In fact, increasing concentrations (25–100 nM) of VEGFR1D2 or VEGFR2D1–D6 completely reversed the effect of 50 nM HPLW (Fig. 5C).

**Activation of VEGF Intracellular Pathways**—VEGF-dependent EC proliferation and rescue from apoptosis are reliant on the activation of specific intracellular pathways (7). To characterize the VEGF-like biological response of peptide HPLW, the phosphorylation state of key enzymes in VEGF intracellular
pathways such as ERK1/2 and AKT was tested. HUVECs treated with HPLW peptide (25 and 100 ng/ml) in serum deprivation conditions for 15 and 30 min displayed ERK1/2 and AKT activation as seen in Western blot analyses by using phosphorylated ERK1/2 and AKT antibodies (Fig. 6). The peptide scHPLW had no effect on ERK1/2 and AKT activation, proving that it is unable to activate these intracellular signaling.

FIGURE 5. Analysis of EC proliferation. A, HUVECs were incubated with VEGF (25 ng/ml), scHPLW (100 ng/ml), and increasing amounts of HPLW. HUVEC proliferation was evaluated at 24, 48, and 72 h. B, ERK1/2 phosphorylation (p-ERK) and RB phosphorylation (p-RB) were evaluated at 24 h after stimulation with the indicated concentrations of HPLW, VEGF, scHPLW, and VEGFR1D2, VEGFR2D1–D6. HUVEC proliferation was evaluated at 48 h. The proliferation was evaluated using CyQUANT NF reagent and measuring fluorescence intensity on a microplate reader. Values were calculated as the percentage of proliferating cells. Mean values ± S.D.

FIGURE 6. Analysis of EC intracellular pathways. HUVECs were incubated with VEGF, HPLW, and scHPLW for 15 and 30 min at 37 °C in starvation medium. A Western blot analysis was performed on the total protein extracted using anti-phospho-ERK1/2 (p-ERK) and anti-phospho-AKT (p-AKT). Anti-ERK1/2, anti-AKT, and anti-GAPDH were used as loading controls.

directed in vivo angiogenesis assay (DIVAA). Fig. 7, top, shows pictures from angioreactors containing buffer (negative control), VEGF (positive control), HPLW (5, 10, and 100 ng/ml), or scHPLW (100 ng/ml) that were removed 21 days after subcutaneous implantation in mice. The presence of ECs in angioreactors was quantified by determining the number of FITC-lec-tin-positive cells observed using a fluorescence microscope to analyze 20 fields for each sample. EC invasion was observed in VEGF- and HPLW-containing angioreactors. scHPLW peptide was used as a negative control and did not show appreciable activity. In particular, HPLW was able to induce angiogenesis in a dose-dependent manner (Fig. 7, bottom). Specifically, the number of ECs found in HPLW-treated angioreactors (100 ng/ml) was 3.8-fold increased in respect to negative controls (HPLW versus scHPLW: 3.8 ± 0.67, p = 0.000835; HPLW versus PBS: 2.4 ± 0.42, p = 0.001477). This experiment confirmed that HPLW displays a VEGF-like activity, working as an inducer of angiogenesis in vivo.

DISCUSSION

VEGFRs have been the target of intense research aimed at developing molecules able to inhibit or stimulate angiogenesis (28). A few years ago, we undertook the structure-based design of small VEGF peptide binders based on a structural mimic approach that consists of reproducing the three-dimensional arrangements of the ligand-interacting residues stabilizing the
VEGF Receptor-binding β-Hairpin Peptide

The designed 15-mer peptide assumes in water a well defined helical conformation and presents a remarkable thermal stability (30, 31). It binds and activates VEGF receptors and shows VEGF-like activity in vitro (29) and in vivo (32, 33). Here, we extended this approach to the β5-β6 hairpin region. This region is involved in receptor recognition as described by studies on the x-ray structure of the complex between VEGF (8) and PlGF (9) with VEGFR1D2, mutagenesis analyses (34), and the recent x-ray structure of the complex VEGFC-VEGFR2D1–D6 (35).

The β5-β6 hairpin region has already attracted the interest of other research groups who reported the design of receptor antagonist peptides based on the β-hairpin region of VEGF (36–38) and vammin (39). The HPLW peptide differs from the previous peptides in the starting sequence, which is based on the PlGF sequence 87–101, and most notably in the biological activity: HPLW is a VEGF receptor agonist. Moreover, our design strategy is based on stabilization of the β-hairpin conformation, keeping fixed the PlGF residues (Gln87, Leu89, Ile91, Pro97, and Tyr99) involved in VEGFR1D2 recognition and the loop residues. To stabilize the β-hairpin by a hydrophobic cluster, we replaced the natural amino acids (Lys90, Ser98, and Val100) with aromatic residues. Differently from Mirassou et al. (39), in HPLW, the aromatic residues were introduced in hydrogen-bonded positions. We were forced to use this approach as the non-hydrogen-bonded positions were occupied by the interacting residues. The stabilizing role of aromatic residues in non-hydrogen-bonded positions of β-hairpins has been widely exploited (40), but it has scarcely been reported for a hydrogen-bonded position (41, 42).

The NMR analysis indicates that the designed peptide folds into a well defined β-hairpin conformation and shows good structural similarity with the PlGF corresponding sequence (Fig. 1C). A well defined β-hairpin structure is the outcome of different contributions, including main chain hydrogen bonding, secondary structure propensity of the turn region, and side chain to side chain interactions (43). In this study, the hydrophobic interaction between the side chain atoms of Leu3, Leu4, and Trp5 and Trp13, Tyr14, and Tyr15 as well as the intramolecular backbone hydrogen bonds followed by several short range NOEs between non-vicinal residues appears to stabilize the β-hairpin conformation. This result indicates that aromatic residues in hydrogen-bonded positions could stabilize a β-hairpin conformation analogously to what has been described for non-hydrogen-bonded positions.

Comparing the HPLW conformation with the corresponding region of PlGF (residues 87–101) and VEGF (residues 79–93), we found that, whereas the backbone atoms of the loops overlap quite well, the turn conformation and the β-hairpin twist are different. VEGF (Pro85-His86) and PlGF (Ser93-Gly94) present a type II β-turn, whereas HPLW assumes a type I β-turn. Furthermore, the two strands in the natural ligands are more twisted than in HPLW. Nevertheless, the HPLW displays the interacting residues in the same three-dimensional arrangement as the natural ligand. This is an important requisite for its biological activity. In fact, it is able to bind to VEGF receptors as demonstrated at the molecular level by NMR and by flow cytometry studies. In particular, the NMR interaction analysis allowed us to depict an interaction map of the VEGFR1D2 residues in contact with the peptide. Notably, they correspond to the residues involved in the molecular recognition of the corresponding PlGF hairpin region (Fig. 2). This experimental evidence is supported by the binding studies on ECs by flow cytometry. Competition experiments in the presence of VEGF, VEGFR1D2, and VEGFR2D1–D6 suggest that the peptide HPLW targets VEGF receptors on the membrane surface of ECs.

The biological characterization of HPLW showed that it is a bioactive peptide with VEGF-like activity (proangiogenic). In fact, the binding to the receptors activated ERK and AKT kinases, which are key enzymes in the VEGF intracellular signaling pathway. VEGF-dependent phosphorylation of ERK1/2 and AKT is correlated with VEGF functions such as survival and proliferation (44, 45). Like VEGF, when HPLW was incubated with ECs in serum-deprived conditions, it was able to rescue ECs from apoptosis and induce EC proliferation in a dose-dependent manner. Finally, this biological behavior was also confirmed by an in vivo angiogenesis assay. We consider VEGF, rather than PlGF, the appropriate control for the HPLW bioactivity. In fact, PlGF is a positive and negative regulator of angiogenesis depending on the cellular context (7), and its biological activity is not well characterized and still a matter of debate (46). Moreover, the effect of PlGF on primary endothelial cells (such as HUVECs, which we used in this work) is controversial. Ziche et al. (47) reported that PlGF can induce...
migration and proliferation of endothelial cells, whereas other authors reported that PlGF is not able to stimulate proliferation of ECs (48, 49).

VEGF receptors are activated after growth factor-induced dimerization and correct dimer orientation (50). The ability of peptide HPLW to activate VEGF receptors is intriguing but not surprising as we previously showed that a small peptide that binds to VEGF receptors can activate VEGF signaling, ending in a proangiogenic response (29). HPLW retains the PlGF-interacting residues in the same spatial orientation as the natural sequence and bound to VEGF receptor in the same region as the natural β-hairpin sequence. These molecular features likely represent the key elements of its bioactivity.

We showed that HPLW peptide binds to VEGFR1, and competition experiments with soluble VEGFR2D1–D6 support the hypothesis that the peptide could also bind to VEGFR2 on the surface of endothelial cells. This supposition is in good agreement with the proangiogenic bioactivity of peptide HPLW. In fact, it is well established that VEGFR2 is the receptor driving the angiogenic response. We verified that VEGFR2 could be activated by HPLW by evaluating its phosphorylation status by immunoprecipitation/Western blotting. Peptide HPLW, but not scHPLW, effectively induced phosphorylation of the intracellular kinase domain of VEGFR2 like VEGF (Fig. 8). These data strongly support the hypothesis that the proangiogenic properties of HPLW are mediated by VEGFR2. Furthermore, HPLW, which was designed to bind to VEGF receptors, is modeled on PlGF, which is a selective ligand for VEGFR1 and does not bind to VEGFR2. This result could be explained by considering that the five interacting residues are very similar between VEGF (Gln79, Met81, Ile83, Gln89, and Ile91) and PlGF (Gln87, Leu89, Ile91, Pro97, and Tyr99) and that the HPLW conformation is comparable with both VEGF 79–91 and PlGF 87–101 β-hairpin sequences. However, further experimental work will be needed to explain how a monomeric peptide can activate the receptor and mimic the function of a dimeric growth factor.

Whereas many molecules are known to inhibit VEGF activity, only a few VEGF receptor agonists have been described so far (29, 52). Therapeutic angiogenesis is a vast area of medical research that includes several socially important diseases. Up to now, only a single drug, the recombinant PDGF, has been approved to treat diabetic foot ulcer. In this scenario, the development of novel molecular entities is urgently needed, and the peptide described in this work is a promising candidate for a future proangiogenic drug.

In conclusion, we have reported the structure-based design of a novel VEGF receptor-binding bioactive peptide. The peptide was modeled on the PIGF β-hairpin region 87–100 and stabilized by an aromatic hydrophobic cluster. The designed peptide assumes in aqueous solution a well folded β-hairpin conformation with high structural similarity with the natural sequence. The NMR interaction analysis showed that the peptide binds to VEGFR1D2 and highlighted at the molecular level the residues involved in the interaction. These residues correspond to receptor residues involved in the PIGF hairpin recognition. The in vitro biological characterization showed that it is a bioactive peptide with VEGF-like activity. Moreover, the peptide was able to induce angiogenesis in vivo. HPLW is one of the few peptides known with proangiogenic activity, making it a candidate for the development of a novel peptide-based drug for medical applications in therapeutic angiogenesis.

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