Molecular Mapping and Functional Characterization of the VEGF164 Heparin-binding Domain*

Received for publication, January 11, 2007, and in revised form, July 10, 2007 Published, JBC Papers in Press, July 10, 2007, DOI 10.1074/jbc.M700319200

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The longer splice isoforms of vascular endothelial growth factor-A (VEGF-A), including mouse VEGF164, contain a highly basic heparin-binding domain (HBD), which imparts the ability of these isoforms to be deposited in the heparan sulfate-rich extracellular matrix and to interact with the prototype sulfated glycosaminoglycan, heparin. The shortest isoform, VEGF120, lacks this highly basic domain and is freely diffusible upon secretion. Although the HBD has been attributed significant relevance to VEGF-A biology, the molecular determinants of the heparin-binding site are unknown. We used site-directed mutagenesis to identify amino acid residues that are critical for heparin binding activity of the VEGF164 HBD. We focused on basic residues and found Arg-13, Arg-14, and Arg-49 to be critical for heparin binding and interaction with extracellular matrix in tissue samples. We also examined the cellular and biochemical consequences of abolishing heparin-binding function, measuring the ability of the mutants to interact with VEGF receptors, induce endothelial cell gene expression, and trigger microvessel outgrowth. Induction of tissue factor expression, vessel outgrowth, and binding to VEGFR2 were unaffected by the HBD mutations. In contrast, the HBD mutants showed slightly decreased binding to the NRP1 ( neuropilin-1) receptor, and analyses suggested the heparin and NRP1 binding sites to be distinct but overlapping. Finally, mutations that affect the heparin binding activity also led to an unexpected reduction in the affinity of VEGF164 binding specifically to VEGFR1. This finding provides a potential basis for previous observations suggesting enhanced potency of VEGF164 versus VEGF120 in VEGFR1-mediated signaling in inflammatory cells.

Vascular endothelial growth factor-A (VEGF-A) plays pivotal roles in regulating both normal and pathological angiogenic processes (1). Its multiple biological activities during embryogenesis and in the adult organism are mediated mainly through interaction with VEGFR1 (Flt-1) and VEGFR2 (Flk-1, KDR), two structurally similar receptor tyrosine kinases expressed on vascular endothelial cells (2, 3). VEGF-A is expressed and secreted as multiple, homodimeric isoforms that are formed as a result of differential splicing of the VEGF-A pre-mRNA (4, 5). The most abundantly expressed isoforms are VEGF120, VEGF164, VEGF188 in mice, and VEGF121 and VEGF165 and VEGF189 in humans. Although all variants contain the same binding sites for VEGFR1 and VEGFR2, they differ in their affinity for heparin due to the presence or absence of two basic heparin-binding domains (HBDs) encoded by exons 6 and 7 (6). In vivo, both domains independently mediate interactions with glycosaminoglycans, the most common of which, heparan sulfate, is linked to core proteins as heparan sulfate proteoglycans (HSPGs), which are immobilized on the cell surface and in the ECM (7). VEGF120, which lacks a heparin-binding domain, is freely diffusible in the extracellular space, whereas VEGF164 and VEGF188 exhibit moderate and high binding affinity, respectively, resulting in partial or complete retention of these isoforms in HSPG-rich compartments (8). In addition, the exon 7-encoded peptide also contains yet unidentified residues that confer to VEGF165 the ability to bind to the VEGF co-receptor NRPI (9).

The significance of the heparin-binding VEGF isoforms was revealed by the finding that mice engineered to express exclusively the non-heparin-binding VEGF120 have defects in VEGF extracellular gradient formation and microvessel branching, resulting in the death of most animals shortly after birth (10, 11). On the other hand, mice that solely express VEGF164 appear normal and healthy (12). Not only do the heparin-binding and VEGF isoforms play different roles in vascular development, they also confer different biological activities in tumor angiogenesis (13–15). Furthermore, VEGF164, but not VEGF120, is preferentially associated with pathological angiogenesis and inflammation in the eye (16, 17). These findings highlight the importance of better understanding the biology of the HBD.

The HBD of the major VEGF isoform, VEGF165, is a lysine- and arginine-rich region comprising the 55 COOH-terminal residues that can be released by plasmin digestion of the full-length protein (Fig. 1A). This region mainly consists of exon 7- and exon 8-encoded sequences that exhibit 96% amino acid identity between the mouse and the human homolog as well as a high degree of sequence homology with other members of the VEGF family of growth factors (supplemental Fig. 1).

Its solution structure has been reported, showing that this domain is formed by a clearly defined NH₂-terminal (residues
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A schematic representation of the VEGF165 monomer and the COOH-terminal amino acid sequence. The 55-residue HBD (VEGF 111–165) located in the COOH-terminal tail (shaded region) is defined by the plasmid cleavage site (arrowhead) and contains the binding sites for heparin and neuropilin-1. The numbers refer to the residue numbers within the whole protein or within the HBD (boldface type), and amino acids that were targeted in this study are highlighted in blue. Intramolecular disulfide bridges are depicted as lines, and illustration of the VEGF165 HBD fragment (Protein Data Bank code 1KMX) as a ribbon diagram (left) to show structural fold and as a surface topology model (right) to show the distribution of electrostatic potential on the molecule surface. Charged residues are color-coded, with basic and acidic amino acids shown in blue and red, respectively. The surface model is shown at two angles rotated by 180° about the vertical axis. The amino acids that were targeted in this study are depicted with their HBD residue numbers. C, designation and partial sequence numbers (in boldface type) are all located within the exon 7-encoded region. Mutant numbers refer to the amino acid numbers within the HBD portion (residues 1–55).

The present study was undertaken to identify clusters of amino acids of mouse VEGF164 involved in heparin binding using site-directed mutagenesis and to better understand the role of this interaction in VEGF function.

EXPERIMENTAL PROCEDURES

Reagents—Human 125I-VEGF165 and tritium-labeled heparin ([3H]heparin) were obtained from GE Healthcare and PerkinElmer Life Sciences, respectively. Heparinase I and III were purchased from Sigma. The expression vectors pPICZαC-VEGF120 and pPICZαC-VEGF164 were used for expression of full-length, untagged VEGF isoforms and mutant VEGF164 variants in Pichia pastoris and were a kind gift of Dr. M. Golding (Cancer Research UK, London, UK).

Site-directed Mutagenesis and Cloning—In vitro site-directed mutagenesis was performed using the QuikChange™ multisite-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the instructions of the manufacturer. Oligonucleotide primers containing the desired mutation (supplemental Table 1) were designed to bind to adjacent sequences or to separate regions on the same strand of the template plasmid (pPICZαC-VEGF164). All mutations were verified by DNA sequence analysis. To generate the recombinant HBD protein, a DNA fragment encoding the COOH-terminal 55-residue fragment of VEGF165 (HBD) was generated by PCR and subcloned in frame with the α-factor signal peptide of the pPICZαC expression vector (Invitrogen).

For recombinant production of wild-type VEGF isoforms and VEGF164 mutants, all expression constructs were transformed into competent P. pastoris yeast cells (strain KM71H) as described by the manufacturer (Invitrogen). Transformants were screened for high secreting colonies using a colony blotting protocol (22).

Recombinant Protein Production and Purification—Recombinant wild-type and mutant VEGF proteins were produced in the methylotrophic yeast P. pastoris according to the manufacturer’s protocol with slight modifications. Briefly, the highest secreting yeast transformants of each protein variant were grown in 500 ml of BMGY medium at 29–30 °C until the culture reached an A 600 of 3. To induce expression, yeast pellets were then resuspended in 100 ml of BMMY containing 0.01%
Antifoam 204 (Sigma). Cultures were grown at 29–30 °C for 36–42 h, and methanol was added to a final concentration of 1% every 12 h to maintain induction of transgene expression. Supernatants were cleared, concentrated, and equilibrated in Ni²⁺-nitrilotriacetic acid chromatography binding buffer (PBS, pH 7.4, with 2.7 mM KCl, 2 mM KH₂PO₄, 8 mM Na₂PO₄, 300 mM NaCl) and incubated with pre-equilibrated Ni²⁺-nitrilotriacetic acid-agarose (Qiagen) for 60 min at 4 °C. The beads were collected by centrifugation and washed with binding buffer. Bound proteins were eluted with elution buffer (PBS containing 150 mM NaCl and 40 mM imidazole) and analyzed by SDS-PAGE and Coomassie staining.

Eluted protein fractions were concentrated and then loaded onto a Superdex 75 10/300 GL column using an AKTA FPLC™ system (GE Healthcare) for buffer exchange and isolation of protein dimers. Proteins were eluted in Tris-buffered saline (20 mM Tris, pH 7.4) at a flow rate of 0.3 ml/min.

**Tissue Factor Induction in Human Umbilical Vein Endothelial Cells**—Human umbilical vein endothelial cells (Cascade Biologics, Bath, UK) were seeded in a 12-well plate at 8 × 10⁴ cells/well in M200 medium (Cascade Biologics). After 24 h, the cells were serum-starved for 4 h in M200 medium containing 1% fetal bovine serum. The cells were rinsed once with serum-free medium, and VEGF variants (0.3 pmol) or medium alone was added to the cells. After incubation at 37 °C for 1 h, the cells were rinsed once with PBS, and total RNA was isolated using the RNeasy mini kit (Qiagen). Reverse transcription of RNA was performed using the TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocols.

Real-time quantitative PCR (Taqpman) was conducted using 1× PCR master mix (Applied Biosystems), 1× Tissue Factor Assay-on-Demand/FAM (Applied Biosystems), and 2 μl of cDNA in a total volume of 10 μl. Glyceraldehyde-3-phosphate dehydrogenase served as the internal control for all reactions and was amplified with human glyceraldehyde-3-phosphate dehydrogenase primers and probe (Applied Biosystems). Reactions were loaded on a 384-well plate and run in the Applied Biosystems Prism 7900HT System. Gene expression levels were quantified by using the comparative threshold (Ct) method as described (23). The assays were performed three times for each condition using duplicate samples.

**Analytical Heparin Affinity Chromatography**—To determine the heparin-binding affinity of VEGF variants, 200 μl of binding buffer (20 mM Tris, 100 mM NaCl, pH 7.4) containing 50 μg of purified protein were loaded onto a 1-ml HiTrap heparin HP column (GE Healthcare) at a flow rate of 0.25 ml/min using the AKTA FPLC™ system. Unbound material was removed by washing with binding buffer, and bound proteins were then eluted with a linear salt gradient (0.1–1 mM NaCl) over 15 column volumes at 0.5 ml/min (NaCl concentration increment per fraction: 0.06 M). Alternatively, proteins were loaded in binding buffer containing 0.15 M NaCl and eluted with a steeper salt gradient (0.15–1.5 M NaCl) over 11 column volumes (NaCl concentration increment 0.123 M). Fractions were subjected to trichloroacetic acid precipitation, and the protein pellets were diluted in SDS sample buffer, separated on a 12% SDS-polyacrylamide gel, and analyzed by Coomassie staining.

or Western blotting using a monoclonal anti-VEGF antibody (C-1; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

**Nitrocellulose Binding Assay**—Binding of heparin to proteins was assessed in solution based on a protocol by Maccarana and Lindahl (24) with slight modifications. Briefly, [³H]heparin (0.05 μM) was incubated with increasing concentrations of VEGF in binding buffer (25 mM Tris, 150 mM NaCl, 0.1% BSA, pH 7.5) for 1 h at 37 °C. Nitrocellulose filters (0.45-μm pore size; Millipore, Billerica, MA) were pre-equilibrated in BSA-free binding buffer and were placed into separate pockets of a vacuum manifold (Millipore). The samples were transferred onto the filters, and unbound [³H]heparin was removed by applying vacuum and washing with BSA-free binding buffer. The amount of bound [³H]heparin retained by the filter was determined by counting in a β-scintillation counter (Micro Beta TriLux; PerkinElmer Life Sciences). Data analysis for saturation and competition binding was performed using GraphPad Prism 4 software (GraphPad Software, San Diego, CA). Binding assays were repeated three times for each protein.

**Competition Binding Assays with Soluble VEGF Receptors**—96-well microtiter plates (Wallac HB Isolate; PerkinElmer Life Sciences) were coated with Fc-specific anti-human IgG (Calbiochem) at a concentration of 5 μg/ml in PBS (pH 7.4) overnight. The wells were blocked with Super Block™ (Pierce) for 15 min, and receptor/Fc chimera proteins (R&D Systems, Minneapolis, MN) in binding buffer (100 μl/well, PBS with 0.1% BSA) were added at a final concentration of 400 pm (VEGFR1/Fc), 2 nm (VEGFR2/Fc), or 1.8 nm (NRP1/Fc). After 1.5 h at room temperature, the plate was washed, and 100 μl of binding buffer containing 0.01 μCi (for VEGFR1) or 0.02 μCi (for VEGFR-2 and NRP1) of ¹²⁵I-labeled VEGF165 and increasing concentrations of cold competitor (VEGF164) were added to the wells. Binding to equilibrium was carried out at 37 °C (VEGFR1/2) or at room temperature (NRP1) for 2 h (VEGFR1/2) or 3 h (NRP1), after which the plate was washed, and binding of ¹²⁵I-VEGF was quantitated in a β-scintillation counter. IC₅₀ values were calculated using the one-site competition binding analysis program (GraphPad Prism 4 Software). All experiments, in duplicate, were performed three times.

**CD Spectrophotometry**—CD spectra were recorded in the wavelength range 198–260 nm with a Jasco J-810 spectropolarimeter (Jasco, Easton, MD) using a 0.1-cm path length stain-free cell. The proteins were analyzed at a concentration of 0.15 mg/ml in Tris-buffered saline at room temperature. Spectra were recorded in millidegrees of ellipticity (θ). The solvent spectrum (Tris-buffered saline) was subtracted from the sample spectra, and the subtracted spectra were converted to mean residue ellipticity (CD signal per amino acid residue, [θ]) in degrees × cm² × dmol⁻¹ by the equation,

\[
[\theta]_{mew} = \theta \times M_{mw} / 10 \times c \times l
\]  

(Eq. 1)

where c represents the protein concentration in mg/ml, l is the cell path length in cm, and \( M_{mw} \) is the mean residue molecular weight of the VEGF164 dimer. All measurements were done twice, with identical results.

**Quantitation of Blood Vessel Growth in the Aortic Ring Assay**—Thoracic aortas were dissected from male Fisher rats (6–10...
weeks) and placed into a dish containing EB medium (Cambrex) supplemented with 2 mM glutamine and penicillin/streptomycin (100 μg/ml). The aortas were carefully cleaned of all surrounding adventitial tissues under a dissecting microscope and cut into 1-mm-wide rings. Each of the rings was embedded in a rat collagen mix (2 mg/ml collagen I, 4 ml of H2O, 1× Dulbecco’s modified Eagle’s medium, 2.34 mg/ml NaHCO3) in 4-well multidishes (Nunc), and the collagen gel was then overlaid with 450 μl of EB medium. The plates were incubated at 37 °C and 5% CO2 in a humidified atmosphere for 7 days, and EB medium was replaced every 48 h with control medium or medium containing purified VEGF variants (4.4 nM). Rings that did not show any signs of vessel growth after 48 h were discarded. After 7 days, the rings were washed and fixed with paraformaldehyde and stained with biotinylated isolecitin B4, and images of aortic rings were captured using a DMI8 epifluorescence microscope (Leica, Wetzlar, Germany) equipped with a Retiga RXi CCD camera (Qimaging, Burnaby, Canada).

To quantify blood vessel growth, RGB images of the rings were converted to 8-bit grayscale images in Photoshop (Adobe), and blood vessels were traced in a new layer using a 9-point pencil tool. The new image layer was then binarized, and the traced lines were skeletonized using Image-J software (National Institutes of Health). All white pixels in the skeletonized image were counted and expressed relative to the circumference of the aortic ring in order to provide a measure of the total number of new blood vessels normalized to the aortic ring size. All data analyses were performed in a masked fashion.

**Binding of VEGF Species to Cells and Biological Matrices**—Porcine aortic endothelial cells were seeded at 3 × 10^5 cells/well in 12-well dishes and were cultured for 24 h. The cells were washed with binding buffer (Ham’s F-12K medium containing 0.1% (w/v) BSA, pH 7.5), and binding of purified mouse VEGF variants (7.14 nM) to the cell surface and matrix was carried out in binding buffer for 30 min at 37 °C. Unbound VEGF was removed, and bound VEGF was enzymatically dissociated from the cells by adding heparinase I and III (0.5 unit/ml) in 20 mM Tris-HCl (pH 7.5), containing 50 mM NaCl, 4 mM CaCl2, and 0.01% (w/v) BSA. After 1 h at 37 °C, the medium of each well was collected with a pipette, and the cells were washed with binding buffer (final wash). The concentration of VEGF in the medium after heparinase treatment and in the final wash was determined by a mouse-specific VEGF enzyme-linked immunosorbent assay (VEGF Quantikine® enzyme-linked immunosorbent assay; R&D Systems). The experiment was performed in duplicates and was repeated three times.

**Binding of the VEGF Variants to Basement Membranes** was carried out using sections of the mouse eye. Eyes from 2-month-old C57bl/6 female mice (Charles River Laboratories) were harvested and fixed in 4% paraformaldehyde overnight. The eyes were then washed in PBS for 3 h and placed in a 10% sucrose solution for 4 h, followed by incubation in a 30% sucrose solution overnight. On the following day, the eyes were placed in OCT embedding compound, frozen on dry ice, and stored at −80 °C until sectioned. The slides were thawed, and sections were circled with a pap pen and then rehydrated in PBS. The sections were incubated in blocking solution (10% goat serum, 1% BSA, 0.05% Triton X-100 in 1× PBS) containing no protein or 10 μM of purified proteins overnight at 4 °C. The samples were incubated with blocking solution for 15 min followed by incubation with goat anti-VEGF antibody (1:100; R&D Systems) for 1 h. The samples were washed and incubated in Alexa-Fluor 633-conjugated donkey anti-goat secondary antibody (1:500; Molecular Probes, Inc., Eugene, OR) for 45 min. The sections were then mounted using Vectashield with 4’,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) and imaged using an epifluorescence microscope (DMIRA; Leica) equipped with a digital CCD camera (Hamamatsu, Hamamatsu City, Japan). Binding to two separate eye sections of the same eye was performed three times for each protein species.

**Statistical Analysis**—All values were expressed as mean ± S.D. unless otherwise indicated. The unpaired Student’s t test was used when two groups were compared. Data were analyzed by using a post hoc comparison test (Bonferroni) when three or more groups were compared. Differences were considered statistically significant when p values were <0.05.

**RESULTS**

**Site-directed Mutagenesis of the VEGF164 HBD**—Sequence alignment has shown that the HBD of VEGF165 and related VEGF family members have no significant sequence homology to other known heparin-binding proteins (19). The absence of definitive heparin-binding consensus sequences has made it difficult to elucidate features within this region that are important for heparin recognition. However, based on solution structure data and, in particular, the surface charge distribution of the COOH-terminal fragment, it has been suggested that a putative heparin-binding site may involve two basic clusters consisting of side chains of residues Arg-13, Arg-14, and Lys-15 and residues Lys-30, Arg-35, Arg-39, and Arg-46. These clusters are located on either side of the NH2-terminal and COOH-terminal subdomain, respectively, forming a pocket that could accommodate a heparin oligosaccharide (18, 25).

Thus, we utilized a structure-guided mutagenesis approach focusing on the involvement of six solvent-exposed arginines and one lysine in the two basic amino acid clusters identified by NMR and assessed their role in heparin binding by replacing these residues with alanine. Double and triple substitutions were primarily examined, since structural and mutational analysis of other heparin binding proteins suggests that multiple basic residues are required to coordinate interaction with the heparin oligosaccharide (26–30). Alanine mutagenesis was also applied to a Lys-26 outside the two basic clusters for control purposes (Fig. 1C and supplemental Fig. 2, A–J). VEGF164 HBD mutants were generated, expressed in P. pastoris, and purified to >90% purity (supplemental Fig. 3).

**Determination of the Bioactivity of VEGF164 HBD Mutants**—As a first step in the analysis of mutants, we sought to determine if the Pichia-derived proteins were active and to establish if mutations or recombinant production led to major structural defects, problems with dimerization, or aggregation that would impair VEGF signaling. During angiogenesis, VEGF induces vessel growth by activating endothelial cells. One endothelial cell gene that is up-regulated in response to VEGF signaling is tissue factor (TF) (31). TF up-regulation is mediated by VEGFR2, a VEGF receptor that can be activated by all VEGF
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FIGURE 2. Ability of recombinant VEGF164 HBD mutants to induce TF mRNA up-regulation in human umbilical vein endothelial cells. Human umbilical vein endothelial cells were treated for 1 h with medium containing PBS (Buffer) or 0.3 pmol/ml of purified VEGF proteins. Total RNA was isolated and reverse-transcribed to yield cDNA, which was subjected to real time PCR analysis. Levels of TF gene expression were determined using the comparative threshold method. The data represent the mean ± S.D. of three independent experiments.

Isoforms and thus is not dependent on a functional HBD (32). If mutants were defective in TF induction, it would suggest that HBD mutations had destabilized the entire VEGF protein, and this would obviously impede further analysis. TF mRNA levels were ~5-fold higher in VEGF164-treated cells than in buffer-treated control cells. The non-heparin-binding VEGF120 was similar to VEGF164, and all VEGF164 HB mutants were active and induced TF gene transcription to levels that were comparable with that induced by VEGF164 (Fig. 2). These results suggest that the mutations did not cause major structural changes in these proteins that would interfere with VEGF-mediated signaling.

Heparin Binding Activity and Secondary Structure Analysis of VEGF164 HBD Mutants—Next, heparin-Sepharose chromatography was employed as a method to screen the heparin-binding affinity of the mutants. A heparin-Sepharose column was loaded with purified protein dimers, and bound proteins were eluted with a linear NaCl gradient. The relative affinities for heparin were assessed by determining the amount of salt required to elute the proteins from the column (33). Heparin-Sepharose chromatography was first conducted at low ionic strength, thus under less stringent conditions, to detect changes in heparin binding activity. As expected, recombinant VEGF164 and HBD proteins bound to the heparin column in the presence of 0.1 m NaCl, whereas VEGF120 did not bind (Fig. 3A). Under the low salt conditions, VEGF164 eluted from the column as a peak spanning a relatively broad range of the salt gradient (0.58–1 m NaCl). The concentration of NaCl in the elution buffer required to displace 50% of VEGF164 from the column (referred to as EC50) was ~0.82 m.

Similar elution characteristics were observed with the single mutants K26A and K30A and the double mutant K15A/H16A (Fig. 3A and data not shown). Targeting of Arg-46 and Arg-49 resulted in a slightly decreased heparin-binding capacity (EC50 = 0.7 m NaCl). Heparin binding was further reduced in the double mutant R13A/R14A (EC50 = 0.52 m NaCl), and the combined quadruple mutant (R13A/R14A/R46A/R49A) exhibited very little heparin binding activity. Additional mutations in this region (R13A/R14A/R49A and R14A/R49A) confirmed the importance of these residues in the interaction of VEGF164 with heparin.

The side chains of the residues Lys-30, Arg-35, Arg-39, and Arg-49 are located in relatively close proximity to each other and form a basic cluster that may act as a docking site for heparin. The quadruple mutant K30A/R35A/R39A/R49A and the triple mutant K30A/R35A/R39A presented similar elution profiles. In both cases, a significant amount of protein was found in the wash and early elution fractions, and a second portion of the sample bound more tightly to the column and eluted closer to wild-type VEGF164 (Fig. 3A). This biphasic elution pattern was also observed with the double mutant R35A/R39A, albeit to a lesser extent.

Low stringency binding conditions enabled prolonged interaction of wild-type and mutant proteins with the heparin-Sepharose, evidenced by the broad elution profiles. Avidity effects resulting from multimerization of the dimeric proteins and nonspecific solid-state interactions with the column matrix under low salt conditions are possible explanations for this phenomenon and were also examined as a basis for the biphasic elution profiles observed with a subset of the mutants. A more stringent chromatographic assessment was performed by increasing both the ionic strength of the binding buffer (0.15 m NaCl) and the steepness of the salt gradient. Under these conditions, VEGF164 eluted as a narrow peak at ~0.9 m NaCl (Fig. 3B). However, similar to the previous findings, the elution profile of K30A/R35A/R39A consisted of a population that did not bind heparin and a second population that bound to heparin as strongly as VEGF164 (Fig. 3B). Rather than avidity or column interaction effects, the biphasic elution profile under stringent conditions suggests that the K30A/R35A/R39A mutant exists in two states: a folded state that interacts normally with heparin and a misfolded configuration that is non-heparin-binding. CD spectroscopy confirmed that the non-heparin-binding fraction had severe structural alterations and that in the absence of this misfolding, the mutant is a competent heparin-binding protein (supplemental Fig. 4C). Thus, analysis of both K30A/R35A/R39A and K30A/R35A/R39A/R49A was no longer pursued.

In contrast, the secondary structure data for R13A/R14A/R49A and R14A/R49A suggest that both mutants exhibit a backbone conformation similar to that of the wild-type protein (supplemental Fig. 4A and 4B). Indeed, analysis of this cluster of mutants under high stringency binding conditions revealed that R13A/R14A/R49A lost its ability to bind to the heparin column at physiological salt concentration, suggesting that the low level binding observed at low ionic strength may have been nonspecific. Heparin binding of R14A/R49A was also severely compromised (Fig. 3B).

In order to assess more accurately the heparin-binding potential of selected VEGF164 HBD mutants, we determined the apparent dissociation constants (Kd) of these interactions in a nitrocellulose binding assay. This assay involves equilibrium binding of radiolabeled heparin with increasing concentrations of VEGF in solution. Saturation binding analysis showed that binding of VEGF164 to heparin occurs with moderate affinity (Kd = 0.157 μM) (Fig. 4), which is in good agreement with the
FIGURE 3. Binding of VEGF164 HBD mutants to immobilized heparin. Elution profiles of VEGF variants after binding to heparin-Sepharose as determined by affinity chromatography. A, purified protein dimers in binding buffer containing 0.1 M NaCl were applied to a heparin-Sepharose affinity column. Flow-through and wash fractions were collected, and bound proteins were eluted over a linear salt gradient (0.1–1 M NaCl). Proteins were separated by SDS-PAGE and detected by Coomassie staining. B, heparin-binding behavior of VEGF164 and selected HBD mutants at physiological salt concentration. Purified protein dimers were injected onto a heparin-Sepharose affinity column in the presence of 0.15 M NaCl. The column was washed, and bound proteins were eluted over a linear salt gradient of 0.15–1.5 M NaCl. The proteins were separated by SDS-PAGE and immunoblotted with an antibody directed against the VEGF NH$_2$-terminal domain (C-1).
dissociation constant reported in the literature ($K_d = 0.165 \mu M$) (34). The single mutant K26A showed a slightly reduced affinity for heparin compared with wild-type VEGF164 ($K_d = 0.415 \mu M$) that was not detected by heparin-Sepharose chromatography, confirming the increased sensitivity of the nitrocellulose binding assay. The binding affinities of the double mutants R13A/R14A ($K_d = 1.595 \mu M$) and R46A/R49A ($K_d = 1.540 \mu M$) were reduced ~10-fold. Strikingly, the greatest effect was demonstrated by the double mutant R14A/R49A and the triple mutant R13A/R14A/R49A, respectively, both lacking any detectable heparin-binding activity ($K_d$ values $> 10 \mu M$) (Fig. 4). These results strongly indicate the presence of a potential heparin-binding surface that encompasses the side chains of Arg-13, Arg-14, and Arg-49 and a minimal heparin-binding element consisting of the two residues, Arg-14 and Arg-49.

**Analysis of VEGF-VEGF Receptor Interactions**—The biological consequences of the loss in heparin binding activity were explored, first by analyzing ligand-receptor interactions. The VEGF isoform-specific receptor NRPI binds to the exon 7-encoded HBD region of VEGF164 (9), but the amino acids mediating this interaction have not yet been identified (35). To evaluate the effect of the HBD mutations on NRPI binding, a plate-based competition binding assay was performed in which increasing concentrations of wild-type and mutant VEGF variants competed with $^{125}$I-VEGF165 for binding to the immobilized NRPI receptor. The binding of radiolabeled VEGF165 to NRPI was inhibited in a dose-dependent manner by VEGF164 with a half-maximal inhibitory concentration (IC$_{50}$) of 0.128 nM, indicating that binding to NRPI occurs with relatively high affinity in the absence of heparin (Fig. 5). All mutants were able to bind NRPI, although to varying and reduced degrees. Compared with VEGF164, the triple mutant R13A/R14A/R49A exhibited a 44-fold reduced binding to NRPI, and the quadruple mutant R13A/R14A/R46A/R49A was 116-fold less effective in displacing $^{125}$I-VEGF165 from NRPI. These results suggested that the heparin and NRPI binding sites may overlap. However, the double mutant (R14A/R49A), which had no detectable heparin binding, showed only a slight reduction (~3.5-fold) in binding to NRPI, suggesting that the most critical binding determinants for these two functions of the HBD were largely independent.

Most of the biological activities of VEGF involve binding to and activation of the VEGF receptors VEGFR1 and VEGFR2. To determine whether the neutralization of residues implicated in heparin binding was affecting the interaction of R14A/R49A and R13A/R14A/R49A with these receptors, competition binding assays were performed. As shown in Fig. 6A, VEGF120 (IC$_{50}$ = 0.071 nM) was as effective as VEGF164 (IC$_{50}$ = 0.081 nM) in competing with $^{125}$I-VEGF165 for binding to VEGFR2, confirming previous experiments using human VEGF isoforms,

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**Table 1:**

| VEGF Variant | IC$_{50}$ (nM) |
|--------------|----------------|
| VEGF164      | 0.128 ± 0.02 nM|
| R35A/R39A    | 0.710 ± 0.16 nM|
| K30A         | 0.621 ± 0.24 nM|
| K26A         | 0.318 ± 0.03 nM|
| R46A/R49A    | 3.080 ± 0.37 nM|
| R13A/R14A    | 1.340 ± 0.24 nM|
| R14A/R49A    | 0.432 ± 0.18 nM|
| R13A/R14A/R49A | 5.610 ± 1.42 nM|
| R13A/R14A/R46A/R49A | 14.80 ± 1.72 nM|
which showed that loss of the COOH-terminal domain does not affect high-affinity binding to VEGFR2 in the absence of heparin (36). Consistent with these findings, the two heparin binding-deficient mutants R14A/R49A (IC50 = 0.099 nM) and R13A/R14A/R49A (IC50 = 0.064 nM) exhibited comparable affinities compared with native VEGF.

VEGFR1, on the other hand, has been shown to bind VEGF165 with higher affinity than VEGF121 (36). Indeed, we observed that the affinity of VEGF164 for VEGFR1 was 3.5-fold higher than that of VEGF120 (IC50 = 0.022 versus 0.077 nM) (Fig. 6B). Mutant R14A/R49A (IC50 = 0.092 nM) and R13A/R14A/R49A (IC50 = 0.151 nM) also exhibited decreased binding to VEGFR1 with IC50 values in the range observed for VEGF120. These results suggest that charged residues within the HBD positively affect high affinity binding of VEGF to VEGFR1.

Proangiogenic Activity of VEGF164 HBD Mutants—Next, the ability of R14A/R49A and R13A/R14A/R49A to induce angiogenesis was assessed. In vivo assessment would be difficult, since it has already been demonstrated that VEGF120 is more readily diffusible and has a reduced residence time in tissues compared with heparin binding VEGFs (11, 37, 38), which would complicate comparison of mutant and wild-type VEGF in the promotion of vessel sprouting. In contrast, rat aortic rings can be stimulated by VEGF to generate microvessel outgrowth and a network composed of branching endothelial tubes (39, 40). This assay recapitulates many of the complex processes of in vivo angiogenesis, including cell proliferation, migration, and lumen formation; thus, it would be well suited to comparing the angiogenic potency of VEGF isoforms.

Treatment of collagen-embedded aortic rings with equimolar concentrations of either VEGF120 or VEGF164 resulted in an increase in total microvessel length that was ∼3.5-fold higher than background levels (Fig. 7, A and B). R14A/R49A...
and R13A/R14A/R49A stimulated a high level of sprouting, yielding 5-fold (R14A/R49A) and 4.6-fold (R13A/R14A/R49A) increases in microvessel length compared with control rings. Differences between wild-type and mutant proteins were not statistically significant, and no gross differences in vascular sprout morphology were observed when rings from different treatment groups were compared.

Binding of VEGF164 HBD Mutants to the Cell Surface and to Biological Matrices—Because endothelial cells express heparan sulfate (and not heparin) on the cell surface and the HSPGs have distinct sulfation patterns and numbers of saccharide repeats, we determined whether deficiency in heparin binding translates into an inability to interact with cell-associated HSPGs. To this end, native porcine aortic endothelial cells, which are known to lack expression of VEGFR1, VEGFR2, and NRP1 (41, 42), were exposed to equimolar amounts of wild-type and mutant VEGF proteins, followed by digestion of HSPGs to release matrix- and cell-surface-bound VEGF. The binding assay was performed at pH 7.5 to avoid VEGF binding to fibronectin (43). Compared with VEGF120, the concentration of VEGF164 that was released into the medium after enzymatic treatment with heparinase I and III was 3.1-fold higher (Fig. 8A). The amount of R14A/R49A and R13A/R14A/R49A mobilized from heparinase-treated cells was similar to that of VEGF120, indicating that the mutant HBDs have lost the ability to promote binding to HSPGs.

Finally, binding of the VEGF164 HBD mutants to more complex extracellular matrix was further evaluated by incubating purified proteins with cross-sections of the retina and choroid taken from the eyes of 8-week-old mice. Bound VEGF was revealed by subsequent immunolabeling with an antibody that binds in a region common and intact within all VEGF proteins tested. As shown in Fig. 8B, endog-
enous VEGF levels were low and variable, but strong anti-VEGF immunoreactivity was observed in Bruch’s membrane and in the inner limiting membrane that forms the vitreoretinal interface after exposure of retinal tissue to VEGF164 but not in tissues exposed to VEGF120. These two retinal layers contain abundant heparan sulfate and chondroitin sulfate proteoglycans in their basement membranes and in surrounding extracellular matrices (44). The failure to detect VEGF164 bound to VEGF receptors in the cellular layers is probably due to the very low level expression of VEGFR1 and VEGFR2 in the adult retina. Similar to VEGF120, the heparin-binding mutants R14A/R49A and R13A/R14A/R49A both failed to bind to these regions, confirming that a functional heparin-binding domain is essential for VEGF to interact with tissue extracellular matrix.

**DISCUSSION**

Several VEGF165-related proteins, including VEGF189, VEGF-B167, VEGF-F, and PIGF-2, bind to heparin via yet unidentified amino acids present in their carboxyl-terminal region (45–48). The lack of a clear heparin-binding motif across the VEGF family has slowed progress in understanding the molecular basis of the interaction and has raised the possibility that binding of VEGF proteins to heparin is not mediated by highly selective binding sites present on the protein molecular surface but rather occurs with little discrimination through general electrostatic interactions of the HBD with sulfate side chains. However, despite the high electropositive charge of the VEGF164 HBD in physiological conditions (the pI for VEGF165 HBD is estimated to be 11.6 (19)), our findings support the concept that the interaction with heparin is mediated by key basic residues.

The mutational analysis carried out in this study indicates that heparin binding depends on a three-arginine cluster and suggests the existence of a principal heparin-binding site centered near Arg-13, Arg-14, and Arg-49. Residues Arg-14 and Arg-49 may represent a minimal binding site, since the neutralization of these two amino acids was sufficient to ablate heparin binding in the solution-based binding assay; however, quantitative analysis of single amino acid substitutions in the cluster will be required in future studies to refine the minimal binding determinants.

The key heparin binding cluster resides along the interface of a clearly defined amino-terminal and carboxyl-terminal subdomain and is noncontiguous in sequence. The proposed heparin-binding region derived from this study is in good agreement with the predicted binding site derived from docking calculations of the interaction between the VEGF165 HBD and short heparin oligosaccharides (49). By using the original HBD solution structure (18), the authors showed that in energetically favorable complexes, a heptasaccharide ligand makes several contacts with basic residues within both subdomains, including Arg-14 and Arg-49 (49). Further support for this model comes from the recently published refined NMR solution structure and a much improved definition of the mutual orientation of the amino-terminal and carboxyl-terminal subdomains (25). In contrast to the original structure, the refinement revealed that the solvent-exposed side chains of the heparin-binding residues located in the amino-terminal (Arg-13 and Arg-14) and the carboxyl-terminal (Arg-49) subdomain are in close contact with each other and form a continuous binding surface. Spatial proximity of key heparin-binding residues within the three-dimensional structure appears to be a common characteristic for several different heparin-binding proteins (28, 50–52).

The difference in binding activity between the triple mutants R13A/R14A/R49A and K30A/R35A/R39A (the heparin-binding fraction) as observed in the column binding assay, also suggests that the degree of binding impairment does not simply correlate with the number of substitutions and, therefore, the overall decrease in electropositive charge. Rather, the selectivity of the VEGF-heparin interaction may be achieved through optimal steric and energetic complementarity between critical functional groups of the binding partners. Nevertheless, it is likely that other residues located in the vicinity of this heparin-binding epitope may participate in binding to heparin and heparan sulfate by contributing to the total binding energy, and this possibility needs to be investigated in more detail.

In addition to interaction with the extracellular matrix, the HBD region of VEGF164 also mediates functional interaction with the VEGFR2 co-receptor, NRP1. Previous information about the requirements of VEGF165 binding to NRP1 at a molecular level include the observation that VEGF and semaphorin 3A competitively bind to the coagulation factor domain b1 of NRP1. Since binding of semaphorin 3A is mediated by its basic COOH-terminal tail, it is thought that competition between semaphorin 3A and VEGF165 results from binding of their basic amino acids to a common electronegative binding surface on NRP1 (53, 54). The data presented here are consistent with the idea that basic residues may play a role. The binding affinity of mutant VEGF proteins to immobilized NRP1 was reduced between 2.5-fold (K26A) and 115-fold (R13A/R14A/R46A/R49A) compared with VEGF164, suggesting that these residues do contribute to the overall free energy of the NRP1-VEGF164 interaction. Nonetheless, all mutants still retained significant binding activity. In fact, the R14A/R49A mutant was only minimally affected in the NRP1 binding assay, suggesting that the key residues required for NRP1 binding to VEGF164 are distinct from those at the core of heparin interactions. Based on the present results, we speculate that the NRP1 binding site is composed of multiple amino acids, including those associated with heparin binding, that function cooperatively in forming an extended binding surface and that may also include residues derived from exon 8 (55, 56). A surprising discovery reported by Shintani et al. (57) was that binding of VEGF to NRP1 is increased due to the presence of heparan sulfate or chondroitin sulfate chains covalently linked to the NRP1 core protein in endothelial and smooth muscle cells. Thus, a detailed analysis of the determinants of VEGF-NRP1 interaction should also consider the possible interaction of the VEGF164 heparin binding determinants with NRP1 glycosaminoglycan modifications.

A number of studies have identified VEGFR2 as the key signaling receptor that mediates the proliferative and migratory effects of VEGF (58–60). Blood vessel growth in the aortic ring assay approximates the in vivo situation in that it involves microvascular sprouting, branching, and capillary tube formation (37, 40). HBD mutants R14A/R49A and R13A/R14A/R49A...
were found to possess an angiogenic activity as potent as that of native VEGF164, supporting previous data suggesting that the VEGF carboxyl-terminal domain is not directly involved in VEGFR2 binding (36). Furthermore, because both mutants exhibited significant reduction in binding to biological matrices, it appears that binding to cell surface- and extracellular matrix-derived HSPGs is not required for ex vivo angiogenesis in the aortic ring model, in which precise branching and network pattern formation of the vasculature would not seem to be critical. However, these studies do not address the purported role of heparin binding in the sequestration of VEGF in tissues, acting as a negative regulator of angiogenesis (61), nor do they address the role of heparin-binding in the deposition of spatial cues and gradients required for proper guidance of developing vessels (11). Additional in vitro and in vivo assays are clearly warranted to address the significance of the HBD domain in the more complex environments associated with the physiological and pathological angiogenesis processes.

Similar to VEGFR2, both the second and third immunoglobulin-like domain of VEGFR1 are necessary and sufficient for high affinity binding of VEGF (62, 63). However, in contrast to VEGFR2, VEGFR1 exhibits differential binding affinities for the soluble and the heparin-binding VEGF isoforms. The increased affinity of this receptor for VEGF164 compared with VEGF120 has been shown to trigger a higher conformation for VEGFR1 binding activity. It still remains to be determined if the contributions within this region resulted in a reproducible decrease in the HBD in VEGF164 (36, 64). The notion that this domain contributes to the interaction with VEGFR1 was directly substantiated in the present study, since the double and triple mutants warranted to address the role of heparin binding in the sequestration of VEGF in tissues, acting as a negative regulator of angiogenesis (61), nor do they address the role of heparin-binding in the deposition of spatial cues and gradients required for proper guidance of developing vessels (11). Additional in vitro and in vivo assays are clearly warranted to address the significance of the HBD domain in the more complex environments associated with the physiological and pathological angiogenesis processes.

Finally, the current findings provide potential insight into the differential potencies of the VEGF isoforms as proinflammatory factors. VEGF165 has been shown to trigger a higher degree of tissue leukostasis and act as a more potent chemotactant for leukocytes, compared with VEGF121. Our current findings would support a model whereby the HBD of VEGF164 (165) potentiates VEGFR1 interactions, signaling, and the consequent leukostasis, thereby acting as a module that enhances the proinflammatory nature of VEGF165, which has been directly linked to the onset of pathological angiogenesis (16, 17). Identification of the determinants involved in heparin binding is a first step in the systematic dissection of the role of the VEGF HBD in both normal development and blood vessel-related pathologies.

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