Synergistic Binding of Vascular Endothelial Growth Factor-A and Its Receptors to Heparin Selectively Modulates Complex Affinity

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Background: Vascular endothelial growth factor (VEGF) requires heparin-like molecules for full activity.

Results: VEGF, VEGF receptor-2, and neuropilin-1 complexes bind heparin synergistically. Neuropilin-1 enhances VEGF signaling and is dependent on heparan sulfate.

Conclusion: Heparin influences VEGF receptor-1, VEGF receptor-2, and neuropilin-1 through distinct mechanisms and regulates VEGF-induced signaling.

Significance: Heparin-like molecules with specific structural features might be used to selectively manipulate the VEGF system to regulate angiogenesis.

Angiogenesis is a highly regulated process orchestrated by the VEGF system. Heparin/heparan sulfate proteoglycans and neuropilin-1 (NRP-1) have been identified as co-receptors, yet the mechanisms of action have not been fully defined. In the present study, we characterized molecular interactions between receptors and co-receptors, using surface plasmon resonance and in vitro binding assays. Additionally, we demonstrate that these binding events are relevant to VEGF activity within endothelial cells. We defined interactions and structural requirements for heparin/HS interactions with VEGF receptor (VEGFR)-1, NRP-1, and VEGF165 in complex with VEGFR-2 and NRP-1. We demonstrate that these structural requirements are distinct for each interaction. We further show that VEGF165, VEGFR-2, and monomeric NRP-1 bind weakly to heparin alone yet show synergistic binding to heparin when presented together in various combinations. This synergistic binding appears to translate to alterations in VEGF signaling in endothelial cells. We found that soluble NRP-1 increases VEGF binding and activation of VEGFR-2 and ERK1/2 in endothelial cells and that these effects require sulfated HS. These data suggest that the presence of HS/heparin and NRP-1 may dictate the specific receptor type activated by VEGF and ultimately determine the biological output of the system. The ability of co-receptors to fine-tune VEGF responsiveness suggests the possibility that VEGF-mediated angiogenesis can be selectively stimulated or inhibited by targeting HS/heparin and NRP-1.

Angiogenesis is a fundamental process by which new blood vessels are formed from pre-existing ones (1, 2). Formation and growth of new vessels is tightly regulated, and loss of control over this process contributes to a number of pathologic conditions (3–6). In some instances, the angiogenic signal is deficient, leading to endothelial cell dysfunction, vessel malformation or regression, and insufficient revascularization, healing, and regeneration (7). In other cases, excessive angiogenesis facilitates tumor growth and metastasis and leads to loss of sight in diabetic retinopathy and wet age-related macular degeneration (4). Inducing and inhibiting angiogenesis is of great clinical interest as a means to stimulate tissue repair (e.g. after myocardial infarction, stroke, diabetic ulcers, etc.) and to inhibit tumor growth and vision loss (8). However, the lack of a detailed understanding of all the factors that control the balance of the angiogenic signal has significantly limited the potential for designing efficient therapies for directing angiogenesis. Defining the various molecular interactions between the major angiogenic regulatory factors will provide insight toward the development of approaches to control and direct angiogenesis.

VEGF-A is the major regulator of angiogenesis in normal and disease states and is critical for the maintenance of vessel homeostasis in adult organisms (9). Alternative splicing of the VEGF-A gene generates several isoforms varying in their ability to bind VEGF receptors, heparan sulfate proteoglycans (HSPGs),2 and NRP-1 (10–12). VEGF165, the predominant isoform in humans, exerts its angiogenic effects by binding and activating two cell surface receptor tyrosine kinases, VEGFR-1 and VEGFR-2, expressed in vascular endothelial cells (13). HSPGs and NRP-1 are required for efficient VEGF signaling (14, 15); nevertheless, the specific mechanistic roles that these co-receptors play remain unclear.

HS is a linear sulfated glycosaminoglycan consisting of repeating disaccharide units containing N-acetyl glucosamine and glucuronic acid that can be extensively modified. Variations in length and structure, including sulfation of O-groups, 2 The abbreviations used are: HSPG, heparan sulfate proteoglycan; NRP, neuropilin; HS, heparan sulfate; SPR, surface plasmon resonance; VEGFR, VEGF receptor; PAE, porcine aortic endothelial cell.

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VEGF Interaction with Receptors Is Regulated by Heparan Sulfate

decaylation, and sulfation of N-groups of glucosamine residues and epimerization of glucuronic acid to iduronic acid, make HS a highly information dense molecule (16, 17). This variability in structure allows for the presence of multiple protein binding sites within HS (18). Heparin, commonly used as a substitute for HS in experimental model systems, is a more abundantly sulfated, mast cell-derived form of HS (19). HSPGs, which consist of one or more HS chains covalently attached to a core protein, are ubiquitously present in the cell surface and extracellular matrices of almost all mammalian cells and have been implicated in controlling the distribution and availability of ligands; more interestingly, HS is also involved in the regulation of several other aspects of receptor-ligand interaction, including complex stability, internalization, and degradation (20). The most well defined example of growth factor regulation by HSPGs is the FGF-2/HS/FGFR-1 system, where HSPGs facilitate ligand-receptor binding and activation (21, 22).

Neuropilins were first identified as transmembrane glycoproteins involved in axonal guidance (23, 24). Subsequently, NRP-1 was established as a VEGF165 binding protein (25) and was proposed as a co-receptor for the VEGF-2-VEGF165 signaling axis. NRP1+/− mice die in utero and display gross cardiovascular and neuronal abnormalities, demonstrating that NRP-1 is required for vascular development. Mice expressing a VEGF isoform unable to bind NRP-1 die before postnatal day 14 because of bleeding in multiple organs or cardiac failure (26–29), further reinforcing the notion that NRP-1-VEGF165 interactions are essential for vascular development. Additionally, recent reports suggest that expression of these co-receptors on adjacent cells (trans) as opposed to cis (same cell) with respect to VEGFR-2 on endothelial cells leads to significant changes in signal transduction upon VEGF165 binding (30, 31).

HS/heparin has been proposed to regulate VEGF biological activity not only by binding VEGF165 directly (32) but also by interacting with receptors and NRP-1 (33–36). However, the data demonstrating direct interaction between VEGF receptors and HS/heparin have yet to be produced or remain incomplete. In this study, we identified new potential mechanisms for the regulation of the VEGF/VEGFR system by HS and NRP-1. We used a combination of surface plasmon resonance (SPR) and other in vitro binding assays to study molecular interactions between the various components of the VEGF system. Our findings indicate that VEGF-1 and NRP-1 bind heparin directly, whereas VEGF-2 does not. Additionally, we demonstrated that heparin has no significant effect on VEGF165 binding to VEGF-1, despite its direct interaction with receptor and VEGF165 (37). Alternatively, heparin enhances VEGF165 binding to the VEGF-2 and appears to be required for VEGF165 binding to NRP-1. Analysis of the size and structural requirements for HS interactions with VEGF-1 and NRP-1, as well as the requirements for the enhanced VEGF165 binding to NRP-1 and VEGF-2, suggest that the presence and structure of HS may ultimately define the specific type of VEGF-VEGFR complexes that form on the cell surface, ultimately controlling VEGF activity. Understanding how specific co-receptors are involved in enhancing and attenuating VEGF165 signaling will provide useful insight for the development of new therapies that aim to manipulate angiogenesis to facilitate tissue repair and prevent disease.

Experimental Procedures

Materials—Recombinant human VEGF165 (293-VE), VEGFR-1 and VEGFR-2 Fc chimeras (321-FL and 357-KD), recombinant rat neuropilin-1 Fc chimera (566-N1), and recombinant human and mouse neuropilin-1 (3870-N1 and 5994-N1) were from R&D Systems (Minneapolis, MN). 125I-Labeled VEGF165 was prepared using a modified Bolton-Hunter procedure (38). 125I-Bolton-Hunter reagent was obtained from PerkinElmer Life Sciences. ProteOn XPR36 neutravidin (NLC) sensor chips were from Bio-Rad. Peroxidase-AffiniPure donkey anti-human IgG, Fc γ fragment specific (709-035-098) was from Jackson ImmunoResearch (West Grove, PA). TMB Microwell peroxidasate substrate system (50-77-00) was from KPL (Gaithersburg, MD). Streptavidin-coated plates and protein A-coated plates were from Pierce. Heparin (12.5 kDa), heparin-derived oligosaccharides: tetrasaccharide (GT8021, 1.2 kDa), hexasaccharide (GT8031, 1.8 kDa), octasaccharide (GT8041, 2.4 kDa), decasaccharide (GT8051, 3 kDa), heparin oligosaccharide I (GT8071, 3.5 kDa) and II (GT8081, 4.2 kDa), and modified heparins: fully de-O-sulfated heparin, 2-O-desulfated heparin, 6-O-desulfated heparin, de-N-sulfated heparin and N-acetylated heparin were from Neoparin Inc. (Alameda, CA). VEGFR-2 (antibody 2478), Y1175-phosphorylated VEGFR-2 (antibody 3770), anti-phospho-ERK1/2 (antibody 9101), ERK1/2 (antibody 4695), and anti-AKT (antibody 9272) antibodies were from Cell Signaling Technologies (Danvers, MA). Anti-rabbit HRP-linked secondary antibody was from Jackson ImmunoResearch. Bovine kidney heparin (B9806), bovine kidney-derived heparan sulfate (H7640), sodium chloride (403016), and all other chemicals were from Sigma-Aldrich.

Surface Plasmon Resonance—All interactions were characterized using the ProteOn XPR36 protein interaction array system from Bio-Rad at 25 °C in binding buffer (PBS with 0.05% Tween 20 and 0.1% BSA) at flow rate of 30–50 μl/min. Biotin–avidin-HRP (B9806) was allowed to interact with the NLC chip surface for 300 s at 25 °C, reaching an immobilization level between 80–120 response units per lane. Biotin (0.5 μg/ml) was immobilized to one lane, obtaining 60 response units of immobilization, and was used as a reference surface. Increasing concentrations of binding partners in running buffer were injected over immobilized heparin until equilibrium was reached and then washed to measure dissociation. Injections of 2 mM NaCl and 5–10 mM NaOH were used to regenerate surfaces after each binding event.

Sensogram Analysis—All sensograms were double-referenced by subtracting buffer injection and a surface containing immobilized bovine. Association and dissociation rate constants (kₐ and kᵈ respectively) were obtained by nonlinear regression of data to a 1:1 Langmuir model (Equations 1–3) using OriginLab, Northampton, MA. In Equation 1, Rₜ represents the response (in response units) at time t, Rₙₓₘₐₓ is the maximal response reached at long time points, and [A] is the ligand concentration in M. Equilibrium affinity constants (Kₛ) were derived from kinetic parameters (Kₛ = Kₘ/Kₐ) or determined from equilibrium analysis (Eq 3).
VEGF Interaction with Receptors Is Regulated by Heparan Sulfate

\[ R_t = \frac{R_{\text{max}}[A]}{K_D} (1 - e^{-k[A] + k_i}) \quad \text{(Eq. 1)} \]

\[ R_t = R_{\text{max}} e^{-k_i t} \quad \text{(Eq. 2)} \]

\[ R_t = \frac{R_{\text{max}}[A]}{[A] + K_D} \quad \text{(Eq. 3)} \]

**Competition Analysis**—SPR competition was used to measure binding affinities of VEGF-1 and NRP-1 with HS, chemically modified heparins, and heparin-derived oligosaccharides. VEGF-1 (1 nM) or NRP-1 (5 nM) Fc chimeras were premixed with a range of concentrations of oligosaccharides and chemically modified heparins in binding buffer. Mixtures were incubated at room temperature for 30 min to allow the protein to bind the oligosaccharide/modified heparin in solution. The mixtures were injected over a biotin-heparin chip surface at 30 \( \mu \text{l/min} \) for 240 s to record maximal response. Regeneration steps were performed as described above. Free protein concentration ([protein]) at each inhibitor (i) concentration was calculated using Equation 4; briefly, the ratio of maximal binding response of protein plus inhibitor (\( R_{\text{max}}(i) \)) to protein alone (\( R_{\text{max}} \)) times the total protein concentration ([protein]_T). The values were plotted against inhibitor concentration and data points were fit to Equation 5 to obtain apparent binding constants (\( K_D(i) \)) for each oligosaccharide and modified heparins as previously described (39).

\[ [\text{protein}]_k = \frac{R_{\text{max}}(i)}{R_{\text{max}}} \times [\text{protein}]_T \quad \text{(Eq. 4)} \]

\[ [\text{protein}]_k = [\text{protein}]_T - \left( \frac{1}{Z} \right) \left( \sqrt{\left( \frac{1}{2} + \frac{[\text{protein}]_T + K_D(i)}{Z} \right)^2 - \frac{1}{2}[\text{protein}]_T} \right) \quad \text{(Eq. 5)} \]

**ELISA Binding Assays**—Biotin-heparin (10 \( \mu \text{g/ml} \), 100 \( \mu \text{l/well} \)) in PBS was adsorbed onto streptavidin-coated 96-well plates overnight at 4 °C. Wells were washed with 200 \( \mu \text{l} \) of binding buffer to remove any unbound heparin. Receptor chimeras (100 \( \mu \text{l/well} \)) at the indicated concentrations in binding buffer were applied to each well and incubated for 2 h at 4 °C. The plate was rinsed three times with binding buffer and blocked in 1.5% BSA 0.2% casein in PBS for 1 h, and the amount of bound receptor was measured using an HRP-linked donkey anti-human IgG (1:5000) in blocking buffer. Wells were washed three times with PBS and 0.05% Tween 20 and three times with PBS before adding 100 \( \mu \text{l} \) of peroxidase substrate to determine the relative amount of receptor bound at each concentration. Color development reactions were stopped after 5–10 min with 1 M sulfuric acid and absorbance at 450 nm (referred to 570 nm) was measured using a spectrophotometer.

**Radioligand Binding Assays**—Receptor Fc chimeras (100 pm, 100 \( \mu \text{l/well} \)) in binding buffer were adsorbed onto a protein A-coated 96-well plate for 1 h at room temperature. \( ^{125} \text{I}-\text{VEGF}_{165} \) was added to wells in the presence or absence of heparin, heparin oligosaccharides, or chemically modified heparins (500 nM in 100 \( \mu \text{l binding buffer/well} \)) and incubated for 1 h at room temperature. The plate was washed three times, and bound \( ^{125} \text{I}-\text{VEGF} \) was extracted using 300 \( \mu \text{l} \) of 1 N NaOH. Radioactivity was quantified using a Cobra Auto-Gamma 5005 counter (Packard Instruments, Meridian, CT).

**Cell Culture**—Mouse-VEGFR-2-expressing porcine aortic endothelial cells (VEGFR-2 PAEs) and empty vector PAEs were kindly provided by Dr. Nader Rahimi (40, 41). Cells were maintained in 10% FBS in DMEM with penicillin/streptomycin and \( \alpha \)-glutamine. All cell culture-related reagents were purchased from Invitrogen.

**Radioligand Binding to Endothelial Cells**—VEGFR-2 and empty vector PAEs were seeded in 24-well dishes (Corning Inc., Corning, NY) in 0.5 ml of 10% dialyzed FBS ± 25 mm chlorate. Cells growing in the presence of chlorate were seeded at a higher density than the control conditions to balance for slower growth under chlorate conditions (20,000 and 15,000 cells/well, respectively). Equilibrium binding assays on confluent cell cultures were carried out as described before (38). Binding buffer consisted of 25 mm HEPES, pH 7.3, in DMEM (without bicarbonate) containing 0.1% BSA. Briefly, cells were washed once with 0.5 ml of ice-cold binding buffer, and 0.15 ml were added to wells and incubated at 4 °C for 10 min to inhibit endocytosis. \( ^{125} \text{I}-\text{VEGF}_{165} \) (0.26 nm) in the presence or absence of soluble NRP-1 (sNRP-1, 5 nm) in binding buffer (preincubated for 2 h at room temperature) was added to cells and allowed bind for 3 h at 4 °C. Unbound \( ^{125} \text{I}-\text{VEGF}_{165} \) was removed by washing cells three times with 0.5 ml of cold binding buffer. Bound \( ^{125} \text{I}-\text{VEGF}_{165} \) was extracted with 0.4 ml of 1 N NaOH, and radioactivity was counted in a Cobra Auto-Gamma 5005 counter (Packard Instruments, Meridian, CT).

**VEGF Signaling**—VEGFR-2-PAEs were seeded on 35-mm² dishes (Corning Inc.) in DMEM containing 10% dialyzed FBS with \( \alpha \)-glutamine in the absence or presence of 25 mm sodium chloride (non-chlorate: 45,000 cells/well; chlorate: 60,000 cells/well). After 2 days, the medium was changed to DMEM containing 0.1% dialyzed FBS ± chlorate overnight and then treated with mixtures of VEGF \(_{165}\) and sNRP-1 (preincubated for 2 h at room temperature) for 10 min. For heparin-treated cells, heparin (10 \( \mu \text{g/ml} \)) was added directly into the media for 1 h at 37 °C before stimulation. Cells were lysed in extraction buffer (1% Triton X-100 150 mm NaCl, 1 mm EDTA, 1 mm EGTA, 0.5% Nonidet P-40, 10 mm Tris, pH 7.5) containing Halt protease and phosphatase inhibitor mixture (Life Technologies, 78440). Lysates were cleared by centrifugation, and samples containing 30 \( \mu \text{g} \) of protein in sample buffer were heated, resolved by 8% SDS-PAGE, and transferred onto nitrocellulose membranes. Membranes were blocked for 1 h at room temperature with 5% milk in TBS-T (10 mm Tris, pH 7.5, 150 mm NaCl, and 0.1% Tween 20), incubated overnight at 4 °C with appropriate primary antibodies in blocking buffer, and subsequently incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Membranes were developed with Clarity ECL Western blotting Substrate reagent (Bio-Rad, 170-5060) and imaged on a ChemiDoc MP system (Bio-Rad). Quantification of immunoblotting signals was performed using Image Lab 5.0 (Bio-Rad).


Results

VEGFR-1 and NRP-1, but Not VEGFR-2, Interact Directly with Heparin—Previous studies indicate that VEGF receptors directly interact with HS/heparin molecules to modulate VEGF function (34–36, 42); however, information about the direct binding events and mechanisms of regulation remain to be determined. The binding kinetics between heparin and VEGF receptors, as well as the co-receptor NRP-1, were evaluated using SPR with heparin-immobilized sensor chips. SPR response curves at increasing concentrations (black and gray lines) of VEGFR-1, VEGFR-2, and NRP-1 Fc chimeras are shown in Fig. 1 (A–C), respectively. Curve fittings to a 1:1 Langmuir binding model are shown as red lines, and the resulting kinetic parameters are summarized in Table 1. VEGFR-1 showed a 5-fold higher affinity (\( K_D \)) obtained from the ratio of the rate constants \( k_C/k_D \) for heparin than did NRP-1 (\( K_D \) values of 11 and 50 nM for VEGFR-1 and NRP-1, respectively). Interestingly, we observed no binding when VEGFR-2 was injected over immobilized heparin. These results suggest that heparin-like molecules might act by selectively interacting with cell surface receptors in the absence of ligand.

Dissociation of VEGFR-1 and NRP-1 Fc chimeras from the heparin layer was found to be too slow to be reliably measured using conventional SPR kinetic experimental methods. Hence, we decided to measure binding of VEGFR-1 and NRP-1 to heparin in solution using a competition approach (43). VEGFR-1 (Fig. 1D) and NRP-1 (Fig. 1E) were premixed with increasing concentrations of heparin as a competitor before flowing the mixture over immobilized heparin at a slow flow rate to favor mass transport. Receptors in complex with heparin in solution are unable to associate with the heparin immobilized on the chip surface resulting in a decrease in SPR response. Free protein concentration at each competitor concentration (squares) was calculated using Equation 4, plotted against inhibitor concentration and fit to Equation 5 (red line). Apparent affinity (\( K_D \)) values for VEGFR-1 and NRP-1 binding to heparin obtained using the competition analysis are shown in Tables 2 and 3, respectively. Binding of receptor Fc chimeras to biotin-heparin coated on streptavidin plates was measured using an ELISA detecting the Fc portion of the chimeras. p values were determined by a two-tailed Student’s t test. *, p of 0.02 versus no receptor. The data are representative of five independent experiments.

**FIGURE 1.** VEGF receptor-1 and neuropilin-1 interact directly with heparin. A–C, SPR sensograms of VEGFR-1 Fc chimera (A, 0.31–5 nM; lower to upper curves) or NRP-1 (B) or VEGFR-2 Fc chimeras (C, 3.1–25 nM; lower to upper curves for B and C) binding to biotin-heparin immobilized on an NLC sensor chip. Dissociation was measured over 1200 s after flow was replaced by buffer (only 360 s of dissociation is shown). The experimentally obtained data are shown as black and gray lines, whereas global fits of these data to a 1:1 Langmuir interaction model are shown in red. The kinetic parameters obtained from nonlinear regression analysis for each interaction are listed in Table 1. Sensograms shown are representative of five individual surfaces on the chip and each experiment was performed three times. D and E, competition analysis of VEGFR-1 Fc chimera (D) or NRP-1 Fc chimera (E) binding to heparin in solution. Briefly, VEGFR-1 (1 nM) or NRP-1 (5 nM) was mixed with increasing concentrations of heparin (competitor) and allowed to incubate for 30 min at room temperature. Mixtures were flowed over a heparin-coated SPR chip and maximal binding responses were recorded (averages of 5 surfaces ± S.D.). Free protein concentration (free [Fc chimera] nm) at each inhibitor concentration (squares) was calculated using Equation 4, plotted against inhibitor concentration and fit to Equation 5 (red line). Apparent affinity (\( K_D \)) or VEGFR-1 Fc chimeras (\( K_D \) values for VEGFR-1 and NRP-1 binding to heparin obtained using the competition analysis are shown in Tables 2 and 3, respectively. F, binding of receptor Fc chimeras to biotin-heparin coated on streptavidin plates was measured using an ELISA detecting the Fc portion of the chimeras. p values were determined by a two-tailed Student’s t test. *, p of 0.02 versus no receptor. The data are representative of five independent experiments.

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**TABLE 1.** Summary of kinetic parameters determined from SPR and competition analysis of binding of receptor Fc chimeras to immobilized heparin.

| Receptor | Free Protein Concentration (nM) | 
|----------|---------------------------------|
| VEGFR-1  | 0.31–5                         |
| NRP-1    | 0.31–5                         |
| VEGFR-2  | 0.31–5                         |

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**TABLE 2.** Competition analysis of VEGFR-1 Fc chimera binding to heparin.

| Heparin Concentration (nM) | Free Protein Concentration (nM) | 
|---------------------------|---------------------------------|
| 0                        | 0.31                            |
| 5                        | 0.31                            |
| 10                       | 0.31                            |
| 20                       | 0.31                            |
| 50                       | 0.31                            |

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**TABLE 3.** Competition analysis of NRP-1 Fc chimera binding to heparin.

| Heparin Concentration (nM) | Free Protein Concentration (nM) | 
|---------------------------|---------------------------------|
| 0                        | 0.31                            |
| 5                        | 0.31                            |
| 10                       | 0.31                            |
| 20                       | 0.31                            |
| 50                       | 0.31                            |
HSPGs have been proposed to regulate VEGF165 signaling through VEGFR-2 (14, 42, 44), but a mechanism for this observation has not been defined. Contrary to previous reports suggesting that VEGFR-2 interacts directly with HSPGs (34, 42), we did not observe VEGFR-2 binding to immobilized heparin (Fig. 1C). To ensure that this observation was not an artifact of our SPR system, we immobilized heparin onto streptavidin-coated plates and measured binding of all three receptor Fc chimeras by an ELISA. We found that under these conditions, VEGFR-1 and NRP-1 showed significant binding, whereas VEGFR-2 did not (Fig. 1F). Together these results suggest that HS/heparin can regulate VEGF function in a complex manner that is dependent on the receptor type present.

Structural and Size Requirements for HS Binding to VEGFR-1 and NRP-1—Because VEGFR-1 and NRP-1 directly interact with heparin, we explored the possibility that there are distinctions in the size and structural requirements for heparin to bind to each receptor. To delineate the minimum heparin chain length required for binding to VEGFR-1 and NRP-1, we screened a series of heparin-derived oligosaccharides using the competition method. VEGFR-1 and NRP-1 Fc chimeras were preincubated with the indicated oligosaccharides for 30 min to allow them to bind in solution, and then mixtures were flowed over immobilized heparin. In Fig. 2, binding of receptor to various oligosaccharides in solution is shown as a percentage of the maximal SPR response observed in the absence of oligosaccharide. Heparin-derived oligosaccharides ranging from 4 (tetrasaccharide) to ~14 saccharide units (oligosaccharide II) were tested for binding VEGFR-1 and NRP-1. Competition of VEGFR-1 binding to heparin was observed exclusively in the presence of oligosaccharides with 10 or more saccharide units. In the case of NRP-1, only moderate binding was observed with heparin oligosaccharides of various lengths as heparin-binding oligosaccharides with 10 or more saccharide units.

Next we investigated the sulfation requirements for heparin binding to VEGFR-1 and NRP-1. Fully de-O-sulfated heparin is a derivative in which all O-sulfate esters have been chemically

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**TABLE 1**

| Binding kinetics of VEGFR-1 and NRP-1 Fc chimera interaction with heparin by SPR |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
|                                  | $k_a$                           | S.E.                           | $k_d$                           | S.E.                           | $K_d$ |
|                                  | $1/M$s                          | $1/M$s                        | $1/s$                           | $1/s$                         |       |
| VEGFR-1                          | $3.81 \times 10^5$              | $9.44 \times 10^4$            | $4.08 \times 10^{-3}$           | $1.87 \times 10^{-3}$         | 11    |
| NRP-1                            | $1.88 \times 10^5$              | $2.69 \times 10^4$            | $9.72 \times 10^{-3}$           | $1.56 \times 10^{-3}$         | 50    |

**TABLE 2**

| Binding affinities of VEGFR-1 Fc chimera for heparin, HS, oligosaccharides, and N-desulfated heparin |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
|                                  | $K_D$                           | S.E.                           | Reduced $\chi^2$ | Adjusted $R^2$ |
|                                  | nM                             | nM                             |                   |                |
| Heparin                          | 1.36                           | 0.09                           | $6.17 \times 10^{-4}$ | 0.995         |
| Heparan sulfate                  | 24.5                           | 2.14                           | 6.93               | 0.986         |
| Oligo I                          | 390                            | 81.7                           | 0.0253             | 0.791         |
| Oligo II                         | 113                            | 19.9                           | 0.0138             | 0.875         |
| N-DS                             | 22.0                           | 1.79                           | 0.000776           | 0.994         |

**TABLE 3**

| Binding affinities of NRP-1 Fc chimera for heparin, HS and oligosaccharide II |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
|                                  | $K_D$                           | S.E.                           | Reduced $\chi^2$ | Adjusted $R^2$ |
|                                  | nM                             | nM                             |                   |                |
| Heparin                          | 2.8                            | 0.8                            | 117                | 1              |
| Heparan sulfate                  | 170                            | 14                             | 0.0442             | 0.985         |
| Oligo II                         | 760                            | 40                             | 17.2               | 0.999         |

**FIGURE 2.** Size and structural requirements for VEGFR-1 and NRP-1 interaction with HS chains. A and B, heparin oligosaccharides of various lengths were screened for the ability to bind VEGFR-1 (A) and NRP-1 (B) Fc chimeras using the competition approach. Oligosaccharides or modified heparins at a single concentration (500 nM) were incubated with the indicated proteins in solution for 30 min at room temperature before measuring binding of the protein to immobilized heparin on an SPR chip. Using the maximal binding responses in the absence of competitor, free protein concentrations in the presence of the indicated oligosaccharide were calculated and presented as the percentages of total protein bound in solution. A high percentage represents a high level of binding between the soluble oligosaccharide and protein (i.e. the SPR signal representing protein binding to the heparin chip was reduced). Bars represent mean ratios ± S.D. of five separate surfaces on the SPR chip, and experiments were repeated two times independently. Oligonucleotides I and II are heparin oligosaccharide I (~14 saccharide units).
VEGF Interaction with Receptors Is Regulated by Heparan Sulfate

removed from heparin without changing the backbone structure. In 2-O-desulfated and 6-O-desulfated heparin, only the O-sulfate groups on C-2 of uronic acid and C-6 of glucosamine, respectively, have been removed. Most of the other sulfate groups remain intact. Heparin lacking all O-sulfate groups showed reduced competition for VEGFR-1 binding to heparin and no competition for NRP-1 binding (Fig. 2, C and D). Binding of heparin to VEGFR-1 and NRP-1 is more dependent on the sulfation of C-6 on glucosamine than on the C-2 position of uronic acid, because the 2-O-desulfated heparin was still able to compete for binding almost as well as heparin. On the other hand, a heparin derivative lacking N-sulfate groups on glucosamine residues was able to bind VEGFR-1 but showed significantly reduced ability to bind NRP-1. Apparent affinities (KD) were calculated by fitting SPR binding inhibition data (Fig. 3) at increasing concentrations of competitor to Equation 5 (red lines) for VEGFR-1 and NRP-1 binding to heparan sulfate (Fig. 3, A and B) and heparin oligosaccharide II (Fig. 3, C and D), and values are shown in Tables 2 and 3. The distinct structural requirements for heparin to bind to VEGFR-1 versus NRP-1 suggest that variations in HS fine structure could function to selectively regulate VEGF binding to one receptor type over another.

VEGF165 Bridges the Interaction between VEGFR-2 and Heparin—The presence of HSPGs is required for effective VEGF165 binding and signaling to endothelial cell surfaces (14, 33, 42); however, the specific mechanisms by which HS molecules assist in VEGF165 signaling remain unknown. Interpretation of results with intact cells has proven challenging because of the number of components involved in the system and the wide range of potential interactions. Consequently, we focused on measuring the influence of heparin on the interactions of VEGF165 with each major cell surface binding partner, VEGFR-1, VEGFR-2, or NRP-1 in isolation. First, we investigated whether VEGF165 could promote receptor interactions with heparin. Fig. 4 shows the response elicited by flowing the indicated receptor Fc chimera (green line), VEGF165 (red line), or a pre-equilibrated equimolar mix of the two (blue line), over immobilized heparin. We did not detect a major change in VEGFR-1 binding to heparin in the presence of VEGF165. A similar result was observed with NRP-1 (Fig. 4C). In contrast, when VEGFR-2 was allowed to interact with VEGF165 in solution, the mixture showed significantly increased binding (Fig. 4B) above the sum of that observed with either protein alone. This result agrees with previous reports showing that VEGF165 promotes VEGFR-2 binding to heparin (45) and suggests that the VEGF165–VEGFR-2 complex may contain a synergistic heparin binding domain that is not present in either protein alone. To determine the extent to which the increase observed could be attributed to VEGFR-2, we used a binding plate assay to measure the amount of receptor bound to heparin in the presence (black bars) or absence (gray bars) of VEGF165. We first allowed the VEGF165 to bind to the heparin-coated plate under static conditions for a prolonged time and then measured
Because there was no significant binding of VEGF165 to NRP-1, the size of oligosaccharide chain able to bind VEGF165 is an octasaccharide (37). To determine the structural requirements for heparin to bind to NRP-1—To gain insight into the roles that HSPGs play in the regulation of VEGF interactions with the each receptor type, we immobilized receptor Fc chimeras onto protein A-coated plates and measured VEGF165 binding to VEGFR-2 and NRP-1. We found that removal of N- or O-sulfate groups reduced, but did not eliminate, the ability to enhance VEGF165 binding compared with unmodified heparin (Fig. 6D). Interestingly, heparin lacking only sulfate groups on the 2-O position was as active as heparin at enhancing binding to NRP-1, whereas 6-O-desulfated heparin showed an effect more comparable with fully de-O sulfated heparin. The data indicate that sulfate groups at the 2-O position are dispensable for the heparin effect on VEGF-NRP-1 interaction. Taken together, these results suggest that changes in the composition of HS chains on cell surfaces might differentially affect interactions between VEGF and its receptors, ultimately modulating cellular responses.

VEGF Interaction with Receptors Is Regulated by Heparan Sulfate

Receptor binding. We found a significant increase in the binding of VEGF165 to the heparin-coated plate in the presence of VEGF165 (Fig. 4D). Interestingly, this effect was not seen with VEGF121, an isoform that is unable to bind heparin and NRP-1 (data not shown).

**Heparin Selectively Increases VEGF<sub>165</sub> Binding to VEGFR-2 and NRP-1**—To better understand how the various components of the VEGF system might act together to regulate VEGF<sub>165</sub> binding, we tested a series of combinations including soluble monomeric form of NRP-1 (sNRP-1), VEGFR-2, and heparin, p values were determined by a Student’s t test. *, p value of 0.01 versus no heparin added. Each data point represents the average of three ± S.D. The experiment was repeated three times.

![Figure 5](image-url)  
**FIGURE 5.** Heparin selectively increases VEGF<sub>165</sub> binding to VEGFR-2 and NRP-1. A–C, VEGF<sub>165</sub>-1 (4), VEGF<sub>2</sub> (B), or NRP-1 (C) Fc chimeras were immobilized onto protein A-coated plates and VEGF<sub>165</sub> binding was quantified in the presence or absence of heparin (10 μg/ml). D, VEGF binding at 20 ng/ml; heparin, *p* values were determined by a Student’s *t* test. *, *p* value of 0.01 versus no heparin added. Each data point represents the average of three ± S.D. The experiment was repeated three times.

![Figure 6](image-url)  
**FIGURE 6.** Size and structural requirements for heparin-induced enhancement of VEGF<sub>165</sub> binding to VEGFR-2 and NRP-1. A, VEGF<sub>165</sub> binding to immobilized VEGF<sub>165</sub> or NRP-1 Fc chimeras was measured in the presence of heparin oligosaccharides (A and C) and chemically modified heparins (B and D). Bars represent the mean ratios of femtomols VEGF<sub>165</sub> bound in the presence of heparin oligosaccharides or modified heparins to VEGF alone ± S.D.
greater than the additive effects of all the single components, as well as any of the two component mixtures (Fig. 7). Under these conditions, none of the individual components showed significant binding to heparin. In particular, sNRP-1, unlike the dimeric form, did not show significant binding to heparin alone but did show binding when preincubated with VEGF165 or VEGFR-2. We suspect that under these conditions, sNRP-1 dimerization is facilitated by the dimeric VEGF165 and VEGFR-2. This would be consistent with previous studies that indicate that dimerization of NRP-1 is a critical element of the mechanism by which NRP-1 modulates VEGF (46).

It is difficult to quantitatively analyze the binding profiles from these mixing reactions because the relative quantity of complexes formed in solution is unknown; however, visual inspection of the dissociation profiles suggests interesting differences in the stability of the various complexes. For instance, VEGF165 interaction with heparin (Fig. 7, light blue line and bar) appears very unstable, showing fast dissociation; however, when in complex with VEGFR-2 (orange), dissociation was much slower. On the other hand, a complex containing sNRP-1 and VEGF165 (green) dissociated more rapidly than VEGF-VEGFR-2 complexes, suggesting that the specific nature of the heparin-complexes that form are dependent on the particular molecular components present. Interestingly, the binding response elicited by mixtures of sNRP-1 and VEGFR-2 (red) showed a profile that was distinct from all other curves, with a relatively reduced association rate and with almost no dissociation. Thus, the formation of each higher order complex appears to have its own particular kinetic properties, suggesting a potential sequence of events that might occur as VEGF interacts with its various binding sites on a cell. However, it is important to recognize that the cell-free binding experiments are not able to capture all aspects of the complexity of these events as they would occur on the surface of a cell.

NRP-1 Enhances VEGF165 Binding and Activation of VEGFR-2 and ERK1/2 in Endothelial Cells—To test the biological implications of the binding synergy observed between co-receptors in vitro, we used PAEs. These cells do not express endogenous VEGFR-2 or NRP-1. We used cells that have been engineered to express full-length VEGF-2 or empty vector (40, 41). We found that soluble NRP-1 significantly enhanced the binding of 125I-VEGF165 to VEGF-2-expressing PAE cell surfaces and to a lesser degree to EV PAEs (Fig. 8A). NRP-1 was not able to enhance binding to PAE cells pretreated with sodium chlorate, a potent inhibitor of proteoglycan sulfation, indicating that this effect was dependent on the presence of HS. Moreover, VEGF binding to VEGF-2-expressing PAEs in the absence of NRP-1 was also significantly reduced by chlorate. These results are consistent with the in vitro binding studies showing that maximal VEGF binding is achieved in the presence of VEGFR-2, NRP-1, and HS. To determine whether the alterations in VEGF binding translated to changes in VEGF-mediated activity, we evaluated VEGF-stimulation of VEGF-2 and ERK1/2 phosphorylation (Fig. 8, B and C). Consistent with the effect on binding, we observed that the addition of NRP-1 enhanced VEGF-2 phosphorylation and to a lesser extent ERK1/2 phosphorylation. Interestingly, the ability of VEGF to stimulate VEGF-2 and ERK1/2 phosphorylation and the ability of NRP-1 to influence these signaling events were abolished in cells treated with chlorate. The addition of exogenous heparin was able to partially rescue the VEGF response in chlorate-treated cells, but not the NRP-1 effect (Fig. 8C).

We have demonstrated that the synergistic binding of VEGF, VEGF-2, NRP-1, and heparin/HS observed in vitro correlates with enhanced endothelial cell responsiveness to VEGF165. Specifically, that the ability of NRP-1 to influence VEGF activity requires sulfated HS chains. This evidence further demonstrates the biological significance of understanding the complex multimeric binding events within the VEGF system. Any attempt to rationally manipulate VEGF activity will require an appreciation of these synergistic binding interactions.

Discussion

Because HSPGs and NRP were identified as VEGF co-receptors, most models of their function have considered that they aid in complex formation by directly associating with either the receptor or ligand to enhance receptor-ligand binding or that they act by forming a stable ternary complex with the receptor

FIGURE 7. VEGF-2, VEGF165, and NRP-1 synergize to bind heparin as a complex. A, VEGFR-2 Fc chimera, sNRP-1, and VEGF165 were preincubated alone or in various equimolar (25 nM) combinations for 2 h at room temperature before being injected over a surface containing immobilized heparin on an SPR chip. Shown are VEGF-2 Fc chimera alone (yellow), sNRP-1 alone (purple), VEGF165 alone (light blue), VEGF-2 Fc chimera with sNRP-1 (red), VEGF-2 Fc chimera with VEGF165 (orange), sNRP-1 with VEGF165 (green), and VEGF-2 Fc chimera with sNRP-1 and VEGF165 (dark blue). The purple and yellow lines overlap somewhat. B, maximal response (response units, RU) for each condition is represented in the bar graph as mean ± S.D. of five separate surfaces on the SPR chip. The experiment was repeated two times independently.
and ligand. However, it seems that these classic models might not effectively capture the full complexity of the VEGF system. Recently, new data have surfaced indicating that the role of co-receptors within the VEGF system is dependent on cellular context (30, 31). Thus, the goal of the present study was to identify potential mechanistic elements for how co-receptors influence VEGF activity. In this regard, the data presented here demonstrate that interactions between VEGF receptors and heparan/HS have distinct structural requirements depending on the receptor in question and that interactions between various binding partners dramatically influence binding to heparan/HS. The differential functions of the signaling receptors, VEGFR-1 and VEGFR-2, in the VEGF system suggest that HS may play a sophisticated role in modulating the angiogenic response by selectively stabilizing particular ligand-receptor complexes based on the specific cellular context.

We conducted a series of binding studies using defined components that revealed multiple possible mechanisms by which heparan/HS might influence the ability of VEGF to form complexes with its receptors. In our proposed model, VEGFR-2 and soluble monomeric NRP-1 do not interact with heparan under these conditions, and VEGF binds only with low affinity (9A). Monomeric NRP-1 can enhance VEGFR-2 binding to heparan but to a lower extent than VEGF_{165}. VEGF can also enhance NRP-1 binding to heparan, but the highest binding to heparan is achieved only in the presence of all three components: VEGF_{165}, VEGFR-2, and NRP-1 monomer (Fig. 9). This model is consistent with previous studies that have shown that maximal binding of VEGF to VEGFR-2 on cells requires cell surface HS (34, 42) and that HSPGs and VEGF-2 appear to associate on cell surfaces (42). Thus, even though VEGFR-2 and HS do not appear to form a stable binary complex in isolation, these molecules in conjunction with NRP-1 appear to synergistically form a high affinity “active” complex. Interestingly, our data indicate mechanisms where heparan/HS can modulate VEGF binding to VEGFR-2. Specifically, the observation that monomeric NRP-1 can synergize with VEGFR-2-VEGF_{165} complexes to enhance binding to heparan suggests a system where all components are required to produce a very high affinity complex (Fig. 9).

![Figure 8: sNRP-1 enhances VEGF_{165} binding and signaling in VEGFR-2 expressing PAEs and it requires sulfation of HS chains.](Image)

**A.** $^{125}$I-VEGF_{165} binding to chlorate-treated PAE cell surfaces in the presence or absence of sNRP-1. Cells were grown in the presence or absence of 25 mM sodium chlorate for 2 days. Wells were washed, and $^{125}$I-VEGF_{165} binding $\pm$ sNRP-1 was measured after incubating for 3 h at 4°C. B, confluent cells were treated with VEGF_{165} $\pm$ sNRP-1 for 10 min. Protein was collected and analyzed by Western blot. Membranes were incubated with the indicated primary antibodies followed by HRP-linked secondary antibodies. The blots show enhanced VEGF_{165}-induced VEGFR-2 phosphorylation in the presence of soluble NRP-1, and this effect is lost when cells are grown in medium containing chlorate. C, the addition of exogenous heparin partially rescued the VEGF_{165}-induced VEGFR-2 phosphorylation but not the NRP-1 effect. Cells were treated with 10 µg/ml heparin before the stimulation with VEGF_{165} $\pm$ sNRP-1.
VEGF Interaction with Receptors Is Regulated by Heparan Sulfate

Figure 9. Synergistic binding model for the VEGFR-2/VEGF165/NRP-1/heparin complex. A, representation of the different interacting partners and their hypothesized complex stability. R2 is VEGFR-2, mN1 is monomeric NRP-1, V is VEGF165, and H is heparin. Arrow size correlates to maximal heparin binding response observed in Fig. 7. Block or small arrow represents no binding or very low affinity binding between components. Large arrows symbolize strong interaction or high complex stability between components. B, model for complex formation between VEGF165, VEGFR-2, NRP-1, and heparan sulfate chains.

these synergistic binding events translate into alterations in endothelial cell responses to VEGF165.

VEGF-targeted therapy has encountered numerous setbacks since its conception. The most successful use is for treatment of the wet form of age-related macular degeneration, where a VEGF-specific antibody is injected directly into the eye to reduce or stop neovascularization of the retina. In less successful cases, like treatment of various types of cancers, an anti-VEGF antibody is injected intravenously, either alone or in combination with chemotherapy, to block vessel recruitment into growing tumors and hence inhibit tumor growth and prevent metastasis (47). Although the conceptual basis for these therapies is reasonable, the results have not been as successful as anticipated. More effective anti-VEGF therapies will likely require the use of a combination approach that targets multiple components of the VEGF system. To achieve this end, a more detailed understanding of how the various components of the VEGF system interact to provide such sophisticated control of angiogenesis is needed.

In this study, we applied a systematic approach to investigate interactions between various components of the VEGF system. Some aspects of these interactions have been described in the past (48), whereas many others remain to be determined. We found that the interactions between heparin/HS and the VEGFRs are specific to each receptor type, suggesting that heparin/HS influences VEGF at multiple levels. Although most studies have focused on identifying the role HSPGs serve in the VEGF165-VEGFR2 signaling axis, the influence of HSPGs on VEGFR-1. We propose that co-receptors provide context-specific regulation of VEGF-VEGFR interactions leading to complex control of angiogenesis.

The results presented in this study support a model where VEGF165 facilitates the association of heparin/HS with VEGFR-2 to generate a high affinity ternary complex. We found that VEGFR-2 did not bind directly to heparin in the absence of VEGF165 whereas VEGFR-1 and dimeric NRP-1 were able to bind to heparin. Binding of heparin to VEGFR-1 did not appear to influence VEGF binding to this receptor, whereas binding of heparin to NRP-1 appeared to be necessary for VEGF to bind NRP-1.

Taking these findings together, we envision a complex system whereby HSPGs on the cell surface might function as a common modulator of VEGF binding to its various receptors/co-receptors and the interactions of these receptors/co-receptors with one another. Moreover, our findings that there are distinct heparin/HS structural requirements for direct binding to VEGFR-1 and NRP-1, as well as for the synergistic binding of VEGF-NRP-1 and VEGF-VEGFR-2, indicate that modifications in HS fine structure might be used to guide VEGF activity within a physiological context. Our cell culture model has begun to address how these complex interactions directly regulate the biological output of endothelial cells in response to VEGF. Additionally, changes in HS sulfation patterns are mediated both through alterations in biosynthesis and by a family of extracellular enzymes, the HS sulfatases, which remove or modify specific sulfate groups in heparan sulfate chains (52). For example, HSulf-2, an endoglycosidase C-6 sulfatase, has been shown to release VEGF from its association with heparin, as well as to play critical roles in modulating other growth factor systems (53). In this study, we used chlorate to inhibit the cellular production of sulfated glycosaminoglycans as a means to evaluate the role of sulfated HS in this system. Thus, our data demonstrating that VEGF response and the ability of NRP-1 to influence VEGF activity were eliminated in chlorate-treated cells yet could be partially rescued by the addition of heparin strongly support a model whereby full VEGF activity is dependent on the presence of HS in endothelial cells. Thus, it is possible that extracellular sulfatases are also involved in regulating other aspects of VEGF function by changing binding properties between HSPGs and cell surface receptors. A number of studies have indicated that HSPGs play critical roles in modulating growth factor activity, yet the specific mechanisms remain somewhat obscure. More structure-specific studies are required to fully understand the implications that changes in HS sulfation have on VEGF function. The data presented here indicate that HSPGs provide high order control of VEGF by participating in multiple interactions with the various components of the system, suggesting that targeted modulation of HSPGs might eventually be an effective means to selectively control VEGF in a context specific manner.

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VEGF Interaction with Receptors Is Regulated by Heparan Sulfate

endothelial growth factor receptor-2 and neuropilin-1 form a receptor complex that is responsible for the differential signaling potency of VEGF165 and VEGF121. J. Biol Chem. 276, 25520–25531

19. Forsten-Williams, K., Chua, C. C., and Nugent, M. A. (2005) The kinetics and the induction of pathological angiogenesis. Annu. Rev. Pathol. 2, 251–275

20. Forsten, K. E., Fannon, M., and Nugent, M. A. (2000) Potential mechanisms for the regulation of growth factor binding by heparin. J. Theor. Biol. 205, 215–230

21. Ibrahim, O. A., Zhang, F., Hisrka, S. C., Mohammadi, M., and Linhardt, R. J. (2004) Kinetic model for FGF, FGFR, and proteoglycan signal transduction complex assembly. Biochemistry 43, 4724–4730

22. Forsten-Williams, K., Chua, C. C., and Nugent, M. A. (2005) The kinetics of FGF-2 binding to heparan sulfate proteoglycans and MAP kinase signaling. J. Theor. Biol. 233, 483–499

23. He, Z., and Tessier-Lavigne, M. (1997) Neuropilin is a receptor for the axonal chemorepellent semaphorin III. Cell 90, 739–751

24. Kolodkin, A. L., Levengood, D. V., Rowe, E. G., Tai, Y.-T., Giger, R. J., and Ginty, D. D. (1997) Neuropilin is a semaphorin III receptor. Cell 90, 753–762

25. Whitaker, G. B., Limberg, B. J., and Rosenbaum, J. S. (2001) Vascular
VEGF Interaction with Receptors Is Regulated by Heparan Sulfate

43. Day, E. S., Capili, A. D., Borysenko, C. W., Zafari, M., and Whitty, A. (2013) Determining the affinity and stoichiometry of interactions between unmodified proteins in solution using Biacore. *Anal. Biochem.* **440**, 96–107

44. Ashikari-Hada, S., Habuchi, H., Kariya, Y., and Kimata, K. (2005) Heparin regulates vascular endothelial growth factor165-dependent mitogenic activity, tube formation, and its receptor phosphorylation of human endothelial cells: comparison of the effects of heparin and modified heparins. *J. Biol. Chem.* **280**, 31508–31515

45. Hamma-Kourbali, Y., Vassy, R., Starzec, A., Le Meuth-Metzinger, V., Oudar, O., Bagheri-Yarmand, R., Perret, G., and Crépin, M. (2001) Vascular Endothelial Growth Factor 165 (VEGF165) Activities are inhibited by carboxymethyl benzylamide dextran that competes for heparin binding to VEGF165 and VEGF165-KDR complexes. *J. Biol. Chem.* **276**, 39748–39754

46. Vander Kooi, C. W., Jusino, M. A., Perman, B., Neau, D. B., Bellamy, H. D., and Leahy, D. J. (2007) Structural basis for ligand and heparin binding to neuropilin B domains. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 6152–6157

47. Ellis, L. M., and Hicklin, D. J. (2008) VEGF-targeted therapy: mechanisms of anti-tumour activity. *Nat. Rev. Cancer* **8**, 579–591

48. Grünewald, F. S., Prota, A. E., Giese, A., and Ballmer-Hofer, K. (2010) Structure-function analysis of VEGF receptor activation and the role of coreceptors in angiogenic signaling. *Biochim. Biophys. Acta* **1804**, 567–580

49. Fong, G.-H., Rossant, J., Gertsenstein, M., and Breitman, M. L. (1995) Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* **376**, 66–70

50. Cao, Y. (2009) Positive and negative modulation of angiogenesis by VEGFR1 ligands. *Sci. Signal.* **2**, re1

51. Hiratsuka, S., Minowa, O., Kuno, J., Noda, T., and Shibuya, M. (1998) Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 9349–9354

52. Morimoto-Tomita, M., Uchimura, K., Werb, Z., Hemmerich, S., and Rosen, S. D. (2002) Cloning and characterization of two extracellular heparin-degrading endosulfatases in mice and humans. *J. Biol. Chem.* **277**, 49175–49185

53. Uchimura, K., Morimoto-Tomita, M., Bistrup, A., Li, J., Lyon, M., Gallagher, J., Werb, Z., and Rosen, S. (2006) HSulf-2, an extracellular endog-lucosamine-6-sulfatase, selectively mobilizes heparin-bound growth factors and chemokines: effects on VEGF, FGF-1, and SDF-1. *BMC Biochem.* **7**, 2