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

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*Running title:
VEGF interaction with receptors is regulated by heparan sulfate

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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.
Conclusions: 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.

ABSTRACT

Angiogenesis is a highly regulated process orchestrated by the VEGF system. Heparin/heparan sulfate (HS) 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 (SPR) and *in vitro* binding assays. Additionally, we demonstrate these binding events are relevant to VEGF activity witihin endothelial cells. We defined interactions and structural requirements for heparin/HS interactions with VEGF receptor-1 (VEGFR-1), NRP-1, and $VEGF_{165}$ in complex with VEGFR-2 and NRP-1. We demonstrate that these structural requirements are distinct for each interaction. We further show that $VEGF_{165}$, 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.

INTRODUCTION

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) and NRP-1 (10-12). $VEGF_{165}$, 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, de-acetylation 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 ECM 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 $VEGF_{165}$ binding protein (25), and was proposed as a co-receptor for the VEGFR-2-$VEGF_{165}$ 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 due to bleeding in multiple organs or cardiac failure (26-29), further reinforcing the notion that NRP-1-$VEGF_{165}$ 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 $VEGF_{165}$ binding (30,31).

HS/heparin has been proposed to regulate VEGF biological activity not only by binding $VEGF_{165}$ 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 SPR and other *in vitro* binding assays to study molecular interactions between the various components of the VEGF system. Our findings indicate that VEGFR-1 and NRP-1 bind heparin directly, while VEGFR-2 does not. Additionally, we demonstrated that heparin has no significant effect on $VEGF_{165}$ binding to VEGFR-1, despite its direct interaction with receptor and $VEGF_{165}$ (37). Alternatively, heparin enhances $VEGF_{165}$ binding to the VEGFR-2 and appears to be required for $VEGF_{165}$ binding to NRP-1. Analysis of the size and structural requirements for HS interactions with VEGFR-1 and NRP-1, as well as the requirements for the enhanced $VEGF_{165}$ binding to NRP-1 and VEGFR-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 $VEGF_{165}$ 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 $VEGF_{165}$ (#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 (#s 3870-N1 and 5994-N1) were from R&D Systems (Minneapolis, MN). ^{125}I-labeled $VEGF_{165}$ was prepared using a modified Bolton-Hunter procedure (38). ^{125}I-Bolton-Hunter reagent was obtained from PerkinElmer Life Sciences (Boston, MA). ProteOn XPR36 neutravidin (NLC) sensor chips were from BIO-RAD (Hercules, CA). Peroxidase-AffiniPure Donkey Anti-Human IgG, Fc (gamma) Fragment Specific (#709-035-098) was from Jackson Immunoresearch (West Grove, PA). TMB Microwell Peroxidase Substrate System (50-77-00) was from KPL (Gaithersburg, MD). Streptavidin-coated plates and protein A-coated plates were from Pierce (Rockford, IL). 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 (#2478), Y1175-phosphorylated VEGFR-2 (#3770), anti-phospho ERK1/2 (# 9101), ERK1/2 (# 4695), and anti-AKT (# 9272) antibodies were from Cell Signaling Technologies, (Danvers, MA). Anti-rabbit HRP-linked secondary antibody was from JacksonImmunoResearch (West Grove, PA). Biotin-heparin (B9806), bovine kidney-derived heparan sulfate (H7640), sodium chlorate (403016) and all other chemicals were from Sigma-Aldrich (St. Louis, MO).

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-heparin (1 µg/mL) was allowed to interact with the NLC chip surface for 300 s at 25 µl/min, reaching an immobilization level between 80-120 RUs per lane. Biotin (0.5

μg/mL) was immobilized to one lane, obtaining 60 RUs 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, then washed to measure dissociation. Injections of 2 M 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 biotin. Association and dissociation rate constants (k_a and k_d, respectively) were obtained by non-linear regression of data to a 1:1 Langmuir model (Equations 1-3) using OriginLab, Northampton, MA. In Equation 1, R_t represents the response (RUs) at time t, R_{max} is the maximal response reached at long time points and [A] is the ligand concentration in M. Equilibrium affinity constants (K_D) were derived from kinetic parameters ($K_D=k_d/k_a$) or determined from equilibrium analysis (Eq 3).

$$R_t=\frac{R_{max}[A]}{K_D}(1-e^{-(k_a[A]+k_d)t}) \quad (1)$$

$$R_t=R_{max}\, e^{-(k_d)t} \quad (2)$$

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

Competition Analysis

SPR competition was used to measure binding affinities of VEGFR-1 and NRP-1 with HS, chemically modified heparins and heparin-derived oligosaccharides. VEGFR-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 (RT) for 30 minutes to allow the protein to bind the oligosaccharide/modified heparin in solution. The mixtures were injected over a biotin-heparin chip surface at 30 μl/min for 240 s to record maximal response. Regeneration steps were performed as described above. Free protein concentration ($[protein]_F$) at each inhibitor (i) concentration was calculated using equation 4; briefly, the ratio of maximal binding response of protein plus inhibitor ($R_{max}i$) to protein alone (R_{max}) times the total protein concentration ($[protein]_T$). Values were plotted against inhibitor concentration and data points were fit to equation 5 to obtain apparent binding constants (K_D) for each oligosaccharide and modified heparins as previously described (39).

$$[protein]_F=\frac{R_{max}(i)}{R_{max}}\times[protein]_T \quad (4)$$

$$[protein]_F=[protein]_T-\left(\frac{[i]+[protein]_T+K_D(i)}{2}\right)+\sqrt{\left(\frac{[i]+[protein]_T+K_D(i)}{2}\right)^2-[i][protein]_T} \quad (5)$$

ELISA Binding Assays

Biotin-heparin (10 μg/mL, 100 μl/well) in PBS was adsorbed onto streptavidin-coated 96-well plates overnight at 4°C. Wells were washed with 200 μl of binding buffer to remove any unbound heparin. Receptor chimeras (100 μl/well) at the indicated concentrations in binding buffer were applied to each well and incubated for two hours at 4°C. The plate was rinsed 3 times with binding buffer, 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 0.05% Tween-20 and three times with PBS before adding 100 μl of peroxidase substrate to determine the relative amount of receptor bound at each concentration. Color development reactions were stopped after 5-10 minutes with 1 M sulfuric acid and absorbance at 450 nm (referenced to 570 nm) was measured using a spectrophotometer.

Radio-ligand Binding Assays

Receptor Fc chimeras (100 pM, 100 μl/well) in

binding buffer were adsorbed onto a Protein-A-coated 96-well plate for 1 h at RT. ^{125}I-VEGF$_{165}$ was added to wells in the presence or absence of heparin, heparin oligosaccharides, or chemically modified heparins (500 nM in 100 µl binding buffer/well) and incubated for 1 h at room temperature. The plate was washed 3 times and bound ^{125}I-VEGF was extracted using 300µl 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 (EV) PAEs were kindly provided by Dr. Nader Rahimi (40,41). Cells were maintained in 10% fetal bovine serum (FBS) in DMEM with PenStrep and L-glutamine. All cell culture-related reagents were purchased from Invitrogen/Gibco (Singapore).

Radio-ligand binding to endothelial cells

VEGFR-2 and EV PAEs were seeded in 24-well dishes (Corning Inc., Corning, NY) in 0.5mL 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}I-labeled VEGF$_{165}$ (0.26 nM) in the presence or absence of soluble NRP-1 (sNRP-1, 5 nM) in binding buffer (pre-incubated for 2 h at RT) was added to cells and allowed bind for 3 h at 4 °C. Unbound ^{125}I-VEGF$_{165}$ was removed by washing cells three times with 0.5 mL cold binding buffer. Bound ^{125}I-VEGF$_{165}$ was extracted with 0.4 mL of 1 N NaOH, and radioactivity was counted in a Cobra Auto-Gamma 5005 γ-counter (Packard Instruments, Meridian, CT).

VEGF Signaling

VEGFR-2-PAEs were seeded on 35 mm^2 dishes (Corning Inc.) in DMEM containing 10% dialyzed FBS with L-glutamine in the absence or presence of 25 mM sodium chlorate (non-chlorate: 45,000 cells/well; chlorate: 60,000 cells/well). After 2 days, media was changed to DMEM containing 0.1% dialyzed FBS +/- chlorate overnight and then treated with mixtures of VEGF$_{165}$ and sNRP-1 (pre-incubated for 2 h at RT) for 10 min. For heparin-treated cells, heparin (10 μg/mL) was added directly into the media for 1 h at 37°C before stimulation. Cells were lysed in extraction buffer (1% Triton-X 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 cocktail (Life Technologies #78440). Lysates were cleared by centrifugation and samples containing 30 µg of protein in sample buffer were heated, resolved by 8% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked for 1 h at RT 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 horse-radish peroxidase (HRP)-conjugated secondary antibodies for 1 h at RT. Membranes were developed with Clarit 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 surface plasmon resonance (SPR) with heparin-immobilized sensor chips. SPR response curves at increasing concentrations (black and grey lines) of VEGFR-1, VEGFR-2 and NRP-1 Fc chimeras are shown in Fig. 1A, B and C, respectively. Curve fittings to a 1:1 Langmuir binding model are shown as red lines and 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_d/k_a) 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 pre-mixed 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 was calculated using the maximal SPR response (R_{max}) in equation 4 (Materials and Methods) and plotted against competitor concentration (Fig. 1D and E). The calculated free protein data points were fit to Equation 5 (red line) to determine apparent affinity (K_D) values for VEGFR-1 and NRP-1 binding to heparin (Fig. 1D and E, and Tables 2 and 3). The use of this approach has been found to yield a more reliable and straightforward analysis of complex interactions (43).

HSPGs have been proposed to regulate $VEGF_{165}$ 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, while 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 receptor type present.

Structural and size requirements for HS binding to VEGFR-1 and NRP-1

Since 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 pre-incubated with the indicated oligosaccharides for 30 minutes 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 approximately 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 an oligosaccharide of 14 saccharide units, indicating that NRP-1 has a more stringent requirement for longer heparin chains compared to VEGFR-1.

We next 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 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. 2C 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, since 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 (K_D) 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. 3A and B) and heparin oligosaccharide II (Fig. 3C 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.

$VEGF_{165}$ bridges the interaction between VEGFR-2 and heparin

The presence of HSPGs is required for effective $VEGF_{165}$ binding and signaling to endothelial cell surfaces (14,33,42); however, the specific mechanisms by which HS molecules assist in $VEGF_{165}$ signaling remain unknown. Interpretation of results with intact cells has proven challenging due to 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 $VEGF_{165}$ with each major cell surface binding partner, VEGFR-1, VEGFR-2 or NRP-1 in isolation. First, we investigated if $VEGF_{165}$ could promote receptor interactions with heparin. Fig. 4 shows the response elicited by flowing the indicated receptor Fc chimera (green line), $VEGF_{165}$ (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 $VEGF_{165}$. A similar result was observed with NRP-1 (Fig. 4C). In contrast, when VEGFR-2 was allowed to interact with $VEGF_{165}$ 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 $VEGF_{165}$ promotes VEGFR-2 binding to heparin (45), and suggests that the $VEGF_{165}$–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 (grey bars) of $VEGF_{165}$. We first allowed the $VEGF_{165}$ to bind to the heparin-coated plate under static conditions for a prolonged time and then measured receptor binding. We found a significant increase in the binding of VEGFR-2 to the heparin-coated plate in the presence of $VEGF_{165}$ (Fig. 4D). Interestingly, this effect was

not seen with $VEGF_{121}$, an isoform that is unable to bind heparin and NRP-1 (data not shown).

Heparin selectively increases $VEGF_{165}$ binding to VEGFR-2 and NRP-1

To gain insight into the roles that HSPGs play in the VEGF system, specifically the role heparin plays in the regulation of $VEGF_{165}$ interactions with the each receptor type, we immobilized receptor Fc chimeras onto Protein A-coated plates and measured ^{125}I-$VEGF_{165}$ binding in the presence or absence of heparin. Heparin had no statistically significant effect on $VEGF_{165}$ binding to VEGFR-1, but increased $VEGF_{165}$ binding to VEGFR-2. Interestingly, binding of $VEGF_{165}$ to NRP-1 appeared to require the presence of heparin (Fig. 5C), as there was no significant binding of $VEGF_{165}$ to NRP-1 in the absence of heparin.

The structural requirements for heparin to bind to NRP-1 are distinct from those for binding $VEGF_{165}$ determined in previous studies (37,39). Thus, we were interested in determining the size and structural features required for heparin to enhance $VEGF_{165}$ binding to VEGFR-2 and NRP-1. It has been shown that *N*-desulfated and 6-*O*-desulfated heparin derivatives are unable to bind $VEGF_{165}$ whereas the absence of 2-*O*-sulfate groups does not appear to affect binding (37), and the minimum size of oligosaccharide chain able to bind $VEGF_{165}$ is an octasaccharide (39). To determine the structural requirements for heparin to enhance $VEGF_{165}$ binding to VEGFR-2 and NRP-1, we measured ^{125}I-$VEGF_{165}$ binding to immobilized Fc chimera receptors on a plate in the presence of a series of heparin oligosaccharides and modified heparins. Only heparin was able to enhance ^{125}I-$VEGF_{165}$ binding to VEGFR-2 (Fig. 6A and B) suggesting that relatively long chains with both *N*- and *O*-sulfation are required for this effect. On the other hand, $VEGF_{165}$ binding to NRP-1 was enhanced by oligosaccharides that are at least 14 saccharide units-long, and not shorter (Fig. 6C), which agrees with the size requirement for heparin binding to NRP-1. We also found that removal of *N*- or *O*-sulfate groups reduced, but did not eliminate, the ability to enhance $VEGF_{165}$ binding compared to 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, while 6-*O*-desulfated heparin showed an effect more comparable to 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.

VEGFR-2, $VEGF_{165}$ and NRP-1 synergize to bind heparin as a complex

To better understand how the various components of the VEGF system might act together to regulate $VEGF_{165}$ binding, we tested a series of combinations including a soluble monomeric form of NRP-1 (sNRP-1), VEGFR-2 and $VEGF_{165}$ for their ability to bind heparin using SPR. We observed intriguing synergy that suggests that these various components interact to form a stable high order molecular complex. Specifically, when $VEGF_{165}$, VEGFR-2 and sNRP-1 were allowed to interact in solution prior to being exposed to the heparin-linked sensor chip, the mixture showed a response 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, mNPR-1, unlike the dimeric form, did not show significant binding to heparin alone but did show binding when pre-incubated with $VEGF_{165}$ or VEGR-2. We suspect that under these conditions sNRP-1 dimerization is facilitated by the dimeric $VEGF_{165}$ 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 since 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, $VEGF_{165}$ interaction with heparin (Fig. 7. light blue line and bar graph) 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 $VEGF_{165}$ (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 $VEGF_{165}$ 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 porcine aortic endothelial cells (PAEs). These cells do not express endogenous VEGFR-2 or NRP-1. We used cells that have been engineered to express full length VEGFR-2 or empty vector (40,41). We found that soluble NRP-1 significantly enhanced the binding of ^{125}I-$VEGF_{165}$ to VEGFR-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 VEGFR-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 if the alterations in VEGF binding translated to changes in VEGF-mediated activity, we evaluated VEGF-stimulation of VEGFR-2 and Erk1/2 phosphorylation (Fig. 8B and C). Consistent with the effect on binding, we observed that the addition of NRP-1 enhanced VEGFR-2 phosphorylation, and to a lesser extent Erk1/2 phosphorylation. Interestingly, the ability of VEGF to stimulate VEGFR-2 and Erk1/2 phosphorylation, and for NRP-1 to influence these signaling events, was 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, VEGFR-2, NRP-1, and heparin/HS observed *in vitro* correlates with enhanced endothelial cell responsiveness to $VEGF_{165}$. Specifically, that 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

Since HSPGs and NRP were identified as VEGF co-receptors, most models of their function

have consider 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 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, data presented here demonstrate that interactions between VEGF receptors and heparin/HS have distinct structural requirements depending on the receptor in question, and that interactions between various binding partners dramatically influence binding to heparin/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 heparin/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 heparin under these conditions, and VEGF binds only with low affinity (9A). Monomeric NRP-1 can enhance VEGFR-2 binding to heparin, but to a lower extent than $VEGF_{165}$. VEGF can also enhance NRP-1 binding to heparin, but the highest binding to heparin is acheived only in the presence of all three components, $VEGF_{165}$, VEGFR-2 and NRP-1 monomer (Fig. 9A big arrow). 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 VEGFR-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 heparin/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 heparin, suggest a system where all components are required to produce a very high affinity complex (Fig. 9). Furthermore, we have demonstrated that these synergistic binding events translate into alterations in endothelial cell responses to $VEGF_{165}$.

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 (AMD), 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), while 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. While most studies have focused on identifying the role HSPGs serve in the $VEGF_{165}$-VEGFR2 signaling axis, the influence of HSPGs on VEGFR-1 has received little attention. The role of VEGFR-1 in regulation of angiogenesis remains controversial. *VEGFR-1*$^{-/-}$ mice show early embryonic lethality due to endothelial-cell overgrowth (49) and, unlike VEGFR-2, VEGFR-1 is more widely expressed in tissues apart from the endothelium, suggesting a more diversified function (50). VEGFR-1 has a low intrinsic kinase activity in response to $VEGF_{165}$, and mice that express a truncated form of VEGFR-1 lacking the entire intracellular domain develop without major vascular defects (51). The differential functions of VEGFR-1 and VEGFR-2 are crucial for vascular homeostasis; however, how these receptors coordinate to regulate receptor activation remains poorly understood. Here, we have begun to describe, in more detail, the interactions between HSPGs and 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 $VEGF_{165}$ 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 $VEGF_{165}$, 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, while 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 endoglucosamine 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 NRP-1's ability 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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FOOTNOTES

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[3]The abbreviations used are: NRP, neuropilin; HS, heparan sulfate; HSPGs, heparan sulfate proteoglycans; SPR, surface plasmon resonance

FIGURE LEGENDS

FIGURE 1. *VEGF receptor-1 and neuropilin-1 interact directly with heparin*
SPR sensograms of (A) VEGFR-1 Fc chimera (0.31 nM – 5 nM; lower to upper curves), (B) NRP-1 or (C) VEGFR-2 Fc chimeras (3.1 nM – 25 nM; lower to upper curves) 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). Experimentally obtained data is shown as black and grey lines while global fits of these data to a 1:1 Langmuir interaction model are shown in red. The kinetic parameters obtained from non-linear regression analysis for each interaction are listed in Table 1. Sensograms shown are representative of 5 individual surfaces on the chip and each experiment was performed three times. 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) were mixed with increasing concentrations of heparin (competitor) and allowed to incubate for 30 min at RT. Mixtures were flowed over a heparin-coated SPR chip and maximal binding responses were recorded. 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) 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. * indicates a *p* of 0.02 vs. no receptor. The data is representative of five independent experiments.

FIGURE 2. *Size and structural requirements for VEGFR-1 and NRP-1 interaction with HS chains*
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 RT before measuring binding of the protein to immobilized heparin on an SPR chip. Using the maximal binding response 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. 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 ± SD of 5 separate surfaces on the SPR chip and experiments were repeated two times independently. Oligo I and II are heparin oligosaccharide I (11-12 saccharides-long 3500 Da) and II (~14 saccharide units 4200 Da). A series of chemically modified heparins were also analyzed for binding to VEGFR-1 (C) or NRP-1 (D) using the competition method described above.

FIGURE 3. *Competition analysis of VEGFR-1 and NRP-1 binding to heparan sulfate and heparin oligosaccharide II*
The indicated receptor Fc chimera was incubated with increasing concentrations of HS (A and B) or heparin oligosaccharide II (C and D) for 30 min before measuring binding to heparin immobilized on an SPR sensor chip. Free protein concentration at each inhibitor concentration was calculated, plotted against inhibitor concentration (mean of 5 independent surfaces ± SD) and fit to equation 5 (red line). Apparent affinity (K_D) values obtained from non-linear regression analysis are shown in Tables 2 and 3.

FIGURE 4. *$VEGF_{165}$ facilitates the interaction between VEGFR-2 and heparin*
VEGFR-1 (A), VEGFR-2 (B) or NRP-1 (C) Fc chimeras were pre-incubated with (green line) or without (blue line) $VEGF_{165}$ at equimolar concentration (25 nM) for 30 min at RT and then injected over a surface containing immobilized heparin on an SPR chip. Red line represents $VEGF_{165}$ interaction with heparin in the absence of receptors. (D) $VEGF_{165}$ (25 nM) or buffer was allowed to interact with biotin-heparin coated on a streptavidin surface for 1 h. Unbound $VEGF_{165}$ was removed and receptor Fc chimeras (10 nM) were added and allowed to incubate for 3 h. Bound Fc chimeras was measured by ELISA. *p* values were determined by a two-tailed Student's *t* test. Asterisk indicates a *p* value of 0.01 vs. no $VEGF_{165}$. Experiment was repeated five times independently.

FIGURE 5. *Heparin selectively increases $VEGF_{165}$ binding to VEGFR-2 and NRP-1*
VEGFR-1 (A), VEGFR-2 (B) or NRP-1 (C) Fc chimeras were immobilized onto Protein A-coated plates and ^{125}I-$VEGF_{165}$ binding was quantified in the presence or absence of heparin (10 μg/mL). Panel D shows VEGF binding at 20 ng/mL +/- heparin. *p* values were determined by a Student's *t* test. Asterisk indicates a *p* value of 0.01 vs. no heparin added. Each data point represents the average of 3 ± SD. Experiment was repeated three times.

FIGURE 6. *Size and structural requirements for heparin-induced enhancement of $VEGF_{165}$ binding to VEGFR-2 and NRP-1*
^{125}I-$VEGF_{165}$ binding to immobilized VEGFR-2 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 ratio of femtomols ^{125}I-$VEGF_{165}$ bound in the presence of heparin oligosaccharides or modified heparins to VEGF alone ± SD.

FIGURE 7. *VEGFR-2, $VEGF_{165}$ and NRP-1 synergize to bind heparin as a complex*
(A) VEGFR-2 Fc chimera, sNRP-1, and $VEGF_{165}$, were pre-incubated alone or in various equimolar (25 nM) combinations for 2 h at RT before being injected over a surface containing immobilized heparin on an SPR chip. VEGFR-2 Fc chimera alone (yellow), sNRP-1 alone (purple), $VEGF_{165}$ alone (light blue), VEGFR-2 Fc chimera with sNRP-1 (red), VEGFR2-R Fc chimera with $VEGF_{165}$ (orange), sNRP-1 with $VEGF_{165}$ (green), and VEGFR-2 Fc chimera with sNRP-1 and $VEGF_{165}$ (dark blue). The purple and yellow lines overlap somewhat. (B) Maximal response (RU) for each condition is represented in the bar graph as mean ± SD of 5 separate surfaces on the SPR chip. Experiment was repeated two times independently.

FIGURE 8. *sNRP-1 enhances $VEGF_{165}$ binding and signaling in VEGFR-2 expressing PAEs and it requires sulfation of HS chains*
(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 +/- sNRP-1 was measured after incubating for 3 h at 4 °C. (B) Confluent cells were treated with $VEGF_{165}$ +/- 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. Blots show enhanced $VEGF_{165}$-induced VEGFR-2 (Y1175) and Erk1/2 phosphorylation in the presence of soluble NRP-1 and this effect is lost when cells are grown in media containing chlorate. Bar

graphs show quantification of the ratio of phospho-VEGFR-2 (pVEGFR-2) to total VEGFR-2 (VEGFR-2) and of the ratio of phospho-ERK (pERK) to total ERK (ERK) relative to untreated for each chlorate-treated and non-chlorate-treated (mean ± s.e.m., n=3 or 4). (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 $VEGF_{165}$ +/- sNRP-1.

FIGURE 9. *Synergistic binding model for the VEGFR-2/$VEGF_{165}$/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 $VEGF_{165}$, 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 $VEGF_{165}$, VEGFR-2, NRP-1 and heparan sulfate chains.

TABLES

TABLE 1. *Binding kinetics of VEGFR-1 and NRP-1 Fc chimera interaction with heparin by SPR.*
Values obtained from non-linear regression of SPR data from Fig. 1A and B (simulated data in red). k_a is the association rate constant, k_d is the dissociation rate and K_D is the affinity constant calculated from rate constants (k_d/k_a). Standard errors (SE) were calculated from 5 independent association and dissociation datasets and combined for the calculation of the K_D.

	k_a (1/Ms)	SE (1/Ms)	k_d (1/s)	SE (1/s)	K_D (nM)	SE (nM)
VEGFR-1	3.81×10^5	9.44×10^4	4.08×10^{-3}	1.87×10^{-3}	11	6
NRP-1	1.88×10^5	2.69×10^4	9.72×10^{-3}	1.56×10^{-3}	50	10

TABLE 2. *Binding affinities of VEGFR-1 Fc chimera for heparin, HS, oligosaccharides and* N-*desulfated heparin.*
Apparent affinity constants (K_D) for VEGFR-1 Fc chimera binding to heparin, HS and a series of oligosaccharides determined by non-linear regression of competition data fit to equation 5 (Fig. 3 red line). Standard error (SE), reduced chi-squared and adjusted R^2 were used to test for goodness of fit. Oligo I and II are heparin oligosaccharide I (11-12 saccharides-long, 3500 Da) and II (~14 saccharide units 4200 Da). N-DS, *N*-desulfated heparin.

	K_D (nM)	SE (nM)	Reduced chi^2	Adj R^2
Heparin	1.36	0.09	6.17×10^{-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.*
Apparent affinity constants (K_D) for NRP-1 Fc chimera binding to heparin, HS and oligosaccharide II were obtained from non-linear regression of competition data (Fig. 3 red lines). Standard error (SE), reduced chi-squared and adjusted R^2 were obtained from fit. Oligo II is heparin oligosaccharide II (~14 saccharide units).

	K_D (nM)	SE (nM)	Reduced chi^2	Adj R^2
Heparin	2.8	0.8	117	1
Heparan sulfate	170	14	0.0442	0.985
Oligo II	760	40	17.2	0.999

Figure 1

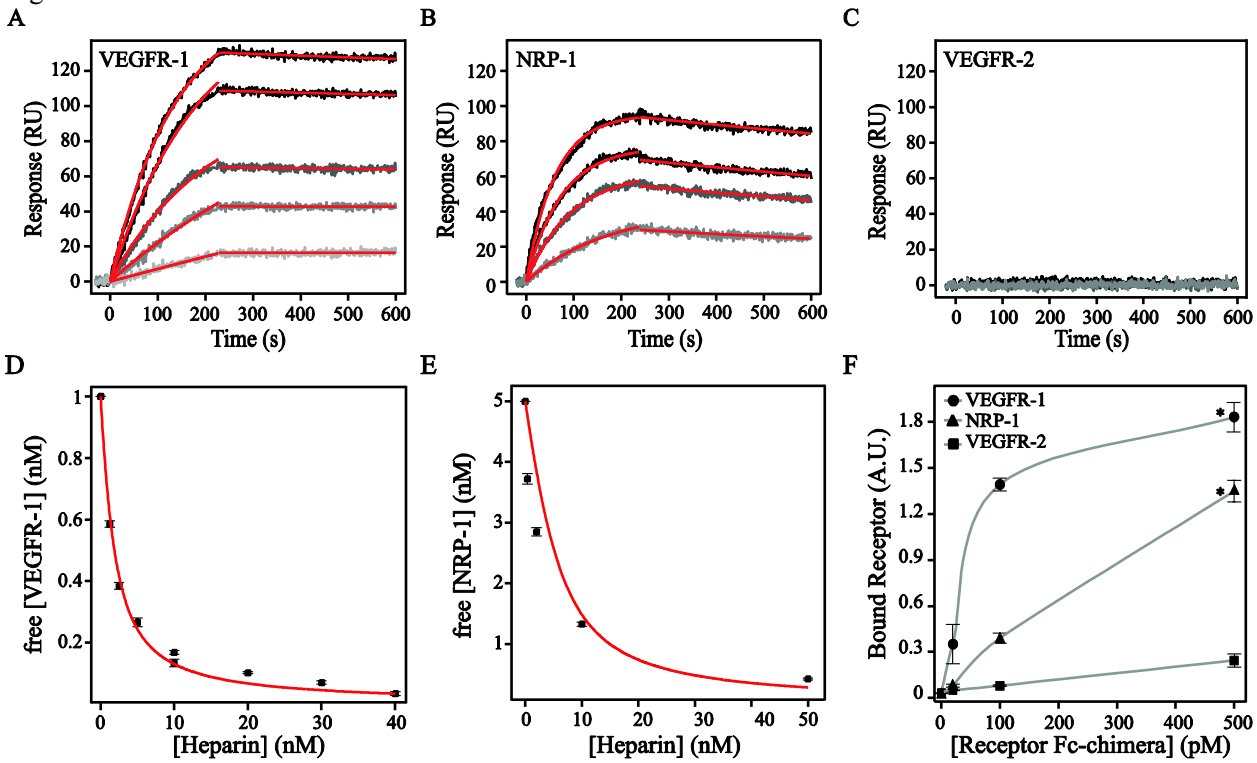


Figure 2

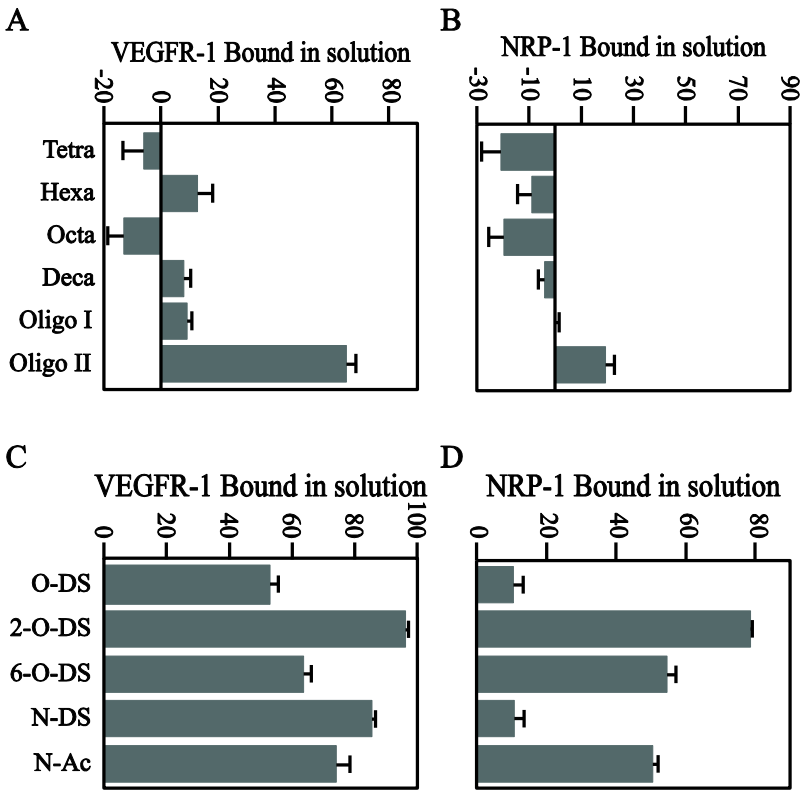
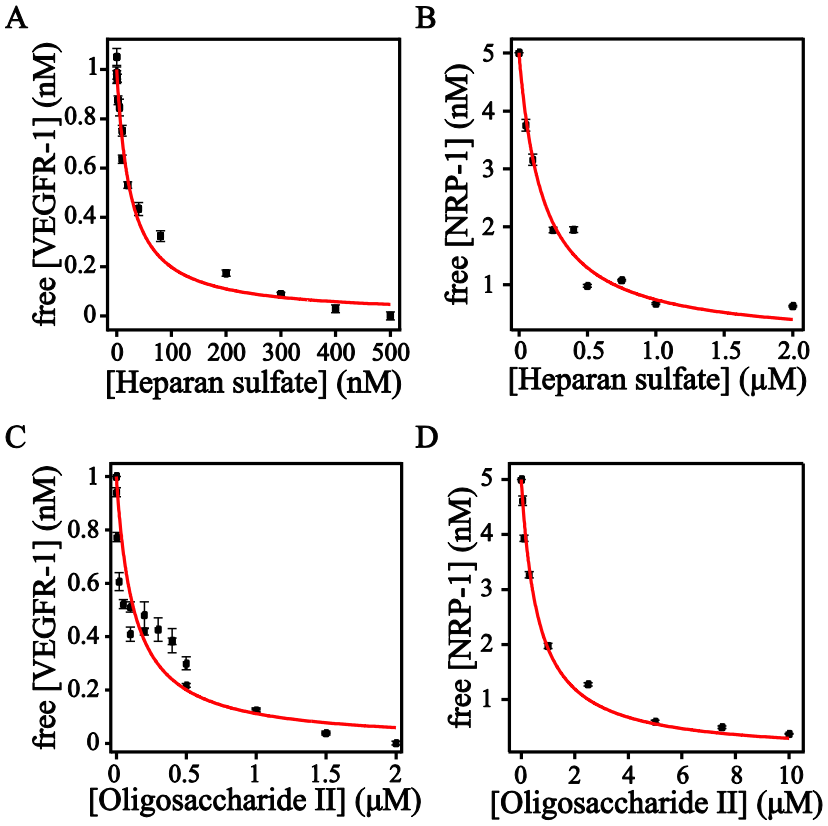


Figure 3



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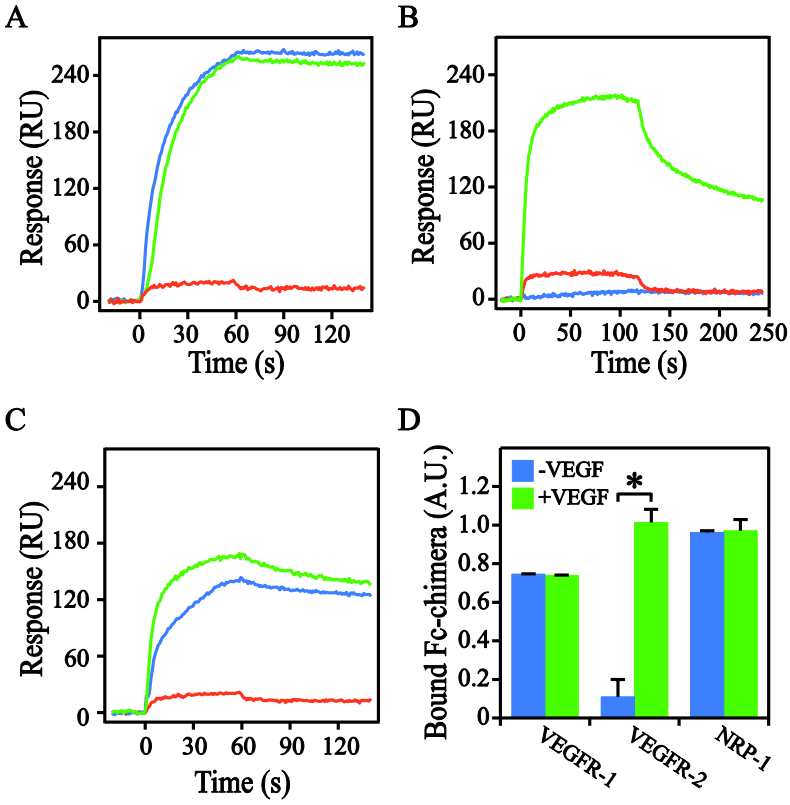


Figure 5

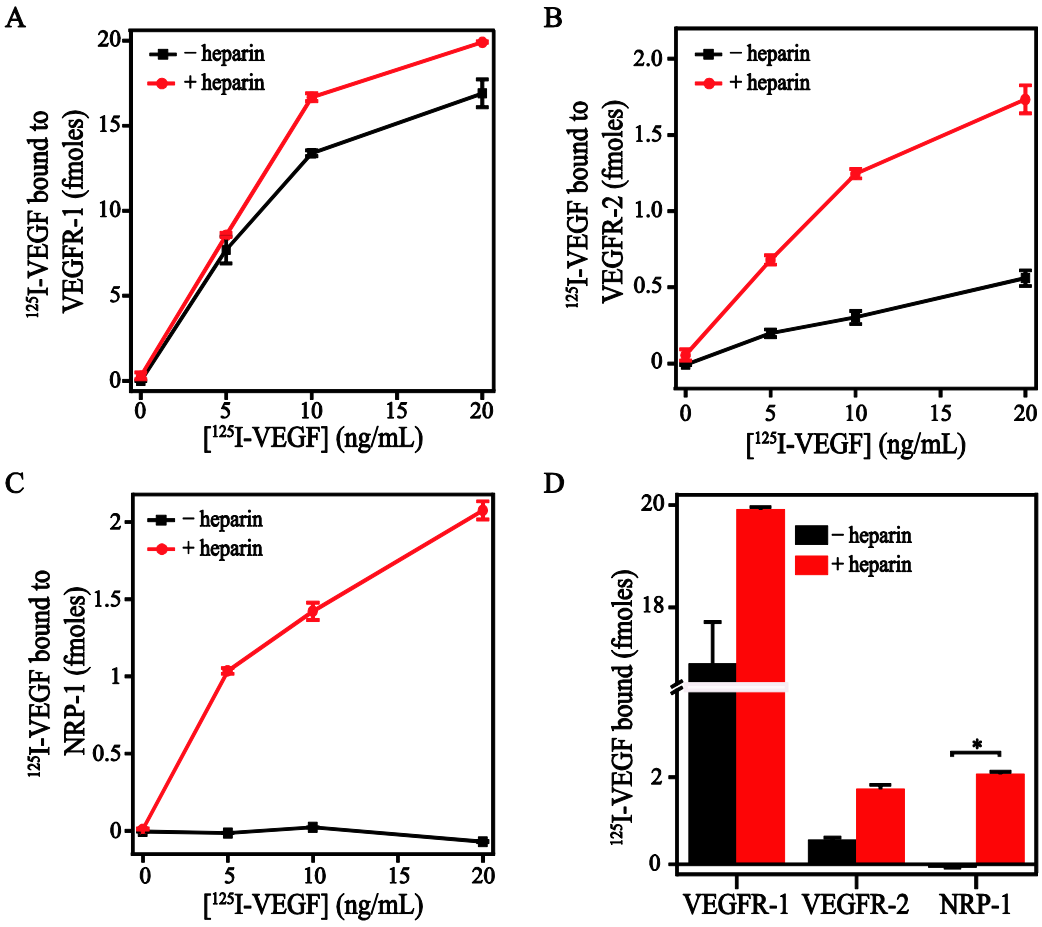


Figure 6

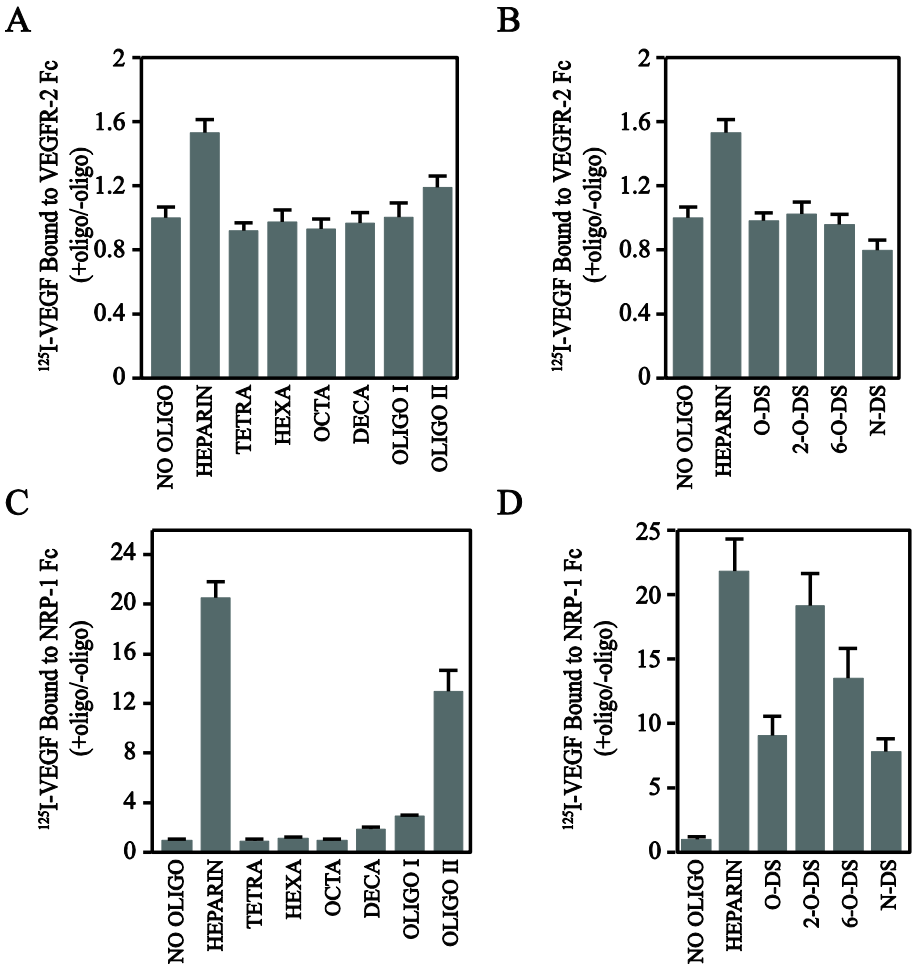


Figure 7

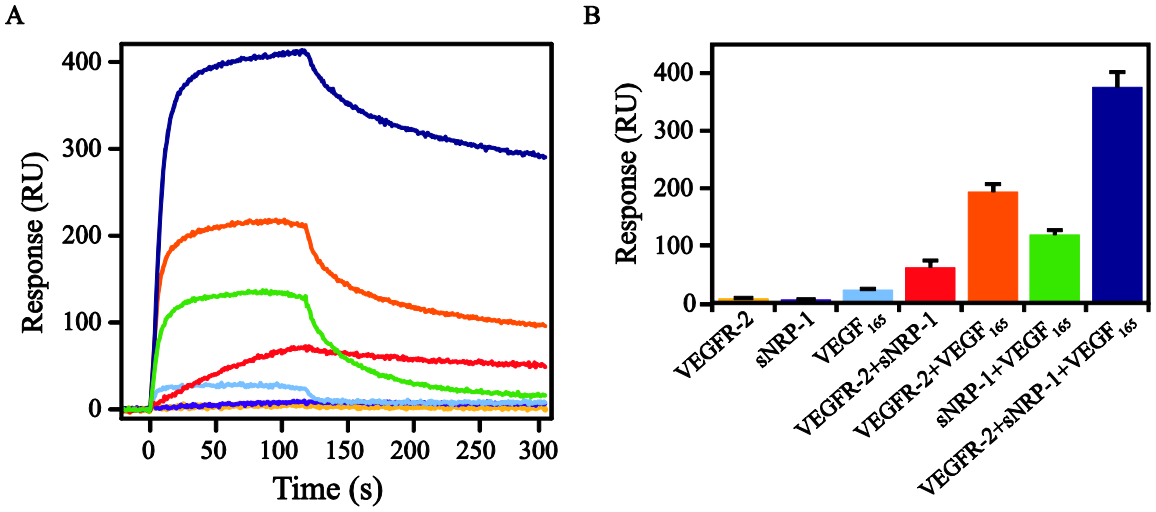


Figure 8

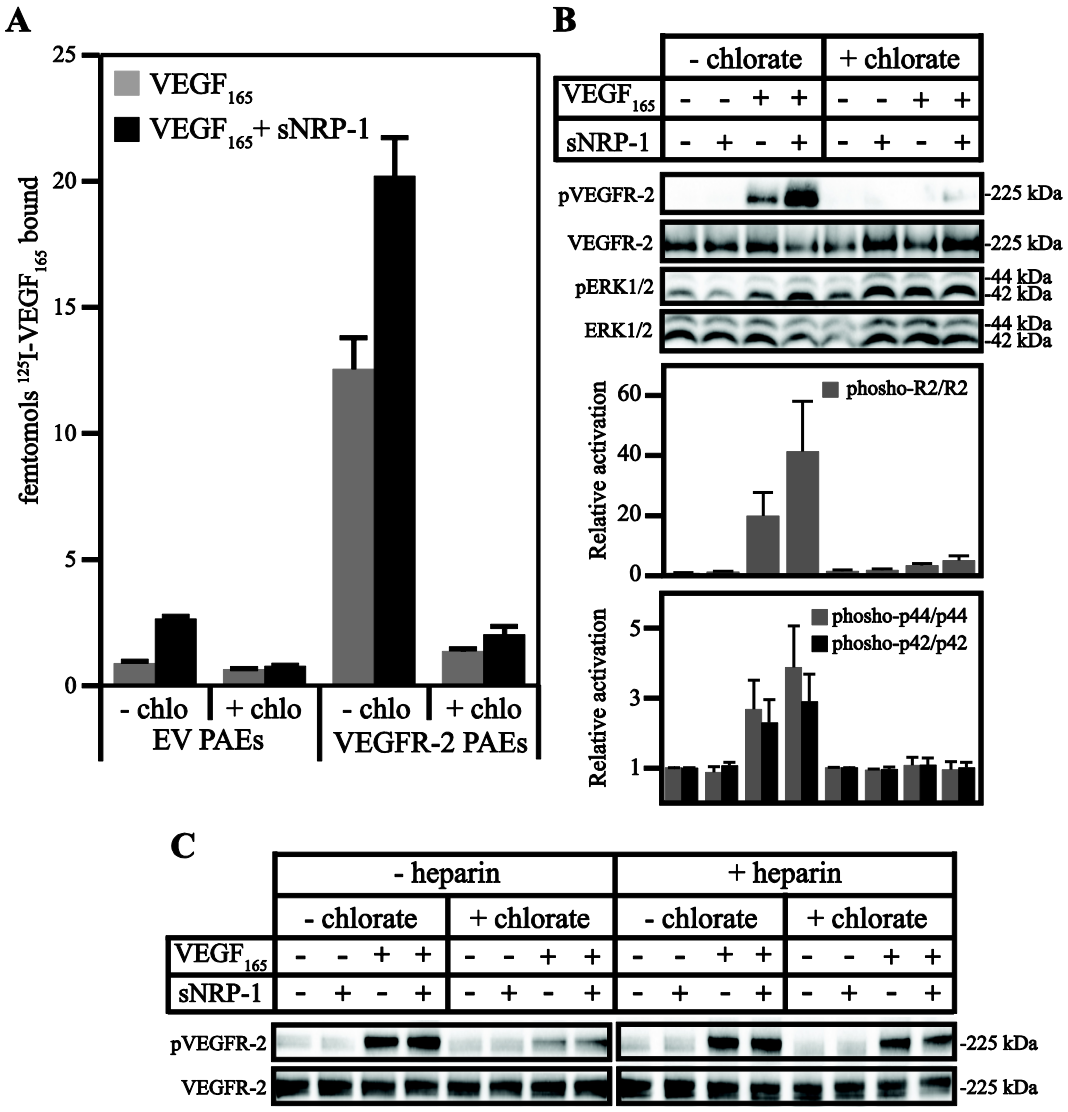
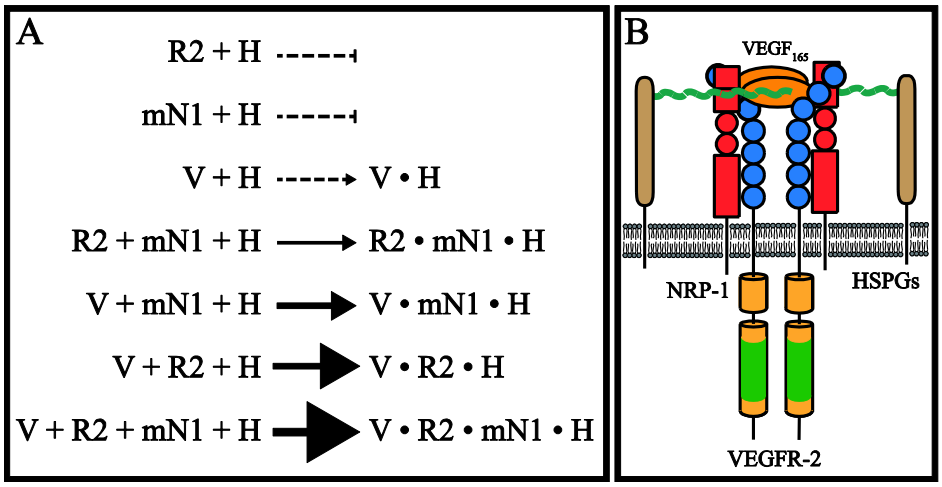


Figure 9



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

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