VEGF<sub>165</sub>-binding Sites within Heparan Sulfate Encompass Two Highly Sulfated Domains and Can Be Liberated by K5 Lyase*\[S\]

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The vascular endothelial growth factor (VEGF) family of proteins controls the formation and growth of blood vessels. The most potent and widely expressed isoform, VEGF<sub>165</sub>, is secreted as a disulfide-linked homodimer with two identical heparin-binding sites. Interactions with heparan sulfate (HS) regulate the diffusion, half-life, and affinity of VEGF<sub>165</sub> for its signaling receptors. We have determined a number of key HS structural features that mediate the specific binding of the VEGF<sub>165</sub> dimer. Carboxylate groups and 2-O-, 6-O-, and N-sulfation of HS contributed to the strength of the VEGF<sub>165</sub> interaction; however, 6-O-sulfates appeared to be particularly important. Cleavage of HS by heparinase, heparitinase, or heparanase severely reduced VEGF<sub>165</sub> binding. In contrast, K5 lyase-cleaved HS retained significant VEGF<sub>165</sub> affinity, suggesting that binding sites for the growth factor are present within extended stretches of sulfation. Binding studies and molecular modeling demonstrated that an oligosaccharide 6 or 7 residues long was sufficient to fully occupy the heparin-binding site of a VEGF<sub>165</sub> monomer. The data presented are consistent with a model whereby the two heparin-binding sites of the VEGF<sub>165</sub> dimer interact simultaneously with highly sulfated S-domain regions of the HS chain that can be linked through a stretch of transition sequence.

Vascular endothelial growth factor (VEGF)<sup>2</sup> plays a key role in most aspects of vascular development and function. In the embryo, VEGF directs the differentiation and assembly of endothelial precursor cells into a primitive vascular plexus (vasculogenesis) (1). Under the influence of VEGF, a process of sprouting growth (angiogenesis) then occurs in which the endothelial cells branch out and are remodeled into a complex network of veins, arteries, and capillaries. In adults, the vasculature is largely quiescent; however, in certain tissues, angiogenesis can be induced by VEGF, e.g. during wound healing and the female reproductive cycle (2). VEGF has also been implicated in a number of pathological conditions that are driven by inappropriate or excessive angiogenesis, such as diabetic retinopathy, rheumatoid arthritis, and the growth of solid tumors (3).

A single VEGF gene gives rise to a number of VEGF isoforms through alternate mRNA splicing (4). The VEGF isoforms differ by the presence or absence of short C-terminal heparin-binding domains (HBDs) encoded by exons 6 and 7 of the VEGF gene (4, 5). These domains mediate interactions with heparan sulfate (HS) proteoglycans (HSPGs) and with neuropilin coreceptors (6). Among the most commonly observed isoforms, VEGF<sub>121</sub>, is not a heparin-binding protein, whereas VEGF<sub>165</sub> is sequestered in the extracellular matrix in an essentially inactive folded state (7). Enzymatic processing of VEGF<sub>165</sub> generates an active form (VEGF<sub>110</sub>) that lacks the HBDs (7). This leaves VEGF<sub>165</sub> as the most widespread and abundantly expressed splice variant that interacts with HSPGs and neuropilins in a biologically active form.

HSPGs are expressed throughout the body on the surface of cells and in the extracellular matrix, where they influence a whole range of cellular activities through their widespread protein interactions (8). HSPGs consist of a protein core upon which several HS polysaccharide chains can be covalently attached. The diffusion, half-life, and bioavailability of protein ligands can all be affected by interactions with the highly sulfated HS chains. However, one of the most intriguing properties of HS is its ability to facilitate protein-protein interactions (9).

HS chains are synthesized as linear repeating polymers of β-D-glucoronic acid and α-L-iduronic acid (GlCUA-GlcNAc)n, typically 50–200 disaccharides in length (10). During chain elongation, N-deacetylase/N-sulfotransferase enzymes act on discrete regions of this heparan precursor, replacing N-acetyl groups with N-sulfates (11). Regions of N-sulfation then act as the substrate for a variety of enzymes that catalyze additional modification steps, most commonly the epimerization of GlcUA to α-L-iduronic acid (IdoUA), 2-O-sulfation (2S) of IdoUA residues, and 6-O-sulfation (6S) of GlcNAc/α-D-N-sulfoglucosamine (GlcNS) (12). However, each enzyme involved acts upon only a fraction of its available substrate sites, thereby creating a domain structure within HS and a significant degree of heterogeneity between HS chains. The current model for the domain organization of HS suggests that sulfated and unmodified regions of roughly equal length (14–18 disaccharides) alternate in a relatively uniform pattern along the HS chain (13). Within the sulfated domains are regions of contiguous IdoUA(2S)-GlcNS disaccharides (S-domains, two to eight disaccharides in length) with varying patterns of 6-O-sulfation (14). Flanking these S-domains are transition regions that consist of alternating N-sulfated and N-acetylated disaccharides in which the levels and patterns of O-sulfation and epimerization are highly variable (15, 16).

The complex structure of HS is the key to its function, as different
patterns of sulfation create binding sites for specific protein ligands. Antithrombin III is an example of a protein with very stringent HS binding requirements. The antithrombin III binding site has been identified as a pentasaccharide sequence that encompasses a relatively rare 3-O-sulfate group (17). Fibroblast growth factor (FGF) 2 binds to short sulfated sequences within HS that are high in N- and 2-O-sulfation (18–20). However, activation of FGF2 requires the presence of one or more critically positioned 6-O-sulfate groups (21). Multimeric proteins such as the chemokine interferon-γ (22) can interact with HS multivalently through several heparin-binding sites. Such interactions are often less dependent on particular primary sequence requirements than on the overall domain organization and conformational aspects of HS structure. Additional studies of HS biosynthesis and structure will help us to appreciate how HS chains from different sources selectively bind protein ligands and control their function.

Heparin and HS have profound effects on VEGF165 function; however, the interpretation of these effects is made difficult by the fact that the neuropilins and VEGF receptors (VEGFRs) are also heparin-binding proteins (23–25). VEGF165 binding to VEGFR1 is dependent on cellular HSPGs; exogenous heparin/HS cannot fully compensate for the loss of cell-surface HS, suggesting that HSPGs play a role in VEGF165 presentation (26, 27). VEGF165 can bind VEGFR2 in the absence of HS, but this interaction is enhanced by cellular or exogenous heparin/HS (26, 28). This effect may be due to the formation of VEGF165/VEGFR2 ternary complexes because VEGFR2 binding to heparin is promoted by VEGF165 (29). Heparin/HS also facilitates VEGF165 interactions with the neuropilin coreceptors, which promote activation of VEGFR2 (23, 30, 31). In addition to their effects on modulating receptor interactions, heparin/HS interactions have wider implications in VEGF function. The different affinities of the VEGF isoforms for HSPGs lead to the establishment of a concentration gradient that guides vascular growth and branching (32). HS interactions also prolong VEGF activity by sequestration away from natural degradatory pathways and by restoring lost function following damage under oxidative conditions (33).

Previous studies on the glycosaminoglycan (GAG) binding properties of VEGF165 have been biased toward the use of heparin; however, because of its abundance, HS will be the major GAG species encountered by VEGF165 in vivo. Therefore, we studied the VEGF165–HS interaction, with the aim of elucidating the structural features within HS that contribute to the specific recognition of VEGF165.

EXPERIMENTAL PROCEDURES

Materials—Recombinant VEGF165 and VEGF121 were kindly provided by the Protein Science Department of ImClone Systems Inc. (New York, NY). Human platelet extract and K5 lysate were provided by Dr. P. Brenchley and Prof. I. Roberts (University of Manchester, Manchester, UK), respectively. Fibroblast HS samples were purified from wild-type and HS2ST−/− mice embryos by Dr. C. Merry (34). Porcine mucosal HS was generously donated by Organon (Oss, The Netherlands). N-Desulfated HS, 2-O-desulfated HS, and selectively 6-O-desulfated HS were prepared from the porcine mucosal HS as described previously (35). Size-defined untreated and completely desulfated/N-recetylated heparin fragments were prepared by high resolution gel filtration of partial heparinase digests (36). Bovine lung heparin, shark cartilage chondroitin sulfate (>90% chondroitin 6-sulfate), bovine mucosal dermatan sulfate, and chondroitin ABC lyase (Proteus vulgaris; EC 4.2.2.4) were from Sigma (Poole, UK). Heparinase I (Flavobacterium hepari- num; heparin lyase, EC 4.2.2.7), heparinase II (F. heparinum; no EC number assigned), and heparinase III (F. heparinum; heparin-sulfate lyase, EC 4.2.2.8) were purchased from Grampian Enzymes (Orkney, UK). Chondroitin ACI lyase (F. heparinum; EC 4.2.2.5) was from Seikagaku Kogyo Co. (Tokyo, Japan). Lysylphosphatase Pronase (Streptomyces griseus) was from Roche Applied Science (Mannheim, Germany). Cell media and donor calf serum were from Invitrogen (Paisley, UK). [3H]Glucosamine hydrochloride (20–45 Ci/mmol) was from PerkinElmer Life Sciences (Stevenage, UK). Cellulose nitrate filters (25-mm diameter, 0.2-μm pore size) were from Sartorius (Göttingen, Germany). Blue dextran 2000, Sepharose CL-6B, DEAE-Sephalac, and Sepharose PD-10 columns were from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Bio-Gel P-10 (fine grade) was from Bio-Rad (Hempestad, UK). The ProPac PA1 HPLC column was from Dionex (Camberley, UK). All other reagents were purchased from BDH-Merck Ltd. (Lutterworth, UK).

Preparation of Metabolically Radiolabeled HS—Murine NIH 3T3 fibroblasts were maintained at 37°C (5% CO2) in Dulbecco’s modified Eagle’s medium supplemented with 10% donor calf serum, 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. GAGs were radiolabeled by incubating near-confluent cells for 24 h with medium containing 10 μCi/mL of [3H]glucosamine hydrochloride. [3H]HS was purified from these cells as described previously (37). Briefly, cells and media were pooled and treated with Pronase, and GAGs were recovered by DEAE-Sephalac anion-exchange chromatography. Hyaluronan and chondroitin sulfate were removed by chondroitin ABC lyase treatment. To remove residual core protein fragments, HS chains were incubated overnight with 1 mM NaBH4 in 50 mM NaOH at room temperature. Samples were then neutralized using acetic acid and desalted on a Sepharose PD-10 column.

Filter Binding Assay—A modified version of the technique described by Macarana and Lindahl (38) was used to study the affinity of various GAG species for VEGF165 and other proteins. [3H]-Radiolabeled GAG samples and growth factors were incubated, along with any nonradioactive competitor GAG species, in 50 mM Tris buffer (pH 7.3) containing 0.13 M NaCl or in the absence of added NaCl. Incubations were typically left for 2 h at room temperature. Following incubation, samples were made up to 300 μL with the incubation buffer and drawn through buffer-equilibrated cellulose nitrate filters held on a 12-well vacuum-assisted manifold filtration apparatus (Millipore Corp.). Proteins and protein-GAG complexes were adsorbed onto the filters. The filters were then washed with 2 × 5 ml of incubation buffer to elute unbound saccharides. The bound fraction either was eluted by washing filters with 2 M NaCl or was step-eluted using Tris buffers of progressively higher salt concentrations. Radiolabeled species were quantified by scintillation counting or desalted on a Sepharose PD-10 column for further analysis. All GAG samples were desalted on a Sepharose PD-10 column prior to use in filter binding assays.

Specific Degradation and Modification of HS Samples—Deaminative cleavage of HS by low pH nitrous acid treatment (followed by neutralization with 2 M NaHCO3) was performed as described by Shively and Conrad (39). Digestion of HS by heparinase I, II, or III was carried out in heparinase buffer (50 mM sodium acetate, 0.5 mM CaAc2, and 0.1 mg/mL bovine serum albumin (pH 7.0)). Typically, aliquots of each enzyme were added to a concentration of 100 mM/L, and the mixture was incubated for 8 h at 37°C. Subsequently, second aliquots of each enzyme were added, and the digestion was left for an additional 16 h at room temperature. HS samples were also degraded by K5 lysate (13) or by the heparinase activity of a human platelet extract as described previously (40).

HS samples were carboxyl-reduced essentially according to the method of Taylor and Conrad (41). Briefly, 500-kdpm samples of [3H]HS were incubated with 0.2 g of N-(3-dimethylaminopropyl)-N-
ethylcarbodiimide hydrochloride in 300 μl of distilled H2O (pH 4.75) for 1 h at room temperature. The activated HS samples were then reduced by the addition of 300 μl of 3 M NaBH4 for 2 h at 50 °C. Reactions were quenched by the addition of 240 μl of glacial acetic acid, neutralized using 5 M NaOH, and desalted on a Sepharose PD-10 column.

**Gel Filtration Chromatography**—Several gel filtration columns were prepared and used to resolve the products of [3H]HS degradation on the basis of chain length. Fractions were collected and analyzed for 3H content by liquid scintillation counting. The void (Ve) and total (Vt) volumes of each column were determined using blue dextran 2000 and sodium dichromate, respectively. The elution volume (Ve) of each fraction could then be used to determine Kav, according to the equation Kav = (Ve – V0)/(Vt – V0).

Intact HS chains and the HS polysaccharide products of heparinase I, K5 lyase, and platelet extract digests were resolved on a Sepharose CL-6B column (1.5 × 100 cm) or a Superdex 75 HR 10/30 fast protein liquid chromatography column. The Sepharose CL-6B column was run at a flow rate of 6 ml/h in 0.25 M (NH4)HCO3, and 1-ml (15 min) fractions were collected. The Kav of the eluted fractions was used with the calibration data of Wasteson (42) to derive estimates of chain length. The Superdex 75 HR 10/30 column was run at 0.5 ml/min in 0.2 M (NH4)HCO3, and with 0.25-ml (0.5 min) fractions were collected. This column was calibrated using untreated heparin saccharides (6–26 degrees of polymerization (dp; the number of monosaccharide units)) as well as completely desulfated N-reacetylated heparin saccharides (dp6–24).

HS saccharides produced by low pH nitrous acid or heparinase III treatment were resolved on a Bio-Gel P-10 column (1 × 120 cm) at a flow rate of 4 ml/h in 0.25 M (NH4)HCO3, and 1-ml (15 min) fractions were collected. For preparative purposes, heparinase III digests of [3H]HS were resolved using two Bio-Gel P-10 columns connected in series and run at 2 ml/h. Aliquots of each 0.5-ml (15 min) fraction were analyzed for 3H content, and saccharides were pooled according to chain length (dp6–16) and then repeatedly lyophilized (more than five times) to remove (NH4)HCO3.

**SAX-HPLC of HS Saccharides**—A ProPac PA1 SAX column (4.6 × 250 mm) was linked to a Hewlett-Packard 1100 series high performance chromatography system. The column was run at a flow rate of 1 ml/min in Milli-Q ultrapure water (Millipore Ltd., Watford, UK) at pH 3.5. Lyophilized samples were resuspended in 1 ml of this buffer and loaded onto the column prior to the application of the NaCl gradient.

[3H]HS samples (100 kdpm) were prepared for disaccharide compositional analysis by exhaustive depolymerization with a combination of heparinases I–III as described previously (37). The resultant disaccharides were resolved on the ProPac PA1 SAX column over a two-step NaCl gradient (0–0.12 M over 90 min, followed by 0.12–1 M over 45 min). Fractions were collected for scintillation counting, and disaccharides were identified by comparison with the elution positions of known standards.

Size-defined heparinase III-derived HS fragments (dp6–12) were resolved on the ProPac PA1 column for analytical and preparative purposes. Samples were desalted (Sepharose PD-10) and loaded onto the column prior to the application of the following NaCl gradients: dp6, 0.0–1.0 M over 80 min; dp8, 0.0–1.2 M over 90 min; and dp10, 0.0–1.2 M over 180 min. The 3H content of each 0.5-ml fraction was quantified by scintillation counting, and oligosaccharide peaks were assigned by comparison with the elution positions of standards sequenced by Merry et al. (14). Samples pooled from the ProPac PA1 column (dp8–10 subpecies) were desalted again (Sepharose PD-10) prior to use in VEGF165 binding assays.

**RESULTS**

**Specificity of the VEGF165 Interaction with HS**—A number of structurally related GAG species are present at the cell surface and in the extracellular matrix, all of which could potentially interact with VEGF165. To investigate whether VEGF165 binds preferentially to HS, a variety of unlabeled GAGs were tested in filter binding assays (FBAs; see “Experimental Procedures”) for their ability to compete with [3H]-labeled NIH 3T3 fibroblast HS (3T3-HS) for VEGF165 (Fig. 1A). Bovine lung heparin was the most effective competitor (IC50 = 36 ng), followed by porcine mucosal HS (PM-HS; IC50 = 1.02 μg). Chondroitin 6-sulfate and dermanan sulfate are sulfated GAGs with some structural similarity to HS, but both were less effective as competitor species (IC50 = 7.33 and 2.46 μg, respectively).

The composition of HS can vary significantly depending on its cellular source (45). Therefore, we compared the VEGF165 affinities of three different metabolically [3H]-labeled HS samples in step-elution FBAs (Fig. 1B). The three HS species all had the same maximum affinity for VEGF165 with just a small proportion (~1%) of chains retained by VEGF165 at 400 mM NaCl. The samples also contained a similar proportion of HS chains (20–40%) that remained unbound even in the presence of a large excess of VEGF165 (data not shown). Despite these similarities, there were clear differences between the samples in the proportion of HS chains that were retained by VEGF165 following each increase in buffer NaCl concentration. Interestingly, these differences did not correlate with the abundance of a particular disaccharide or with overall levels of HS sulfation (Table 1). Bovine aortic endothelial HS (BAE-HS) had a lower proportion compared with 3T3-HS of all the sulfated disaccharide types, including the trisulfated disaccharide UA(2S)-GlcNS(6S); but in 130 mM NaCl buffer, 69% of the BAE-HS chains were VEGF165-bound compared with 42% of the 3T3-HS chains. In the same assay, heparin bound VEGF165 more strongly than did HS (up to 700 mM NaCl), and our VEGF121 negative control had no affinity for HS or heparin (data not shown). Metabolically radiolabeled NIH 3T3 cell dermanan sulfate was also assayed, but was fully eluted by the 250 mM NaCl buffer step, demonstrating a weaker affinity for VEGF165 compared with HS (data not shown).

To determine whether HS chain length plays a role in VEGF165 affinity, we fractionated 3T3-HS into VEGF165-bound and -unbound species using FBAs and then resolved them, along with an unfraccionated 3T3-HS sample, on a Sepharose CL-6B gel filtration column (Fig. 1C). The unfraccionated 3T3-HS material covered a broad size range from >50 to <2.5 kDa, with the peak of counts eluting over 20–28 kDa (mass estimates derived from the calibration data of Wasteson (42)). There was a clear correlation between HS chain length and VEGF165 affinity.
for the fractionated material. Most 3T3-HS chains larger than 40 kDa were bound to VEGF$_{165}$, whereas chains smaller than 8 kDa had essentially no VEGF$_{165}$ affinity.

**Role of HS Sulfate and Carboxylate Groups in VEGF$_{165}$ Binding**—To determine the role that HS sulfate plays in the VEGF$_{165}$ interaction, we assayed the ability of unlabeled selectively desulfated PM-HS species to compete with $[^3]$H]3T3-HS for VEGF$_{165}$ binding (Fig. 2A). The disaccharide composition of these desulfated PM-HS species has been analyzed previously (35). The N-desulfated/N-reacetylated and 2-O-desulfated samples contained just 3.6% (from 43.4%) and 3.3% (from 19.9%) target sulfates/disaccharide, respectively, with negligible loss of sulfation at other positions. The preferentially 6-O-desulfated PM-HS species had been stripped of >50% of its 6-O-sulfation (9.8% 6-O-sulfates/disaccharide from 21.9%), with negligible loss of 2-O- and N-sulfates. All three selectively desulfated HS species were less efficient at competing with $[^3]$H]3T3-HS for VEGF$_{165}$ binding compared with the parent PM-HS species (IC$_{50}$ = 0.73 µg). The N-desulfated/N-reacetylated sample was the least effective competitor, with an IC$_{50}$ in excess of 100 µg. The 2-O-desulfated and preferentially 6-O-desulfated samples were more efficient competitors, with IC$_{50}$ values of 38.97 and 6.71 µg, respectively. Although the preferentially 6-O-desulfated sample retained almost half of the wild-type level of 6-O-sulfation (a loss of just 15.9% of the total sulfates), it was still a much poorer competitor compared with the parent PM-HS species. These data show that sulfate groups at all three positions within HS contribute to the strength of VEGF$_{165}$ binding, but that 2-O-sulfate groups are less important than N- and 6-O-sulfate groups.

We investigated further the relative contribution of 2-O-sulfate groups by assaying the VEGF$_{165}$ affinity of HS samples from wild-type and homozygous HS2ST$^-$ (heparan-sulfate 2-O-sulfotransferase gene knockout murine fetal fibroblasts) (46). A disaccharide analysis shows that this HS is completely deficient in 2-O-sulfates (Table 1) (34); however, a modest increase in N-sulfation and a large increase in 6-O-sulfation maintain the overall charge density. When assayed in step-elution FBAs, there appeared to be no significant difference in VEGF$_{165}$ affinity between the wild-type and HS2ST$^-$/- cell HS (Fig. 2B). This contrasts with the reduced affinity of this mutant HS type for FGF1 and FGF2 (34). Our data suggest that 2-O-sulfation is not required for the high affinity interaction of HS with VEGF$_{165}$.

To determine the role of carboxylate groups in the VEGF$_{165}$-HS interaction, step-elution FBAs were performed on 3T3-HS and BAE-HS samples that had been chemically treated to reduce carboxylates to uncharged primary alcohol groups (Fig. 2C). Both of the treated HS samples had severely reduced VEGF$_{165}$ affinity in comparison with untreated controls (75–90% lower binding in 0.13 M NaCl buffer). Charged carboxylate groups could be directly involved in VEGF$_{165}$ interactions, or they may be important in maintaining a specific conformation of HS necessary for VEGF$_{165}$ binding.

**Importance of HS Domain Structure in VEGF$_{165}$ Binding**—We digested aliquots of $[^3]$H]BAE-HS with various specific enzymes and tested their affinity for VEGF$_{165}$ (Fig. 3A). The samples were analyzed by gel filtration chromatography to confirm that enzyme digests had gone to completion (HS chains cleaved at all available substrate sites) (data not shown). Heparinase 1 cleaved predominantly within S-domains, generating BAE-HS fragments around dp16 in length. These saccharides had essentially no affinity for VEGF$_{165}$, whereas heparanase III digested most of the HS chain, liberating short S-domain fragments from BAE-HS (dp6–14), only a very small proportion of which were capable of binding VEGF$_{165}$. A platelet extract purified for its heparanase activity was also used to cleave BAE-HS (40). Despite being relatively large (7 kDa, dp26–36), the resultant fragments retained very little affinity for VEGF$_{165}$.

The K5 lyase enzyme cleaves HS only at sites devoid of sulfation (13). K5 lyase-derived fragments contain both S-domain and transition sequences and were found to be around dp16 (6–7 kDa) in size for BAE-HS. HS samples treated with K5 lyase bound VEGF$_{165}$ more strongly compared with all other HS digests investigated (Fig. 3A). Although this binding appeared to be lower than that observed for intact BAE-HS chains, it should be noted that K5 lyase-derived HS fragments are much smaller than their parent HS chains (6 kDa compared with 55 kDa, ~11%) and so emit proportionally less radioactivity per bound component.
We calibrated a Superdex 75 HR 10/30 column with both untreated and completely desulfated heparin oligosaccharides (dp6–26) so that we could accurately size the K5 lyase-resistant HS fragments. K5 lyase-treated samples of BAE-HS were fractionated into VEGF165-bound and -unbound material using FBAs and then resolved on the calibrated column (Fig. 3B). The bulk of the K5 lyase-treated material (~90%) eluted in the VEGF165-unbound fraction. On the Superdex 75 HR 10/30 column, these HS chains had both a mode and median average length of around dp22–26, but fragments ranging in size down to disaccharides could be observed. The VEGF165-bound fraction on the same column contained essentially no chains shorter than dp22; the proportion of bound chains then increased with chain length. Given the nature of the K5 lyase breakdown of HS (13), these fragments are likely to have one to two unsulfated disaccharides at each end and therefore may be sulfated only on 14–18 central residues.

Interaction of VEGF165 with Short HS S-domain Oligosaccharides—VEGF165 is secreted from cells as disulfide-linked homodimers, and so

### TABLE 1
Disaccharide composition of [3H]HS samples used in this study

Approximately 100 kdpm of each sample was exhaustively digested with heparinases I–III. The resultant disaccharides were resolved by SAX-HPLC on a ProPac PA1 column and quantified by liquid scintillation counting. Data are shown as a percentage of the total disaccharides and represent the mean of two repeats. Data for wild-type and 2-O-sulfotransferase-deficient murine fibroblast HS samples were provided by Merry et al. (34). UA, GlcUA/IdoUA.

| [3H]BAE-HS | [3H]CHO-HS | [3H]3T3-HS | Wild-type fetal fibroblast [3H]HS | HS2ST−/− fetal fibroblast [3H]HS |
|------------|------------|------------|-----------------------------------|----------------------------------|
| UA–GlcNAc  | 58.0       | 54.4       | 46.8                              | 43.6                             |
| UA–GlcNS   | 22.8       | 16.5       | 24.8                              | 25.5                             |
| UA–GlcNAc(6S) | 3.3       | 5.9        | 3.4                               | 10.3                             |
| UA–GlcNAc(6S) | 1.4       | 2.5        | 3.5                               | 3.1                              |
| UA–GlcNS(6S) | 0.7       | 2.6        | 2.5                               | 4.7                              |
| UA(2S)–GlcNS | 12.2      | 8.3        | 12.6                              | 9.6                              |
| UA(2S)–GlcNS(6S) | 1.7      | 9.9        | 6.5                               | 3.1                              |
| NS disaccharides | 37.3   | 37.3       | 46.4                              | 42.9                             |
| 2S disaccharides  | 15.3   | 20.7       | 22.5                              | 15.8                             |
| 6S disaccharides  | 5.7    | 18.4       | 12.4                              | 19.1                             |

![FIGURE 2. Role of HS sulfate and carboxylate groups in VEGF165 binding.](image)

A, competitive inhibition of [3H]HS binding to VEGF165 by various unlabeled selectively desulfated HS samples. FBAs were carried out as described in the legend to Fig. 1A. The competitor species used were untreated PM-HS (○), N-desulfated/N-reacetylated PM-HS (□), 2-O-desulfated PM-HS (▲), and preferentially 6-O-desulfated PM-HS (◆). B, interaction of VEGF165 with HS from HS2ST knockout cells. Samples (10 kdpm) of wild-type (gray bars) and HS2ST−/− murine fetal fibroblast [3H]HS (white bars) were incubated with 5 μg of VEGF165 in 100 μl of buffer and then split into unbound (▵) and bound (●) fractions using FBAs. These fractions were resolved on a Superdex 75 HR 10/30 column. The arrow indicates the elution position of a dp22 saccharide based on calibration of the column with both untreated and fully desulfated heparin oligosaccharide standards.

![FIGURE 3. Relative affinity of various enzyme-cleaved HS samples for VEGF165.](image)

A, comparison of the VEGF165 affinities of HS samples following treatment with various HS-degrading enzymes using step-elution FBAs as described in the legend to Fig. 1B. Data represent the mean of three repeats, with S.D. shown as error bars. The BAE-HS samples were heparinase I-digested (black bars), heparinase III-digested (white bars), platelet extract-digested (hatched bars), and K5 lyase-digested (gray bars). B, gel filtration of K5 lyase-resistant HS fragments following fractionation by VEGF165. K5 lyase-digested [3H]BAE-HS (50 kdpm) was incubated with 5 μg of VEGF165 in 100 μl of buffer and then split into unbound (▵) and bound (●) fractions using FBAs. These fractions were resolved on a Superdex 75 HR 10/30 column. The arrow indicates the elution position of a dp22 saccharide based on calibration of the column with both untreated and fully desulfated heparin oligosaccharide standards.
each dimer contains two identical HBDs (47). To elucidate the minimum HS oligosaccharide length capable of binding to a single VEGF<sub>165</sub> HBD, we purified S-domain-derived oligosaccharides according to length (dp6–16) from heparinase III digests of [3H]3T3-HS. Incubations were set up in NaCl-free buffer (50 mM Tris-HCl (pH 7.3)), and VEGF<sub>165</sub>-bound species were recovered using buffers of increasing NaCl concentration in step-elution FBAs (Fig. 4). In this assay, dp6 saccharides showed essentially no affinity for VEGF<sub>165</sub>, even in the absence of NaCl. In contrast, dp8 saccharides clearly had some affinity for VEGF<sub>165</sub> in salt-free buffer (40% bound); however, complete elution of dp8 saccharides could be achieved in the presence of 100 mM NaCl. Various heparin-derived octasaccharides have previously been reported to interact weakly with VEGF<sub>165</sub> (48). We found a small fraction (~5%) of dp10 saccharides to be the shortest S-domain fragments capable of interacting with VEGF<sub>165</sub> in buffers at physiological NaCl (Fig. 4B). For oligosaccharides larger than dp10, the levels of VEGF<sub>165</sub> binding increased with chain length, with the dp14 and dp16 saccharides still showing appreciable affinity for VEGF<sub>165</sub> in 200 mM NaCl buffer (11 and 25% binding, respectively). HS fragments derived from heparinase III treatment rarely carry sulfate groups on the two terminal monosaccharide units and are only occasionally 2-O-sulfated on the uronate residue adjacent to the reducing end (14). Therefore, VEGF<sub>165</sub> interactions with dp10 saccharides, for example, may involve just 7 or 8 internal sulfated residues.

Two control assays were set up for the oligosaccharide binding assay: one in which VEGF<sub>165</sub> was incubated with short sulfated iduronate-containing oligosaccharides (dp6–10) derived from chondroitin AC II lyase digestion of dermatan sulfate and the other in which the VEGF<sub>121</sub> isoform was incubated with the HS S-domain saccharides (data not shown). In both cases, no interactions were observed even in the absence of NaCl. These data show that VEGF<sub>165</sub> binds specifically to sulfated regions within HS and that this interaction is mediated by the exon 7-derived HBD.

**VEGF<sub>165</sub> Interactions with HS Oligosaccharides of Defined Sequence—**

Our laboratory has previously sequenced a number of heparinase III-resistant HS oligosaccharides (Ref. 14). The dp8g saccharide has not been sequenced; however, disaccharide analyses confirmed that this saccharide was 6-O-sulfated (data not shown). Because a proportion (~5%) of our dp10 sample bound VEGF<sub>165</sub> at physiological ionic strength (Fig. 4), we fractionated the dp10 sample into VEGF<sub>165</sub>-bound and -unbound saccharides when incubated in NaCl-free Tris buffer; 8, 10 kdpm of dp10 (fractionated into VEGF<sub>165</sub>-bound and -unbound saccharides when incubated in Tris buffer containing 130 mM NaCl). Our data revealed an absolute dependence on 6-O-sulfation for VEGF<sub>165</sub> binding (Fig. 5A). The octasaccharides with no 6-O-sulfation (dp8a–c) failed to interact, whereas those containing one or more 6-O-sulfate groups (dp8d–g) were all capable of binding (see Ref. 14 for sequencing data). The dp8g saccharide has not been sequenced; however, disaccharide analyses confirmed that this saccharide was 6-O-sulfated (data not shown). Because a proportion (~5%) of our dp10 sample bound VEGF<sub>165</sub> at physiological ionic strength (Fig. 4), we fractionated the dp10 sample into VEGF<sub>165</sub>-bound and -unbound saccharides in 50 mM Tris-HCl (pH 7.3) containing 130 mM NaCl. Only the dp10a (no 6-O-sulfation) and dp10d (one 6-O-sulfate group) saccharides had been sequenced previously (14). (Note that dp10d is referred to as dp10c in Ref. 14.) Most of the dp10 sample (>90%) was recovered in the unbound fraction, including the dp10a and dp10d saccharides (Fig. 5B). The major species in the VEGF<sub>165</sub>-bound fraction of the dp10 sample were dp10i and dp10j, which we predicted from
and desalted on a Sepharose PD-10 column. 1.25 kdpm of each 3H-labeled octasaccharide was applied to a ProPac PA1 SAX-HPLC column. The prominent peaks identified in Fig. 5 were pooled and concentrated. We purified these oligosaccharides separately to further characterize their binding to VEGF165. The dp8a-g octasaccharides were purified, and their elution profiles to contain two and three 6-sulfate groups were determined. All data represent the mean of at least three repeats. The octa- and decasaccharides used were as follows: a (A), b (B), c (C), d (D), e (E), f (F), and g (G).

FIGURE 6. Step elution of heparinase III-derived HS dp8 and dp10 subspecies from VEGF165. Heparinase III-resistant [3H]3T3-HS octa- and decasaccharides were resolved on a ProPac PA1 SAX-HPLC column. The prominent peaks identified in Fig. 5 were pooled. The effect of NaCl on the elution of these HS chains was determined by step-elution FBAs. The proportion of HS that remained associated with VEGF165 following each buffer wash is shown as a percentage of the total counts added. All data represent the mean of at least three repeats. The octa- and decasaccharides used were as follows: a (A), b (B), c (C), d (D), e (E), f (F), and g (G).

Because of its elution position on the SAX-HPLC column, we predicted that dp8g is the most highly sulfated S-domain octasaccharide and contains two 6-sulfate groups. Because SAX-HPLC resolved the heparinase III-resistant derived S-domain dp8 and dp10 samples into their major constituent species, we purified these oligosaccharides separately to further characterize their binding to VEGF165. The dp8a-g octasaccharides were purified, and their relative affinities for VEGF165 were determined by step-elution FBAs. The proportion of HS that remained associated with VEGF165 following each buffer wash is shown as a percentage of the total counts added. All data represent the mean of at least three repeats. The octa- and decasaccharides used were as follows: a (A), b (B), c (C), d (D), e (E), f (F), and g (G).

We found that the octasaccharides with no 6-O-sulfation (dp8a–c) had essentially no affinity for VEGF165. The dp8d–f species all contained one 6-O-sulfate group and had a moderate affinity for VEGF165. The most strongly bound octasaccharide, dp8g, remained associated with VEGF165 until the 100 mM NaCl buffer was applied. Because of its elution position on the SAX-HPLC column, we predicted that dp8g is the most highly sulfated S-domain octasaccharide and contains two 6-O-sulfate groups. In the same assay, the three major dp10 species (dp10a, dp10d, and dp10g) were tested for VEGF165 affinity (Fig. 6B). Of the sequenced decasaccharides (14), dp10a (no 6-O-sulfation) had low VEGF165 affinity, whereas dp10d (one 6-O-sulfate group) had moderate affinity. The dp10g saccharide has yet to be sequenced, but is predicted to contain two 6-O-sulfate groups and had the strongest VEGF165 affinity of the purified decasaccharides we tested. None of the three most common dp10 species remained associated with VEGF165 at physiological ionic strength (130 mM NaCl).

Modeling the VEGF165 Heparin-binding Domain in Complex with Heparin/HS—The solution structure of the HBD (residues 111–165) of VEGF165 has been published (Protein Data Bank code 2VGH) (43). We took this structure and modeled its interaction with short heparin oligosaccharides (dp5, dp7, and dp11) to try to determine the best oligosaccharide size fit for the predicted heparin-binding groove. The modeled complexes consistently predicted that the oligosaccharide lies in a shallow groove orthogonal to the main axis of the protein structure (Fig. 7). This groove is lined with basic residues: Arg124, His126, and Lys140 on the N-terminal side and Arg145, Arg149, Arg156, and Arg159 on the C-terminal side. These residues were observed to make numerous contacts with sulfate and carboxylate groups of the oligosaccharide probes. The dimensions of the groove were such that the pentasaccharide probe did not completely fill the heparin-binding cleft, whereas several of the terminal residues of the heparin endocamer did not interact with 2VGH. For the heptasaccharide ligand, close contacts between oligosaccharide and protein were predicted for six of the monosaccharide units (residues 1–6 or 2–7). Of the 10 lowest energy complexes (−2584 to −2542 kcal/mol), seven had the oligosaccharide oriented with its reducing end residues interacting with Arg145 and Arg149 and its nonreducing end residues toward Arg124. In the other three complexes, the oligosaccharide adopted the opposite orientation, with Arg124 engaging the reducing end. Arg159 interacted with acidic groups on residues in the center of the ligand oligosaccharide. A hydrophobic residue in the base of the groove, Leu147, made close contact with the sugar rings of these central residues and may be a significant determinant for the position of the oligosaccharide in the predicted complex.

**DISCUSSION**

HS interactions regulate VEGF165 bioactivity through a number of distinct mechanisms, including extending the half-life, controlling diffusion, and differentially modulating interactions with the receptor tyrosine kinases (6). We set out to define the structure and sequence elements within HS chains that are responsible for binding VEGF165. This information might allow us to exploit the interaction with HS to inhibit or potentiate VEGF165 bioactivity.

Because a variety of structurally related GAG species are present at the cell surface, we initially investigated the specificity of the VEGF165 interaction with HS. Using a competitive binding assay (Fig. 1A), we determined that VEGF165 interacted preferentially with the GlcNS-containing GAGs heparin and HS rather than with chondroitin 6-sulfate and dermatan sulfate. Heparin chains tend to be more extensively sulfated compared with HS (12), and the strong interaction between VEGF165 and heparin may reflect a preference of this growth factor for highly sulfated GAG sequences. HSPGs are abundant in the matrix and on the surface of most cells, whereas heparin is produced only by mast cells. Therefore, interactions with HS are more likely to influence VEGF165 bioactivity in vivo. The composition of HS has been shown to vary significantly depending on its cellular source (45). We compared three different HS species and found that all had a similar maximum affinity for VEGF165 (Fig. 1B). In addition, all three HS samples contained a proportion of chains (20–40%) that consistently showed no affinity for VEGF165. It appears that, although the HS biosynthetic machinery in each cell type generated very high affinity binding sites for VEGF165 in a small number of HS chains (<5%), many HS chains lacked some key element for VEGF165 recognition. Interestingly, the proportion of chains that bound VEGF165 at physiological NaCl varied widely between the HS types (42–69%) (Fig. 1B). HS chains might contain a number of different VEGF165-binding sequences with varying levels of affinity. We found no correlation between VEGF165 affinity and either the abundance of a particular disaccharide or the overall level of HS sulfation (Table 1). VEGF165 affinity might be determined more by how HS is organized in terms of its domain structure: how long the S-domains and transition sequences are and how they are dispersed along the HS chain.

Because the covalent VEGF165 homodimer contains two identical HBDs, it is possible that both can be occupied by the same HS chain. This mode of HS interaction appears to be common for multimeric interactions.
proteins (interferon-γ (22), platelet factor-4 (49), interleukin-8 (50), and macrophage inflammatory factor-1α (40)). Long HS sequences consisting of S-domain regions linked by stretches of relatively unmodified N-acetyl-rich sequence (termed SAS domains (51)) have been reported as the binding species for protein ligands that contain multiple heparin-binding sites. Cleavage of HS with either heparinase I or III resulted in an almost complete loss of VEGF165 affinity in buffer at physiological NaCl (Fig. 3A). This indicates that the VEGF165-binding site within HS is also complex, involving both S-domain and non-S-domain sequences. The longest stretches of S-domain sequence observed within our HS samples were typically just dp14–16. However, Soker et al. (52) reported that heparin fragments dp22–24 in length were required to potentiate VEGF165 receptor binding. Using the novel HS-cleaving enzyme K5 lyase, we were able to isolate VEGF165-binding fragments from HS (Fig. 3A), a proportion of which bound to VEGF165 almost as strongly as the uncleaved parent HS chains (Fig. 3A). This indicates that the VEGF165-binding site within HS is also complex, involving both S-domain and non-S-domain sequences. The longest stretches of S-domain sequence observed within our HS samples were typically just dp14–16. However, Soker et al. (52) reported that heparin fragments dp22–24 in length were required to potentiate VEGF165 receptor binding. Using the novel HS-cleaving enzyme K5 lyase, we were able to isolate VEGF165-binding fragments from HS (Fig. 3A), a proportion of which bound to VEGF165 almost as strongly as the uncleaved parent HS chains (Fig. 1B). VEGF165 binding ability was observed only for K5 lyase-generated HS fragments dp22 or larger in size (Fig. 3B), identical to the heparin size requirements reported to enhance VEGF165 binding to VEGFRs (52) and neuropilin-1 (23). Our data (Fig. 3A) demonstrate that high affinity VEGF165-binding sites can be accommodated within K5 lyase-resistant BAE-HS fragments, but not within the equivalently sized but structurally distinct fragments produced by heparinase and heparanase. K5 lyase is specific for stretches of unsulfated sequence of HS and uniquely liberates S-domains along with their flanking transition sequences (13). K5 lyase-derived HS fragments will encompass structures such as TST and TSTST (where S is S-domain (contiguous N-sulfated disaccharides) and T is transition region (alternating N-sulfated disaccharides)). Therefore, S-domains embedded within stretches of transition sequence (STS sequences) could interact with the two HBDs of the VEGF165 dimer (Fig. 8).

Our data show that S-domain stretches alone are unlikely to mediate high affinity binding of VEGF165. However, it would be these short highly sulfated domains within STS sequences that would presumably show the highest affinity for the HBDs of VEGF165 (Fig. 4). HS fragments derived from heparinase III treatment rarely carry sulfate groups on the two terminal monosaccharide units and are only occasionally 2-O-sulfated on the uronate residue adjacent to the reducing end (14). Therefore, although the dp10 samples were the shortest oligosaccharides capable of binding VEGF165 in physiological saline buffer (Fig. 4), the actual interaction may have involved just 7 or 8 internal sulfated residues. We also modeled the binding of a range of sized heparin saccharides onto the protein structure of VEGF165 residues 111–165 (Protein Data Bank code 2VGH) (43). This approach revealed that a heptasaccharide would be sufficient to fully occupy the heparin-binding cleft of VEGF165 (Fig. 7). Heptasaccharide docking calculations predicted that seven of the 10 most favorable complexes with 2VGH had the saccharide oriented with its reducing end toward Arg145 and Arg149, with three in the reverse orientation. This sug-

FIGURE 7. Model of a heparin/HS heptasaccharide docked with the VEGF165 heparin-binding domain. A, ribbon diagram of the VEGF165 HBD (residues 111–165; Protein Data Bank code 2VGH) with a docked heparin-derived dp7 saccharide (space-filling representation: carbon (gray), oxygen (red), sulfur (yellow), nitrogen (blue), and hydrogen (white)). Basic residues lining the shallow binding groove are shown in a stick representation (green). One residue, Arg145, is hidden behind the heparin heptasaccharide. The hydrophobic residue Leu127 (orange) is also obscured. The figure depicts the most favorable intermolecular interaction energy (−2584 kcal/mol) calculated by AutoDock and shows the saccharide oriented with its reducing end to the left. A rotating version of this figure is supplied as supplemental material. B, the same complex with 2VGH depicted as a protein surface. Arg and Lys residues are shown in blue, and Glu and Asp residues are shown in red. The heparin dp7 saccharide is shown in a stick representation. The atomic coloration is the same as described for A, except that carbons are shown in green.
the dp8σ-δ and dp10σ oligosaccharides (Fig. 6). These saccharides contain a common pentasaccharide sequence with a central 6-O-sulfated glucosamine residue (14). Therefore, sequences of the type GlcNS-IdoUA(2S)-GlcNS(6S)-IdoUA(2S)-GlcNS could potentially mediate the interaction of HS at each HBD of the VEGF165 dimer. Such sequences are sufficiently abundant within HS that two or more could be present within the same chain. S-domains consist of stretches of contiguous GlcNS-IdoUA(2S) disaccharides with varying levels of 6-O-sulfation (14). We predict that the number and position of 6-O-sulfate groups within S-domains will be a critical factor in determining HS affinity for VEGF165. Additionally, the spacing of S-domains along the HS chain will be crucial to allow the simultaneous interaction with both subunits of the covalent VEGF165 dimer (Fig. 8). How far apart these two S-domains need to be positioned remains to be determined. However, our observation that K5 lyase-derived HS fragments as short as dp22 interact with VEGF165 (Fig. 3B) confirmed that such sequences could be situated very close to one another. K5 lyase can be used to isolate intact VEGF165-binding sites from HS chains and may be a useful tool in future studies to refine our model for the VEGF165-HS interaction.

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FIGURE 8. Model of the VEGF165 interaction with its binding site in HS. Shown is a schematic representation of a K5 lyase-resistant HS fragment (white and hatched boxes) bound to the VEGF165 homodimer (gray). The N and C termini of VEGF165 and the reducing (R) and nonreducing (NR) ends of the HS chain are indicated. The two VEGF165 subunits are held by disulfide linkages in an antiparallel “side-by-side” orientation. The C-terminal HBD (Fig. 7) of each VEGF165 monomer can be released by plasmin cleavage at the sites indicated (arrows). Basic residues within each heparin-binding cleft are shown (+). The two clefts are occupied by separate S-domain stretches (white boxes) within the same HS chain. Because the VEGF165 monomers are antiparallel, they are depicted interacting with the S-domains in different orientations with respect to the direction of the HS chain; molecular modeling indicates that this may occur. The S-domains are at least dp6 in length and are 6-O-sulfated. They are connected through a stretch of less highly sulfated sequence (hatched box) that is susceptible to cleavage by heparinase III, but not by K5 lyase.

gests that VEGF165 may have a certain degree of flexibility in how it binds to HS, with the heparin-binding grooves able to accommodate S-domain regions in both orientations (Fig. 8). This property of heparin-binding sites has been described previously for FGF1 (53). The importance of the VEGF165 residues most strongly implicated in amin-binding sites has been described previously for FGF1 (53). The S-domain regions in both orientations (Fig. 8). This property of hep-

protein-saccharide interactions. Nevertheless, potentially important VEGF165 interactions with 2-O-, 6-O-, and N-sulfates as well as with carboxylates groups were observed. Data from competitive binding assays (Fig. 2A) confirmed that sulfate groups at all three positions within HS contribute to the strength of VEGF165. However, N- and 6-O-sulfation in particular appeared to be more important. Ono et al. (54) found that N- and 6-O-sulfation of heparin, but not 2-O-sulfation, are necessary for VEGF165 binding. Using mutant HS from HS2ST knockout cells (34), we also found that high affinity interactions with VEGF165 did not require 2-O-sulfation (Fig. 2B). However, competition assays (Fig. 2A) showed that 2-O-sulfation of wild-type HS chains did contribute to the strength of the VEGF165 interaction. A number of S-domain octa- and decasaccharides had been purified from HS and sequenced by our group previously (14). The affinity of these saccha-

rides for VEGF165 was directly correlated with their 6-O-sulfate content (Fig. 6), demonstrating the importance of S-domain 6-O-sulfation to the VEGF165 interaction. In buffers at physiological NaCl, dp10σ and dp10σ (Fig. 5B) were the shortest HS-derived saccharides capable of binding VEGF165. These two saccharides have yet to be sequenced; however, their elution positions on the SAX-HPLC column (Fig. 5B) suggest that they are very highly sulfated. These structures were not prevalent enough within intact 3T3-HS chains to account for the observed levels of VEGF165 binding.

Our data suggest that VEGF165 binding through a single HBD to HS is unlikely to result in a stable VEGF165-HS complex. However, the bidentate interaction of VEGF165 with two S-domains on the same HS chain could be very stable. A moderate affinity for VEGF165 was observed for
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