Identification of Common and Specific Growth Factor Binding Sites in Heparan Sulfate Proteoglycans†

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ABSTRACT: The structural complexity within heparan sulfate has suggested that it contains multiple protein-specific binding sites. To evaluate the selectivity of growth factor binding to heparan sulfate, we conducted a detailed study of the intercompetition of fibroblast growth factor-2 (FGF-2) and heparin-binding epidermal growth factor-like growth factor (HB-EGF) binding to heparan sulfate (HS) on bovine aortic smooth muscle cells. Radioligand binding assays were conducted, and an analytical method was developed for determining the apparent binding constants and numbers of specific and shared binding sites within HS. These studies revealed the presence of two general classes of HS-binding sites for FGF-2 and HB-EGF. The major class ($\sim 10^6$ sites per cell) was able to bind to either growth factor with relatively low affinity ($K_d = 12$ and 44 nM for FGF-2 and HB-EGF, respectively) and was termed "common" binding sites. However, both FGF-2 and HB-EGF also showed specific high affinity (0.6 and 6.1 nM for FGF-2 and HB-EGF, respectively) binding to a minor subset (118 000 and 28 000 sites per cell for FGF-2 and HB-EGF, respectively) of "unique" binding sites, which were unable to bind the other growth factor. These studies indicate that growth factor binding to HS involves multiple binding sites of variable affinity, density, and selectivity. The approach outlined in this study could be applied to aid in the evaluation of the relative biological roles of these selective and nonselective growth factor binding domains within HS.

Heparan sulfate (HS) represents a class of complex macromolecules that are members of the glycosaminoglycan family of linear polysaccharides (*1*−*3*). Significant evidence has accumulated in recent years indicating that HS plays critical roles in modulating cell function within the subkingdom of metazoan (*1*, *4*). For example, numerous genetic and biochemical studies have demonstrated key functions for HS in the development, maintenance, and repair of differentiated tissues in nematodes, fruit flies, frogs, mice, and humans (*5*−*9*). In most of these situations, it is believed that the function of HS is mediated by the ability to bind and modulate extracellular regulatory proteins such as growth factors. Indeed, well over 200 growth factors, cytokines, hormones, and extracellular matrix proteins have been shown to bind to HS with high affinity, and this interaction has been demonstrated to have functional consequences for a number of these proteins (*10*, *11*).

The ability to bind to such a vast array of proteins is likely attributed to the complex nature of the HS structure. HS chains are linear polymers of repeating disaccharide units of uronic (either iduronic or glucuronic) acid and *N*-acetylated, sulfated, or unsubstituted glucosamine, which can be O-sulfated on the 2 position of the uronic acid and on the 6 and 3 positions of the glucosamine. When the variable modifications of HS are taken together, they predict as many as 48 distinct disaccharides, which, when aligned in specific sequences, can provide a highly information dense polymer (*12*−*14*). HS chains are physically positioned on the cell surface, within the extracellular matrix, or in soluble form, through covalent linkage to core proteins as heparan sulfate proteoglycans (HSPGs).[1] The physical location and specific HS structure allow HSPG to alternatively inhibit or promote protein interactions with cells (*15*−*17*).

Only a limited number of protein−HS interactions have been analyzed in detail at the physical and chemical level (*11*). From these studies, a view has begun to emerge suggesting that the criteria for HS binding vary significantly from one protein to another. In some instances, such as with the pentasaccharide sequence that binds to antithrombin III, the requirements for binding are highly restrictive, suggesting the presence of protein-specific binding sites within HS (*18*−*23*). In contrast to antithrombin III, several other proteins (i.e., growth factors) appear to show more relaxed criteria such that a range of binding sites might exist within HS. For example, high-affinity binding of FGF-2 to HS requires 2-O-sulfation of the uronic acid and does not require 6-O-

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[1] Abbreviations: BAEC, bovine aortic endothelial cell; CS, calf serum; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FGF-2, fibroblast growth factor 2; HB-EGF, heparin-binding epidermal growth factor-like growth factor; HSPG, heparan sulfate proteoglycan; SEM, standard error of the mean; SMC, smooth muscle cell, VEGF165, vascular endothelial growth factor.

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sulfation of the glucosamine (*24*−*26*). However, 6-O-sulfation does not appear to interfere with FGF-2 binding, suggesting that binding sites with and without these residues can interact with FGF-2. Surprisingly, cells deficient in the key HS 2-*O*-sulfotransferase (from *Hs2st*$^{-/-}$ mice) showed apparently normal signaling in response to FGF-2, indicating that 2-O sulfates are not absolutely required for this process (*27*). Moreover, ionic interactions only account for ~30% of the FGF-2-HS binding energy, with the majority of the binding energy involving hydrogen bonding and van der Waals forces (*28*, *29*).

In a recent study, using a heparin-derived octasaccharide library containing varying O-sulfation, the binding requirements for a number of growth factors were evaluated revealing the potential for significant overlap between binding sites (*30*). Hence, the presence of one heparin-binding growth factor would likely impact the availability of select sites on HS for other heparin-binding growth factors. As one example, FGF-10, which requires 6-O-sulfation of glucosamine residues for binding to HS, would be expected to compete for FGF-2 binding to sites that contain 6-O- and 2-O-sulfation but not to sites that contain 2-O-sulfation without 6-O-sulfation. In this way, the presence of FGF-10 could enhance the selectivity of FGF-2 for a subclass of binding sites on HS. The importance of this type of ligand intercompetition, as well as the possible role for multiple classes of protein-binding sites on HS, is poorly understood.

To begin to develop a framework for analyzing the significance of this level of binding complexity, we have conducted a focused intercompetition binding analysis with two heparin-binding growth factors [FGF-2 and heparin-binding epidermal growth factor-like growth factor (HB-EGF)] that utilize distinct receptor systems on bovine aortic smooth muscle cells (SMCs). SMCs were used for these studies because they express receptors for FGF-2 and HB-EGF. In addition, SMCs express relevant HS sequences for both growth factors, which are required for maximal biological response to these growth factors (*31*−*33*). A series of radioligand binding assays were conducted, and an analytical method was developed for determining the apparent binding constants and numbers of specific and shared binding sites within HS expressed by these cells. These studies revealed the presence of two general classes of HS-binding sites for FGF-2 and HB-EGF. The major class was able to bind to either growth factor with relatively low affinity and was termed "common" binding sites. However, both FGF-2 and HB-EGF showed specific high-affinity binding to a subset of "unique" binding sites, which were unable to bind the other growth factor. Hence, these studies indicate that growth factor binding to HS is complex, involving multiple binding sites of variable affinity, density, and selectivity. While the significance of multiple classes of HS-binding sites for growth factors is not known, the present study outlines a relatively simple approach for defining the binding properties for these HS sites.

MATERIALS AND METHODS

Materials. Recombinant human FGF-2 was a generous gift from Chiron, Inc. (Mountain View, CA). Murine recombinant epidermal growth factor (EGF) was from Invitrogen Corp. (Carlsbad, CA), and recombinant human HB-EGF was from R&D Systems (Minneapolis, MN). Human recombinant vascular endothelial growth factor 165 (VEGF165) was from R&D Systems and from the NCI Bulk Cytokine and Monoclonal Antibody Preclinical Repository (Frederick, MD). ^{125}I-FGF-2, ^{125}I-HB-EGF, and ^{125}I-VEGF165 were prepared using a modified Bolton−Hunter procedure (*34*). ^{125}I-EGF was made using Iodobeads (Pierce Endogen; Rockford, IL) (*35*). [^{125}I]-Bolton−Hunter and Na^{125}I were from Perkin−Elmer (Boston, MA). Heparinase III, from *Flavobacterium heparinum*, was a gift from Dr. E. Denholm at Biomarin Technologies (Montreal, Canada).

Cell Culture. Bovine aortic vascular SMCs were obtained from Coriell Cell Repositories (Camden, NJ), and bovine aortic endothelial cells (BAECs) were a gift from Dr. Elazer Edelman at MIT (Cambridge, MA). For experiments, SMCs (passages 6−15) and BAECs (passages 8−12) were used at confluence and maintained as described previously (*33*, *36*−*40*). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM low glucose, Invitrogen), supplemented with 10% bovine calf serum (CS, Hyclone), penicillin (100 units/mL), streptomycin (100 μg/mL), and glutamine (2 mM). The cell number was determined with a Coulter Counter (Miami, FL), and cell viability was determined by trypan blue exclusion.

Growth Factor Cell-Surface Equilibrium Binding. Equilibrium cell-surface binding of ^{125}I-labeled growth factors was conducted essentially as described (*17*, *33*, *36*, *37*, *39*, *40*). SMCs or BAECs were plated at 75 000 cells/2 cm^2 (24-well plate) and cultured for 2 days. Before growth factor addition, cells were washed once with ice-cold binding buffer (DMEM low glucose, 0.05% gelatin, and 25 mM HEPES at pH 7.4), and incubated on ice for 10 min in fresh cold binding buffer (0.5 mL/well) to inhibit internalization. Unlabeled and radiolabeled growth factors were added directly to the binding buffer, and the cells were incubated for 2.5 h at 4 °C. Unbound growth factor was removed by washing the cells with ice-cold binding buffer 3 times. To release HSPG-bound growth factor, cells were subjected to a brief (10 s) high salt, neutral pH extraction (2 M NaCl and 20 mM HEPES at pH 7.4) followed by a PBS rinse (*17*, *34*, *41*). To remove receptor-bound FGF-2 and HB-EGF, a high salt, low pH extraction (2 M NaCl and 20 mM sodium acetate at pH 4) was conducted for 5 min followed by a PBS rinse. Cell-bound EGF was extracted by solubilizing the cells in 1 N NaOH. ^{125}I-radiolabeled growth factor was quantitated using an Auto-Gamma Cobra II series γ counter (Packard Instruments, Meriden, CT). To characterize the affinity and specificity of HSPG-binding sites for FGF-2 and HB-EGF in SMCs, we carried out a series of binding assays with several concentrations of radiolabeled growth factor (0.028−0.56 nM for ^{125}I-FGF-2 and 0.042−0.83 nM for ^{125}I-HB-EGF) in the presence of a wide range of concentrations of the other unlabeled growth factor (11.1−556 nM for FGF-2 and 16.7−833 nM for HB-EGF). To evaluate HSPG binding of FGF-2 and VEGF165 in BAECs, binding assays were conducted with a single concentration of growth factor (0.1 nM; ^{125}I-FGF-2 and ^{125}I-VEGF165) in the presence of a range of unlabeled growth factor (0.1−50 nM; FGF-2 and VEGF165).

Intercompetition Theoretical Considerations. To begin to characterize subclasses of growth factor binding sites within HSPG on SMCs, we defined the following terms for the

FGF-2/HB-EGF intercompetition experiments. The fraction of the cell-surface binding of ^{125}I-HB-EGF or ^{125}I-FGF-2 that is competed for by a large excess of the same unlabeled ligand and is extracted with high salt (2 M NaCl) (salt releasable fraction) is defined as the total specific HSPG-binding component for that ligand. The fraction of the specific binding for each ligand that is competed for by the other ligand (i.e., ^{125}I-FGF-2 binding competed for by unlabeled HB-EGF) is defined as the "common" binding component. The remaining fraction of specific binding that is only competed for by the same ligand (i.e., ^{125}I-FGF-2 binding competed for by unlabeled FGF-2 but not HB-EGF) represents the "unique" binding component. Thus, we propose that these heparin-binding growth factors bind to two general classes of sites within HSPG; one fraction represents "common" sites that do not distinguish between the two ligands, while the other fraction represents "unique" sites that select for one ligand over the other.

To determine the relevant binding constants and numbers of HSPG-binding sites for HB-EGF and FGF-2 on SMCs, we applied the following rules to model the different classes of HSPG sites for FGF-2 and HB-EGF:

(1) HB-EGF and FGF-2 bind to HSPG expressed on the surface of SMCs, which is released by extraction in high salt (2 M NaCl) (*17*, *34*, *41*).

(2) For each ligand, "unique" sites exist that bind only one of the ligands.

(3) Both ligands bind to "common" HSPG sites in a mutually exclusive manner.

(4) The total number of "common" sites is identical for HB-EGF and FGF-2 (by definition).

(5) The ligands may have different K_d values for the "common" sites.

(6) Total specific binding to HSPG for each ligand represents the sum of that bound to the "unique" and "common" sites.

(7) The "unique" and "common" sites do not interact (i.e., relative occupancy of one class of site does not alter the observed K_d for the other. Specifically, occupancy of the "common" sites by HB-EGF does not affect the binding of FGF-2 to its "unique" sites and vice versa).

Model Abbreviations

$HSPG_c$	represents "common" HSPG-binding sites
$HSPG_{uF}$	represents "unique" sites for FGF-2
$HSPG_{uH}$	represents "unique" sites for HB-EGF
Kd_{cF}	represents the K_d for FGF-2 binding to the "common" sites
Kd_{cH}	represents the K_d for HB-EGF binding to the "common" sites
Kd_{uF}	represents the K_d for FGF-2 binding to its "unique" sites
Kd_{uH}	represents the K_d for HB-EGF binding to its "unique" sites

Process. Excess concentrations of unlabeled like ligand were used to define the total specific binding at each concentration of ^{125}I-labeled ligand. The fraction of specific binding that was not competed for by the nonlike ligand represented the amount of ^{125}I-labeled ligand bound to its unique sites. The amount of ^{125}I-ligand bound to its unique sites was determined at several concentrations of ^{125}I-ligand. The K_{d_u} and number of unique sites($HSPG_u$) were determined by fitting [unique bound] versus [free ligand] data to the following equation:

$$[\text{unique bound}] = \frac{[\text{free ligand}][HSPG_u]}{K_{d_u} + [\text{free ligand}]} \quad (1)$$

and solving for the two unknown terms using the nonlinear least-squares method (KaliedaGraph version 3.6, Synergy Software), where [free ligand] represents the amount of ^{125}I-ligand that is not bound to the cells in the presence of a large excess of the non-like ligand and $[HSPG_u]$ represents the total number of unique sites.

A similar analysis could be conducted to determine the parameters for the common sites; however, a higher range of ^{125}I-ligand concentrations would be required because the K_d for these sites appears much greater than the concentrations used (0.5, 1.0, 2.0, 5.0, and 10 ng/mL). Thus, saturation was not approached, and the [bound] versus [free] curves for these sites were in the linear phase.

Alternatively, the K_{d_c} values were determined by the non-like ligand competition curves based on the following relation:

$$[HSPG_c]_{TOT} = [HSPG_c]_{free} + [HSPG_cFGF2] + [HSPG_cHB\text{-}EGF] \quad (2)$$

where $[HSPG_cFGF\text{-}2]$ and $[HSPG_cHB\text{-}EGF]$ represent the amount of FGF-2 and HB-EGF bound to the common sites, respectively. When the ^{125}I-ligand is present at concentrations that are below its K_{d_c} and the non-like ligand is present at concentrations that are above its K_{d_c}, several simplifications can be applied such that

$$\frac{[^{125}\text{I-ligand bound w/o competitor}]}{[^{125}\text{I-ligand bound w/ competitor}]} = 1 + \frac{[\text{competitor}]}{K_{d_c}} \quad (3)$$

Thus, plotting of the common sites bound without the competitor divided by that with the competitor against the competitor concentration yields a straight line that can be fit to eq 3 to yield a value for the K_{d_c} for the competing non-like ligand binding to the common sites (note: the derivation of eq 3 is shown in the Appendix). This was done for each data set (i.e., at each concentration of ^{125}I-radiolabeled growth factor used), and the averaged value was used as the K_{d_c} for the competitor.

The number of $HSPG_c$ sites was then determined from the five values of common bound versus free ^{125}I-ligand concentrations by fitting that data set to eq 4 for a single unknown ($[HSPG_c]$) as

$$[\text{common bound}] = \frac{[\text{free-ligand}][HSPG_c]}{K_{d_c} + [\text{free-ligand}]} \quad (4)$$

where the K_{d_c} determined from eq 3 is entered as a constant and the data are fit to a single unknown. In this situation, the [free-ligand] is equal to the total amount of radioligand present minus that which bound to the cells when no non-like ligand was present. [common bound] values are determined by subtracting unique bound from total salt releasable values.

RESULTS

FGF-2 and HB-EGF have been shown to bind to HSPG on SMCs, and this interaction has been demonstrated to increase the relative affinities of these ligands for their specific cell-surface receptors (*31*, *33*). As evidence of the role of HSPG in these two growth factor systems, SMCs were treated with or without heparinase III prior to conducting radioligand binding assays with ^{125}I-FGF-2, ^{125}I-HB-EGF, and ^{125}I-EGF (Figure 1). For both FGF-2 and HB-EGF, specific binding to HSPG, as defined by that fraction of bound growth factor that was released by high salt extraction, was completely eliminated by pretreatment with heparinase III, while specific binding to the respective receptors was reduced but not eliminated. In contrast to these heparin-binding growth factors, binding of EGF was unaffected by heparinase III. Thus, we used the high salt extraction method to evaluate the intercompetition of heparin-binding growth factors for HSPG sites on SMCs.

We conducted competition binding assays where binding of ^{125}I-FGF-2 or ^{125}I-HB-EGF to HSPG on SMCs was measured in the presence of a large excess of unlabeled growth factor (Figure 2). Over the range of labeled ligand used, both growth factors were able to compete for a large fraction of HSPG binding. In both instances, the "like" ligand was a more effective competitor of binding (i.e., FGF-2 competed ^{125}I-FGF-2 binding more effectively than did HB-EGF), suggesting that, while a certain fraction of the binding sites on HSPG are not able to discriminate between these two growth factors, a subclass exist that is specific for each over the other. Across this concentration range, FGF-2 binding to HSPG appeared to approach saturation, while HB-EGF binding remained below saturation, suggesting that FGF-2 has a higher relative binding affinity for HSPG on SMCs than does HB-EGF. To evaluate the relative selectivity of the binding of FGF-2 and HB-EGF to HSPG in more detail, we conducted binding assays with ^{125}I-growth factors in the presence of a range of concentrations of unlabeled growth factor (Figure 3). EGF was included as a control in these experiments because it does not bind HSPG yet can compete for HB-EGF binding to EGF receptors. As expected, EGF showed no detectable competition for ^{125}I-FGF-2 binding to HSPG; however, HB-EGF competed for a large fraction (30−40%) of ^{125}I-FGF-2 binding. In a similar manner, unlabeled FGF-2 competed for a portion (40−50%) of ^{125}I-HB-EGF binding to HSPG on SMCs. As expected, EGF and HB-EGF but not FGF-2 competed for ^{125}I-EGF binding to EGF receptors on SMCs.

The partial intercompetition of FGF-2 and HB-EGF for one another's binding sites on HSPG suggest that at least two distinct classes of binding sites exist for these two heparin-binding growth factors: a "common" class of sites that can bind either growth factor and a class of "unique" sites that show exclusivity. To characterize these two classes of sites, detailed titrations of unlabeled growth factor against five separate concentrations of labeled growth factor were conducted. Figure 4 shows a representative competition profile at one concentration of ^{125}I-FGF-2 (Figure 4A) and ^{125}I-HB-EGF (Figure 4B). In both instances, we observed a progressive decrease in the level of ^{125}I-growth factor specifically bound to HSPG with an increasing concentration of the competing ligand. Also shown is the maximal competition for each ^{125}I-growth factor that was observed with excess of the "like" unlabeled ligand (total specific bound).

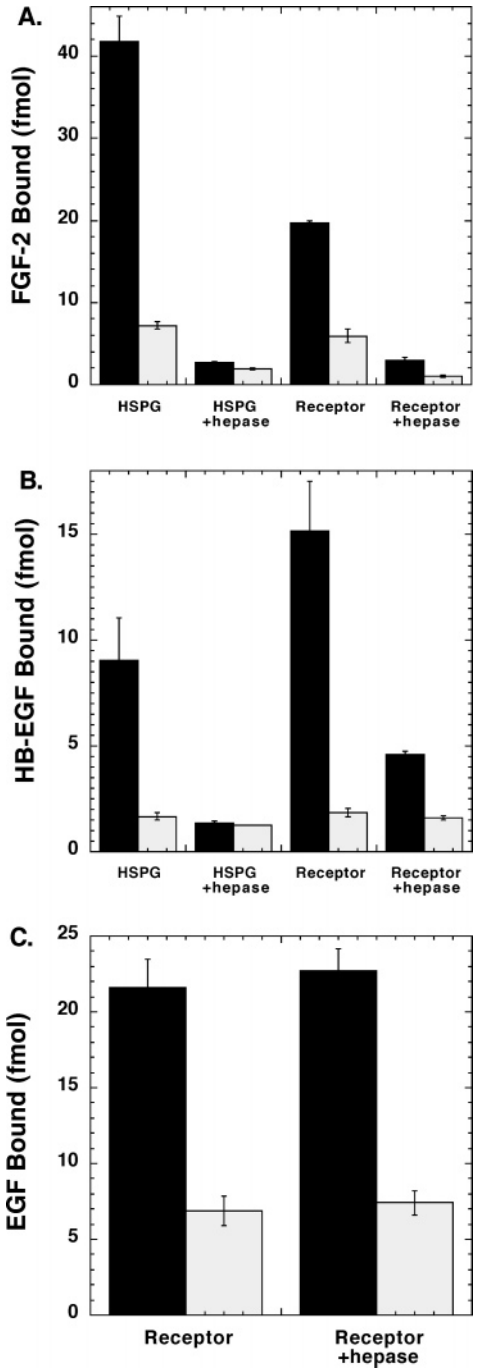

FIGURE 1: Heparinase III eliminates binding of FGF-2 and HB-EGF to HSPG. Confluent SMCs were treated with and without heparinase III (+hepase, 0.1 unit/mL) for 60 min at 37 °C. After hepase treatment, the cells were washed with binding buffer to remove the HS degradation products, and equilibrium binding of 0.28 nM ^{125}I-FGF-2 (A), 0.42 nM ^{125}I-HB-EGF (B), and 0.83 nM ^{125}I-EGF (C) was conducted for 2.5 h at 4 °C in the presence (shaded bars) and absence (filled bars) of a large excess (5 μg/mL) of unlabeled growth factor. After the binding period, cells (A and B) were subjected to sequential extraction with high salt (HSPG) or low pH buffer (receptor) as described under the Materials and Methods. ^{125}I-EGF bound to cells (C) was determined by extracting the entire cell layer in 1 N NaOH (30 min at RT). Data represent the average ± SEM of triplicate determinations.

The ratio of bound ^{125}I-growth factor without and with competitor ligand was calculated and plotted versus the

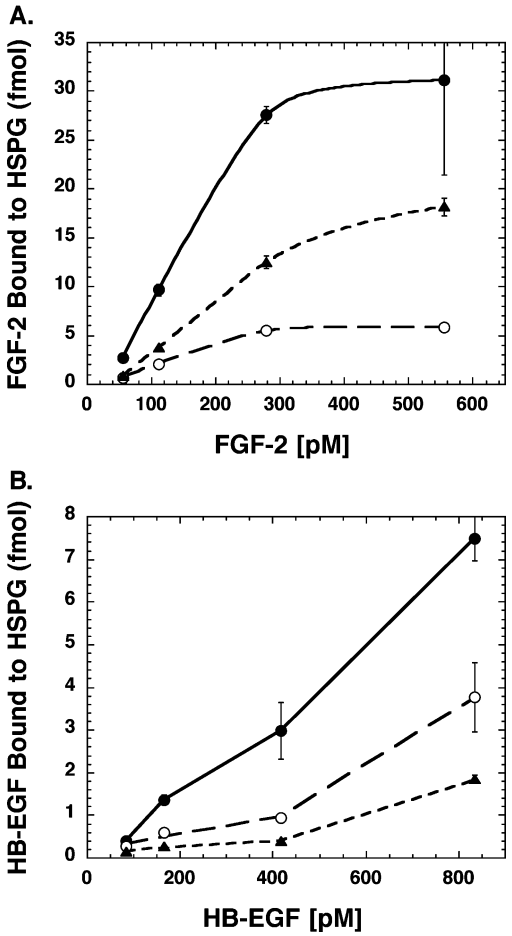

FIGURE 2: Binding of FGF-2 and HB-EGF to HSPG on SMCs. Increasing concentrations of ^{125}I-FGF-2 (A, 0.01−0.28 nM) or ^{125}I-HB-EGF (B, 0.02−0.42 nM) in the presence or absence of unlabeled FGF-2 (1111 nM) or HB-EGF (1667 nM) were added to confluent SMCs. Cell surface binding was conducted for 2.5 h at 4 °C. Total HSPG bound growth factor released by high salt in the absence of any unlabeled growth factor (●), in the presence of unlabeled FGF-2 (○), and that in the presence of unlabeled HB-EGF (▲) were measured in triplicate wells, and the average SEM is presented.

concentration of the competitor ligand (Figure 5). The K_{d_c} of the competitor ligand was calculated using eq 3 at each of five radiolabeled growth factor concentrations. For 1.0 ng/mL (0.056 nM) ^{125}I-FGF-2, the K_{d_cH} was approximately 27 nM. From all concentrations of radiolabeled ^{125}I-FGF-2, the average K_{d_cH} was 44 nM (Table 1). For 1.0 ng/mL (0.083 nM) ^{125}I-HB-EGF, the K_{d_cF} was approximately 19 nM, and for the data set shown in Figure 5B (10 ng/mL; 0.83 nM), the K_{d_cF} was 5.5 nM. From all concentrations of radiolabeled ^{125}I-HB-EGF, the average K_{d_cF} was 12 nM (Table 1). Using eq 4, the number of common sites $[HSPG]_c$ was calculated. From radiolabeled ^{125}I-FGF-2 bound versus free data, the number of common sites was calculated as 837 000 sites/cell. From radiolabeled ^{125}I-HB-EGF bound versus free data, the number of common sites was calculated as 1 560 000 sites/cell. By definition, the number of common sites for both growth factors must be the same; hence, the difference observed represents the intrinsic error of the analysis. Interestingly, FGF-2 binds with higher affinity than HB-EGF to the common sites.

The level of "unique" bound growth factor (that fraction of bound growth factor that was not competed for by the other growth factor) was measured at five concentrations of ^{125}I-FGF-2 and ^{125}I-HB-EGF, and these values were plotted against the concentration of free growth factor (unbound in solution at equilibrium) according to eq 1 (Figure 6). The

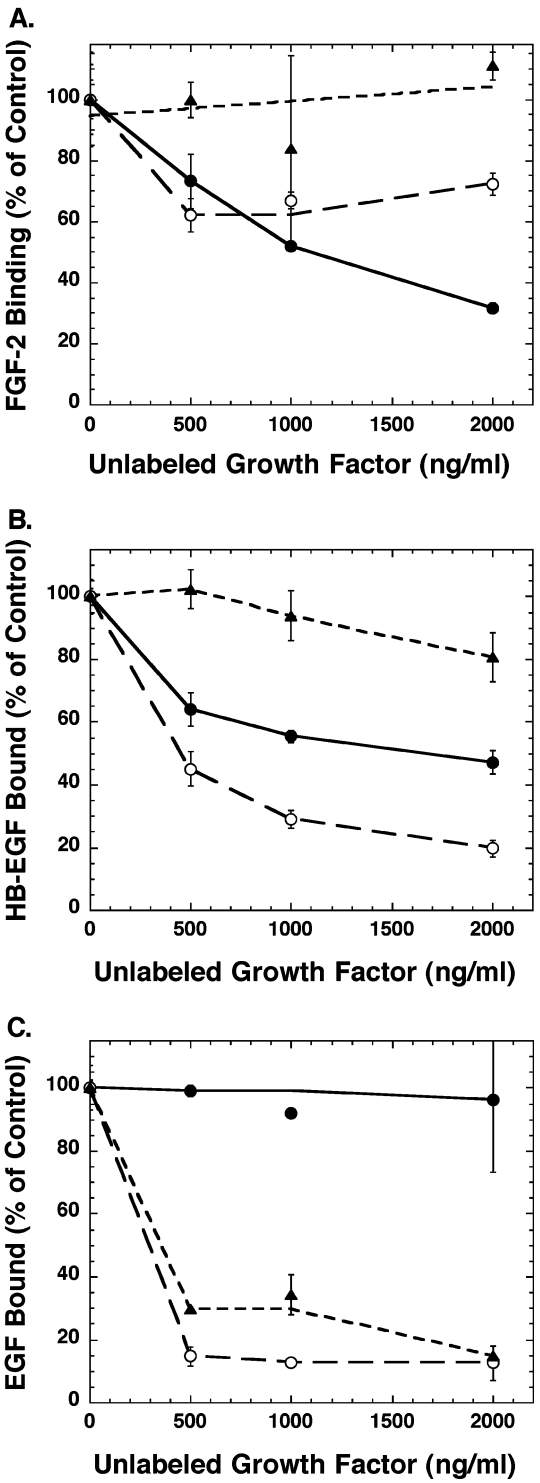

FIGURE 3: Intercompetition binding of FGF-2, HB-EGF, and EGF to SMCs. Growth factor binding was conducted with confluent SMCs at 4 °C for 2.5 h with ^{125}I-FGF-2 (0.28 nM) (A), ^{125}I-HB-EGF (0.42 nM) (B), or ^{125}I-EGF (0.83 nM) (C) in the presence of unlabeled FGF-2 (●), HB-EGF (○), or EGF (▲) (range of 500−2000 ng/mL). The amount of ^{125}I-FGF-2 (A) and ^{125}I-HB-EGF (B) bound to HSPG (i.e., released into high salt) is shown as a percentage of that in the absence of any unlabeled competitor (% control). Total ^{125}I-EGF (C) binding to SMC (1 N NaOH extraction) is shown as a percentage of that in the absence of any competitor. All data represent the average ± SEM of triplicate determinations.

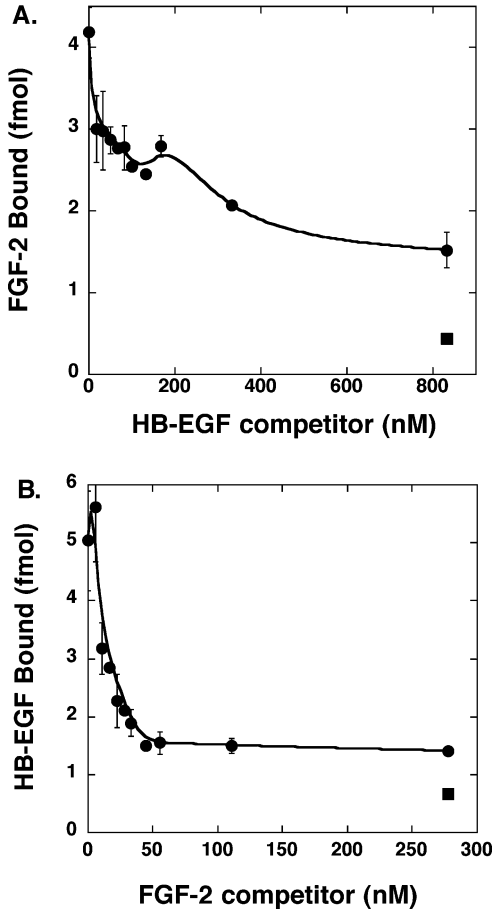

FIGURE 4: Extensive competition of FGF-2 and HB-EGF for HSPG-binding sites. Binding of ^{125}I-FGF-2 (A; 1.0 ng/mL, 0.056 nM) and ^{125}I-HB-EGF (B; 10 ng/mL, 0.83 nM) to HSPG sites on confluent SMCs was conducted in the presence of a wide range of unlabeled HB-EGF (A) or unlabeled FGF-2 (B) shown as ● (note: concentrations of ^{125}I-growth factors that resulted in similar amounts of HSPG binding were chosen for presentation). Also shown is the maximal inhibition observed with the "like" ligand (FGF-2 against ^{125}I-FGF-2; HB-EGF against ^{125}I-HB-EGF) present at 5 μg/mL indicated by ■ on each plot. All date are the average ± SEM of triplicate determinations.

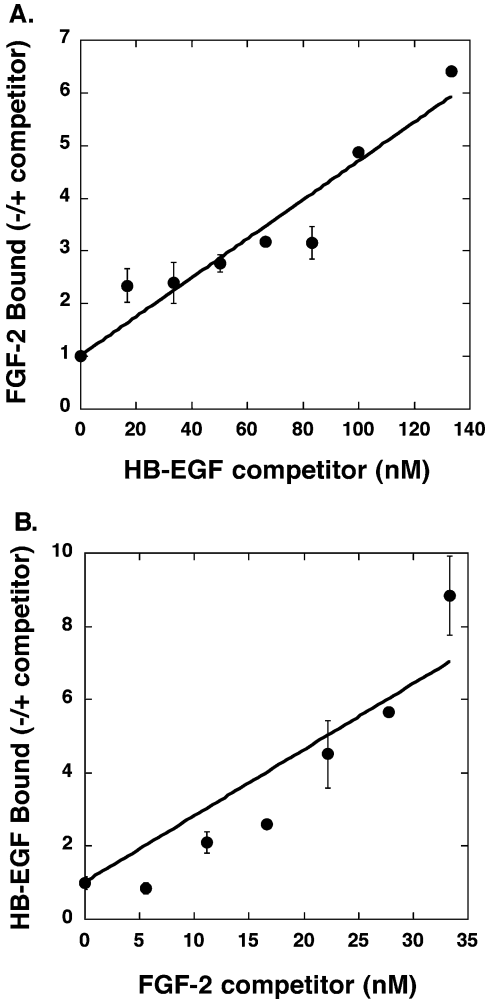

FIGURE 5: Analysis of "common" HSPG-binding site affinity. ^{125}I-FGF-2 and ^{125}I-HB-EGF bound to common HSPG sites were calculated from the data sets described in Figure 4 at each concentration of unlabeled ligand by subtracting the amount bound in the presence of the maximal amount of the unlabeled ligand. The amount bound to "common" sites in the absence of the competitor divided by that in the presence of each concentration of competitor was plotted against the concentration of competitor ligand. The data were fit to eq 3. (A) Competition of FGF-2 binding by HB-EGF for this data set yielded a $K_{dcH} = 27.1 \pm 1.8$ nM with an $R = 0.95$. (B) Competition of HB-EGF binding by FGF-2 produced a $K_{dcF} = 5.5 \pm 0.66$ nM with an $R = 0.91$. Data represent the average ± SEM of triplicate determinations.

number of unique sites $[HSPG]_u$ and the affinity of the unique binding sites for each ligand [K_{d_uF}, K_{d_uH}] were calculated by fitting to eq 1. For FGF-2, there were 118 000 unique sites/cell with a binding affinity of 0.6 nM (Table 1). For HB-EGF, there were ~80% fewer unique sites with nearly 10-fold lower affinity as compared to FGF-2 (28 000 sites/cell; $K_{d_uH} = 6.1$ nM).

To evaluate the consequences of the existence of "unique" and "common" binding sites, we conducted a theoretical analysis of the relative occupancy of these two sites by FGF-2 and HB-EGF on SMCs under steady-state conditions. Figure 7A shows that a considerable fraction of the FGF-2 bound to HSPG (45−65%), over a biologically relevant range of concentrations, would be bound to the "unique" sites. Moreover, the dose dependence for FGF-2 binding to its "unique" sites closely parallels that reported for stimulation of DNA synthesis and activation of Erk1/2 in these cells (*33*, *38*, *42*), indicating that these sites might be the critical mediators of FGF-2 biological activity. The binding of HB-EGF to its "unique" sites on HSPG, on the other hand, is predicted to represent only a small fraction (10−15%) of the total bound to HSPG over the same range of concentrations (Figure 7C). Interestingly, this analysis indicates that both growth factors show significantly increased relative binding to their "unique" sites when the other growth factor is present (parts B and D of Figure 7). Thus, if these two general classes of binding sites are involved in mediating distinct biological functions for FGF-2 and HB-EGF, then

Table 1: Apparent Binding Parameters for FGF-2 and HB-EGF[a]

	"common" binding sites		"unique" binding sites	
	K_{dc} (nM)	sites/cell	K_{du} (nM)	sites/cell
FGF-2	12	1 560 000	0.6	118 000
HB-EGF	44	837 000	6.1	28 000

[a] Binding data as described in Figures 4−6 were subjected to the analysis described under the Materials and Methods to generate the apparent binding parameters to describe FGF-2 and HB-EGF binding to HS sites on SMC.

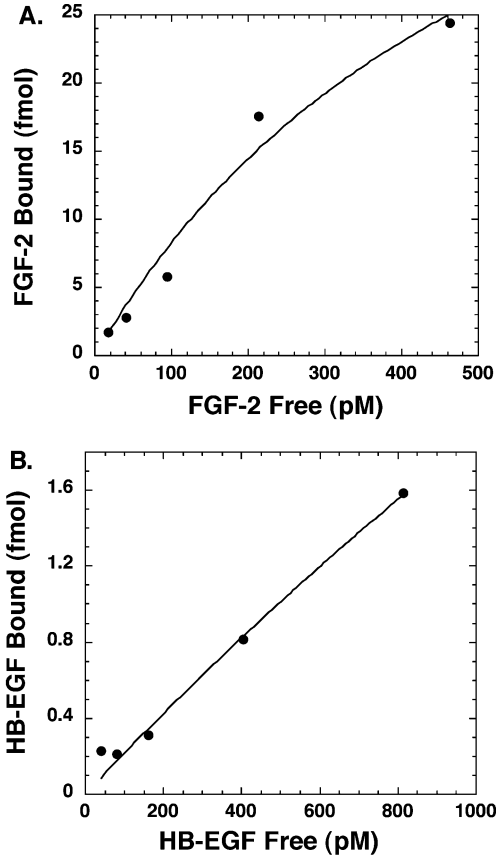

FIGURE 6: Binding of FGF-2 and HB-EGF to "unique" sites on HSPG. ^{125}I-FGF-2 (A) and ^{125}I-HB-EGF (B) bound to unique HSPG sites were calculated at each of five radioligand concentrations as the difference between the maximal competition with the like versus the unlike ligand (i.e., in Figure 4; the difference between ■ and the highest concentration represented by ●). The data were fit by the nonlinear least-squares method to a single binding site model (eq 1), For FGF-2 (A), the fit produced a $K_{duF} = 0.6$ nM with 118 000 sites/cell ($R = 0.98$). For HB-EGF (B), a $K_{duH} = 6.1$ nM with 28 000 site/cell ($R = 0.99$) was calculated. Data represent the average ± SEM of triplicate determinations.

the presence of competing factors would be predicted to alter the biological activity of these growth factors.

To determine if the partial competition between different growth factors for cell-associated HSPG is a general characteristic of heparin-binding growth factors, we conducted an analysis of FGF-2 and VEGF165 intercompetition with BAECs. These cells respond to both FGF-2 and VEGF165 and have been demonstrated to contain HSPG-binding sites for these growth factors (*32*, *39*, *40*, *43*). Specific binding for each labeled ligand at 0.1 nM was determined in the presence of 250 nM of the same unlabeled "like" ligand. In addition, unlabeled FGF-2 (2−50 nM) was titrated against ^{125}I-VEGF165 (0.1 nM), and unlabeled VEGF165 (2−50 nM) was titrated against ^{125}I-FGF-2 (0.1 nM). The relative amount of specific bound ^{125}I-FGF-2 and ^{125}I-VEGF165 are plotted against the unlabeled competitor concentration (Figure 8) showing that both growth factors were able to compete for a fraction of the other's binding to HSPG on endothelial cells. While more extensive experiments need to be conducted to fully evaluate the relative binding affinities for FGF-2 and VEGF165 to the endothelial HSPG, these data indicate that the ability of distinct heparin-binding growth factors to show partial overlap in binding to HSPG on cells is not unique to FGF-2 and HB-EGF on SMCs. As a further extension of this process, we have also investigated the relative intercompetition within members of the FGF family for binding to HS sites on pulmonary epithelial cells, where we have observed partial competition between FGF-2, FGF-7, and FGF-10 [(*44*), data not shown]. These findings are consistent with a recent study demonstrating that similar structures within HS account for the majority of the binding for a range of FGF family members (*45*). Thus, it appears that the ability to bind to a range of HS structures is a general property for heparin-binding growth factors.

DISCUSSION

The discovery of a large number of heparin-binding proteins in conjunction with the potential for enormous complexity within HS structure has suggested the possibility that protein-specific binding sites exist within HS (*1*, *11*, *12*, *46*). However, to date, there has been little evidence that the majority of heparin-binding proteins bind to specific structures within HS. On the contrary, with the exception of antithrombin III, which binds to a particular pentasaccharide sequence, most proteins appear to possess general binding properties that do not argue for the presence of highly selective binding sites within HS. In particular, several growth factors have been shown to have certain minimal requirements for interacting with HS. These minimal requirements are not highly stringent, suggesting that many proteins can bind to highly sulfated regions of HS with little discrimination (*30*, *45*). Thus, one consequence of this low stringency is the possibility that the binding of heparin-binding proteins to HS on cells would be sensitive to the amount of other heparin-binding proteins present within the extracellular environment (*47*). However, it is important to note that this lack of selectivity likely represents binding sites containing minimal requirements for binding to these proteins. Therefore, the possibility that small subclasses of highly selective sites exist within this field of HS-binding sites has not been evaluated in detail. To investigate these possibilities, we conducted a detailed analysis of the intercompetition of heparin-binding growth factors for HS-binding sites on responsive cells. In particular, we observed that both FGF-2 and HB-EGF showed indiscriminant binding to a large class of sites on HS with relatively low affinity. However, a minor fraction (1−10%) of the HS binding for each of these growth factors represented interactions with a subclass of "unique" binding sites, which showed high selectivity and affinity (Figure 9). Moreover, it appears that the presence of "common" and "unique" growth factor binding sites is likely to be a generalized phenomenon, because we also noted evidence of a similar process with FGF-2 and VEGF165 binding to HS on endothelial cells. It is possible that these minor "unique" sites represent important functional binding sites that dictate biological response to their particular growth factor, while the more general "common" sites might function to facilitate growth factor access to the "unique" sites. Alternatively, the "common" sites could function to sequester growth factors to limit their activity or directly participate in mediating certain growth factor biological responses. Identification of the potential roles and importance of these multiple binding sites will require significantly more experimentation.

Because HS was first identified as an important coreceptor for FGF-2 binding and activation of its tyrosine kinase

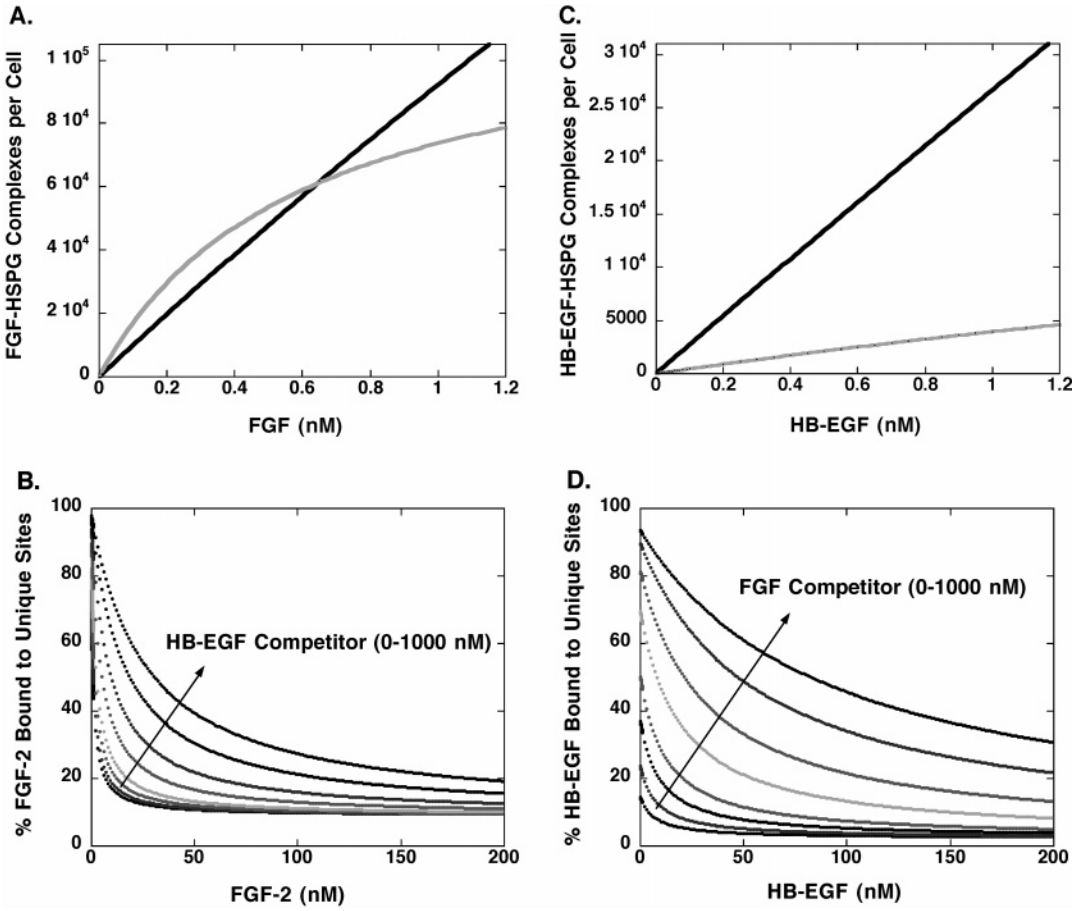

FIGURE 7: Calculated binding of FGF-2 and HB-EGF to "common" and "unique" sites. The number of growth factor occupied "common" (dark line) and "unique" (shaded line) binding sites was calculated using eqs 1 and 4 for FGF-2 (A) and HB-EGF (C) using the parameters presented in Table 1 at the range of free growth factor concentrations shown on the *x* axes. The ability of competing ligand to shift the relative binding of FGF-2 (B) and HB-EGF (D) from "common" to "unique" sites was evaluated by calculating the amount of each growth factor bound to "common" and "unique" sites at a range of concentrations of the competing factor using eqs 1 and 9. The percentage bound to the unique sites was calculated by dividing the amount of growth factor bound to its unique sites by the sum of that bound to common and unique sites (×100). The line closest to the origin in each graph represents the case with no competitor present. Each additional line represents the results obtained with the competitor present at 10, 30, 60, 150, 300, 600, and 1000 nM, respectively. At the lowest concentration of FGF-2 evaluated (0.001 nM, B), the amount bound to "unique" sites increased from 66% in the absence of the competitor to 98% in the presence of 1000 nM HB-EGF. For HB-EGF (0.001 nM; D), the amount bound to unique sites increased from 14% with no FGF-2 present to 93% in the presence of 1000 nM FGF-2.

receptors (*48*, *49*), the underlying mechanisms of this process have been subjected to intense study. While the role of HS in this process appeared to be partially explained by a mechanism of binding avidity enhancement through the generation of a bivalent (receptor and HS) interaction (*34*, *50*−*52*), a considerable number of studies noted that the effects of heparin and HS on FGF-2 binding and activity did not always directly correlate (*25*, *33*, *53*, *54*). Moreover, analysis of the HS structural requirements for FGF-2 binding and receptor activation revealed that both 2-O- and 6-O-sulfation are required for activity, while only 2-O-sulfation is needed to bind to FGF-2, providing a basis for the presence of inhibiting and stimulating sequences within HS (*25*, *55*, *56*). However, it is important to note that these requirements for specific sulfation groups are not absolute because studies with cells from *Hs2st*$^{-/-}$ mice have revealed FGF-2 binding and activity in the absence of 2-O-sulfation (*27*). Consequently, heparin and HS in certain situations function as potent inhibitors of FGF-2 binding and activity (*17*, *25*, *32*, *43*, *57*). Similarly, enzymes that target HS for degradation (i.e., heparinases or heparanase) have been shown to stimulate and inhibit FGF-2 activity in a variety of settings (*58*−*60*), suggesting that the relative amounts of the various functional HS types (stimulatory or inhibitory) may define the function of HS. In a similar fashion, the presence of various heparin-binding proteins could compete for the access of FGF-2 to particular subclasses of binding sites depending on the characteristics of the competing protein. Thus, it is clear that one needs to consider the amount and relative density of HS-binding sites in conjunction with the array of heparin-binding proteins present within a given system as a network of interacting components before activity of heparin-binding growth factors can be effectively predicted within living tissue environments.

These considerations are likely to be particularly important in eventually understanding the role of HS as a modulator of heparin-binding growth factors within vascular SMCs. Many previous studies have investigated the effects of heparin, HS, and heparinases in modulating FGF-2 binding and activity in vascular cells and have produced a rather complicated array of data with multiple interpretations. For example, in SMC cultures and *in vivo*, heparin and HS have been shown to be potent inhibitors of FGF-2 (*32*, *43*, *61*−*64*) while also being implicated as critical mediators of

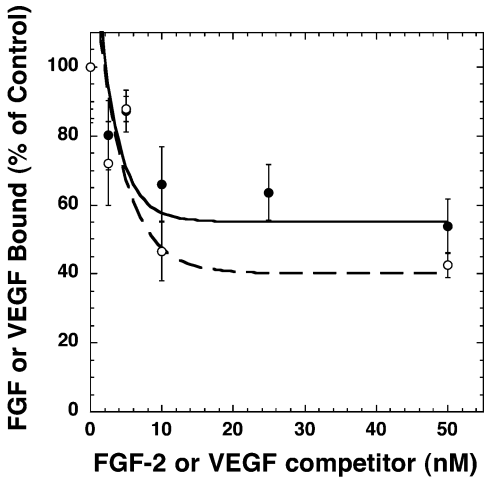

FIGURE 8: Intercompetition binding of FGF-2 and VEGF165 for HSPG sites on endothelial cells. Equilibrium binding of ^{125}I-FGF-2 (●; 0.1 nM) and ^{125}I-VEGF165 (○; 0.1 nM) to confluent bovine aortic endothelial cells was conducted at 4 °C for 2.5 h in the presence of the indicated concentration of the other unlabeled ligand [unlabeled VEGF165 (●) and unlabeled FGF-2 (○)]. Specific binding to HSPG was determined for each growth factor by subtracting the level of ^{125}I-growth factor bound in the presence of excess unlabeled "like" ligand (250 nM). Data are presented as the percentage of specific binding observed in the absence of the competing unlabeled growth factor. Data are the averages ± SEM of triplicate determinations.

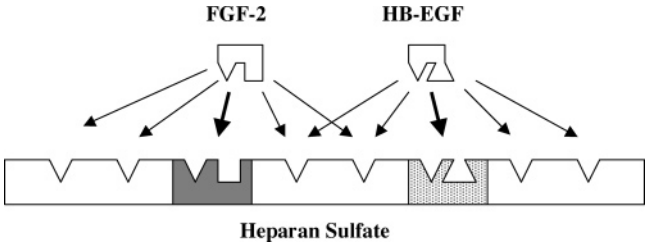

FIGURE 9: Schematic model of "common" and "unique" growth factor binding sites on heparan sulfate. Heparan sulfate, represented by the long notched bar, is considered to contain multiple "common" binding sites (indicated by the single notch) that can bind to FGF-2 and HB-EGF with relatively low affinity and fewer "unique" binding sites (indicated by the shaded regions), which contain properties that allow selective binding of one or the other growth factor. The model is not meant to indicate that all "common" binding sites must be identical nor does the model require that the interaction between each growth factor and the common sites is the same. In this scheme, the "unique" sites are indicated as containing characteristics in addition to the common sites to allow for enhanced affinity. However, it is important to note that the binding analysis provides no information regarding the structural nature of these binding sites nor does it allow for calculations of stoichiometry (i.e., the number of binding sites per heparan sulfate chain was not determined).

FGF-2 activity (*33*, *36*, *38*, *42*, *58*, *65*, *66*). To a certain extent, these apparently conflicting results are indicative of the ability of distinct structures within vascular HS to either stimulate of inhibit FGF-2 action (*65*). These inhibitory and stimulatory HS structures might relate to the "common" and "unique" binding sites described here. A more complete understanding of the role of the HS structure in modulating SMC response to FGF-2 would provide valuable insight into the potential use of heparin/HS to modulate these cells in clinical situations.

In the present study, we outlined a relatively simple routine for binding site analysis that can be used to determine the relative amounts and affinities of "common" and "unique" binding sites for heparin-binding growth factors on cells in culture. This method can be applied to aid in the evaluation of the relative roles of these selective and nonselective domains within HS. As an example, it would be interesting to use this approach in conjunction with biological activity measurements to determine the particular function of each binding site and alternatively to evaluate how various agents might selectively modulate one class of binding site over another. Hence, the impact of treatments that result in limited changes in the total amount of HS binding of a particular growth factor that alter its activity could potentially be revealed by focusing on the specific effects on the minor "unique" sites. This type of regulation might not only act at the HS biosynthetic level but might also involve direct modification of existing HS chains on the cell surface and in the extracellular matrix through the action of enzymes such as the 6-*O*-sulfatases (*67*, *68*). Thus, the presence of multiple classes of overlapping and specific growth factor binding sites within HS provides additional insight into the mechanisms underlying the role that this class of polysaccharides plays in tightly controlling cellular response to growth factors during tissue development and repair.

APPENDIX

The relationship presented as eq 3 in the Materials and Methods is derived on the basis of the following process.

Starting from eq 2 (note: [H] is used to denote terms containing [HSPG])

$$[H_c]_{tot} = [H_c]_{free} + [H_cFGF] + [H_cHB\text{-}EGF] \quad (2)$$

in the condition where FGF-2 is the radiolabeled ligand and HB-EGF is the unlabeled competitor, the following expressions define the binding of each ligand to the "common" sites as

$$K_{d_cH} = \frac{[HB\text{-}EGF]_{free}[H_c]_{free}}{[H_cHB\text{-}EGF]} \quad (5)$$

$$K_{d_cF} = \frac{[FGF]_{free}[H_c]_{free}}{[H_cFGF]} \quad (6)$$

Solving eq 5 for $[H_cHB\text{-}EGF]$ and substituting into eq 2 to give

$$[H_c]_{tot} = [H_c]_{free} + [H_cFGF] + \frac{[HB\text{-}EGF]_{free}[H_c]_{free}}{K_{d_cH}} \quad (7)$$

and solving eq 7 for $[H_c]_{free}$ as

$$[H_c]_{free} = \frac{[H_c]_{tot} - [H_cFGF]}{\left(1 + \frac{[HB\text{-}EGF]_{free}}{K_{d_cH}}\right)} \quad (8)$$

then substituting eq 8 into eq 6 and solving for $[H_cFGF]$ to give

$$[H_cFGF] = \frac{1}{\left(K_{d_cF} + \frac{[FGF]_{free}}{\left(1 + \frac{[HB\text{-}EGF]_{free}}{K_{d_cH}}\right)}\right)} \cdot \frac{[FGF]_{free}[H_c]_{tot}}{\left(1 + \frac{[HB\text{-}EGF]_{free}}{K_{d_cH}}\right)} \quad (9)$$

where $K_{d_cF} + [FGF]_{free}/((1 + [HB\text{-}EGF]_{free}/K_{d_cH})) \approx K_{d_cF}$ when $[FGF]_{free} \ll K_{d_cF}$ or $[HB\text{-}EGF]_{free} \gg K_{d_cH}$ and $[HB\text{-}EGF]_{free} \approx [HB\text{-}EGF]$, then

$$[H_cFGF] = \frac{[FGF][H_c]_{tot}}{K_{d_cF}} \cdot \frac{1}{\left(1 + \frac{[HB\text{-}EGF]}{K_{d_cH}}\right)} \quad (10)$$

where $[H_cFGF]$ is the amount of FGF-2 bound to "common" sites at each given concentration of HB-EGF and $[FGF]-[H_c]_{tot}/K_{d_cF}$ is the amount of FGF-2 bound to "common" sites without the HB-EGF competitor, thus, producing the form of eq 3 for ^{125}I-FGF-2 and unlabeled HB-EGF competitor

$$\frac{\text{FGF bound w/o competitor}}{\text{FGF bound w/ competitor}} = 1 + \frac{[HB\text{-}EGF]}{K_{d_cH}} \quad (11)$$

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