

## Current Topics

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### Kinetic and Structural Studies on Interactions between Heparin or Heparan Sulfate and Proteins of the Hedgehog Signaling Pathway<sup>†</sup>

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**ABSTRACT:** Heparan sulfate (HS) proteoglycans (PGs) interact with a number of extracellular signaling proteins, thereby playing an essential role in the regulation of many physiological processes. These interactions are important for both normal signal transduction and regulation of the tissue distribution of signaling molecules. In this study, we use surface plasmon resonance (SPR) to study interactions of HS and structurally related heparin with proteins in the Hedgehog signaling pathway. SPR analysis shows that heparin binds with different affinities to active fragments of the proteins Hedgehog (Hh), Interference Hedgehog (Ihog), Cam-related/Down-regulated by Oncogenes (CDO), and Sonic Hedgehog (Shh). Solution competition studies show that the minimum size of a heparin oligosaccharide capable of interacting with Ihog is larger than a tetrasaccharide and for interacting with Shh is larger than an octasaccharide. In comparison with heparin, Ihog and Shh exhibited a lower affinity for HS than for heparin, and CDO and Hh exhibit negligible binding to HS. This study clearly demonstrates Shh and Ihog are heparin and HS binding proteins and that both molecules preferentially bind heparin or HS having a high level of sulfation.

Hedgehog (Hh)<sup>1</sup> is a secreted signaling protein involved in key tissue patterning events in both vertebrates and invertebrates (*1*). The name Hedgehog derives from the

appearance of *Drosophila* embryos deficient in *hedgehog* gene function, which lack the typical segmented bristle pattern and develop a uniform distribution of bristles reminiscent of a hedgehog's coat (*2*). A single Hh homologue exists in the *Drosophila* genome, but three Hh homologues, termed Sonic, Desert, and Indian Hedgehog (Shh, Dhh, and Ihh, respectively), are found in vertebrates. Loss of Ihh and Shh function in mice results in severe developmental abnormalities (*3, 4*), and several human developmental malformations arise from impaired Shh function (*5*). In addition to roles in embryonic development, Shh is involved

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<sup>1</sup> Abbreviations: HS, heparan sulfate; HP, heparin; PGs, proteoglycans; SPR, surface plasmon resonance; Hh, Hedgehog; Ihog, Interference Hedgehog; CDO, Cam-related/Down-regulated by Oncogenes; Shh, Sonic Hedgehog; Dhh, Desert Hedgehog; Ihh, Indian Hedgehog; Ptc, Patched; Smo, Smoothed; HSPGs, heparan sulfate proteoglycans;

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Dlp, Dally-like protein; FNIII, fibronectin type III; Boi, Brother of Ihog; BOC, Brother of CDO; GAG, glycosaminoglycan; ECM, extracellular matrix; TEV, tobacco etch virus; SA, streptavidin; FC, flow cell; LMW heparin, low-molecular weight heparin;  $\beta$ -ME,  $\beta$ -mercaptoethanol.

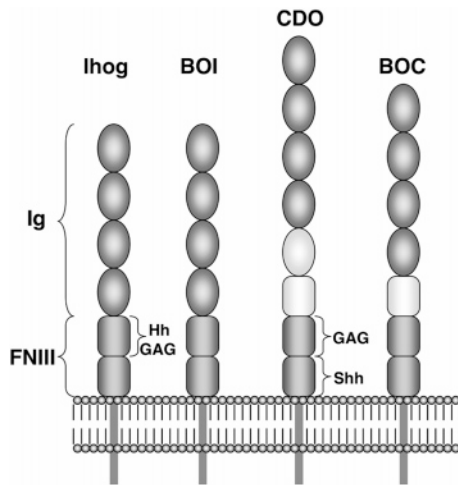


FIGURE 1: Domain architectures of the extracellular regions of Ihog, BOI, CDO, and BOC are shown relative to a schematic membrane bilayer. Ovals represent Ig domains, and rounded rectangles represent FNIII domains. CDO and BOC contain an extra Ig+FNIII or single FNIII repeat, respectively, relative to Ihog/BOI. Sequence analysis suggests that the first FNIII repeats of CDO and BOC and the last Ig repeat of CDO lack direct homologues in Ihog/BOI, and these repeats are represented in a lighter color. The FNIII domains to which Hh, Shh, and GAGs have been shown to bind are shown.

in regulating the formation and maintenance of stem cells (6), and abnormal Shh signaling has been implicated in several human cancers (7). This role in cancer has made the Hh signaling pathway a target for the development of inhibitors that may have therapeutic value (8).

Hh proteins influence tissue patterning by acting as classical morphogens. A gradient of decreasing Hh concentrations is formed from sites of Hh secretion and exerts concentration-dependent effects on tissue differentiation (6). Consequently, the range and strength of the Hh signal are tightly regulated. The Hh protein itself undergoes two lipid modifications, which restrict the range of Hh signaling and are essential for normal Hh function (9), and several membrane-associated proteins have been identified that transmit or modulate Hh signals in receiving cells (10). A central event in Hh signaling is the interaction of Hh with Patched (Ptc), a 12-pass transmembrane protein homologous to resistance-nodulation-division (RND) transporters (11). Ptc normally inhibits the activity of Smoothened (Smo), a seven-pass transmembrane protein, but the presence of Hh inhibits the ability of Ptc to inhibit Smo and leads to activation of the Hh signaling pathway (11). Dally-like protein (Dlp), a member of the glypican family of heparan sulfate proteoglycans (HSPGs) and attached to the membrane through a glycosylphosphatidylinositol (GPI) anchor, has also been shown to modulate Hh signaling, although the mechanism remains unclear (12–14). A role for HSPGs in Hh signaling is further implied by restriction in the range of Hh signaling in cells lacking the function of the *tout velu* gene, which encodes a HS copolymerase (15).

More recently, members of the Interference Hedgehog (Ihog) family (Figure 1) of type I integral membrane proteins have been found to be components of the Hh signaling pathway (16–18). Ihog and its homologue Brother of Ihog (Boi) are *Drosophila* proteins that consist of an extracellular region of four immunoglobulin-like (Ig) domains followed

by two fibronectin type III (FNIII) domains, a single membrane-spanning region, and a cytoplasmic tail of no known function (16). Two Ihog homologues exist in vertebrates, Cam-related/Down-regulated by Oncogenes (CDO) and Brother of CDO (BOC), which contain five and four Ig domains, respectively, followed by three FNIII domains (17, 18). Amino acid sequence alignments indicate that the second and third FNIII repeats of CDO and BOC are homologous to the first and second FNIII repeats of Ihog, respectively. Pulldown assays demonstrate that Ihog interacts with Hh through its first FNIII repeat (IhogFn1) but that the vertebrate Ihog homologues interact with Shh through their third FNIII repeats, a curious migration of the Hh interaction site between vertebrate and invertebrate Ihog homologues (16, 18). How interactions among Hh, Ihog, and Ptc are coordinated remains to be determined, but a formation of a ternary complex of these molecules in which Ihog functions to enhance cellular affinity for Hh has been suggested as the simplest model consistent with all observations (19).

Both IhogFn1 and the N-terminal signaling domain of Hh (HhN) bind individually to heparin, and recent results demonstrate that heparin is required to promote high-affinity interactions between Hh and Ihog (19, 26–28). This latter observation led to crystallization of a complex between HhN and a fragment of Ihog encompassing the two FNIII repeats (IhogFn1-2). The structure of this complex revealed a 2:2 IhogFn1-2–HhN complex likely to reflect a physiological interaction (19). Heparin is not visualized in this complex; however, a contiguous region of positive charge spans the Hh–Ihog interface, and mutations within this basic region greatly reduce the strength of interactions among Ihog, Hh, and heparin. It has thus been suggested that heparin is likely to promote interactions between Hh and Ihog by binding and spanning the Hh–Ihog complex (19). Heparin (Figure 2) is an intracellular, highly sulfated glycosaminoglycan (GAG) that is not present in Hh-responding cells. Thus, it is unlikely that heparin is the physiological mediator of interactions between Hh and Ihog. HS, an extracellular GAG structurally related to heparin, is ubiquitously found on the surface of animal cells, including *Drosophila*, and in the surrounding extracellular matrix (ECM), however, and could supply this function.

To better understand the role of GAG chains in Hh signaling, surface plasmon resonance (SPR) was used to investigate the effects of chain length, saccharide composition, and sulfo group substitution on the ability of GAG chains to mediate interactions between Hh and Ihog. The binding of CDO to Shh and the influence of GAG chains on this interaction were also characterized.

## EXPERIMENTAL PROCEDURES

### Materials

The GAGs used were porcine intestinal heparin (16 kDa), low-molecular mass (LMW) heparin (4.8 kDa), and porcine intestinal heparan sulfate (Celsus Laboratories, Cincinnati, OH), chondroitin sulfate A (20 kDa) from porcine rib cartilage (Sigma, St. Louis, MO), dermatan sulfate (also known as chondroitin sulfate B, 30 kDa, from porcine intestine, Sigma), dermatan disulfate (4,6-disulfo DS, 33 kDa, Celsus) prepared through the chemical 6-*O*-sulfonation of dermatan sulfate (20), chondroitin sulfate C (20 kDa, from

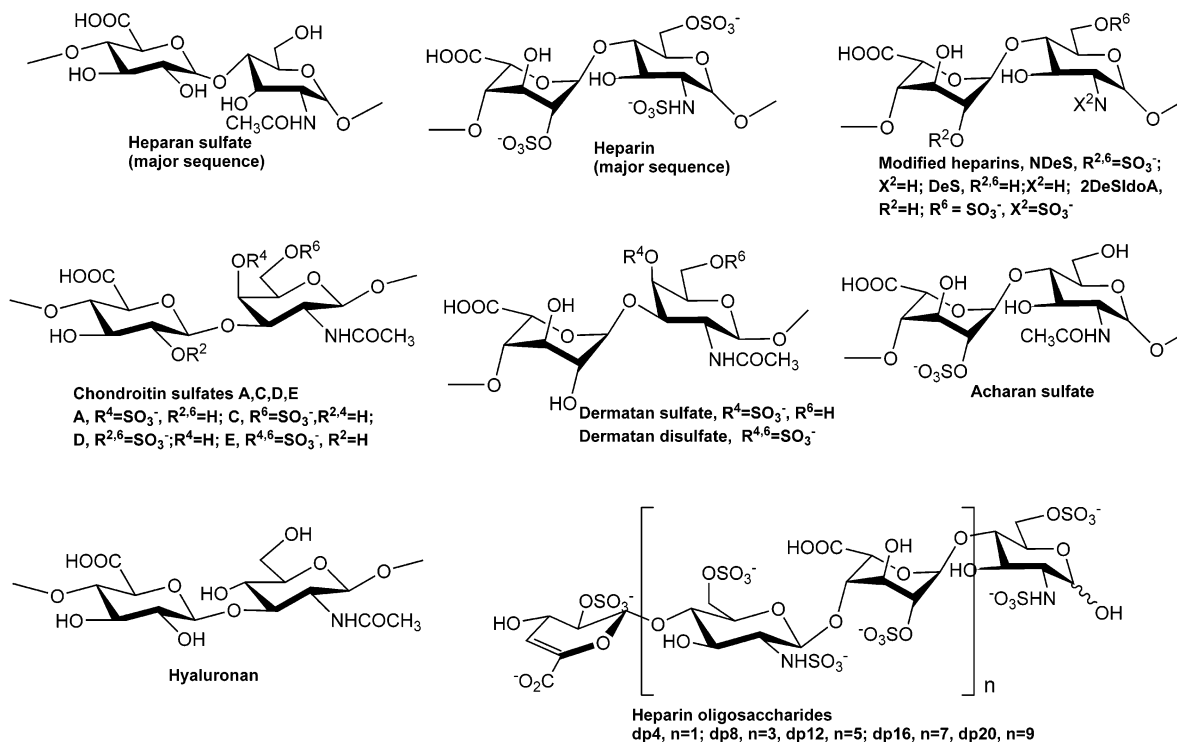


FIGURE 2: Chemical structures of GAGs, modified GAGs, and heparin-derived oligosaccharides.

shark cartilage, Sigma), chondroitin sulfate D (20 kDa, from whale cartilage, Seikagaku, Tokyo, Japan), chondroitin sulfate E (20 kDa from squid cartilage, Seikagaku), and hyaluronic acid sodium salt (100 kDa, from *Streptococcus zooepidemicus*, Sigma). Acharan sulfate (29 kDa from *Achatina fulica*) was provided by Y.-S. Kim (Seoul National University, Seoul, South Korea). Fully desulfated heparin (14 kDa), *N*-desulfated heparin (14 kDa), and 2-*O*-desulfated IdoA heparin (13 kDa) were all prepared via the method of Yates et al. (21). Heparin oligosaccharides included tetrasaccharide (dp4), octasaccharide (dp8), deca-saccharide (dp10), dodecasaccharide (dp12), hexadecasaccharide (dp16), and eicosaccharide (dp20) and were prepared from a controlled partial heparin lyase 1 treatment of bovine lung heparin (Sigma) followed by size fractionation (22). Chemical structures of these GAGs are shown in Figure 2. The sensor SA chips and CM5 chips were from BIAcore (Biacore AB, Uppsala, Sweden). SPR measurements were performed on a BIAcore 3000 operated using BIAcore 3000 control and BIAevaluation software (version 4.0.1).

### Methods

**Protein Expression and Purification.** DNA fragments encoding IhogFn1-2 (residues 466–679), HhN (residues 85–248), mShhN (residues 26–189), hCDOFn2-3 (residues 722–924), hCDOFn2 (residues 722–820), and hCDOFn3 (residues 826–924) were cloned into the SalI and NotI restriction sites of the pT7HMT expression vector (23). IhogFn1-2 and HhN proteins were expressed in B834(DE3) cells and purified to homogeneity as described previously (19). Soluble CDO fragments were expressed in *Escherichia coli* and purified using a Ni<sup>2+</sup>-NTA column followed by tobacco etch virus (TEV) protease cleavage to remove affinity tags. The cleaved proteins were further purified by ion-exchange and size-exclusion chromatography, followed

by dialysis against phosphate-buffered saline (PBS) and 20 mM  $\beta$ -mercaptoethanol (ME).

**Preparation of a Heparin or HS Biochip.** Biotinylated heparin or HS was immobilized on streptavidin (SA) chips on the basis of the manufacturer's protocol. Biotinylated heparin or HS was prepared by reacting sulfo-*N*-hydroxy-succinimide long chain biotin (Pierce, Rockford, IL) with free amino groups of unsubstituted glucosamine residues in the polysaccharide chain following a published procedure (24). The biotin–GAG conjugate contained one or two biotin residues/GAG chain as determined by <sup>1</sup>H NMR spectroscopy. A solution of the heparin–biotin conjugate (0.1 mg/mL) in HBS-EP running buffer was injected over flow cell 2 (FC2) or FC4 of the SA chip at a flow rate of 5  $\mu$ L/min. The successful immobilization of heparin was confirmed by the observation of a 100–200 resonance unit (RU) increase in the sensor chip. Control flow cell 1 or 3 (FC1 or FC3) on the chip was prepared by injecting 30  $\mu$ L of saturated biotin. HS biochips were prepared with SA sensor chips using the same protocol.

**Preparation of a Protein Biochip.** The protein sensor chip was prepared by immobilization of Ihog (IhogFn1-2) on a research grade CM 5 chip (Biosensor AB, Uppsala, Sweden) through its primary amino groups using EDC/NHS according to the standard amine coupling protocol. Following activation with EDC/NHS, successful immobilization of Ihog ( $\Delta$ RU  $\sim$  1500) was achieved by diluting 200  $\mu$ g/mL Ihog in PBS with 20 mM  $\beta$ -ME buffer.

**Measurement of the Strength of the Interaction of Proteins with a Heparin/HS Biochip Using BIAcore.** The protein samples were diluted in PBS and 20 mM  $\beta$ -ME buffer. Different dilutions of protein samples in the buffer were injected at a flow rate of 30  $\mu$ L/min. At the end of the sample injection, the same buffer (PBS and 20 mM  $\beta$ -ME) was passed over the sensor surface to facilitate dissociation. After

a 3 min dissociation, the sensor surface was regenerated by injection with 30  $\mu\text{L}$  of 50 mM NaOH in 1 M NaCl to obtain a fully regenerated surface. The response was monitored as a function of time (sensorgram) at 25  $^{\circ}\text{C}$ .

**Solution Competition SPR Study.** Ihog (IhogFn1-2) or Shh (mShhN) proteins (1000 nM) premixed with different concentrations of heparin, LMW heparin, tetrasaccharide (dp4), octasaccharide (dp8), decasaccharide (dp10), dodecasaccharide (dp12), hexadecasaccharide (dp16), and eicosaccharide (dp20) in PBS and 20 mM  $\beta$ -ME buffer were injected over a heparin chip at a flow rate of 30  $\mu\text{L}/\text{min}$ . After each run, the dissociation and regeneration were performed as described above. For each set of competition experiments on SPR, a control experiment (only protein without added heparin or oligosaccharides) was performed to make certain the surface was completely regenerated and that the results obtained between runs were comparable.

Similar procedures were performed on a HS biochip to determine the solution competition of other GAGs for interaction of Ihog and Shh with HS. Ihog or Shh protein (500 nM), mixed with different concentrations with each of the GAGs (Figure 2) in PBS and 20 mM  $\beta$ -ME buffer, was injected over the HS biochip at a flow rate of 30  $\mu\text{L}/\text{min}$ .

## RESULTS

**Kinetics of Interactions between IhogFn1-2 or HhN and Heparin or Heparan Sulfate.** *Drosophila* IhogFn1-2 and HhN both interact with heparin, and these interactions are important for normal Hh signaling (19, 26–29). The strengths of interactions between Hh and Ihog and either heparin or HS have not been rigorously determined, however, and we thus used SPR to characterize the binding of IhogFn1-2 and HhN to both heparin and HS immobilized independently on SA sensor chips. Although heparin is commonly used to study interactions between signaling molecules and GAGs, cell surface HS is the most likely physiological binding partner. Sensorgrams of IhogFn1-2–heparin, IhogFn1-2–HS, HhN–heparin, and HhN–HS interactions are shown in Figure 3. Except for the HhN–HS sensorgrams, they fit well to a Langmuir 1:1 binding model, and the binding kinetics are presented in Table 1. The SPR data show that IhogFn1-2 binds to heparin and HS with dissociation constants of  $\sim 7$  and  $\sim 18$   $\mu\text{M}$ , respectively, and that HhN binds to heparin with a dissociation constant of  $\sim 48$   $\mu\text{M}$ . Binding of HhN to HS was too weak to measure ( $\geq 100$   $\mu\text{M}$ ). Curiously, the on rate for binding of HhN to heparin was  $\sim 730$ -fold slower than that for IhogFn1-2, suggesting that HhN may exist in an equilibrium between forms more and less accessible to interactions with heparin.

**Importance of Heparin in Ihog and Hh Interactions.** An IhogFn1-2-coupled CM5 sensor chip was prepared to study the interaction between IhogFn1-2 and HhN. IhogFn1-2 was immobilized to the CM5 sensor chip through its surface amino groups in a random ensemble of orientations. No interaction between IhogFn1-2 and HhN was observed when 1 and 5  $\mu\text{M}$  HhN were injected onto the IhogFn1-2 chip (data not shown). When 1  $\mu\text{M}$  heparin was added to the injected HhN, however, a sensorgram could be recorded from the Ihog biochip demonstrating some fraction of the immobilized IhogFn1-2 was in the proper orientation for binding HhN in the presence of heparin (Figure 4A). The

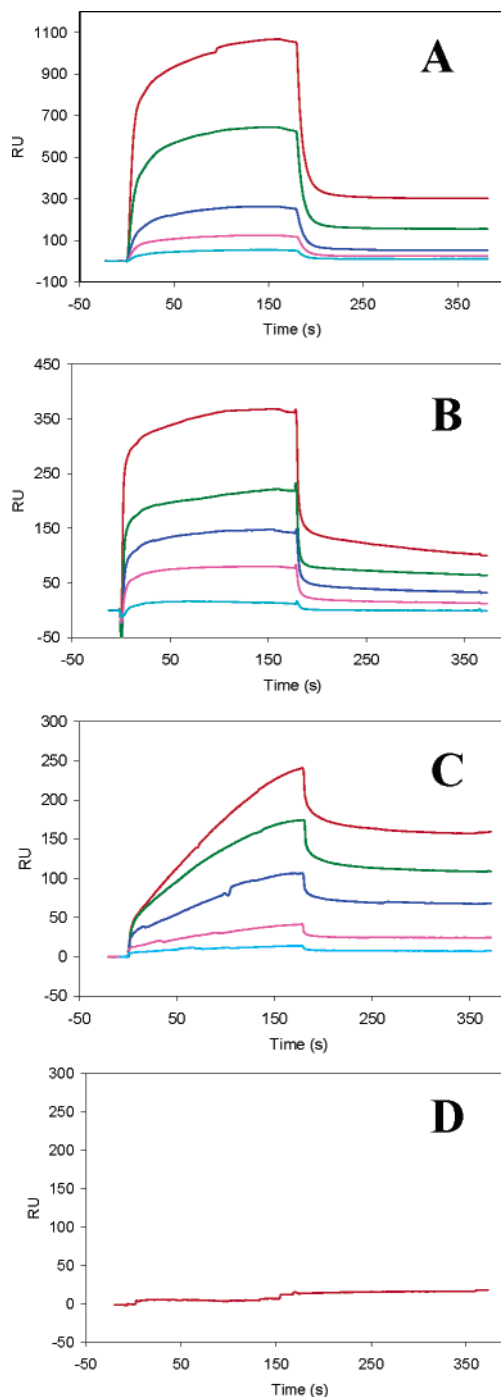


FIGURE 3: SPR sensorgrams of protein–heparin or –HS interactions. (A) Ihog (IhogFn1-2)–heparin interaction. Concentrations of IhogFn1-2 (from top to bottom) were 500, 250, 100, 50, and 25 nM, respectively. (B) Ihog (IhogFn1-2)–HS interaction. Concentrations of IhogFn1-2 (from top to bottom) were 1000, 750, 500, 200, and 100 nM, respectively. (C) Hh (HhN)–heparin interaction. Concentrations of HhN (from top to bottom) were 5, 4, 3, 2, and 1  $\mu\text{M}$ , respectively. (D) Hh (HhN)–HS interaction (no detectable binding). The concentration of HhN was 5  $\mu\text{M}$ .

binding affinity of IhogFn1-2 for HhN in the presence of 1  $\mu\text{M}$  heparin was 1.8  $\mu\text{M}$  ( $K_D$ ) as determined by SPR. The binding of heparin to the immobilized Ihog surface is negligible in the absence of Hh. A control experiment was conducted by injecting 1  $\mu\text{M}$  heparin, 1  $\mu\text{M}$  HS, or a micromolar concentration of dermatan disulfate in the absence of Hh onto the Ihog chip; only a weak response (10–20 RU) was detected. Thus, the concentration-dependent



Table 1: Summary of Kinetic Data of Protein–Heparin or –HS Interactions

interaction	$k_{\text{on}}$ ( $\text{M}^{-1} \text{s}^{-1}$ )	$k_{\text{off}}$ ( $\text{s}^{-1}$ )	$K_{\text{D}}$ (M)
Ihog (IhogFn1-2)–HP	$1.56 \times 10^4$	0.104	$6.66 \times 10^{-6}$
Hh (HhN)–HP	21.4	$1.02 \times 10^{-3}$	$4.77 \times 10^{-5}$
CDO (hCDOFn2-3)–HP	$1.58 \times 10^5$	0.243	$1.53 \times 10^{-6}$
Shh (mShhN)–HP	$2.69 \times 10^4$	$1.8 \times 10^{-3}$	$6.71 \times 10^{-8}$
CDO (hCDOFn2)–HP	$6.00 \times 10^4$	0.360	$6.00 \times 10^{-6}$
Shh (mShhN)–HS	$5.16 \times 10^2$	$1.63 \times 10^{-2}$	$3.16 \times 10^{-5}$
Ihog (IhogFn1-2)–HS	$5.45 \times 10^3$	$9.70 \times 10^{-2}$	$1.78 \times 10^{-5}$
IhogFn1-2–HhN	$8.48 \times 10^2$	$1.50 \times 10^{-3}$	$1.77 \times 10^{-6}$

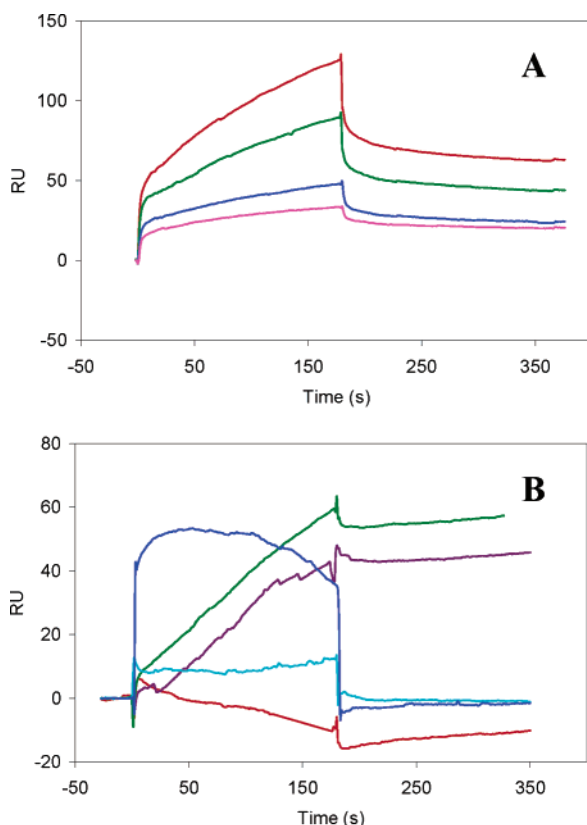


FIGURE 4: (A) SPR sensorgrams of IhogFn1-2 (immobilized) and HhN interaction with the addition of  $1 \mu\text{M}$  heparin. Concentrations of HhN (from top to bottom) were 8, 5, 2, and  $1 \mu\text{M}$ , respectively. (B) SPR sensorgrams of IhogFn1-2 and HhN interaction with the addition of  $1 \mu\text{M}$  HS or dermatan disulfate: purple for  $1 \mu\text{M}$  Hh and  $1 \mu\text{M}$  HS, green for  $2 \mu\text{M}$  Hh and  $1 \mu\text{M}$  HS, turquoise for  $1 \mu\text{M}$  Hh and  $1 \mu\text{M}$  dermatan disulfate, blue for  $3 \mu\text{M}$  Hh and  $1 \mu\text{M}$  dermatan disulfate, and red for  $1 \mu\text{M}$  Hh only (control).

responses in the sensorgrams (Figure 4A) of heparin–Ihog–Hh interactions are due to specific binding of Hh to Ihog.

SPR experiments were next performed to compare the effect of  $1 \mu\text{M}$  HS and  $1 \mu\text{M}$  dermatan disulfate on the Ihog–Hh interaction. The results showed that dermatan disulfate had an effect similar to that of HS in enhancing binding of Hh to Ihog (Figure 4B) and that these interactions were concentration-dependent. However, the shapes of the sensorgrams were different. The HS–Ihog–Hh interaction exhibited a slow on rate (association) and a very slow off rate (dissociation), while the dermatan disulfate Ihog–Hh interaction resulted in a fast on rate and a fast off rate.

**Characterization of Binding of CDO and Shh to Heparin and Heparan Sulfate.** CDO, a mammalian homologue of Ihog, has not been shown to bind to heparin, but a region of positive charge important for binding of IhogFn1 to heparin

is conserved in CDOFn2 (because of an inserted FNIII repeat in CDO relative to Ihog, CDOFn2 is homologous to IhogFn1) (19). SPR analysis shows that CDOFn2-3 (homologous to IhogFn1-2) binds to a heparin-coupled biochip with a dissociation constant of  $\sim 1.5 \mu\text{M}$  (Figure 5A). The kinetics of binding of CDOFn2-3 and IhogFn1-2 to heparin are similar and exhibit a fast on rate and a fast off rate that result in dissociation constants ( $K_{\text{D}}$ ) in the low to midmicromolar range (Table 1). To map the heparin-binding region of CDO, two CDO fragments (CDOFn2 and CDOFn3) were constructed. SPR sensorgrams clearly demonstrate that CDOFn2 binds to heparin ( $K_{\text{D}} = 6 \mu\text{M}$ ), while CDOFn3 shows no appreciable binding (Figure 5B,C). This result indicates that CDOFn2 accounts for virtually all of the binding affinity of CDOFn2-3 for heparin, which is consistent with conservation in CDOFn2 of the basic region shown to mediate heparin binding in IhogFn1.

Shh binds to heparin through both high-affinity and low-affinity sites, and a Cardin–Weintraub consensus sequence for heparin binding at the N-terminus of Shh has been shown to mediate high-affinity Shh–heparin interactions (27–29). SPR analysis of binding of Shh to heparin and HS-coupled biochips revealed dissociation constants of  $\sim 67 \text{ nM}$  and  $32 \mu\text{M}$ , respectively (Figure 5D,E). The  $\sim 500$ -fold tighter binding of Shh to heparin compared to that of HS likely reflects a strong preference of Shh for more highly sulfated regions or forms of HSPGs. The  $> 1000$ -fold tighter binding of Shh to heparin relative to binding of heparin by HhN arises primarily through a  $> 1000$ -fold increase in the on rate (Table 1).

**Estimation of the Heparin Binding Size in the Interaction of Heparin with Ihog and Shh Proteins.** Solution–surface competition experiments were performed by SPR to examine the effect of saccharide chain size of heparin on the heparin–Ihog interaction. In the first set of experiments, different amounts of heparin or LMW heparin were added to a solution of IhogFn1-2 before injection onto a heparin biochip. Increased concentrations of competing heparin and LMW heparin decreased the observed level of binding of Ihog to the surface heparin (Figure 6). The  $\text{IC}_{50}$  values (concentration of competing analyte resulting in a 50% decrease in the response) for heparin and LMW heparin were 200 and 800 nM, respectively. Similar competition studies conducted with Shh (data not shown) resulted in  $\text{IC}_{50}$  values for heparin and LMW heparin of 50 and 700 nM, respectively.

Heparin-derived oligosaccharides of a defined length (from dp4 to dp20) were used in a second competition study to estimate the minimum size of the binding site in heparin for IhogFn1-2 and Shh. A single concentration ( $1 \mu\text{M}$ ) of each heparin oligosaccharide was added to either IhogFn1-2 or Shh prior to its injection over a heparin biochip. The heparin tetrasaccharide (dp4) exhibited no competition for Ihog binding, but the remaining heparin oligosaccharides (dp8–dp20) exhibited a monotonic increase in their level of competition with heparin for Ihog binding (Figure 7A,B). The chain length dependence of competition for IhogFn1-2 binding suggests that the minimum oligosaccharide size required to observe measurable binding to IhogFn1-2 is larger than a tetrasaccharide. Shh competition studies (Figure 7C,D) also show chain length-dependent binding. In this case, however, neither the tetrasaccharide nor the octasaccharide effectively competed with heparin for Shh binding. Instead,

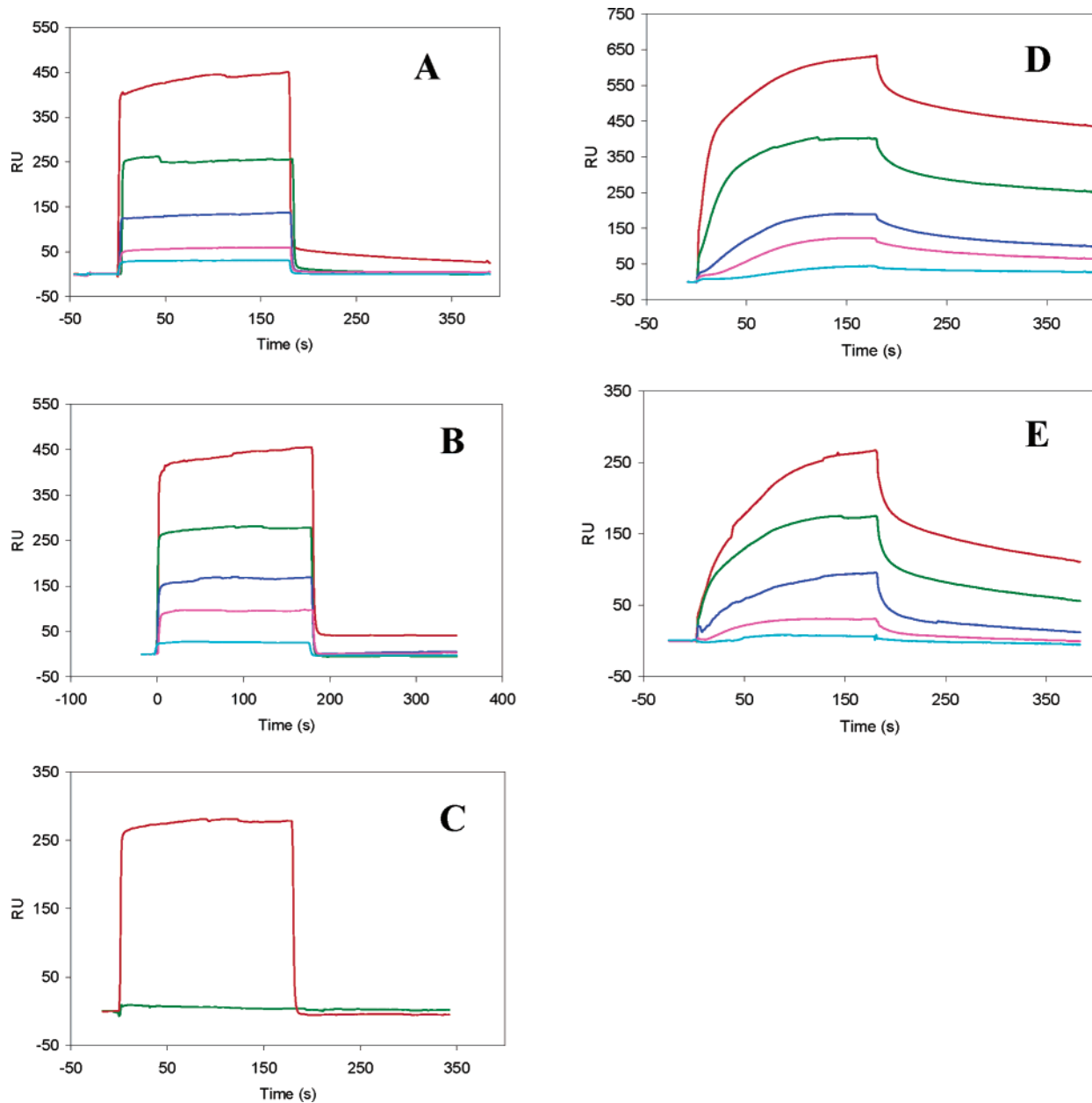


FIGURE 5: SPR sensorgrams of protein–heparin or –HS interactions. (A) CDO (hCDOFn2-3)–heparin interaction. Concentrations of hCDOFn2-3 (from top to bottom) were 2000, 1000, 500, 200, and 100 nM, respectively. (B) hCDOFn2–heparin interaction. Concentrations of hCDOFn2 (from top to bottom) were 2000, 1000, 500, 250, and 100 nM, respectively. (C) Comparison of hCDOFn2–heparin and hCDOFn3–heparin interaction: red for the sensorgram of the hCDOFn2–heparin interaction and green for the sensorgram of the hCDOFn3–heparin interaction. (D) Shh (mShhN)–heparin interaction. Concentrations of mShhN (from top to bottom) were 1000, 500, 200, 100, and 50 nM, respectively. (E) Shh (mShhN)–HS interaction. Concentrations of mShhN (from top to bottom) were 1000, 750, 500, 200, and 100 nM, respectively.

the minimum size for Shh binding appears to be larger than an octasaccharide.

*Specificity of the HS GAG for Signaling Proteins.* Genetic experiments have identified HSPGs as important regulators of the tissue distribution of extracellular signaling molecules such as members of the Hh, Wingless (Wnt/Wg), bone morphogenetic protein (BMP), and fibroblast growth factor (FGF) families (26). HSPGs consist of a core protein to which one or more GAG chains are attached. The specificity of IhogFn1-2 and Shh for HS was next examined by competition experiments involving other GAGs (the affinity of HhN for HS was too weak to perform these experiments with HhN). In these experiments, different concentrations

of natural and chemically modified GAGs (Figure 2) were added to IhogFn1-2 or Shh before injection over the surface HS biochip. The inhibition behavior of each GAG toward Ihog and Shh binding to surface HS was very similar, and the  $IC_{50}$  values for all the GAGs that were tested are summarized in Table 2. Heparin and dermatan disulfate exhibited the strongest inhibition, binding tighter to both proteins than did HS. Weak inhibitory activity was observed in the following order: chondroitin sulfate E > heparan sulfate > chondroitin sulfate B. Chondroitin sulfates A, C, and D, hyaluronic acid, and acharan sulfate were unable to compete with surface HS at the concentrations that were examined.

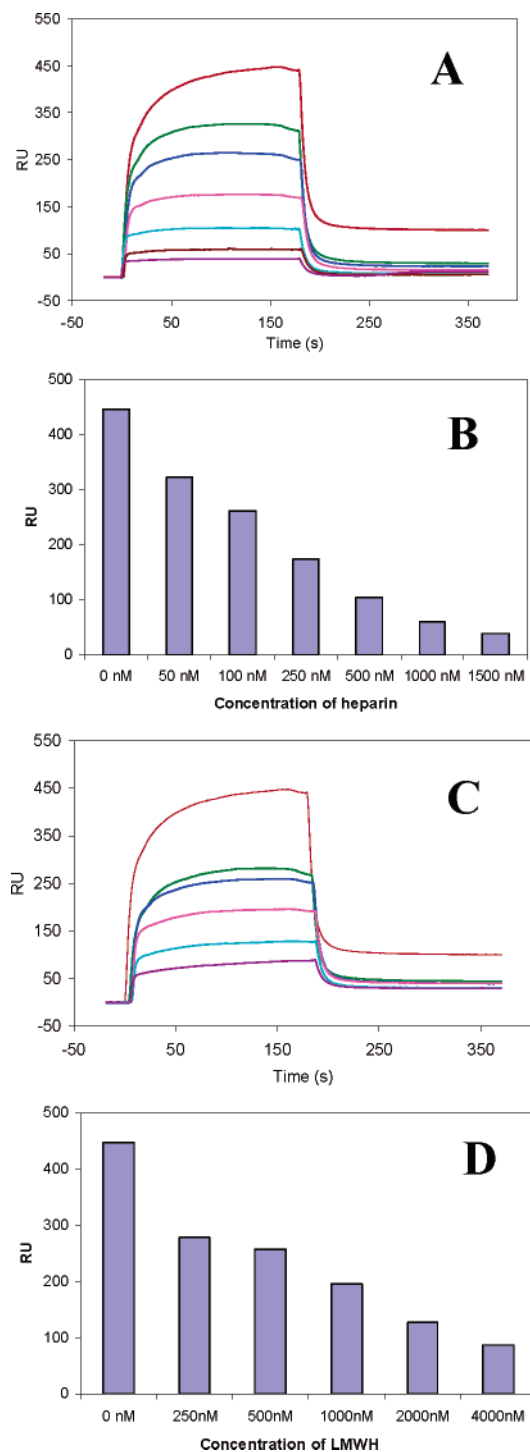


FIGURE 6: Solution heparin or LMW heparin–surface heparin competition. (A) The Ihog (IhogFn1-2) concentration was 500 nM, and concentrations of heparin in solution (from top to bottom) were 0, 50, 100, 250, 500, 1000, and 1500 nM, respectively. (B) RU for binding of Ihog to surface heparin via competition with different concentrations of heparin in solution. (C) The Ihog concentration was 500 nM, and concentrations of LMW heparin in solution (from top to bottom) were 0, 50, 100, 250, 500, 1000, and 1500 nM, respectively. (D) RU for binding of Ihog to surface LMW heparin via competition with different concentrations of heparin in solution.

## DISCUSSION

HSPGs consist of several families of core proteins covalently linked to large glycosaminoglycan (GAG) chains and are found on the cell surface and in the ECM. Intensive

biochemical and genetic studies have shown that HSPGs play crucial roles in regulating key developmental signaling pathways, such as the Wnt, Hedgehog, transforming growth factor- $\beta$ , and fibroblast growth factor pathways (30). In the Hh signaling pathway, HSPGs are essential for proper Hh distribution, stabilization, and signaling activity (1, 31). HSPGs are also believed to facilitate the presentation of Hh ligand to responding cells and participate as part of a larger receptor complex (30).

It is well-established that Shh, *Drosophila* IhogFn1-2, and HhN interact with heparin and that these interactions are important for normal Hh signaling (19, 26–29). However, the kinetics for these interactions between hedgehog proteins and heparin or HSPGs have not previously been determined. Furthermore, although heparin is a model GAG commonly used to study GAG–signaling molecule interactions, cell surface HS is the most likely physiological binding partner for these signaling molecules. SPR analysis showed heparin bound to IhogFn1-2, HhN, CDO, and Shh with different affinities. Shh exhibited the highest heparin binding affinity (67 nM), while each of the other proteins interactions with heparin was in the micromolar range. In contrast, the putative natural ligand, HS, showed a micromolar binding affinity for Ihog and Shh, but binding of HS to Hh and CDO was too weak to detect. These data suggest that HS may be involved in regulating the distribution of the Shh ligand promoting its interaction with the CDO/BOC receptors.

Moreover, SPR analysis of the interaction of IhogFn1-2 with HhN showed that it required heparin (or HS) to be present, suggesting that this GAG family promotes the binding of these signaling molecules. These results are consistent with previous findings that purified HhN and IhogFn1-2 do not interact appreciably unless heparin is present (19). The binding affinity of HhN for IhogFn1-2 in the presence of heparin determined by SPR ( $K_D = 1.8 \mu\text{M}$ ) matches well with the binding affinity ( $K_D = 0.4\text{--}8 \mu\text{M}$ ) determined by analytical ultracentrifugation (19). Interestingly, another SPR experiment showed that although dermatan disulfate binds Ihog tighter than HS and also promotes interaction of IhogFn1-2 with HhN, the shape of the resulting sensorgrams and, hence, the binding kinetics are markedly different from those of HS. This suggests that the heparin–HS GAG structure influences the nature of Ihog–HhN interactions.

SPR solution–surface competition experiments were used (1) to explore the affect of heparin saccharide chain size on the heparin–Ihog and heparin–Shh interactions, (2) to test the specificity of the heparin/HS GAGs in promoting these interactions, and (3) to investigate the impact of GAG structure (e.g., sulfation level) on these interactions. The competition study with heparin and LMW heparin showed different  $\text{IC}_{50}$  values (200 and 800 nM, and 50 and 700 nM for Ihog and Shh, respectively), suggesting the binding was dependent on chain length. This was supported by competition SPR using defined chain length heparin-derived oligosaccharides. The chain length dependence observed in the competition SPR study suggested that the minimum heparin oligosaccharide for IhogFn1-2 and Shh binding was greater than a tetrasaccharide and octasaccharide (dp4 and dp8), respectively. Similar chain length requirements for heparin/HS protein interactions have been reported for binding to other proteins (32, 33). Moreover, minimum GAG chain

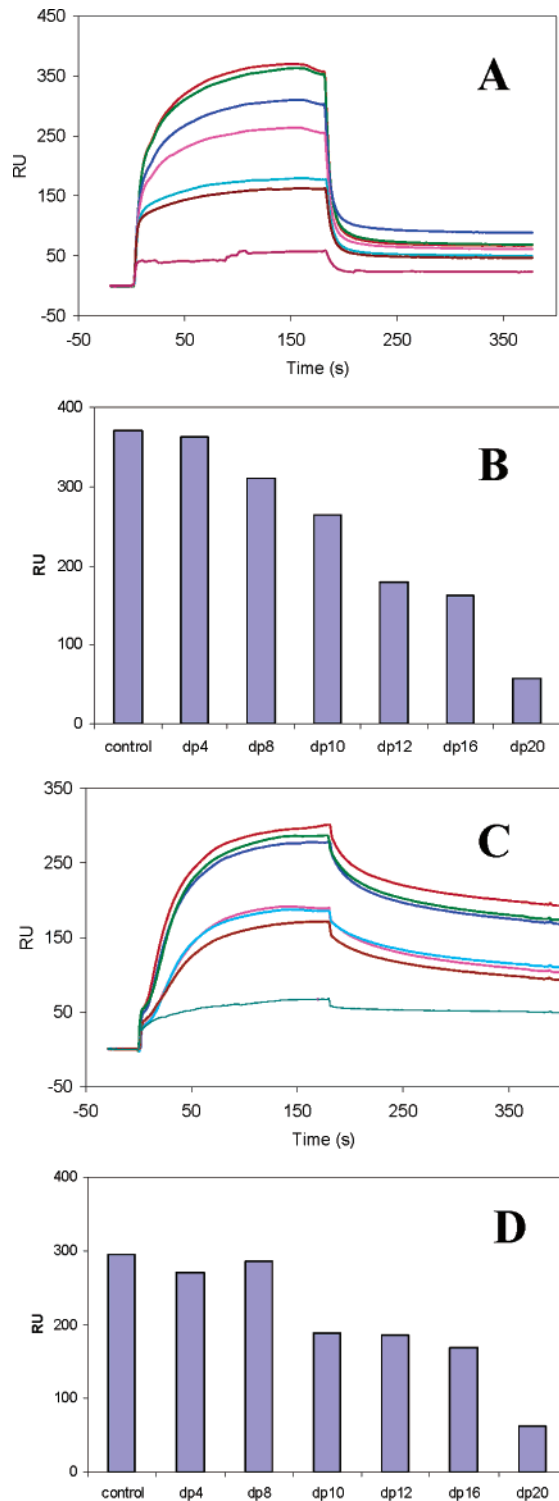


FIGURE 7: (A) Sensorgrams of solution heparin oligosaccharide–surface heparin competition (Ihog concentration of 500 nM and concentration of the heparin oligosaccharide solution of 1000 nM). Sensorgrams from top to bottom are as follows: Ihog only control, Ihog and dp4, Ihog and dp8, Ihog and dp10, Ihog and dp12, Ihog and dp16, and Ihog and dp20, respectively. (B) RU for binding of Ihog to surface heparin via competition with different sizes of heparin oligosaccharides in solution. (C) Sensorgrams of solution heparin oligosaccharide–surface heparin competition (Shh concentration of 1000 nM and concentration of the heparin oligosaccharide solution of 1000 nM). Sensorgrams from top to bottom are as follows: Shh only control, Shh and dp4, Shh and dp8, Shh and dp10, Shh and dp12, Shh and dp16, and Shh and dp20. (D) RU for binding of Shh to surface heparin via competition with different sizes of heparin oligosaccharides in solution.

Table 2: IC<sub>50</sub> Values of GAGs to Inhibit Ihog or Shh Binding to Surface HS

GAG	IC <sub>50</sub> (nM) for Ihog	IC <sub>50</sub> (nM) for Shh
chondroitin sulfate A	ni <sup>a</sup>	ni <sup>a</sup>
chondroitin sulfate C	ni <sup>a</sup>	ni <sup>a</sup>
chondroitin sulfate E	1000	500
chondroitin sulfate D	ni <sup>a</sup>	ni <sup>a</sup>
hyaluronic acid, sodium salt	ni <sup>a</sup>	ni <sup>a</sup>
dermatan sulfate	3000	ni <sup>a</sup>
dermatan disulfate	100	100
heparan sulfate	1000	1500
acharan sulfate	ni <sup>a</sup>	ni <sup>a</sup>
NDeS-heparin	ni <sup>a</sup>	ni <sup>a</sup>
DeS-heparin	ni <sup>a</sup>	ni <sup>a</sup>
2-desulfated IdoA heparin	500	70
heparin	25	10

<sup>a</sup> No inhibition.

lengths can be critical for accommodation of the binding of two proteins for steric reasons and because shorter saccharide chains may not suffice in neutralizing the positively charged heparin-binding regions in proteins and avoiding strong electrostatic repulsion between heparin-binding proteins.

The SPR solution–surface competition experiments with natural and chemically modified GAGs clearly showed that the inhibitory activity of heparin is greatly reduced when it is desulfated, and the inhibitory activity of dermatan sulfate increases when it is sulfonated to prepare dermatan disulfate. These data indicate that the interactions between IhogFn1-2 and GAGs or Shh and GAGs, while structure-dependent, are influenced by the level of GAG sulfation. This result is consistent with the preference of both IhogFn1-2 and Shh for the more highly sulfated heparin than the less sulfated HS. It also consistent with the inhibitory activity pattern of GAGs: heparin (2.8 mol of sulfate per disaccharide)  $\gg$  chondroitin sulfate E (1.5–2 mol of sulfate per disaccharide)  $>$  heparan sulfate (1.2 mol of sulfate per disaccharide)  $>$  chondroitin sulfate B (1 mol of sulfate per disaccharide). On the other hand, acharan sulfate (1 mol of sulfate per disaccharide), chondroitin sulfates A, C, and D ( $<$ 1 mol of sulfate per disaccharide), and hyaluronic acid (0 mol of sulfate per disaccharide) were unable to compete with surface HS at the concentrations that were examined. Interestingly, NDeS-heparin exhibited no detectable inhibitory activity, suggesting that the *N*-sulfo group is critical for the interaction between heparin and Ihog or Shh. The *N*-sulfo group is found only in the heparin/HS GAG family, further supporting the specific reliance of the Hh signaling pathway on the HS family of PGs.

In summary, SPR analysis shows that Hh, Shh, Ihog, and CDO all bind heparin but with differing affinities. The SPR solution competition study shows that the minimum length of a heparin chain to show measurable binding to IhogFn1-2 is greater than a tetrasaccharide, and the minimum length required to bind Shh is greater than an octasaccharide. Also, Shh shows the highest affinity for heparin of all the proteins that have been examined. HS shows a lower affinity for IhogFn1-2 and Shh and no detectable binding to CDOFn2-3 and HhN. Higher sulfation levels of GAGs enhance their binding affinities for all proteins that have been tested, suggesting that high sulfation domains in HS are responsible for mediating Hh signaling interactions.



## REFERENCES

1. Ingham, P. W., and McMahon, A. P. (2001) Hedgehog signaling in animal development: Paradigms and principles, *Genes Dev.* 15, 3059–3087.
2. Nusslein-Volhard, C., and Wieschaus, E. (1980) Mutations affecting segment number and polarity in *Drosophila*, *Nature* 287, 795–801.
3. Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H., and Beachy, P. A. (1996) Cyclopia and defective axial patterning in mice lacking *Sonic hedgehog* gene function, *Nature* 383, 407–413.
4. Bitgood, M. J., Shen, L., and McMahon, A. P. (1996) Sertoli cell signaling by Desert hedgehog regulates the male germline, *Curr. Biol.* 6, 298–304.
5. Muenke, M., and Beachy, P. A. (2000) Genetics of ventral forebrain development and holoprosencephaly, *Curr. Opin. Genet. Dev.* 10, 262–269.
6. Teleman, A. A., Strigini, M., and Cohen, S. M. (2001) Shaping morphogen gradients, *Cell* 105, 559–562.
7. Taipale, J., and Beachy, P. A. (2001) The Hedgehog and Wnt signalling pathways in cancer, *Nature* 411, 349–354.
8. Kiselyov, A. S. (2006) Targeting the hedgehog signaling pathway with small molecules, *Anticancer Agents Med. Chem.* 6, 445–449.
9. Mann, R. K., and Beachy, P. A. (2004) Novel lipid modification of secreted protein signals, *Annu. Rev. Biochem.* 73, 891–923.
10. Wilson, C. W., and Chuang, P. T. (2006) New “hogs” in Hedgehog transport and signal reception, *Cell* 125, 435–438.
11. Taipale, J., Cooper, M. K., Maiti, T., and Beachy, P. A. (2002) Patched acts catalytically to suppress the activity of Smoothed, *Nature* 418, 892–897.
12. Desbordes, S. C., and Sanson, B. (2003) The glypican Dally-like is required for Hedgehog signalling in the embryonic epidermis of *Drosophila*, *Development* 130, 6245–6255.
13. Lum, L., Yao, S., Mozer, B., Rovescalli, A., Von Kessler, D., Nirenberg, M., and Beachy, P. A. (2003) Identification of Hedgehog pathway components by RNAi in *Drosophila* cultured cells, *Science* 299, 2039–2045.
14. Han, C., Belenkaya, T. Y., Wang, B., and Lin, X. (2004) *Drosophila* glypicans Dally and Dally-like shape the extracellular Wingless morphogen gradient in the wing disc, *Development* 131, 601–611.
15. The, I., Bellaiche, Y., and Perrimon, N. (1999) Hedgehog movement is regulated through tout velu-dependent synthesis of a heparan sulfate proteoglycan, *Mol. Cell* 4, 633–639.
16. Yao, S., Lum, L., and Beachy, P. (2006) The ihog cell-surface proteins bind Hedgehog and mediate pathway activation, *Cell* 125, 343–357.
17. Zhang, W., Kang, J. S., Cole, F., Yi, M. J., and Krauss, R. S. (2006) Cdo functions at multiple points in the Sonic Hedgehog pathway, and Cdo-deficient mice accurately model human holoprosencephaly, *Dev. Cell* 10, 657–665.
18. Tenzen, T., Allen, B. L., Cole, F., Kang, J. S., Krauss, R. S., and McMahon, A. P. (2006) The cell surface membrane proteins Cdo and Boc are components and targets of the Hedgehog signaling pathway and feedback network in mice, *Dev. Cell* 10, 647–656.
19. McLellan, J. S., Yao, S., Zheng, X., Geisbrecht, B. V., Ghirlando, R., Beachy, P. A., and Leahy, D. J. (2006) Structure of a heparin-dependent complex of hedgehog and Ihog, *Proc. Natl. Acad. Sci. U.S.A.* 103, 17208–17213.
20. Brister, S. J., Buchanan, M. R., Griffin, C. C., Van Gorp, C. L., and Linhardt, R. J. (1999) Dermatan disulfate, an inhibitor of thrombin and complement activation, U.S. Patent 5,922,690.
21. Yates, E. A., Santini, F., Guerrini, M., Naggi, A., Torri, G., and Casu, B. (1996) <sup>1</sup>H and <sup>13</sup>C NMR spectral assignments of the major sequences of twelve systematically modified heparin derivatives, *Carbohydr. Res.* 294, 15–27.
22. Pervin, A., Gallo, C., Jandik, K. A., Han, X.-J., and Linhardt, R. J. (1995) Preparation and Structural Characterization of Large Heparin-Derived Oligosaccharides, *Glycobiology* 5, 83–95.
23. Geisbrecht, B. V., Bouyain, S., and Pop, M. (2006) An optimized system for expression and purification of secreted bacterial proteins, *Protein Expression Purif.* 46, 23–32.
24. Hernaiz, M., Liu, J., Rosenberg, R. D., and Linhardt, R. J. (2000) Enzymatic modification of heparan sulfate on a biochip promotes its interaction with antithrombin III, *Biochem. Biophys. Res. Commun.* 276, 292–297.
25. Stringer, S. E., and Gallagher, J. T. (1997) Heparan sulfate, *Int. J. Biochem. Cell Biol.* 29, 709–714.
26. Hacker, U., Nybakken, K., and Perrimon, N. (2005) Heparan sulphate proteoglycans: The sweet side of development, *Nat. Rev. Mol. Cell Biol.* 6, 530–541.
27. Bumcrot, D. A., Takada, R., and McMahon, A. P. (1995) Proteolytic processing yields two secreted forms of sonic hedgehog, *Mol. Cell Biol.* 15, 22294.
28. Lee, J. J., Ekker, S. C., von Kessler, D. P., Porter, J. A., Sun, B. I., and Beachy, P. A. (1994) Autoproteolysis in hedgehog protein biogenesis, *Science* 266, 1528.
29. Rubin, J. B., Choi, Y., and Segal, R. A. (2002) Cerebellar proteoglycans regulate sonic hedgehog responses during development, *Development* 129, 2223.
30. Lin, X. (2004) Functions of heparin sulfate proteoglycans in cell signaling during development, *Development* 131, 1563.
31. Lin, X., and Perrimon, N. (2002) Developmental roles of heparan sulfate proteoglycans in *Drosophila*, *Glycoconjugate J.* 19, 363.
32. Linhardt, R. J., and Toida, T. (2004) Role of glycosaminoglycans in cellular communication, *Acc. Chem. Res.* 37, 431.
33. Capilla, I., and Linhardt, R. J. (2002) Heparin-protein interactions, *Angew. Chem., Int. Ed.* 41, 390.

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