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Structural determinants of heparan sulfate interactions with Slit proteins

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Abstract

We have previously demonstrated that the Slit proteins, which are involved in axonal guidance and related processes, are high-affinity ligands of the heparan sulfate proteoglycan glypican-1. Glypican–Slit protein interactions have now been characterized in greater detail using two approaches. The ability of heparin oligosaccharides of defined structure (ranging in size from disaccharide to tetradeccasaccharide) to inhibit binding of a glypican-Fc fusion protein to recombinant human Slit-2 was determined using an ELISA. Surface plasmon resonance (SPR) spectroscopy, which measures the interactions in real time, was applied for quantitative modeling of heparin–Slit binding on heparin biochips. Heparin was covalently immobilized on these chips through a pre-formed albumin–heparin conjugate, and the inhibition of Slit binding by heparin, LMW heparin, and heparin-derived oligosaccharides (di-, tetra-, hexa-, and octa-) was examined utilizing solution competition SPR. These competition studies demonstrate that the smallest heparin oligosaccharide competing with heparin binding to Slit was a tetrasaccharide, and that in the ELISA maximum inhibition (~60% at 2 μM concentration) was attained with a dodecasaccharide.

Keywords: Heparan sulfate; Heparin; Slit proteins; Glypican-1; ELISA; Surface plasmon resonance spectroscopy

We previously described a major heparan sulfate proteoglycan of nervous tissue [1–3] that we later cloned and identified as the rat homologue of glypican-1 [4], which was the initial member of a family of glycosylphosphatidylinositol-anchored heparan sulfate proteoglycans that is currently composed of six vertebrate proteins. The glypicans share an N-terminal signal sequence followed by a globular domain containing a characteristic pattern of 14 cysteine residues, a presumably more extended domain with the heparan sulfate attachment sites (suggesting that the HS chains are deployed close to the cell surface), and a hydrophobic C-terminal sequence that is involved in the formation of the GPI anchor structure. Glypican-1 has a 56 kDa core protein and 3–4 heparan sulfate chains. Northern analysis demonstrated high levels of glypican-1 mRNA in brain and skeletal muscle, and in situ hybridization histochemistry showed that glypican-1 mRNA is especially prominent in cerebellar granule cells, large motor neurons in the brain stem, and CA3 pyramidal cells of the hippocampus [5]. From this work and parallel immunocytochemical studies we concluded that glypican-1 is predominantly a neuronal product in the late embryonic and postnatal rat nervous system. Genetic studies provide additional support for a role of glypicans in cell growth and development [6–8]. We have also demonstrated a novel nuclear localization of glypican-1 in nervous tissue, suggesting that it may be involved in the regulation of cell division and survival by direct participation in nuclear processes [9].

We later determined that the Slit proteins, that are known to regulate axonal guidance, branching, dendritic development, and neural migration [10–13], are high-affinity ligands of glypican-1, and in situ hybridization histochemistry showed that these glypican-1 ligands are synthesized by neurons, such as hippocampal pyramidal cells and cerebellar granule cells, where we previously also demonstrated glypican-1 mRNA and immunoreactivity [14,15]. Recombinant human Slit-2 protein and

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the N- and C-terminal portions generated by in vivo proteolytic processing were used in an ELISA to measure binding of a glypican-Fc fusion protein [15]. Saturable and reversible high-affinity binding (which did not require the presence of divalent cations) was seen to the full-length protein and to the C-terminal portion that can be released from the cell membrane, with dissociation constants in the 80–110 nM range, whereas only a relatively low level of binding was detected to the larger N-terminal segment. Co-transfection of 293 cells with Slit and glypican-1 cDNAs followed by immunoprecipitation demonstrated that these interactions also occur in vivo. The binding affinity of the glypican core protein to Slit is an order of magnitude lower than that of the glycanated protein, and *O*-sulfate groups on the heparan sulfate chains play a critical role in the interaction. These findings suggest that glypican binding to the releasable C-terminal portion of Slit may serve as a mechanism for regulating the biological activity of Slit and/or the proteoglycan. In view of the potential importance of glypican–Slit interactions both in normal developmental processes and for repair following CNS injury, in the present study we have obtained more detailed information concerning the molecular determinants affecting the interactions of Slit proteins with glypican-1 heparan sulfate chains.

Materials and methods

Materials. Heparin (MW: 14,000) from porcine intestinal mucosa, sodium salts, was prepared by Celsus Laboratories (Cincinnati, OH). LMW heparin from porcine intestinal mucosa, sodium salt (MW: 5000) prepared using nitrous acid was purchased from Calbiochem–Novabiochem (La Jolla, CA). Heparin lyase I (heparinase, EC 4.2.2.7) was purchased from Sigma Chemical (St. Louis, MO). Gel permeation chromatography was performed on Bio-Gel P2, P6 (superfine) from Bio-Rad (Richmond, CA) or Sephadex G10 from Sigma Chemical (St. Louis, MO). Pioneer Sensor C1 Chip, *N*-hydroxysuccinimide (NHS), and *N*-ethyl-*N*-(dimethyaminopropyl)carbodiimide (EDC) were obtained from BIAcore (Biosensor AB, Uppsala, Sweden). Bovine serum albumin (BSA) was obtained from Amresco (Solon, Ohio).

Preparation of glypican-1-Fc and human Slit-2 fusion proteins. Human embryonic kidney 293 cells were transfected with a glypican-1-Fc fusion protein construct [9] using Lipofectamine 2000 and grown in serum-free DMEM containing 1% ITS$^+$. To separate the glycanated form of the proteoglycan (which was used for all studies) from unglycanated core protein, the conditioned medium was applied to a 0.9×8 cm column of DEAE–Sephacel equilibrated with 150 mM NaCl, 50 mM Tris–HCl, pH 8.0 [2]. After elution with 50 mM Tris–HCl (pH 8.0) containing 0.6 M NaCl, the glycanated glypican-1-Fc was bound to protein A–Sepharose beads and eluted with 0.1 M glycine, pH 3.0.

293 cells were transfected with the pSecTagB vector (Invitrogen, Carlsbad, CA) containing cDNA for the His-tagged uncleavable variant of human full-length Slit-2, in which the nine amino acids encompassing the proteolytic processing site were deleted, producing an uncleavable full-length protein [16]. One molar NaCl extracts of the 293 cells were incubated with nickel–agarose beads for 2 h at 4 °C, and after washing, bound protein was eluted with 10 mM Hepes (pH 7.5) containing 250 mM imidazole and 1 M NaCl. Protein concentrations were determined by the Bradford assay [17].

ELISA. Ninety-six-well plates (Corning Costar #9018) were coated overnight with the human full-length Slit 2 at a saturating concentration of 5 μg/well in PBS. After removing the unbound protein by washing with TBST (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20), the wells were blocked with 10% FBS in TBST for 2 h and then incubated for 18 h at room temperature with glypican-1-Fc (1 μg/well) in PBS. Bound glypican was detected using a biotinylated anti-human Fc antibody (Jackson Immunoresearch; 1:250,000 in TBST, for 2 h), followed by incubation for 20 min with HRP-conjugated streptavidin (1:20,000 in TBST). The colorimetric reaction product from the *o*-phenylenediamine substrate was measured at 450 nm using a Dynatech MRX ELISA plate reader. Non-specific binding was calculated as the binding of glypican-1-Fc to wells coated with 100 μg BSA. From serial dilutions of a known concentration of glypican-1-Fc directly coated on the wells and the corresponding immunoreactivity absorbance, a standard curve was created and used to quantitate the amount of glypican-1-Fc bound.

Preparation and characterization of heparin-derived oligosaccharides. Heparin-derived oligosaccharides were prepared enzymatically. Ten grams of heparin (MW: 14,000, from porcine intestinal mucosa, Celsus Laboratories, Cincinnati, OH) in 160 ml of 50 mM sodium phosphate buffer (pH 7.0) containing 2 mg/ml BSA was sterile filtered through a 0.22 μm filter unit. With the addition of heparin lyase I (Sigma, heparinase, EC 4.2.2.7; 92 mU was added twice at 0 and 24 h), the digestion was carried out at 30 °C. When the absorbance at 232 nm indicated that the digestion was 30% completed, the digestion was stopped by heating at 100 °C for 1 min to inactivate the enzyme. Undigested heparin was removed by ultra-filtration with a membrane (5000 MWCO). Next, the low molecular heparin oligosaccharide (MW, <5000) mixture obtained was fractionated on a Bio-Gel P-6 column (4.8×100 cm) using 200 mM sodium chloride as the running buffer. Fractions consisting of disaccharides, tetrasaccharides, hexasaccharides, octasaccharides, and higher oligosaccharides were obtained for the further purification.

Before HPLC purification, samples of molecular weight <2000 (disaccharide, tetrasaccharide, hexasaccharide, and octasaccharides) were desalted on a Sephadex G-10 column. Charge separation of the sized oligosaccharides was then carried out by a semi-preparative SAX-HPLC on a 5 μm, 0.46×25 cm column (Waters) using a linear gradient from 0.2 to 2 M of sodium chloride (pH 3.5) at a flow rate of 1.0 ml/min. The major peaks were pooled, freeze-dried, and desalted on a Sephadex G-10 column. The purified oligosaccharides were analyzed by analytical SAX-HPLC, capillary electrophoresis (CE), gradient PAGE, and ^{1}H NMR to confirm the purity and structures of each oligosaccharide.

Preparation of heparin biochip. Two batches of albumin–heparin conjugate (from Sigma or synthesized in our laboratory) were used for the preparation of heparin biochips. Covalently bound conjugate of albumin–heparin prepared in our laboratory relied on the condensation reaction of albumin and heparin using *N*-ethyl-*N*-(dimethyaminopropyl) carbodiimide (EDC) [18]. Unreacted albumin and heparin were removed by diethylaminoethyl (DEAE)–cellulose and Cibacron blue Sepharose chromatography, respectively. The biochip with immobilized heparin–BSA conjugate prepared in our laboratory afforded a higher RU (resonance units), making it possible to perform competition studies with this chip.

The heparin biochip was prepared by covalently immobilizing the albumin–heparin conjugate to the biosensor surface through its primary amino groups on a C1 chip (Biosensor AB, Uppsala, Sweden) [19]. Briefly, carboxymethyl groups on the C1 chip surface were first activated using an injection pulse of 50 μl (flow rate, 5 μl/min) of an equimolar mixture of NHS/EDC (final concentration 0.05 M, mixed immediately prior to injection). An albumin–heparin solution (200 μg/ml in sodium acetate buffer with 2 M guanidine hydrochloride, pH 4.0) was applied to the chip surface. Excess unreacted sites on the sensor surface were blocked with a 40 μl injection of 1 M ethanolamine. Successful immobilization was confirmed by the observation of a

~300 RU response increase. To prepare the control flow cell, bovine serum albumin was immobilized on the surface using a similar coupling procedure.

SPR experiments were performed on the BIAcore 3000 (Biosensor AB, Uppsala, Sweden) apparatus operated using BIAcore 3000 version software. Buffers used in SPR were filtered and degassed.

Kinetic measurements of heparin and Slit protein interaction using surface plasmon resonance. For kinetic studies of Slit interactions with heparin, measurements were performed on a BIAcore 3000. Different concentrations (50, 100, 200, 300, and 500 nM) of Slit protein in buffer (1 mM sodium phosphate, pH 7) were injected over both the albumin–heparin (Sigma) and control albumin surfaces simultaneously at a flow rate of 10 μl/min. At the end of each sample injection, the same buffer was flowed over the sensor surface to facilitate dissociation studies. After a 3 min dissociation time, the sensor surface was regenerated by injection of 20 μl of 2 M NaCl. The response was monitored as a function of time (sensorgram) at 25 °C. The control cell was used to subtract the contribution of non-specific interactions with the immobilized albumin on the surface. Kinetic parameters were evaluated using the BIA Evaluation software (Version. 3.1, 1999).

Solution competition study between Slit protein and heparin, LMW heparin, and heparin-derived oligosaccharides using surface plasmon resonance. Slit protein (80 nM) mixed with different concentrations of heparin, LMW heparin, disaccharide, tetrasaccharide, hexasaccharide, and octasaccharide in HBS-EP buffer (0.01 M Hepes, 0.15 M NaCl, 3 mM EDTA, and 0.005% polysorbate, pH 7.4) (BIAcore) was injected over both the albumin–heparin (prepared) and the control albumin surfaces at a flow rate of 25 μl/min. After each run, the dissociation and regeneration were performed as described above. For each set of competition experiments using surface plasmon resonance (SPR), a control experiment (only Slit protein without any heparin or oligosaccharides) was performed to make sure that the surface was completely regenerated and that the results obtained between runs were comparable.

Results and discussion

ELISAs of the interactions of glypican-1 with Slit

Using ELISAs in which we quantitated the inhibition of glypican-1 binding to Slit-2 by heparin oligosaccharides of defined structure [20], it was found that maximum inhibition of binding begins to be seen with a heparin decasaccharide, and is somewhat more extensive with dodecasaccharides and tetradecasaccharides (i.e., 6–7 disaccharide units; Fig. 1). Using the same test system, it was found that the small hexasulfonated molecule suramin (MW 1429 for the sodium salt) at a concentration of 5 μM produced >40% inhibition of glypican-1 binding, whereas the same concentration of sucrose octasulfate had no effect (data not shown). These findings suggest that small molecule inhibitors that can be produced in economical quantities may be effective drugs for perturbing glypican–Slit interactions, and that there is a considerable degree of structural specificity for these effects beyond such properties as net charge.

Kinetic measurement of Slit protein interaction with heparin

The structure of heparin is very similar to the sulfated regions of heparan sulfate, and it has therefore been used as an excellent molecular model for heparan sulfate–protein interaction studies [21]. Most heparin (or heparan sulfate)–protein interactions take place at the cell surface [22]. Consequently, SPR biosensors are widely used to study heparin–protein interactions. These experiments require the immobilization of either heparin or a heparin-binding protein on the surface of a biosensor chip, over which its binding partner, a heparin-binding protein (or heparin), is passed. In natural biological systems, heparan sulfate is immobilized on the cell surface through its core protein [23] and captures heparin-binding proteins that flow over the cell surface. Thus, for SPR studies of heparin–protein interactions, immobilizing

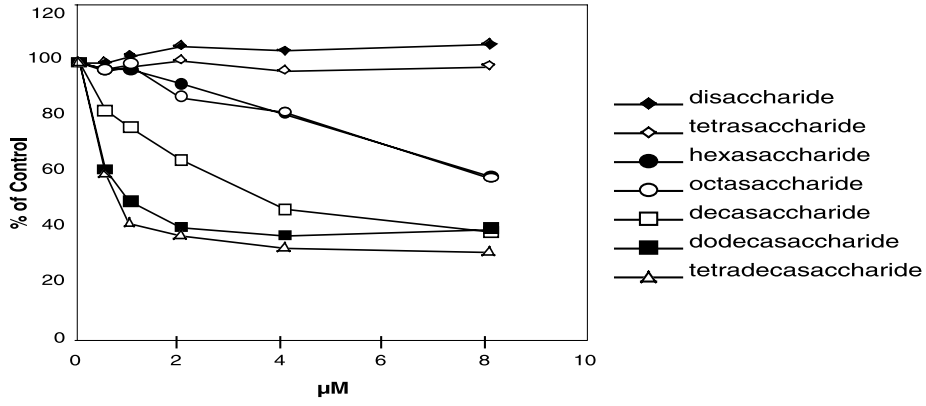

Fig. 1. Inhibition of glypican-1 binding to Slit-2 protein in the presence of heparin oligosaccharides. ELISA wells were coated with purified human Slit 2 protein at a concentration of 5 μg/well. The wells were washed and blocked with 10% FBS/TBST for 2 h and then incubated with glypican-1-Fc (1 μg/well) in the presence of varying concentrations of heparin oligosaccharides. Bound glypican, detected by an anti-human Fc antibody, was measured at equilibrium (18 h) using binding buffer containing 150 mM NaCl. The degree of inhibition is expressed as percent bound compared to control in the absence of oligosaccharides, and was calculated from the total microgram bound minus microgram bound to wells coated with BSA. The points shown in the figure are averages of duplicate determinations, with standard errors that are too low to be visibly shown by error bars.

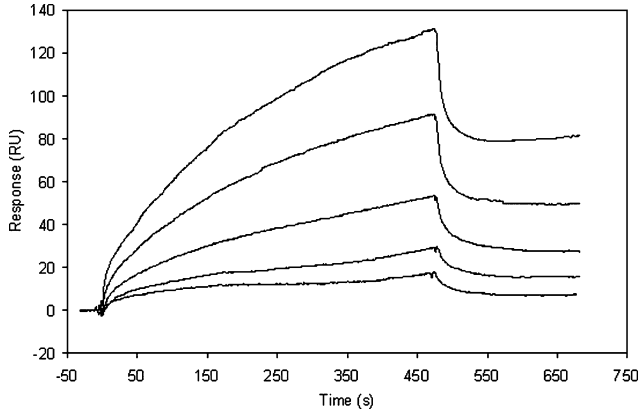

Fig. 2. SPR sensorgrams of Slit protein–heparin interactions. Varying concentrations of Slit protein (50, 100, 200, 300, and 500 nM) were injected over a BIAcore chip with heparin immobilized on the surface.

heparin to the surface of biochips is a better way to naturally mimic heparan sulfate on the cell surface. Here, we used SPR to obtain information concerning the kinetics of binding of heparin to Slit and its inhibition by heparin and heparin oligosaccharides. This approach can also be helpful in establishing both the minimum and optimal size of the binding domain in heparin for Slit.

Sensorgrams for the binding of Slit protein to immobilized heparin are shown in Fig. 2. Kinetic analysis of the interaction between Slit protein and heparin afforded a k_d value of $1.1 \times 10^{-3}\,s^{-1}$, a k_a value of $3.3 \times 10^{3}\,M^{-1}\,s^{-1}$, and a K_D of $3.3 \times 10^{-7}\,M$. These binding kinetics data are comparable to our previous report [15] on glypican–Slit interactions which indicated a single class of high-affinity binding sites with dissociation constants of 80–100 nM for full-length Slit and its C-terminal portion, and 300 nM for the N-terminal construct. It has also recently been shown that glypican-1 is a high-affinity ligand ($K_d = 10$ nM) of the novel epidermal growth factor-related peptide, Cripto-1, which activates the tyrosine kinase c-Src as a result of this specific interaction [24].

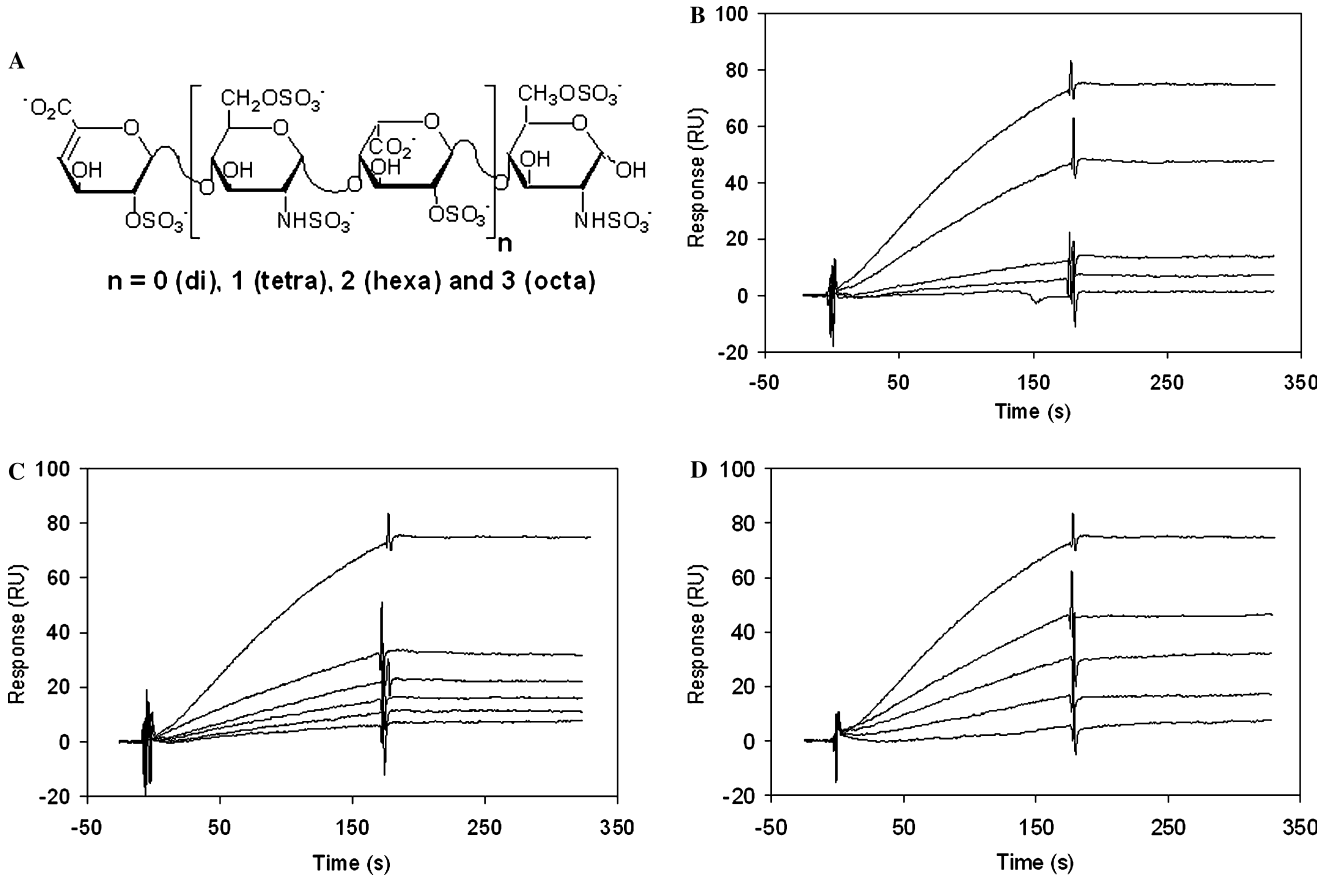

Fig. 3. Structures of heparin-derived oligosaccharides and SPR sensorgrams of competition studies with immobilized heparin for Slit protein binding. (A) Structures of heparin-derived oligosaccharides. (B) Solution heparin/surface heparin competition (concentrations of heparin in solution were 0, 0.02, 0.1, 0.5, and 2 nM, respectively). (C) Solution LMW heparin/surface heparin competition (concentrations of LMW heparin in solution were 0, 20, 100, and 500 nM and 5 and 20 μM, respectively). (D) Solution octasaccharide/surface heparin competition (concentrations of octasaccharide in solution were 0, 100, 200, 300, and 500 μM, respectively). The concentration of Slit protein was 80 nM for all experiments, and varying amounts of heparin, LMW heparin, and oligosaccharides were added to the protein solution in HBS-EP buffer.

Solution competition study between Slit and heparin, LMW heparin, and heparin-derived oligosaccharides using SPR

To examine the effect of saccharide chain size of heparin on the Slit protein interaction, solution/surface competition experiments were performed by SPR. In each competition experiment, different amounts of heparin, LMW heparin, and heparin-derived saccharide (from di- to octa-) of defined structures (Fig. 3A) were added in the analyte (Slit protein) solution. When different concentrations of heparin disaccharide were present in the Slit protein/heparin interaction solution, no competition effect was observed (not shown). In all other cases, increasing concentrations of competing analytes (heparin, LMW heparin, tetrasaccharide, hexasaccharide, and octasaccharide) decreased the observed binding of Slit protein (Figs. 3 and 4). For example, when the concentration of octasaccharide was 100 μM (Fig. 3D), the interaction decreased to approximately 50% of the control value (no competing analyte present). At a concentration of octasaccharide of 500 μM, virtually no binding was observed. The IC_{50} value is commonly defined as the concentration of competing analyte resulting in 50% of the response observed in the absence of competing analyte. The IC_{50} values for heparin, LMW heparin, tetrasaccharide, and hexasaccharide were calculated from these curves and are shown in Table 1. The variation in IC_{50} values observed suggests that the interaction between Slit protein and heparin is chain-length dependent, and that the minimum heparin oligosaccharide size that competes with heparin binding to Slit is a tetrasaccharide.

Table 1
IC_{50} for different oligosaccharides, heparin, and LMWH in solution in Slit–heparin interaction

Heparin/LMWH/oligosaccharides	IC_{50}
Heparin	~0.02 nM
LMWH	~20 nM
Octa-	~100 μM
Hexa-	~400 μM
Tetra-	~600 μM

Conclusions

The studies described here have provided additional information on the fine structural features required for the heparan sulfate-mediated binding of glypican-1 to Slit proteins, and also demonstrated that significant inhibition of these interactions can be obtained by small sulfated molecules whose structures are entirely unrelated to those of heparin and heparan sulfate. In addition to earlier evidence for the role of cell surface heparan sulfate in the repulsive guidance activities of Slit-2 protein [25], it has recently been reported that both Slit-2 and glypican-1 mRNA are strongly up-regulated and co-expressed in the reactive astrocytes of injured adult brain [26,27], suggesting a possible function of Slit proteins and glypican-1 in the adult CNS (where few axon guidance events occur) as significant components of the inhibitory environment after injury. It is therefore possible that glypican-1 and Slit proteins, either acting alone or as a complex, are a significant factor in preventing axonal regeneration after spinal cord injury. Although significant amounts of full-length unprocessed Slit are present in nervous tissue (ac-

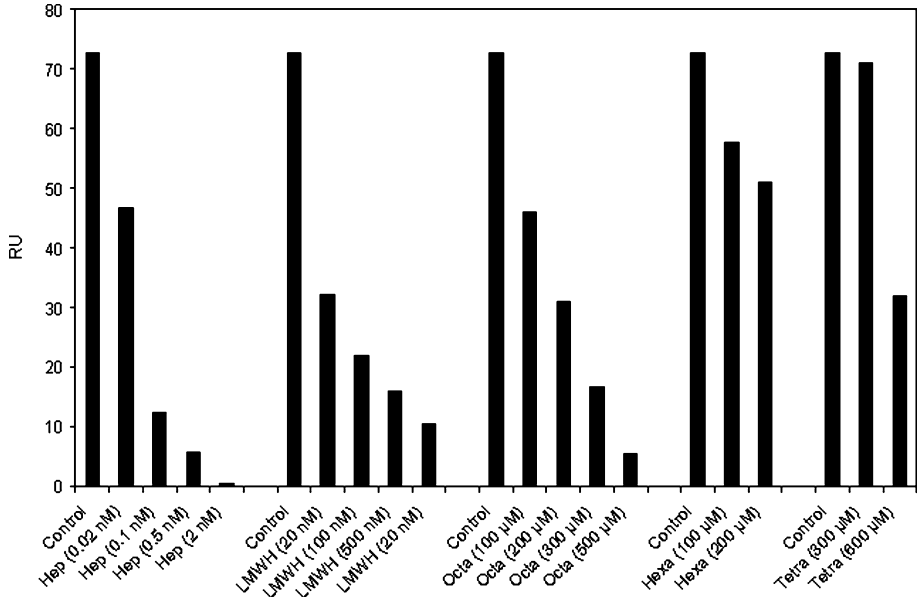

Fig. 4. Graphical summary of solution surface heparin competition studies.

counting for its original identification as a glypican-1 ligand in the form of the 200 kDa protein; [14]), because the smaller C-terminal proteolytic processing product binds with high affinity to glypican-1 this would prevent its diffusion from sites of central nervous system injury. Whether any adverse effects on axonal regeneration are due to a glypican–Slit complex or the retention of C-terminal Slit protein fragments at the injury site, it is reasonable that by inhibiting their interaction heparin-like compounds could limit the functional consequences of spinal cord injury. We are therefore currently exploring the possibility that relatively low molecular weight oligosaccharides of defined structure or other small polysulfated molecules such as suramin could prove useful in inhibiting interactions of glypican-1 with Slit proteins or other ligands, and thereby serve as a pharmacological means for promoting recovery in conditions such as spinal cord injury.

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