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Analysis of the Interaction between Heparin and Follistatin and Heparin and Follistatin−Ligand Complexes Using Surface Plasmon Resonance

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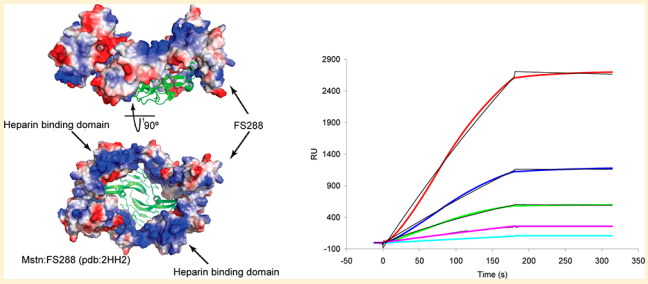
ABSTRACT: Heparin and related heparan sulfate interact with a number of cytokines and growth factors, thereby playing an essential role in many physiological and pathophysiological processes by involving both signal transduction and the regulation of the tissue distribution of cytokines/growth factors. Follistatin (FS) is an autocrine protein with a heparin-binding motif that serves to regulate the cell proliferative activity of the paracrine hormone, and member of the TGF-β family, activin A (ActA). Follistatin is currently under investigation as an antagonist of another TGF-β family member, myostatin (Mstn), for the promotion of muscle growth in diseases associated with muscle atrophy. In this study, we employ surface plasmon resonance (SPR) spectroscopy to dissect the binding interactions between the heparin polysaccharide and both free follistatin (FS288) and its complexes (FS288−ActA and FS288−Mstn). FS288 complexes show much higher heparin binding affinity than FS288 alone. SPR solution competition studies using heparin oligosaccharides showed that the binding of FS288 and its complex to heparin is dependent on chain length. Full chain heparin or large oligosaccharides, having 18−20 sugar residues, show the highest binding activity for FS288 and the FS288−ActA complex, whereas smaller heparin molecules could interact with the FS288−Mstn complex. These interactions were also analyzed in normal physiological buffers and at different salt concentrations and pH values. Unbound follistatin was much more sensitive to all salt concentrations of >150 mM. The binding of heparin to the FS288−ActA complex was disrupted at 500 mM salt, whereas it was actually strengthened for the FS288−Mstn complex. At acidic pH values, binding of heparin to FS288 and the FS288−ActA complex was enhanced. While slightly acidic pH values (pH 6.2 and 5.2) enhanced the binding of the FS288−Mstn complex to heparin, at pH 4 heparin binding was inhibited. Overall, these studies demonstrate that binding of a specific ligand to FS288 differentially regulates its affinity and behavior for heparin molecules.



Heparin and heparan sulfate (HS) are structurally related members of a family of polyanionic, polydisperse, linear polysaccharides called glycosaminoglycans (GAGs), which perform a variety of critical biological functions. They consist of repeating disaccharide subunits of 1→4-linked hexuronic acid, β-D-glucuronic acid (GlcA) or α-L-iduronic acid (IdoA) and glucosamine, α-D-*N*-acetylglucosamine (GlcNAc), or α-D-*N*-sulfoglucosamine (GlcNS).[1,2] Heparin and HS are biosynthesized as proteoglycans (PGs) using the same biosynthetic pathway.[3] While heparin PGs are stored in mast cell granules, HS PGs are ubiquitously expressed on the cell surface of most cell types and in the extracellular matrix (ECM). Interactions between heparin or HS and proteins mediate diverse biological processes, including development, angiogenesis, anticoagulation, inflammation, cancer, and microbial and viral pathogenesis.[4−9] Intensive biochemical and genetic studies have shown that HSPGs play crucial roles in regulating key developmental signaling pathways, such as the pathways for fibroblast growth factors (FGFs), hedgehog (Hh), and transforming growth factor-β (TGF-β).[10] In the FGF signaling pathway, dimerization of the FGF receptor (FGFR) with FGF requires the association of heparin or highly sulfated HS polysaccharide chains of HSPGs; e.g., FGF1 signaling is transmitted across the cell membrane through the formation of a ternary complex of FGF1, FGFR1, and HS.[11−13] In the Hh signaling pathway, HSPGs are essential for proper Hh distribution, stabilization, and signaling activity.[14] HSPGs are also believed to facilitate Hh ligand presentation to responding

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cells and participate as part of a larger receptor complex.[10,15] Thus, an understanding of heparin− and HS−protein interactions at the molecular level is of fundamental importance to biology and will aid in the development of highly specific therapeutic agents for these pathways.[4,9]

Transforming growth factor- β (TGF-β) is the prototypical molecule of a superfamily of ligands that includes TGF-β isoforms, activins, bone morphogenetic proteins (BMPs), and growth and differentiation factors (GDFs).[16,17] Ligands of the TGF-β superfamily of growth factors initiate signal transduction through a bewildering complexity of ligand−receptor interactions, which regulate a diverse set of cellular and physiological functions, including early embryonic development, cellular growth and proliferation, differentiation, migration, and death.[18,19] Myostatin (Mstn), also known as growth and differentiation factor-8 (GDF-8), is a TGF-β family member that has been identified as a strong inhibitor of muscle growth.[20] Mstn knockout mice exhibit muscles that are 2−3 times larger than those of wild-type (WT) mice.[21] A similar phenotype was observed by transgenic overexpression of Mstn inhibitors (antagonists), such as follistatin (FS).[22] FS is a secreted polypeptide that regulates several signaling pathways through its ability to inactivate TGF-β-like growth factor molecules such as activin or bone morphogenetic proteins by sequestering ligands in a tight, nearly irreversible inactive complex.[23] Previous biochemical studies indicated that the different follistatin isoforms (FS288, FS303, and FS315) have different binding affinities for heparin, which potentially influence the physiological functions and locations of these isoforms.[23] It was proposed that FS288 represents a predominantly cell-bound bioneutralizing antagonist because FS288 has the highest affinity for heparin. Structural studies have determined that FS288 completely surrounds the ligand and blocks both type I and type II receptor binding sites. Furthermore, our structural studies of myostatin in complex with the antagonist FS288 revealed a unique continuous electropositive surface created when Mstn binds FS288.[20] This feature significantly increases the affinity of the complex for heparin over FS288 alone or the FS288−ActA complex. As such, this is likely the reason that FS288−Mstn complexes are degraded more readily when applied to the cell surface than the corresponding FS288−ActA complexes. [20]

The goal of this study is to analyze the molecular interactions of heparin with FS288 and its complexes with ActA and Mstn (FS288−ActA and FS288−Mstn, respectively), under normal physiological and nonphysiological conditions (high salt concentrations and low pH). The Biacore system (BIAcore 3000) was employed for the study. The system utilizes the surface plasmon resonance (SPR) phenomena and allows a direct quantitative analysis of the label-free molecular interactions in real time.

EXPERIMENTAL PROCEDURES

Materials. Sensor SA Chip was from GE Healthcare (Uppsala, Sweden). Heparin sodium salt and low-molecular weight heparin (LMWH) were obtained from porcine intestinal mucosa (Celsus Laboratories, Cincinnati, OH). Heparin oligosaccharides (dp2 to dp20) were prepared from controlled partial heparin lyase 1 treatment of bovine lung heparin (Sigma) followed by size fractionation.[24] SPR measurements were performed on a BIAcore 3000 (GE Healthcare, Uppsala, Sweden) operated using the version software. Buffers were filtered and degassed for the SPR assay.

Protein Expression and Purification. FS288, ActA and Mstn, proteins were expressed utilizing stable CHO cells and purified as described previously.[20,25] Briefly, FS288 was purified through binding a heparin affinity column followed by binding a cation exchange column. ActA was purified through affinity chromatography using an NHS-Sepharose column coupled with FS288. ActA was eluted with 50 mM glycine and 0.03% Tween 80 (pH 2.5). Mstn was purified through sequential anion and cation exchange columns followed by a C4 reverse phase step. Purified proteins were concentrated and buffer exchanged into 10 mM HEPES and 150 mM NaCl (pH 7.5).

FS288−ActA and FS288−Mstn were prepared by mixing FS288 at a 2.5:1 molar ratio with each ligand and separating the complexes from unbound FS through application of samples on an S200 Hiload Sephacryl column.

Preparation of the Heparin Biochip. Biotinylated heparin was prepared by reacting sulfo-*N*-hydroxysuccinimide long-chain biotin (Pierce, Rockford, IL) with free amino groups of unsubstituted glucosamine residues in the polysaccharide chain following a published procedure.[26] The biotinylated heparin was immobilized to the streptavidin (SA) chip based on the manufacturer's protocol. The successful immobilization of heparin was confirmed by the observation of an ∼250 resonance unit (RU) increase in the sensor chip. The control flow cell (FC1) was prepared by a 1 min injection with saturated biotin.

Kinetic Measurement of the Interaction between Heparin and Protein using BIAcore. The protein samples were diluted in HBS-EP buffer [0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, and 0.005% surfactant P20 (pH 7.4)]. Different dilutions of protein samples were injected at a flow rate of 40 μL/min for 3 min. At the end of the sample injection, the same buffer was passed over the sensor surface to facilitate dissociation. After a 2 min dissociation time, the sensor surface was regenerated by injection with 40 μL of 2 M NaCl to obtain a fully regenerated surface. The response was monitored as a function of time (sensorgram) at 25 °C.

SPR Solution Competition Study of Heparin Oligosaccharides. To measure the chain length dependence of the heparin−protein interactions, an SPR solution competition study was performed by pre-equilibrating different heparin oligosaccharides with protein followed by subsequent injection of this solution over the heparin-immobilized sensor chip. Different chain lengths of heparin [full size, LMWH, and heparin oligosaccharides with different degrees of polymerization (from dp2 to dp20)] at 50 nM were premixed with 5 nM FS288 or FS288−ActA complex in HBS-EP buffer and injected over the heparin chip at a flow rate of 40 μL/min. After each run, the dissociation period and regeneration protocol were performed as described above. The SPR experiments were performed in triplicate.

Effect of Buffer Conditions on Heparin−Protein Interactions. To measure the effect of buffer conditions on heparin−protein interactions, samples of the standard SPR HBS-EP buffer [0.01 M HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20 (pH 7.4)] were modified to contain 300, 500, and 1000 mM NaCl, or adjusted to pH 6.2, 5.2, or 4.0. Three protein samples, FS288, FS288−ActA, and FS288−Mstn, were diluted (1:9) under the five buffer conditions and injected at a flow rate of 40 μL/min for 3 min. At the end of the sample injection, the same buffer was passed over the sensor surface to facilitate dissociation. After a 2 min dissociation time,

the sensor surface was regenerated by injection with 40 μL of 10 mM glycine and 2 M NaCl to fully regenerate the surface.

RESULTS AND DISCUSSION

Kinetic Measurement of Heparin−FS288 and Heparin−FS288 Ligand Complex Interactions. The profiles of binding kinetics were measured for FS288 and its complexes with immobilized heparin using SPR to provide a quantitative picture of these interactions. Sensorgrams of heparin−FS288, −FS288−ActA, and −FS288−Mstn interactions are shown in Figure 1. The parameters of binding kinetics (Table 1) were determined by globally fitting the sensorgram curves to a 1:1 Langmuir model (black fitting lines in Figure 1) from BIAevaluation. FS288 and FS288 complexes showed very different heparin binding kinetics and affinities (Figure 1 and Table 1). The FS288 complexes showed 100-fold greater heparin binding affinity than FS288 alone (K_D = 4.7 × 10^{-10} and 4.0 × 10^{-10} M vs K_D = 5.6 × 10^{-8} M). The enhanced heparin affinity of the complexes can be ascribed to a k_{off} much slower than that observed for FS288. The high binding affinity of the FS288−Mstn complex for heparin is consistent with previous findings demonstrating the electrostatic surface potential of the FS288−Mstn complex was remarkably electropositive.[20] The results are also consistent with the data

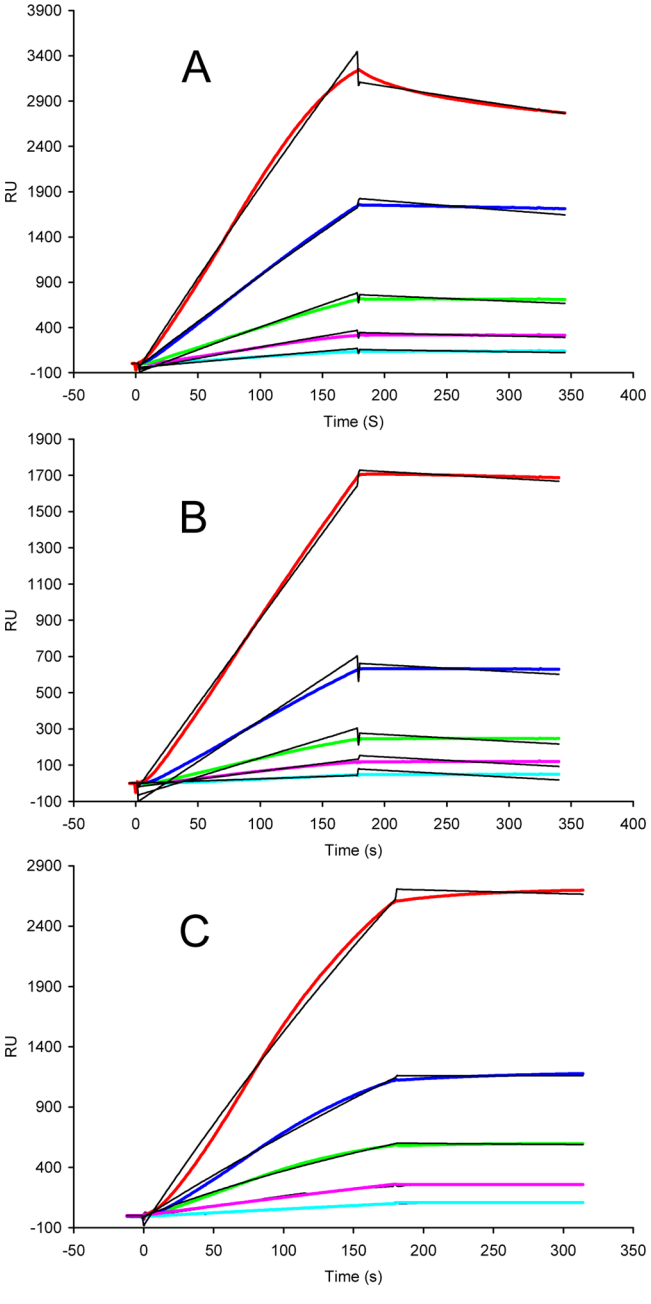

Figure 1. (A) SPR sensorgrams of FS288−heparin interaction. Concentrations of the protein (from top to bottom) were 20, 10, 5, 2.5, and 1.25 nM, respectively. (B) SPR sensorgrams of the interaction of FS288−ActA with heparin. Concentrations of the protein (from top to bottom) were 20, 10, 5, 2.5, and 1.25 nM, respectively. (C) SPR sensorgrams of the interaction of Mstn−FS288 with heparin. Concentrations of the protein (from top to bottom) were 20, 10, 5, 2.5, and 1.25 nM, respectively.

Table 1. Summary of Kinetic Data of Protein−Heparin Interactions[a]

protein	k_{on} (ms^{-1})	k_{off} (s^{-1})	K_D (M)
FS288	1.1 × 10^4 (±669)	6.0 × 10^{-4} (±1.4 × 10^{-5})	5.6 × 10^{-8}
FS288−ActA	3.3 × 10^3 (±118)	1.6 × 10^{-6} (±5.2 × 10^{-7})	4.7 × 10^{-10}
FS288−Mstn	4.85 × 10^4 (±3.68 × 10^4)	1.96 × 10^{-5} (±2.5 × 10^{-5})	4.0 × 10^{-10}

[a]The data in parentheses are the standard errors (SE) from the global fitting of sensorgrams from different concentrations for protein binding.

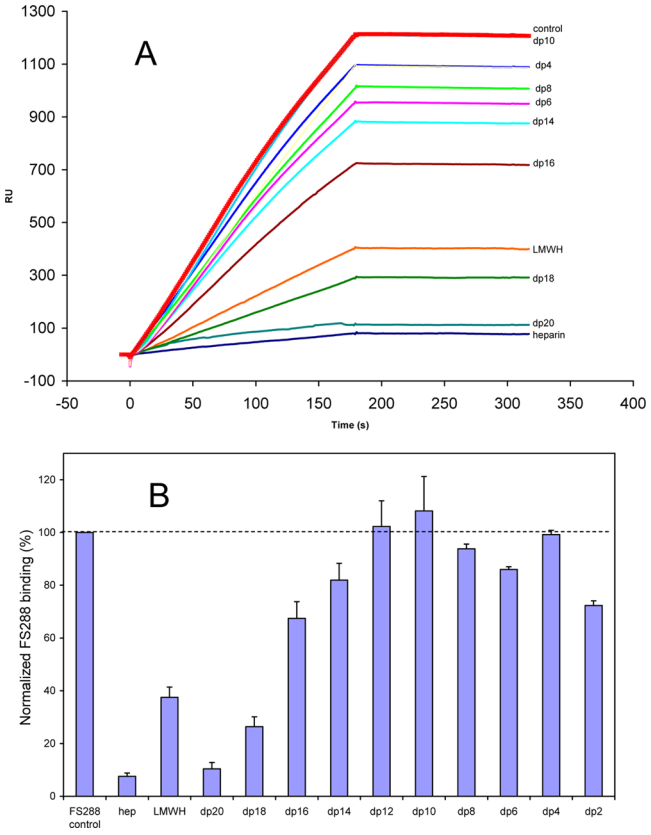

Figure 2. (A) Preferences for binding of FS288 to heparin of various chain lengths using a SPR solution competition assay. (B) Bar graphs (based on triplicate experiments) displaying the inhibition activity of different sized heparins (in solution) on binding of FS288 to immobilized heparin (on chip surface).

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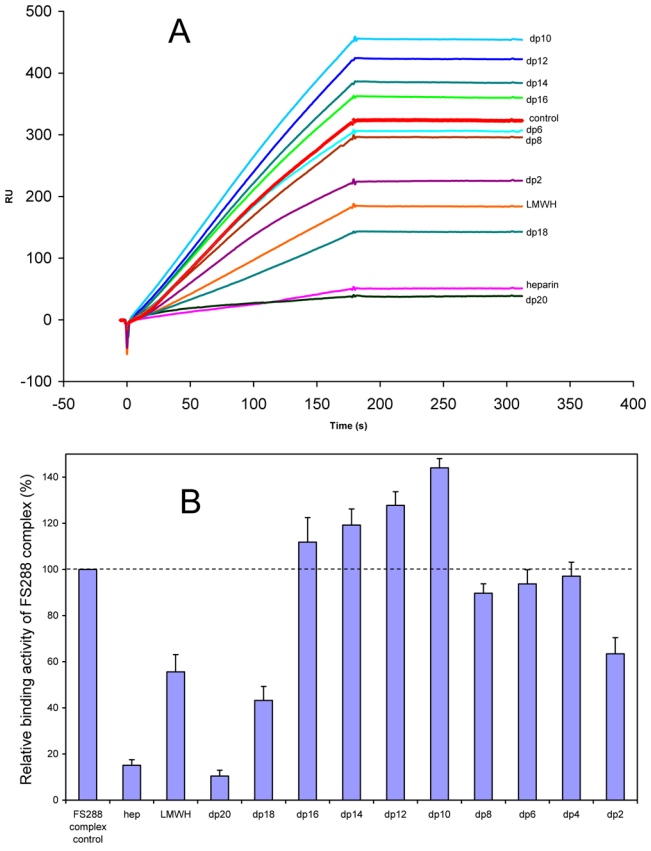

Figure 3. (A) Preferences for binding of the FS288−ActA complex to heparin of various chain lengths using a SPR solution competition assay. (B) Bar graphs (based on triplicate experiments) displaying the inhibition activity of different sized heparins (in solution) on binding of the complex to immobilized heparin (on chip surface).

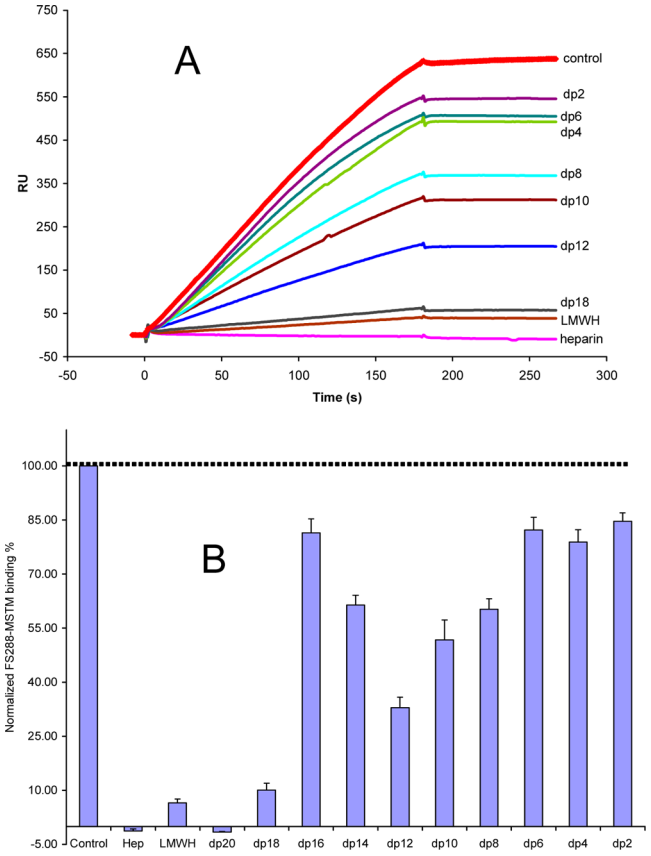

Figure 4. (A) Preferences for binding of the FS288−Mstn complex to heparin of various chain lengths using a SPR solution competition assay. (B) Bar graphs (based on triplicate experiments) displaying the inhibition activity of different sized heparins (in solution) on binding of the complex to immobilized heparin (on chip surface).

from our earlier study showing that FS288 was released from a heparin column prior to the FS288−Mstn complex.[20]

SPR Solution Competition Study of Heparin Oligosaccharides. While the amino acid residues of FS involved in heparin binding have been identified,[27] little is known about the structural requirements of heparin molecules that bind to FS and the structure of the ligand-bound state is unclear.[20] An SPR competition assay was used to examine the chain length dependence of heparin critical for binding. FS288 and its complexes were each mixed with heparin-derived oligosaccharides of various sizes in HBS-EP buffer and injected over a heparin chip. Once the active binding site on FS288 (or FS288 complexes) is occupied by a heparin-derived oligosaccharide in the solution, its level of binding to the surface-immobilized heparin should decrease, resulting in a reduction in the magnitude of the SPR signal. Heparin-derived oligosaccharides of different sizes, identified for the number of sugar residues in each (dp2 to dp20), were used in this competition experiment. The results of this SPR solution competition study are shown in Figures 2−4. The results show that the binding of FS288 and its FS288−ActA and FS288−Mstn complexes to heparin is dependent on chain length. In all cases, heparin, LMWH, and large oligosaccharides of dp18 and dp20 strongly inhibited the binding of FS288 and FS288−ActA and FS288−Mstn complexes to heparin immobilized on the chip. These findings about the heparin size requirement for binding are in good agreement with our previous structural measurements.[20] In the FS288−Mstn complex, the structure suggests that a composite heparin-binding site, corresponding to a continuous electropositive crevice that spans the complex, is generated in which a single heparin molecule could bind. The crevice measures ∼60 Å wide and would fit a heparin molecule ∼14−16 sugars in length, similar to that reported in the FGF−FGF receptor heparin complex.[28] Of particular interest was the disparity observed for the ability of dodecasaccharide (dp12) to block binding to the heparin chip. For the FS288−Mstn complex, more than half of the binding interaction was lost in the presence of the dp12 form, whereas Fs288 and FS288−ActA were unaffected. This suggests that the overall size requirement of heparin binding might be unique for different FS−ligand complexes.

Effect of Buffer Conditions on the Interactions. Binding buffers with different salt (NaCl) concentrations (300, 500, and 1000 mM) at different pH values (6.2, 5.2, and 4.0) were used for the SPR analysis to assess the effect of buffer on heparin−protein interactions. The results of heparin−protein interactions in various buffers are shown in Figures 5 and 6. A high salt concentration (1 M) inhibited all the binding of the protein or complex to heparin, suggesting that this is primarily an electrostatically driven interaction. At 300 mM salt, binding of FS288 to heparin was inhibited, but binding of FS288−ActA and FS288−Mstn complexes to heparin was promoted. Surprisingly, the strongest interaction of the FS288−Mstn complex with heparin was observed at 500 mM salt, whereas this concentration of salt abolished binding of both FS288 and FS288−ActA to heparin. This observation of

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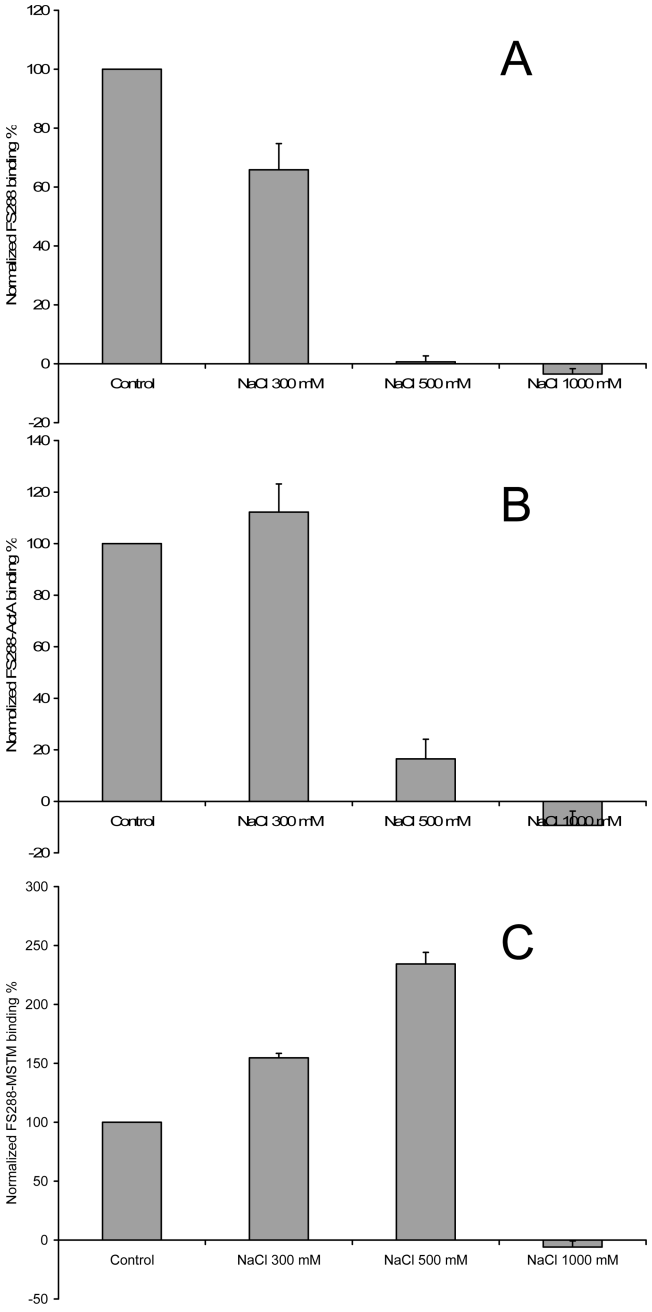

Figure 5. (A) Preference for binding of FS288 at various NaCl concentrations. Bar graphs (based on triplicate experiments) displaying the binding of FS288 to immobilized heparin (on chip surface). (B) Preference for binding of the FS288−ActA complex at various NaCl concentrations. Bar graphs (based on triplicate experiments) displaying the binding of the FS288−ActA complex to immobilized heparin (on chip surface). (C) Preference for binding of the FS288−Mstn complex at various NaCl concentrations. Bar graphs (based on triplicate experiments) displaying the binding of the FS288−Mstn complex to immobilized heparin (on chip surface).

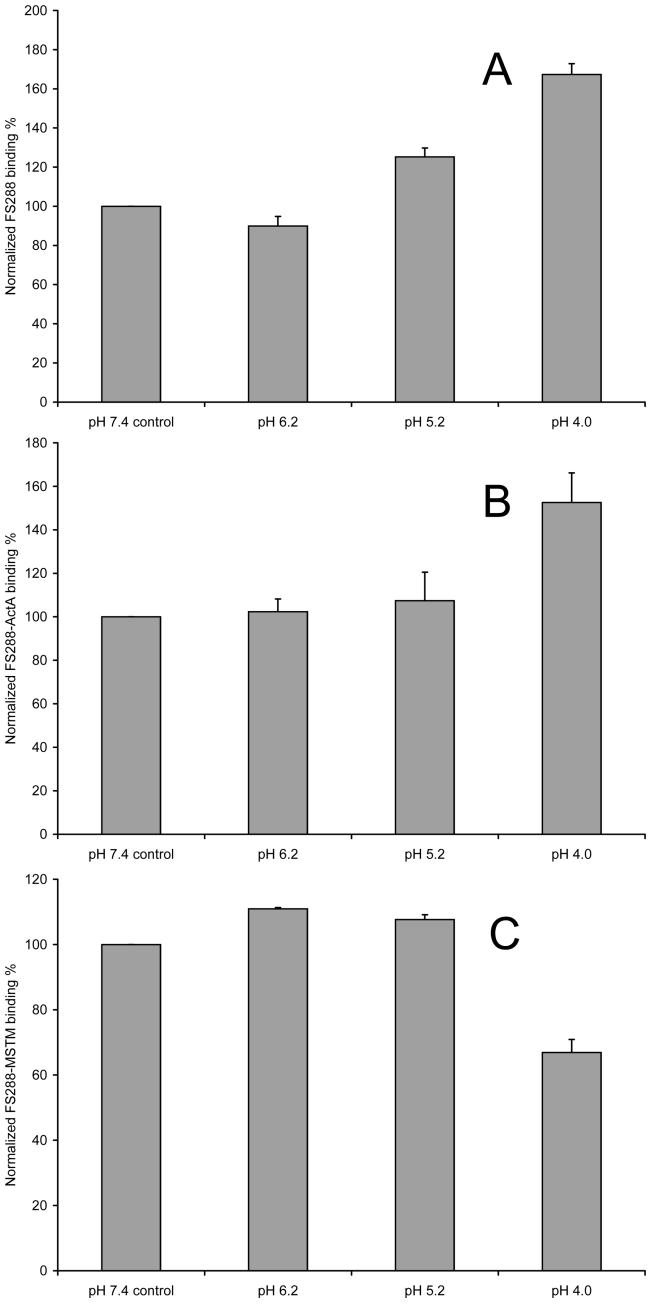

Figure 6. (A) Preference for binding of FS288 in various pH buffers. Bar graphs (based on triplicate experiments) displaying the binding of FS288 to immobilized heparin (on chip surface). (B) Preference for binding of the FS288−ActA complex in various pH buffers. Bar graphs (based on triplicate experiments) displaying the binding of the FS288−ActA complex to immobilized heparin (on chip surface). (C) Preference for binding of the FS288−Mstn complex in various pH buffers. Bar graphs (based on triplicate experiments) displaying the binding of the FS288−Mstn complex to immobilized heparin (on chip surface).

binding of FS288 and FS288−ActA to heparin in the presence of NaCl is consistent with previous fluorescence polarization measurements.[29]

The impact of pH on these interactions was also assessed. The results showed that acidic pH improved FS288 and FS288−ActA binding. Lower pH values cause more amino acids to become positively charged. For example, at pH <6, histidines become protonated and hence carry a positive charge. This favors electrostatic interactions with the negatively charged heparin.[30] For FS288−Mstn, the results showed that slightly acidic pH values (6.2 and 5.2) improved its binding to heparin, but at a lower pH value (4), the binding was inhibited. This could result from the protonation of carboxyl groups on heparin required for protein interaction or alteration of protein conformation. There are a number of heparin− and HS−protein interactions that are regulated by pH, such as those of

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β-amyloid peptide (Aβ),[31] selenoprotein P,[32] and stromal cell-derived factor-1 (SDF-1),[33] although some of these effects might be attributable to proteins localized in acidic cell compartments like the lysozymes.

FS plays an important role in the bioneutralization of ActA and Mstn. This is partially a result of FS mediating ligand degradation through heparin-dependent cell surface binding events. Previously, we identified heparin binding differences when FS was in complex Mstn. Here we further define these differences and provide a quantitative analysis of interactions of heparin with FS288 and FS288 complexes. Overall, knowledge of these differences might augment efforts to further modify follistatin to specifically antagonize Mstn therapeutically.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

FS, follistatin; Mstn, myostatin; ActA, activin A; SPR, surface plasmon resonance; GAG, glycosaminoglycan; HS, heparan sulfate; HP, heparin; LMWH, low-molecular weight heparin; TGF-β, transforming growth factor-β; FGFs, fibroblast growth factors; Hh, hedgehog; ECM, extracellular matrix; SA, streptavidin; FC, flow cell; RU, resonance unit.

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