Glycosaminoglycans Bind Factor Xa in a Ca^{2+}-Dependent Fashion and Modulate Its Catalytic Activity†

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ABSTRACT: Recent studies have demonstrated the existence of a Ca^{2+}-dependent heparin-binding site on factor Xa. To characterize this heparin-binding site, the extrinsic fluorescence of fluorescein-labeled, active site-blocked factor Xa was monitored as it was titrated with glycosaminoglycans of various sulfate content and chain length. The binding of glycosaminoglycans to factor Xa appears to be charge-dependent because affinity is correlated with degree of glycosaminoglycan sulfation. All glycosaminoglycans bind factor Xa with higher affinity in the presence of Ca^{2+} than in its absence. In contrast, when Gla-domainless factor Xa was substituted for factor Xa, glycosaminoglycans bound with similar affinities in the absence and presence of Ca^{2+}. These results support the hypothesis that the anionic Gla domain impairs glycosaminoglycan binding in the absence of Ca^{2+}. The changes in fluorescence intensity of factor Xa when titrated with glycosaminoglycans suggest that glycosaminoglycans induce conformational changes in the active site environment of factor Xa. To explore the consequences of these conformational changes, the effect of glycosaminoglycans on the catalytic activity of factor Xa was examined. Glycosaminoglycans influenced the ability of factor Xa to cleave chromogenic substrates and attenuated the capacity of factor Xa to activate factor VII. The potency of glycosaminoglycans in these assays reflected their affinity for factor Xa. These studies suggest that glycosaminoglycan binding perturbs exosites on the surface of factor Xa, potentially modifying interactions with cofactors or substrates.

Activated factor X (factor Xa or f.Xa)[1] is a vitamin K-dependent clotting enzyme found at the convergence of the intrinsic and extrinsic pathways of coagulation. As the catalytic moiety in the prothrombinase complex, f.Xa converts prothrombin to thrombin (*1*). In addition to its essential role in thrombin generation, f.Xa also amplifies coagulation by activating other coagulation factors, including factors V, VII, and VIII (*2*, *3*). Because excess f.Xa generation leads to thrombosis, whereas insufficient f.Xa formation can result in bleeding, f.Xa levels must be closely regulated. Antithrombin, one of the most important physiologic inhibitors of f.Xa, maintains f.Xa at appropriate levels (*4*).

Heparin and low molecular weight heparin (LMWH) are glycosaminoglycans that act as anticoagulants by catalyzing the inhibition of f.Xa and thrombin by antithrombin. The interaction of heparin and LMWH with antithrombin is mediated by a unique pentasaccharide sequence found on one-third and one-fifth of the chains of heparin and LMWH, respectively (*5*). Binding of this pentasaccharide to antithrombin induces a conformational change in the serpin that renders it more reactive with f.Xa, thereby enhancing the rate of f.Xa inactivation by antithrombin by 2−3 orders of magnitude (*4*, *6*).

In contrast to f.Xa, pentasaccharide-induced conformational changes in antithrombin have little effect on the rate at which it inactivates thrombin. To catalyze this reaction, heparin must bind not only to antithrombin via its pentasaccharide sequence but also to thrombin (*4*, *7*, *8*). Binding studies using heparin chains of varying length indicate that only pentasaccharide-containing chains comprised of 18 or more saccharide units, which correspond to a molecular weight of 5400 or more, are of sufficient length to provide this bridging function (*9*−*13*). With a mean molecular weight of about 5000, at least half of the chains of LMWH are too short to bridge antithrombin to thrombin. Consequently, LMWH produces less catalysis of thrombin inhibition by antithrombin than f.Xa inhibition. In contrast, with a mean molecular weight of 15000, almost all of the chains of unfractionated heparin (heparin) are long enough to bridge antithrombin to thrombin, a feature that explains why heparin produces greater catalysis of thrombin inhibition by antithrombin than does LMWH.

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[1] Abbreviations: $C_{0.5}$, concentration to produce half-maximal effect; DS, dermatan sulfate; DX, dextran sulfate; f.Xa, factor Xa; f.VIIa, factor VIIa; f.Va, factor Va; FPRck, D-Phe-Pro-Arg chloromethyl ketone; f-FPR, fluorescein-labeled FPR; Gla, γ-carboxyglutamic acid; GD, Gla-domainless; HS-LMWH, hypersulfated LMWH; *I*, intensity; LMWH, low molecular weight heparin; ND-LMWH, N-desulfated LMWH; PCPS, phosphatidylcholine−phosphatidylserine; rTAP, recombinant tick anticoagulant peptide; SD, standard deviation; *V*, absorbance/time.

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Heparin binds to a positively charged domain on thrombin known as exosite 2 (*14*, *15*). This domain, which is distinct from the active site of thrombin, is composed of 11 basic amino acids. Recent studies indicate that f.Xa also possesses a heparin-binding exosite at a position on the catalytic domain analogous to its counterpart on thrombin (*16*−*18*). Whereas heparin binding to exosite 2 on thrombin is Ca^{2+}-independent, heparin only binds to factor Xa in the presence of Ca^{2+}. When Ca^{2+} is present, heparin chains with a molecular weight over 6000 produce a 10−20-fold higher rate of f.Xa inhibition by antithrombin than heparin chains of lower molecular weight (*18*, *19*). These findings suggest that, in the presence of Ca^{2+}, longer heparin chains can bridge antithrombin to f.Xa, thereby producing a modest increase in the rate of f.Xa inhibition by antithrombin.

Previous studies have shown that the Gla domain of f.Xa has a disordered conformation in the absence of Ca^{2+}, a finding attributed to like-charge repulsion between Gla residues (*20*). By neutralizing this repulsion, Ca^{2+} induces conformational changes that expose the heparin-binding site on f.Xa. If Gla-domainless f.Xa is substituted for full-length f.Xa, the heparin-catalyzed rate of f.Xa inhibition by antithrombin is the same in the absence of Ca^{2+} as it is in its presence (*18*). These findings suggest that Ca^{2+} is no longer necessary for heparin binding when the Gla domain is removed.

Although the heparin-binding domain on f.Xa has been identified, it is not known whether the negative charge of heparin determines its affinity for this site or if glycosaminoglycans other than heparin and LMWH also bind to this domain. Likewise, it is unknown whether heparin binding to f.Xa induces conformational changes in the active site of the enzyme that modulate the catalytic activity of f.Xa against low molecular weight or macromolecular substrates. This is of particular interest because heparin binding to exosite 2 on thrombin evokes conformational changes at its active site that may influence thrombin's catalytic functions (*21*). The purpose of this study was to clarify these issues.

EXPERIMENTAL PROCEDURES

Materials

Reagents. Human f.Xa, protein C, and factor VII (f.VII) were obtained from Enzyme Research Laboratories (South Bend, IN). Human Gla-domainless f.Xa (GD-f.Xa) and 5′-fluorescein-D-Phe-Pro-Arg-chloromethyl ketone (f-FPRck) were purchased from Haematologic Technologies Inc. (Essex Junction, VT). The purity of proteins was confirmed by SDS−polyacrylamide gel electrophoresis using 4−15% gradient gels (Ready-Gel; Bio-Rad, Mississauga, Ontario, Canada) (*22*). L-α-Phosphatidylcholine (type III-E from egg yolk), L-α-phosphatidyl-L-serine (from bovine brain), unfractionated grade 1 sodium heparin (174 units/mg) from porcine intestinal mucosa, Polybrene, and dextran sulfate (DX) with a mean molecular weight of 5000 were obtained from Sigma-Aldrich Canada. Unilamellar phosphatidylcholine−phosphatidylserine (PCPS) vesicles (75%/25% w/w) were prepared and characterized as previously described (*23*). Hypersulfated LMWH (HS-LMWH) with a mean molecular weight of 5000 was provided by Agen Biomedical (Brisbane, Australia). Dermatan sulfate (DS) was obtained from Mediolanum Farmaceutici (Milan, Italy), and enoxaparin was supplied by Rhône-Poulenc-Santé (Paris, France). N-Desulfated LMWH (ND-LMWH) was prepared from enoxaparin using a solvolytic technique (*24*). Chromogenic substrates S-2765 (*N*-α-Z-D-Arg-Gly-Arg-*p*-nitroanilide) and S-2366 (PyrGlu-Pro-Arg-*p*-nitroanilide) were from Chromogenix (West Chester, OH), and Chromozym-TH (tosyl-D-Gly-Pro-Arg-*p*-nitroanilide; Chz-TH) was from Roche Applied Sciences Canada. Pefachrome-f.VIIa 5979 (CH_3SO_2-D-CHA-But-Arg-*p*-nitroanilide), Protac, and Prionex were obtained from Centerchem (Stamford, CT). Recombinant tissue factor and recombinant tick anticoagulant peptide (rTAP) were generously provided by Dr. G. Vlasuk, Corvas International, Inc. (San Diego, CA).

Preparation of Active Site-Blocked F.Xa and GD-f.Xa. F.Xa or GD-f.Xa (22 μM) was incubated with a 3.3-fold molar excess of f-FPRck at 23 °C. Chromogenic activity of 44 nM f-FPR-f.Xa with 0.2 mM S-2765 was monitored, and the incubation was terminated when no further activity was detected. The sample was then dialyzed against 500 mL of 20 mM Tris-HCl, pH 7.4, and 150 mM NaCl (TS) at 4 °C with two changes of buffer over 18 h, and the concentration of active site-blocked f.Xa or GD-f.Xa was determined by absorbance using $\epsilon_{0.1\%}^{280}$ of 1.2 (*25*), after correcting for light scatter at 320 nm using the relationship $A_{280}^{corr} = A_{280} - 1.7A_{320}$ (*23*). The absence of residual f-FPRCK was confirmed by incubating 10 μL of 3.73 μM f-FPR-f.Xa or f-FPR-GD-f.Xa with 10 μL of 100 nM thrombin for 10 min. Chromogenic activity was comparable to that of a thrombin control lacking f-FPR-f.Xa.

Methods

Affinity of Glycosaminoglycans for f-FPR-f.Xa or f-FPR-GD-f.Xa. The extrinsic fluorescence of 100 nM f-FPR-f.Xa or f-FPR-GD-f.Xa in 900 μL of TS containing 1 mM EDTA or 4 mM $CaCl_2$ was continuously monitored using a LS50B luminescence spectrometer (Perkin-Elmer, Etobicoke, Ontario, Canada) with $\lambda_{ex} = 492$ nm, $\lambda_{em} = 535$ nm, cutoff filter = 515 nm, and slit widths set to 5−7 nm. The contents of the 10 × 4 mm semi-micro quartz cuvette were stirred with a microstir bar, and the temperature was maintained at 25 °C using a circulating water bath. The fluorescence intensity of f-FPR-f.Xa was measured before (I_0) and after (I) the addition of 1−10 μL aliquots of 400 μM glycosaminoglycan (heparin, HS-LMWH, DX, DS, ND-LMWH, or LMWH) containing 100 nM f-FPR-f.Xa or f-FPR-GD-f.Xa and 1 mM EDTA or 4 mM $CaCl_2$. The signal was allowed to stabilize prior to each addition, and the titration was continued until there was no further change in fluorescence. I/I_0 values were plotted versus the heparin concentration, and the K_d was calculated by nonlinear regression analysis (Table Curve; Jandel Scientific, San Rafael, CA) using the equation (*26*):

$$\frac{I}{I_0} = 1 + \frac{\alpha}{2}\left(1 + \frac{K_d + L_0}{nP_0} - \sqrt{\left(1 + \frac{K_d + L_0}{nP_0}\right)^2 - 4\frac{L_0}{nP_0}}\right) \quad (1)$$

where L_0 and P_0 are the input concentrations of heparin and f-FPR-f.Xa, respectively, and n and α are the stoichiometry and maximum change in emission intensity, respectively.

Effect of Glycosaminoglycans on F.Xa Chromogenic Activity. The effect of 1 μM heparin on the activity of f.Xa in concentrations ranging from 2 to 25 nM with chromogenic

substrates S-2765 and Chz-TH was determined in the presence of 2 mM EDTA or $CaCl_2$. Chromogenic substrate concentrations ranged from 10 to 1000 μM. Substrate hydrolysis was monitored by measuring absorbance at 405 nm over a 5 min period using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Absorbance values were converted to the concentration of *p*-nitroaniline using an extinction coefficient determined empirically for the plate reader. Parameters of K_m and k_{cat} were determined by linear regression analysis of Hanes plots of [substrate]/rate versus [substrate].

Effect of Glycosaminoglycans on F.Xa-Mediated Activation of F.VII and Protein C. F.VII activation was measured by incubating 1 μM f.VII with 20 μM PCPS vesicles, 5 mM $CaCl_2$, 0.5 nM tissue factor, 1 nM f.Xa, and varying concentrations of glycosaminoglycan (ranging from 0 to 1.2 mM). After 20 min incubation at 37 °C, the reaction was terminated by addition of 400 nM rTAP and 300 mM EDTA. Activation of f.VII was assessed by measuring the hydrolysis of the f.VIIa-directed substrate, Pefa-5979 in the presence of 300 nM rTF. The concentration of f.VIIa generated in the presence of glycosaminoglycan (V) relative to that generated in its absence (V_0) was used as an index of glycosaminoglycan-induced inhibition of f.VII activation. V/V_0 values were plotted versus the glycosaminoglycan concentration to determine the concentration of glycosaminoglycan required to produce 50% of the maximum change in f.Xa-mediated activation of f.VII.

Activation of protein C was monitored using a chromogenic assay for activated protein C. Reactions containing 2.5 μM protein C, 200 μM PCPS vesicles, and 5 mM $CaCl_2$ or EDTA were incubated in TS buffer in the presence of heparin in concentrations ranging from 0 to 26 μM. Activation was initiated by addition of f.Xa to 24 or 120 nM (Ca^{2+} or EDTA, respectively), and the reactions were allowed to proceed for 1 h at 23 °C. F.Xa was inactivated by a 2 min incubation with antithrombin and heparin (5 μM and 20 units/mL, respectively). Activated protein C chromogenic substrate S-2366 (350 μM) containing 8 mg/mL Polybrene was added to the wells, and activity was monitored in a plate reader. Rates measured in the presence of heparin were divided by those determined in its absence, and the relative values were plotted against heparin concentration. Data were analyzed by nonlinear regression, as described above. Experiments were then repeated using Protac, a snake venom-derived protein C activator, in place of f.Xa. In these experiments, PCPS vesicles were omitted, and the concentration of Protac was 0.08 unit/mL. Activation times were 3 h in the presence of $CaCl_2$ and 5 min in the presence of EDTA.

Statistical Analyses. All values represent the mean ± standard deviation (SD) of at least three separate experiments. SD was calculated using Quattro Pro version 5.0 (Borland International Inc., Scotts Valley, CA).

RESULTS

Affinity of Glycosaminoglycans for F.Xa and GD-f.Xa. The affinity of heparin for f.Xa was measured by monitoring changes in the extrinsic fluorescence of f-FPR-f.Xa as it was titrated with heparin in the absence or presence of Ca^{2+}. Titrations resulted in concentration-dependent and saturable decreases in fluorescence of f-FPR-f.Xa, and K_d values were determined by nonlinear regression (Figure 1, Table 1). The affinity of heparin for f-FPR-f.Xa was 126-fold lower in the absence of Ca^{2+} than in its presence (K_d values of 50 and 0.4 μM, respectively). This K_d value of heparin for f.Xa in the presence of Ca^{2+} is comparable to the value of 0.33 μM obtained by surface plasmon resonance (*27*). The experiment was then repeated using f-FPR-GD-f.Xa in place of f-FPR-Xa to explore the influence of the Gla domain of f.Xa on heparin binding (data not shown). Heparin bound f-FPR-GD-f.Xa with similar affinity in the absence or presence of Ca^{2+} (K_d values of 1.5 ± 0.2 μM and 1.3 ± 0.4 μM, respectively), consistent with results inferred from functional assays (*18*). These data support the hypothesis that the heparin-binding site on f.Xa is partially blocked by the anionic Gla domain in the absence of Ca^{2+}.

The affinity of LMWH for f-FPR-f.Xa was measured to determine whether the length of heparin chains influences their interaction with f.Xa. LMWH binds f-FPR-f.Xa with an affinity similar to that of heparin in the absence of Ca^{2+}. In contrast, the affinity of LMWH for f-FPR-f.Xa is 17-fold lower than that of heparin in the presence of Ca^{2+} (Table 1). This may be related to the finding that longer heparin

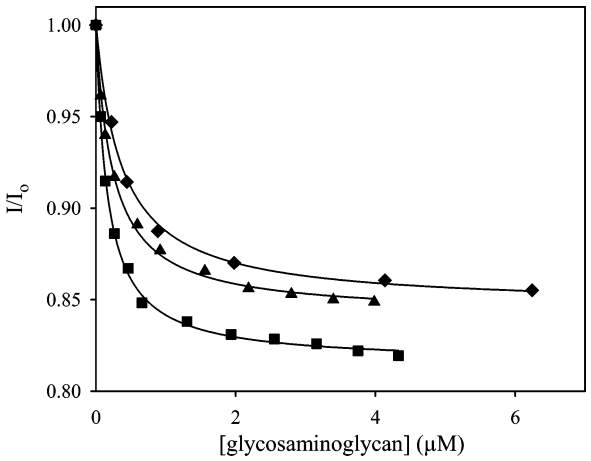

FIGURE 1: Interaction of glycosaminoglycans with f-FPR-f.Xa in the presence of Ca^{2+}. Fluorescein-labeled, active site-blocked f.Xa (100 nM) was titrated with heparin (◆), HS-LMWH (■), or DX (▲). Fluorescence intensity was measured before (I_0) and after (I) each glycosaminoglycan addition, and I/I_0 was calculated. Data were analyzed by nonlinear regression analysis, as represented by the solid lines.

Table 1: Comparison of the Affinity of Various Glycosaminoglycans for F.Xa in the Presence of 1 mM EDTA or 4 mM $CaCl_2$[a]

glycosaminoglycan	K_d (μM)		$CaCl_2$-induced increase in affinity
	EDTA	$CaCl_2$	
ND-LMWH	NB[b]	151.2 ± 84.9	
DS	81.0 ± 34.9	60.7 ± 1.7	1.3
LMWH	52.5 ± 28.0	6.9 ± 1.9	7.6
heparin	50.4 ± 20.3	0.4 ± 0.1	126.0
HS-LMWH	1.5 ± 0.3	0.2 ± 0.1	7.5
DX	1.8 ± 0.2	0.2 ± 0.1	9.2

[a] Dissociation constants were determined by titrating 100 nM f-FPR-f.Xa with glycosaminoglycans in the presence of 1 mM EDTA or 4 mM $CaCl_2$. Changes in fluorescence were quantified, and K_d values were determined by nonlinear regression. The $CaCl_2$-induced increase in affinity is the ratio of K_d values in the presence of $CaCl_2$ to those in the presence of EDTA. Experiments were performed at least three times, and values represent the mean ± SD. [b] NB indicates that no binding was observed.

chains bridge antithrombin to f.Xa more effectively than shorter ones (*28*).

To begin to examine whether the affinity of glycosaminoglycans for f.Xa depends on their charge, the affinities of N-desulfated LMWH (ND-LMWH) and dermatan sulfate (DS), glycosaminoglycans that are less sulfated than heparin (*29*), for f-FPR-Xa were determined (data not shown). In the presence of Ca^{2+}, ND-LMWH and DS bound to f-FPR-f.Xa with affinities 378- and 152-fold lower than that of heparin (Table 1). In the absence of Ca^{2+}, the affinity of DS for f-FPR-f.Xa was 1.6-fold lower than that of heparin, whereas the affinity of ND-LMWH for f-FPR-f.Xa was too low to be measured under the experimental conditions employed. These studies suggest that glycosaminoglycans that are less negatively charged than heparin bind f.Xa with lower affinity. To further explore this concept, the affinities of HS-LMWH and DX, glycosaminoglycans that are more negatively charged than heparin (*29*), for f-FPR-f.Xa also were determined. In the absence of Ca^{2+}, HS-LMWH and DX bound f-FPR-f.Xa with K_d values of 1.5 μM and 1.8 μM, respectively, affinities 34- and 28-fold higher than that of heparin. In the presence of Ca^{2+}, the K_d values were both 0.2 μM, a reduction of 50% compared with that of heparin (Figure 1, Table 1). The effect of sulfation is most evident with HS-LMWH, which demonstrated 33-fold higher affinity for f-FPR-f.Xa than LMWH. These results indicate that (a) glycosaminoglycans other than heparin bind to f.Xa, (b) the affinity of glycosaminoglycans for f.Xa depends on their negative charge, and (c) all glycosaminoglycans bind to f.Xa with higher affinity in the presence of Ca^{2+} than in its absence. These studies also suggest that glycosaminoglycan binding to f.Xa is independent of the pentasaccharide sequence that mediates the binding of heparin and LMWH to antithrombin because DX, a glycosaminoglycan that does not contain this sequence, binds f.Xa with an affinity similar to that of HS-LMWH.

Effect of Glycosaminoglycans on F.Xa-Mediated Hydrolysis of Synthetic Substrates. The influence of heparin on f.Xa-mediated hydrolysis of chromogenic peptidyl substrates was examined in the presence of Ca^{2+} and EDTA to determine whether heparin influences the catalytic activity of f.Xa. Heparin increased the activity of f.Xa with two substrates, S-2765 and Chz-TH, by about 20% in the presence of Ca^{2+}. In contrast, heparin had little effect on f.Xa-mediated hydrolysis of these substrates in the presence of EDTA. To elucidate the mechanism of modulation, kinetic parameters for hydrolysis of the chromogenic substrates by f.Xa were determined in the absence and presence of heparin. Heparin was used at 1 μM in these experiments so that, in the presence of Ca^{2+}, its concentration exceeded the K_d value for f.Xa−heparin interaction, whereas it was well below the K_d of heparin for f.Xa in the presence of EDTA. This was done to overcome potential nonspecific effects of heparin on the chromogenic substrates.

With S-2765, the catalytic efficiency of f.Xa increased 16% in the presence of heparin (Table 2). Heparin produced a 27% reduction in K_m (from 57 to 42 μM) and a 15% reduction in k_{cat} (from 169 to 142 s^{-1}). In contrast, in the presence of EDTA, heparin caused only a 2% increase in catalytic efficiency. With Chz-TH, heparin produced a 23% increase in the catalytic efficiency in the presence of Ca^{2+}, whereas only a 2% increase occurred in the presence of EDTA. In the presence of Ca^{2+}, there was an ~2-fold reduction in K_m (from 206 μM in the absence of heparin to 97 μM in its presence). The observation that heparin influences the K_m of f.Xa for peptidyl chromogenic substrates is consistent with the direct binding data illustrating that the environment of an active site-bound fluorophore is altered when heparin binds to f.Xa. Taken together, these findings support the concept that heparin binding to f.Xa influences the active site of the enzyme.

Table 2: Effect of Heparin on Kinetic Parameters of Chromogenic Activity of F.Xa[a]

	K_m (μM) ± heparin		k_{cat} (s^{-1}) ± heparin		k_{cat}/K_m (μM^{-1} s^{-1}) ± heparin	
	0	1 μM	0	1 μM	0	1 μM
S-2765	57	42	169	144	2.93	3.41
Chz-TH	206	97	79	46	0.38	0.47

[a] F.Xa (2−25 nM) was incubated with S-2765 or Chz-TH at concentrations ranging up to 1000 μM in TS buffer containing 2 mM $CaCl_2$ in the absence or presence of 1 μM heparin. Chromogenic activity was monitored in a plate reader. Rates of chromogenic substrate cleavage were calculated, and kinetic parameters were determined by Hanes analysis.

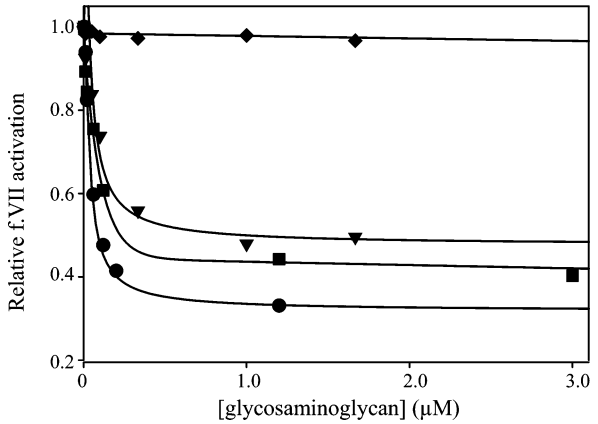

FIGURE 2: Effect of glycosaminoglycans on f.Xa-mediated activation of f.VII. F.VII (1 μM) was activated by 1 nM f.Xa in the presence of 0.5 nM tissue factor, 20 μM PCPS vesicles, 5 mM $CaCl_2$, and varying concentrations of DX (■), HS-LMWH (●), or heparin (▼). After 20 min incubation, the reaction was terminated by addition of 400 nM rTAP and 1 mM EDTA. F.VIIa generation was monitored by measuring the hydrolysis of Pefa-5979. The extent of f.VIIa generation in the presence of glycosaminoglycan was divided by that measured in the absence of glycosaminoglycan. Incubation of f.VIIa with heparin had no effect on f.VIIa-mediated hydrolysis of Pefa-5979 (◆).

Effect of Glycosaminoglycans on the F.Xa-Mediated Activation of F.VII and Protein C. To determine whether glycosaminoglycans modulate the activity of f.Xa with a macromolecular substrate, the rate of activation of tissue factor-bound f.VII, as monitored by Pefa-5979 hydrolysis, was determined in the absence or presence of glycosaminoglycans. As illustrated in Figure 2, heparin, LMWH, HS-LMWH, and DX reduced FVII activation in a concentration-dependent and saturable fashion. In contrast, ND-LMWH and DS, the least sulfated glycosaminoglycans, had no effect on f.VII activation (data not shown). The most sulfated glycosaminoglycans, HS-LMWH and DX, were 2- and 1.5-fold more potent than heparin at inhibiting f.Xa-mediated f.VII activation, whereas LMWH was 197-fold less potent than heparin (Table 3). Control experiments were performed

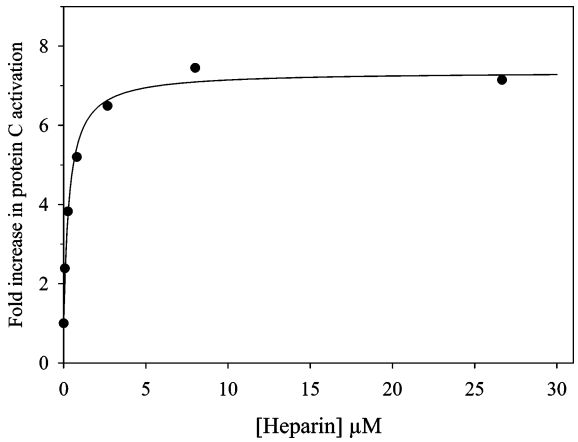

FIGURE 3: Effect of heparin on protein C activation by f.Xa. Protein C (2.5 μM) was incubated for 60 min at 23 °C with 24 nM f.Xa in the presence of 5 mM $CaCl_2$, 200 μM PCPS vesicles, and 0−26 μM heparin. F.Xa was then inactivated with antithrombin and heparin, and activated protein C was detected with S-2366. Rates of chromogenic substrate cleavage were determined in a plate reader. The *x*-fold increase in protein C activation was calculated as a ratio of that produced in the absence of heparin.

Table 3: Concentrations of Various Glycosaminoglycans Required To Produce a 50% Reduction in F.Xa-Mediated Activation of F.VII[a]

glycosaminoglycan	$C_{0.5}$ (μM)
ND-LMWH	NC[b]
DS	NC[b]
LMWH	23.60 ± 14.70
heparin	0.12 ± 0.02
HS-LMWH	0.06 ± 0.01
DX	0.08 ± 0.01

[a] Activation of 1 μM f.VII was performed with 0.5 nM TF, 1 nM f.Xa, 20 μM PCPS, and 5 mM $CaCl_2$ in the presence of various concentrations of glycosaminoglycans. After 20 min incubation, reactions were terminated with rTAP and EDTA, and f.VIIa activity was determined with a chromogenic substrate. Concentrations of glycosaminoglycans that produced 50% of the maximal reduction in f.VII activation ($C_{0.5}$) were determined by nonlinear regression. Values represent the mean ± SD of three experiments. [b] NC indicates no change in f.VII activation.

to demonstrate that f.Xa produced minimal hydrolysis of the f.VIIa-directed chromogenic substrate under the experimental conditions employed and that high concentrations of glycosaminoglycans had no effect on f.VIIa-mediated hydrolysis of Pefa-5979. The absence of an inhibitory effect of heparin on f.VIIa chromogenic activity is consistent with its lack of inhibition of f.X activation by f.VIIa/tissue factor (*30*). The effect of glycosaminoglycans on f.VII activation also was verified using SDS−PAGE analysis (not shown).

As another index of the effect of heparin on f.Xa activity, activation of protein C was examined. Although much lower than the rate of protein C activation by the thrombin−thrombomodulin complex, the observation that heparin promotes f.Xa-mediated activation of protein C suggests another avenue by which heparin exerts its anticoagulant activity (*31*). In these experiments, protein C was incubated with f.Xa for 60 min, and activation was monitored by chromogenic assay for activated protein C. Reactions were performed with varying concentrations of heparin, and the amount of activated protein C generated was compared with that obtained in the absence of heparin (Figure 3). The heparin dose−response curve revealed up to a 7-fold enhancement in the rate of protein C activation by f.Xa. Half-maximal response occurred with a heparin concentration of 330 nM, a value comparable to the K_d of heparin for f.Xa. The experiment was repeated using Protac, a snake venom-derived protein C activator, in place of f.Xa (not shown). Although heparin-dependent stimulation also was observed, half-maximal enhancement required 6 μM heparin, a concentration 20-fold higher than that needed for stimulation of protein C activation by f.Xa. These results provide further support for the concept that heparin modulates the activity of f.Xa with macromolecular substrates.

DISCUSSION

A Ca^{2+}-dependent heparin-binding site has recently been identified on f.Xa (*18*, *32*). This site was previously overlooked in inhibition studies done in plasma systems where Ca^{2+} chelators were added to eliminate the potential for feedback activation of coagulation (*33*, *34*). In the absence of Ca^{2+}, it is postulated that the heparin-binding site in the catalytic domain of f.Xa is blocked because f.Xa assumes a disordered conformation as a consequence of like-charge repulsion between the 11 negatively charged γ-carboxyglutamic acid (Gla) residues within the Gla domain (*16*, *18*, *20*). Ca^{2+} neutralizes the anionic Gla domain and induces a conformational change in f.Xa that exposes the heparin-binding site. By simultaneously binding to the heparin-binding site on f.Xa and to antithrombin, heparin serves as a template to bridge the enzyme to the inhibitor. This phenomenon rationalizes the recent observation that, in the presence of Ca^{2+}, the rate of f.Xa inactivation by antithrombin in the presence of heparin is 20-fold higher than that measured in the presence of heparin-derived pentasaccharide (*18*, *32*). Our studies have extended previous work in two important ways. First, we have demonstrated that the affinity of heparin derivatives for f.Xa is dependent on their negative charge and that glycosaminoglycans other than heparin can bind to the Ca^{2+}-dependent heparin-binding site on f.Xa. Second, our studies indicate that glycosaminoglycan binding to f.Xa induces conformational changes in the active site of the enzyme that modulate its catalytic activity against low molecular weight and macromolecular substrates. This provides another potential mechanism by which heparin may exert its anticoagulant activity.

Glycosaminoglycan Binding. Binding of heparin to f.Xa was monitored by the fluorescence change of fluorescein covalently attached to the active site. In the presence of Ca^{2+}, heparin bound to f.Xa with a K_d value of 0.4 μM, comparable to the K_d value of 0.37 μM inferred from kinetic assays and 0.33 μM determined by surface plasmon resonance (*17*, *27*). This value also is comparable to the affinity of heparin for thrombin, as determined in direct binding assays [0.12 μM (*35*)] and kinetic experiments [0.8 μM (*36*)]. In the absence of Ca^{2+}, binding of heparin to f.Xa was 126-fold weaker, with a K_d value of 50 μM. LMWH bound to f.Xa with the same affinity as heparin in the absence of Ca^{2+} but only demonstrated an 8-fold increase in affinity in the presence of Ca^{2+} (K_d value of 6.9 μM). When GD-f.Xa was used in place of f.Xa, heparin binding was independent of Ca^{2+} (K_d values of 1.3 and 1.5 μM in the absence and presence of Ca^{2+}, respectively), and its affinity was comparable to that for f.Xa in the presence of Ca^{2+}. These observations support the concept that it is the anionic Gla domain that blocks the

glycosaminoglycan-binding site on f.Xa and that Ca^{2+} serves to stabilize this domain and expose the heparin-binding site (*32*). However, binding is detected even in the absence of Ca^{2+}, suggesting that the glycosaminoglycan-binding site on f.Xa is partly exposed under these conditions but becomes fully accessible when Ca^{2+} is present. The ability of f.Xa to bind heparin in the absence of Ca^{2+} is consistent with f.X retention on heparin−agarose, a technique used in f.X purification (*37*).

We next examined the extent to which glycosaminoglycan sulfation dictates their affinity for f.Xa in the absence and presence of Ca^{2+}. Glycosaminoglycans that are more sulfated than heparin have higher affinity for f.Xa, whereas less sulfated glycosaminoglycans have lower affinity. These observations suggest that the net negative charge of glycosaminoglycans determines their affinity for f.Xa, a finding in agreement with other studies demonstrating that increased glycosaminoglycan sulfation increases affinity for positively charged domains on other clotting factors or on inhibitors (*29*, *38*−*40*). DX binds to f.Xa with an affinity comparable to that of HS-LMWH, indicating that glycosaminoglycans other than heparin can bind to f.Xa and that binding is independent of the pentasaccharide sequence that mediates the interaction of heparin and LMWH with antithrombin. HS-LMWH and DX bind f.Xa with relatively high affinity in the absence of Ca^{2+} (K_d values of 1.5 and 1.8 μM, respectively), resulting in lower ratios of Ca^{2+}-induced increase in affinity. These findings raise the possibility that access of more sulfated glycosaminoglycans to the heparin-binding site on f.Xa is less hampered by the Gla domain in the absence of Ca^{2+}. It is possible that more highly sulfated glycosaminoglycans utilize additional residues in the heparin-binding domain of f.Xa, residues that are less accessible to glycosaminoglycans with lower net charge.

Chromogenic Activity of F.Xa. Compared with thrombin, the lack of surface loop insertions in f.Xa leaves its catalytic triad more accessible (*41*). Consequently, the repertoire of substrates for f.Xa is wider than that for thrombin (*42*). There is mounting evidence that the specificity of f.Xa arises from its interactions with inhibitors, cofactors, or substrates at sites distinct from the active site. Hypothesizing that occupation of the glycosaminoglycan-binding exosite on f.Xa may influence the catalytic activity of f.Xa, we examined the effect of heparin on f.Xa-mediated hydrolysis of two chromogenic substrates. Rates of hydrolysis of S-2765 and Chz-TH increase in the presence of heparin. These effects are specific for heparin binding because no effect of heparin was observed in the presence of EDTA, a condition that attenuates the interaction of heparin with f.Xa. Examining the effect of a lower concentration of heparin in the absence of Ca^{2+}, Barrowcliffe et al. failed to observe any change in the amidolytic activity of f.Xa (*33*). Our studies indicate that this effect requires higher concentrations of glycosaminoglycan when experiments are done in the absence of Ca^{2+}. The concept that binding of heparin influences activity is not unique to f.Xa. Thus, binding of a ligand to exosite 2 on thrombin also alters the enzyme's reactivity toward chromogenic substrates (*21*).

Modulation of f.Xa-mediated substrate hydrolysis by glycosaminoglycans may reflect glycosaminoglycan-induced steric hindrance of the active site or allosteric changes at the active site that influence substrate hydrolysis by altering the interaction of the substrate with the catalytic groove or active site of the enzyme. The observation that heparin increases the rate of hydrolysis of S-2765 and Chz-TH argues against steric hindrance as the mechanism by which heparin modulates chromogenic activity. To enhance catalytic activity, heparin may remodel a f.Xa subsite to improve alignment between the substrate and the catalytic triad of f.Xa. Specifically, it has been shown that Tyr99 at the S2 site of f.Xa influences chromogenic substrate specificity and this residue is thought to be a key site by which heparin modulates f.Xa reactivity with antithrombin (*43*). Therefore, alteration of f.Xa-mediated hydrolysis of chromogenic substrates may be a marker of heparin-induced modulation of enzyme specificity.

The influence of heparin on the activity of f.Xa with peptide substrates contrasts with the lack of an observed effect of f.Va on f.Xa chromogenic activity (*42*). This is somewhat surprising given that heparin and f.Va bind to the same domain on f.Xa. This discrepancy could reflect the presence of subdomains, where different residues in the binding domain contribute to the interaction with different ligands. Support for this possibility comes from the demonstration that mutations in the heparin-binding site differentially affect f.Va and heparin binding (*17*).

Activity of F.Xa toward Macromolecular Substrates. There is abundant evidence that the catalytic activity of f.Xa for macromolecular substrates is enhanced by interactions between f.Xa and its cofactors or substrates. One such example is the 300000-fold increase in prothrombin activation rate that occurs when f.Xa is assembled within the prothrombinase complex (*1*, *44*). The residues critical for f.Va recognition by f.Xa have been localized to the basic amino acids that comprise the heparin-binding site (*17*, *32*). It is postulated that f.Va binding to this site induces conformational changes at the surface of f.Xa, thereby exposing secondary exosites that interact with substrate (*45*, *46*). Supporting this concept, synthetic peptides that bind to various domains on the f.Xa surface inhibit cofactor−substrate complex formation (*47*).

Several studies indicate that f.Xa within the prothrombinase complex is protected from inhibition by the heparin/antithrombin complex because, once it is assembled in the prothrombinase complex, the affinity of f.Xa for prothrombin is higher than that for heparin (*1*, *48*−*50*). However, a highly sulfated glycosaminoglycan can inhibit prothrombinase in an antithrombin-independent fashion, likely by interrupting the f.Xa−f.Va interaction (*29*). Consistent with this concept, our data indicate that HS-LMWH binds f.Xa with high affinity.

Our studies show that glycosaminoglycans produce concentration-dependent inhibition of f.Xa-mediated activation of f.VII. Heparin caused ~50% reduction of f.Xa-mediated activation of f.VII compared with that measured in the absence of glycosaminoglycan. The $C_{0.5}$ value of 0.12 μM is comparable to the affinity of heparin for f.Xa, as measured in direct binding studies (K_d of 0.4 μM). The values for the half-maximal effect for HS-LMWH and DX were 50% lower, consistent with their higher affinities for f.Xa, as measured in direct binding studies. These data support the concept that ligand binding at the heparin-binding site can modulate the activity of f.Xa with macromolecular substrates. Further substantiation of this concept comes from the

observation that f.Va, which binds to the same site on f.Xa as heparin, also reduces f.VII activation by f.Xa (*51*).

As another index of the effect of heparin on f.Xa activity, activation of protein C also was examined. Heparin promotes f.Xa-mediated protein C activation 7-fold, with a half-maximal effect at 330 nM, a value similar to the K_d value for the f.Xa−heparin interaction. Although heparin had opposing effects on f.VII and protein C activation, the similar dose responses and the fact that the heparin effect is abrogated in the presence of EDTA suggest that these phenomena are the result of the interaction of heparin with f.Xa. The differential responses could reflect interactions of the substrates with different residues or subsites in the vicinity of the active site of f.Xa.

Tyr99, which occupies the S2 subsite of the active site, is thought to be one of the key mediators of f.Xa specificity (*43*). The presence of this bulky residue likely explains the occurrence of a Gly residue at P2 in antithrombin and most physiological substrates of f.Xa (*41*). Mutation of Tyr99 to a Thr residue reduced the activity of f.Xa with prethrombin 1 and antithrombin, although reactivity with antithrombin could be restored in the presence of heparin (*43*). These findings suggest a direct connection between heparin binding and substrate specificity of f.Xa and may explain why heparin modulates f.Xa activity against f.VII, protein C, and chromogenic substrates. From a structural viewpoint, it is notable that Lys96, which forms part of the heparin-binding site (*32*), also populates the S4 subsite in the active site (*41*). The proximity of residues in the heparin-binding site to those in the active site of f.Xa may help to explain why heparin produces allosteric modulation of f.Xa activity.

Taken together, these data suggest that glycosaminoglycan binding to f.Xa influences the catalytic activity of the enzyme, possibly by perturbing an exosite on the surface of f.Xa and modifying its interactions with its substrates. This concept is supported by studies with the f.Xa-directed monoclonal antibody, αBF.X-2b (*52*). This antibody binds f.Xa within the prothrombinase complex and inhibits prothrombin activation, but αBF.X-2b has no effect on f.Xa-mediated hydrolysis of peptidyl substrates or on f.Xa inhibition by tick anticoagulant peptide, an active site-directed inhibitor of f.Xa. Our results provide additional evidence that f.Xa exosite interactions modulate the enzyme's catalytic activity. They also suggest that, because they utilize the same binding domain, heparin and f.Va elicit similar allosteric effects on f.Xa.

In summary, these results demonstrate that heparin and other glycosaminoglycans have the potential to modulate the activity of f.Xa. This complements previous studies showing that heparin can impair association of the inhibitor, NAPc2 (*53*), and the cofactor, f.Va (*29*), with f.Xa. Therefore, the heparin-binding domain on f.Xa may do more than mediate interactions with f.Va and the heparin−antithrombin complex. These observations add another level of complexity to f.Xa regulation and support the concept that, like thrombin, f.Xa possesses exosites that modulate the function of the active site of the enzyme.

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