In the Presence of Phospholipids, Glycosaminoglycans Potentiate Factor Xa-Mediated Protein C Activation by Modulating Factor Xa Activity†

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ABSTRACT: Although the thrombin/thrombomodulin complex is considered the physiological activator of protein C, factor Xa (f.Xa) can also activate protein C in a reaction that is potentiated by glycosaminoglycans. To explore this phenomenon, we first examined the effect of glycosaminoglycans of varying degrees of sulfation on the kinetics of protein C activation by f.Xa in the presence of Ca^{2+} and phosphatidylcholine−phosphatidylserine vesicles (PCPS). Heparin increased the rate of protein C activation by f.Xa by 4-fold. In contrast, N-desulfated heparin had no effect on activation, whereas dextran sulfate, which is more sulfated than heparin, increased catalytic efficiency 21-fold. These data suggest that the capacity of glycosaminoglycans to catalyze protein C activation by f.Xa depends on their degree of sulfation. The affinities of individual glycosaminoglycans for protein C and f.Xa were measured in the absence or presence of PCPS by monitoring changes in extrinsic fluorescence when fluorescein-labeled f.Xa or protein C was titrated with the various glycosaminoglycans. Heparin binds protein C with low affinity in the absence or presence of PCPS. In contrast, the affinity of heparin for f.Xa is 86-fold higher in the presence of PCPS compared to that in the absence of PCPS. Similar results were obtained using surface plasmon resonance. These findings suggest that a high affinity glycosaminoglycan binding site is exposed when f.Xa binds to PCPS. The observation that heparin promotes f.Xa-mediated activation of prethrombin 1 only in the presence of phospholipid suggests that glycosaminoglycan binding modulates the active site of f.Xa. This study reveals that when f.Xa interacts with anionic phospholipids, glycosaminoglycans bind f.Xa more tightly, allosterically modulate its active site, and enhance its capacity to activate protein C.

The protein C pathway serves as an endogenous anticoagulant mechanism that comes into play when the coagulation system is activated (*1*, *2*). Anticoagulation is effected by activated protein C (APC)[1], which attenuates thrombin generation by proteolytically inactivating activated factors VIII and V, key cofactors in the f.X and prothrombin activation complexes, respectively. Activation of protein C is thought to occur on the endothelial cell surface and is mediated by the thrombin/thrombomodulin complex. Thrombomodulin, a transmembrane receptor, binds thrombin and alters its substrate specificity by converting it from a procoagulant enzyme into a potent activator of protein C (*3*−*5*). This reaction is facilitated by the endothelial cell protein C receptor, which binds protein C and presents it to the thrombin/thrombomodulin complex. The binding of protein C to the receptor is mediated by its γ-carboxyglutamic acid (Gla) domain, and is Ca^{2+}-dependent (*1*).

Although the thrombin/thrombomodulin complex is regarded as the physiological activator of protein C, recent studies have demonstrated that activated f.X (f.Xa) also can activate protein C in a reaction that is thrombomodulin independent (*6*, *7*). The activation of protein C by f.Xa requires Ca^{2+} and a negatively charged phospholipid surface, and the rate of this reaction is enhanced by negatively charged glycosaminoglycans (GAGs), such as unfractionated heparin (UFH) or dextran sulfate (DX). However, the mechanism by which these GAGs enhance f.Xa-mediated protein C activation is unknown. Protein C and f.Xa both possess GAG-binding domains (*8*, *9*), but it is unclear whether potentiation of their interaction requires GAG binding to one or both of these proteins. GAGs of higher molecular weight have been reported to enhance protein C activation by f.Xa to a greater extent than those of lower molecular weight, raising the possibility that GAGs serve as a template to bridge f.Xa and protein C together (*6*).

Because the potentiation of protein C activation by f.Xa may represent an accessory anticoagulant role of UFH and

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[1] Abbreviations: APC, activated protein C; DX, dextran sulfate; f., factor; FITC, fluorescein isothiocyanate; FPRck, Phe-Pro-Arg chloromethyl ketone; GAG, glycosaminoglycan; GD, Gla-domainless; Gla, γ-carboxyglutamic acid; HS-LMWH, hypersulfated LMWH; *I*, fluorescence intensity; LMWH, low molecular weight heparin; ND, N-desulfated; OD, optical density; PCPS, phosphatidylserine−phosphatidylcholine vesicles; SD, standard deviation; sTM, soluble TM; TM, thrombomodulin; TM456, TM domains 4, 5, and 6; UFH, unfractionated heparin.

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other GAGs, we set out to explore the mechanism by which GAGs potentiate this reaction. The GAGs studied included UFH, which has a mean molecular weight of about 15,000, and enoxaparin, a low-molecular-weight heparin (LMWH) with a mean molecular weight of about 5,000. To explore the importance of GAG sulfation with regard to the capacity to promote protein C activation by f.Xa, we also studied hypersulfated LMWH (HS-LMWH) and DX, both of which have mean molecular weights of 5,000, as well as N-desulfated UFH (ND-UFH). In addition, the affinities of these GAGs for f.Xa and protein C were measured in the absence or presence of negatively charged phospholipid vesicles.

EXPERIMENTAL PROCEDURES

Materials

Reagents. Human f.Xa, Gla-domainless f.Xa (GD-f.Xa), α-thrombin, prothrombin, APC, and protein C were obtained from Enzyme Research Laboratories (South Bend, IN). Prethrombin 1 was prepared by thrombin-mediated cleavage of prothrombin, as described (*10*). Protein C was depleted of benzamidine by dialysis against 500 mL of 20 mM Tris-HCl at pH 7.4 and 150 mM NaCl (TS). Fluorescein-D-Phe-Pro-Arg-chloromethyl ketone (fluorescein-FPRck) and factor Va (f.Va) were purchased from Haematologic Technologies, Inc. (Essex Junction, VT). Human antithrombin was from Affinity Biologicals, Inc. (Ancaster, Canada). L-α-Phosphatidylcholine (type III-E from egg yolk) was from Avanti Polar Lipids (Alabaster, AL). L-α-Phosphatidyl-L-serine (from bovine brain), unfractionated grade 1 sodium heparin (UFH) derived from porcine intestinal mucosa (186 units/mg), ND-UFH, polybrene (hexadimethrine bromide), fluorescein-isothiocyanate (FITC), and DX, with a mean molecular weight of 5000, were all obtained from Sigma-Aldrich, Canada. Unilamellar phosphatidylcholine−phosphatidylserine (PCPS) vesicles (75%/25% w/w, respectively) were prepared and characterized as previously described (*11*). HS-LMWH with a mean molecular weight of 5000 was synthesized as described (*11*). Dermatan sulfate was obtained from Mediolanum Farmaceutici (Milan, Italy), whereas LMWH enoxaparin was supplied by Rhone-Poulenc-Sante (Paris, France). The APC-directed chromogenic substrate S-2366 (PyroGlu-Pro-Arg-*p*-nitroanilide) and the f.Xa-directed chromogenic substrate S-2765 (*N*-α-*Z*-D-Arg-Gly-Arg-*p*-nitroanilide 2HCl) were obtained from Chromogenix (West Chester, OH), whereas Chromozym-TH (tosyl-Gly-Pro-Arg-*p*-nitroanilide acetate), a thrombin-directed substrate, was from Roche Diagnostics (Laval, QC). Prionex was from Centerchem (Stamford, CT). Soluble thrombomodulin (sTM) and a TM-derived fragment composed of the epidermal growth factor-like domains 4, 5, and 6 of thrombomodulin (TM456; residues 348−465) (*12*) were kind gifts from Dr. Charles Esmon (University of Oklahoma).

Preparation of Fluorescein-FPR-f.Xa and Fluorescein-FPR-GD-f.Xa. f.Xa or GD-f.Xa (22 μM) was incubated with a 3.3-fold molar excess of fluorescein-FPRck at 23 °C (*13*). Chromogenic activity of 44 nM fluorescein-FPR-f.Xa or fluorescein-FPR-GD-f.Xa with 0.2 mM of the f.Xa-directed substrate, S-2765, was monitored, and the incubation was terminated when no further substrate hydrolysis was detected. The sample was then dialyzed against 500 mL of TS at 4 °C with two changes of buffer over 18 h. The concentration of fluorescein-FPR-f.Xa or fluorescein-FPR-GD-f.Xa was then determined by absorbance using $\epsilon_{0.1\%}^{280}$ of 1.2, after correction for light scatter at 320 nm using the relationship $A_{280}^{corr} = A_{280} - 1.7 \times A_{320}$ (*14*, *15*). The absence of residual fluorescein-FPRck was confirmed by incubating 10 μL of 3.73 μM fluorescein-FPR-f.Xa or fluorescein-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 fluorescein-FPR-f.Xa.

Preparation of FITC-Protein C. Protein C was subjected to dialysis in a 0.1 M sodium phosphate buffer at pH 7.5. FITC was made in dry dimethyl sulfoxide to a stock concentration of 6.1 mM. To 200 μL of 24 μM protein C, 8 μL of 6.1 mM FITC was added to give a final concentration of 240 μM FITC (10-fold molar excess). After incubation for 1.5 h at 23 °C in the dark with gentle end-over-end mixing, the reaction was terminated by the addition of NH_4-Cl to 0.1 M. FITC-labeled protein C (FITC-protein C) was separated from free FITC using a PD10 column (Amersham Biosciences) equilibrated with TS (*13*). The concentration of FITC-protein C was determined by measuring absorbances at 280 and 492 nm and using extinction coefficients of 1.45 mL/mg/cm (Enzyme Research Lab) and 68,000 M^{-1} cm^{-1} (*16*), respectively. The molar ratio of FITC to protein C was 0.5:1.

Methods

Effect of GAGs on Protein C Activation by f.Xa. Protein C activation was monitored using the APC-directed substrate S-2366. All reactions were performed in TS containing 0.5% Prionex in 96-well microtiter plates, with a final reaction volume of 15 μL. Protein C (1 μM), 0 or 100 μM PCPS vesicles, and the various GAGs, in final concentrations ranging from 0 to 26.7 μM, were initially placed into the well. After a 1 h incubation, activation was initiated by the addition of f.Xa (at final concentrations of 24 or 240 nM). Reactions contained $CaCl_2$ or EDTA at concentrations of 3 or 5 mM, respectively. The reactions were incubated for 60 min at 23 °C. Prior to monitoring protein C activation, f.Xa was inactivated by a 2 min incubation with antithrombin and UFH (final concentrations of 1.6 and 7.4 μM, respectively). Then, 175 μL of 400 μM S-2366 containing 8 mg/mL polybrene was added, and substrate hydrolysis over 5 min was monitored at 405 nm using a microplate spectrophotometer (Thermomax, Molecular Devices, Sunnyvale, CA). Rates of chromogenic substrate cleavage were determined by instrument software. Final APC concentrations were then determined by reference to a standard curve constructed with known concentrations of APC ranging from 0 to 20 nM. Less than 25% of the total amount of protein C was activated in any individual reaction. To examine the effect of f.Va on f.Xa-mediated protein C activation, the experiment was repeated in the presence of f.Va (in concentrations ranging from 0 to 60 nM) and 8 μM UFH, with the reagents incubated for 60 min prior to the addition of protein C.

To determine the K_m and k_{cat} values for f.Xa-mediated protein C activation, protein C (in concentrations ranging from 0 to 9.3 μM) was incubated with 100 μM PCPS and 3 mM $CaCl_2$ in TS in the presence of different GAGs (all at a concentration of 8.8 μM). Activation was initiated by the

addition of 24 nM f.Xa. After incubation for 1 h at 23 °C, APC generation was quantified as described above. Up to 45% of total protein C was activated in individual reactions containing the lowest starting protein C concentration of 0.4 μM, with less than 5% of protein C activated in reactions containing the highest starting concentration of 9.3 μM. The rate of APC generated per min was then divided by the f.Xa concentration in the reaction, and this result was then used to calculate K_m and k_{cat} values from the Michaelis−Menten equation by nonlinear regression using Table Curve (Jandel Scientific, San Rafael, CA).

Kinetic Parameters for Thrombin-Mediated Protein C Activation in the Absence or Presence of TM. Thrombin-mediated protein C activation was examined in the absence or presence of TM. In the absence of TM, reactions were initiated by the addition of 100 nM thrombin (final concentration) to wells containing protein C (in concentrations ranging from 0 to 9.3 μM) and 3 mM $CaCl_2$ in the absence or presence of the various GAGs in TS. After incubation for 150 min at 23 °C, APC concentrations were quantified, and the kinetic parameters were calculated as described above. The same procedures were used to study thrombin-mediated protein C activation in the presence of 48.6 nM sTM or TM-456 except that reactions were initiated by the addition of thrombin to 10 nM, and the incubation time was shortened to 20 min. Less than 20% of total protein C was activated by thrombin with or without TM at all starting concentrations of protein C.

Affinity of GAGs for Fluorescein-FPR-f.Xa, Fluorescein-FPR-GD-f.Xa, and FITC-Protein C. The extrinsic fluorescence of 50 nM fluorescein-FPR-f.Xa, fluorescein-FPR-GD-f.Xa, or FITC-protein C in TS and 3 mM $CaCl_2$ (final volume of 900 μL) was continuously monitored using a LS50B luminescence spectrophotometer (Perkin-Elmer, Etobicoke, ON, Canada) with $\lambda_{ex} = 492$ nm, $\lambda_{em} = 535$ nm, cutoff filter = 515 nm, and slit widths set to 5−15 nm (*13*). 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. To determine the affinity of GAGs for fluorescein-FPR-f.Xa, fluorescein-FPR-GD-f.Xa, or FITC-protein C, their fluorescence intensities were measured prior to (I_o) and after (I) the addition of 1 to 10 μL aliquots of 200 μM UFH, 100 μM DX, 100 μM HS-LMWH, or 600 μM LMWH. Studies were done in the absence or presence of 100 μM PCPS. The signal was allowed to stabilize prior to each addition. Titrations were continued until there was no change in fluorescence with subsequent additions. I/I_o values were calculated and plotted versus GAG concentration. Assuming a 1:1 stoichiometry, K_d values were determined by nonlinear regression analysis using the following equation (*13*), where L is the GAG concentration, P_o is the concentration of fluorescein-FPR-f.Xa, fluorescein-FPR-GD-f.Xa, or FITC-protein C, and α is the maximum change in emission intensity.

$$\frac{I}{I_o} = 1 + \frac{\alpha}{2}\left(1 + \frac{K_d + L}{P_o} - \sqrt{\left(1 + \frac{K_d + L}{P_o}\right)^2 - 4 \times \frac{L}{P_o}}\right)$$

Surface Plasmon Resonance. Binding of f.Xa to heparin also was examined by surface plasmon resonance using a BIAcore 1000 (Piscataway, NJ). Heparin was conjugated with biotin and adsorbed onto the flow cells of a streptavidin-coated SA sensor chip. To prepare biotin−heparin, deaminated heparin (Sigma) was dissolved in 0.1 M sodium sulfate and subjected to size exclusion chromatography on a Bio-Sep SEC-S3000 preparative column (Phenomenex, Torrance, CA) using a Beckman 32 Karat high-pressure liquid chromatography system (Beckman-Coulter Inc., Fullerton, CA). Fractions were dialyzed against water and rechromatographed on a Bio-Sep S2000 column (Tosoh BioScience, LLC, Montgomeryville, PA) that had been calibrated with heparin derivatives of known molecular weight. The fraction corresponding to a peak molecular weight of 15,000 with a range of 9000−20,000 was selected. A 1.4 mg aliquot of this preparation was dissolved in 100 μL of water and labeled with biotin by mixing with 1.3 mg of biotinamidohexanoic acid-hydrazide (Sigma) dissolved in 100 μL of 0.1 M sodium acetate at pH 5.5. The reaction was allowed to proceed for 2.5 h at 23 °C before being applied to a PD-10 column equilibrated with water. Fractions of 0.5 mL were collected, and the absorbance at 210 nm was monitored. The peak heparin-containing fractions were pooled and lyophilized.

For SPR analysis, 12 μL of 0.25 μg/mL biotin−heparin was injected at 5 μL/min into a flow cell containing an SA sensor chip (prewashed with 10 μL of 50 mM NaOH and 1 M NaCl). After equilibration with 10 mM Hepes-OH at pH 7.4, 150 mM NaCl, and 2 mM $CaCl_2$ (HBSC buffer), 30 response units of heparin was bound to the chip. The cells were then washed with HBSC buffer containing 1 M NaCl to remove nonspecifically bound biotin−heparin. Using the kinetic injection mode, serial dilutions of f.Xa, in concentrations ranging from 15 to 2000 nM in HBSC buffer, were passed through the cell at a flow rate of 40 μL/min for 3 min in the absence or presence of 30 μM PCPS. Between each injection, the flow cell was regenerated with HBSC containing 1 M NaCl and then re-equilibrated with HBSC buffer. For each titration, a parallel flow cell lacking biotin−heparin was titrated with f.Xa or f.Xa/PCPS as a control. Data were analyzed with Scrubber 2 software (Biologic Software. Pty., Campbell, Australia) using a single 1:1 interaction model or with BIAevaluation software from BIAcore.

Effect of PCPS or GAGs on f.Xa-Mediated Activation of Prethrombin 1. The effect of 100 nM heparin and/or 26 μM phospholipid vesicle on the activation of 1 μM prethrombin 1 by 50 nM f.Xa was examined by chromogenic assay. Studies were performed in TBS containing 2 mM $CaCl_2$ at 23 °C. Aliquots were removed at 10 min intervals into microplate wells containing 200 μM Chromozym-TH and 10 mg/mL polybrene. Thrombin activity was monitored at 405 nm by determining the rate of chromogenic substrate cleavage.

Statistical Analyses. All values represent the mean ± standard deviation (SD) of at least two measurements. These values were calculated using Microsoft Excel or Minitab Release 14 statistical software (Minitab Inc., State College, PA). Using Minitab, mean K_d values for the binding of individual GAGs to either f.Xa or protein C in the absence of PCPS were compared with those measured in the presence of PCPS using a 2-sample Student's t-test. A p value of <0.05 was considered statistically significant.

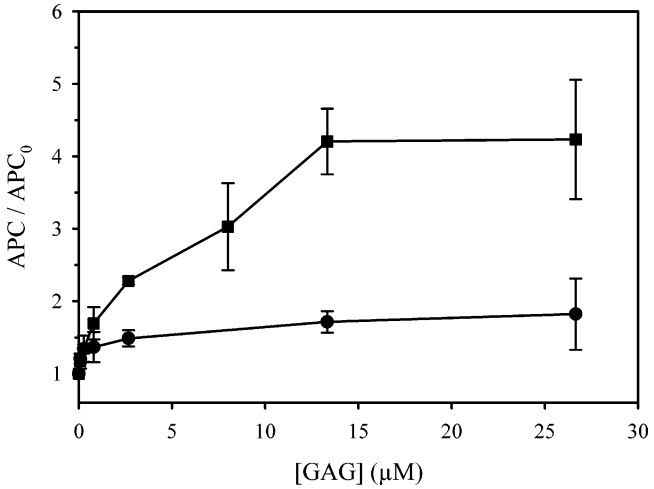

FIGURE 1: Effect of GAGs on protein C activation by f.Xa in the absence of PCPS. Protein C (1 μM) was incubated with 240 nM f.Xa and 0 to 26.7 μM UFH (●) or DX (■) for 60 min at 23 °C in a buffer containing 5 mM $CaCl_2$ in the absence of PCPS. f.Xa was then inactivated with antithrombin and UFH, and APC generation was quantified using a chromogenic assay. APC/APC_0, calculated by dividing the amount of APC generated in individual reactions by that generated in the absence of GAG, is plotted against GAG concentration. The data points represent the mean of at least two experiments, whereas the bars reflect the standard deviation.

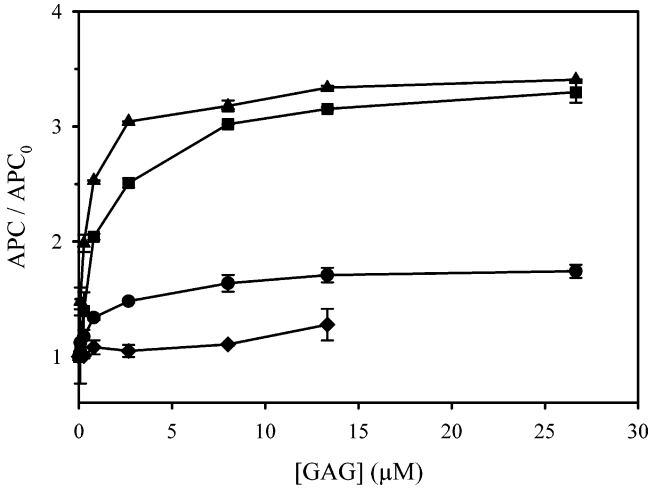

FIGURE 2: Effect of GAGs on protein C activation by f.Xa in the presence of PCPS. Protein C (1 μM) was incubated for 60 min at 23 °C with 24 nM f.Xa in the presence of 5 mM $CaCl_2$, 100 μM PCPS, and 0−26.7 μM UFH (●), DX (■), HS-LMWH (▲), or LMWH (♦). Reactions were terminated by the addition of antithrombin and UFH, and APC generation was quantified. APC/APC_0, calculated by dividing the amount of APC generated in individual reactions by that generated in the absence of GAG, is plotted against GAG concentration. The data points represent the mean of at least two experiments, whereas the bars reflect the standard deviation.

RESULTS

Effect of GAGs on f.Xa-Mediated Activation of Protein C. The rate of protein C activation by f.Xa was determined in the absence or presence of various GAGs. In the absence of PCPS and with a f.Xa concentration of 240 nM, DX and to a lesser extent UFH increased f.Xa-mediated protein C activation in a concentration-dependent and saturable fashion (Figure 1). At optimum GAG concentrations, UFH produced a 1.8-fold increase in the basal rate of activation (0.0038 min^{-1}), whereas the more highly sulfated DX produced about a 4-fold increase. This experiment was then repeated in the presence of PCPS (Figure 2). Similar effects of GAGs were observed but protein C activation was effected with a 10-fold lower concentration of f.Xa. Thus, in the presence of PCPS, UFH, DX, and HS-LMWH increased protein C activation by 24 nM f.Xa in a concentration-dependent and saturable fashion. At optimum concentrations, UFH produced a 1.7-fold increase in the basal activation rate (0.034 min^{-1}), whereas DX and HS-LMWH, which are more sulfated than UFH, produced 3- to 3.5-fold increases in the activation rate. LMWH had minimal stimulatory effect, and no increases in activation rates were seen with less sulfated GAGs, ND-UFH, and dermatan sulfate (data not shown). GAG enhancement of protein C activation by f.Xa is Ca^{2+}-dependent because neither DX nor UFH (at concentrations up to 27 μM) increased the rate of protein C activation, with or without PCPS, when 3 mM EDTA was present (data not shown). To rule out potential contamination of the f.Xa preparation with thrombin, the rate of protein C activation by f.Xa in the presence of DX and PCPS was measured in the absence or presence of hirudin. Hirudin had no effect on the rate of protein C activation, thereby excluding the presence of thrombin in the f.Xa preparation (not shown).

To begin to explore the mechanism by which GAGs influence protein C activation by f.Xa in the presence of PCPS, kinetic analyses were performed using protein C in concentrations ranging from 0 to 9.3 μM. As shown in Table 1, the more sulfated GAGs produce up to a 20-fold increase in the catalytic efficiency of protein C activation by f.Xa. The increase in catalytic efficiency in the presence of GAGs predominantly results from a 6- to 8-fold reduction in the K_m (from 3.3 μM in the absence of GAGs) with DX or HS-LMWH and a 2.3-fold decrease in K_m with UFH. In contrast, no change in K_m is seen with LMWH. The more sulfated GAGs also produce 2.0- to 2.5-fold increases in k_{cat} values (from 0.09 min^{-1} in the absence of GAG to 0.21 or 0.24 min^{-1} in the presence of DX or HS-LMWH, respectively).

Table 1: Kinetic Parameters for Protein C Activation by f.Xa and PCPS and in the Absence or Presence of GAGs[a]

GAG	K_m μM	k_{cat} min^{-1}	k_{cat}/K_m $M^{-1}\,min^{-1}$	fold increase[b]
none	3.3 ± 0.9	0.09 ± 0.03	(0.27 ± 0.01) × 10^5	1.0
LMWH	3.2 ± 0.2	0.12 ± 0.008	(0.38 ± 0.01) × 10^5	1.4
UFH	1.4 ± 0.09	0.15 ± 0.002	(1.07 ± 0.01) × 10^5	3.9
HS-LMWH	0.54 ± 0.07	0.21 ± 0.01	(3.88 ± 0.05) × 10^5	14.3
DX	0.40 ± 0.06	0.24 ± 0.01	(5.80 ± 0.09) × 10^5	21.4

[a] Kinetic constants were determined by incubating protein C (0 to 9.3μM) with 24 nM f.Xa, 100 μM PCPS, and 3 mM $CaCl_2$ in the absence or presence of the various GAGs (all at 8.8 μM) for 60 min at 23 °C. The concentrations of APC generated were determined by a chromogenic assay. K_m and k_{cat} values are the mean ± SD of at least two experiments. [b] Fold increase is the increase relative to the k_{cat}/K_m measured without GAG.

To determine whether f.Xa incorporated into the prothrombinase complex retains its capacity to activate protein C, we examined the effect of f.Va addition on the rate of protein C activation by f.Xa in the presence of PCPS and 8 μM UFH. The addition of f.Va to 60 nM, a concentration twice that of f.Xa, had no influence on the rate of protein C activation. Under these conditions, over 90% of the f.Xa should be bound to f.Va. These data suggest that f.Xa retains

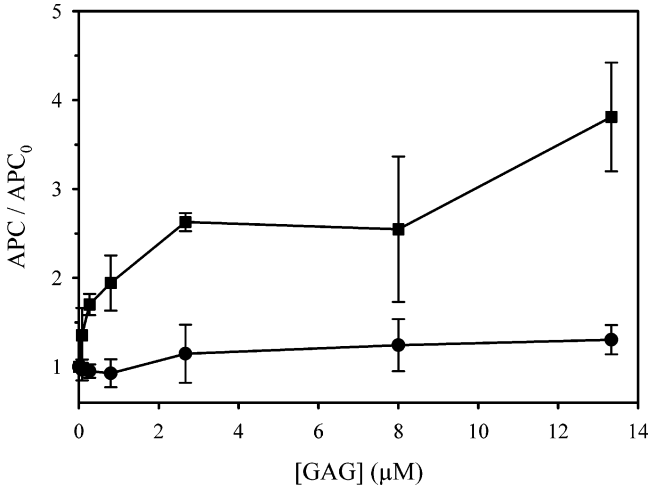

FIGURE 3: Effect of UFH and DX on protein C activation by GD-f.Xa. Protein C (1 μM) was incubated with 240 nM GD-f.Xa in the presence of 5 mM $CaCl_2$, 100 μM PCPS, and 0 to 13 μM UFH (●) or DX (■) for 60 min at 23 °C. Reactions were terminated by the addition of antithrombin and UFH, and APC generation was quantified. APC/APC_0, calculated by dividing the amount of APC generated in individual reactions by that generated in the absence of GAG, is plotted against GAG concentration. The data points represent the mean of at least two experiments, whereas the bars reflect the standard deviation.

its capacity to activate protein C even when f.Xa is incorporated into the prothrombinase complex and that GAGs can enhance this reaction.

Effect of GAGs on Protein C Activation by GD-f.Xa. To determine the extent to which protein C activation by f.Xa requires the interaction of f.Xa with negatively charged phospholipid membranes, GD-f.Xa was used in place of f.Xa. At a GD-f.Xa concentration of 24 nM, no increase in the rate of protein C activation was seen in the presence of UFH or DX (not shown). When the concentration of GD-f.Xa was increased to 240 nM, DX increased the rate of protein C activation 3.8-fold, whereas UFH still had little effect (Figure 3).

Protein C Activation by Thrombin. To compare the catalytic efficiency of protein C activation by f.Xa relative to that of thrombin, we studied the thrombin-mediated activation of protein C in the absence or presence of TM. In the absence of TM, rates of activation failed to reach saturation at a protein C concentration of 9.3 μM. This suggests that the K_m is above 10 μM. The catalytic efficiency, estimated from the slope of the linear portion of the plot, was 0.011×10^5 M^{-1} min^{-1} (Table 2). This is comparable to previous reports and is lower than that of f.Xa in the absence of DX. With the addition of sTM or TM-456, the catalytic efficiency of protein C activation by thrombin is enhanced 600-fold to a value comparable to those reported in the literature (*17*, *18*). The enhancement results from both a reduction in K_m and an increase in k_{cat} values (*3*). As observed previously, the catalytic efficiency of protein C activation by the thrombin/TM complex is comparable to that of f.Xa in the presence of sulfated GAGs (*6*).

Affinity of GAGs for Fluorescein-FPR-f.Xa or Fluorescein-FPR-GD-f.Xa in the Absence or Presence of PCPS. Because GAGs had different effects in the presence of PCPS than in their absence, the affinity of GAGs for f.Xa was quantified both in the absence and presence of phospholipids. The

Table 2: Kinetic Parameters for Protein C Activation by Thrombin in the Absence or Presence of TM456 or sTM[a]

activator	K_m μM	k_{cat} min^{-1}	k_{cat}/K_m M^{-1} min^{-1}
thrombin	ND	ND	$(0.011 \pm 0.001) \times 10^5$
thrombin + TM456	4.7 ± 0.7	5.3 ± 0.2	$(10.8 \pm 0.2) \times 10^5$
thrombin + sTM	4.2 ± 0.4	3.9 ± 0.1	$(9.2 \pm 0.1) \times 10^5$

[a] Kinetic constants for the thrombin activation of protein C were determined by incubating 100 nM thrombin with 0 to 9.3 μM protein C and 3 mM $CaCl_2$ for 150 min at 23 °C. When the thrombin/TM456 or thrombin/TM complex was used as the activator, the same conditions were employed except that the thrombin concentration was 10 nM, TM456 or sTM was added at 48 nM, and the incubation time was 20 min. The concentrations of APC generated were determined by a chromogenic assay. K_m and k_{cat} values are the mean ± SD and reflect the results of at least two experiments. ND, not determined.

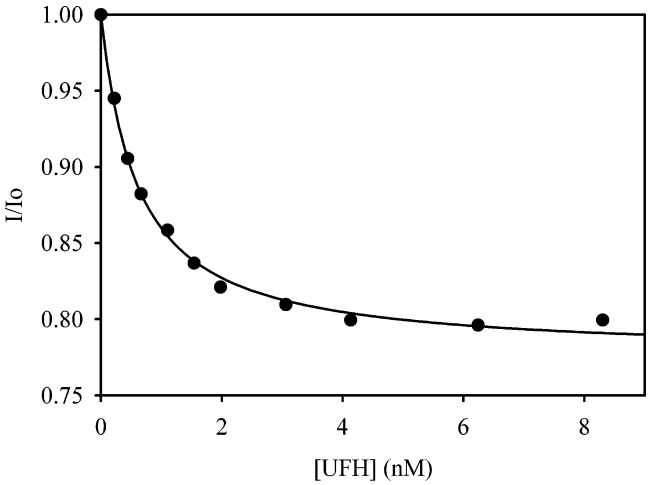

FIGURE 4: Binding of UFH to fluorescein-FPR-f.Xa. Fluorescein-FPR-f.Xa (50 nM) was titrated with UFH in the presence of 2 mM $CaCl_2$, and fluorescence was monitored at 535 nm using an excitation wavelength of 492 nm and an emission filter of 515 nm. Fluorescence intensity was measured before (I_0) and after (I) the addition of UFH, and I/I_0 values were calculated. Data were analyzed by nonlinear regression analysis (solid line) yielding a K_d value of 0.57 μM.

affinity of the various GAGs for f.Xa in the presence of 3 mM $CaCl_2$ was measured by monitoring changes in the extrinsic fluorescence of fluorescein-FPR-f.Xa as it was titrated with the respective GAGs in the absence or presence of 100 μM PCPS. Titrations resulted in concentration-dependent and saturable decreases in fluorescence intensity, and K_d values were determined by nonlinear regression analysis (Figure 4). In the absence of phospholipid, the extent of sulfation of the various GAGs appears to influence their affinity for f.Xa (Table 3). Thus, in comparison with UFH ($K_d = 0.61$ μM), the more highly sulfated GAGs, DX and HS-LMWH, have 2-fold higher affinity for f.Xa, whereas the affinity of LMWH for f.Xa is 2-fold lower. Notably, DX and HS-LMWH also were the most potent stimulators of protein C activation by f.Xa (Table 1).

With the exception of LMWH, the addition of PCPS was associated with a statistically significant increase in the affinity of all GAGs for f.Xa (Table 3). The affinity of UFH for f.Xa increased almost 100-fold in the presence of PCPS (from a K_d value of 0.61 to 0.007 μM), whereas the affinities of DX and HS-LMWH for f.Xa increased 10-fold, and the affinity of LMWH for f.Xa increased 1.5-fold. The fact that increases in affinity also were obtained with short-chained

Table 3: Affinity of Various GAGs for f.Xa or GD-f.Xa in the Absence or Presence 100 μM PCPS[a]

		K_d (μM)		
	GAG	no PCPS	PCPS	*p*-value
F.Xa	LMWH	1.46 ± 0.21	0.95 ± 0.21	0.1
	UFH	0.61 ± 0.04	0.007 ± 0.001	<0.01
	DX	0.29 ± 0.05	0.025 ± 0.004	0.02
	HS-LMWH	0.22 ± 0.03	0.021 ± 0.009	<0.01
GD-f.Xa	UFH	0.499 ± 0.112	0.248 ± 0.025	0.06

[a] The dissociation constants were determined by titrating 50 nM fluorescein-FPR-f.Xa or fluorescein-FPR-GD-f.Xa with GAGs in the absence or presence of 100 μM PCPS. All titrations were done in the presence of 3 mM $CaCl_2$. Changes in fluorescence were quantified, and K_d values were determined by nonlinear regression analysis. The values represent the mean ± SD of at least two experiments. The significance of differences in K_d values with or without PCPS was calculated using Student's *t*-test.

GAGs suggests that the GAG-mediated bridging of multiple f.Xa molecules is unlikely to be the mechanism through which binding is increased. No binding of UFH to f.Xa could be detected in the presence of 5 mM EDTA, with or without PCPS (data not shown). Within the limits of sensitivity of the techniques used, no difference in binding of UFH to fluorescein-FPR-GD-f.Xa could be detected in the absence or presence of 100 μM PCPS. These findings suggest that when f.Xa binds to negatively charged phospholipid via its Gla-domain, it undergoes a conformation change that heightens its affinity for GAGs.

Determination of Heparin Affinity for f.Xa by Plasmon Resonance. To confirm the observation that the affinity of f.Xa for heparin increases in the presence of phospholipid, plasmon resonance binding studies were performed. Biotin-labeled heparin was adsorbed onto a streptavidin-coated flow cell. Increasing concentrations of f.Xa were passed through the flowcell in the absence or presence of 30 μM PCPS (Figure 5). As a control, proteins were injected into a flow cell lacking heparin. The K_d values of f.Xa binding to immobilized heparin were 900 and 8.5 nM in the absence and presence of PCPS, respectively. These values were derived from the association and dissociation rates obtained by the instrument software. Similar values were obtained when the data were analyzed by the maximum response values. The K_d values compare closely to those determined by fluorescence (Table 3) and reveal a 106-fold higher affinity of f.Xa for heparin in the presence of PCPS compared to that in its absence. This effect was maintained even with low concentrations of f.Xa, where the possibility of UFH bridging multiple f.Xa molecules is remote. Thus, at 15 nM f.Xa and 30 μM PCPS, where the molar ratio of f.Xa to vesicles is near unity, the K_d value is 4 nM, a value comparable to the K_d value of 7 nM obtained when all concentrations of f.Xa are considered. No interaction of PCPS with biotin−heparin was observed in the absence of f.Xa. Thus, the plasmon resonance studies provide additional support for the concept that phospholipid-bound f.Xa has higher affinity for heparin than free f.Xa.

Affinity of GAGs for FITC-Protein C. To examine the possibility that GAGs were affecting activation by interacting with the substrate, GAG binding to protein C also was quantified. The affinities of the various GAGs for protein C were determined by monitoring changes in the extrinsic

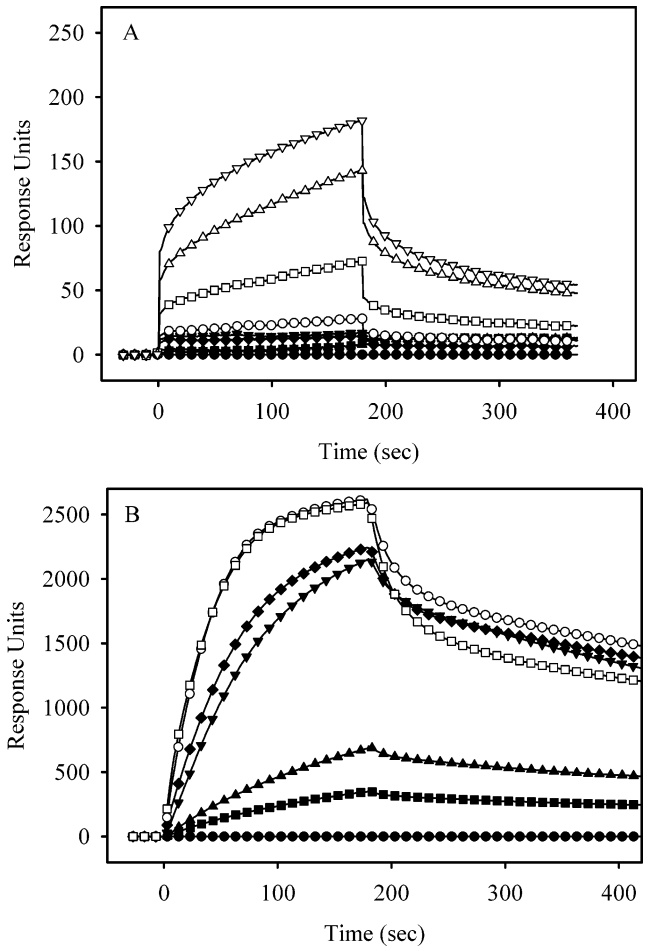

FIGURE 5: Surface plasmon resonance analysis of f.Xa binding to biotin−heparin in the absence or presence of PCPS. Biotin−heparin (30 response units) was adsorbed onto a streptavidin-coated sensor chip. f.Xa, in concentrations of 0, 15.6, 31.3, 62.5, 125, 250, 500, 1000, or 2000 nM (●, ■, ▲, ▼, ♦, ○, □, △, ▽, respectively), was passed over the chip in the absence (panel A) or presence (panel B) of 30 μM PCPS. This was followed by buffer to monitor dissociation. A parallel flow cell lacking biotin−heparin was used to correct for the background signal. Corrected response units are plotted vs time. The vertical scale in panel B is 10-fold greater than that in panel A.

fluorescence of 50 nM FITC-protein C as it was titrated with the respective GAGs in the presence of 3 mM $CaCl_2$. Affinities were measured in the absence or presence of 100 μM PCPS. With this concentration of PCPS, the bulk of protein C will be bound to the phospholipid because the K_d value for this interaction is 3 μM (*19*). As illustrated in Table 4, DX and HS-LMWH, the more highly sulfated GAGs bind protein C with higher affinity than LMWH, a less sulfated GAG (K_d values of 1.0, 0.9, and 5.9 μM, respectively). In contrast to the results obtained with f.Xa, only UFH bound to protein C with significantly higher affinity in the presence of PCPS compared to that in its absence (K_d values of 1.0 and 2.0 μM, respectively). The affinity of UFH for FITC-protein C is comparable to that reported for APC (*9, 20*).

Effect of UFH and/or PCPS on f.Xa Activation of Prethrombin 1. To determine whether the enhancing effect of heparin on f.Xa activity applies only to protein C activation, the effect of heparin on f.Xa activation of a second macromolecular substrate also was examined. Prethrombin 1, a thrombin-derived cleavage product of prothrombin, was

Table 4: Affinity of Various GAGs for Protein C in the Absence or Presence of 100 μM PCPS[a]

GAG	K_d (μM)		*p*-value
	No PCPS	PCPS	
LMWH	5.92 ± 0.18	5.24 ± 0.68	0.3
UFH	1.98 ± 0.33	0.99 ± 0.10	0.01
DX	1.01 ± 0.08	0.89 ± 0.28	0.6
HS-LMWH	0.94 ± 0.09	0.89 ± 0.06	0.6

[a] The dissociation constants were determined by titrating 50 nM FITC-PC with various GAGs in the absence or presence of 100 μM PCPS. All titrations were done in the presence of 3 mM $CaCl_2$. Changes in fluorescence were quantified, and K_d values were determined by nonlinear regression analysis. The values represent the mean ± SD of at least two experiments. The significance of differences in K_d values with or without PCPS was calculated using Student's *t*-test.

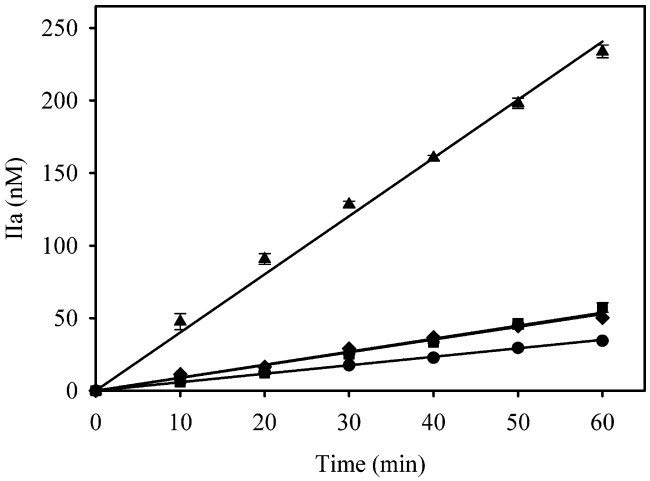

FIGURE 6: Influence of heparin and phospholipid on the activation of prethrombin 1 by f.Xa. Prethrombin 1 (1 μM) was activated by 50 nM f.Xa in the presence of 2 mM $CaCl_2$ (●) and in the presence of either 26 μM PCPS (■), 100 nM heparin (♦), or both (▲). At 10 min intervals, the aliquots were removed, and thrombin generation was quantified by chromogenic activity.

selected because it lacks the phospholipid-binding Gla-domain. Because the K_m for prethrombin 1 activation by prothrombinase is about 10 μM (*21*), a full kinetic study was not performed. Prethrombin 1 was activated by f.Xa in the absence or presence of heparin and/or PCPS. On their own, neither heparin nor phospholipid affected the rate of f.Xa-mediated activation of prethrombin 1 (Figure 6). When added in combination, however, the rate of activation increased 7-fold. These findings support the concept that the influence of heparin on f.Xa activity is magnified in the presence of phospholipids and is not restricted to protein C activation.

DISCUSSION

The possibility that f.Xa could activate protein C first arose from the results of animal studies demonstrating that the infusion of f.Xa and PCPS produced a bleeding diathesis in healthy dogs. Investigations into the mechanism showed the associated activation of protein C and the inactivation of factors Va and VIIIa (*22*, *23*). Subsequent studies confirmed the ability of f.Xa to activate human protein C in a reaction requiring Ca^{2+} and negatively charged phospholipid but independent of thrombomodulin (*6*, *24*). Both f.Xa and protein C possess heparin-binding sites (*8*, *9*, *20*, *25*), and separate studies have demonstrated that the rate of activation of protein C by f.Xa is enhanced by negatively charged GAGs, such as UFH and DX (*6*, *24*). However, the mechanism by which GAGs potentiate this reaction is uncertain. A template mechanism has been proposed on the basis of the observations that oligosaccharides composed of fewer than 10 saccharide units do not accelerate the reaction, whereas the K_m for the reaction is reduced by longer polysaccharides (*6*). However, a bell-shaped concentration-dependence curve for heparin acceleration was not observed, casting doubt on a template role for heparin. The purpose of the current study was to explore the mechanism by which GAGs accelerate the rate of protein C activation by f.Xa. This is important because potentiation of this reaction may contribute to the anticoagulant activity of heparin and other GAGs.

Because both f.Xa and protein C possess heparin-binding sites, it is possible that heparin serves a template role, binding both enzyme and substrate, thereby promoting protein C activation. The heparin-binding site of f.Xa consists of basic residues located in the catalytic domain of f.Xa and is similar in structure and location to the heparin-binding exosite 2 of thrombin (*25*). f.Xa assumes a disordered conformation in the absence of Ca^{2+} (*26*), and it has been hypothesized that the anionic Gla-domain of f.Xa blocks the GAG-binding site under these circumstances (*8*, *25*). Heparin binds to APC via an anion-binding exosite consisting of basic residues clustered on three exposed surface loops in a location that coincides with exosite 1 of thrombin (*20*). The binding of heparin to APC also is Ca^{2+}-dependent, and again, it has been suggested that the Gla-domain of the protein interferes with the anion-binding exosite, if not stabilized by Ca^{2+} (*20*, *26*).

We demonstrate that sulfated GAGs increase the rate of protein C activation by f.Xa in a concentration-dependent and saturable fashion. As in previous studies, the reaction was Ca^{2+}-dependent (*6*, *24*). The ability of individual GAGs to increase the rate of protein C activation by f.Xa is charge-dependent, in that the highly sulfated GAGs, DX and HS-LMWH, had maximal effect, UFH had an intermediate effect, and less sulfated GAGs had little or no activity. These findings are in agreement with earlier observations that more highly charged polysaccharides bind f.Xa with higher affinity and are more efficient at promoting protein C activation by f.Xa (*13*, *24*). Molecular charge appears to be a greater determinant of catalytic effect rather than molecular weight because the activity of GAGs of similar molecular weight varied depending on their charge. It is likely, therefore, that low net molecular charge explains the previously observed inability of small oligosaccharides to catalyze f.Xa activation of protein C, as opposed to the failure of these compounds to serve as a template (*6*). Because highly sulfated LMWH was a potent stimulator of protein C activation by Xa, these results suggest that bridging is not the principle mechanism by which GAGs promote protein C activation by f.Xa.

The K_d value of 0.61 μM for UFH binding to f.Xa determined by fluorescence is comparable to values that were previously obtained using a variety of methods (*5*, *8*, *13*). In this study, the affinity of UFH for f.Xa increases 100-fold when PCPS vesicles are present. Lesser but still statistically significant increases in the affinities of DX and HS-LMWH for f.Xa also were seen. These observations suggest that when f.Xa binds to negatively charged phospholipid via its Gla-domain, either there is a conformational

change at the heparin-binding site of f.Xa that optimizes its interaction with UFH or there is exposure of a novel high-affinity heparin-binding site. The importance of the protein−membrane interaction is demonstrated by the observations that GAG-mediated promotion of protein C activation is impaired with Gla-domainless forms of either f.Xa (Figure 3) or protein C (*24*). The smaller increase in affinity observed with more highly sulfated GAGs may result from the higher initial affinities in the absence of phospholipid or may result from the interaction with different basic residues on f.Xa compared to UFH, as has been previously suggested (*13*, *25*). A more modest increase in the binding affinity of UFH for protein C was seen in the presence of PCPS, with the binding of other GAGs showing no significant change. These findings suggest that the heparin-binding site of protein C undergoes little or no conformational change when protein C binds to phospholipid.

Previously, we demonstrated that heparin modulates the activity of f.Xa with chromogenic or macromolecular substrates, suggesting that heparin causes a conformational change at the active site of f.Xa (*13*). Because the previous experiments were performed in the absence of phospholipids, the potential effect of heparin on the activity of f.Xa may have been underestimated. To explore this possibility, we performed a kinetic evaluation of protein C activation by f.Xa in the absence or presence of GAGs. In the presence of 100 μM PCPS, the predominant effect of GAG is a reduction of the K_m of protein C for f.Xa. In contrast, with thrombin-mediated activation of protein C, the major effect of TM is to increase the k_{cat} value (Table 2) (*3*). To verify that GAGs specifically modulate the active site of f.Xa, we examined the effect of UFH on f.Xa-mediated activation of prethrombin 1. This prothrombin derivative was selected because it retains its f.Xa cleavage sites, but (a) it does not bind phospholipid, and (b) it does not bind heparin because exosite 2 is masked by fragment 2. On their own, neither heparin nor phospholipid affected the rate of f.Xa-mediated prethrombin 1 activation. In contrast, when added together, the rate of activation increased 7-fold. Similar to the findings with protein C activation, these data suggest that heparin enhancement of f.Xa activity is phospholipid-dependent. Taken together, our data suggest that GAG binding to f.Xa induces conformational changes in the active site of the enzyme. These heparin-induced conformational changes are maximized when f.Xa is bound to phospholipids. It is possible that the conformational changes in phospholipid-bound f.Xa induced by heparin are analogous to those that occur when f.Xa binds to f.Va within prothrombinase. Heparin binds to the same site on f.Xa as does f.Va, and like f.Va (*27*), heparin binds f.Xa with higher affinity in the presence of phospholipid and stimulates the catalytic activity of f.Xa with macromolecular substrates. Thus, heparin may mimic the cofactor role that f.Va plays in prothrombinase.

The proposed allosteric effect of heparin binding on the active site of f.Xa could also modulate f.Xa inhibition by antithrombin. In the presence of Ca^{2+}, the heparin-catalyzed rate of inhibition of f.Xa by antithrombin is increased compared to that observed with the antithrombin-binding pentasaccharide. Although this phenomenon has been attributed to the bridging of f.Xa to antithrombin by longer heparin molecules (*6*), the current findings raise the possibility that heparin-induced allosteric changes at the active site of f.Xa may enhance its reactivity with antithrombin. Consistent with this concept is the proposal that antithrombin interacts with a putative novel exosite prior to reacting with the active site of its target proteases (*28*).

The capacity of heparin to modify the activity of f.Xa adds to the complexity of the regulation of f.Xa activity. Like f.Va, heparin modulates f.Xa activity. Interestingly, these two effectors promote the opposing processes of coagulation and anticoagulation. It may not be coincidental, therefore, that these effectors are directed toward the same exosite on f.Xa. The relative abundance of f.Va and sulfated GAGs could be a switch point in the balance between procoagulant and anticoagulant processes. This is similar to the role of thrombomodulin, which redirects thrombin from a procoagulant to an anticoagulant via protein C activation. Activation of protein C by f.Xa may be another example of redundancy wherein the last two enzymes in the coagulation pathway are both capable of initiating this potent anticoagulant system. It is noteworthy that protein C activation by either f.Xa or thrombin could be localized to the endothelium where TM and endogenous GAGs reside. Further support for an anticoagulant role of heparin expressed through f.Xa comes from the observation that GAGs impair the f.Xa-mediated activation of f.VII (*13*). The relative contribution of the f.Xa-mediated activation of protein C may vary depending on the location in the vasculature. Overall, protein C activation by the thrombin−TM complex is likely to predominate over that by f.Xa because activation by this complex is further stimulated by the endothelial protein C receptor (*29*). However, in areas where TM is less abundant, such as the macrovasculature (*30*), f.Xa contribution to protein C activation may become significant.

In summary, the mechanism by which GAGs promote protein C activation by f.Xa appears to be an allosteric modulation of the enzyme. Supporting this conclusion are the observations that (a) sulfated, low molecular weight GAGs promote protein C activation and that (b) heparin promotes f.Xa-mediated activation of prethrombin 1, a substrate that does not bind heparin. The effect of heparin on f.Xa activity is phospholipid-dependent and similar to the cofactor role played by f.Va. The physiological significance of protein C activation by f.Xa remains uncertain. However, our data indicate that sulfated GAGs are potential stimulators of this reaction. These agents highlight a new avenue to pursue in the development of more effective anticoagulants.

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