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Factor XI Anion-Binding Sites are required for Productive Interactions with Polyphosphate

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Abstract

Background—Conversion of factor XI (FXI) to factor XIa (FXIa) is enhanced by polymers of inorganic phosphate (polyP). This process requires FXI to bind to polyP. Each FXIa subunit contains anion-binding sites (ABSs) on the apple 3 (A3) and catalytic domains that are required for normal heparin-mediated enhancement of FXIa inhibition by antithrombin.

Objective—To determine the importance of FXI ABSs to polyP–enhancement of FXI activation.

Methods—Recombinant FXI variants lacking one or both ABSs were tested in polyP-dependent purified protein systems, plasma clotting assays, and a murine thrombosis model.

Results—In the presence of polyP, activation rates for FXI lacking either ABS were reduced compared to wild type FXI, and FXI lacking both sites had an even greater defect. In contrast to heparin, polyP binding to FXIa did not enhance inhibition by antithrombin, and did not interfere with FXIa activation of factor IX. FXI lacking one or both ABSs does not reconstitute FXI-deficient plasma as well as wild type FXI when polyP was used to initiate coagulation. In FXI-deficient mice, FXI lacking one or more ABSs was inferior to wild type FXI in supporting arterial thrombus formation.

Conclusion—The ABSs on FXIa that are required for expression of heparin's cofactor activity during protease inhibition by antithrombin are also required for expression of polyP cofactor activity during FXI activation. These sites may contribute to FXI-dependent thrombotic processes.

Keywords

Factor XI; factor XIa; blood coagulation; polyphosphates; heparin

INTRODUCTION

Factor XI (FXI) is the zymogen of a protease, FXIa, that contributes to blood coagulation by activating factor IX (FIX) [1,2]. FXI is a 160 kDa protein with two subunits [3–5], each containing four apple domains (A1 to A4) and a catalytic domain. Each FXI subunit is activated by cleavage of the Arg369-Ile370 bond by factor XIIa (FXIIa) or thrombin [5–7]. *In vitro*, rates for these reactions are low [7,8], suggesting that a cofactor is required *in vivo*.

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Choi and coworkers demonstrated that polyphosphates (polyP) released from platelets enhance FXI activation by thrombin, and promote FXI activation by FXIa, through a mechanism requiring protease and substrate to bind polyP. [9]. Geng et al. showed that polyP enhance FXI activation by α-thrombin and FXIIa by ~3000 and ~30-fold respectively [8].

PolyP are linear polymers of orthophosphate linked by phosphoanhydride bonds that are widely distributed in nature [10]. PolyP in human platelets contain 60–100 phosphate groups, and demonstrate procoagulant and pro-inflammatory properties [11–14]. FXI binding to polyP appears to be integral to the mechanism for enhancement of FXI activation [8,9]. Each FXI subunit has electropositive areas on the A3 and catalytic domains that are required for heparin-mediated enhancement of FXIa inhibition by antithrombin (AT) [15,16]. Here we show that these anion-binding sites (ABSs) are required for polyP-mediated enhancement of FXI activation, and contribute to FXI activity in a thrombosis model.

EXPERIMENTAL PROCEDURES

Materials

Corn trypsin inhibitor (CTI), FXIIa, β-FXIIa; Enzyme Research Laboratories. α-Thrombin, β-thrombin, AT, FXI, FXIa, factor IX; Hematologic Technologies. Dextran sulfate (avg 500,000 Daltons), heparin (avg. 12,000 Daltons), bovine serum albumin (BSA), polyP, and diisopropylfluorophosphate (DIP); Sigma-Aldrich. PolyP of 70–85 phosphate units was prepared by preparative polyacrylamide gel electrophoresis [8,14]. Previously, polyP concentrations were given in total phosphate units [8,9,12–14]. Since the ternary mechanism describe in this study requires polymers of >55 phosphate units [9], we use polymer concentration based on an average size of 80 units to facilitate comparisons with heparin.

Recombinant FXI

In human FXI, Arg250, Lys252, Lys253 and Lys255 in the A3 domain comprise one ABS [15], while Lys529, Arg530 and Arg532 form a second ABS on the catalytic domain [16] (Fig. 1A). cDNAs for wild type FXIWT, FXI with alanine replacing Arg250, Lys252, Lys253 and Lys255 (FXIABS-1), FXI with alanine replacing Lys529, Arg530 and Arg532 (FXIABS-2), or both sets of substitutions (FXIABS-1/2) were inserted into pJVCMV. FXI was expressed in HEK293 cells and purified from media (Cellgro Complete, Mediatech) using anti-FXI-IgG 1G5.12 [17]. Protein concentrations were determined by colorimetric assay (BioRad) and purity by SDS-PAGE. FXI was incubated with 500 μM DIP on ice for 1 hr to inhibit traces FXIa, followed by dialysis into 50 mM Tris-HCl pH 7.4, 100 mM NaCl (TBS). FXI was activated with FXIIa (20:1 FXI:FXIIa) at 37 °C for 24 hrs.

Recombinant thrombin

Wild type α-thrombin (rIIaWT) and α-thrombin with Arg89 replaced by Glu (rIIaR89E) were prepared as described [18]. Prothrombin forms of the proteins were expressed in BHK cells, and purified from medium by barium sulfate precipitation and chromatography on DEAE-Sephacel. α-Thrombin was generated by incubating Prothrombin with *Oxyuranus scutellatus* venom, and purified by ion-exchange chromatography. Thrombin active sites were titrated with hirudin.

FXI activation in the presence of polyanions

FXI (120 nM subunits) was incubated with dextran sulfate (1 μg/ml), heparin (400 nM), or polyP (50 nM) in 30 mM HEPES, pH 7.4, 50 mM NaCl, 0.1% BSA (HBSA) at 37°C. At

various times, 18 μl samples were mixed with 4 μg/ml Polybrene and 500 μM S-2366 (Pyroglutamyl-L-prolyl-L-arginine-p-nitroanilide [Diapharma]). Conversion of FXI to FXIa was determined by monitoring ΔOD 405 nm (0.3 cm path length) on a microplate reader, and comparing results to a FXIa standard curve.

FXI activation by α-thrombin or FXIIa

In the absence of polyP, FXI (200 nM subunits) was incubated with FXIIa (10 nM) or thrombin (50 nM) in HBSA at 37 °C. Reactions in the presence of polyP (50 nM) contained FXI (120 nM subunits) and 3 nM FXIIa or thrombin. At various times, aliquots were supplemented with 1.5 μM CTI (FXIIa) or hirudin (thrombin), then mixed with equal volumes of HBSA containing 1 mM S-2366. ΔOD 405 nm were monitored. Reactions with polyP were terminated with a mixture of CTI or hirudin and 4 μg/ml Polybrene.

Factor IX activation by FXIa

Factor IX (100 nM) in 50 mM HEPES, pH 7.4, 125 mM NaCl, 5 mM $CaCl_2$, 1 mg/ml polyethylene glycol 8000 was incubated at RT with FXIa (3 nM active sites) with vehicle, 1mg/ml dextran sulfate, 400 nM heparin, or 50 nM polyP. At various times, aliquots were removed into non-reducing SDS-sample buffer, size fractionated on 17% polyacrylamide gels, and stained with GelCode Blue (Pierce). Gels were imaged under infrared wavelengths, and conversion of factor IX to factor IXα and factor IXaβ was assessed by densitometry as described [17].

Inhibition of FXIa by antithrombin

FXIa (6 nM active sites) was incubated with AT (130 nM), and heparin (10^0 to 10^4 nM) or polyP (10^{-3} to 10^4 nM) in TBS with 0.1% BSA (TBSA) at 37°C. At various times, 50 μl was removed into 50 μl TBSA containing 1 mM S2366 and 4 μg/ml Polybrene. FXIa activity was measured by monitoring ΔOD405 nm. Progress curves of residual FXIa activity (E/E0) were analyzed by direct nonlinear least squares fitting to a first-order decay equation, $E/E_0 = e^{-k_{obs}.t}$ in which k_{obs} is the first-order inactivation rate constant (s^{-1}) and t is time (s). The semi-logarithmic dependence of k_{obs} on [heparin] was analyzed with a model in which heparin forms a ternary complex with FXIa and AT [19]. The data for poly-P were analyzed by a quadratic equation $k_{obs}=k_{obs,unc}+F(k_{obs,lim}-k_{obs,unc})$, where

$$F=\frac{([fXIa]_o+[P]_o+K_{fXIa,P})-\sqrt{([fXIa]_o+[P]_o+K_{fXIa,P})^2-4[fXIa]_o[P]_o}}{2[fXIa]_o}$$

$k_{obs,unc}$ is the rate constant for AT inactivation in the absence of poly-P, and $k_{obs,lim}$ is the limiting rate constant at high poly-P concentration. $[FXIa]_o$ and $[P]_o$ are concentrations of FXIa active sites and polyP, and $K_{FXIa,P}$ is the apparent dissociation constant for poly-P binding to the FXIa subunit, assuming 1 binding site for the poly-P chain on each FXIa subunit.

FXI activity in plasma

FXI-deficient plasma (30 μl, George King) was mixed with 30 μl FXI (5 μg/ml) in TBSA. Contact activation was initiated with 30 μl PTT-A reagent (Diagnostic Stago) or a mixture of 40 μM phosphatidylcholine-phosphatidylserine [PC:PS 7:3] vesicles and 200 nM polyP. After incubation at 37°C for 5 min, 30 μl $CaCl_2$ (25 mM) was added, and time to clot formation was determined on an ST4 coagulometer (Diagnostica Stago). In separate experiments, 30 μl FXI (5 μg/ml) in TBSA was mixed with 30 μl FXI-deficient plasma

containing 8 μM CTI, 40 μM PC/PS and 200 nM polyP. β-thrombin (1.5 nM) was added and incubated for 3 min at 37°C. Clotting was initiated with 30 μl of $CaCl_2$ (25 mM)5

Transient expression of human FXI in mice by hydrodynamic tail vein injection (HTI)

C57Bl/6 FXI deficient ($FXI^{-/-}$) mice [8,20] (25 gm) were anesthetized with pentobarbital (50 mg/kg intraperitoneal). Lactated Ringer solution (2 ml) containing 15 μg pJVCMV/FXI cDNA constructs were infused rapidly into the tail vein [8]. Plasma was prepared from blood samples (50 μl) collected 24 hrs post-HTI, and FXI concentration was determined by ELISA (Affinity Biologicals). Values were compared to dilutions of normal human plasma, and purified human plasma FXI. Western blots of FXI in plasma were prepared using anti-FXI IgG 14E11 for detection [20].

Thrombosis model

Twenty-four hours post-HTI, mice were anesthetized. A Model 0.5 VB Doppler flow probe (Transonic) was placed around the right common carotid artery. Thrombus formation was induced by applying two 1 × 1.5-mm filter papers (GB003; Schleicher & Schuell) saturated with 3.5% $FeCl_3$ to opposite sides of the artery for 3 minutes, followed by rinsing with saline [20]. Flow was monitored for 30 minutes. At the end of the experiment, blood was collected for measurement of plasma FXI.

RESULTS

FXI activation by polyanions

Recombinant FXI migrated at the appropriate 160 (unreduced) and 80 (reduced) kDa positions on SDS-PAGE (Fig. 1B). After conversion to FXIa, the proteases cleaved the substrate S-2366 with similar K_m and k_{cat} (not shown) [15,16], indicating the active sites were intact. Dextran sulfate-, heparin- or polyp-induced FXI activation requires FXI to bind to the polyanion [7,8,21]. Activation is probably initiated by traces of FXIa in the FXI. DFP-treated FXI preparations cleave S2366 at a rate suggesting 120 nM zymogen contains ~0.3 pM FXIa. During FXIWT activation with heparin (Fig. 2A), there is an initial lag phase followed by accelerated activation. A similar response was obtained with polyP (Fig. 2B). With heparin, FXIWT and FXIABS-2 were activated similarly, while FXIABS-1 and FXIABS-1/2 showed activation defects (Fig. 2A). Comparable results were obtained with dextran sulfate (not shown), indicating the A3 domain ABS, but not the catalytic domain ABS, is required for polyanion binding. With polyP, FXIABS-1 and FXIABS-2 both demonstrated longer lag phases than FXIWT, while FXIABS-1/2 failed to activate (Fig. 2B), indicating polyP interacts with both ABSs to promote activation.

FXI activation by thrombin and FXIIa

In the absence of a polyanion, second order rate constants for FXIWT activation by α-thrombin (Fig. 3A, 240 M-1sec-1) or FXIIa (Fig. 3B, 16,600 M-1sec-1) were roughly comparable to those for FXI ABS mutants (Table 1). PolyP enhanced FXIWT activation by α-thrombin ~3000-fold (Fig. 3C, 713,000 M-1sec-1) and by FXIIa ~30-fold (Fig. 3D, 543,000 M-1sec-1). Rates were moderately reduced for FXIABS-1 activation by α-thrombin (34%) and FXIIa (42%). There were greater reductions for FXIABS-2 (5-fold for α-thrombin, 3-fold for FXIIa). With FXIABS-1/2, rate constants were ~20- and ~7-fold lower, respectively, confirming the importance of both FXI ABSs to polyP enhancement of FXI activation.

PolyP-dependences of FXI activation by α-thrombin (Fig. 4A) or FXIIa (Fig. 4B) have bell-shaped distributions, consistent with a ternary mechanism requiring protease and substrate to

bind polyP. In the absence of polyP, FXI is activated comparably by rIIaWT and α-thrombin with a key residue in the heparin-binding site changed from Arg to Glu (rIIaR89E) (Fig. 4C. 177 and 124 M-1sec-1, respectively). With polyP (Fig. 4D), the rate constant for rIIaR89E (16,500 M-1sec-1) was ~35-fold lower than with rIIaWT (573,000 M-1sec-1) demonstrating the importance of α-thrombin binding to polyP [22]. β-FXIIa, a degradation product of FXIIa lacking the anion-binding heavy chain, activated FXI at ~40% of the rate of FXIIa (7,300 and 17,100 M-1sec-1, respectively) without polyP (Fig. 4E), but showed a more profound defect with polyP (Fig. 4F. 13,600 and 293,000, respectively; a >20-fold difference), consistent with the template mechanism requirement that FXIIa bind to polyP.

Effects of polyP on FXIa activity

Heparin reduces FXIaWT-mediated cleavage of the tripeptide S-2366, probably through an allosteric effect (Fig. 5A). PolyP produces a similar, though less pronounced effect (Fig. 5B). This process requires the catalytic domain ABS, as polyanions do not affect S-2366 cleavage by FXIABS-2 or FXIABS-1/2. Lack of the A3 domain site (FXIABS-1) reduces inhibition somewhat, perhaps by changing the manner in which polyanions bind to the catalytic domain site. The results are consistent with the premise that heparin and polyP engage both ABSs on FXIa.

FXIaWT activates factor IX by cleavage after Arg145 to produce factor IXα (Fig. 5C), then after Arg180 to form factor IXaβ (Fig. 5D) [2,17]. The catalytic efficiency of the second cleavage is greater than the first, so factor IXα accumulation is normally limited (Fig. 5C). Key to this mechanism is a factor IX binding site on the FXIa A3 domain [17]. When factor IX is activated by FXIaWT in the presence of heparin or dextran sulfate there is significant factor IXα accumulation (Fig. 5C) and reduced rates of factor IXaβ formation (Fig. 5D), indicating the polyanions interfere with factor IX binding to the A3 exosite. PolyP, in contrast, did not alter factor IX activation, suggesting that it interacts differently with FXIa than dextran sulfate or heparin despite using the same binding sites.

Effects of polyanions on AT-mediated inhibition of FXIa

AT is one of the plasma protease inhibitors that regulates FXIa activity [15,16,23,24]. Heparin enhanced FXIa inhibition by AT 40-fold [15]. Heparin-dependence of AT inhibition of FXIa (Fig. 5E) is bell-shaped, indicating FXIa and AT bind to heparin [15,19]. PolyP has little effect on AT inhibition of FXIa (Fig. 5E). At polyP >1 μM a modest saturable inhibition was seen (Fig. 5F), consistent with an allosteric effect due to polyP binding to FXIa. AT up to 6 μM did not bind polyP in surface plasmon resonance studies (S.A. Smith and J.H. Morrissey, unpublished observation), consistent with results reported by Church and coworkers [25].

FXI in plasma assays

In a partial thromboplastin time (PTT) assay triggered by adding silica to FXI deficient plasma, FXIWT and FXIABS-1 restored clotting times comparably, while FXIABS-2 and FXIABS-1/2 gave slightly longer clotting times (1.2–1.35 times FXIWT) (Fig. 6A). Replacing silica with polyP resulted in longer clotting times (Fig. 6B) because polyP of 80 units are relatively weak FXII activators [8]. With polyP, differences in clotting times between plasma supplemented with the FXI variants are evident, and in line with results in Fig. 3D. In Fig. 6C, FXIIa activity is blocked with CTI, and FXI is activated in the presence of polyP with β-thrombin. β-thrombin, a cleavage product of α-thrombin, has reduced capacity to convert fibrinogen to fibrin because it lacks a properly formed anion-binding exosite I [6]. Because it interacts weakly with fibrinogen, β-thrombin is a more potent FXI activator in plasma than α-thrombin [6]. As with polyP-enhanced FXIIa activation of FXI

(Fig. 6B), polyP-dependent β-thrombin activation of FXI in plasma is compromised by loss of the FXI ABSs.

Thrombus formation in vivo

FXIWT [8] and FXI ABS mutants can be transiently expressed in $FXI^{-/-}$ mice by HTI (Fig. 7A). Twenty-four hours post-HTI, plasma FXI concentration of ABS mutants are comparable to normal human plasma (30 nM), while FXIWT was modestly lower (Fig. 7B). Carotid arteries of wild type mice occlude within 15 minutes of exposure to 3.5% $FeCl_3$, while $FXI^{-/-}$ mice are resistant to occlusion [20,26]. All $FXI^{-/-}$ mice expressing FXIWT developed artery occlusion after $FeCl_3$ exposure (Fig. 7C). However, despite plasma FXI levels ~40% higher on average than FXIWT expressing mice, only about half of mice expressing FXI lacking one or both ABSs developed occlusion (Fig. 7C).

DISCUSSION

Heparin enhances coagulation protease inhibition by AT through allosteric and ternary (template) mechanisms [19,27]. The latter typically involves binding of the catalytic domain to heparin through electrostatic interactions involving ABSs containing lysine and arginine residues [2,18,35]. The α-thrombin heparin-binding site (anion binding exosite II) is comprised of Arg89, Arg98, Arg245, Lys248 and Lys252 (chymotrypsin numbering) [2,16,36]. For FXIa, Lys529, Arg530 and Arg532 on the catalytic domain 170-loop bind heparin [16]. FXIa also has a novel heparin-binding site on its A3 domain (Arg250, Lys252, Lys253 and Lys255) [15]. PolyP binds to α-thrombin through anion binding exosite II (K_d ~5 nM) [22]. As polyP-mediated enhancement of FXI activation by α-thrombin involves a template mechanism [8,9], it follows that FXI also has sites that bind polyP. Here we assessed the importance of the known FXI heparin-binding sites to the polyP interaction.

FXI is a homolog of a more ancient protease, prekallikrein (PK) [29]. Like FXI, PK contains basic residues in the 170-loop (Fig. 5A) [30] and, similar to FXIa, cleavage of a chromogenic substrate by activated PK (α-kallikrein) is reduced by heparin [31]. However, PK lacks an A3 domain ABS [30], suggesting this feature supports specific FXI functions. The data presented here demonstrate that both FXI ABSs are required for optimal polyP enhancement of FXI activation by thrombin, FXIIa or autoactivation. The ABSs are positioned on the FXI zymogen subunit in a manner that would allow a single polymer to span both sites (Fig. 1A). An examination of the FXI structure does not immediately suggest why heparin (which engages both ABSs [15,16]) or dextran sulfate only require the A3 ABS to promote activation. This may reflect differences in the structures of the polyanions or their affinities for FXI. Although it has the capacity to enhance FXI activation specifically, and contact activation in general [32,33], heparin produces a net anticoagulant effect in plasma because it serves as a cofactor for AT-mediated protease inhibition. PolyP does not bind AT, and predictably lacks this activity (Fig. 5E), consistent with its proposed role as a promoter of coagulation.

The FXIa A3 and catalytic domains serve key roles in factor IX activation [17], so it is not surprising that heparin interferes with this reaction. In the normal mechanism for factor IX activation by FXIa, the rate of the initial cleavage after Arg145 is 7-fold slower than for the second cleavage after Arg180, limiting accumulation of the intermediate factor IXα [17]. Disruption of factor IX binding to the A3 domain has a greater deleterious effect on the second cleavage, leading to factor IXα accumulation. Heparin, and to an greater extent dextran sulfate, reduce the rate of factor IX activation by FXIa, with marked factor IXα accumulation, possibly by sterically interfering with factor IX binding to the FXIa. PolyP, in contrast, did not inhibit factor IX activation, consistent with the impression that polyP and

heparin interact with FXIa somewhat differently, and in support of the premise that polyP is, on balance, a procoagulant.

PolyP appears to contribute to thrombus formation in the mouse model used in this study [12]. The prothrombotic effect of $FeCl_3$-injury can be reduced by treating mice with alkaline phosphatase to destroy polyP [12], or by administering compounds that neutralize polyP [34,35]. RNA and DNA are polymers with phosphate backbones that also contribute to thrombosis in animal models [36,37]. Chromatin released from activated neutrophils (neutrophil extracellular traps - NETs) have been identified in thrombi [37,38], and it will be interesting to see if this material enhances FXI activation. Based on the importance of the FXI ABSs to interactions with polyanions, loss of one or both sites *in vivo* could result in (1) enhanced sensitivity to $FeCl_3$-induced thrombosis because of reduced AT-mediated inhibition, (2) decreased sensitivity to $FeCl_3$-induced thrombosis due to decreased FXI activation, or (3) a combination of effects leading to an intermediate result. The results presented here indicate, on balance, that loss of the FXI ABSs produces an antithrombotic effect, supporting the notion that targeting the interaction between FXI and polyanions such as polyP may be a useful antithrombotic strategy.

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ADDENDUM

Authorship: Y. Geng performed experiments on the effects of polyp on FXI activation and wrote the manuscript. I.M. Verhamme contributed to design of experiments of FXI activation *in vitro*, performed kinetic analyses of data, and conducted some experiments on AT inhibition of FXIa. S.A Smith prepared and characterized poly-P, conducted studies of AT binding to polyP, and contributed to experimental design. Q.C. conducted HTI experiments. M-f. Sun prepared and characterized recombinant FXI/XIa and their inhibition by AT. J.P. Sheehan prepared and characterized recombinant thrombin, and contributed to experimental design. J.H. Morrissey contributed to experimental design, and to writing of the final manuscript. D. Gailani was responsible for oversight of the project and preparation of the manuscript.

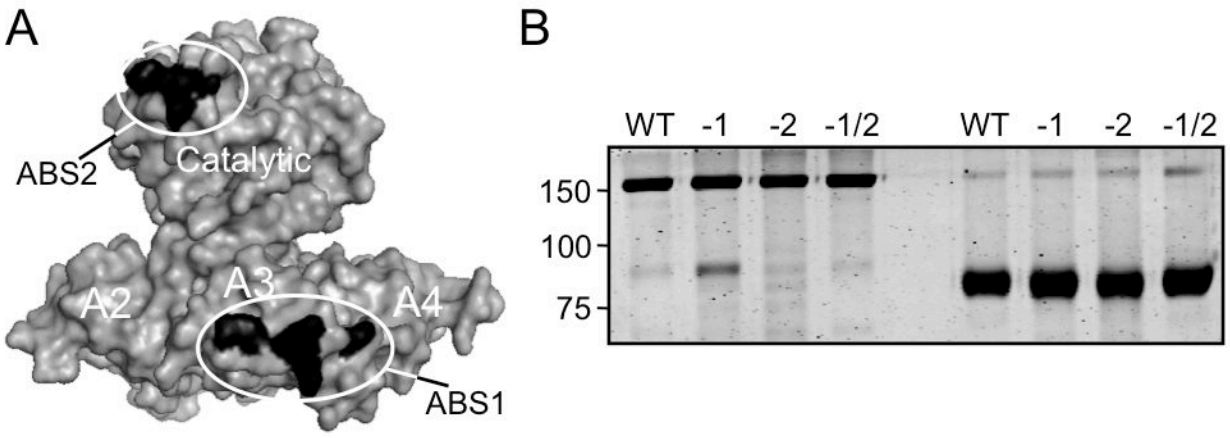

Figure 1. Recombinant FXI

(A) Space filling model of a FXI subunit (generated with PyMole) showing positions of lysine and arginine residues (black) involved in polyanion binding. **(B)** Purified FXIWT, FXIABS-1, FXIABS-2 and FXIABS-1/2 run under non-reducing (left) and reducing (right) conditions on a 10% polyacrylamide-SDS gel. Positions of molecular mass standards in kilodaltons are on the left.

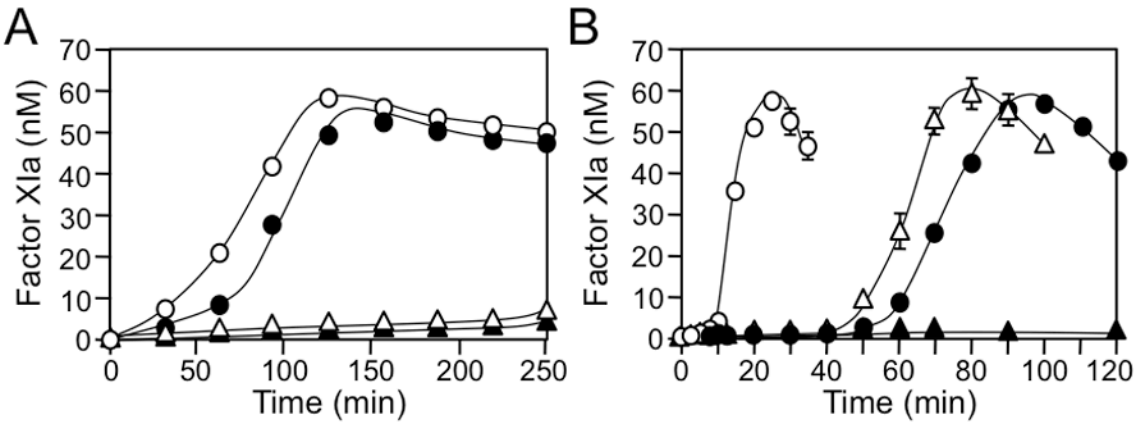

Figure 2. FXI activation in the presence of heparin or polyP

FXIWT (○), FXIABS-1 (△), FXIABS-2 (●) or FXIABS-1/2 (▲) incubated with **(A)** 400 nM heparin or **(B)** 50 nM polyP. FXIa generation was determined by chromogenic assay.

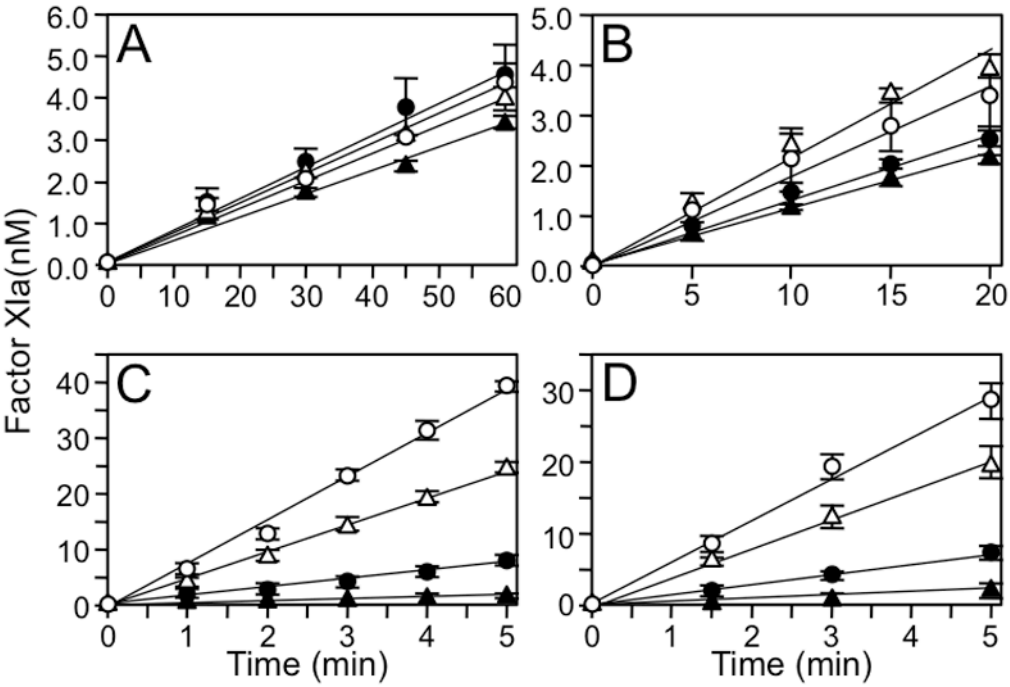

Figure 3. FXI activation by α-thrombin or FXIIa

(A and B) FXIWT (○), FXIABS-1 (△), FXIABS-2 (●) or FXIABS-1/2 (▲), 200 nM subunits, incubated with **(A)** 50 nM α-thrombin or **(B)** 5 nM FXIIa. **(C and D)** Activation of 120 nM subunit of FXI (symbols same as in panels A and B) by 3 nM **(C)** α-thrombin or **(D)** FXIIa in the presence of 50 nM polyP. FXIa was measured by chromogenic assay.

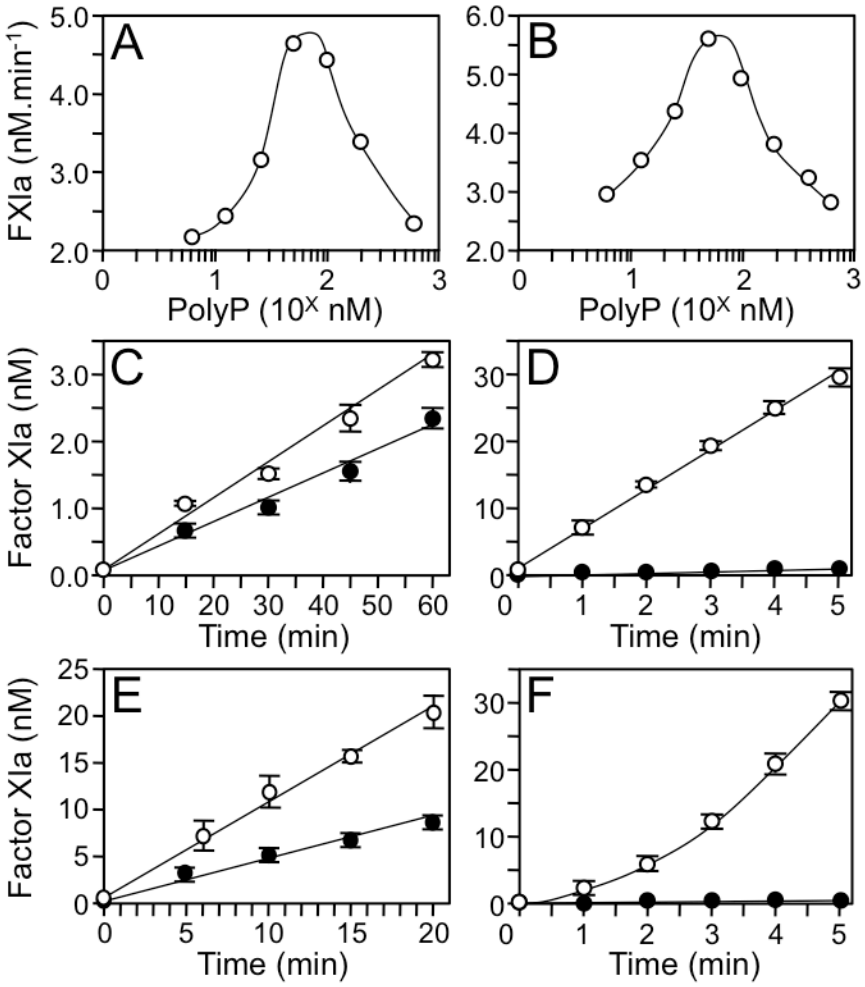

Figure 4. FXI activation by thrombin or FXIIa variants

(A and B) Concentration dependence of polyP-enhanced FXIWT (120 nM subunits) activation by 3 nM **(A)** α-thrombin or **(B)** FXIIa. **(C)** FXIWT (200 nM subunits) incubated with 50 nM (○) rIIaWT or (●) rIIaR89E. **(D)** FXIWT (120 nM subunits) was activated by 3 nM (○) rIIaWT or (●) rIIaR89E in the presence of 50 nM polyP. **(E)** FXIWT (200 nM subunits) incubated with 10 nM (○) FXIIa or (●)β-FXIIa. **(F)** FXIWT (120 nM subunits) incubated with 3 nM (○) FXIIa or (●)β-FXIIa in the presence of 50 nM polyP. FXIa was measured by chromogenic assay.

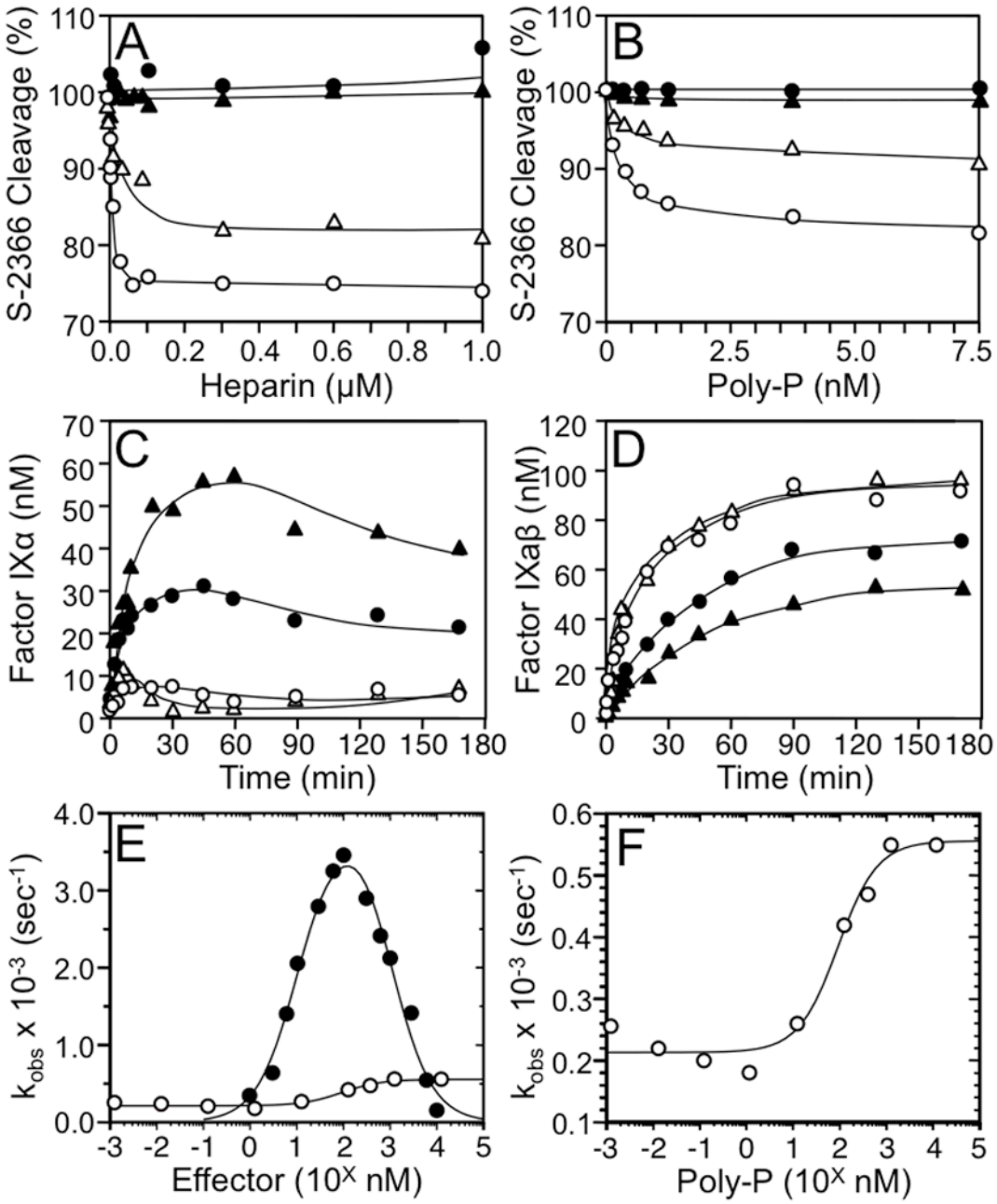

Figure 5. Effects of polyanions on FXIa activity

(A and B) FXIaWT (○), FXIaABS-1 (△), FXIaABS-2 (●) or FXIaABS-1/2 (▲), 6 nM active sites, was supplemented with **(A)** heparin or **(B)** polyP, then tested for its ability to hydrolyze S2366. **(C and D)** Factor IX (100 nM) was incubated in Assay Buffer at RT with 3 nM (active sites) FXIaWT with (○) vehicle, (●) 400 nM unfractionated heparin, (▲) 1ug/ml dextran sulfate or (△) 50 nM polyP. Samples were removed into nonreducing SDS-sample buffer and size-fractionated on 17% polyacrylamide-SDS. Conversion of factor IX to (**C**) factor IXα and (**D**) factor IXaβ was determined by densitometry of stained gels. **(E and F)** FXIaWT (6 nM active sites) incubated n TBSA with antithrombin (130 nM) and (●) unfractionated heparin or (○) polyP. k_{obs} for each polyanion concentration was determined as described under **Experimental Procedures**.

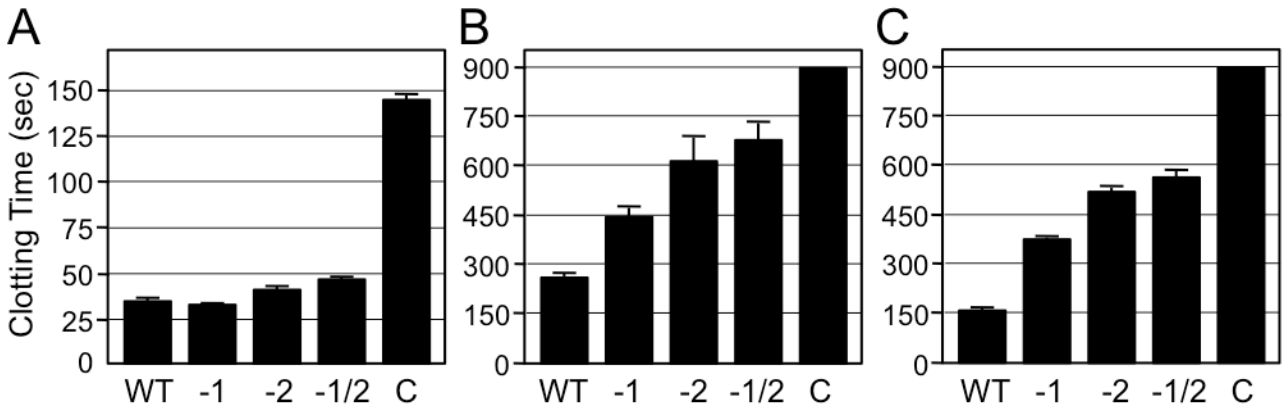

Figure 6. Plasma clotting assays

(A) FXI-deficient plasma supplemented with 30 nM FXIaWT, FXIaABS-1, FXIaABS-2, FXIaABS-1/2, or vehicle (C) incubated with a PTT reagent. After recalcification, time to clot formation was determined. **(B)** Clotting times of FXI-deficient plasma supplemented with FXI as in panel A incubated for 3 min with 50 nM polyP and 10 μM PC/PS vesicles. **(C)** Clotting times of FXI-deficient plasma supplemented with FXI incubated for 3 min with 50 nM polyP, 10 μM PC/PS vesicles, and 1.5 nM β-thrombin. CTI (4 μM) was used to inhibit FXIIa. For all panels, proteins were tested in triplicate. Error bars represent one standard deviation.

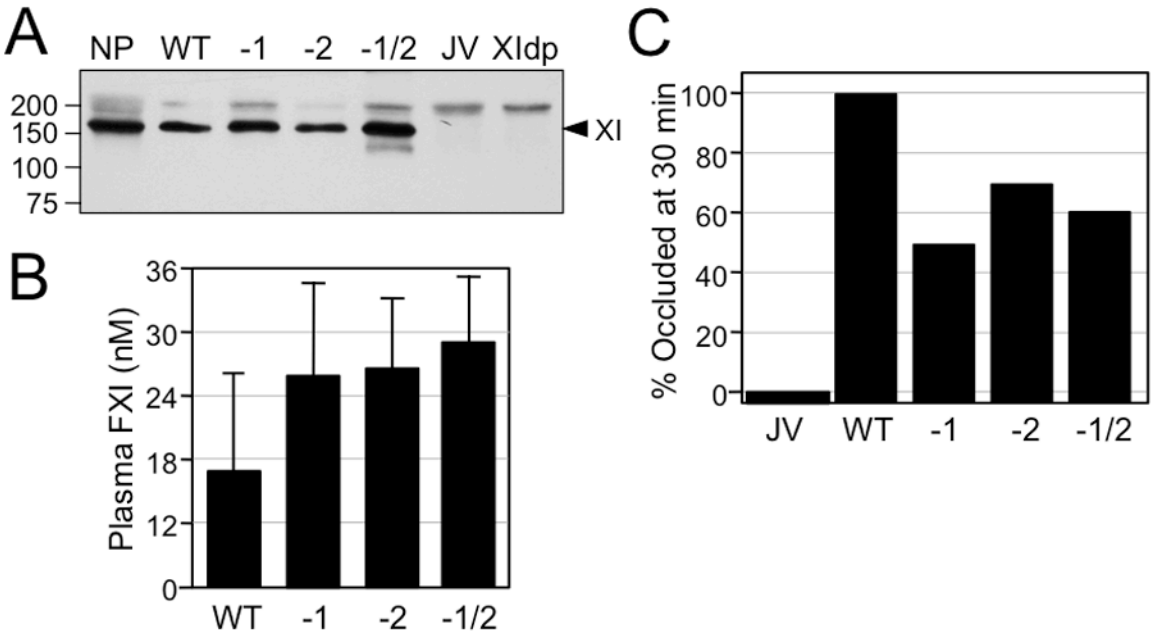

Figure 7. FeCl3-induced carotid artery occlusion in FXI$^{-/-}$ mice expressing human FXI

(A) FXI western blot of mouse plasmas 24 hours post-HTI with constructs for FXIaWT, FXIaABS-1, FXIaABS-2, FXIaABS-1/2 or empty vector (JV). Normal human plasma (NP) and FXI-deficient mouse plasma (XIdP) serve as controls. Positions of molecular mass standards (kDa) are shown on the left, and FXI is indicated on the right. **(B)** FXI concentrations in mouse plasma 24 hours post-HTI. Error bars indicate 1 SD (n=10). **(C)** FXI$^{-/-}$ mice underwent HTI with FXI constructs (n=10 per construct). 24 hours post-HTI, mice were tested in a model in which thrombus formation in the carotid artery is induced with 3.5% $FeCl_3$. Bars indicate percent of mice with occluded arteries 30 min post-$FeCl_3$ application. Data were analyzed by χ^2-contingency analysis and a Fisher's protected least significant difference approach. Results for mice expressing empty vector ($p<0.005$), FXIaABS-1 ($p=0.01$) and FXIaABS-1/2 ($p=0.025$) were significantly different than FXIaWT. Differences between FXIaWT and mice expressing FXIaABS-2 did not reach statistical significance ($p=0.06$).

Table 1

Effects of polyP on FXI activation

Second order rate constants (M-1sec-1) derived from curves in Figure 3 for FXI activation by α-thrombin or FXIIa with or without 50 nM polyP.

	α-Thrombin			FXIIa		
Protein	**No PolyP (M-1sec-1)**	**With PolyP (M-1sec-1)**	**Fold-Increase with polyP**	**No PolyP (M-1sec-1)**	**With PolyP (M-1sec-1)**	**Fold-Increase with PolyP**
FXIWT	240	714,000	2975	16,600	543,000	33
FXIΔA3	230	450,000	1957	20,000	375,000	19
FXIΔCD	270	153,000	567	12,100	135,000	11
FXIΔA3/CD	190	29,000	152	10,600	49,000	5

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