

Anticoagulant Protein S Targets the Factor IXa Heparin-Binding Exosite to Prevent Thrombosis

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Objective—PS (protein S) is a plasma protein that directly inhibits the coagulation FIXa (factor IXa) in vitro. Because elevated FIXa is associated with increased risk of venous thromboembolism, it is important to establish how PS inhibits FIXa function in vivo. The goal of this study is to confirm direct binding of PS with FIXa in vivo, identify FIXa amino acid residues required for binding PS in vivo, and use an enzymatically active FIXa mutant that is unable to bind PS to measure the significance of PS–FIXa interaction in hemostasis.

Approach and Results—We demonstrate that PS inhibits FIXa in vivo by associating with the FIXa heparin-binding exosite. We used fluorescence tagging, immunohistochemistry, and protein–protein crosslinking to show in vivo interaction between FIXa and PS. Importantly, platelet colocalization required a direct interaction between the 2 proteins. FIXa and PS also coimmunoprecipitated from plasma, substantiating their interaction in a physiological milieu. PS binding to FIXa and PS inhibition of the intrinsic Xase complex required residues K132, K126, and R170 in the FIXa heparin-binding exosite. A double mutant, K132A/R170A, retained full activity but could not bind to PS. Crucially, Hemophilia B mice infused with FIXa K132A/R170A displayed an accelerated rate of fibrin clot formation compared with wild-type FIXa.

Conclusions—Our findings establish PS as an important in vivo inhibitor of FIXa. Disruption of the interaction between PS and FIXa causes an increased rate of thrombus formation in mice. This newly discovered function of PS implies an unexploited target for antithrombotic therapeutics. (*Arterioscler Thromb Vasc Biol.* 2018;38:00-00. DOI: 10.1161/ATVBAHA.117.310588.)

Key Words: blood platelets ■ factor IXa ■ hemostasis ■ protein S ■ thrombosis

To prevent undesirable thrombotic complications, the natural anticoagulants antithrombin, APC (activated protein C), tissue factor pathway inhibitor, and PS (protein S) regulate coagulant activity.^{1–6} Deficiencies in these anticoagulants are associated with increased risks of venous thromboembolism and higher rates of stroke and myocardial infarction.^{1,7–10} Homozygous PS deficiency causes fatal neonatal purpura fulminans, a dramatic example of the critical role of PS in regulation of human blood coagulation.^{11,12} Similarly, heterozygous PS deficiency is associated with increased familial risk of venous thromboembolism.^{13–15} Consistent with these clinical manifestations, gene ablation of PS in mice causes embryonic lethality related to consumptive coagulopathy and intracranial hemorrhage.^{16,17}

Despite the biological significance of PS, to now, there has not been definitive identification of the in vivo biochemical mechanism(s) for PS function. PS was originally identified as a cofactor for APC in the inactivation of factor Va.¹ Subsequent studies demonstrated APC-independent anticoagulant activity for PS, notably as a cofactor for tissue factor pathway inhibitor in the inhibition of factor Xa.^{9,11} Additionally, FXa was directly

inhibited by plasma PS that contained Zn²⁺.¹⁸ Likewise, direct binding of PS to FVIII resulted in decreased anticoagulant activity.¹⁹ These disparate PS mechanisms require re-examination in a physiological context because multimeric PS structures effectively inhibit phospholipid-dependent reactions at low phospholipid concentrations (ie, <50 μmol/L), and therefore interfere during functional analysis of PS.²⁰

Ex vivo modeling of blood coagulation demonstrates that FX (factor X) activation by the intrinsic Xase complex (FIXa [factor IXa]–FVIIIa [factor VIIIa]) is the rate-limiting step for thrombin generation.^{21,22} FIXa binds substrates and inhibitors poorly because of a partially occluded active site; incorporation of FIXa into the intrinsic Xase complex results in a dramatic enhancement in catalytic activity.^{23–25} The FIXa heparin-binding exosite (HBE) is key to regulation of protease activity by cofactor and inhibitors. Mutagenesis of this exosite modestly reduces FIXa–FVIIIa affinity by destabilizing the critical interaction with the FVIIIa A2 domain.²⁶ The HBE is targeted by select glycosaminoglycans for antithrombin-independent inhibition of intrinsic Xase activity in serpin-deficient human plasmas,^{27–30} and the HBE interacts directly with the

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Nonstandard Abbreviations and Acronyms

PS	protein S
FIXa	factor IXa
FX	factor X
PhosSer	phosphatidylserine
APC	activated protein C
TFPI	tissue factor pathway inhibitor
FV	factor V
FVIIIa	factor VIIIa
DEGR	Dansyl-Glu-Gly-Arg chloromethyl ketone
HBE	heparin-binding exosite

heparin component of the antithrombin–heparin complex to accelerate FIXa inhibition.^{31,32}

We demonstrated previously that, in vitro, PS functions as an independent regulator of the intrinsic coagulation pathway; PS inhibits both free FIXa and the Xase complex, thereby directly inhibiting FX activation.³³ The goals of the present study were to define the PS binding site(s) on FIXa and establish the contribution of PS-mediated FIXa inhibition to in vivo regulation of blood coagulation. Westmark et al³² characterized recombinant FIX (rFIX) proteins possessing mutations in the HBE. Their results demonstrated a significant disparity between protease activity of purified proteins and plasma-based thrombin generation activity, suggesting involvement of an unidentified plasma inhibitor of FIXa. We show that the FIXa HBE is critical for PS binding and inhibition of FIXa in physiological environments, including the activated platelet surface, in human plasma and in murine thrombosis models. Particularly, we identified a FIXa HBE double mutant, K132A/R170A that did not bind PS yet retained full enzymatic activity. Critically, this FIXa mutant caused an increased rate of thrombus formation in hemophilia B mice, a result that confirmed that PS maintains hemostasis and prevents thrombosis by inhibiting FIXa.

Materials and Methods

Materials

Reagents

Human PS, FX, FIXa, α -thrombin, and a Dansyl-Glu-Gly-Arg chloromethyl ketone active site blocked-FIXa (DEGR-FIXa) were purchased from Hematologic Technologies Inc (Essex Junction, VT). DEGR forms of the mutant FIXa proteins were produced in-house according to Lollar and Fass.³⁴ The FXa-specific substrate *N*-2-benzyloxycarbonyl-*D*-arginyl-*L*-arginine *p*-nitroanilide dihydrochloride (S-2765) was purchased from DiaPharma (West Chester, OH). 1,2-dioleoyl-3-sn-phosphatidylcholine and 1,2-dioleoyl-sn-glycerol-3-phospho-*L*-serine (PS) were obtained from Avanti Polar Lipids (Alabaster, AL). All solvents were high-performance liquid chromatography grade.

A plasmid with a wild-type (WT) human FIX gene was a generous gift from Valder R. Arruda (University of Pennsylvania, Pennsylvania, PA). Gla-deleted FIXa was prepared by enzymatic digestion with chymotrypsin.³⁵ Gla-EGF1-deleted FIXa was provided by Jim Huntington (Cambridge Research Institute, United Kingdom)³⁶; its concentration was determined by titrating with antithrombin as described.³⁷ A sheep antihuman PS polyclonal antibody and a sheep antihuman FIX polyclonal antibody, which binds both FIX and FIXa (PAHFIX-S), were purchased from Hematologic Technologies Inc. A

rat antimouse PS monoclonal antibody (MAB4976) was purchased from R&D Systems (Minneapolis, MN).

Methods

Expression and Purification of Recombinant Proteins

HEK293 cells stably transfected with a plasmid encoding FIX R170L was used for expression.³⁸ This rFIX protein was purified from conditioned media using a FIX Select column.³⁹ Uncarboxylated rFIX was generated by withholding vitamin K from the growth and expression media. Recombinant human FIX WT, K126A, N129A, K132A, R150A, R165A, R170A, K126A/K132A, and K132A/R170A proteins (chymotrypsin numbering) were expressed in VKOR/HEK293 cells (stably transfected with vitamin K epoxide reductase) and purified to homogeneity from conditioned media as described.^{26,32} FIX protein concentrations were determined by absorbance at 280 nm using an extinction coefficient ($\epsilon_{0.1\%}$) of 1.32. rFIX proteins (1 μ g) produced a single band on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels stained with Gel-Code Blue as previously demonstrated.³² Purified rFIX was activated to rFIXa with human FXIa (150:1 substrate:enzyme molar ratio) at 37°C for 1.5 hours, followed by depletion of factor XIa by immunoabsorption with polyclonal anti-FXIa antisera crosslinked to Affi-gel beads in a spin column. FIXa active sites were quantitated by titration with antithrombin as described.⁴⁰ PS was purchased from Hematologic Technologies Inc, devoid of the zinc that affects PS activity in vivo.⁴¹

Binding of FIXa and PS With Activated Platelets

An ex vivo experiment with Alexa-Fluor-647–labeled FIXa and Alexa-Fluor-488–labeled PS was performed to detect binding of these 2 proteins to activated platelets. Washed platelets (5×10^8) were diluted 1:10 in Tyrode's buffer containing 2 mmol/L CaCl₂ and activated for 15 minutes with FIXa or PS and 500 ng/mL convulxin and 500 μ mol/L Par 4 agonist. Annexin V staining was included as a positive control. Samples were immediately analyzed by flow cytometry (N=4) as described.⁴²

Coimmunoprecipitation of FIXa and PS From Human FIX-Deficient Plasma

WT or mutant FIXa (1 nmol/L) was added to 5 mL of citrated FIX-deficient plasma that was thawed at 37°C. The plasma was incubated on a shaker at 4°C for 30 minutes and precleared with Poly-AG beads. Plasma was then centrifuged at 10,000g and 5 μ g of FIXa antibody (PAHFIX-S) was added to the supernatant. FIXa was immunoprecipitated with a FIXa antibody (PAHFIX-S) and immunoblotted for PS with a sheep polyclonal anti-PS antibody.⁴³

Direct Binding of PS to DEGR-FIXa

To assess the interaction between PS and different mutant forms of FIXa, the steady-state fluorescence of active site–labeled FIXa was measured in the presence of increasing concentrations of PS. FIXa was labeled with 1,5-Dansyl-Glu-Gly-Arg to yield DEGR-FIXa.⁴⁴ PS was titrated into a reaction containing 75 nmol/L DEGR-FIXa and 50 μ mol/L phosphatidylserine/phosphatidylcholine vesicles in 20 mmol/L Tris-HCl (pH 7.4), 5 mmol/L Ca²⁺, and 150 mmol/L NaCl. Fluorescence intensity was measured at 23°C with a Spex FluoroLog-3 spectrofluorometer (Jobin Yvon Inc, Edison, NJ), using an excitation wavelength of 340 nm (bandpass 5 nm) and an emission wavelength of 540 nm (bandpass 5 nm). Average intensity was recorded over a 10-second period. The fluorescence intensity data were fit to a hyperbola (see Data Analysis section below) to determine the mutant FIXa K_d s for binding to PS.

Competitive Binding of Mutant Human FIXa to PS in the Presence of DEGR-FIXa

To further verify the interaction between PS and the mutant forms of FIXa, a competitive binding assay utilizing fluorescence anisotropy was performed with DEGR-FIXa and untagged FIXa mutants. A total of 25 nmol/L DEGR-FIXa was incubated with 60 nmol/L PS in 20 mmol/L Tris-HCl (pH 7.4), 5 mmol/L Ca²⁺, and 150 mmol/L NaCl. Because concentrations of the FIXa mutants were varied from 0 to 350

nmol/L, anisotropy was measured at 23°C with a Spex FluoroLog-3 spectrofluorometer (Jobin Yvon Inc, Edison, NJ), using an excitation wavelength of 340 nm (bandpass 8 nm) and an emission wavelength of 540 nm (bandpass 8 nm); anisotropic values were derived from the average of 10 readings. The data were fit to a sigmoidal curve and K_i was determined for each protein as described below in Data Analysis.

FIX Activation by FXIa

A total of 5 μ mol/L FIX was incubated with 25 nmol/L FXIa with and without 7.5 μ M PS at 37°C in 20 mmol/L Tris-HCl, 150 mmol/L NaCl, and 5 mmol/L CaCl_2 at pH 7.4. Samples containing 3 μ g total cleaved and uncleaved FIX were taken at 1-, 5-, 10-, 15-, 30-, 45-, 60-, 90-, and 120-minute time points and were quenched with 2.5 mmol/L ethylenediaminetetraacetic acid and 15 mg/mL SBTI on ice for 5 minutes. 6 \times reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer was then added and the samples were immediately heated at 95°C. The samples were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, and the cleaved bands were quantitated by densitometry on a GE Amersham Imager 600.

FX Activation by FIXa-Phospholipid

FIXa (20 nmol/L WT or mutant) was incubated for 5 minutes at 37°C with 200 μ mol/L (25:75) phosphatidylserine:phosphatidylcholine vesicles, 0.4 mmol/L chromogenic substrate (S-2765), and 0 to 300 nmol/L PS, in separate wells of a clear bottom microplate containing 50 mmol/L Tris-HCl (pH 7.4), 5 mmol/L Ca^{2+} , 175 mmol/L NaCl, and 0.6% polyethyleneglycol.^{45,46} Reactions were initiated by addition of 150 nmol/L FX, and the absorbance was recorded for 30 minutes at 405 nm.^{45,47} For the immunoblotting analysis, FX activation by FIXa was determined by incubating 20 nmol/L FIXa in assay buffer (20 mmol/L Tris-HCl at pH 7.4, 150 mmol/L NaCl, 5 mmol/L CaCl_2) with 200 μ mol/L phosphoSer-containing vesicles in the presence or absence of PS (300 nmol/L) at 37°C for 5 minutes, followed by addition of FX (150 nmol/L). Aliquots withdrawn at various times and reactions were terminated by addition of ethylenediaminetetraacetic acid (to 10 mmol/L final) and sodium dodecyl sulfate (to 1%); the samples were resolved on 10% polyacrylamide-sodium dodecyl sulfate gels, followed by immunoblotting with anti-FX antibody.

FX Activation by the Xase Complex

FIXa (0.5 nmol/L WT or mutant) was incubated for 10 minutes at 37°C with 200 μ mol/L 25:75 phosphatidylserine:phosphatidylcholine vesicles, 0.4 mmol/L chromogenic substrate (S-2765), and 0 to 250 nmol/L PS, in separate wells of a clear bottom microplate containing the same buffer as the experiment with FIXa alone.³³ Simultaneously, to produce FVIIIa, 50 nmol/L of a B-domain-deleted FVIII (from Bayer) was incubated with 5 nmol/L α -thrombin for 4 minutes at room temperature.³³ This reaction was stopped by adding 40 nmol/L hirudin and the mixture was left to stand for 1 minute. During this time, 150 nmol/L FX was added to the wells followed by 0.4 nmol/L FVIIIa to initiate the reaction. The absorbance at 405 nm was recorded for 5 minutes.^{33,48,49} The rate of activation of FX was plotted against PS concentration and the data were fit by SigmaPlot to a hyperbolic decay equation to determine the K_i for each FIXa mutant.

Data Analysis

From each kinetic assay, the mean values ($n=3-4$) were taken from the data collected at various PS concentrations in binding or activity studies. These data were fit to a nonlinear regression by means of a hyperbolic, sigmoid, or exponential decay function. This method generated values that described the concentration at which either the reaction was 50% inhibited with respect to rate of FIXa activity (Xa generation assays), 50% of DEGR-FIXa was displaced from PS by untagged FIXa (competitive anisotropy), or 50% of FIXa was bound to PS (fluorescence binding), that is, the values: K_i , IC_{50} , and K_d , respectively. SigmaPlot was used to calculate the standard deviation and associated error for these values.

Statistics

All the experiments were performed 3 times and the data were presented as mean \pm standard deviation. Figure 1A data were analyzed

by 1-way analysis of variance followed by Student's *t* test and the *P* values <0.05 were considered statistically significant.

Modified aPTT Utilizing PS-FIX Double-Depleted Plasma

A total of 40 μ L of PS-FIX double-depleted plasma was added to a 96-well microplate. A total of 5 μ L of FIXa (≈ 0.1 nM, final), 5 μ L of PS (150/300 nmol/L, final), and 40 μ L of Pacific Hemostasis KONTACT reagent were added; the solution was shaken and incubated at 37°C for 5 minutes. The reaction was initiated with 40 μ L of 25 mmol/L CaCl_2 and absorbance was measured at 405 nm. The clotting times were then converted to FIXa activity by measuring the clotting time of each parameter,⁵⁰ FIXa and PS separately and together in PS-FIX double-depleted plasma. The FIX-PS double immunodepleted plasma was supplemented with various concentrations of WT FIXa, R150A, and K132A/R170A mutant FIXa, in the absence or presence of 150 or 300 nmol/L PS, and aPTT clotting time was determined as described above. A standard curve was generated by plotting clotting times versus log FIXa concentration and fitted to a linear plot. Using this standard curve, clotting times were converted to equivalent FIXa coagulant activity. Thus, clotting times determined in the presence and absence of PS were converted into relative FIXa coagulant activity. Relative FIX coagulant activity was determined from the ratio of FIXa+PS coagulant activity to FIXa alone coagulant activity and plotted as average \pm standard deviation (Figure VIB in the online-only Data Supplement). Baseline clotting times were obtained by supplementation of 100 pmol/L FIXa WT, R150A, or K132A/R170A into FIX-PS double immunodepleted plasma in the absence and presence of 150 and 300 nmol/L PS.

Thrombin Generation Assay Utilizing PS-FIX Double-Depleted Plasma

A total of 40 μ L of PS-FIX double-depleted plasma was added to a 96-well microplate. A total of 10 μ L of FIXa (≈ 0.8 nmol/L, final), 10 μ L of PS (0-250 nmol/L, final), and 10 μ L of Technothrombin Thrombin Generation Assay Reagent B were added, shaken, and incubated at 37°C for 5 minutes. The reaction was initiated with 50 μ L of a mixture of 15 mmol/L CaCl_2 and z-Gly-Gly-Arg-AMC (0.5 mmol/L final), and fluorescence was measured on a SpectraMax plate reader with emission and excitation wavelengths of 360 and 460 nm, respectively.

Murine Electrolytic Model of Venous Thrombosis

For in vivo investigation of the localization of FIX, FIXa, and PS in an active thrombus, a murine model of venous thrombosis was used.⁵¹ Adult, male WT C57Bl/6 mice were anesthetized with intraperitoneal pentobarbital (50 mg/kg body weight). Before thrombus induction, anti-factor IX/IXa labeled with Alexa-Fluor-647 (Invitrogen) and human PS (Enzyme Research Laboratories, South Bend, IN) labeled with Alexa-Fluor-532 were coinjecting into the jugular vein (≈ 10 ng of each) of the anesthetized mice. A 75- μ m-diameter iron-containing wire was used to deliver a brief (30 seconds) electrolytic injury to the surface of the surgically exposed, undissected femoral vein, using an anodal voltage of 1.5 volts. The site was strobe-irradiated with green and red beam-expanded laser light (532 and 650 nm, respectively), with time-lapse image capture in a 2.4 \times 3.2 mm field. A growing thrombus developed, with peak growth at 20 to 60 minutes. A low-light digital camera (DVC 1412) with filter wheel was used for image capture of fluorescence emissions over the course of 60 minutes.

Recombinant FIX (BeneFix, Pfizer) was injected intravenously into hemophilia B mice (C57Bl/6 mice, backcrossed 13X) at a dose of 250 IU/kg, either unlabeled or labeled with the ultraviolet (UV) light-activated crosslinking molecule sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino) hexanoate (sulfo-SANPAH; Thermo Scientific, Cat No. 22589). The infrarenal inferior vena cava was exposed after 5 minutes of rFIX injection, and an electrolytic injury was applied to the outer surface of the vein at a point just proximal to the iliac bifurcation, delivering by direct contact 3 volts of anodal direct current for 6 to 8 minutes through a blunted 25-gauge steel cannula. Fifteen minutes after the induction, the exposed vessel and thrombus site were irradiated with a UV source

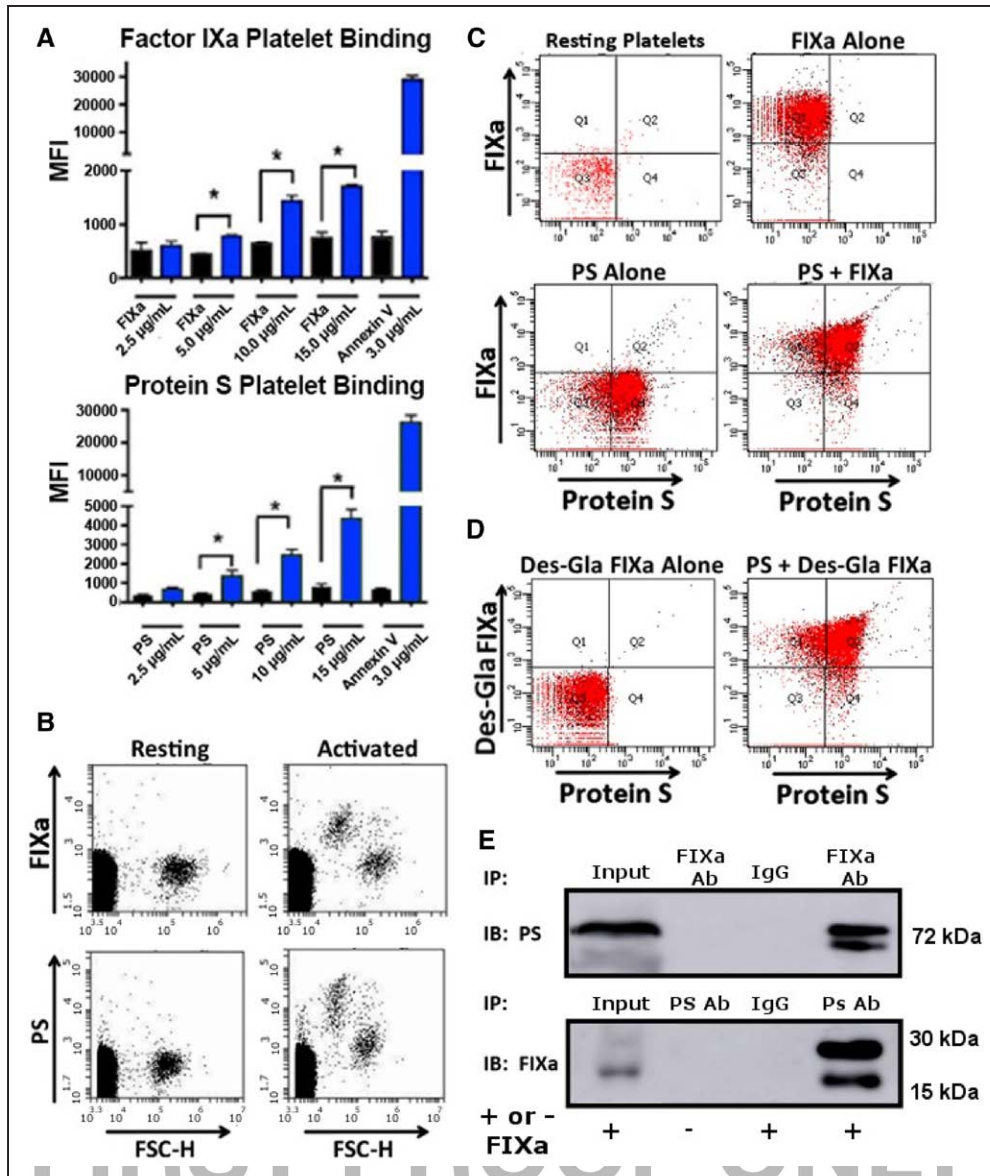


Figure 1. FIXa (factor IXa) interacts with PS (protein S) in vitro and ex vivo. **A**, Flow cytometry was used to quantify the binding of fluorescently labeled FIXa and PS to resting platelets (black bars) and convulxin/PAR-4 peptide-activated platelets (blue bars). Minimal interactions with Gla-containing proteins are seen on the resting platelets. In contrast, binding of both FIXa and PS is observed on platelet activation, as reflected by increased mean fluorescent intensity (MFI). **B**, A forward scatter of resting and activated platelets exposed to fluorescently tagged FIXa (**top**) and PS (**bottom**) demonstrated that platelet size decreases on activation. These smaller, “coated,” platelets showed an increase in fluorescence when binding to FIXa or PS. **C**, Flow cytometry studies of resting and activated platelets were performed with fluorescently tagged FIXa (1 nmol/L) and PS (140 nmol/L; labeled above each plot). A fluorescence shift into Q1 indicates FIXa binding to platelets, whereas a shift into Q4 suggests PS binding. Movement into Q2 indicates colocalization of the 2 proteins on the same platelet (see plot of PS+FIXa). All panels in **C** were derived from experiments that utilized activated platelets, except for the **upper left panel**, which describes resting platelets exposed to both FIXa and PS. **D**, The flow cytometry studies in **C** were repeated on activated platelets with des-Gla FIXa in place of wild-type FIXa. A shift to Q2 is observed in the Des-Gla FIXa+PS experiment, despite the fact that des-Gla FIXa alone is unable to bind platelets; this result demonstrated that PS was required to recruit des-Gla FIXa to the platelet surface. **E**, Coimmunoprecipitation of FIXa with PS, and vice versa, revealed that FIXa interacts with PS in plasma. FIXa (1 nmol/L) was added (lanes with +) or was not added (lanes with -) to FIX-deficient plasma. This plasma was immunoprecipitated with an IgG (immunoglobulin G) control antibody (both blots IgG lane) or with an anti-FIXa or anti-PS antibody (**top blot** and **bottom blot**, respectively), and immunoblotted for PS or FIXa (**top blot** and **bottom blot**, respectively). Initial plasma samples which were supplemented with FIXa are shown on both blots in the Input lane. Molecular weights of bands (kDa) are shown to the right of the blots. The values in **A** are expressed as mean±standard deviation (n=3 replicates). **P*<0.05 vs resting platelet (by *t* test).

(350–380 nm) continuously for 30 minutes to induce protein–protein crosslinking. The thrombus was then harvested from the inside of the vessel and frozen for protein analysis. Clots were dissolved in RIPA buffer, and equivalent protein concentrations from each sample, with or without SANPAH, were immunoblotted with

FIX and PS antibodies. Fluorescence blotting was performed in a similar manner; however, either human FIX or a sulfo-SANPAH tagged human FIX, both pre-labeled with Alexa-Fluor-647, along with human PS pre-labeled with Alexa-Fluor-532, were injected into WT mice before clot formation.

FIXa Infusion-Induced Clotting in Hemophilia B Mice

For the locally infused active FIX proteins, a 30-gauge needle connected to a catheter and syringe was inserted into an ankle vein of an anesthetized FIX knockout mouse. The syringe was attached to a syringe injector, with slow infusion at a rate of 3 $\mu\text{L}/\text{min}$. An antifibrin antibody (59D8) isolated from ascites with specificity to fibrin and not fibrinogen⁵¹ (permission for use kindly provided by Dr Marschall Runge) labeled with Alexa-Fluor-647 and rhodamine 6G (to label platelets) was injected via the jugular vein before thrombus induction, for labeling and subsequent imaging of fibrin and platelets, respectively. An electrolytic injury was generated on the surface of the ipsilateral femoral vein just distal to the inguinal ligament, using the blunt end of a microsurgical needle touching the outer surface of the vein for 30 seconds with a 1.5-volt anodal voltage delivery, conferring a free radical injury within the vein as a nidus for thrombus development. The induction site was subsequently imaged with 532- and 650-nm laser excitation, capturing fluorescence emissions in time-lapse videos for 60 minutes, with subsequent off-line analysis and normalization of fluorescence intensities for interanimal comparisons, as described.⁵¹ FIXa WT (Benefix FIXa) or R150A was infused upstream from the thrombus induction site via an ankle vein at a rate of 2 $\mu\text{g}/\text{h}$, or FIXa K132A/R170A at a rate of 1.3 $\mu\text{g}/\text{h}$; controls received infusion of physiological saline. Infusions were started at the time of onset of the electrolytic injury and were maintained at a flow rate of 3 $\mu\text{L}/\text{min}$ for 60 minutes. Enzymatically equivalent doses of Benefix FIXa, FIXa R150A, and K132A/R170A for infusion were determined by the rate of FX activation in an intrinsic Xase assay containing 0.1 nmol/L FIXa, 1 nmol/L FVIIIa, 150 nmol/L FX, and 200 $\mu\text{mol}/\text{L}$ of 25% phosphatidylserine vesicles (data not shown). The injury site was imaged for 60 minutes under fluorescence image capture with time-lapse acquisition.⁵¹

Results

Interaction of FIXa and PS on Platelets and in Plasma

We used flow cytometry to evaluate the ability of FIXa and PS to bind to the surface of activated platelets. We incubated

activated platelets with increasing concentrations of fluorescently labeled FIXa or PS and observed a steady increase in the mean fluorescence intensity which indicated individual binding of the proteins to platelets (Figure 1A, blue bars). Resting platelets demonstrated a negligible response (Figure 1A, black bars).⁵²

A forward side-scatter (Figure 1B) showed that activation with convulxin/PAR-4 peptide resulted in a population of smaller (lower FSC-H) platelets binding both FIXa and PS. These smaller platelets are characteristic of “coated platelets,” produced by exposure to multiple strong agonists.^{53,54} A hallmark of coated platelets is surface exposure of phosphatidylserine, a negatively charged phospholipid that facilitates the assembly of the membrane-bound enzyme complexes of coagulation. Furthermore, the PS- and FIX-positive platelets stained positive with annexin V (Figure 1A), which selectively binds membranes containing exposed phosphatidylserine. We also used annexin V and unlabeled PS to compete with fluorescently labeled PS in binding to activated platelets. On addition of different concentrations of either annexin V or unlabeled PS, we observed a gradual but marked decrease in fluorescently labeled PS binding to the platelets, indicating that PS binds to phosphatidylserine-positive platelets (Figure 1 in the [online-only Data Supplement](#)).

We continued to assess in more detail the colocalization of the proteins on platelets. No significant binding was detected with resting platelets (Figure 1C, top left), but colocalization of FIXa and PS was observed on activated platelets (Figure 1C, bottom right panel).^{52,55} To assess whether colocalization occurred by interaction between the proteins, we used a form of FIXa lacking the membrane-binding Gla domain (des-Gla-FIXa). Labeled des-Gla-FIXa did not bind

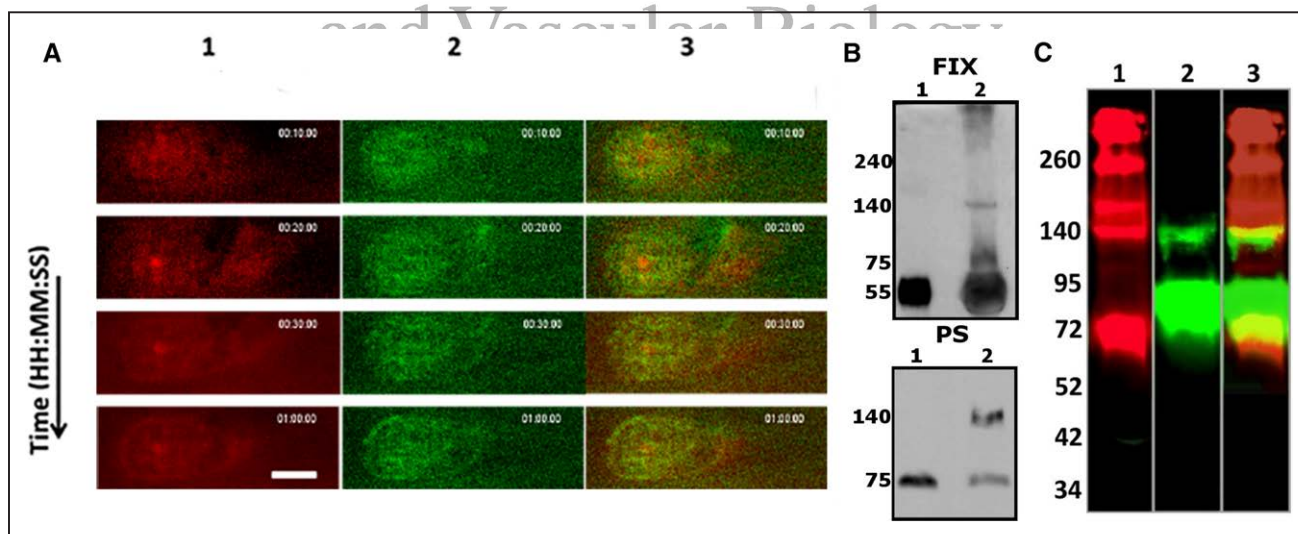


Figure 2. FIX (factor IX) interacts with PS (protein S) in vivo. **A**, After the injection of Alexa-Fluor-647-labeled FIXa (anti-factor IXa; **panel 1**) and Alexa-Fluor-532-labeled human PS (**panel 2**) into wild-type mice, a thrombus was induced by a brief electrolytic injury to the femoral vein; diffuse partial colocalization of the fluorescent molecules was observed in the merged image (3). A continuous correlated increase in fluorescence intensity was evident during the clotting process. **B**, Femoral vein clots formed in hemophilia B mice, supplemented with FIX or FIX-sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino) hexanoate (SANPAH), were exposed to ultraviolet (UV) light to crosslink FIX-SANPAH to associated proteins. Immunoblots of clots from (1) FIX-injected mice and (2) FIX-SANPAH-injected mice were probed for FIX (**top blot**) and PS (**bottom blot**). Higher molecular weight forms (molecular weight in kDa is shown to the left of the blots) are seen in both blots for FIX-SANPAH, but not for FIX, suggesting that FIX associated with PS. **C**, A fluorescent blot of plasma from a hemophilia B mouse, injected with FIX-SANPAH that was conjugated with Alexa-Fluor-647, and PS that was conjugated with Alexa-Fluor-532 is (1) probed for FIX and (2) probed for PS. **Panel 3** shows an overlay of lanes 1 and 2, exhibiting a band at ≈ 140 kDa, in similar fashion to the blots from **B**. Color has been added to highlight the overlap of bands, denoted in yellow. Scale bar, 300 μm .

to activated platelets in the absence of PS (Figure 1D, left panel). However, when activated platelets were incubated with both labeled des-Gla FIXa and PS, the proteins colocalized on the platelet surface, an effect suggesting that the platelet binding-competent PS pulled des-Gla FIXa down to the platelet surface (Figure 1D, right panel). The median fluorescence intensity for PS+FIXa was 4336, whereas the median fluorescence intensity for PS+des-Gla FIXa was 3997 (Figure 1C and 1D). These results indicated that the Gla domain of FIXa is not required for binding to PS.

The ability of FIXa and PS to interact in human plasma was evaluated by coimmunoprecipitation. We added WT FIXa (1 nmol/L) to FIX-deficient plasma and found that the FIXa coimmunoprecipitated with PS (Figure 1E, top blot lane 4);

we did not detect PS in the immunoprecipitate from plasma that either lacked FIXa (Figure 1E, top lane 2) or that was incubated with a control IgG antibody (Figure 1E, top blot, lane 3). The failure to immunoprecipitate PS under the latter 2 conditions indicated that PS had not bound adventitiously to the immunoabsorbent. Finally, reverse coimmunoprecipitation of this plasma with antihuman PS antibody confirmed PS binding to FIXa in plasma (Figure 1E, bottom blot).

Interaction of FIXa and PS in Mouse Thrombi

Fluorescently labeled human anti-FIX antibody (red) and human PS (green) were injected into WT mice, and an electrolytic injury was applied to the femoral vein to induce thrombi. The monoclonal anti-FIX/FIXa antibody was checked to

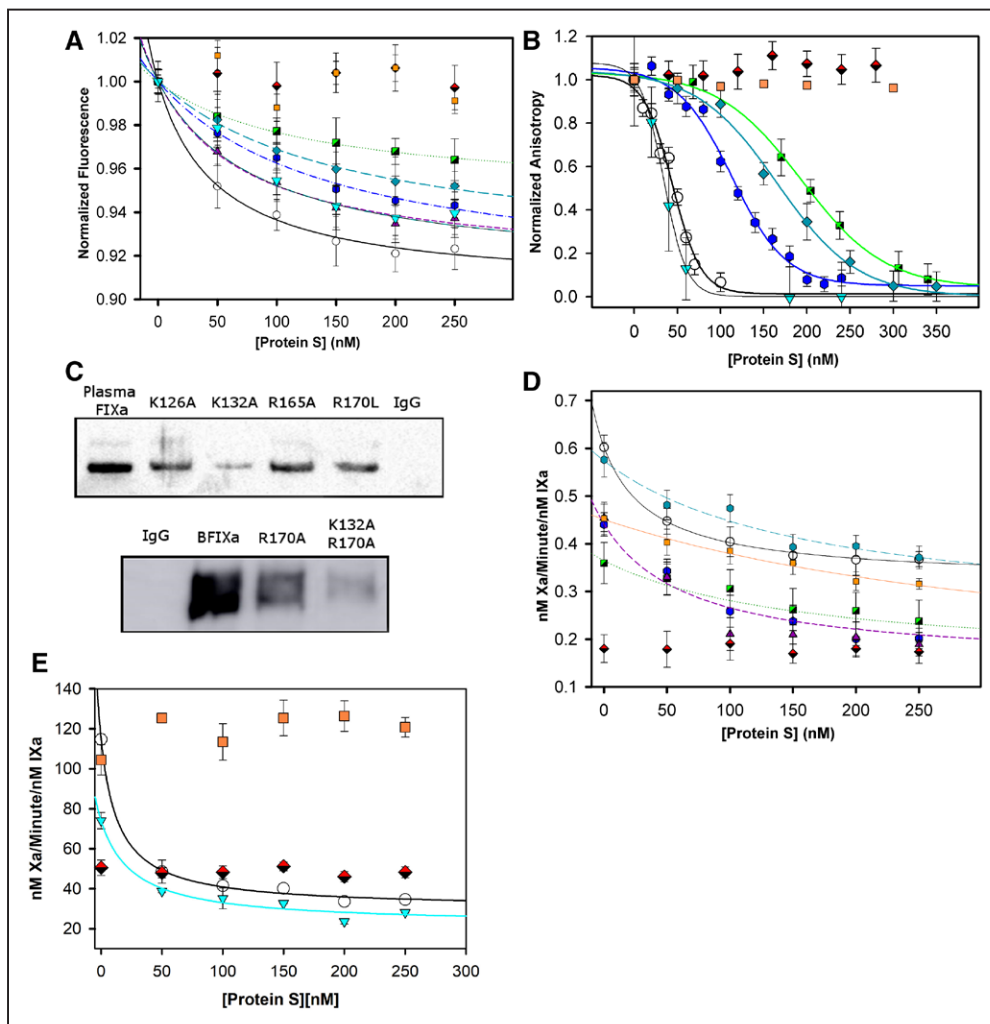


Figure 3. FIXa (factor IXa) protease domain single-site mutant kinetics. **A**, Dansyl-Glu-Gly-Arg chloromethyl ketone (DEGR)-FIXa mutant binding fluorescence: 75 nmol/L DEGR-FIXa was titrated with increasing concentrations of PS (protein S); fluorescence was measured and normalized. **B**, DEGR-FIXa fluorescence anisotropy binding competition: 50 nmol/L DEGR-FIXa was incubated with 80 nmol/L PS, then titrated with unlabeled FIXa constructs at concentrations from 0 to 300 nmol/L; the anisotropy was measured and normalized. **C**, 1 nmol/L of each FIXa construct was added to FIX-deficient plasma, immunoprecipitated with anti-FIX antibody, and immunoblotted for PS. The **top blot** was generated using X-ray film, whereas the **bottom blot** was scanned with an Amersham Imager 600. The particular FIXa construct that was supplemented into the FIX-deficient plasma is labeled above each blot. **D**, Effect of PS on FXa generation by the FIXa-phospholipid complex: 20 nmol/L FIXa proteins were incubated with 0.4 mmol/L S-2765 chromogenic substrate and 0 to 250 nmol/L PS at 37°C. The reactions were initiated by the addition of 150 nmol/L FX to each well and absorbance was measured at 405 nm. **E**, Effect of PS on FXa generation by the intrinsic Xase complex: performed as above in the presence of FIXa (0.5 nmol/L) and FVIIIa (0.4 nmol/L), complexed in the physiologically relevant Xase complex. Error bars represent standard deviation on all plots ($n=3$). No significant differences (compared with FIXa wild type [WT]) were observed in the inhibition kinetics of FIXa R150A or R165A by PS (data not shown). FIXa WT: , K126A: , N129A: , K132A: , R150A: , R165A: not plotted, R170L: , K132A/R170A: , K126A/K132A: .

confirm that it does not affect FIXa activity. The labeled proteins colocalized over the entire area of a clot for the 20 minutes after injury (Figure 2A). The fluorescence intensities exhibited a correlated increase, suggesting that FIXa and PS colocalized during the process of thrombus formation.⁵¹ Labeled control antibody or albumin did not localize in the thrombus.⁵¹

In vivo association between FIXa and PS was further assessed by protein–protein crosslinking in hemophilia B mice. We injected rFIX, covalently modified with the UV-inducible crosslinker SANPAH, after which we applied an electrolytic injury to the infrarenal vena cava, followed by UV irradiation and immunoblotting of resulting thrombi. Immunoblots demonstrated an intense FIX band at ≈ 60 kDa and a higher molecular weight species at ≈ 140 kDa. The higher molecular weight band represented crosslinked material because we observed only a single FIX band when we used unmodified FIX (Figure 2B, upper panel). The band at ≈ 140 kDa represents a complex between FIXa and PS because PS binds to FIX ≈ 5 -fold weaker (as described below) than with FIXa, and it is probable that FIXa will form a complex with PS in the clot. Similarly, immunoblotting for PS after FIX–SANPAH injection revealed 2 bands, the free form of PS at ≈ 80 kDa and a crosslinked form of PS at ≈ 140 kDa. We did not observe the 140-kDa species in mice injected with unmodified FIX (Figure 2B, lower panel). The 140-kDa FIXa and PS bands were evaluated in UV-irradiated mouse plasma that contained fluorescently labeled, SANPAH-tagged FIXa, and fluorescently labeled PS (Figure 2C). When immunoblotted for FIXa, these plasma samples showed a similar ≈ 140 kDa band (Figure 2C, left lane), in addition to multiple, unidentified higher molecular weight species that we attribute to differences between the plasma and thrombus environments. We performed non-SANPAH controls for FIXa and PS and the figure is provided as a supplemental figure (Figure VIII in the [online-only Data Supplement](#)).

Direct and Competitive Binding of FIXa Mutants to PS

We previously demonstrated that titrating a DEGR-FIXa-PS complex with unfractionated heparin reduced the fluorescence anisotropy (Figure II in the [online-only Data Supplement](#)), suggesting that the binding sites for heparin and PS overlap within FIXa. Thus, we labeled with DEGR the active sites of previously characterized FIXa mutants having alanine substitutions in the HBE^{26,32,40} to evaluate the contribution of the HBE in PS binding. The interactions of these labeled FIXa mutants with PS were detected in a direct binding assay by changes in fluorescence intensity.⁴⁷ We observed an exponential decay in fluorescence with addition of PS, suggesting a conformational change in DEGR-FIXa on PS binding (Figure 3A). FIXa WT exhibited a $K_d \approx 51 \pm 7$ nmol/L.³³ Several FIXa proteins with mutations in the HBE demonstrated marked reductions in PS affinity (ie, increased K_d), including FIXa K126A (118 \pm 20 nmol/L), K132A (148 \pm 39 nmol/L), and K132A/R170A and K126A/K132A (binding not detectable; Table 1). Additionally, the FIXa Padua mutant (R170L) bound PS with significantly reduced affinity (157 \pm 33 nmol/L).

Table 1. FIXa Protease Mutant Construct Activity and Binding Kinetics

FIXa Protease Mutant	K_d of FIXa Binding by Protein S, nmol/L	FIXa IC ₅₀ in Anisotropy Competition, nmol/L	K_i of FIXa Activity Inhibition by Protein S, nmol/L
WT	51 \pm 12	63 \pm 27	35 \pm 26
K126A	118 \pm 34	140 \pm 24	100 \pm 52
N129A	77 \pm 64	92 \pm 45	72 \pm 31
K132A	148 \pm 67	213 \pm 79	132 \pm 52
R150A	48 \pm 14	43 \pm 29	48 \pm 33
R165A	54 \pm 12	68 \pm 36	33 \pm 19
R170L	157 \pm 57	163 \pm 47	175 \pm 90
K132AR170A	ND	ND	271 \pm 119
K126A K132A	ND	ND	<1% activity

Various kinetic parameters for PS binding to FIXa WT and mutants (protease variants) are shown. The table displays mean apparent K_d (direct binding of Dansyl-Glu-Gly-Arg chloromethyl ketone-FIXa with protein S by fluorescence intensity), IC₅₀ (competition of FIXa with DEGR-FIXa toward binding to protein S by fluorescence anisotropy), or K_i (inhibition of FX activation by FIXa in the presence of increasing concentration of protein S by proteolysis of synthetic substrate S-2765) \pm standard deviation (n=3 replicates of each), which were determined by fitting curves to data (average of 3 data sets) collected in the described fluorescent or activity assays. FIXa indicates factor IXa; ND, not determinable, because of weak binding or low activity (<1% activity); and WT, wild type.

We used a fluorescence anisotropy-based binding assay to assess competition by mutant FIXa proteins in DEGR-FIXa binding to PS (Figure 3B). The IC₅₀ values obtained from these competition assays followed the trend of binding affinities obtained from the direct binding assays (FIXa WT>K126A>R132A>R170L>K132A/R170A and K126A/K132A). PS binding to the R150A mutant, which demonstrates markedly reduced inhibition by antithrombin, was similar to its binding of WT FIXa. Furthermore, the zymogen form of FIX showed an IC₅₀ value ≈ 200 nmol/L (data not shown), indicating a significantly weaker interaction with PS compared with FIXa. In sum, the general agreement between the direct and competitive binding assays demonstrated that the FIXa HBE contributes to the interaction with PS.

We also measured the ability of FIXa mutants to coimmunoprecipitate PS from plasma (Figure 3C). The relative intensities of the PS immunoprecipitation bands inversely correlated with the K_d values determined in the fluorescence binding assays, with the faintest bands in the co-IPs corresponding to the highest dissociation constants (Figure 3C). Importantly, as demonstrated by immunoblotting, the various FIXa mutants showed negligible differences in antibody affinity (Figure III in the [online-only Data Supplement](#)). These data confirmed that the effects of the FIXa HBE mutations on PS binding affinity in vitro were retained in the plasma environment.

Effect of PS on FX Activation by FIXa Mutants

We assessed the effect of select mutations in the FIXa HBE on the ability of PS to inhibit FX activation by the FIXa–phospholipid and the intrinsic Xase (FIXa–FVIIIa) complexes. Titration of the FIXa–phospholipid complex with PS resulted

Table 2. FIXa Gla Deletion Construct Activity and Binding Kinetics

FIXa Gla Construct	K_d of FIXa Binding by Protein S, nmol/L	FIXa IC_{50} in Anisotropy Competition, nmol/L	K_i of FIXa Activity Inhibition by Protein S, nmol/L
Des-Gla	124±66	135±21	<1% (active Xase $K_i=120±93$)
Des-Gla-EGF1	ND	ND	<1% activity
Uncarboxylated	65±26	77±28	<1% (active Xase $K_d=46±41$)

Various kinetic parameters for protein S binding to FIXa domain variants are shown. The table displays mean apparent K_d (direct binding of Dansyl-Glu-Gly-Arg chloromethyl ketone-FIXa with protein S by fluorescence intensity), IC_{50} (competition of FIXa with Dansyl-Glu-Gly-Arg chloromethyl ketone-FIXa toward binding to protein S by fluorescence anisotropy), or K_i (inhibition of FX activation by FIXa in the presence of increasing concentration of protein S by proteolysis of synthetic substrate S-2765) ±standard deviation (n=3 replicates), which were determined by fitting curves to data (average of 3 data sets) collected in the described fluorescent or activity assays. FIXa indicates factor IXa; and ND, not determinable, because of weak binding or low activity (<1% activity).

in partial inhibition of FX activation ($\approx 45\%$ maximal inhibition for WT FIXa) with resistance to PS inhibition observed for multiple FIXa HBE mutants (Figure 3D). Furthermore, the relative K_i values for the FIXa mutants correlated with the dissociation constants (K_d) observed in the PS binding studies

(Table 1). Similarly, titration of the intrinsic Xase complex with PS also resulted in $\approx 65\%$ inhibition of FX activation by WT FIXa, a result that agreed with our previous study.³³ Thus, PS binds not only to free FIXa but it also binds as an allosteric inhibitor to the physiologically relevant Xase complex (Figure 3E). The mechanism of Xase inhibition is similar to the inhibition of the FIXa–phospholipid complex because the FIXa HBE mutants resistant to PS inhibition demonstrated a similar trend when incorporated into the Xase complex. Under these conditions, PS did not inhibit FX activation by either FIXa K132A/R170A or K126A/K132A, findings consistent with the loss of PS binding to these mutant proteases. Moreover, the double mutant K132A/R170A showed 1.5-fold (data not shown) higher activity than WT FIXa in the absence of PS, but we did not detect a change in the activity of K132A/R170A after PS addition (Figure 3E). We did monitor the activation of FX by FIXa in the presence of phospholipid vesicles in sodium dodecyl sulfate gel (see Methods) at different time intervals, and our data indicated that PS inhibits the formation of FXa (determined by the appearance of heavy chain; Figure V in the [online-only Data Supplement](#)).

Effect of Gla and EGF1 Domain Deletions on FIXa Binding to PS

We examined the ability of PS to bind FIXa lacking either the Gla domain (des-Gla)(amino acid numbering, 40–415), the

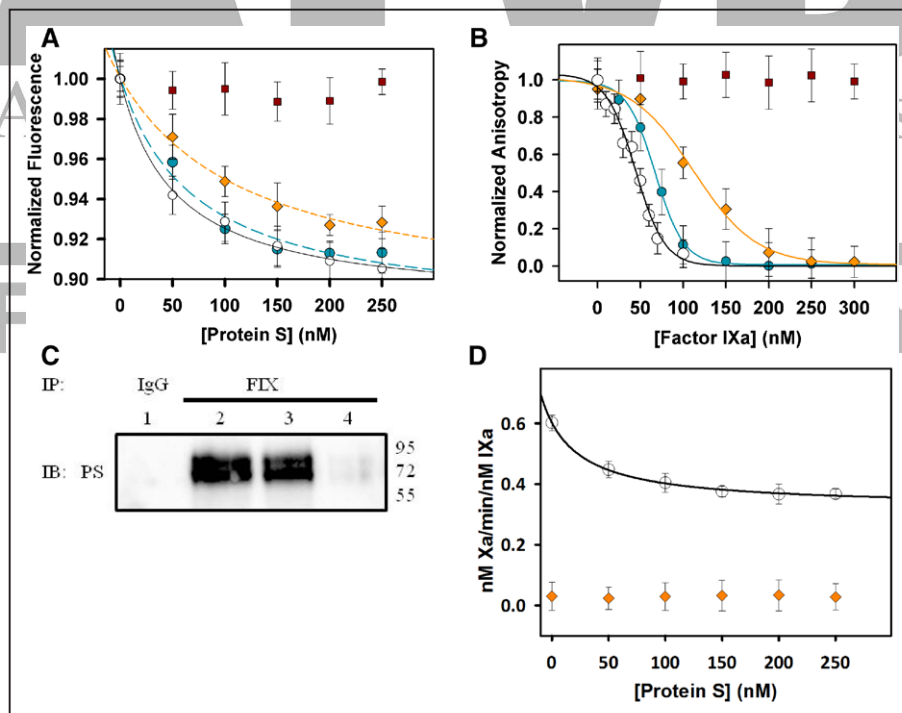


Figure 4. Identification of additional FIXa (factor IXa) domains necessary for FIX–PS (protein S) interaction. **A**, Dansyl-Glu-Gly-Arg chloromethyl ketone (DEGR)–FIXa mutant binding fluorescence: 75 nmol/L DEGR-FIXa and its deletions constructs were titrated with various concentrations of PS; fluorescence was measured and normalized. **B**, DEGR-FIXa fluorescence anisotropy binding competition: 50 nmol/L DEGR-FIXa was incubated with 80 nmol/L PS, then titrated with unlabeled FIXa deletion constructs having concentrations from 0 to 300 nmol/L and anisotropy was measured. **C**, FIXa constructs (labeled above blot), at a final concentration of 1 nmol/L, were added to FIX-deficient plasma, immunoprecipitated with anti-FIX antibody, and immunoblotted for PS. **D**, FXa generation assay by Xase complex: 0.5 nmol/L FIXa constructs were incubated with 0.4 mmol/L S-2765 chromogenic substrate and 0 to 250 nmol/L PS at 37°C. Reactions were initiated by the addition of 150 nmol/L FX and 0.4 nmol/L FVIIIa to each well; absorbance was measured at 405 nm. Error bars represent standard deviation on all plots (n=3). (Protein or Co-IP control: symbol [Co-IP number]): IgG (immunoglobulin G) control: Co-IP only (1), FIXa-deficient plasma: Co-IP only (2), FIXa wild type (WT): (3), uncarboxylated FIXa: (4), Des-Gla FIXa: (5), Des-Gla-EGF1: (6).

Gla and EGF1 domains (des-Gla-EGF1)(amino acid numbering, 88–425), or γ -carboxylation. In both the direct and competitive binding assays, uncarboxylated FIXa demonstrated only slightly weaker binding (K_d , 65 ± 15 nmol/L), whereas des-Gla FIXa had a more pronounced reduction (K_d , 124 ± 38 nmol/L) in PS affinity (Table 2, Figure 4A and 4B).⁵⁶ Both des-Gla and uncarboxylated FIXa retained the ability to coimmunoprecipitate PS from plasma (Figure 4C). In contrast, the des-Gla-EGF1 mutant neither bound to PS (Figure 4A and 4B) nor coimmunoprecipitated PS from plasma (Figure 4C). Enzymatic analysis of FX activation could be obtained only for the uncarboxylated WT derivative in the Xase complex (Figure 4D), likely because of the important role of the N-terminal Gla domains in the productive assembly of membrane-bound coagulation complexes.^{57,58} The binding and immunoprecipitation data suggested that while the Gla domain was largely dispensable for the PS interaction, we could not exclude a contribution of the EGF-1 domain to FIXa–PS binding. A summary of the putative intermolecular interactions of FIXa with PS is displayed on a representative structure in Figure 5. Each residue or domain analyzed is displayed on a gradient from green to yellow to red (untested residues in blue), where red and green indicate, respectively, the most and least necessary residues in the FIXa–PS interaction.

Effect of PS on FIX Activation by FXIa

To assess other consequences of the PS–FIXa and PS–FIX interactions, we examined the effect of PS on the rate of FXIa-mediated activation of FIX. We monitored activation of FIX by FXIa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of samples over time with and without PS present. Densitometric quantification of protein band volume indicated that there was no effect of PS on the rate of FIX activation, which showed >95% complete cleavage by 90 minutes without or with PS at a 1:200 molar ratio of protease to zymogen (Figure IV in the [online-only Data Supplement](#)). These results suggested that the inhibitory effect of PS is primarily because of direct interaction with FIXa.

Modified aPTT and Thrombin Generation in FIX–PS Double-Immunodepleted Plasma

The effect of PS on aPTT-based coagulant activity for FIXa WT, FIXa K132A/R170A, and R150A was examined in FIX/PS double-immunodepleted plasma (FIX–PS-ID plasma) supplemented with 0, 150, or 300 nmol/L PS (described in Methods). We measured the coagulant activity of FIXa with no PS present relative to coagulant activity in the presence of 150 and 300 nmol/L PS; the data are presented in Table 3 and Figure VIA in the [online-only Data Supplement](#). At 300 nmol/L PS, the activities of WT and R150A FIXa decreased by $\approx 90\%$, whereas the activity of K132A/R170A decreased by 58%. The resistance of FIXa K132A/R170A relative to WT and R150A with respect to prolongation of clotting time by PS was consistent with the reduced PS binding affinity and reduced inhibitor potency of PS for FIXa K132A/R170A (Table 1).

The relative resistance of FIXa K132A/R170A coagulant activity to inhibition by PS in an aPTT-based assay mirrored

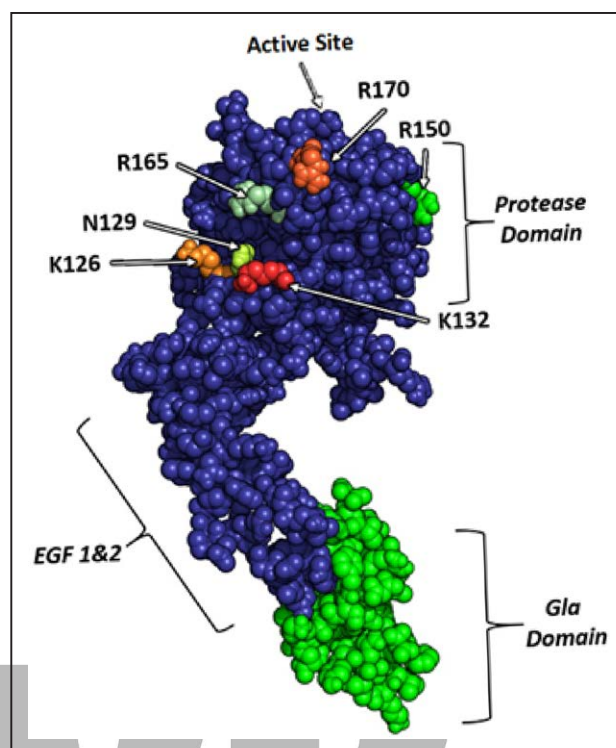


Figure 5. Structural identification of binding site of PS (protein S) on FIXa (factor IXa). The porcine FIXa structure (PDBID: 1PFX) is displayed with relevant amino acid residues designated by a color gradient from green to red: Green indicates residues not required for PS interaction, yellow indicates residues that contribute weakly to PS interaction, and red indicates residues required for strong interaction with PS. Domains are indicated in italics. Porcine and human FIX share 84% peptide sequence identity, and each of the relevant residues that were mutated in the protease domain are conserved between the porcine and human proteins.

the inability of FIXa K132A/R170A to bind PS. To further examine the physiological consequence of this impaired interaction, we evaluated the ability of FIXa WT, R150A, and K132A/R170A to support plasma thrombin generation in the FIX–PS-ID plasma. Assays were initiated with limiting TF (0.4 pmol/L) in the presence of 10 pmol/L FIXa and increasing PS concentrations (Figure 6). Titration of PS into the plasma caused a decrease in peak thrombin for FIXa WT, FIXa R150A, and K132A/R170A (Figure 6A through 6C).

Table 3. Clotting Activity Relative to No PS of FIXa at Varied PS Concentrations in FIX–PS Double-Immunodepleted Plasma

FIXa Construct	0 nmol/L PS	150 nmol/L PS	300 nmol/L PS
WT FIXa	1 \pm 0.074	0.288 \pm 0.006	0.121 \pm 0.002
R150A	1 \pm 0.008	0.310 \pm 0.005	0.098 \pm 0.004
K132A/R170A	1 \pm 0.065	0.677 \pm 0.025	0.417 \pm 0.019

The table shows relative FIXa coagulant activity with respect to no PS binding obtained from titration of FIX–PS double-immunodepleted plasma with 100 pmol/L FIXa WT, R150A, and K132A/R170A in the absence and presence of PS. The values represent the relative coagulant activity of 100 pmol/L FIXa WT, in the absence of PS compared with activity after supplementation of 150 or 300 nmol/L PS into the FIX–PS double-depleted plasma. Results are reported as mean relative coagulation activity fraction \pm standard deviation (n=3 replicates for each). FIXa indicates factor IXa; PS, protein S; and WT, wild type.

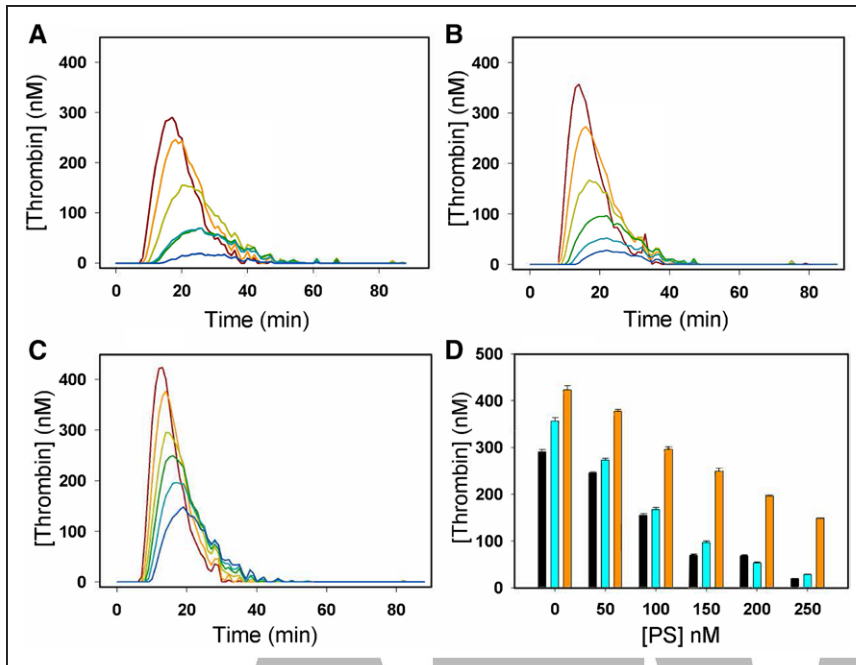


Figure 6. Thrombin generation in FIX (factor IX)–PS (protein S) double-immunodepleted plasma. Thrombin generation assays were performed in FIX–PS–ID plasma supplemented with 10 pmol/L wild-type (WT) FIXa (A), R150A FIXa (B), or K132A/R170A FIXa (C), along with concentrations of PS ranging from 0 to 250 nmol/L (thrombin generation assay was performed at least 3 times for each protein). Reactions were performed in the presence of a limiting concentration of tissue factor (TF) to selectively generate thrombin through the intrinsic pathway. The addition of PS resulted in reduced thrombin generation as demonstrated by peak thrombin values (D). FIXa K132A/R170A demonstrated a relatively smaller reduction in peak thrombin generation in the presence of PS compared with WT, likely because of its reduced ability to bind PS. Legend: (A–C): 0 nmol/L, 50 nmol/L, 100 nmol/L, 150 nmol/L, 200 nmol/L, 250 nmol/L PS. D, WT, R150A, K132A/R170A.

FIXa K132A/R170A exhibited only a 41% decrease in peak thrombin generation in the presence of 150 nmol/L PS, compared with FIXa WT which demonstrated a 76% decrease, confirming the contribution of the FIXa HBE to the anticoagulant activity of PS. The decrease in peak thrombin for FIXa R150A at 150 nmol/L PS was similar to FIXa WT (73%), suggesting that reduced FIXa inhibition by antithrombin does not significantly enhance thrombin generation under these conditions. The peak thrombin and endogenous thrombin potential values for FIXa WT, K132A/R170A, and R150A FIXa at 0, 150 nmol/L, and 300 nmol/L PS are presented in Tables I and II in the [online-only Data Supplement](#), respectively.

SEMs (unidirectional, to avoid too much noise; data points taken every 10 seconds in the graphs). The enhanced fibrin accumulation for the PS-resistant FIXa mutant indicates enhanced *in vivo* activity, consistent with its specific resistance to PS-dependent inhibition in the aPTT and thrombin generation assays. Thus, a protease with minimal affinity for PS, FIXa K132A/R170A, demonstrated enhanced *in vivo* coagulant activity after an electrolytic femoral vein injury in hemophilia B mice. Differences in platelet accumulation did not achieve significance ($P=0.292$). These results compellingly indicate the existence of a physiologically significant interaction between PS and the HBE of FIXa.

FIXa Infusion–Induced Clotting in Hemophilia B Mice

Because of its markedly reduced affinity for PS (Table 1) and resistance to PS anticoagulant effects in plasma (Table 3, Figure 6), we used FIXa K132A/R170A to probe *in vivo* PS function in a murine thrombosis model. Hemophilia B mice were continuously infused with FIXa WT, R150A, or K132A/R170A upstream of an electrolytic injury. FIXa WT (BenefIXa) or R150A were infused at a rate of 2 $\mu\text{g/h}$, and FIXa K132A/R170A was infused at 1.3 $\mu\text{g/h}$ because, in the FX activation assay, K132A/R170A had 1.5-fold higher activity compared with WT and R150A FIXa. Importantly, FIXa K132A/R170A demonstrated increased fibrin accumulation relative to FIXa WT or R150A; this increase was seen at all time points beyond 10 minutes to the end of the experiment (60 minutes). The fibrin data analyzed with analysis of variance were significant ($P<0.005$). In post hoc Fisher least significant difference tests, the vehicle-infused thrombi had less fibrin accumulation than each of the other groups ($P<0.005$); infusion of the double mutant resulted in significantly higher fibrin accumulation compared with infusion of the other 2 proteins ($P<0.05$). The graphs (Figure 7B and 7C) are averages, with the colored error bars showing

Discussion

Our objectives were to define the PS binding site(s) on FIXa and establish PS inhibition of FIXa as an authentic component of the regulation of blood coagulation. We used FIXa proteins with amino acid substitutions and domain deletions to identify a region extensively overlapping the FIXa HBE as critical for PS interaction. Direct and competition binding assays and coimmunoprecipitation from human plasma identified FIXa residues critical for interaction with PS, including K132>R170>K126 (chymotrypsinogen numbering; Figure 3; Table 1). Similarly, the resistance to PS inhibition of FIXa with these HBE mutations, either as free FIXa or in the intrinsic Xase complex, was proportional to the effects of the mutations on FIXa–PS affinity (Figure 3D). FX activation by FIXa, monitored in gel (Figure V in the [online-only Data Supplement](#)) indicated direct PS effects on FIXa rather than FXa in the Xase studies using purified components as FXa formation was clearly inhibited in the presence of PS (Figure V in the [online-only Data Supplement](#)). In contrast, the FIXa Gla domain was not required for PS interaction because deletion of the domain or a lack of γ -carboxylation had little or no effect on PS binding affinity and coimmunoprecipitation from plasma (Figures 1 and 4; Table 2). Deletion of both

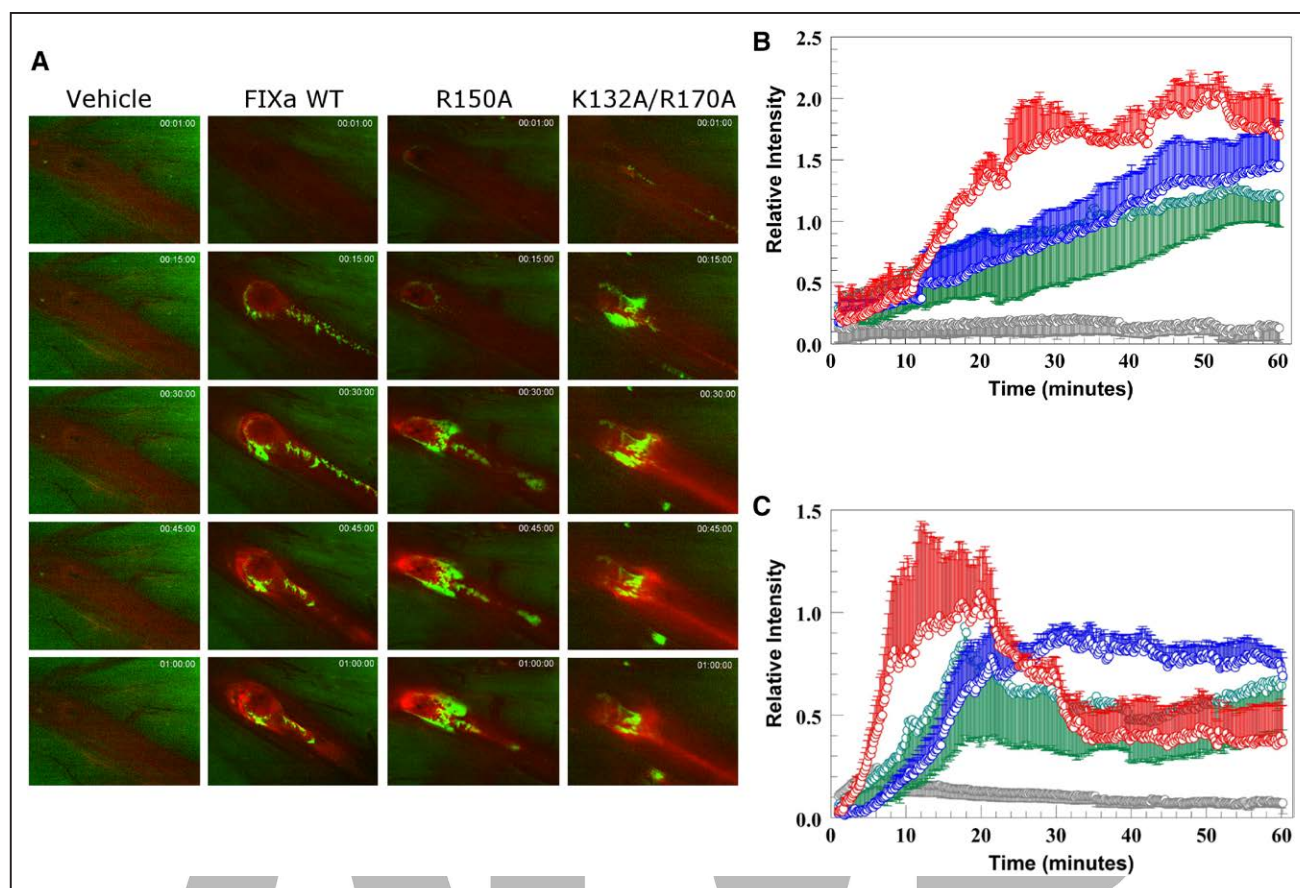


Figure 7. FIXa (factor IXa) infusion-induced clot formation in hemophilia B mice. **A**, Representative image capture of thrombus induction site during local, upstream infusion of vehicle, wild type (WT), R150A, and K132A/R170A FIXa at 1, 15, 30, 45, and 60 minutes. Each video (see supplemental materials in the [online-only Data Supplement](#)) shows a frame of 2.4×3.2 mm, with the femoral vein flowing from upper left to lower right and with the thrombus induction site, where the thrombus is most concentrated on the left. Platelets appear green and antifibrin antibody (showing fibrin accumulation) is in red. These data were quantitated for fluorescence intensity of fibrin (**B**) and platelet accumulation (**C**) over the 60-minute imaging run. Data were normalized for the amount of injected fluorophore and animal weight (surrogate of blood volume), from images captured every 10 seconds. Lines are averages with error bars showing standard deviation ($n=3$ /data set).

the Gla and EGF1 domains resulted in a complete lack of PS–FIXa binding and coimmunoprecipitation, suggesting that EGF1 contributes to this interaction (Figure 4). The EGF domains function as spacers to position the active site of FIX/FIXa and related coagulation proteases at a suitable distance from the biological membrane for interaction with activators, cofactors, and substrates.^{59,60} As the tertiary structure of the EGF2–protease domain protein is intact,³⁶ these results suggest that FIXa and PS may have an extended protein–protein interaction on the membrane surface. Finally, the affinity of PS for FIXa ($K_d \approx 50$ nmol/L) is consistent with the expected physiological concentration of free PS (≈ 150 nmol/L) in human plasma and at least 4-fold greater than the affinity for the FIX zymogen.

To establish that the inhibition of FIXa by PS is a bona fide component of the regulation of blood coagulation, we compared the activities of WT FIXa and FIXa K132A/R170A in the presence of PS in multiple physiological environments. FIXa K132A/R170A was the key to these studies because it had dramatically reduced PS binding but full enzymatic activity (Figures 3D, 3E, and 6 and Table 3). We used the active protease forms of the rFIX proteins to exclude potential

differences in zymogen activation. In plasma immunodepleted of both FIX and PS, FIXa K132A/R170A was relatively resistant to the anticoagulant effect of PS (compared with WT FIXa) in both aPTT-based coagulant activity (Table 3) and thrombin generation assays (Figure 6). In contrast, FIXa R150A behaved similarly to WT protease, suggesting that FIXa inhibition by antithrombin does not substantially influence these end points. These results clearly demonstrated the contribution of the FIXa HBE to regulation of coagulation by PS in these physiological environments. Further, we confirmed the FIXa HBE–PS interaction in mice.⁵¹ In the electrolytic injury model, direct imaging of thrombi demonstrated colocalization of fluorescently labeled FIXa and PS (Figure 2A). In the infrarenal inferior vena cava injury model, immunoblot analysis of thrombi demonstrated crosslinking of SANPAH–FIXa with PS, a result that further substantiated the *in vivo* occurrence of this protein–protein interaction (Figure 2B and 2C). Finally, the ability of these proteases to initiate thrombus formation was assessed in hemophilia B mice. After injury, FIXa K132A/R170A-injected mice produced significantly greater total fibrin accumulation compared with mice injected with FIXa WT and R150A ($P=0.001$; analysis of variance and

post hoc Fisher least significant difference test), whereas differences in platelet accumulation were insignificant ($P=0.292$; Figure 7). These results confirm the in vivo relevance of interaction between PS and FIXa in regulation of coagulation. Our data from in vivo and in vitro experiments, in the presence of plasma PS concentration of 150 nmol/L, further suggest that the natural PS plasma concentration exerts an anticoagulant function by directly inhibiting FIXa.

The FIXa HBE contributes to cofactor stability and allosteric communication within the intrinsic Xase complex, antithrombin-independent inhibition of intrinsic Xase activity and plasma thrombin generation by select glycosaminoglycans, and inhibition by antithrombin-heparin.^{23–32,61} Our findings establish that interaction of PS with the FIXa HBE is another important APC-independent regulatory mechanism for the propagation phase of coagulation. This critical regulatory role of the FIXa HBE may provide alternative approaches to therapeutic anticoagulation. Currently available treatments include small molecules that either directly target the active site of FXa or thrombin (eg, rivaroxaban or dabigatran) or indirectly target coagulation protease active sites via activation of antithrombin (unfractionated and low molecular weight heparins).^{62,63} While these anticoagulants are efficacious, they are all associated with a clinically significant risk of bleeding. As an alternative to targeting active sites, the functional exosites of coagulation proteases are an underexplored set of therapeutic targets. Here, we have shown that PS targets the HBE of FIXa. Thus, we suggest that our study has established the basis for exploring a novel, exosite-based therapeutic strategy for the management of thrombosis based on the FIXa–PS interaction.

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Disclosures

None.

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Highlights

- Protein S contributes significantly to hemostasis by binding the protease domain of FIXa (factor IXa), thereby directly inhibiting FIXa.
- The heparin-binding exosite of FIXa is critical for protein S binding.
- A double mutation in FIXa, R132A/R170A abolishes protein S binding.
- Disruption of the protein S–FIXa interaction in mice causes an increased rate of thrombosis.

Arteriosclerosis, Thrombosis, and Vascular Biology



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Anticoagulant Protein S Targets the Factor IXa Heparin-Binding Exosite to Prevent Thrombosis

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Supplemental Figures

Figure I:

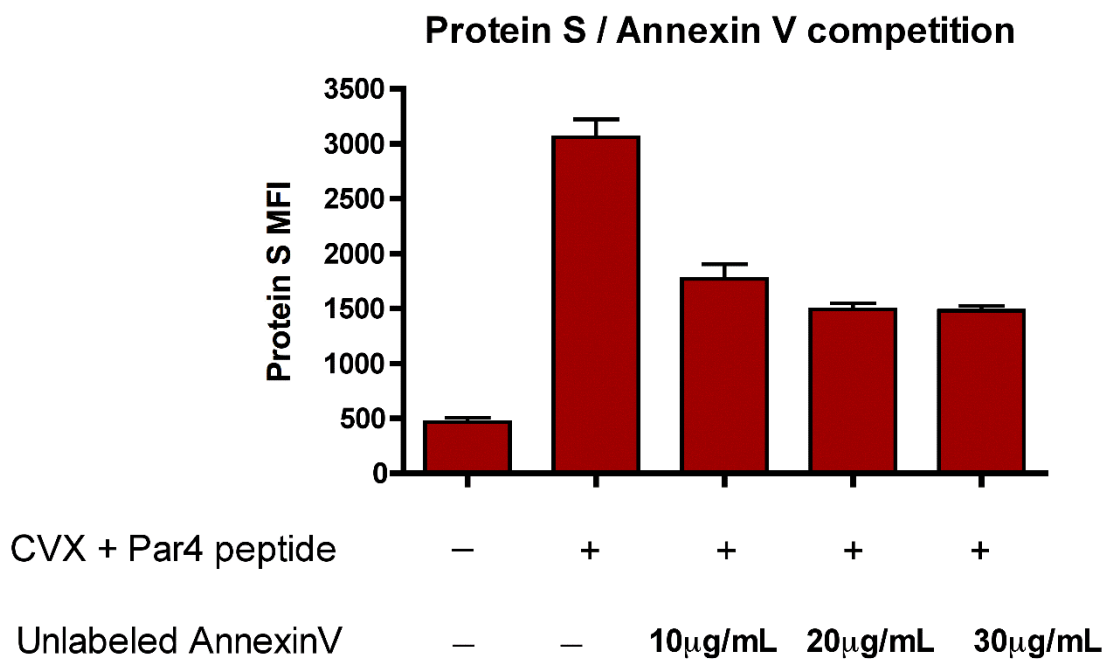


Figure I:

Flow cytometry study with fluorescently labeled PS and unlabeled annexin. The platelets were activated with convulxin/PAR-4 and the change in binding of the fluorescently labeled PS with activated platelets were measured with the addition of increasing concentration of annexin.

Figure II:

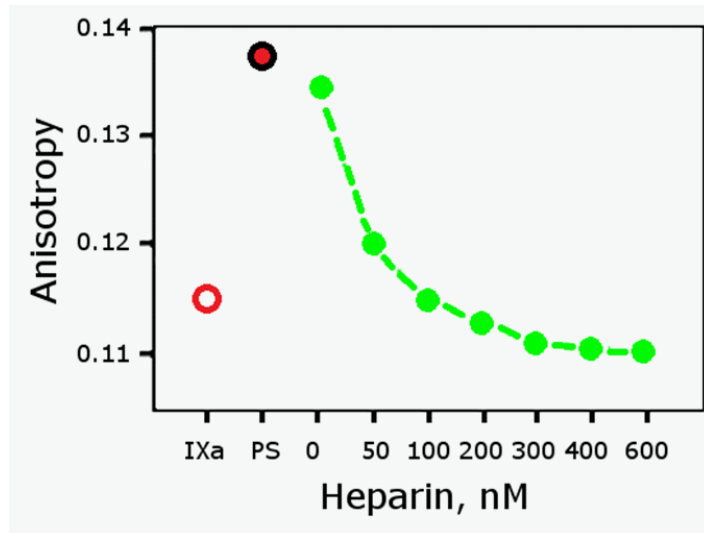


Figure II:

5nM DEGR-IXa in the presence of 25:75 PS:PC vesicles (red open circle) and 10nM PS (closed red circles) binds to heparin (green circles). Fitting the curve by a hyperbola gives a $K_d \sim 40$ nM.

Figure III:

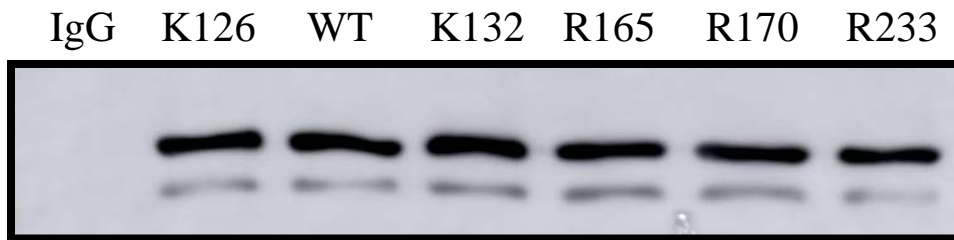


Figure III:

Equal masses of various FIXa constructs were immunoblotted for, utilizing the same FIXa antibody, as we used in our FIXa co-immunoprecipitations. The differences seen between the bands are negligible when compared with the differences observed when pulled down with PS.

Figure IV:

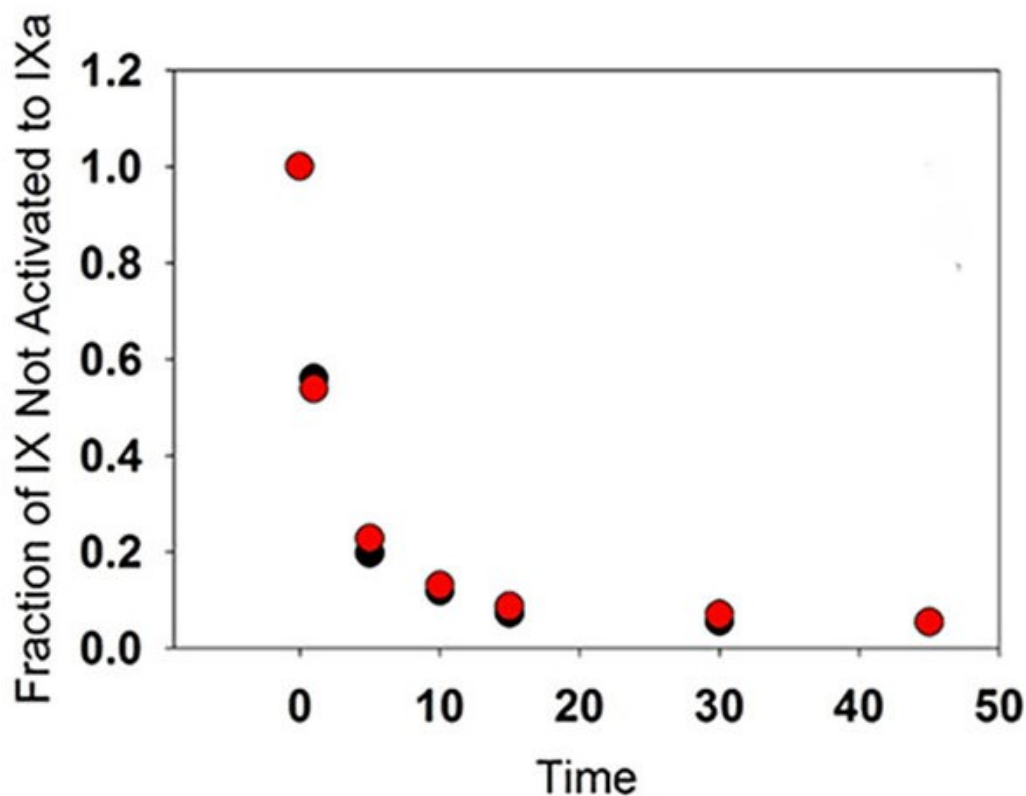


Figure IV:

5 μ M WT FIX(black) or K132A/R170A FIX(red) was incubated with 25nM XIa, in 20mM Tris-HCl (pH 7.4), 150mM NaCl and 5mM CaCl₂ at 37°C. Samples were taken and their reactions were stopped using soybean trypsin inhibitor and EDTA, followed by addition of 6x SDS-PAGE loading dye into the samples. SDS-PAGE was performed and the protein bands were stained with coomassie blue. Band volume of zymogenic FIX was taken in each lane, utilizing the densitometric function of an Amersham 600 imager. These volumes were normalized and plotted, demonstrating negligible changes in the rate of activation of IX by XIa due to mutations within the FIXa protease domain.

Figure V:

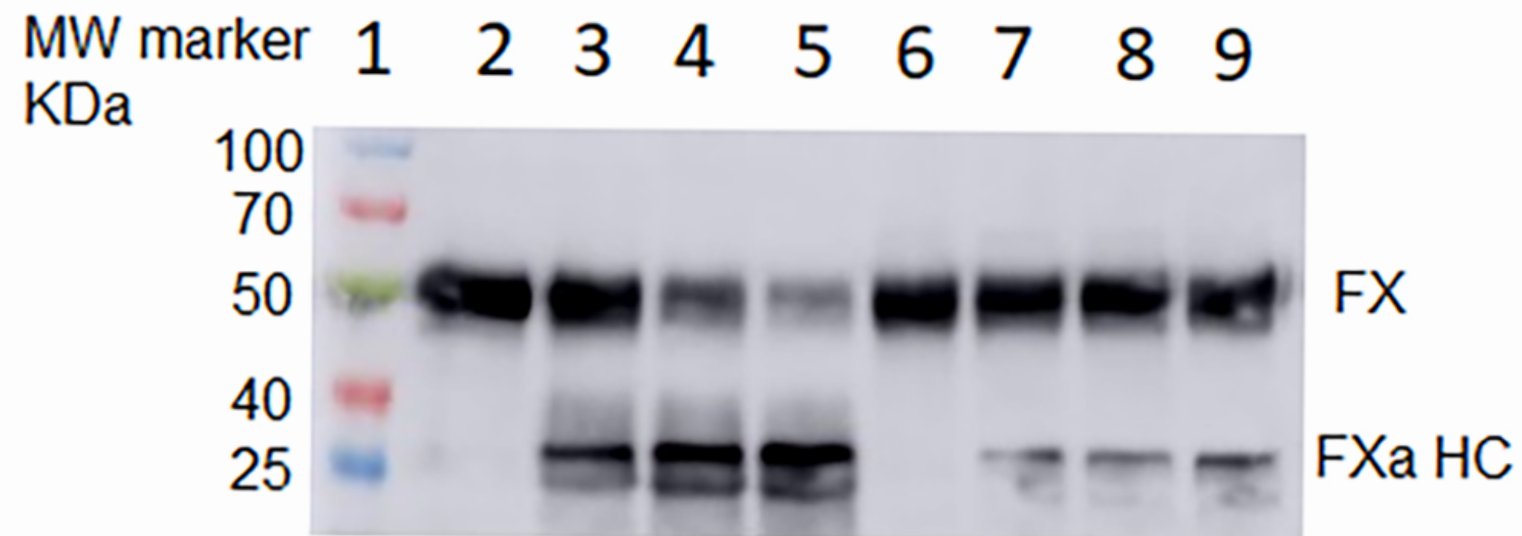


Figure V:

Effect of PS on FX activation by FIXa monitored by Western blotting. FX (150 nM) was activated by FIXa (20 nM) in presence of 200 μ M phosSer vesicles with or without PS (300 nM) in buffer containing 20 mM Tris-HCl at pH mM NaCl, 5 mM CaCl₂, 0.6% PEG at 37 °C. Lane 1: Molecular weight marker, Lane 2-5: FX digested by FIXa at 0, 30, 60 and 120 min respectively, Lane 6-9: FX digested by FIXa in presence of Protein S at 0, 30, 60 and 120 min respectively

Figure VI:

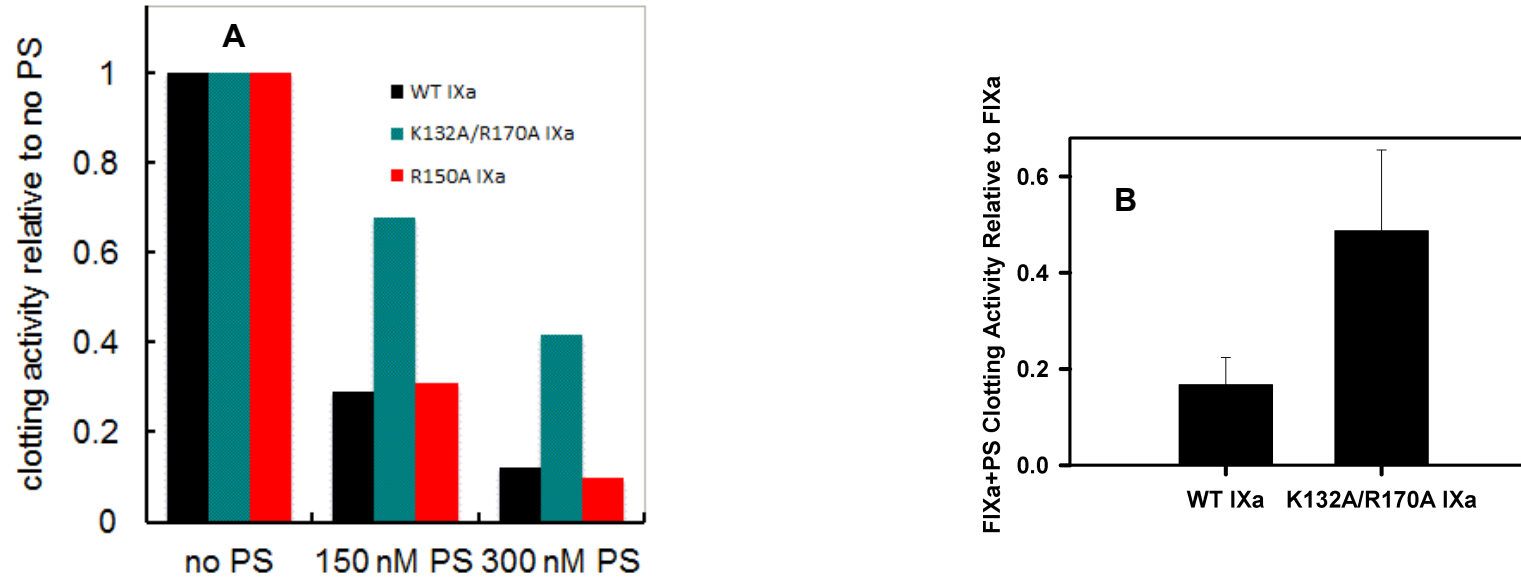


Figure VI:

Relative Clotting activity of WT and Mutant (R150A and K132A/R170A) FIXa at various PS concentrations in FIX-PS Double Immunodepleted Plasma. aPTT clotting time was determined from the FIX-PS double immuno-depleted plasma supplemented with various concentrations of WT and mutant (R150A and K132A/R170A) FIXa in the presence and absence of PS as described in method. A) relative clotting activity of 100 pm WT and mutants (R150A and K132A/R170A) FIXa in the presence of 150 and 300 nM PS, B) Average clotting activity relative to FIXa of the WT and K132A/R170A mutant FIXa in the presence of 150 nM PS was determined from the linear plot of aPTT clotting time vs log of FIXa concentration as mentioned in the methods.

Figure VII:

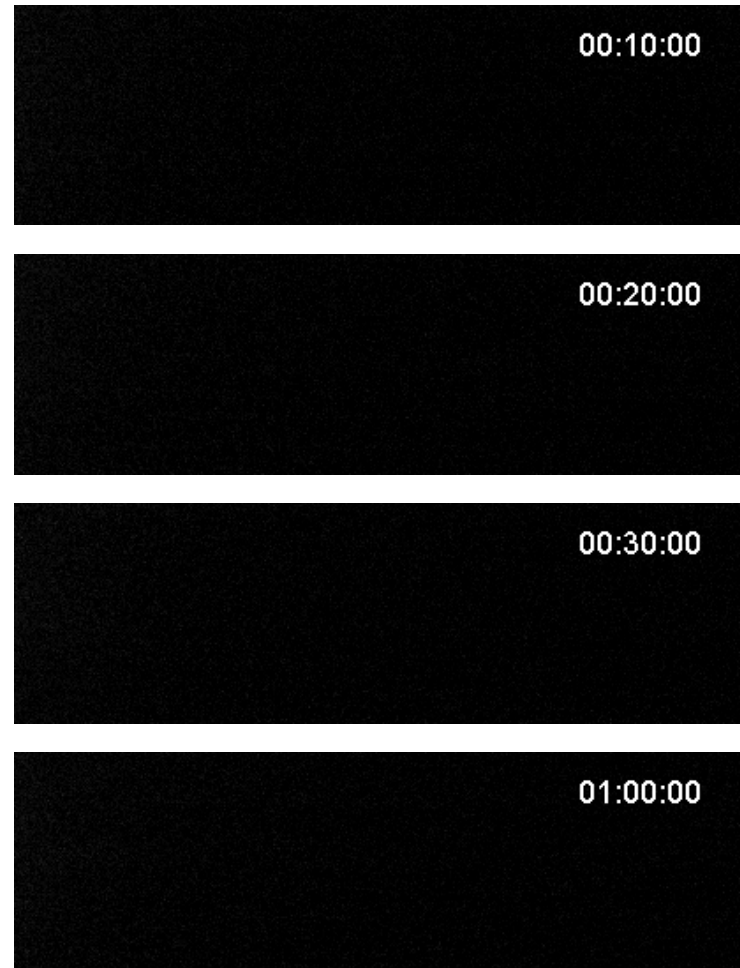


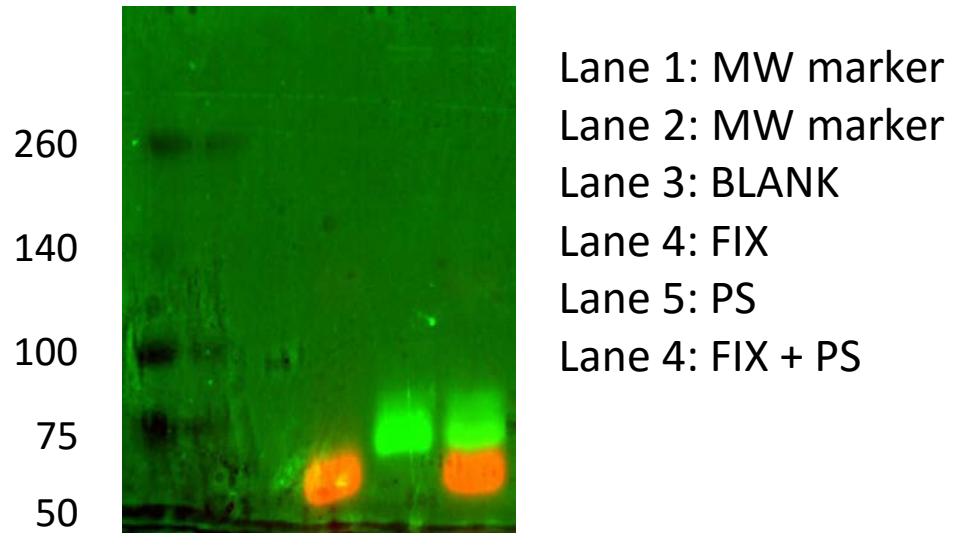
Figure VII: Control images without an electrolytic injury to the vein as negative controls to Figure 1.

Femoral vein without electrolytic injury under identical conditions of imaging and fluorophore circulation.

Scale bar = 300 μm .

Figure VIII:

1 2 3 4 5 6



Lane 1: MW marker
Lane 2: MW marker
Lane 3: BLANK
Lane 4: FIX
Lane 5: PS
Lane 4: FIX + PS

Figure:VIII: Non-SANPAH controls of FIX labeled with Alexa Flour 647 and Protein S labeled with Alexa-Flour 488 showed no complex formation between FIX and PS. (1) FIX, (2) PS (3) FIX + PS.

Table I: Peak IIa of thrombin generation from WT and mutant IXa (R150A, K132A/K170A) supplemented with FIX-PS double immunodepleted plasma

PS nM	WT Peak IIa, nM	R150A Peak IIa, nM	K132A/R170A Peak IIa, nM
0	292.37 ± 2.19	348.06 ± 12.47	412.79 ± 15.67
50	245.97 ± 0.36	264.49 ± 26.56	365.48 ± 16.58
100	175.29 ± 29.04	191.36 ± 20.84	270.99 ± 34.90
150	156.46 ± 0.01	156.54 ± 51.73	233.66 ± 22.56
200	92.68 ± 33.35	109.58 ± 49.23	182.73 ± 19.33
250	33.27 ± 20.14	61.66 ± 30.73	147.06 ± 1.74
300			96.12 ± 28.07

The values represent Peak IIa in nM ± STDEV

Table II: ETP of thrombin generation from WT and mutant IXa (R150A, K132A/K170A) supplemented with FIX-PS double immunodepleted plasma

PS nM	WT ETP, nM	R150A ETP, nM	K132A/R170A ETP, nM
0	4013.04 ± 80.76	4081.86 ± 104.00	4111.16 ± 54.12
50	3767.05 ± 109.75	3905.10 ± 144.89	4014.88 ± 24.36
100	2841.72 ± 278.09	3481.97 ± 20.69	3577.95 ± 127.55
150	1614.10 ± 177.25	3050.27 ± 300.05	3362.54 ± 47.35
200	1189.79 ± 365.85	2662.41 ± 177.01	2829.39 ± 113.13
250	388.75 ± 55.17	1426.58 ± 345.49	2286.94 ± 31.63
300			1640.03 ± 549.68

The values represent ETP in nM ± STDEV.

Legends for supplemental Video Files:

The effect on FIXa- PS affinity on in vivo thrombus formation in a murine model of venous thrombosis. Prior to thrombus induction by electrolytic injury, anti-fibrin antibodies, labeled with Alexa-Fluor-647 and rhodamine 6G for labeling platelets were injected via the jugular vein of an anesthetized Hemophilia B mouse with either (1) a saline vehicle control, (2) Benefix Factor Xa, (3) the FIXa mutant R150A, or (4) FIXa double mutant K132A/R170A. The doses of the wild type and the mutant proteins were adjusted based on their activities in the tenase assay(as described in the results) Thrombus images were captured by a low-light digital camera (DVC 1412) with a filter wheel. Platelets appear green, and anti-fibrin antibody (showing fibrin accumulation) is in red in the video images.