BIOCHEMISTRY
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Biochemistry **2010,** *49,* 9997–10005 9997
DOI: 10.1021/bi100906q

The Regulation of Factor IXa by Supersulfated Low Molecular Weight Heparin[†]

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Received June 4, 2010; Revised Manuscript Received September 13, 2010

ABSTRACT: Supersulfated low molecular weight heparin (ssLMWH) inhibits the intrinsic tenase (factor IXa−factor VIIIa) complex in an antithrombin-independent manner. Recombinant factor IXa with alanine substitutions in the protease domain (K126A, N129A, K132A, R165A, R170A, N178A, R233A) was assessed with regard to heparin affinity in solution and ability to regulate protease activity within the factor IXa−phospholipid (PL) and intrinsic tenase complexes. In a soluble binding assay, factor IXa K126A, K132A, and R233A dramatically (10−20-fold) reduced ssLMWH affinity, while factor IXa N129A and R165A moderately (5-fold) reduced affinity relative to wild type. In the factor IXa−PL complex, binding affinity for ssLMWH was increased 4-fold, and factor X activation was inhibited with a potency 7-fold higher than predicted for wild-type protease−ssLMWH affinity in solution. In the intrinsic tenase complex, ssLMWH inhibited factor X activation with a 4-fold decrease in potency relative to wild-type factor IXa−PL. The mutations increased resistance to inhibition by ssLMWH in a similar fashion for both enzyme complexes (R233A > K126A > K132A/R165A > N129A/N178A/wild type) except for factor IXa R170A. This protease had ssLMWH affinity and potency for the factor IXa−PL complex similar to wild-type protease but was moderately resistant (6-fold) to inhibition in the intrinsic tenase complex based on increased cofactor affinity. These results are consistent with conformational regulation of the heparin-binding exosite and macromolecular substrate catalysis by factor IXa. An extensive overlap exists between the heparin and factor VIIIa binding sites on the protease domain, with residues K126 and R233 dominating the heparin interaction and R165 dominating the cofactor interaction.

Heparin is a complex, heterogeneous mixture of oligosaccharide chains that possess both antithrombin-dependent and -independent mechanisms of action, the latter of which includes direct inhibition of factor X activation by the intrinsic tenase complex (factor IXa−factor VIIIa) (*1*). Heparin oligosaccharides inhibit the intrinsic tenase complex via interaction with an exosite on factor IXa, which antagonizes cofactor (factor VIIIa) activation without complete disruption of the enzyme complex (*2*). Factor IXa mutations that reduce protease affinity for immobilized heparin are associated with increased resistance to inhibition by low molecular weight heparin (LMWH)[1] or partially depolymerized fucosylated chondroitin sulfate in the intrinsic tenase complex (*3*, *4*). Supersulfated low molecular weight heparin (ssLMWH) is prepared from LMWH by treatment with sodium periodate to destroy high-affinity antithrombin binding, followed by O-sulfation with trimethylamine sulfur trioxide. These modifications result in 9-fold increased affinity for factor IXa relative to low-affinity LMWH and the ability to inhibit both intrinsic tenase and prothrombinase complexes with purified components (*5*). The enhanced affinity of ssLMWH facilitates analysis of binding interactions with both the free protease and factor IXa incorporated into membrane-bound enzyme complexes.

An extensive protein−protein interaction between factor VIIIa and factor IXa in the intrinsic tenase complex is suggested based on evidence from type II hemophilia B mutations, in vitro characterization of site-directed factor IXa mutants, and modeling of the cofactor−protease interaction (*6*−*12*). Cofactor interactions have been demonstrated for the Gla (*13*), EGF (*14*, *15*), and protease domains (*7*, *9*) of factor IXa. The extensive interaction of the factor VIIIa light chain (A3-C1-C2) with the factor IXa EGF domains makes a dominant contribution to cofactor affinity but completely lacks cofactor activity (*16*). In contrast, the interaction of the unstable factor VIIIa A2 domain with the protease domain is required for cofactor activity (*17*). In particular, based on the effect of the hemophilia B mutation R165Q, the side chain of R165 is critical for binding of the isolated A2 domain (*9*). Thus, prevention of the factor VIIIa dependent increase in catalytic efficiency of factor IXa in the intrinsic tenase complex does not require disruption of the entire protease−cofactor interaction, since specific targeting of the intrinsically unstable factor IXa−A2 domain interaction should be sufficient.

The heparin-binding site on the factor IXa protease domain appears to be a critical regulator of hemostasis, based on the ability of glycosaminoglycans to inhibit intrinsic tenase activity and the rate of plasma thrombin generation (*2*, *3*, *18*, *19*). Likewise, selected mutations in the protease domain (R170A and R233A) demonstrate opposing effects on cofactor affinity, thrombin generation in human plasma, and murine models of bleeding and thrombosis following saphenous vein injury (*18*). The critical nature of the

[†]This research was supported by National Institutes of Health Grant HL080452.

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[1]Abbreviations: BSA, bovine serum albumin; DHG, depolymerized holothurian glycosaminoglycan; EGF, epidermal growth factor; Fl-ssLMWH, fluoresceinated supersulfated low molecular weight heparin; Gla, γ-carboxylated glutamic acid; HBSC, 20 mM HEPES, 0.15 M NaCl, 2 mM $CaCl_2$, pH 7.4; HEK, human embryonic kidney; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IdoA, α-L-iduronic acid; GlcNS, 2-deoxy-2-sulfamido-α-D-glucopyranosyl; LMWH, low molecular weight heparin; PC, phosphatidylcholine; PEG-8000, polyethylene glycol (average MW 8000); pFIXa, plasma-derived factor IXa; PL, phospholipid; pNA, *p*-nitroaniline; PS, phosphatidylserine; ssLMWH, supersulfated low molecular weight heparin.

Published on Web 10/15/2010

factor IXa−A2 domain interaction and the ability of heparin oligosaccharides to inhibit factor X activation via interaction with this protease exosite suggest that detailed knowledge of these overlapping binding sites will yield insight into regulation of the intrinsic tenase complex. Supersulfated low molecular weight heparin (ssLMWH) binds factor IXa with increased affinity, facilitating comparison of protease−heparin interactions in solution versus within the membrane-bound enzyme complex. A series of well-characterized mutant proteins with alanine substitutions in the protease domain were employed to determine the contribution of specific residues to protease−ssLMWH binding in solution and regulation of protease activity by ssLMWH within the factor IXa−phospholipid (PL) and intrinsic tenase complexes. These results provide a detailed picture of the heparin-binding exosite, its contribution to allosteric regulation of macromolecular substrate catalysis, and the extent of overlap with the cofactor interactive site on the factor IXa protease domain.

EXPERIMENTAL PROCEDURES

Materials. Human plasma-derived factor X, IXa, XIa, and thrombin were purchased from Enzyme Research (South Bend, IN). Recombinant human factor VIII (Kogenate FS) was generously provided by Andreas Mueller-Beckhaus of the Bayer Corp. (Berkeley, CA). Recombinant B-domainless factor VIII and D519V/E665V B-domainless factor VIII were generously provided by Phil Fay (Rochester, NY) (*20*). Fluorescein-labeled and unlabeled supersulfated low molecular weight heparins (Fl-ssLMWH and ssLMWH, average MW = 5000) were generously provided by Jeffrey Weitz of McMaster University (Hamilton, Ontario, Canada). The LMWH (average MW = 5000) used for sulfation was obtained from an unfractionated heparin preparation by nitrous acid depolymerization, and its affinity for antithrombin was reduced by sodium periodate oxidation (*5*). The LMWH was then ultrafiltrated, O-sulfated with trimethylamine sulfur trioxide, lyophilized, and subjected to elemental analysis to determine the number of sulfate residues/disaccharide. Fluorescein labeling of ssLMWH was performed using fluorescein isothiocyanate (*5*). The ssLMWH and Fl-ssLMWH preparations used in these experiments contained five sulfates per disaccharide. Depolymerized holothurian glycosaminoglycan (DHG) was generously provided by Kazuhisa Minamiguchi of Taiho Pharmaceuticals (Saitama, Japan). Low molecular weight heparin (LMWH, dalteparin, average MW = 5000) was obtained from Pharmacia and UpJohn Co. (Kalamazoo, MI). Recombinant hirudin, bovine serum albumin (BSA), and poly(L-lysine) were purchased from Sigma (St. Louis, MO). Pefachrome FIXa (CH_3O_2-D-CHG-Gly-Arg-*p*-nitroaniline (pNA)) was purchased from Centerchem, Inc. (NorWalk, CT). Chromogenic substrate, S-2765 (*N*-α-benzyloxycarbonyl-D-Arg-Gly-Arg-pNA), was purchased from DiaPharma (Franklin, OH). Phosphatidylserine (PS) and phosphatidylcholine (PC) were purchased from Avanti Lipids (Alabaster, AL). Cholesterol was purchased from Calbiochem (San Diego, CA). Phosphatidylcholine−phosphatidlylserine−cholesterol (molar ratio 75:25:20) phospholipid (PL) vesicles were prepared by extrusion through a 100 nm polycarbonate filter (*21*). Cholesterol was included in the vesicles to increase stability at room temperature (*22*−*24*). The molar concentration of PL was determined with an elemental phosphorus assay (*25*).

Expression and Purification of Recombinant Factor IX. An HEK 293 cell line stably transfected with human factor IX R170A was provided by Darrel Stafford (University of North Carolina—Chapel Hill) (*26*). Stable HEK 293 cell lines expressing human factor IX wild type, K126A, N129A, K132A, R165A, R170A, N178A, or R233A were constructed as described (*3*, *12*). Factor IXa amino acid residues are identified using chymotrypsinogen numbering. Recombinant factor IX proteins were purified from conditioned media and quantified by absorbance at 280 nm using an extinction coefficient ($\varepsilon_{0.1\%}$) of 1.32 (*3*). Proteins were >90% pure based on Coomassie-stained SDS−PAGE (data not shown). Purified factor IX was activated with human factor XIa, and factor IXa catalytic sites were quantified by titration with antithrombin III (*3*, *12*).

Heparin-Binding Assay. The affinity of factor IXa for fluorescein-labeled supersulfated LMWH (Fl-ssLMWH) was determined by measuring the change in fluorescence anisotropy for 0.2 nM Fl-ssLMWH in HBSC buffer (20 mM HEPES, 0.15 M NaCl, 2 mM $CaCl_2$, pH 7.4) upon addition of 0−500 nM factor IXa. The assay was performed at room temperature using black 96-well flat-bottom plates (Corning Life Sciences, Corning, NY). After addition of factor IXa to Fl-ssLMWH, the plates were shaken for 30 s, and fluorescence anisotropy was measured (excitation 485 nm/emission 535 nm) in a TECAN GENios Pro fluorescent microplate reader. The signal was maximal at 10 min and remained stable for at least 1 h. Factor IXa concentration was plotted versus anisotropy, and the data were fit by nonlinear regression to a single site binding model to determine the apparent K_d (*27*). Competition binding experiments were done at final concentrations of 0.2 nM Fl-ssLMWH, 75 nM plasma factor IXa, and 0−1000 μM ssLMWH (supersulfated LMWH) or LMWH, or 0−30 μM depolymerized holothurian glycosaminoglycan (DHG), under identical assay conditions. Data were fit to a four-parameter logistic equation to determine the EC_{50} (*27*, *28*):

$$Y = (a - d)/(1 + (X/c)^b) + d \quad (1)$$

where y = response, X = the arithmetic dose, a = the response when $X = 0$, d = the response for an "infinite" dose, $c = EC_{50}$, and b = a slope factor that describes the steepness of a curve. Direct binding studies for Fl-ssLMWH and factor IXa were also performed in the presence of 50 μM PL vesicles and 30% ethylene glycol.

Heparin Inhibition of Factor IXa Chromogenic Substrate Hydrolysis. The rate of Pefachrome IXa hydrolysis was determined at room temperature in a reaction containing final concentrations of 80 nM recombinant factor IXa, 700 μM Pefachrome IXa, 50 μM PL vesicles, and 30% ethylene glycol in the presence of ssLMWH (0−32 μM) (*12*). Ethylene glycol stimulates the rate of cleavage for selected chromogenic substrates with hydrophobic moieties in the S3 position and factor X activation (*29*, *30*). PL vesicles, ssLMWH, ethylene glycol, and tenase buffer (20 mM HEPES, 0.15 M NaCl, 2 mM $CaCl_2$, pH 7.4, 1 mg/mL BSA, 0.1% PEG-8000) were added to a 96-well flat-bottom plate (Greiner Bio-One, Longwood, FL). Factor IXa was added just prior to initiating the reaction by addition of the Pefachrome IXa substrate. Absorbance at 405 nm was measured every 30 s for 10 min to determine the initial rate of substrate hydrolysis.

Heparin Inhibition of Factor IXa−PL Activity. The rate of factor X activation by the factor IXa−PL complex in the presence of ssLMWH or LMWH (0−20 μM) was determined over 20 min at room temperature in an 80 μL reaction containing final concentrations of 5 nM recombinant factor IXa, 150 nM factor X, 50 μM PL vesicles, and 30% ethylene glycol (*3*). The reaction was terminated after 20 min by addition of 16 μL of stop solution (0.25 M EDTA/1.1 mg/mL Polybrene). Activity was measured in a 96-well flat-bottom plate plate (Greiner Bio-One, Longwood, FL) by addition of 100 μL of 0.48 mM S-2765

substrate to 40 μL aliquots of the quenched reaction mixture with comparison to a factor Xa standard curve. Since both unfractionated and low molecular weight heparin have previously been found to behave as a partial noncompetitive inhibitors of the intrinsic tenase complex (*1*, *2*), the rate of factor X generation was plotted versus ssLMWH or LMWH concentration, and the data were fit by nonlinear regression to the equation for partial noncompetitive inhibition to determine the apparent K_i (*1*, *31*).

Heparin Inhibition of Intrinsic Tenase. The rate of factor X activation by the intrinsic tenase in the presence of ssLMWH or LMWH (0−40 μM) was determined over 60 s at room temperature in an 80 μL reaction containing final concentrations of 0.2 nM recombinant factor IXa, 1 nM factor VIIIa, 200 nM factor X, and 50 μM PL vesicles (*3*). Thrombin was used to activate factor VIII immediately prior to addition to the assay, and factor X was added to start the reaction. The reaction was stopped after 60 s by addition of stop solution, and activity was measured in a 96-well flat-bottom plate (Greiner Bio-One, Longwood, FL) as described above for the factor IXa−PL complex. The rate of factor Xa generation was plotted versus ssLMWH or LMWH concentration, and the data were fit by nonlinear regression to the equation for partial noncompetitive inhibition to determine the apparent K_i (*1*, *31*).

Docking of Heparin to Factor IX. Docking simulations of a heparin pentasaccharide to the heparin-binding exosite of the human factor IXa EGF2−protease domain fragment (PDB code: 1RFN) were performed using Autodock 4.2 and Autodock Tools 1.5.4 rev21 as described using Autodock's Lamarckian genetic algorithm (*32*). The 1RFN receptor used for docking simulations was reduced to a 20 Å sphere centered in the exosite which extends from R170 in the 162−170 α-helix to at least R233 in the C-terminus α-helix and is defined by the residues K126, N129, K132, R165, N178, and R233. A heparin pentasaccharide with the sequence 4dIdoA(2S)-(1−4)GlcNS(6S)-(1−4)IdoA-(2S)-(1−4)GlcNS(6S)-(1−4)IdoA(2S) was extracted from the crystal structure of annexin A2 complexed with heparin (PDB code: 2HYV). The pentasaccharide had 32 active torsions during the docking runs, a charge of −3, and an initial binding pose centered in the heparin-binding exosite. The grid box of size 60 × 50 × 50 (*x*, *y*, *z* points), defined with a constant grid point spacing of 0.375 Å, was centered around the pentasaccharide. The docking was subjected to 200 search runs, using a population of 200 individuals and 10 million energy evaluations.

RESULTS

Recombinant Factor IXa Proteins. The interaction of factor IXa with periodated, supersulfated (ssLMWH) and unmodified LMWH was examined in solution binding and enzymatic assays employing the free protease, factor IXa−PL, and intrinsic tenase complexes. A series of recombinant factor IXa proteins containing alanine substitutions for conserved basic and neutral residues in the serine protease domain including K126, N129, K132, R165, R170, N178, and R233 (chymotrysinogen numbering) were chosen based on their proximity or contribution to the predominant electropositive surface on the protease domain. Recombinant factor IX proteins were expressed in HEK 293 cells, purified from conditioned media, activated, and characterized as described previously (*12*).

Affinity of Factor IXa for Fluoresceinated LMWH. A soluble binding assay was used to measure the relative heparin affinity of the recombinant factor IXa mutants for Fl-ssLMWH.

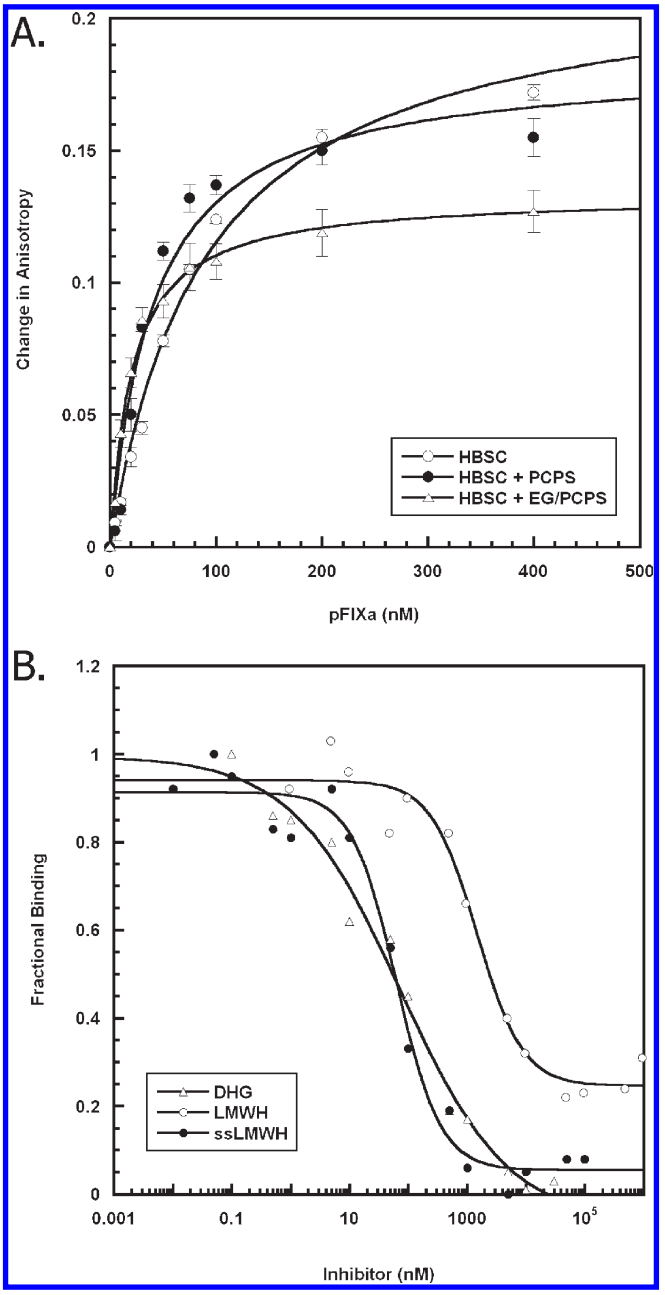

FIGURE 1: Plasma-derived factor IXa binding to Fl-ssLMWH. (A) Binding curves were constructed by expressing the observed fluorescence anisotropy after subtraction of baseline anisotropy for 0.2 nM Fl-ssLMWH in the presence of 0−500 nM plasma-derived factor IXa in either HBSC alone, HBSC + 50 μM PL vesicles, or HBSC + 30% ethylene glycol and 50 μM PL vesicles. Mean data for $n = 4-7$ experiments are presented. (B) Specificity of the binding response was examined by competition with DHG, ssLMWH, and LMWH. DHG, ssLMWH, or LMWH was titrated into assays containing 75 nM plasma factor IXa and 0.2 nM Fl-ssLMWH. The change in anisotropy at each concentration was divided by the maximal change in anisotropy (in the absence of competitor) to obtain the fractional binding value.

This highly sulfated LMWH is 32-fold more potent than unmodified LMWH in the inhibition of factor X activation by the intrinsic tenase complex (*5*). Titration of 0.2 nM Fl-ssLMWH with 0−500 nM plasma-derived factor IXa (pFIXa) in HBSC demonstrated a saturable binding curve with a 6-fold increase in anisotropy (Figure 1A). These data were fit by nonlinear regression to a single site binding model to determine the apparent K_d of 91 ± 6 nM (±SEM, $n = 7$). The specificity of this binding

signal was examined by competition with unlabeled ssLMWH, LMWH, and DHG. Titration of reactions containing 75 nM plasma-derived factor IXa and 0.2 nM Fl-ssLMWH with 0−1000 μM ssLMWH or LMWH resulted in complete or partial competition of the binding signal, respectively, with EC_{50} values of 60 ± 10 or 1000 ± 100 nM (±SEM, n = 3), respectively (Figure 1B). Under these conditions, the $EC_{50} \sim K_I$ for the competing heparin derivatives, suggesting the affinity of ssLMWH for factor IXa was ~17-fold higher than unmodified LMWH, consistent with previous work (*5*). Furthermore, LMWH incompletely (~75%) competed this binding signal, suggesting that ssLMWH interacts with a partial overlap on the protease surface. The higher affinity of ssLMWH for factor IXa is consistent with previous reports (*5*). Depolymerized holothurian glycosaminoglycan (DHG), a fucosylated chondroitin sulfate that inhibits factor X activation by the intrinsic tenase complex in both purified and plasma-based systems, also completely competed the Fl-ssLMWH binding signal with an EC_{50} value of 100 ± 20 nM, consistent with the overlapping binding sites for DHG and ssLMWH on factor IXa (*2*, *4*).

Effect of Phospholipid and Ethylene Glycol on Factor IXa−ssLMWH Affinity. To evaluate the factor IXa−ssLMWH interaction under the conditions used in the enzymatic assays, the effect of 50 μM PL vesicles and 30% ethylene glycol on apparent heparin affinity was examined (Figure 1A). PS-containing PL surface enhances factor X activation by factor IXa alone or in the factor IXa−factor VIIIa complex (*33*, *34*). In the presence of 50 μM PL vesicles, the factor IXa−Fl-ssLMWH interaction demonstrated an apparent K_d of 41 ± 9 nM, a 2-fold reduction from the baseline value. In the absence of cofactor, addition of 30% ethylene glycol significantly enhances the low basal activity of factor IXa−PL toward both chromogenic substrates and factor X (*29*, *30*). In the presence of both 30% ethylene glycol and 50 μM PL vesicles, the plasma-derived factor IXa−Fl-ssLMWH interaction demonstrated an apparent K_d of 21 ± 2 nM (±SEM, n = 4), a 4-fold increase in affinity.

Effect of Factor IXa Mutations on Protease−LMWH Affinity. The fluorescence-based heparin-binding assay was similarly used to assess the effect of the alanine substitutions on the affinity of recombinant factor IXa for Fl-ssLMWH. Binding curves were constructed as described for plasma-derived FIXa (Figure 2), and the data were fit by nonlinear regression to a single site binding model to determine the apparent K_d for each recombinant factor IXa (Table 1). The apparent K_d for wild-type factor IXa was 22 ± 1 nM. Factor IXa K126A, K132A, and R233A demonstrated severely reduced affinity for Fl-ssLMWH, with apparent K_d values 10−20-fold higher than wild-type protease (apparent K_d values for K126A, K132A, and R233A were not statistically different). Factor IXa N129A and R165A demonstrated moderately reduced affinity for Fl-ssLMWH, with apparent K_d values 5-fold higher than the wild-type protease. Factor IXa N178A demonstrated similar affinity to wild-type protease for Fl-ssLMWH. Factor IXa R170A uniquely demonstrated a 2-fold *greater* affinity for Fl-ssLMWH than factor IXa wild type. Thus, the relative affinities of the factor IXa mutants for Fl-ssLMWH ranked as follows: R170A > (wild type, N178A) > (N129A, R165A) > (K126A, K132A, R233A).

Modeling the Interaction between Human Factor IXa and a Heparin Pentasaccharide. Autodock 4.2 was used to model the docking of a heparin pentasaccharide, 4dIdoA(2S)-(1−4)GlcNS(6S)-(1−4)IdoA(2S)-(1−4)GlcNS(6S)-(1−4)IdoA(2S) (structure extracted from PDB: 2HYV), to the heparin-binding exosite of human factor IXa EGF2−protease domain (PDB

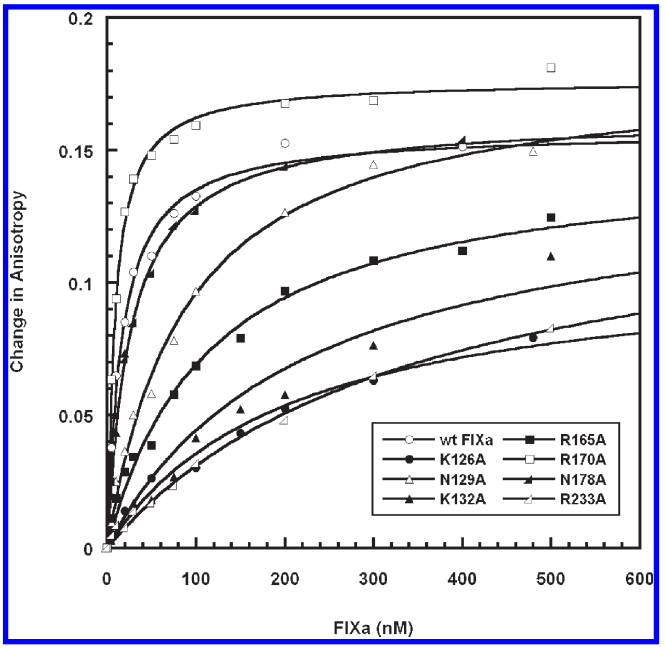

FIGURE 2: Recombinant factor IXa binding to Fl-ssLMWH. Binding curves were constructed by expressing the observed fluorescence anisotropy after subtraction of baseline anisotropy for 0.2 nM Fl-ssLMWH in HBSC in the presence of 0−500 nM recombinant factor IXa. Representative data are presented.

Table 1: Effect of Factor IXa Mutations on Affinity for ssLMWH[a]

factor IXa	app K_d (nM)	fold increase
wild type	22 ± 3	1
K126A	229 ± 40	11
N129A	98 ± 7	5
K132A	431 ± 75	20
R165A	103 ± 7.0	5
R170A	10 ± 2	0.5
N178A	29 ± 4	1
R233A	293 ± 46	14

[a]The interaction of 0−500 nM recombinant factor IXa with 0.2 nM Fl-ssLMWH was detected by change in anisotropy. Data are presented as the mean apparent K_d ± SEM (n = 4−5), and fold increase was determined by comparison to wild-type protease.

structure: 1RFN). Due to the large size of the ligand and the 32 active torsions, no single docking was identified. A total of 129 different dockings were observed overall in the 200 docking runs. The docking presented in Figure 3 is the best of nine dockings in the lowest energy conformational cluster. It had an estimated free energy of binding equal to −4.34 kcal/mol.. Hydrogen bonds were predicted between the heparin pentasaccharide and residues K126, N129, K132, K230, R233, and R165 of factor IXa. The IdoA(2S) on the nonreducing end of the pentasaccharide formed hydrogen bonds (H-bonds) via its 3-OH, 4-OH, 6-CO_2, and 2-OSO_3 moieties with the ε-NH_3 and α-NH_2 of K126, the γ-NH_2 of N129, and the ε-NH_3 of K132. The 6-OSO_3 moiety of the second saccharide of the chain, GlcNS(6S), formed H-bonds with the δ-NH and ε-NH_2 of R233. The 6-CO_2 of the third saccharide, IdoA(2S), formed H-bonds with the ε-NH3 of K230, while the 6-OSO_3 of the fourth saccharide, GlcNS(6S), H-bonded with the ε-NH_2 of R165 and K230. Therefore, the sulfate groups on the pentasaccharide were responsible for the primary interactions between the pentasaccharide and the amino groups of the electropositive residues of the heparin-binding exosite.

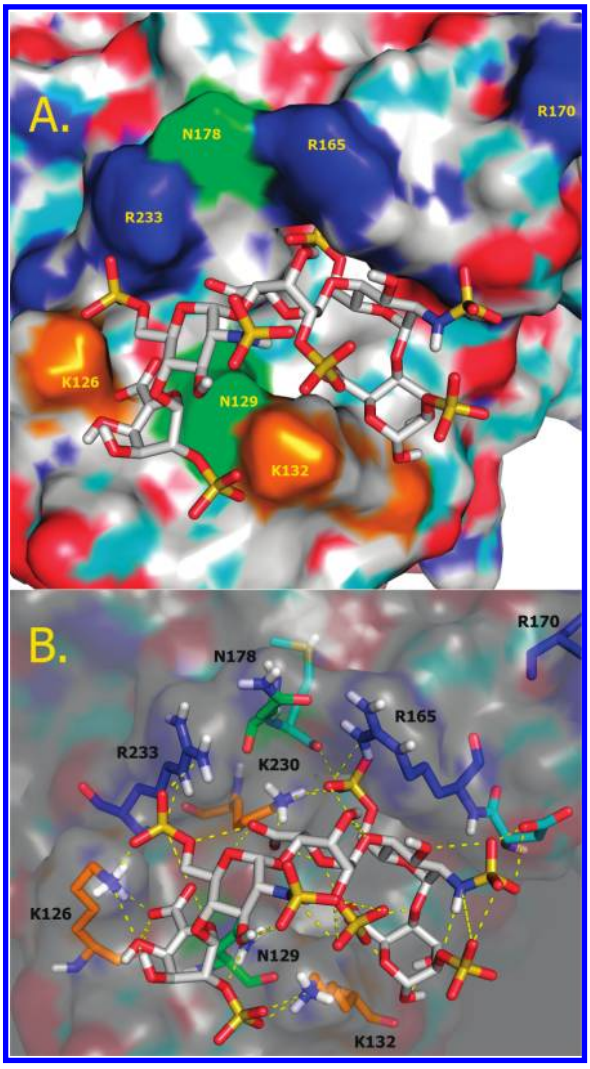

FIGURE 3: Preferred binding of a heparin pentasaccharide with the human factor IXa−EGF2 protease fragment. Autodock 4 was used to model the docking of a heparin pentasaccharide (extracted from PDB code: 2HYV) to the heparin-binding exosite of human factor IXa (PDB code: 1RFN). (A) Surface model of human factor IXa protease domain is shown with surface residues labeled. The pentasaccharide is shown as a stick figure. (B) Predicted hydrogen bonds between human factor IXa residues and the heparin pentasaccharide are shown as yellow dashed lines. The side chains of selected factor IXa residues and the heparin pentasaccharide are illustrated as stick figures.

Effect of ssLMWH on Chromogenic Substrate Hydrolysis by the Factor IXa−PL Complex. The effect of increasing concentrations of ssLMWH on the ability of 80 nM plasma-derived factor IXa to cleave the peptide substrate Pefachrome IXa (CH_3O_2-D-CHG-Gly-Arg-pNA) was examined in the presence of 30% ethylene glycol. No inhibition of activity was observed upon addition of 0−32 μM ssLMWH or LMWH (data not shown).

Effect of LMWH Derivatives on Factor X Activation by the Factor IXa−PL Complex. The effect of increasing concentrations of the LMWH derivatives on factor X activation by the factor IXa−PL complex was examined in the presence of 30% ethylene glycol to enhance protease activity (Figure 4) (*29*). The apparent K_i values were determined by fitting the data to the equation for partial, noncompetitive inhibition (Table 2). The ssLMWH demonstrated 90% inhibition of factor X activation by factor IXa wild type with an apparent K_i of 3.0 ± 0.2 nM.

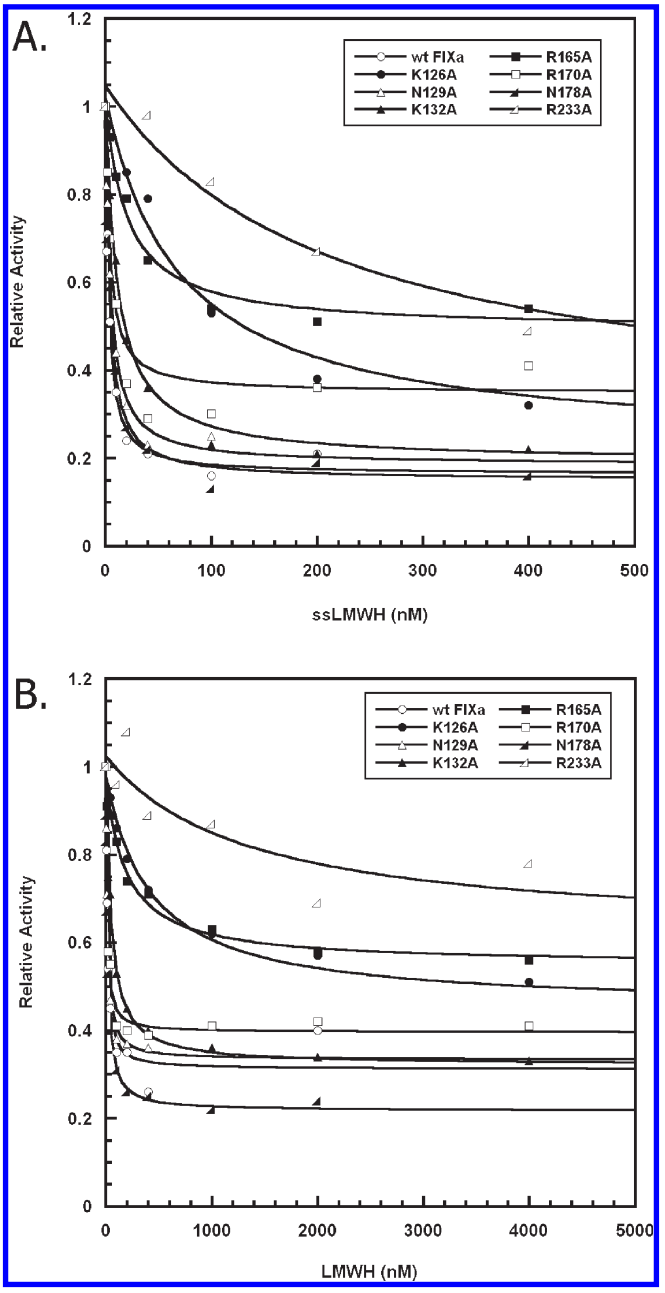

FIGURE 4: LMWH inhibition of factor X activation by the factor IXa−PL complex. The inhibition of factor IXa−PL activity by ssLMWH and LMWH was evaluated in a reaction containing 5 nM recombinant factor IXa, 150 nM factor X, 50 μM PL vesicles, 30% ethylene glycol, and 0−40 μM ssLMWH or LMWH. Representative data are presented. Representative starting activity was 0.75, 0.43, 0.81, 0.49, 0.31, 0.65, 0.45, and 0.39 nM FXa/min for wild-type, K126A, N129A, K132A, R165A, R170A, N178A, and R233A FIXa, respectively.

LMWH demonstrated ~80% inhibition of factor IXa wild-type activity with an apparent K_i of 12.0 ± 2.0 nM, consistent with previous results in the absence of 30% ethylene glycol (*2*).

Effect of Factor IXa Mutations on Inhibition of Factor IXa−PL Activity by LMWH Derivatives. The effect of alanine substitutions in the factor IXa protease domain on the ability of ssLMWH and LMWH to inhibit factor X activation by the factor IXa−PL complex was compared to wild-type protease (Figure 4). Factor IXa R233A and K126A were highly resistant to inhibition by both ssLMWH and LMWH, with a 25-fold or greater increase in apparent K_i relative to factor IXa wild type.

Table 2: LMWH Inhibition of Factor X Activation by the Factor IXa−PL Complex[a]

factor IXa	ssLMWH K_i (nM)	fold increase	LMWH K_i (nM)	fold increase
wild type	3.0 ± 0.2	1	12.0 ± 2.0	1
K126A	72 ± 4	24	320 ± 50	26
N129A	4.2 ± 0.4	1	10 ± 1	1
K132A	10 ± 1	3	49 ± 1	4
R165A	17 ± 2	6	160 ± 20	13
R170A	2.5 ± 0.8	1	12 ± 3	1
N178A	3.9 ± 0.3	1	14.1 ± 0.5	1
R233A	170 ± 30	56	1470 ± 230	120

[a]Inhibition of factor IXa−PL activity by ssLMWH and LMWH was evaluated in a reaction containing 5 nM recombinant factor IXa, 150 nM factor X, 50 μM PL vesicles, 30% ethylene glycol, and 0−10 μM ssLMWH or 0−20 μM LMWH. Data are presented as the mean apparent K_i ± SEM (n = 3−4), and fold increase was determined by comparison to wild-type protease.

Factor IXa R233A was significantly more resistant to inhibition than K126A. Factor IXa K132A and R165A were moderately resistant to inhibition with a 3−13-fold increase in apparent K_i relative to wild-type factor IXa, while factor IXa wild type, N129A, R170A, and N178A demonstrated similar apparent K_i values. The relative potency for inhibition of the recombinant proteases by ssLMWH in the factor IXa−PL complex ranked as follows: (wild type, N129A, R170A, N178A) > (K132A, R165A) > (K126A) > (R233A). Inhibition of the mutant factor IXa−PL complexes by LMWH follows the same trend as ssLMWH inhibition, but the apparent K_i values were significantly greater (3−10-fold) in all cases (Table 2).

Effect of LMWH Derivatives on Factor X Activation by the Intrinsic Tenase Complex. The effect of increasing concentrations of the LMWH derivatives on factor X activation by the intrinsic tenase complex (factor IXa−factor VIIIa) was examined (Figure 5). The rate of factor Xa generation was plotted versus LMWH concentration, and the data were fit by nonlinear regression to the equation for partial, noncompetitive inhibition to determine the apparent K_i as previously described (Table 3). ssLMWH demonstrated complete inhibition of factor IXa wild-type activity in the intrinsic tenase complex, with an apparent K_i of 11.0 ± 1.0 nM. In contrast, LMWH demonstrated partial inhibition (~90%) of the wild-type protease with a 45-fold decrease in inhibitor potency (apparent K_i = 490 ± 70 nM) relative to ssLMWH.

Effect of Factor IXa Mutations on Inhibition of Intrinsic Tenase Activity by LMWH Derivatives. The effect of alanine substitutions in the factor IXa protease domain on the ability of ssLMWH and LMWH to inhibit factor X activation by the intrinsic tenase complex was compared to wild-type protease (Figure 5). Factor IXa K126A and R233A were highly resistant to inhibition by both LMWH forms in the intrinsic tenase complex, with a 30-fold or greater increase in apparent K_i relative to wild-type protease. Factor IXa K132A, R165A, and notably R170A were moderately resistant to inhibition in the intrinsic tenase complexes with a 4−9-fold increase in K_i relative to factor IXa wild type. Factor IXa N129A and N178A had heparin sensitivities similar to the factor IXa wild type in the intrinsic tenase complex. Thus, the relative potency of ssLMWH inhibition for the recombinant proteases within the intrinsic tenase complex ranked as follows: wild type > (N129A, N178A) > (K132A, R165A, R170A) > (K126A, R233A). The relative potency of LMWH for each of the recombinant proteases within the intrinsic tenase complex ranked similarly; however, the apparent K_i values were > 30-fold higher for LMWH in all cases (Table 3).

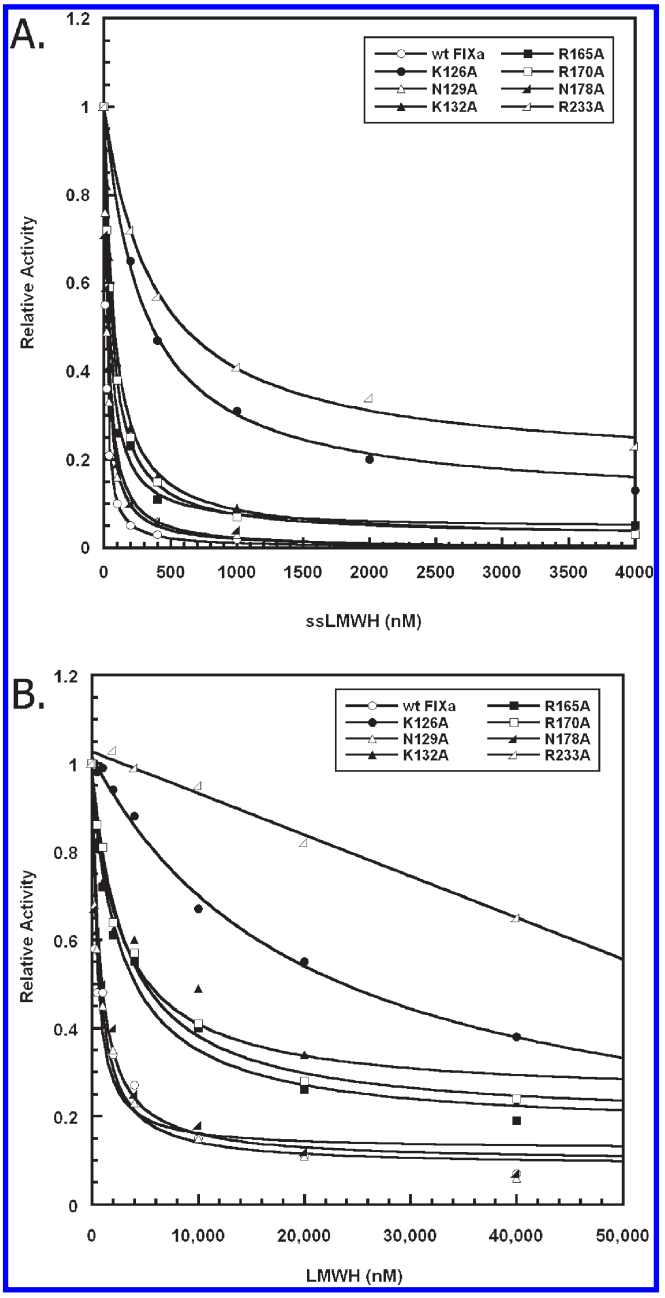

FIGURE 5: LMWH inhibition of factor X activation by the intrinsic tenase complex. Inhibition of intrinsic tenase activity by ssLMWH and LMWH was evaluated in a reaction containing 0.2 nM recombinant factor IXa, 1 nM thrombin-activated factor VIIIa, 200 nM factor X, 50 μM PL vesicles, and 0−40 μM ssLMWH or LMWH. Representative data are presented. Representative starting activity was 14.0, 7.1, 11.4, 7.2, 6.7, 28.4, 12.1, and 5.7 nM FXa/min for wild-type, K126A, N129A, K132A, R165A, R170A, N178A, and R233A FIXa, respectively.

Effect of Factor VIIIa Stability on LMWH Inhibition of Factor X Activation by the Intrinsic Tenase Complex. Recombinant factor VIIIa is intrinsically unstable due to dissociation of the A2 subunit (*35*). To assess the contribution of accelerated A2 domain dissociation to the inhibition mechanism for LMWH, the recombinant B-domainless factor VIIIa D519V/E665V was compared to wild-type protease (*20*). Factor VIIIa D519V/E665V demonstrates enhanced stability as judged by four parameters (factor VIII thermal and chemical stability, factor

Table 3: LMWH Inhibition of Factor X Activation by the Intrinsic Tenase Complex[a]

factor IXa	ssLMWH K_i (nM)	fold increase	LMWH K_i (nM)	fold increase
wild type	11 ± 1	1	490 ± 70	1
K126A	320 ± 50	29	15500 ± 2800	32
N129A	20 ± 1	2	680 ± 10	1
K132A	74 ± 5	7	4160 ± 1010	8
R165A	39 ± 3	4	3690 ± 630	8
R170A	61 ± 7	6	4460 ± 460	9
N178A	25 ± 2	2	680 ± 80	1
R233A	385 ± 9	35	> 40000	> 80

[a]Inhibition of the intrinsic tenase activity by ssLMWH and LMWH was evaluated for each factor IXa mutant in a reaction containing 0.2 nM recombinant factor IXa (20 nM for factor IXa R165A only), thrombin-activated 1 nM factor VIIIa, 200 nM factor X, 50 μM PL vesicles, and 0−20 μM ssLMWH or 0−40 μM LMWH. Data are presented as the mean apparent K_i ± SEM (n = 3−5), and fold increase was determined by comparison to wild-type protease.

VIIIa stability, and thrombin generation capacity) due to the removal of detrimental interdomain interactions at the interface of the A2 domain with A1 (D519) or A3 (E665) domains. The apparent K_i was 410 ± 20 nM LMWH using stable D519V/E665V B-domainless factor VIIIa versus 320 ± 20 nM LMWH for B-domainless factor VIIIa wild-type control. Since the potency of inhibition by LMWH derivatives was similar whether the stable or unstable form of factor VIIIa was employed, factor VIIIa stability does not play a large role in heparin inhibition of the intrinsic tenase.

DISCUSSION

Heparin affinity was determined for a series of alanine substitutions in the factor IXa protease domain via a soluble binding assay that utilizes a fluoresceinated supersulfated LMWH (Fl-ssLMWH) (*5*). The binding assay revealed that the mutations K126A, K132A, and R233A dramatically (10−20-fold) reduced factor IXa heparin affinity, while the mutations N129A and R165A moderately (5-fold) reduced affinity (Table 1). These results suggest that the core heparin-binding site is located in the groove between R233 in the C-terminus α-helix and residues K126 and K132 in the c126−132 α-helix, with additional contributions from N129 and the R165 side chain (c164−170 α-helix). The significant contribution of the R233 side chain to heparin affinity is in agreement with previous reports analyzing the interaction of factor IXa with immobilized heparin (*3*, *36*). In contrast to studies employing immobilized heparin, our solution binding assays demonstrated that the side chains of K132 and K126 dominated the interaction relative to R165 and that R170 did not significantly contribute to the binding of ssLMWH (*3*, *36*). Modeling the interaction of a heparin pentasaccharide with this protease exosite using Autodock 4 revealed a preferred binding mode consistent with the binding and inhibition data (Figure 3). Hydrogen bonding between the sulfate and carboxylate oxygens of heparin and the amino groups of K126, N129, K132, R165, and R233 was predicted. The model also predicted that residue K230, located in the bottom of the ligand-binding pocket, participates in heparin binding. This prediction agrees with the previous report that K230 contributed to Gla-domainless factor IXa binding to immobilized heparin (*36*). This pocket likely represents the minimal size of the heparin-binding site. LMWH, with an average molecular weight of 5000, is much longer than the pentasaccharide and may also bind to adjacent residues. The binding of heparin oligosaccharide in this manner appears well positioned to block important cofactor interactions with the c164−170 α-helix, including the critical interaction between the factor VIIIa A2 domain and the side chain of R165 (*12*).

Similar to other heparin oligosaccharides, ssLMWH demonstrated the ability to directly inhibit factor X activation by the factor IXa−PL complex activated with ethylene glycol. The ssLMWH did not significantly affect the rate of *p*-nitroanilide cleavage from the substrate, CH_3SO_2-D-CHG-Gly-Arg-pNA, consistent with a lack of oligosaccharide effect on the S3−S1 subsites. In contrast, factor X activation by the factor IXa−PL complex was inhibited by ssLMWH with an apparent affinity (K_I) approximately 7-fold greater than the wild-type protease−heparin interaction in solution. Addition of PL vesicles ± ethylene glycol increased protease−heparin affinity 2−4-fold (Figure 1), partially explaining the increased affinity within the enzyme complex. In principle, factor X may also contribute to the apparent increased affinity via direct oligosaccharide binding or indirectly via effects on protease conformation. Since the affinity of the factor Xa−ssLMWH interaction appears almost 5-fold less than the factor IXa−ssLMWH interaction (*5*), a conformational effect on the protease appears more likely. Alanine substitutions within the protease exosite resulted in increased resistance to inhibition of factor X activation by the LMWH derivatives (Table 2), but their specific contributions to the inhibition were significantly different from the results of protease−ssLMWH binding in solution. In particular, K132 and N129 demonstrated a significantly reduced contribution to the ssLMWH interaction within the factor IXa−PL complex, while R233 continued to make a dominant contribution. The effect of alanine substitutions for R233 and K126 was relatively greater in the enzyme complex compared to protease−LMWH affinity in solution, while the effect of R165 was relatively consistent. The enhanced overall affinity for ssLMWH and reduced contribution of the c129−132 α-helix to the interaction within the factor IXa−PL complex suggests that the heparin-binding exosite undergoes a significant conformational change upon incorporation into the membrane-bound enzyme complex.

LMWH derivatives demonstrated significantly lower apparent affinity for factor IXa within the intrinsic tenase versus the factor IXa−PL complex. Factor IXa wild type demonstrated K_I values nearly 4-fold higher for ssLMWH in the intrinsic tenase compared to the factor IXa−PL complex. With the notable exception of R170A (see below), this relationship was consistent for the remainder of factor IXa mutants which displayed K_I values 2−7-fold higher for ssLMWH in the intrinsic tenase relative to the factor IXa−PL complex (Tables 2 and 3). These results are consistent with the conclusion that LMWH derivatives and cofactor compete for binding to the factor IXa protease domain (*2*). Factor IXa R170, similar to wild-type protease with regard to affinity for ssLMWH in solution and inhibitor potency in the factor IXa−PL complex, demonstrated moderate resistance to inhibition by ssLMWH in the intrinsic tenase complex. The relative increase in K_I values between the factor IXa−PL and intrinsic tenase complexes for factor IXa R170A was significantly larger than the other mutant proteases: over 24-fold for ssLMWH. Thus, the effect of the R170A mutation on apparent heparin affinity was cofactor specific. This result is consistent with the 4-fold increase in factor IXa−cofactor affinity reported for this protease, enhancing the ability of cofactor to compete with LMWH for binding to the protease domain (*3*). In summary, LMWH derivatives interact

with the factor IXa−PL and intrinsic tenase complexes in a similar fashion, and the presence of cofactor reduces factor IXa−LMWH affinity based on competition for overlapping binding sites on the protease domain.

In principle, inhibition of the intrinsic tenase complex by heparin oligosaccharide may result from disruption of the factor IXa−cofactor interaction, destabilization of the cofactor within the enzyme complex (loss of the A2 domain), or allosteric modulation of factor IXa proteolytic activity. To address the contribution of cofactor destabilization, the ability of LMWH derivatives to inhibit intrinsic tenase activity was examined in the presence of a B-domainless factor VIIIa containing mutations that stabilize the A2 domain within the activated cofactor (*20*, *37*, *38*). The stabilized cofactor had only a minor effect on inhibitor potency relative to wild-type protein, suggesting that cofactor destabilization does not contribute significantly to this inhibition mechanism. Similarly, an allosteric effect of heparin oligosaccharide on factor X catalysis may contribute to the inhibition (based on results with the factor IXa−PL complex); however, the relative contribution of this mechanism is likely to be dwarfed by the effect of disrupting the cofactor−protease domain interaction.

These results provide a detailed view of the molecular target for antithrombin-independent inhibition of the intrinsic tenase complex by heparin oligosaccharides. A recent crystal structure of the complex between the human factor IXa protease−EGF2 fragment and pentasaccharide-activated antithrombin III contains an additional pentasaccharide−factor IXa interaction that is located on the periphery of the binding site identified by our results, utilizing some but not all of the implicated residues (*39*). Intermolecular contacts were observed for K126, R165, and R233 as predicted, but not N129 or, more importantly, K132, which had profound effects on heparin affinity when mutated to alanine. On the contrary, crystal contacts were observed for N178, which did not affect heparin binding or inhibition when mutated to alanine. While our results suggest that this interaction does not represent the favored binding mode of a pentasaccharide in solution, the complete extent of the heparin-binding site is not addressed in our studies and potentially includes the region identified in the crystal structure. Additionally, a degree of flexibility in the protease−heparin interaction is suggested by the differences observed between the binding of free factor IXa in solution versus the factor IXa−PL complex. These results suggest that the heparin-binding exosite undergoes a conformational change within the enzyme complex.

The factor IXa−factor VIIIa complex represents an extensive, high-affinity interaction that involves nearly the full length of the protease (Figure 6) (*17*). Interaction between the factor IXa EGF domains and the factor VIIIa light chain (A3-C1-C2 domains) contributes substantially to the overall affinity but completely lacks cofactor activity (*16*, *17*). In contrast, the interaction between the protease domain and the isolated factor VIIIa A2 domain is relatively low affinity but required for cofactor activity (*17*). Analysis of alanine substitutions within the protease domain heparin-binding exosite suggests an extensive cofactor interactive site that includes contributions from K126, N129, K132, R165, and R233 (*3*, *12*). Our current results demonstrate substantial overlap between the heparin and cofactor binding sites on the protease domain, which includes residues critical to the A2 domain interaction (Figure 6) (*12*, *17*). Mutagenesis of this protease exosite modulates cofactor affinity, with dramatic effects on recombinant factor IX phenotype in the setting of

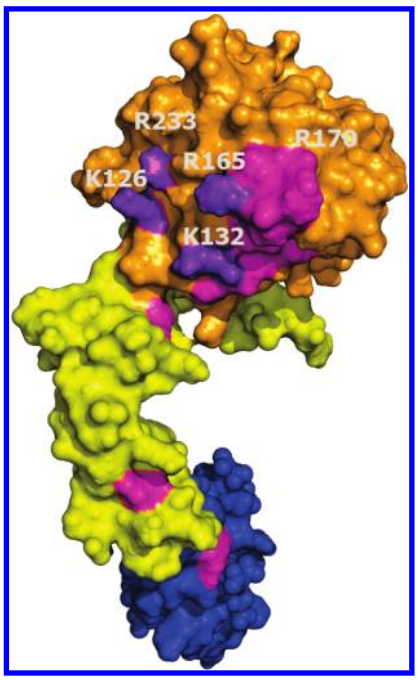

FIGURE 6: Comparison of the factor VIIIa and heparin-binding sites on factor IXa. A surface model of porcine factor IXa (PDB code: 1PFX) is shown with the heparin-binding site (purple regions, including K126, K132, R165, and R233) and previously identified factor VIIIa binding sites (magenta regions, plus K132 and R165) indicated. The factor IXa protease domain is in orange, the EGF domains are in yellow, and the Gla domain is in blue.

tissue factor stimulated thrombin generation in human plasma and venous thrombosis following saphenous vein injury in the hemophilia B mouse (*18*). Allosteric communication between this protease exosite and the catalytic machinery of factor IXa is suggested by the role of the R165 side chain in cofactor-induced protease activation and the ability of LMWH to both inhibit factor X activation by the factor IXa−PL complex and modify accessibility of the factor IXa active site to bovine pancreatic trypsin inhibitor (*9*, *12*, *40*). Thus, the critical but unstable interaction between this protease domain exosite and the cofactor A2 domain represents a highly vulnerable protein−protein interaction that may be therapeutically exploited.

ACKNOWLEDGMENT

We thank Darrell Stafford for providing the human factor IX cDNA and factor IX wild-type cell line; Jeffrey Weitz for providing fluoresceinated and unlabeled supersulfated low molecular weight heparin (ssLMWH); Phil Fay for providing the recombinant B-domainless factor VIII wild type and D519V/E665V proteins; Andreas Mueller-Beckhaus of the Bayer Corp. for providing recombinant factor VIII (Kogenate FS); Kazuhisa Minamiguchi of Taiho Pharmaceuticals for providing DHG; Ken Satyshur for assistance with Autodock; and Erik Walke for assistance with cell culture and protein purification.

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