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The Heparin-Binding Exosite Is Critical to Allosteric Activation of Factor IXa in the Intrinsic Tenase Complex: The Role of Arginine 165 and Factor X[†]

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ABSTRACT: Heparin inhibits the intrinsic tenase complex (factor IXa−factor VIIIa) via interaction with a factor IXa exosite. To define the role of this exosite, human factor IXa with alanine substituted for conserved surface residues (R126, N129, K132, R165, N178) was characterized. Chromogenic substrate hydrolysis by the mutant proteases was reduced 20−30% relative to factor IXa wild type. Coagulant activity was moderately (N129A, K132A, K126A) or dramatically (R165A) reduced relative to factor IXa wild type. Kinetic analysis demonstrated a marked reduction in apparent cofactor affinity (23-fold) for factor IXa R165, and an inability to stabilize cofactor activity. Factor IXa K126A, N129A, and K132A demonstrated modest reductions (∼2-fold) in apparent cofactor affinity, and accelerated decay of intrinsic tenase activity. In the absence of factor VIIIa, factor IXa N178A and R165A demonstrated a defective $V_{max(app)}$ for factor X activation. In the presence of factor VIIIa, $V_{max(app)}$ varied in proportion to the predicted factor IXa−factor VIIIa concentration. However, factor IXa R165A had a 65% reduction in the k_{cat} for factor X, suggesting an additional effect on catalysis. The ability of factor IXa to compete for physical assembly into the intrinsic tenase complex was enhanced by EGR-chloromethylketone bound to the factor IXa active site or addition of factor X, and reduced by selected mutations in the heparin-binding exosite (N178A, K126A, R165A). These results suggest that the factor IXa heparin-binding exosite participates in both cofactor binding and protease activation, and cofactor affinity is linked to active site conformation and factor X interaction during enzyme assembly.

In vitro and *ex vivo* modeling of the coagulation cascade indicates that factor X activation by the intrinsic tenase complex (factor IXa−factor VIIIa) is the rate-limiting step for thrombin generation (*1*, *2*). The protease factor IXa is poorly reactive with both substrates and inhibitors in the basal state, but undergoes a dramatic 10^6-fold enhancement in catalytic efficiency for factor X upon incorporation into the intrinsic tenase complex (*3*, *4*). The mechanism(s) for cofactor activation of factor IXa within the tenase complex remain poorly understood. Factor VIIIa is a complex multidomain protein with unstable cofactor activity due to loss of the noncovalently bound A2 domain, which is partially stabilized by formation of the protease−cofactor complex on the phospholipid surface (*5*, *6*). The factor VIIIa light chain (A3-C1-C2 domains) appears to provide the majority of the binding affinity for factor IXa, but completely lacks cofactor activity (*7*, *8*). In contrast, the factor IXa−A2 domain interaction directly modulates the catalytic activity of factor IXa, and this effect is enhanced by A1 domain, markedly increasing the k_{cat} for factor X activation (*9*). Thus, the factor IXa−A2 domain interaction appears to be the critical protein−protein interaction for cofactor enhancement of factor X activation. The isolated A2 domain binds with relatively low affinity to factor IXa, but contributes significantly to protease−cofactor affinity in the membrane-bound enzyme complex (*10*). Clustering of hemophilia B type II mutations and kinetic analysis of site-directed factor IXa mutants suggest that the residues 132−133 and 162−170 (chymotrypsinogen numbering system) are cofactor interactive sites (*11*, *12*). Furthermore, the hemophilia B mutation R165Q disrupts the interaction of factor IXa with the isolated A2 domain, suggesting that at least part of this critical protein−protein interaction involves the 162−170 helix of factor IXa (*13*).

Antithrombin-independent inhibition of intrinsic tenase activity by heparin results from oligosaccharide binding to an exosite on factor IXa, which disrupts a critical cofactor interaction without complete dissociation of the enzyme complex (*14*). Mutagenesis of full-length and Gla-domainless[1] forms of factor IXa demonstrate that heparin binds to an exosite on the factor IXa protease domain that is homologous to the heparin-binding exosites on factor Xa and

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[1] APTT, activated partial thromboplastin time; BSA, bovine serum albumin; DME, Dulbecco's modified Eagle's media; Gla, γ-carboxyglutamic acid; factor IXa-Fl-EGR, fluorescein-labeled factor IXa modified with EGR-chloromethylketone; factor IXa-EGR, factor IXa modified with EGR-chloromethylketone; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; LMWH, low molecular weight heparin; PC:PS, phosphatidylcholine:phosphatidylserine; PEG-8000, polyethylene glycol (average MW 8000); PL, phospholipids; tenase buffer, 0.15 M NaCl, 20 mM HEPES, pH 7.4, 2 mM $CaCl_2$, 1 mg/mL BSA, 0.1% PEG-8000.

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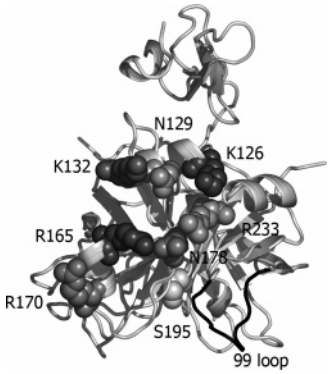

FIGURE 1: Representation of the crystal structure of human factor IXa EGF2-protease fragment. The crystal structure of the factor IXa-EGF fragment (1RFN) is represented by a ribbon diagram created with PyMOL (*47*). The EGF2 domain is positioned on top of the protease domain, with the active site oriented downward. The active site S195 (light gray) and surface residues K126, N129, K132, R165, R170, N178, and R233 (dark gray) are labeled and highlighted with a space-filling representation of their respective side chains. The 99-loop (black) is also labeled. All residues are labeled based on chymotrypsinogen numbering.

thrombin (*15*−*18*). This exosite represents the predominant electropositive surface on the protease domain, and extends from R170 in the 162−170 α-helix to at least R233 in the C-terminus α-helix (Figure 1). Factor IXa mutants with decreased heparin affinity demonstrate increased resistance to inhibition of factor X activation by heparin, thereby defining the molecular target for antithrombin-independent inhibition of the intrinsic tenase complex (*18*). Depolymerized holothurian glycosaminoglycan (DHG), a structurally unrelated glycosaminoglycan that lacks the antithrombin-dependent activities of heparin, inhibits intrinsic tenase activity by interacting with this exosite in a similar fashion (*19*). Characterization of factor IXa R233A indicates that this residue also contributes to factor VIIIa binding and stability within the intrinsic tenase complex, suggesting that the cofactor interactive site extends away from the 162−170 helix, and overlaps substantially with the heparin-binding site (*18*). These findings indicate that the heparin-binding exosite on factor IXa overlaps the critical cofactor interactive site for binding and stabilization of the factor VIIIa A2 domain.

To address more fully the role of the heparin-binding exosite in activation of factor IXa within the intrinsic tenase complex, a series of alanine substitutions were introduced into conserved basic and neutral residues surrounding the surface residue R233. These residues were chosen based on their proximity or contribution to the predominant electropositive surface on the protease domain. Kinetic and equilibrium binding analyses of the recombinant proteases were undertaken with regard to protease−cofactor affinity, cofactor stability, cofactor-independent and −dependent catalysis, and the effects of macromolecular substrate and active site modification on intrinsic tenase assembly. These results demonstrate a critical role for this exosite in cofactor binding, stabilization of factor VIIIa A2 domain, and allosteric activation of the protease. Furthermore, these results suggest that macromolecular substrate binding and active site conformation are linked to cofactor affinity during assembly of the tenase complex.

EXPERIMENTAL PROCEDURES

Materials. Human plasma-derived factor X, IXa, XIa, and thrombin were purchased from Enzyme Research (South Bend, IN). Human plasma-derived factor IXa modified at the active site with the tripeptide EGR-chloromethylketone (factor IXa-EGR) and fluorescein labeled factor IXa modified with EGR-chloromethylketone (factor IXa-Fl-EGR) were purchased from Haematologic Technologies (Essex Junction, VT). Recombinant human factor VIII (Kogenate FS) was generously provided by Andreas Mueller-Beckhaus of the Bayer Corp. (Berkeley, CA). Recombinant hirudin, bovine serum albumin, and poly-L-lysine were purchased from Sigma (St. Louis, MO). Chromogenic substrates were purchased as follows: S-2765 (*N*-α-benzyloxycarbonyl-D-Arg-Gly-Arg-pNA) from DiaPharma (Franklin, OH), and Pefachrome IXa (CH_3SO_2-D-CHG-Gly-Arg-pNA) from Centerchem, Inc. (Stamford, CT). 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 75:25:1) phospholipid vesicles (PC:PS vesicles) were prepared by extrusion through a 100 nm polycarbonate filter (*20*). The molar concentration of phospholipid was determined with an elemental phosphorus assay (*21*). FuGENE 6 was obtained from Roche Diagnostics (Indianapolis, IN). Geneticin (G418) was purchased from Gibco-BRL (Gaithersburg, MD). Affinity purified goat anti-human factor IX peroxidase conjugate was from Enzyme Research (South Bend, IN) and goat anti-human FXI was from Haematologic Technologies Inc (Burlington, VT).

Mutagenesis and Expression of Recombinant Human Factor IX. pCMV5 vector containing the human factor IX cDNA (pCMV5-FIX) and a HEK 293 cell line stably transfected with the factor IX wild type construct were generously provided by Darrel Stafford (University of North Carolina) (*22*). Alanine substitutions were introduced into the factor IX sequence by PCR-based mutagenesis using the QuickChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). The constructs FIX-K126A, FIX-N129A, FIX-K132A, FIX-R165A, and FIX-N178A (amino acid residues identified by chymotrypsinogen numbering, Figure 1) were generated using the wild type pCMV5-FIX plasmid as a template, and constructs were confirmed by full-length sequencing of the cDNA. The FIX mutant cDNAs were shuttled into the pcDNA3.1 (+) vector using *Eco*RI restriction sites prior to transfection of HEK 293 cells. Additional HEK 293 cells were transfected with mutant pcDNA3.1-FIX plasmids using FuGENE 6 lipid. Stably transfected cell lines for each mutant construct were selected by limiting dilution in the presence of 1.0 mg/mL Geneticin as described (*18*).

Purification and Activation of Recombinant Factor IX. Recombinant human factor IX was purified from conditioned media by conventional anion exchange chromatography and calcium-dependent elution from a Resource Q FPLC column (*18*). Protein concentrations were initially determined by absorbance at 280 nm using an extinction coefficient ($\epsilon_{0.1\%}$) of 1.32. Purified factor IX was activated with human factor XIa (150:1 substrate:enzyme molar ratio) at 37 °C for 1.5 h, followed by depletion of factor XIa by incubation with polyclonal anti-human factor XI antisera cross-linked to Affi-

gel beads in a spin column (*18*). Factor IXa catalytic sites were quantitated by titration with antithrombin III as described (*23*), except that the factor IXa−antithrombin−heparin incubation step was prolonged (90 min). The active protease concentrations determined by antithrombin titration (±SEM) were 18.0 ± 1.1 μM for wild type, 6.6 ± 0.5 μM for K126A, 6.6 ± 0.5 μM for N129A, 43.1 ± 1.3 μM for K132A, 18.8 ± 1.2 μM for R165A, and 5.1 ± 0.3 μM for N178A factor IXa.

Chromogenic Substrate Hydrolysis and Plasma Coagulant Activity of the Recombinant Factor IXa. The rate of hydrolysis of Pefachrome IXa was determined at room temperature in a reaction containing final concentrations of 40 nM recombinant factor IXa in tenase buffer (0.15 M NaCl, 20 mM HEPES, pH 7.4, 2 mM $CaCl_2$, 1 mg/mL BSA, 0.1% PEG-8000), 30% ethylene glycol, and 125 to 500 μM Pefachrome IXa as described (*18*). The initial rate of substrate cleavage between 125 and 500 μM Pefachrome IXa was determined, and the slope of the substrate vs velocity plot used to estimate the specificity constant (k_{cat}/K_M) of each recombinant factor IXa for the peptide substrate. Coagulant activity was determined in an activated partial thromboplastin time (APTT) with addition of recombinant factor IXa to factor IX-deficient plasma just prior to recalcification (*18*).

Kinetic Determination of the Affinity (Apparent K_D) for Factor IXa−Factor VIIIa Complex Formation. Affinity of the factor IXa_B−factor VIIIa complex was assessed by monitoring intrinsic tenase complex activity at a limiting concentration of factor VIIIa as described (*14*). Thrombin-activated factor VIII (0.15 nM) was incubated with increasing amounts of factor IXa (0−61 nM for R165A or 0−25.5 nM for wild type and the other mutants), in the presence of 50 μM PC:PS vesicles, and 200 nM factor X. Factor IXa concentration was plotted versus the rate of factor Xa generation, and the data fit by nonlinear regression to a single site binding model to determine the $K_{D(app)}$. The factor IXa−factor VIIIa complex concentration for each recombinant factor IXa under assay conditions was calculated using the experimentally determined $K_{D(app)}$ to solve the quadratic equation as described (*14*).

Determination of the Stability of the Intrinsic Tenase Complex and the in Vitro Half-Life of Factor VIIIa. The stability of the intrinsic tenase was determined in a reaction containing 1.0 nM thrombin-activated factor VIII, 0.2 nM recombinant factor IXa, and 50 μM PC:PS vesicles in tenase buffer, by varying the time of factor X (200 nM) addition between 0 and 12.25 min. The *in vitro* half-life of factor VIIIa activity was determined for cofactor alone, cofactor plus PC:PS vesicles, and cofactor plus PC:PS vesicles and factor IXa as described (*18*). Briefly, 20 nM factor VIII was activated with 40 nM thrombin for 30 s, neutralized with 60 nM hirudin, followed by a 1:2 dilution into buffer alone, buffer plus 100 μM PC:PS vesicles, or buffer plus 100 μM PC:PS vesicles and 40 nM recombinant factor IXa. Aliquots were removed from the factor VIIIa incubation mixtures over time, and diluted 25-fold into the intrinsic tenase assay containing 1.5 nM plasma-derived factor IXa, and 200 nM factor X to determine residual factor VIIIa activity.

Factor X Activation by the Factor IXa−Phospholipid and Factor IXa−Factor VIIIa Complexes. Factor X activation was determined by chromogenic assay as previously described (*14*). Factor X activation by factor IXa−phospholipid in the absence of cofactor was determined over 20 min at room temperature in a reaction containing 5 nM recombinant human factor IXa, 0−700 nM factor X, 50 μM PC:PS vesicles, and 30% ethylene glycol in tenase buffer (*18*). Factor IXa−PL and factor X were dissolved separately in 30% ethylene glycol/tenase buffer prior to addition in the enzymatic reaction to avoid incomplete mixing. For reactions performed in the absence of ethylene glycol, 40 nM factor IXa was employed. Factor X activation by the factor IXa−factor VIIIa−phospholipid complex was determined in a reaction containing 0.1 nM recombinant human factor IXa, 1.0 nM thrombin-activated factor VIII, and 50 μM PC:PS phospholipid vesicles, in tenase buffer. The reaction was initiated by addition of factor X immediately after factor VIIIa, and incubated 15 s for determination of factor X kinetics (or 30 s for other assays) at room temperature, and then terminated by addition of 0.25 M EDTA/1.1 mg mL^{-1} Polybrene. The amount of factor Xa was determined by comparing the rate of S-2765 substrate hydrolysis to a standard curve as described (*14*). Incubation times were restricted to conditions under which less than 20% total factor X cleavage occurred. Kinetic constants for factor X activation were obtained by plotting the rate of factor Xa generation (nM/min) versus substrate concentration, and fitting the data by nonlinear regression to the Michaelis−Menten equation.

Determination of Factor IXa−Factor VIIIa Binding Affinity (K_D) on the Phospholipid Surface. The binding affinity of factor IXa for cofactor in the presence and absence of factor X was examined in both direct and competition binding assays employing fluorescein-labeled factor IXa (factor IXa-Fl-EGR). All fluorescence measurements were obtained on a TECAN GENios Pro microplate reader (Salzburg, Austria), using an excitation wavelength of 485 nm, an emission wavelength of 535 nm, and a G-factor of 1.0142. Samples were prepared in Corning 96-well solid black flat bottom polystyrene microplates (Corning Life Sciences, Corning, NY) at 23 °C. Direct binding was monitored by change in anisotropy upon titration of factor IXa-Fl-EGR with increasing amounts of factor VIIIa (0−25 nM) (*24*). Recombinant human factor VIII was activated with a 1.6-fold molar excess of thrombin in 0.15 M NaCl, 20 mM HEPES pH 7.2, and 2 mM $CaCl_2$ for 30 s, and immediately added to a binding reaction containing 0.15 M NaCl, 20 mM HEPES pH 7.4, 5 mM $CaCl_2$, 50 μM PCPS vesicles, and 5 nM factor IXa-Fl-EGR, in the absence or presence of 125 nM factor X. Anisotropy values were determined in duplicate reactions, with measurements initiated 10 and 55 s after addition of factor VIIIa. Maximum anisotropy signals were 0.203 ± 1 in the presence of factor X (±SEM), and 0.163 ± 1 in the absence of factor X (±SEM). Baseline anisotropy in the absence of factor VIIIa (0.105 ± 0.5) was subtracted from each experimental value to determine the change in anisotropy. Titration of factor IXa-Fl-EGR with factor X alone produced no significant change in anisotropy, and the mean change in fluorescent intensity was less than 10% in all binding studies. The change in anisotropy was plotted versus factor VIIIa concentration, and the data were fit by nonlinear regression to a simple binding model.

Competition binding studies were performed in identical fashion by adding increasing amounts of plasma-derived factor IXa, unlabeled factor IXa-EGR, or recombinant factor

IXa (0−250 nM for R165A, or 0−100 nM for wild type and the other mutants) to a binding reaction containing 5 nM factor IXa-Fl-EGR, 8 nM factor VIIIa, and 50 μM PC:PS vesicles, in the absence or presence of 125 nM factor X. Data were plotted as the concentration of competitor factor IXa versus the fractional change in maximal anisotropy (in the absence of competitor). The maximal signal was 0.203 ± 1 (±SEM), while the baseline signal was 0.108 ± 1 (±SEM). The EC_{50} was determined by fitting the data to the equation

$$B = \frac{(EC_{50})^n}{(EC_{50})^n + I^n}$$

in which B represents the fractional specific binding, *I* represents the concentration of factor IXa used as competitor, EC_{50} represents the concentration of unlabeled factor IXa that causes a 50% reduction in the anisotropy response, and *n* represents the pseudo Hill coefficient (*25*). The pseudo Hill coefficients were approximately 0.8 for plasma-derived and wild type factor IXa, and ranged from 0.5 to 0.8 for the mutant proteases. The EC_{50} values were converted to $K_{I(app)}$ values using the method of Cheng and Prusoff (*26*) and Chou (*27*) as described in the equation

$$K_I = EC_{50}/[1 + (D/K_D)]$$

where *D* is the concentration of labeled factor IXa and K_D is 1.7 nM as determined above using the direct binding assay in the presence of factor X.

Statistical Analysis. The individual experiments were fit by nonlinear regression to determine the fitted parameters ($K_{D(app)}$, B_{max}, etc.). The parameter values obtained from the individual experiments were pooled and expressed as the mean of the fitted parameter ± the standard error of the mean (SEM). The significance of differences in the measured parameters was determined by employing the Student's *t* test. The differences stated in the discussion are all significant at the 95% confidence level.

RESULTS

Expression, Activation, and Active Site Titration of Recombinant Factor IXa. The putative heparin-binding exosite on factor IXa was targeted by substituting alanine for conserved basic (Arg, Lys) or neutral (Asn) surface residues (Figure 1). Targeted residues included K126A, N129A, and K132A in the 126−132 α-helix, R165A in the 162−170 α-helix, and N178A in the 172−178 insertion loop (chymotrypsinogen numbering system). The targeted residues surround R233, which was recently demonstrated to play a role in the heparin and cofactor interactions of factor IXa (*18*). Recombinant factor IX proteins expressed in HEK 293 cells were purified to homogeneity from conditioned media, and exhibited indistinguishable chromatographic behavior during calcium elution from anion-exchange resin (*28*, *29*). The recombinant FIX proteins demonstrated high purity by Coomassie Blue staining, with a single band of approximately 56,000 Da when analyzed by 10% SDS−PAGE under nonreducing conditions (data not shown). Recombinant factor IX proteins were activated to factor IXa by incubation with human factor XIa, and titrated with antithrombin in the presence of unfractionated heparin to determine the number of active sites present.

Table 1: Coagulant and Peptide Substrate Activity for Recombinant Factor IXa

enzyme (FIXa)	coagulant activity (%)	Pefachrome IXa k_{cat}/K_M ($mM^{-1}\,s^{-1}$)
WT	100	2.23 ± 0.06
K126A	41 ± 2	1.80 ± 0.05
N129A	88 ± 4	1.89 ± 0.03
K132A	80 ± 4	1.76 ± 0.09
R165A	1.0 ± 0.1	1.53 ± 0.32
N178A	116 ± 4	1.65 ± 0.14

The initial rate of Pefachrome IX cleavage by 40 nM factor IXa was determined in tenase buffer with 30% ethylene glycol present. Coagulant activity was determined by APTT in factor IX deficient plasma. Data are expressed as the mean (±SEM) of 3−6 experiments.

Chromogenic Substrate Cleavage and Coagulant Activity of Recombinant Factor IXa. The ability of active site-titrated recombinant factor IXa to cleave the chromogenic substrate Pefachrome IXa was examined in the presence of 30% ethylene glycol (*30*). The recombinant mutant proteases demonstrated a ∼20−30% reduction in the k_{cat}/K_M for Pefachrome IXa relative to factor IXa wild type (Table 1). These results suggest that the active site and S1−S3/4 subsites of these recombinant proteases were intact, but possessed modestly reduced catalytic efficiency for the peptide substrate.

The coagulant activity of the recombinant factor IXa was determined by APTT in factor IX deficient plasma. Factor IXa N178A demonstrated relatively normal clotting activity, while factor IXa N129A and K132A demonstrated a 10−20% decrease in coagulant activity relative to the wild type protein, and factor IXa K126A demonstrated a 60% decrease. In contrast, factor IXa R165A demonstrated a profound reduction in clotting activity (99%), suggesting that this residue has a vital role in factor IXa coagulant activity (Table 1).

Effect of Factor IXa Mutations on Apparent Factor IXa−Factor VIIIa Affinity. The ability of these recombinant proteases to interact with cofactor was assessed by kinetic determination of factor IXa−factor VIIIa affinity (Figure 2). Factor IXa wild type and N178A demonstrated similar apparent affinity ($K_{D(app)}$) for factor VIIIa, suggesting that this mutation did not disrupt factor IXa−factor VIIIa assembly on PC:PS vesicles, while the $K_{D(app)}$ of factor IXa K126A, N129A, and K132A for cofactor was increased less than 2-fold compared to wild type, suggesting that these mutations had modest effects on intrinsic tenase assembly (Table 2). This reduction in cofactor affinity is consistent with the decreased coagulant activity of these proteins. In contrast, the $K_{D(app)}$ of factor IXa R165A for factor VIIIa was markedly increased (23-fold) relative to wild type protease, suggesting that this mutation significantly disrupts assembly of a productive enzyme complex, consistent with the markedly decreased coagulant activity of this protein (Table 1).

The maximal activity of the saturated cofactor (B_{max}) was similar for factor IXa K132A relative to factor IXa wild type, suggesting that this protease was fully active in the intrinsic tenase complex. The B_{max} for factor IXa K126A, N129A, and N178A was moderately decreased (16−24%) relative

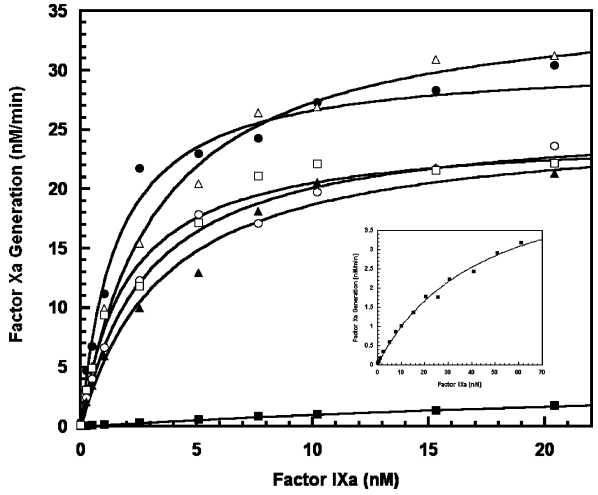

FIGURE 2: Effect of factor IXa mutations on the apparent affinity of the factor IXa−factor VIIIa complex on PC:PS vesicles. The apparent affinity ($K_{D(app)}$) of the factor IXa−factor VIIIa interaction was determined using enzymatic activity to detect complex formation. The rate of factor X activation by factor IXa−factor VIIIa was determined by adding increasing concentrations of factor IXa into a reaction containing 0.15 nM factor VIIIa, 200 nM factor X, and 50 μM PC:PS vesicles in tenase buffer as described in Experimental Procedures. Representative curves for wild type (●), K126A (○), N129A (▲), K132A (△), R165A (■), and N178A (□) factor IXa are presented. The R165A factor IXa data (■) is also presented in the inset to illustrate the goodness of the fit.

Table 2: Cofactor Interactions for Recombinant Factor IXa[a]

enzyme (FIXa)	FIXa−FVIIIa $K_{D(app)}$ (nM)	FIXa−FVIIIa B_{max} (nM/min)	FIXa−FVIIIa k_{obs} (min^{-1})	FIXa-Fl-EGR $K_{I(app)}$ (nM)
WT	1.8 ± 0.1	32.1 ± 0.7	0.132 ± 0.005	6.5 ± 0.2
K126A	3.2 ± 0.4	27.0 ± 0.5	0.163 ± 0.002	14.1 ± 0.7
N129A	2.8 ± 0.3	24.5 ± 0.8	0.142 ± 0.002	7.2 ± 1.3
K132A	3.4 ± 0.4	35.4 ± 1.3	0.157 ± 0.003	5.6 ± 0.6
R165A	41 ± 8	5.4 ± 0.8	ND	106 ± 13
N178A	1.9 ± 0.1	25.2 ± 1.0	0.132 ± 0.008	10.4 ± 0.2

[a] The $K_{D(app)}$ and B_{max} for the factor IXa−factor VIIIa complex were determined by titration of 0.15 nM factor VIIIa, 50 μM PC:PS vesicles, and 200 nM FX with factor IXa in tenase buffer. The data are presented as the mean $K_{D(app)}$ and B_{max} (±SEM), where $n = 4-6$ experiments. The rate of intrinsic tenase decay (k_{obs}) was determined by varying the time of addition of 200 nM factor X to 0.2 nM factor IXa, 1.0 nM factor VIIIa, and 50 μM PC:PS vesicles in tenase buffer. The data are presented as the mean k_{obs} (±SEM), where $n = 4-6$ experiments. The ability of recombinant factor IXa to compete for cofactor binding was assessed by monitoring the fractional change in anisotropy with increasing amounts of unlabeled protease in a reaction containing 5 nM factor IXa-Fl-EGR, 8 nM factor VIIIa, and 50 μM PC:PS vesicles. The data are presented as the mean $K_{I(app)}$ (±SEM), where $n = 3-10$ experiments. ND: not determined.

to wild type protease, consistent with reduced activity of these proteases in the assembled enzyme complex. Remarkably, factor IXa R165A demonstrated a 6-fold reduction in the B_{max} of the factor IXa−factor VIIIa interaction compared to factor IXa wild type, suggesting that the activity of this protease was markedly defective in the assembled tenase complex.

Effect of Factor IXa Mutations on the Ability To Stabilize Factor VIIIa Activity. The cofactor activity of factor VIIIa degrades in a first-order manner due to the loss of the noncovalently associated A2 domain, which is partially stabilized in the presence of factor IXa and phospholipid vesicles (*6*, *31*). To assess the effect of these mutations on

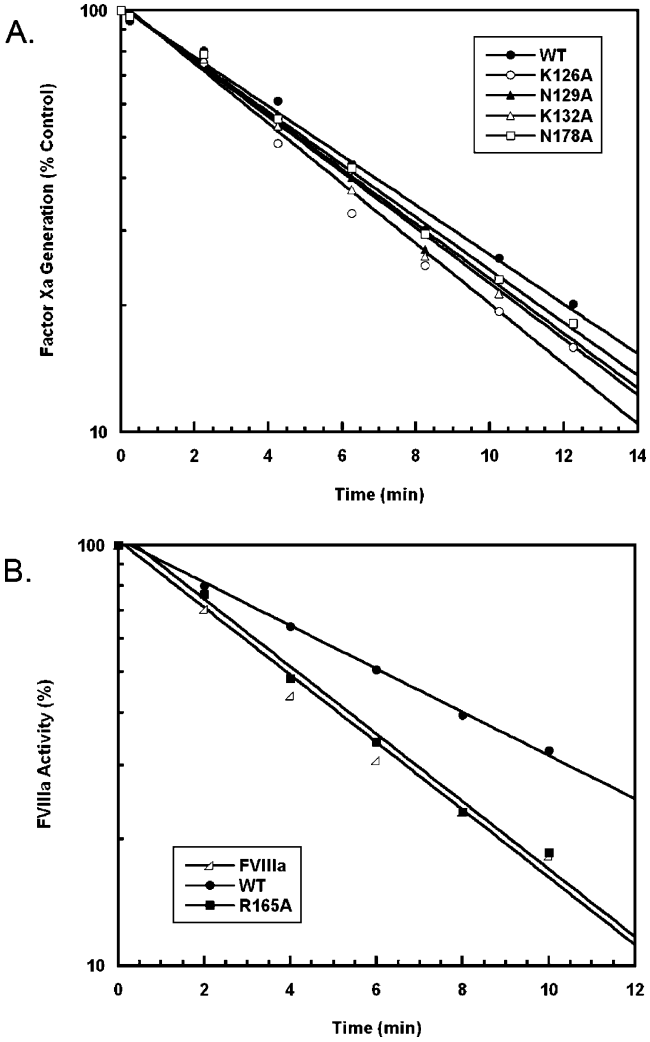

FIGURE 3: Effect of factor IXa mutations on the stability of the intrinsic tenase complex (A) and the *in vitro* half-life of factor VIIIa (B). (A) Factor X (200 nM) was added at intervals between 15 s and 12.25 min after cofactor addition to 0.2 nM recombinant factor IXa, 1 nM factor VIIIa, 50 μM PC:PS vesicles in tenase buffer. The rate of factor X activation was determined in a chromogenic assay as described in Experimental Procedures. (B) Factor VIII was activated with 40 nM thrombin, neutralized with 60 nM hirudin, and immediately diluted 1:2 into tenase reaction buffer in the absence or presence of 100 μM PC:PS vesicles and 40 nM factor IXa wild type or R165A. Residual factor VIIIa activity was determined by sampling into the intrinsic tenase assay as described in Experimental Procedures. Representative curves are presented.

stability of the factor IXa−factor VIIIa−phospholipid complex, the rate of intrinsic tenase decay was examined by varying the time of factor X addition (Figure 3A). Factor IXa wild type and N178A demonstrated similar rates of intrinsic tenase decay (k_{obs}), suggesting that this mutation does not destabilize cofactor activity within the intrinsic tenase complex (Table 2). Factor IXa K126A, N129A, and K132A demonstrated modestly increased rates of decay. The rate of decay for factor IXa R165A could not be adequately evaluated under these conditions due to the markedly reduced baseline activity of this protease. To assess the effect of this mutation, thrombin-activated factor VIII was incubated with a 2-fold molar excess of either factor IXa wild type or R165A in the presence of phospholipid vesicles, and sampled into the tenase assay over time to determine residual cofactor activity. The k_{obs} for loss of factor VIIIa activity in the

Table 3: Kinetics of Factor X Activation by Recombinant Factor IXa[a]

	FIXa−PL (30% EG) factor X activation			FIXa−FVIIIa−PL factor X activation		
enzyme (FIXa)	$K_{M(app)}$ (nM)	$V_{max(app)}$ (nM/min)	k_{cat} (s^{-1}) × 10^3	$K_{M(app)}$ (nM)	$V_{max(app)}$ (nM/min)	k_{cat} (s^{-1})
WT	55 ± 8	0.40 ± 0.01	1.34 ± 0.03	9.6 ± 0.7	11.7 ± 0.1	5.51 ± 0.06
K126A	89 ± 4	0.60 ± 0.05	1.99 ± 0.16	7.3 ± 0.4	7.3 ± 0.1	5.2 ± 0.1
N129A	61 ± 11	0.40 ± 0.01	1.34 ± 0.03	8.5 ± 2	8.7 ± 0.5	5.6 ± 0.3
K132A	48 ± 4	0.53 ± 0.01	1.78 ± 0.03	8.5 ± 0.3	7.4 ± 0.1	5.47 ± 0.09
R165A	67 ± 5	0.30 ± 0.02	1.00 ± 0.05	5.2 ± 2	0.28 ± 0.04	1.9 ± 0.3
N178A	80 ± 16	0.26 ± 0.01	0.60 ± 0.14	10.0 ± 0.5	10.2 ± 0.1	5.08 ± 0.04

[a] The kinetics of factor X activation by 5 nM factor IXa with 50 μM PC:PS vesicles was determined in tenase buffer with 30% ethylene glycol present. The kinetics of factor X activation by the intrinsic tenase complex (0.1 nM factor IXa, 1.0 nM factor VIIIa, 50 μM PC:PS) was determined in tenase buffer. The data are presented as the mean $K_{M(app)}$, $V_{max(app)}$, and k_{cat} (±SEM), where $n = 4-6$ experiments.

presence of 50 μM PC:PS vesicles was 0.173 ± 0.002 min^{-1} for cofactor alone, 0.112 ± 0.004 min^{-1} for cofactor plus 20 nM factor IXa wild type, and 0.174 ± 0.001 min^{-1} for cofactor plus 20 nM factor IXa R165A (Figure 3B). Thus, while incubation with factor IXa wild type significantly reduced the observed rate constant for A2 dissociation, factor IXa R165A failed to prolong the half-life of factor VIIIa activity relative to phospholipid vesicles alone.

Effect of Factor IXa Mutations on the Kinetics of Factor X Activation in the Absence of Factor VIIIa. The kinetics of factor X activation by these recombinant proteases was assessed in both the factor IXa−phospholipid and the factor IXa−factor VIIIa−phospholipid complexes. In the absence of cofactor, analysis of factor X kinetics is problematic due to the poor catalytic activity of factor IXa−phospholipid. Ethylene glycol stimulates factor IXa catalytic activity by interacting with a binding site between residues 60 and 90 (*32*, *33*). Thus, 30% ethylene glycol was added to the buffer to accelerate factor X activation by factor IXa under these conditions (*30*, *34*). The apparent substrate affinity ($K_{M(app)}$) for factor X under these conditions was similar to factor IXa wild type for factor IXa N129A, K132A, N178A, and R165A (Table 3). Factor IXa K126A demonstrated a modest increase in $K_{M(app)}$, suggesting that this mutation reduces the affinity of the factor IXa−factor X interaction on the phospholipid surface.

These mutations had diverse effects on the $V_{max(app)}$ for factor X activation by the factor IXa−phospholipid complex under these conditions (Figure 4). Interestingly, the $V_{max(app)}$ for factor K126A and K132A was significantly increased relative to wild type protease. Factor IXa wild type and N129A demonstrated similar maximal rates of factor X activation, while factor IXa R165A and N178A had moderately reduced rates, suggesting a catalytic defect for these proteases (Table 3). In the absence of ethylene glycol, the maximal rate of factor X activation was similar for all the recombinant proteases except factor IXa R165A and N178A, which were reduced by 70% and 50%, respectively (data not shown).

Effect of Factor IXa Mutations on the Kinetics of Factor X Activation in the Presence of Factor VIIIa. The kinetics of factor X activation was similarly examined for each recombinant protease assembled in the intrinsic tenase complex (Figure 5). In the presence of cofactor, none of the mutant proteases demonstrated an increase in the $K_{M(app)}$ for factor X relative to factor IXa wild type (Table 3). Thus, these mutations did not appear to significantly disrupt the initial interaction of the protease−cofactor complex with factor X, or protease with the factor X−factor VIIIa complex on the phospholipid surface (*35*).

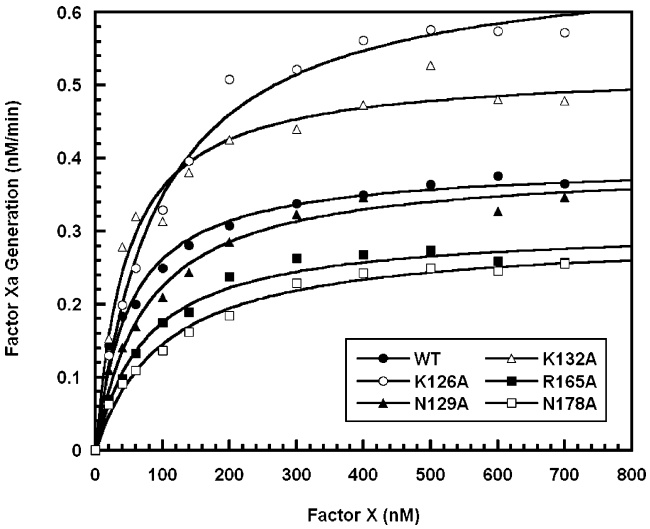

FIGURE 4: Effect of factor IXa mutations on the kinetics of factor X activation by factor IXa−phospholipid in the presence of 30% ethylene glycol. The rate of factor X activation by factor IXa was determined in reactions containing 5 nM factor IXa, 0−700 nM factor X, and 50 μM PC:PS vesicles in tenase buffer containing 30% ethylene glycol. Representative curves are presented.

In contrast, the $V_{max(app)}$ for factor X activation was decreased to varying degrees for the mutant proteases in the intrinsic tenase complex. The maximal rate of factor X activation was modestly reduced (13%) for factor IXa N178A; moderately reduced (26−38%) for factor IXa K126A, N129A, and K132A; and profoundly reduced for factor IXa R165A (98%) relative to wild type protease (Table 3). The catalytic rate (k_{cat}) for factor X activation by each recombinant protein in the intrinsic tenase complex was calculated by dividing $V_{max(app)}$ by the predicted enzyme concentration under these conditions based on the apparent affinity ($K_{D(app)}$) of the factor IXa−factor VIIIa complex (Figure 2, Table 2). Factor IXa K126A, N129A, K132A, and N178A demonstrated k_{cat} values within 92% of factor IXa wild type, suggesting the absence of a significant defect in the cofactor-dependent catalytic rate for factor X (Table 3). In contrast, the k_{cat} value for factor IXa R165A was only 35% of the wild type protein, indicating a substantial defect in factor X catalysis.

Effect of Active Site Modification and Factor X on the Affinity of Factor IXa−Factor VIIIa Interaction. To assess the effect of active site modification (chloromethylketone inhibitor) and factor X on the physical assembly of the factor

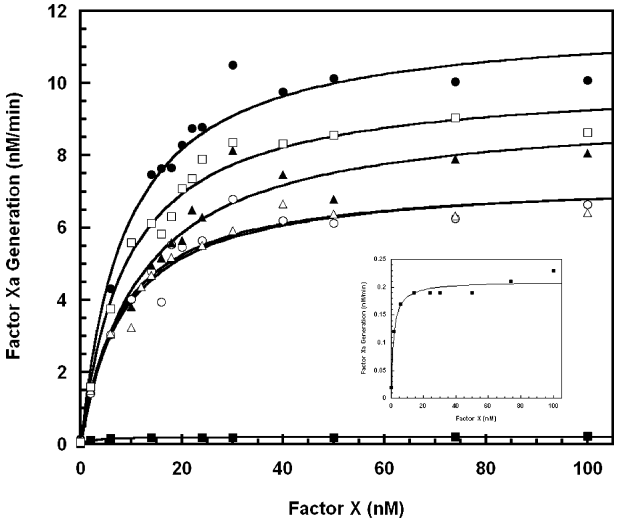

FIGURE 5: Effect of factor IXa mutations on the kinetics of factor X activation by the factor IXa−factor VIIIa−phospholipid complex. The rate of factor X activation by each recombinant factor IXa was determined in reactions containing 1.0 nM factor VIIIa, 0.1 nM factor IXa, 0−100 nM factor X, and 50 μM PC:PS vesicles in tenase buffer. Representative curves for wild type (●), K126A (○), N129A (▲), K132A (△), R165A (■), and N178A (□) factor IXa are presented. The R165A factor IXa data (■) is also presented in the inset to illustrate the goodness of the fit.

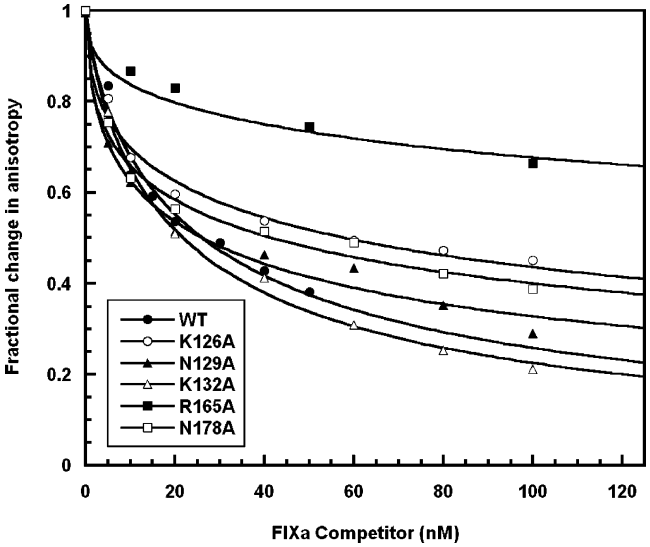

FIGURE 6: Effect of mutations on the ability of recombinant factor IXa to compete for factor IXa−factor VIIIa binding in the presence of factor X. The ability of the recombinant factor IXa to compete for factor IXa−factor VIIIa binding was determined by titrating increasing amounts of factor IXa competitor into a reaction mixture containing 5 nM factor IXa-Fl-EGR, 8 nM factor VIIIa, 125 nM factor X, and 50 μM PC:PS vesicles in 0.15 M NaCl, 20 mM HEPES, pH 7.4, and 5 mM $CaCl_2$ as described in Experimental Procedures. The maximal anisotropy signal was 0.203 ± 1 (±SEM), while the baseline signal was 0.108 ± 1 (±SEM). Representative curves are presented.

IXa−factor VIIIa complex, direct and competition binding assays were performed with factor IXa-Fl-EGR. As reported, titration of factor IXa-Fl-EGR with thrombin-activated factor VIII demonstrated a saturable increase in anisotropy, which was enhanced in the presence of factor X (*24*, *36*). Fitting these titrations to a simple binding model with 1:1 stoichiometry resulted in K_D values of 4.1 ± 0.6 nM and 1.7 ± 0.2 nM for cofactor binding to factor IXa-Fl-EGR in the absence and presence of 125 nM factor X, respectively. Although these values represent the physical association of factor IXa−factor VIIIa complex, they must be considered apparent affinities due to instability of the factor VIIIa ligand. The increase in factor IXa−factor VIIIa affinity in the presence of factor X is consistent with the reported effects of substrate on kinetically determined $K_{D(app)}$ values (*37*). Addition of factor X to factor IXa-Fl-EGR in the absence of cofactor did not result in any significant change in anisotropy. However, the presence of factor X dramatically improved the ability of the unmodified factor IXa to compete for binding with factor IXa-Fl-EGR. The $K_{I(app)}$ value (±SEM) for unmodified factor IXa was 4.9 ± 0.1 nM in the presence of factor X compared to 154 ± 10 nM in the absence of factor X. In contrast, the $K_{I(app)}$ for factor IXa-EGR was only modestly decreased from 10 ± 2 nM to 4.7 ± 0.2 nM by the addition of factor X.

Effect of Factor IXa Mutations on the Affinity of Factor IXa−Factor VIIIa Complex in the Presence of Factor X. To assess the effect of these mutations on physical assembly of the intrinsic tenase complex, the ability of the recombinant proteins to compete for cofactor binding with factor IXa-Fl-EGR was examined in the presence of factor X (Figure 6). The $K_{I(app)}$ values were similar for factor IXa wild type, N129A, and K132A (Table 2). $K_{I(app)}$ values were modestly increased for factor IXa N178A (1.6-fold) and K126A (2.2-fold), while factor IXa R165A demonstrated a 16.3-fold increase in $K_{I(app)}$ relative to factor IXa wild type. The results suggest that the mutations N178A, K126A, and particularly R165A disrupt physical assembly of the intrinsic tenase complex.

DISCUSSION

The role of the heparin-binding exosite in activation of factor IXa within the intrinsic tenase complex was examined using a series of alanine substitutions for conserved basic (K126, K132, R165) and neutral (N129, N178) residues surrounding the surface residue R233 (Figure 1). R233A is critical for protease-heparin binding, and contributes to cofactor binding and stability (*18*). Analysis of these recombinant proteases demonstrated effects on coagulation and intrinsic tenase activity, suggesting that mutations in the heparin-binding exosite may have distant effects on the factor IXa catalytic mechanism.

The most dramatic effects on factor IXa activity were observed with the R165A mutation. The coagulant activity of factor IXa R165A was profoundly decreased relative to wild type (Table 1) consistent with the severe hemophilia B phenotype of the type II mutation R165Q (*38*). In a functional binding assay, factor IXa R165A demonstrated a 23-fold decrease in factor VIIIa affinity and markedly defective activity in the saturated complex (B_{max}) (Figure 2). It also failed to stabilize factor VIIIa activity (Figure 3B). Since the decay of intrinsic tenase activity primarily depends on the rate of A2 domain dissociation from factor VIIIa (*39*), R165A may play an essential role in the factor IXa−A2 domain interaction (*13*).

In addition to its effect on factor VIIIa binding, R165A affected the catalytic mechanism for factor X activation. In the absence of factor VIIIa, factor IXa R165A demonstrated a decreased maximal rate of factor X activation relative to

wild type protease in the presence (Table 3) or absence of ethylene glycol (not shown). In the presence of factor VIIIa, R165A did not appear to disrupt the interaction of the factor IXa−factor VIIIa complex with factor X, or factor IXa with the factor VIIIa−factor X complex on the phospholipid surface (Table 3) (*35*). However, factor IXa R165A demonstrated a 65% decrease in the k_{cat} for factor X activation in the presence of factor VIIIa relative to wild type protease (Table 3), suggesting that this residue is critical to cofactor-enhanced catalysis in the intrinsic tenase complex. Thus, R165 contributes to both factor VIIIa-independent and factor VIIIa-dependent catalytic mechanisms.

The other mutations showed mild to moderate effects on factor IXa activity. Factor IXa K126A, N129A, and K132A had modest, but significant, decreases in coagulant activities (12−59%), catalytic efficiency (k_{cat}/K_M) for Pefachrome IXa (15−21%), cofactor affinity in the functional binding assay (1.5−2 fold), and ability to stabilize factor VIIIa activity (Tables 1−3). In contrast, these proteases demonstrated a normal (or increased) k_{cat} for factor X in the absence or presence of factor VIIIa (Table 3). Thus, surface residues K126, N129, and K132 contribute to the cofactor interactive site and stabilization of the A2 domain, without significant effects on the factor X catalytic mechanism. Factor IXa N178A demonstrated modest reductions in the efficiency of peptide substrate cleavage (26%) and the k_{cat} for factor X activation (34%) in the absence of cofactor (Tables 1 and 3), suggesting a mild catalytic defect. The coagulant activity of factor IXa N178A was intact relative to wild type protein (Table 1), contrasting with the observed mild hemophilia phenotype of the N178D mutation (*33*). Likewise, the ability of factor IXa N178A to stabilize factor VIIIa activity within the factor IXa−factor VIIIa complex, and the k_{cat} for factor X in the intrinsic tenase complex were comparable to wild type protease (Tables 2 and 3). Thus, surface residue N178 does not contribute significantly to stabilization of the A2 domain, but the N178A mutation results in a mild defect in factor X catalysis that is largely corrected in the presence of factor VIIIa.

Active site modification, factor X substrate binding, and mutations in the heparin-binding exosite of factor IXa affected physical assembly of the intrinsic tenase complex. Similar to studies with porcine factor IXa-Fl-FPR, the maximal change in anisotropy for human factor IXa-Fl-EGR, and the affinity of the enzyme-cofactor interaction were enhanced in the presence of factor X (*24*, *36*). Covalent modification of the factor IXa active site with EGR-chloromethylketone markedly improved the ability of the protease to compete for factor VIIIa binding. Since factor VIIIa does not interact directly with the factor IXa active site, this modification must increase cofactor affinity by inducing conformational change at distant binding site(s). The reduced rate of macromolecular and peptide substrate catalysis by factor IXa R165A, and the ability of heparin to modify substrate and inhibitor interactions with the active site, support an allosteric link between the heparin-binding exosite and the active site of factor IXa (*14*, *40*, *41*). Further, factor X markedly improved the ability of unmodified factor IXa to compete with factor IXa-Fl-EGR for binding to factor VIIIa, suggesting that macromolecular substrate binding also increases protease−cofactor affinity. Factor X may modify cofactor affinity by formation of the factor VIIIa−factor X complex (*35*) and/or by a direct effect on factor IXa conformation. In contrast, factor X had a limited effect on the ability of factor IXa-EGR to compete for cofactor binding, suggesting that this active site modification may replicate, in part, the effect of macromolecular substrate binding.

Factor IXa N178A, K126A, and R165A demonstrated modest to marked reductions in ability to compete for physical assembly into the intrinsic tenase complex relative to wild type protease. These results confirm the contribution of the factor IXa heparin-binding exosite to assembly of the enzyme complex. The effect of the R165A mutation relative to wild type protease was more pronounced in the functional binding assay (23-fold increase in $K_{D(app)}$) than the physical competition assay (16-fold increase in the $K_{I(app)}$) (Table 2). This result further emphasizes that the profound catalytic defect of factor IXa R165A is not adequately explained by effects on physical assembly alone. Thus, R165 plays a critical role in both cofactor binding and allosteric activation of factor IXa within the tenase complex, consistent with the significantly reduced k_{cat} for factor X activation by factor IXa R165A in the presence of factor VIIIa.

Mutagenesis of factor IXa demonstrates that the heparin-binding exosite represents the critical cofactor interactive site for protease activation, with the residues K126, N129, K132, R165, and R233 contributing to stabilization of the factor VIIIa A2 domain within the enzyme complex. These findings are consistent with the participation of the 162−170 helix, and particularly R165, in direct binding to the isolated A2 domain (*13*, *42*). The extensive overlap between cofactor and heparin-binding sites is similar to the role of the factor Xa heparin-binding exosite in the prothrombinase complex (*15*, *43*). The observation that mutations in the heparin-binding exosite have distant effects on the factor IXa catalytic mechanism is consistent with the ability of low molecular weight heparin to enhance the reactivity of the factor IXa active site with basic pancreatic trypsin inhibitor (*40*), and inhibit cofactor-independent factor X activation by the factor IXa−PL complex (*14*). The allosteric effect of heparin is modulated, at least in part, through rearrangement of K98 in the 99-loop, which partially obstructs the active site of factor IXa (*41*). The 99-loop restricts substrate access to the S2−S4 subsites, demonstrates alternative conformations in *p*-aminobenzamide versus FPR-chloromethylketone-inhibited factor IXa structures, and is hypothesized to undergo substrate-induced rearrangement in the activated enzyme complex (*32*, *44*−*46*). Cofactor interaction with the heparin-binding exosite of factor IXa may likewise modulate conformation of the 99-loop, contributing to the dramatic enhancement in factor X catalysis within the physiologic intrinsic tenase complex.

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REFERENCES

1. Lawson, J. H., Kalafatis, M., Stram, S., and Mann, K. G. (1994) A model for the tissue factor pathway to thrombin. I. An empirical study, *J. Biol. Chem. 269*, 23357−23366.

2. Rand, M. D., Lock, J. B., van't Veer, C., Gaffney, D. P., and Mann, K. G. (1996) Blood clotting in minimally altered whole blood, *Blood 88*, 3432−3445.
3. Duffy, E. J., and Lollar, P. (1992) Intrinsic pathway activation of factor X and its activation peptide-deficient derivative, factor Xdes-143-191, *J. Biol. Chem. 267*, 7821−7827.
4. McRae, B. J., Kurachi, K., Heimark, R. L., Fujikawa, K., Davie, E. W., and Powers, J. C. (1981) Mapping the active sites of bovine thrombin, factor IXa, factor Xa, factor XIa, factor XIIa, plasma kallikrein, and trypsin with amino acid and peptide thioesters: development of new sensitive substrates, *Biochemistry 20*, 7196−7206.
5. Fay, P. J., Beattie, T. L., Regan, L. M., O'Brien, L. M., and Kaufman, R. J. (1996) Model for the factor VIIIa-dependent decay of the intrinsic factor Xase. Role of subunit dissociation and factor IXa-catalyzed proteolysis, *J. Biol. Chem. 271*, 6027−6032.
6. Lollar, P., Knutson, G. J., and Fass, D. N. (1984) Stabilization of thrombin-activated porcine factor VIII:C by factor IXa phospholipid, *Blood 63*, 1303−1308.
7. Fay, P. J., and Koshibu, K. (1998) The A2 subunit of factor VIIIa modulates the active site of factor IXa, *J. Biol. Chem. 273*, 19049−19054.
8. Lenting, P. J., Donath, M. J., van Mourik, J. A., and Mertens, K. (1994) Identification of a binding site for blood coagulation factor IXa on the light chain of human factor VIII, *J. Biol. Chem. 269*, 7150−7155.
9. Fay, P. J., Koshibu, K., and Mastri, M. (1999) The A1 and A2 subunits of factor VIIIa synergistically stimulate factor IXa catalytic activity, *J. Biol. Chem. 274*, 15401−15406.
10. Jenkins, P. V., Dill, J. L., Zhou, Q., and Fay, P. J. (2004) Contribution of factor VIIIa A2 and A3−C1−C2. subunits to the affinity for factor IXa in factor Xase, *Biochemistry 43*, 5094−5101.
11. Kolkman, J. A., Lenting, P. J., and Mertens, K. (1999) Regions 301−303 and 333−339 in the catalytic domain of blood coagulation factor IX are factor VIII-interactive sites involved in stimulation of enzyme activity, *Biochem. J. 339* (Part 2), 217−221.
12. Mathur, A., and Bajaj, S. P. (1999) Protease and EGF1 domains of factor IXa play distinct roles in binding to factor VIIIa. Importance of helix 330 (helix 162 in chymotrypsin) of protease domain of factor IXa in its interaction with factor VIIIa, *J. Biol. Chem. 274*, 18477−18486.
13. Bajaj, S. P., Schmidt, A. E., Mathur, A., Padmanabhan, K., Zhong, D., Mastri, M., and Fay, P. J. (2001) Factor IXa:factor VIIIa interaction. helix 330−338 of factor IXa interacts with residues 558-565 and spatially adjacent regions of the A2 subunit of factor VIIIa, *J. Biol. Chem. 276*, 16302−16309.
14. Sheehan, J. P., Kobbervig, C. E., and Kirkpatrick, H. M. (2003) Heparin inhibits the intrinsic tenase complex by interacting with an exosite on factor IXa, *Biochemistry 42*, 11316−11325.
15. Rezaie, A. R. (2000) Identification of basic residues in the heparin-binding exosite of factor Xa critical for heparin and factor Va binding, *J. Biol. Chem. 275*, 3320−3327.
16. Sheehan, J. P., and Sadler, J. E. (1994) Molecular mapping of the heparin-binding exosite of thrombin, *Proc. Natl. Acad. Sci. U.S.A. 91*, 5518−5522.
17. Yang, L., Manithody, C., and Rezaie, A. R. (2002) Localization of the heparin binding exosite of factor IXa, *J. Biol. Chem. 277*, 50756−50760.
18. Yuan, Q. P., Walke, E. N., and Sheehan, J. P. (2005) The factor IXa heparin-binding exosite is a cofactor interactive site: mechanism for antithrombin-independent inhibition of intrinsic tenase by heparin, *Biochemistry 44*, 3615−3625.
19. Sheehan, J. P., and Walke, E. N. (2006) Depolymerized holothurian glycosaminoglycan and heparin inhibit the intrinsic tenase complex by a common antithrombin-independent mechanism, *Blood 107*, 3876−3882.
20. MacDonald, R. C., MacDonald, R. I., Menco, B. P., Takeshita, K., Subbarao, N. K., and Hu, L. R. (1991) Small-volume extrusion apparatus for preparation of large, unilamellar vesicles, *Biochim. Biophys. Acta 1061*, 297−303.
21. Chen, P., Toribara, T., and Warner, H. (1956) *Anal. Chem. 28*, 1756−1758.
22. Chang, J., Jin, J., Lollar, P., Bode, W., Brandstetter, H., Hamaguchi, N., Straight, D. L., and Stafford, D. W. (1998) Changing residue 338 in human factor IX from arginine to alanine causes an increase in catalytic activity, *J. Biol. Chem. 273*, 12089−12094.
23. Lozier, J. N., Monroe, D. M., Stanfield-Oakley, S., Lin, S. W., Smith, K. J., Roberts, H. R., and High, K. A. (1990) Factor IX New London: substitution of proline for glutamine at position 50 causes severe hemophilia B, *Blood 75*, 1097−1104.
24. Duffy, E. J., Parker, E. T., Mutucumarana, V. P., Johnson, A. E., and Lollar, P. (1992) Binding of factor VIIIa and factor VIII to factor IXa on phospholipid vesicles, *J. Biol. Chem. 267*, 17006−17011.
25. Limbird, L. (1995) *Cell Surface Receptors: A Short Course on Theory and Methods*, 2nd ed., Kluwer Academic Publishers, Boston.
26. Cheng, Y., and Prusoff, W. H. (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction, *Biochem. Pharmacol. 22*, 3099−3108.
27. Chou, T. (1974) Relationships between inhibition constants and fractional inhibition in enzyme-catalyzed reactions with different numbers of reactants, different reaction mechanisms, and different types and mechanisms of inhibition, *Mol. Pharmacol. 10*, 235−247.
28. Cote, H. C., Stevens, W. K., Bajzar, L., Banfield, D. K., Nesheim, M. E., and MacGillivray, R. T. (1994) Characterization of a stable form of human meizothrombin derived from recombinant prothrombin (R155A, R271A, and R284A), *J. Biol. Chem. 269*, 11374−11380.
29. Yan, S. C., Razzano, P., Chao, Y. B., Walls, J. D., Berg, D. T., McClure, D. B., and Grinnell, B. W. (1990) Characterization and novel purification of recombinant human protein C from three mammalian cell lines, *Biotechnology (NY) 8*, 655−661.
30. Sturzebecher, J., Kopetzki, E., Bode, W., and Hopfner, K. P. (1997) Dramatic enhancement of the catalytic activity of coagulation factor IXa by alcohols, *FEBS Lett. 412*, 295−300.
31. Lollar, P., and Parker, C. G. (1990) pH-dependent denaturation of thrombin-activated porcine factor VIII, *J. Biol. Chem. 265*, 1688−1692.
32. Sichler, K., Banner, D. W., D'Arcy, A., Hopfner, K. P., Huber, R., Bode, W., Kresse, G. B., Kopetzki, E., and Brandstetter, H. (2002) Crystal structures of uninhibited factor VIIa link its cofactor and substrate-assisted activation to specific interactions, *J. Mol. Biol. 322*, 591−603.
33. Sichler, K., Kopetzki, E., Huber, R., Bode, W., Hopfner, K. P., and Brandstetter, H. (2003) Physiological fIXa activation involves a cooperative conformational rearrangement of the 99-loop, *J. Biol. Chem. 278*, 4121−4126.
34. Sheehan, J. P., and Phan, T. M. (2001) Phosphorothioate oligonucleotides inhibit the intrinsic tenase complex by an allosteric mechanism, *Biochemistry 40*, 4980−4989.
35. Panteleev, M. A., Ananyeva, N. M., Greco, N. J., Ataullakhanov, F. I., and Saenko, E. L. (2006) Factor VIIIa regulates substrate delivery to the intrinsic factor X-activating complex, *FEBS J. 273*, 374−387.
36. Lollar, P., Parker, E. T., Curtis, J. E., Helgerson, S. L., Hoyer, L. W., Scott, M. E., and Scandella, D. (1994) Inhibition of human factor VIIIa by anti-A2 subunit antibodies, *J. Clin. Invest. 93*, 2497−2504.
37. Mathur, A., Zhong, D., Sabharwal, A. K., Smith, K. J., and Bajaj, S. P. (1997) Interaction of factor IXa with factor VIIIa. Effects of protease domain Ca2+ binding site, proteolysis in the autolysis loop, phospholipid, and factor X, *J. Biol. Chem. 272*, 23418−23426.
38. Green, P. M., Giannelli, F., Sommer, S. S., Poon, M.-C., Ludwig, M., Schwaab, R., Rietsma, P. H., Goossens, M., Yoshioka, A., Figueiredo, M. S., Tagariello, G., and Brownlee, G. G. (2004) Haemophilia B mutation database, version 13 ed., www.kcl.ac.uk/ip/petergreen/haemBdatabase.html.
39. Fay, P. J., Beattie, T. L., Regans, L. M. O., Brien, L. M., and Kaufman, R. J. (1996) Model for the factor VIIIa-dependent decay of the intrinsic factor Xase. Role of subunit dissociation and factor IXa-catalyzed proteolysis, *J. Biol. Chem. 271*, 6027−6032.
40. Neuenschwander, P. F. (2004) Exosite occupation by heparin enhances the reactivity of blood coagulation factor IXa, *Biochemistry 43*, 2978−2986.
41. Neuenschwander, P. F., Williamson, S. R., Nalian, A., and Baker-Deadmond, K. J. (2006) Heparin modulates the 99-loop of factor IXa: effects on reactivity with isolated Kunitz-type inhibitor domains, *J. Biol. Chem. 281*, 23066−23074.
42. Rohlena, J. (2004) in *Department of Plasma Proteins of Sanquin Research*, pp 1−111, University of Utrecht and Sanquin Research at CLB, Amsterdam.

43. Rudolph, A. E., Porche-Sorbet, R., and Miletich, J. P. (2001) Definition of a factor Va binding site in factor Xa, *J. Biol. Chem. 276*, 5123−5128.
44. Brandstetter, H., Bauer, M., Huber, R., Lollar, P., and Bode, W. (1995) X-ray structure of clotting factor IXa: active site and module structure related to Xase activity and hemophilia B, *Proc. Natl. Acad. Sci. U.S.A. 92*, 9796−9800.
45. Hopfner, K. P., Brandstetter, H., Karcher, A., Kopetzki, E., Huber, R., Engh, R. A., and Bode, W. (1997) Converting blood coagulation factor IXa into factor Xa: dramatic increase in amidolytic activity identifies important active site determinants, *EMBO J. 16*, 6626−6635.
46. Hopfner, K. P., Lang, A., Karcher, A., Sichler, K., Kopetzki, E., Brandstetter, H., Huber, R., Bode, W., and Engh, R. A. (1999) Coagulation factor IXa: the relaxed conformation of Tyr99 blocks substrate binding, *Structure 7*, 989−996.
47. DeLano, W. L. (2006), DeLano Scientific, San Carlos, CA.

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