The Second Epidermal Growth Factor-like Domain of Human Factor IXa Mediates Factor IXa Binding to Platelets and Assembly of the Factor X Activating Complex†

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ABSTRACT: Factor IXa binding to the activated platelet surface is required for efficient catalysis of factor X activation. Platelets possess a specific binding site for factor IXa, occupancy of which has been correlated with rates of factor X activation. However, the specific regions of the factor IXa molecule that are critical to this interaction have not yet been fully elucidated. To assess the importance of the second epidermal growth factor (EGF2) domain of factor IXa for platelet binding and catalysis, a chimeric protein (factor IXa_{Xegf2}) was created by replacement of the EGF2 domain of factor IX with that of factor X. Competition binding experiments showed 2 different binding sites on activated platelets (∼250 each/platelet): (1) a specific factor IXa binding site requiring the intact EGF2 domain; and (2) a shared factor IX/IXa binding site mediated by residues G_4-Q_{11} within the Gla domain. In kinetic studies, the decreased V_{max} of factor IXa_{Xegf2} activation of factor X on the platelet surface (V_{max} 2.90 ± 0.37 pM/min) versus normal factor IXa (37.6 ± 0.15 pM/min) was due to its decreased affinity for the platelet surface (K_d 64.7 ± 3.9 nM) versus normal factor IXa (K_d 1.21 ± 0.07 nM), resulting in less bound enzyme (functional complex) under experimental conditions. The hypothesis that the binding defects of factor IXa_{Xegf2} are the cause of the kinetic perturbations is further supported by the normal k_{cat} of bound factor IXa_{Xegf2} (1701 min^{-1}) indicating (1) an intact catalytic site and (2) the normal behavior of bound factor IXa_{Xegf2}. The EGF2 domain is not a cofactor binding site since the mutant shows a normal rate enhancement upon the addition of cofactor. Thus, the intact EGF2 domain of factor IXa is critical for the formation of the factor X activating complex on the surface of activated platelets.

Blood coagulation factor IX is critical for normal blood coagulation since its deficiency results in a severe bleeding disorder (*1*). A single-chain glycoprotein (M_r 57 000) containing 18% carbohydrate and consisting of 415 amino acids (*1*−*3*), it circulates in plasma as a zymogen that is activated by limited proteolysis either by the factor VIIa−tissue factor complex (extrinsic or initiating pathway) or by factor XIa (intrinsic or sustaining pathway) (*1*−*5*). Factor IXa (M_r 45 000) results from cleavage of two peptide bonds and the formation of an activation peptide (M_r ∼11 000) and two-chain factor IXa, which consists of disulfide-linked, heavy (M_r ∼28 000) and light (M_r ∼18 000) chains (*4*). The factor IX gene (∼34 kb) contains 7 introns and 8 exons coding for distinct structural domains that are highly conserved among the other homologous vitamin K-dependent plasma coagulation proteins (factor X, factor VII, prothrombin, protein C, and protein S) (*6*). Exon I encodes the signal peptide whereas exons II−III encode the propeptide (which is cleaved from the mature protein prior to secretion through the Golgi apparatus) and the γ-carboxyglutamic acid (Gla)[1] domain (comprising residues 1−46, including 12 glutamic acid residues that are posttranslationally modified to the dicarboxylic Gla form by a vitamin K-dependent carboxylase) (*7*). Exons IV and V encode two nonidentical epidermal growth factor (EGF)-like domains (residues 47−145), each of which contains six cysteines. The specific functions of the EGF domains are unknown, but the EGF1 domain of factor IX contains one high-affinity Ca^{2+} binding site (*7*−*10*). Exon VI encodes an activation peptide (residues 146−180, containing two carbohydrate binding sites), and exons VII−VIII (and the 3′ portion of exon VI) code for residues 181−415, which comprise the trypsin-like, heavy-chain, catalytic domain of factor IXa (*6*−*9, 11*). Factor IXa activates

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[1] Abbreviations: Gla, γ-carboxyglutamic acid; EGF, epidermal growth factor; BSA, bovine serum albumin; ACD, acid citrate dextrose; PRP, platelet-rich plasma; chromogenic substrate S2238, H-D-F-Pip-R-pNA; chromogenic substrate S2765, *N*-α-Cbo-R-G-R-pNA; factor IXa_{PD}, plasma-derived factor IXa; factor IXa_{wt}, wild-type-derived factor IXa; factor IXa_{Xegf2}, chimeric-derived factor IXa; PAGE, polyacrylamide gel electrophoresis; HEPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; DMEM, Dulbecco's modification of Eagle's medium; k_{cat}, turnover number; B_{max}, maximum number of binding sites; V_{max}, maximum velocity.

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factor X by cleaving a specific peptide bond (Arg 52−Ile 53) in the amino-terminal region of the factor X heavy chain (*12*, *13*). It recognizes factor X as its normal macromolecular substrate and can also cleave and activate factor VIII but less effectively than thrombin or factor Xa (*14*, *15*).

Factor IXa is a component of the factor X activating complex (*1*, *2*) which forms on the surface of activated platelets in conjunction with factor VIIIa, a cofactor involved in the conversion of factor X to factor Xa (*16*−*18*). A discrete number of high-affinity, saturable receptors for factor IXa are expressed on the surface of thrombin-activated platelets in the presence of calcium ions (*17*). In the absence of factor VIII and factor X, factor IXa can occupy ∼500−600 binding sites per platelet of which ∼250−300 can also be occupied by factor IX, the zymogen (*17*). Although in the absence of factor VIII and factor X the affinity of binding of factor IX and factor IXa are similar (K_d ∼2.5 nM), in the presence of factor VIII and factor X the affinity of factor IXa binding is increased 5-fold (K_d ∼0.5 nM), whereas the binding of factor IX is unaffected (*17*). Nesheim et al. (*19*) have demonstrated the presence on thrombin-activated platelets of ∼450 binding sites per platelet for factor VIII with a K_d ∼3.0 nM (i.e., with a stoichiometry and affinity similar to those that describe factor IXa binding). Although unactivated platelets have no significant effect on the kinetics of factor X activation by factor IXa either in the presence or in the absence of thrombin-activated factor VIII, thrombin-activated platelets decrease the K_m by more than 200-fold and permit factor VIIIa to increase the k_{cat} more than 50 000-fold (*18*). Thus, the combined effects of thrombin-activated platelets and factor VIIIa result in an overall increase in catalytic efficiency (k_{cat}/K_m) of more than 2×10^7-fold.

To identify platelet binding domains within factor IX and factor IXa, we have employed a variety of biochemically modified, mutant and chimeric proteins as well as synthetic peptides. These studies have shown that the Gla-containing domain of factor IX/IXa is essential for its normal binding to activated platelets (*20*), and that specific factor IX/IXa binding to platelets is mediated at least in part by residues 3−11 within the Gla domain of factor IX (*21*). However, recent studies with a conformationally constrained synthetic peptide comprising residues G_4−Q_{11} demonstrate that the Gla domain mediates the binding of factor IXa to only 250−300 of the total 500−600 sites per platelet (*22*), leaving the remaining 250−300 sites that are functionally active in factor X activation still occupied by factor IXa. Moreover, studies were carried out with chimeric factor IXa molecules after replacing the first or second growth factor domain (EGF1 or EGF2) with the corresponding polypeptide region of factor X (*23*, *24*). The studies with factor IXa_{Xegf1} suggest either that the EGF1 domain of factor IXa is not involved in factor IXa binding to platelets or that the EGF1 domain from factor X, when inserted into the factor IXa molecule, suffices to promote normal factor IXa binding (*23*). The studies with factor IXa_{Xegf2} suggest that an intact EGF2 domain may be important for specific, high-affinity factor IXa binding to platelets in the presence of factor VIIIa and factor X (*24*). To define the role of the EGF2 domain of factor IXa in the assembly of the factor X activating complex, we have now carried out detailed kinetic and equilibrium binding studies with factor IXa_{Xegf2}.

EXPERIMENTAL PROCEDURES

Materials

Heparin from porcine intestinal mucosa (168 units/mg), Sepharose 2B-CL, and ELISA-grade bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Sephadex G-50 was purchased from Pharmacia Biotech, Inc. (Piscataway, NJ). Nucleopore polycarbonate membranes were purchased from Costar Corp. (Cambridge, MA). Bovine brain phosphatidylserine and L-α-dioleoylphosphatidylcholine were purchased from Avanti Polar Lipids (Birmingham, AL). Nucleopore polycarbonate membranes (100 nm pore diameter) were purchased from Costar Corp. Centri-prep 3 concentration units were purchased from Amicon Division, W. R. Grace & Co. (Danvers, MA). The Iodo-Gen iodination reagent (1,3,4,6-tetrachloro-3α,6α-diphenylglycuril) was obtained from the Pierce Chemical Co. (Rockford, IL).

Human factor IX and the mutant factor IX_{Xegf2} cDNA were obtained as a generous gift from Dr. Darrel W. Stafford from the University of North Carolina, Chapel Hill. Restriction enzymes were obtained from Promega (Madison, WI) and were used according to manufacturer's specifications. Taq DNA polymerase was supplied by Perkin-Elmer Cetus (Foster City, CA) and thermocycling done in a MJ Research Inc. thermocycler (Cambridge, MA). Sequenase version 2.0 and reagents were supplied by the United States Biochemical Corp. (Cleveland, OH), and sequencing gels were run on a Bio-Rad bio-gel apparatus. The mammalian expression vector pCMV5 was a generous gift from Dr. David W. Russel from the University of Texas Southwestern Medical Center (Dallas, TX). Plasmids were transformed and overexpressed in *E. coli* of the DH5a f′ strain. Supercoiled plasmid DNA was purified using cesium chloride density gradient purification (*25*) and then transfected into 293 human embryo kidney cells using the calcium chloride transfection method (*25*). Antibodies were either purchased from Enzyme Research Laboratories (South Bend, IN) or purified from serum or ascites using caprylic acid and ammonium sulfate precipitation (*26*). A HRP chemiluminescent substrate was purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD).

Dulbecco's modification of Eagle's medium was purchased from Mediatech (Herndon, VA). Fetal bovine serum and Fast Flow Q-Sepharose were purchased from Sigma. The insulin/transferrin/selenite supplement was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Acid citrate dextrose (ACD) anticoagulant for blood collection consisted of trisodium citate·$2H_2O$ (12 mM), citric acid monohydrate (10 mM), and dextrose (15 mM). HEPES-buffered Tyrode's solution consisted of *N*-(2-hydroxethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES, 15 mM), 3 mM KCl, 1 mM $MgCl_2$, 0.4 mM monosodium phosphate, pH 6.5 or 7.4 with BSA, 2 mg/mL.

Human factors IX, X, and XIa and thrombin were obtained from Enzyme Research Laboratories (South Bend, IN) in Tris-buffered saline + 1 mM benzamidine. High-purity recombinant factor VIII (3058 units/mL) was obtained as a generous gift from Baxter Healthcare Corp. (Duarte, CA). The chromogenic substrates S2238 (H-D-F-Pip-R-pNA) and S2765 (*N*-α-Cbo-R-G-R-pNA) were purchased from AB

Kabi Diagnostica (Stockholm, Sweden). Phospholipid vesicles were composed of bovine brain phosphatidylserine and phosphatidylcholine (1:3) from Avanti Polar Lipids (Birmingham, AL) and were made by extrusion through a 100 nm membrane at 500 psi. A conformationally constrained synthetic peptide (CPGKLDEFVQPC) comprising G_4-Q_{11} of the Gla domain of factor IX was synthesized and characterized as previously reported (*22*) by the Protein Chemistry Laboratory (Dr. John Lambris) of the University of Pennsylvania (Philadelphia, PA). The thrombin receptor agonist peptide SFLLRN-amide was synthesized as previously described (*27*).

Methods

Factor X Preparation. Human factor X was passed through a soybean trypsin inhibitor column to remove any serine protease contamination. The factor X was subsequently dialyzed against HEPES−Tyrode's buffer to remove any residual benzamidine in the preparation. The factor X concentration was then determined spectrophotometrically by measuring its absorbance at 280 nm using an extinction coefficient of 1.16 $mL \cdot mg^{-1} \cdot cm^{-1}$ (*28*). The purified factor X contained <0.01% contamination by factor Xa as measured by chromogenic substrate cleavage.

Expression of Mutant Protein. The chimeric and wild-type constructs were purified using a cesium chloride gradient to separate genomic and nicked DNA from supercoiled plasmid DNA (*25*). Driven by a CMV promoter in the vector pCMV5 along with the selection vector pSV2Neo, they were then transfected into 293 cells (human embryo kidney cells) which were selected using G418 at a concentration of 600 mg/mL of media. Stable cell lines were established and individual clones were assayed for factor IX expression with an ELISA utilizing an affinity-purified goat anti-human factor IX polyclonal antibody as the capture antibody. The secondary antibody was a polyclonal goat anti-factor IX antibody conjugated to horseradish peroxidase. Detection by hydrolysis of the chromogenic substrate *O*-phenylaminediamine was measured at 490 nm spectrophotometrically. Measured values were compared to a standard curve.

Western Blot of Cell Culture Supernatants. To ensure that the recombinant protein was not being degraded, serum-free culture medium from stable cell lines following transfection was analyzed using Western blots with a polyclonal goat antibody directed against human factor IX. Detection was done using a chemiluminescent substrate from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD).

Production and Purification of Recombinant Factor IX. The stable cell lines expressing the highest amounts of recombinant factor IX as detected by ELISA were isolated and grown to confluence in four 100 mm dishes supplemented with DMEM. Next, the cells were trypsinized, and the cells from each dish were transferred into 850 cm^3 tissue culture roller bottles and then grown to approximately 70% confluence in 100 mL of supplemented DMEM in a Wheaton bottle roller at less than 1 rpm. At 70% confluence, the supplemented DMEM was removed and replaced with serum-free DMEM containing 5 mg/L insulin, 5 mg/L sodium selenite, 5 mg/L transferrin, 2 mM glutamine, 100 μg/mL penicillin, and 50 μg/mL streptomycin. The cells were grown in the serum-free DMEM for 24 h, and the medium then was discarded and replaced with fresh serum-free media. This serum-free medium was then harvested every 24−48 h for use in the factor IX purification protocol. EDTA (4 mM) and benzamidine hydrochloride (5 mM) were added to the collected medium which was then transferred to 500 mL centrifuge bottles and centrifuged at 5000 rpm in a Sorvall GS-3 rotor for 10 min at 4 °C to pellet cellular debris. The supernatant containing the factor IX was then carefully removed and filtered through a 0.22 mm pore size surfactant-free cellulose acetate filter. The secreted recombinant proteins were purified using "pseudo-affinity" chromatography on fast flow Q Sepharose as described by Yan et al. (*29, 30*). The fully carboxylated recombinant protein was eluted with TBS + 5 mM $CaCl_2$. All proteins (factor IX_{PD}, factor IX_{wt}, and factor IX_{Xegf2}) were >97% pure, showing a single band at 69 kDa upon SDS−PAGE. The protein-containing fractions were pooled and concentrated ~60-fold and then dialyzed against HEPES−Tyrode's buffer, pH 7.4, for 24 h at 4 °C.

Characterization of Plasma-Derived and Recombinant Proteins by Gla Analysis. Since factor IX contains 12 Gla residues that are critical for normal factor IXa function, Gla analysis of the plasma-derived and recombinant factor IX molecules was performed utilizing the modified alkaline hydrolysis method of Przysiecki et al. (*31*). These analyses were generously performed by Dr. Peter Larson of the Children's Hospital of Philadelphia (Philadelphia, PA) for each factor IX-containing $CaCl_2$ fraction. An aliquot (20 μg) of each fraction was dialyzed against 50 mM $NaHCO_3$ before analysis. The factor IX_{wt} that was eluted at 5 mM $CaCl_2$ contained 9.6 Gla residues per molecule, whereas the molecules that were eluted at 10 mM $CaCl_2$ contained 6.65 Gla residues per molecule, and the molecules that were eluted at 15 mM $CaCl_2$ contained 2.8 Gla residues per molecule. The plasma-derived factor IX contained 9.8 Gla residues per molecule and the factor IX_{Xegf2} chimera contained 9.9 Gla residues per molecule using the same analysis, indicating that the fraction eluted at 5 mM $CaCl_2$ concentration yielded identically modified recombinant wild-type factor IX and factor IX_{Xegf2} chimera compared to plasma-derived factor IX. Since it is known that the catalytic functions of factor IX remain intact despite incomplete modification of some normally carboxylated residues (*32*), and since the recombinant and plasma-derived factor IX molecules were modified to a similar extent, the differences between the chimeric, wild-type, and plasma-derived proteins observed in this study were regarded as unlikely to result from differences in carboxylation.

Activation of Factor IX. The recombinant and plasma-derived factor IX was activated with factor XIa to factor IXa using a 200:1 molar ratio of factor IX to factor XIa. The reaction was carried out in HEPES−Tyrode's buffer + 5 mM $CaCl_2$ +1 mg/mL PEG 8000 at 37 °C for 1 h. Complete activation was observed upon SDS−PAGE. The heavy chain migrated at M_r ~28 000 and the poorly staining light chain at ~23 000, in agreement with results of other investigators (*33*).

Determination of Factor IXa Concentration. Activated plasma-derived and recombinant factor IXa was analyzed by SDS−PAGE, and then the protein concentration was determined by densitometric scanning of the heavy chain against a purified factor IXa standard that was previously quantitated by its absorbance at 280 nm using an extinction

coefficient of 1.32 mL·mg^{-1}·cm^{-1} as previously described (*5*).

Active Site Titration of Factor IXa with Antithrombin III. Thrombin was active site titrated with FMGB-HCl (*34*, *35*). This active site titrated thrombin was used to standardize human antithrombin III by active site titration. The standardized antithrombin III was diluted from 0 to 100 nM in 20 nM increments in HEPES−Tyrode's buffer + 5 mM $CaCl_2$ + 20 μg/mL heparin, incubated with factor IXa diluted to ~100 nM, mixed (1:1 v/v) with each of the antithrombin III dilutions, and finally incubated for 15 min at 37 °C. The reaction was stopped by 10-fold dilution in HEPES−Tyrode's buffer. The remaining factor IXa activity was measured in a factor Xa generation assay. Twenty microliters of the diluted antithrombin III/factor IXa reaction was mixed with 20 μL of HEPES−Tyrode's buffer containing 5 mM $CaCl_2$, 1.2 μM factor X, 12 units/mL factor VIII, and 60 μM PS/PC. The reaction was started by the addition of 20 μL of 10 units/mL thrombin in HEPES−Tyrode's buffer and incubated at 37 °C for 2 min. The reaction was stopped by the addition of 60 μL of cold 175 mM NaCl, 50 mM HEPPS, 0.5 mg/mL BSA, 20 mM EDTA, pH 8.1. The factor Xa thus formed was quantitated by incubation with an equal volume of 700 μM S2765 diluted in stopping solution (50 μL). Substrate cleavage was monitored kinetically at 405 nm in a Molecular Devices ThermoMax microplate reader (Menlo Park, CA) at 37 °C. The factor Xa activity was plotted against the antithrombin III concentration of the initial incubations. A straight line was fit to all points with a nonzero velocity using Kaleidagraph, and the *x*-intercept was determined, which is equal to the concentration of active factor IXa in the initial dilution. Active site titration yielded a ratio of 1.22 antithrombin III molecules per factor IXa_{wt} molecule with a corresponding value of 1.46 for factor IXa_{Xegf2}. Therefore, it is concluded that the concentrations of factor IXa_{wt} and factor IXa_{Xegf2} from $A_{280\ nm}$ determinations underestimated the protein concentration. The enzyme concentrations were thereby adjusted on the basis of the active site titration values, and the initial estimates of enzyme concentrations based on protein concentration were adjusted accordingly. The maximal activity of factor IXa_{wt} was used as the 100% activity standard for all subsequent measurements.

*Phospholipid Vesicle*s. Phospholipid vesicles were composed of bovine brain phosphatidylserine and phosphatidylcholine (1:3) from Avanti Polar Lipids (Birmingham, AL) and were prepared by extrusion through three stacked polycarbonate 100 nm membranes at 500 psi which was repeated 5 times (*36*).

Purification of Platelets. Citrated blood (43 mL of blood + 7 mL of ACD) was centrifuged at 1000 rpm for 20 min at room temperature. The top layer of platelet-rich plasma (PRP) was carefully removed and recentrifuged at 1000 rpm (~150*g*) for 5 min to remove any residual red blood cells and leukocytes. The platelets were pelleted from the PRP by centrifugation at 1700 rpm (~300*g*) for 15 min. The supernatant was removed and the pellet was carefully resuspended in 7 mL of HEPES−Tyrode's buffer + 1 mg/mL BSA, pH 6.5. The platelets were allowed to stand for 30 min at 37 °C. The platelets were repelleted by centrifugation at 1700 rpm (~300*g*) for 15 min. The supernatant was removed, and the pellet was carefully resuspended in 3 mL of HEPES−Tyrode's buffer + 1 mg/mL BSA, pH 6.5. The platelets were allowed to stand for 30 min at 37 °C. The platelets were loaded on a 50 mL Sepharose CL-2B column preequilibrated with HEPES−Tyrode's buffer + 1 mg/mL BSA, pH 7.4, and 2 mL fractions were collected. The middle fractions containing the most concentrated platelets were pooled and allowed to stand for 30 min at 37 °C. The platelet suspension was counted on a Coulter Counter to determine concentration.

Iodination of Factor IX. Factor IX was iodinated using the Iodo-Gen method (Pierce Chemical Co., Rockford, IL), as previously described (*16*). Specific radioactivity was ~5 × 10^6 cpm/μg of protein as determined by counting γ-emission of known dilutions of the radiolabeled factor IX in a Wallac 1470 Wizard gamma counter (Gaithersburg, MD). The ^{125}I-labeled factor IX was activated with factor XIa as previously described under *Activation of Factor IX*. Complete activation was observed upon SDS−PAGE and autoradiography. Greater than 95% of factor IX functional activity was retained as measured in a factor X activation assay.

Competition Binding Experiments. Binding experiments were performed in 1.5 mL microcentrifuge tubes in a final reaction volume of 100 μL, as previously described (*16*, *17*).

Measurement of Kinetic Constants. Velocity of factor Xa generation was plotted against varying factor X concentrations in order to determine V_{max} and K_m. Gel-filtered platelets (~3 × 10^8/mL for experiments without factor VIII; ~5 × 10^7/mL with factor VIII) in HEPES−Tyrode's buffer, pH 7.4, were activated using the thrombin receptor peptide SFLLRN-amide (25 μM). Either activated platelets or PS/PC vesicles (20 μM for experiments without factor VIII; 500 pM for experiments with factor VIII) were then incubated with factor IXa (10 nM in the absence of factor VIII; 500 pM with factor VIII) and 5 mM $CaCl_2$ for 6 min at 37 °C. Varying concentrations of factor X were added to each reaction vessel and incubated at 37 °C for 20 min for experiments without factor VIII. Alternatively, in a separate tube, factor VIII (500 units/mL) in HEPES−Tyrode's buffer containing 10 mM $CaCl_2$ was activated with thrombin (0.05 unit/mL, 45 s, 37 °C) and added to the reaction mixture at a final concentration of 15 units/mL. The reaction was allowed to proceed for 3 min (during which time the rates of factor Xa formation were linear). The reaction was stopped by the addition of EDTA to a final concentration of 10 mM. The amount of factor Xa formed was measured in a Molecular Devices ThermoMax microplate reader (Menlo Park, CA) at 405 nm following the addition of the chromogenic substrate S2765 at 37 °C. The values obtained were compared to a standard curve to determine the factor Xa concentration and to calculate rates of factor Xa formed, which were linear during the time course of the experiment.

Factor IXa Titrations. Rates of factor Xa formation were plotted against various factor IXa concentrations using previously described conditions in the presence of factor VIIIa and excess factor X (20 × K_m) on an activated platelet surface or phospholipid surface.

The apparent dissociation constant (K_d) for factor IXa binding to platelets or phospholipid vesicles was determined using the equation:

$$v = V^*[\text{factor IXa}]/(K_d + [\text{factor IXa}]) \qquad (1)$$

where

$$V^* = \frac{k_{cat}[\text{factor X}]}{K_m + [\text{factor X}]}[\text{factor IXa}]B_{max} \quad (2)$$

V* is not a true V_{max} but rather a derived binding constant that is proportional to V_{max} (derivation not shown). K_d was determined by a nonlinear least-squares fit to eq 1 using the program Kaleidagraph on a Macintosh Quadra 900 computer (Apple Computer, Cupertina, CA).

Determination of k_{cat} for Bound Enzyme on the Activated Platelet Surface. Using the equation:

$$[\text{factor IXa}]_{bound} = \frac{[\text{enzyme}]B_{max}}{K_d + [\text{enzyme}]} \quad (3)$$

the amount of bound factor IXa was determined using a B_{max} value of 282 sites/platelet. The V_{max} values were then divided by the concentration of bound enzyme to obtain a k_{cat} value adjusted for bound enzyme.

Determination of k_{cat} for Bound Enzyme on the Phospholipid Surface. The same method was used as for the platelet surface except since the B_{max} value was not known the k_{cat} value was left as a factor of the B_{max} per vesicle.

RESULTS

Factor IX/IXa Binding

Contribution of the EGF2 Domain of Factor IXa for Binding to Activated Platelets. To determine whether the EGF2 domain might play a part in the binding of factor IX or factor IXa to activated platelets and to ascertain which class of binding sites (i.e., the shared factor IX/IXa binding site or the specific factor IXa binding site) might require an intact EGF2 domain, competition binding studies with the factor IXa_{Xegf2} chimera were performed (Figure 1). The platelet binding experiments were performed using ^{125}I-labeled factor IXa in the presence of various unlabeled competitor molecules in the absence of the cofactor, factor VIIIa. The results show that the zymogen factor IX can occupy 306 ± 16 sites per platelet (Figure 1A) and can compete with (Figure 1B) approximately half of the total factor IXa binding sites on activated platelets with a K_i of 2.6 ± 0.25 nM (data not shown). Factor IXa can also occupy (Figure 1A) and compete with (Figure 1B) all 565 ± 138 factor IXa binding sites per platelet with a $K_i = 1.9 \pm 0.36$ nM (data not shown). Similarly, the zymogen factor IX_{Xegf2} chimera occupied approximately half of the factor IXa binding sites (340 ± 72 sites/platelet; Figure 1A) and displaced ~50% of factor IXa_{wt} (Figure 1B) with a K_i of 1.33 ± 0.61 nM (data not shown), indicating normal interaction of the chimeric protein with the zymogen binding site. However, upon activation of the EGF2 chimera to factor IXa_{Xegf2}, no additional sites were exposed (Figure 1A), and the factor IXa_{Xegf2} chimera was able to bind only 175 ± 141 sites/platelet. We therefore conclude that the EGF2 chimera possesses an intact shared factor IX/IXa binding site and is lacking the specific factor IXa binding site that is exposed upon activation from zymogen to active enzyme.

Factor IXa Competition Binding Experiments. To differentiate between the specific factor IXa binding site and shared factor IX/IXa binding site interactions, competition binding studies were performed in the presence of excess factor IX to establish a standardized value that did not include ^{125}I-labeled factor IXa bound to the shared factor IX/IXa binding site. In effect, only specific factor IXa binding was allowed by saturating the shared factor IX/IXa site with unlabeled zymogen. Identical methods were used in this experiment as in the previous binding experiment with the exception that excess unlabeled zymogen or Gla peptide (10 × K_i) was added to each reaction. The results are shown in Figure 1B. Addition of excess factor IX alone could compete for approximately half of the factor IXa binding sites, leaving 258 ± 22 sites/platelet occupied by factor IXa. However, in the presence of excess factor IX competitor, the addition of the factor IXa_{Xegf2} chimera had no additional effect upon factor IXa binding, whereas, as expected, factor IXa_{wt}

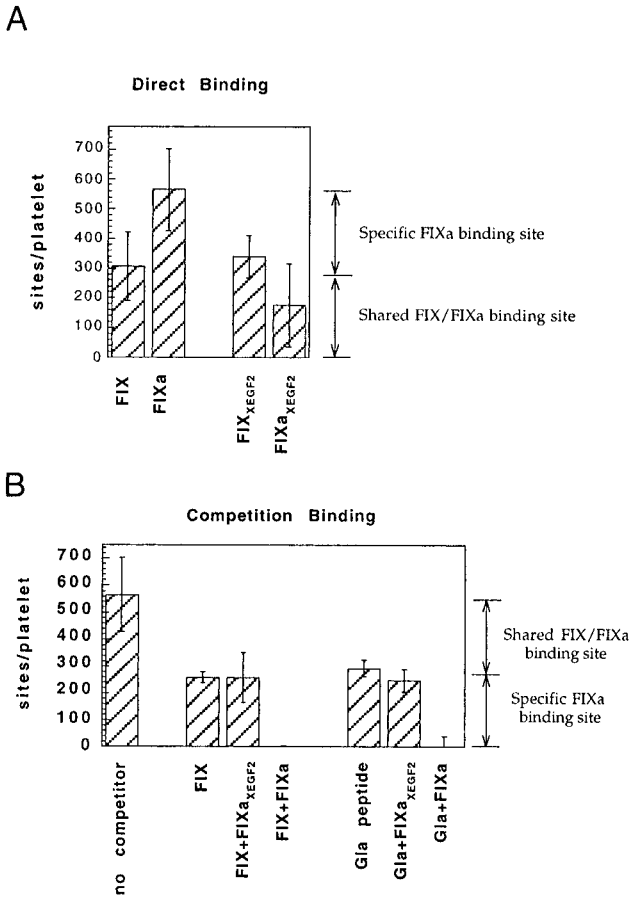

FIGURE 1: Direct and competition binding experiments with ^{125}I-labeled factor IXa to activated platelets. (A) The number of factor IXa binding sites per platelet was first measured by determining the amount of ^{125}I-factor IXa_{PD} bound. Either factor IX_{PD}, factor IXa_{PD}, factor IX_{Xegf2}, or factor IXa_{Xegf2} was added in excess, and the amount of ^{125}I-factor IXa_{wt} remaining was subtracted from the total bound in the absence of competitor to obtain an estimate of the number of sites occupied by each competitor. The data are expressed as direct binding data to facilitate clarity of presentation. Ten nanomolar ^{125}I-labeled factor IXa was incubated with ~4 × 10^8 activated platelets/mL in the presence and absence of 60 nM cold competitor, and binding was assayed as detailed under Experimental Procedures. (B) Competition binding experiments with ^{125}I-labeled factor IXa_{wt} and activated platelets in the presence of excess unlabeled factor IX_{PD} zymogen or Gla peptide (G_4−Q_{11}). Ten nanomolar radiolabeled factor IXa was incubated with ~4 × 10^8 activated platelets/mL in the presence of 60 nM unlabeled factor IX zymogen plus 60 nM unlabeled competitor or 1 mM Gla peptide plus 60 nM unlabeled competitor. Each column represents the mean of three independent experiments each performed in duplicate on separate days. Error bars represent the standard error of the mean.

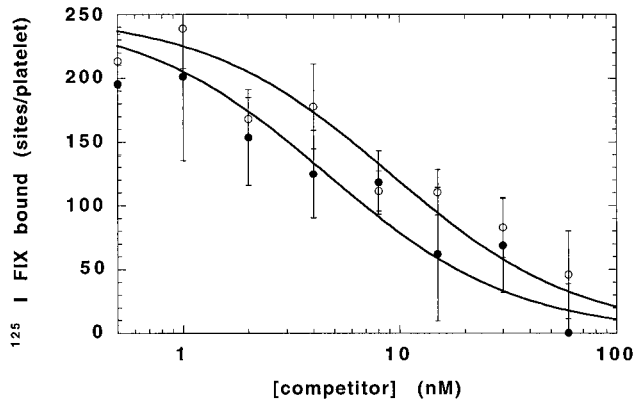

FIGURE 2: Competition by factor IXa_{PD} or factor IXa_{Xegf2} for binding of ^{125}I-labeled factor IX to activated platelets. Increasing concentrations of factor IXa_{PD} (●) or factor IXa_{Xegf2} (○) were titrated against 10 nM ^{125}I-factor IX and ~4 × 10^8 activated platelets/mL. Each point represents the mean of two independent experiments each carried out in duplicate. Error bars represent the standard error of the mean.

displaced all the remaining ^{125}I-labeled factor IXa molecules. Thus, the factor IXa_{Xegf2} chimera failed to compete for any of the specific factor IXa binding. The chimera appears to possess the same platelet binding properties as the zymogen factor IX; that is, they both bind to the shared factor IX/IXa site with similar affinity and stoichiometry. It has been previously shown that the Gla domain, specifically residues G_4-Q_{11}, mediates binding of factor IXa to the shared factor IX/IXa site (*22*). To exclude the possibility that the EGF2 domain is involved in this interaction, a peptide comprising residues G_4-Q_{11} was used instead of zymogen. This peptide, which is not an effective inhibitor in a functional assay (*22*), can still compete for ~50% of factor IXa binding to platelets (Figure 1B). Similarly, in the presence of excess Gla peptide, the factor IXa_{Xegf2} chimera failed to compete for any additional sites with radiolabeled factor IXa. Since the Gla domain peptide appears to possess the same platelet binding properties as factor IX, we conclude that the shared factor IX/IXa binding site is mediated by residues G_4-Q_{11} within the Gla domain and is distinct from the site in which the EGF2 domain is involved.

Factor IX Competition Binding Experiments. To demonstrate that the shared factor IX/IXa binding site is intact in factor IXa_{Xegf2}, competition binding experiments were performed with ^{125}I-labeled factor IX zymogen and activated platelets using various concentrations of unlabeled competitor (Figure 2). The factor IXa_{Xegf2} chimera ($K_i = 1.9 \pm 0.36$ nM) was nearly as effective in competing with the zymogen as the normal factor IXa control ($K_i = 1.0 \pm 0.18$ nM). Thus, factor IXa_{Xegf2} appears to bind normally to the shared factor IX/IXa binding site. Since we have previously shown that the Gla domain of factor IXa binds to the shared factor IX/IXa binding site, we conclude from the above data that factor IXa_{Xegf2} is defective in its interaction with the specific, high-affinity factor IXa binding site on the plasma membrane of activated platelets, and that the Gla domain (G_4-Q_{11}) mediates binding to the shared factor IX/IXa binding site.

Kinetic Studies

Activated Platelet Surface. The kinetic constants (V_{max} and K_m) for factor X activation by factor IXa_{Xegf2}, by factor IXa_{wt}, and by factor IXa_{PD} were measured on thrombin-activated platelets in the absence of cofactor to determine whether the intact EGF2 domain is critical for factor X activating complex function, and, more specifically, for platelet binding (Figure 3). The kinetic constants derived from these experiments are shown in Table 1. The apparent K_m for factor IXa_{Xegf2} was 12-fold higher than normal, suggesting that the affinity of the enzyme for the substrate was decreased. However, at saturating substrate concentrations, the V_{max} of factor IXa_{Xegf2} was also decreased 10-fold compared to the normal and wild-type molecules. The latter observation shows that the decreased substrate affinity of the chimera is not the sole cause of the velocity perturbations since saturating substrate still results in a kinetic defect (V_{max}). These data suggest that an intact EGF2 domain of factor IXa is essential for normal catalysis in the absence of cofactor (factor VIII), i.e., for the assembly of the factor X activating complex on the platelet surface.

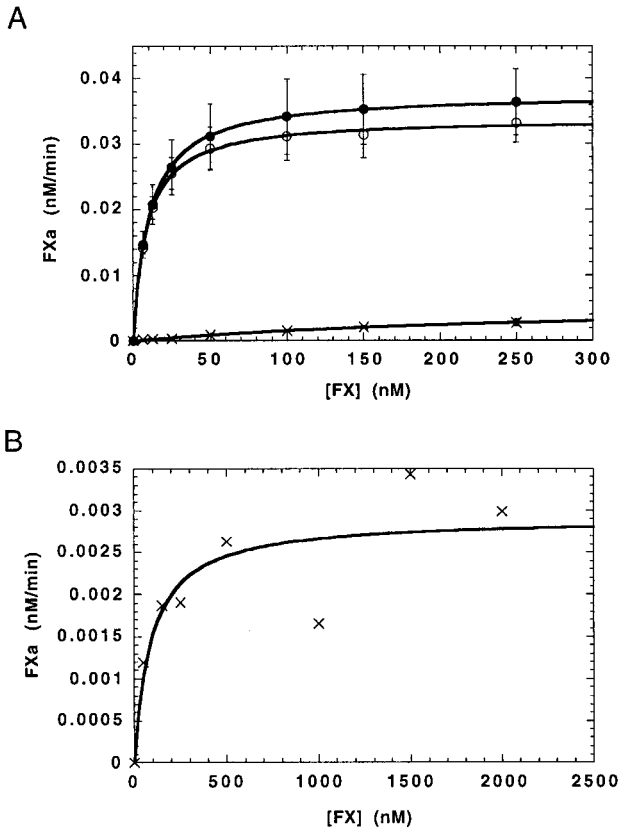

FIGURE 3: Rates of factor Xa formation by factor IXa on activated platelets in the absence of factor VIIIa. (A) Rates of factor Xa formation were measured as a function of varying concentrations of factor X using 10 nM plasma-derived (●), wild-type (○), or EGF2 chimeric (×) factor IXa molecules. The reactions were carried out as described under Methods using 3 × 10^8 activated platelets/mL in HEPES−Tyrode's buffer, pH 7.4 with 5 mM $CaCl_2$. Each point represents the mean of four independent experiments each performed in duplicate on separate days. Error bars represent the standard error of the mean. (B) Rates of factor Xa formation were measured against higher concentrations of factor X using 10 nM factor IXa_{Xegf2} (×) to achieve saturation with substrate. Each point represents the mean of duplicate determinations. Curves were drawn by a nonlinear least-squares fit of the data to the Michaelis−Menten equation using Kaleidagraph.

Phospholipid Surface. To determine whether the kinetic abnormalities seen with the EGF2 domain would be observed on phospholipid vesicles, the kinetic constants (V_{max} and K_m)

Table 1: Summary of Kinetic Parameters of Various Factor IXa Molecules on Activated Platelets or Phospholipid Vesicles in the Absence or Presence of Factor VIIIa[a]

condition	enzyme	K_m (nM)	V_{max}	V_{max}/K_m	k_{cat}[c] (relative)	k_{cat}[b] (s^{-1})	$K_{dkinetic}$ (nM)
activated platelets	factor IXa_{PD}	10.1 ± 0.20	37.6 ± 0.15 (pM/min)[d]	3.7	–	–	–
	factor IXa_{wt}	8.4 ± 0.33	33.9 ± 0.26	4.0	–	–	–
	factor IXa_{Xegf2}	91.2 ± 58.4	2.90 ± 0.37	0.03 (0.8%)	–	–	–
activated platelets and factor VIIIa	factor IXa_{PD}	17.8 ± 0.26	4.8 ± 0.022 (nM/min)[d]	269.7	16.5[c]	793[c]	1.21 ± 0.072
	factor IXa_{wt}	18.2 ± 0.35	4.4 ± 0.026	241.8	19.3[c]	799[c]	1.38 ± 0.174
	factor IXa_{Xegf2}	471 ± 116	0.27 ± 0.023	0.57 (0.2%)	35.2[c]	1701[c]	64.7 ± 3.9
phospholipids	factor IXa_{PD}	75.3 ± 4.8	96.7 ± 2.4 (pM/min)[d]	1.3	–	–	–
	factor IXa_{wt}	73.0 ± 3.6	112.7 ± 2.2	1.5	–	–	–
	factor IXa_{Xegf2}	566.1 ± 88.4	33.6 ± 2.2	0.06	–	–	–
phospholipids and factor VIIIa	factor IXa_{PD}	23.3 ± 1.5	13.8 ± 0.25 (nM/min)[d]	592.3	34.0[c]	–	0.660 ± 0.034
	factor IXa_{wt}	22.8 ± 1.6	13.2 ± 0.25	578.9	27.3[c]	–	0.863 ± 0.103
	factor IXa_{Xegf2}	54.8 ± 21.3	0.27 ± 0.037	4.9 (0.8%)	19.6[c]	–	35.7 ± 6.77

[a] The kinetic constants shown were calculated as described under Methods and represent the means ± SEM of data presented in Figures 3 through 8. [b] Calculated as described under Methods by dividing V_{max} values by the concentration of bound enzyme assuming 282 binding sites/platelet. [c] Calculated as a factor of the concentration of the maximum number of binding sites per platelet or vesicle. [d] The numbers in parentheses represent the values of V_{max}/K_m (catalytic efficiency) for the factor IXa_{Xegf2} chimera, expressed as a percentage of the mean V_{max}/K_m value for the factor IXa_{PD} and the factor IXa_{wt}.

for factor X activation by factor IXa_{Xegf2}, by factor IXa_{wt}, and by factor IXa_{PD} were determined (Figure 4 and Table 1). Similar to results with platelets (Figure 3), the apparent K_m of factor X activation by factor IXa_{Xegf2} was 10-fold higher than normal, and at saturating substrate concentrations, the V_{max} of factor IXa_{Xegf2} was also decreased 4-fold compared to the normal and wild-type molecules.

Activated Platelet Surface in the Presence of Cofactor. To determine whether a defective cofactor interaction is a cause of the observed velocity perturbation of the EGF2 chimera, the kinetic behavior of the chimera was determined in the presence of cofactor on the platelet surface (Figure 5 and Table 1). A 20-fold decrease in V_{max} was observed with factor IXa_{Xegf2} compared to factor IXa_{PD} and factor IXa_{wt} controls, which was similar to the decrease in the absence of factor VIII (i.e., 12-fold). The V_{max} enhancement observed in the presence of factor VIIIa was ~100-fold for all factor IXa molecules (factor IXa_{Xegf2}, factor IXa_{PD}, and factor IXa_{wt}). A 25-fold increase in K_m (compared with factor IXa_{wt} or factor IXa_{PD}) was found for the EGF2 chimera in the presence of factor VIIIa compared to a 10-fold increase in K_m (compared with factor IXa_{wt} or factor IXa_{PD}) in the absence of cofactor. These data provide no evidence that the EGF2 domain is involved in cofactor binding, and strongly suggest that the abnormalities in the kinetics of factor X activation by the EGF2 chimera on the activated platelet surface are not a consequence of deficient binding of factor VIII.

Phospholipid Surface in the Presence of Factor VIIIa. Kinetic experiments were performed in the presence of factor VIIIa on phospholipid vesicles (Figure 6 and Table 1). The results showed a 2-fold increase in K_m and a 50-fold decrease in V_{max} with factor IXa_{Xegf2} compared to factor IXa_{PD} and factor IXa_{wt}. The enhancement of V_{max} observed in the presence of factor VIIIa was ~10-fold for the chimera on PS/PC vesicles compared to an increase of ~100-fold for the normal controls, suggesting a defective cofactor interaction for the chimera on the PS/PC surface.

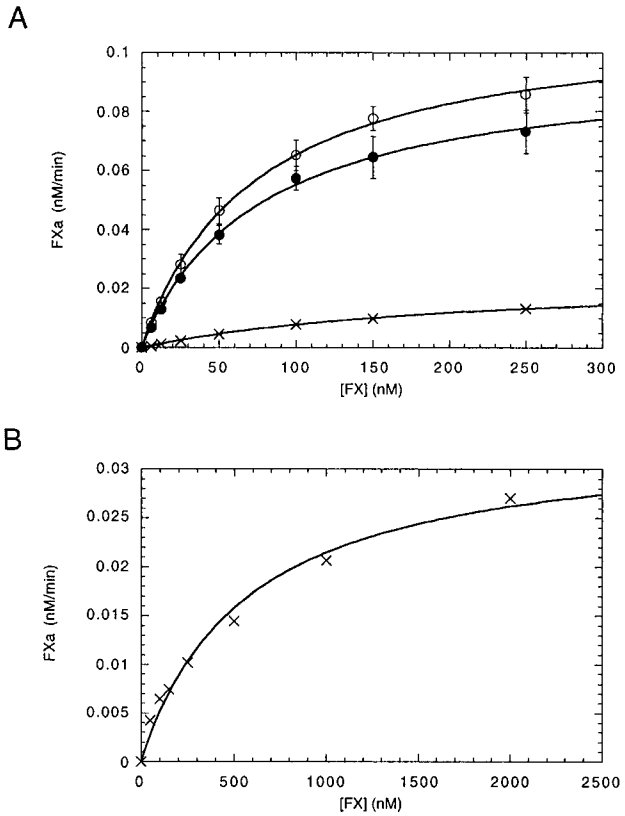

FIGURE 4: Rates of factor Xa formation by factor IXa on PS/PC vesicles in the absence of factor VIIIa. (A) Rates of factor Xa formation were measured as a function of varying concentrations of factor X using 10 nM plasma-derived (●), wild-type (○), or EGF2 chimeric (×) factor IXa molecules. The reactions were carried out as described under Methods using 20 μM PS/PC vesicles in HEPES−Tyrode's buffer, pH 7.4 with 5 mM $CaCl_2$. Each point represents the mean of three independent experiments each performed in duplicate on separate days. Error bars represent the standard error of the mean. (B) Rates of factor Xa formation were measured against higher concentrations of factor X using 10 nM factor IXa_{Xegf2} (×) to achieve saturation with substrate. Each point represents the mean of duplicate determinations. Curves were drawn by a nonlinear least-squares fit of the data to the Michaelis−Menten equation using Kaleidagraph.

Kinetic Studies of Factor IXa Binding to Platelets

Affinity of Factor IXa for the Functional Platelet Binding Site. A possible explanation for the complex kinetic abnormalities observed (i.e., increased K_m and decreased V_{max}) with factor IXa_{Xegf2} is the failure of this chimeric protein to bind

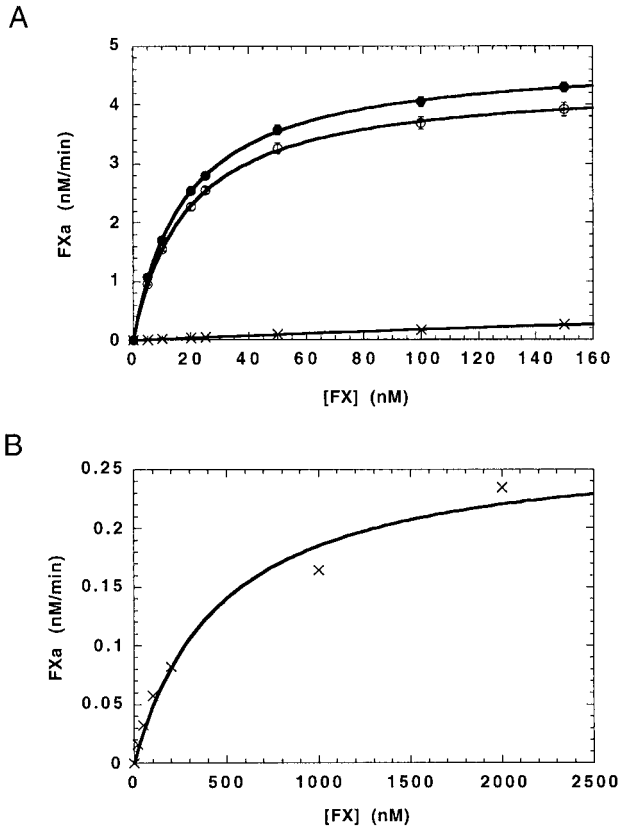

FIGURE 5: Rates of factor Xa formation by factor IXa on activated platelets in the presence of cofactor. (A) Rates of factor Xa formation were measured against varying concentrations of factor X using 500 pM plasma-derived (●), wild-type (○), or EGF2 chimeric (×) factor IXa molecules. The reactions were carried out as described under Methods using 5×10^7 activated platelets/mL in HEPES−Tyrode's buffer, pH 7.4 with 5 mM $CaCl_2$, and 15 units/mL factor VIIIa. Each point represents the mean of five independent experiments each performed in duplicate on separate days. Error bars represent the standard error of the mean. (B) Rates of factor Xa formation were measured against higher concentrations of factor X using 500 pM factor IXa_{Xegf2} (×) to achieve saturation with substrate. Each point represents the mean of duplicate determinations. Curves were drawn by a linear least-squares fit of the data to the Michaelis−Menten equation using Kaleidagraph.

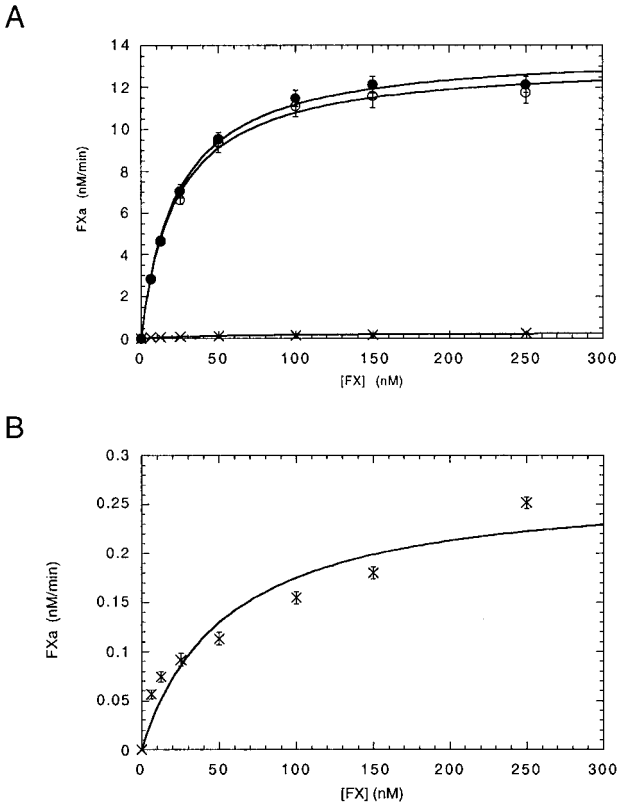

FIGURE 6: Rates of factor Xa formation by factor IXa on PS/PC vesicles in the presence of cofactor. (A) Rates of factor Xa formation were measured against varying concentrations of factor X using 500 pM plasma-derived (●), wild-type (○), or EGF2 chimeric (×) factor IXa molecules. The reactions were carried out as described under Methods using 200 nM PS/PC vesicles in HEPES−Tyrode's buffer, pH 7.4 with 5 mM $CaCl_2$, and 10 units/mL factor VIIIa. Each point represents the mean of four independent experiments each performed in duplicate on separate days. Error bars represent the standard error of the mean. (B) Rates of factor Xa formation were measured against higher concentrations of factor X using 500 pM factor IXa_{Xegf2} (×) to achieve saturation with substrate. Each point represents the mean of duplicate determinations. Curves were drawn by a linear least-squares fit of the data to the Michaelis−Menten equation using Kaleidagraph.

with normal affinity to the platelet receptor, occupancy of which is essential for assembly of the factor X activating complex. To explore this possibility, rates of factor Xa formation were determined at varying factor IXa concentrations in order to calculate values of the apparent affinity (K_d^{app}) for binding of factor IXa or factor IXa_{Xegf2} to the functional enzyme binding site (Figure 7). The calculated K_d^{app} values are summarized in Table 1. The K_d^{app} of factor IXa_{Xegf2} was 64.7 ± 3.9 nM, and 1.21 ± 0.072 and 1.38 ± 0.174 nM for factor IXa_{PD} and factor IXa_{wt}, respectively. Similar results were obtained with phospholipid vesicles (Figure 8 and Table 1): The K_d^{app} of factor IXa_{Xegf2} was 35.7 ± 6.77 nM, compared with 0.660 ± 0.034 and 0.863 ± 0.103 nM for factor IXa_{PD} and factor IXa_{wt}, respectively. The 60-fold higher values of K_d^{app} determined in the presence of either platelets or phospholipids for factor IXa_{Xegf2} compared with factor IXa_{wt} or factor IXa_{PD} indicate that the EGF2 chimera binds to platelets or phospholipids with decreased affinity, which could account for the observed kinetic abnormalities.

Determination of k_{cat}. As previously discussed, the concentrations of enzyme molecules (factor IXa_{PD}, factor IXa_{wt}, and factor IXa_{Xegf2}) used in this study were determined by active site titration. This ensures that equal concentrations of catalytically competent normal and chimeric factor IXa molecules are compared. Therefore, the decreased value of V_{max} obtained for factor IXa_{Xegf2} either could be a consequence of catalytically incompetent factor IXa molecules bound normally to the macromolecular factor X activating complex or, alternatively, could be due to deficient binding of factor IXa molecules that are functionally normal when assembled (albeit with decreased affinity) within the complex. To distinguish between these two possibilities, we calculated the k_{cat} values (turnover number) for factor IXa_{Xegf2}, factor IXa_{wt}, and factor IXa_{PD}, based upon the measured V_{max} values ($nM \cdot min^{-1}$) divided by the concentration (nM) of bound factor IXa molecules, determined as described under Methods. The k_{cat} values are presented in Table 1. Values for k_{cat} of 793 ± 47 and 799 ± 100 min^{-1} were obtained for factor IXa_{PD} and factor IXa_{wt}, respectively. Factor IXa_{Xegf2} yielded a k_{cat} of 1701 ± 176 min^{-1}. To determine whether these differences were significant, the *t*-distribution between the k_{cat} values of the chimera and wild-type and plasma-derived controls was calculated. The *t*-

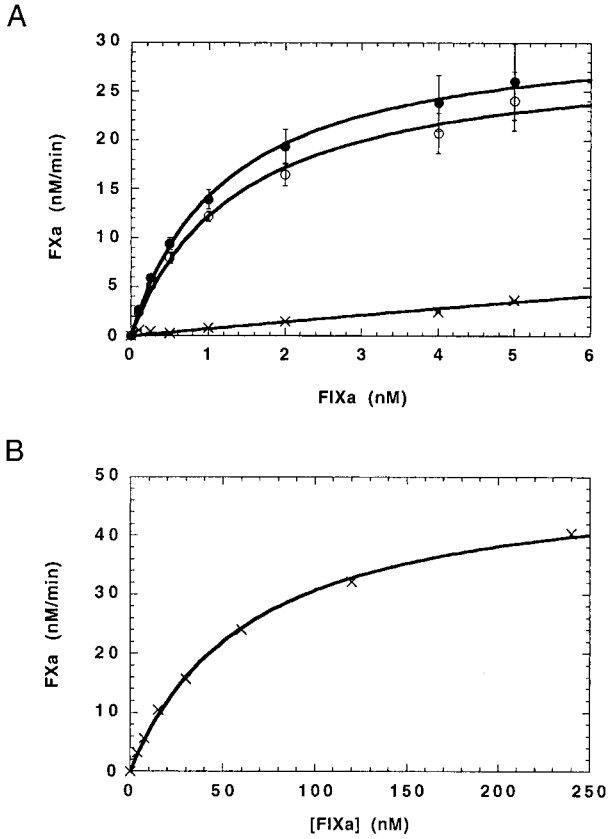

FIGURE 7: Determination of K_d^{app} of various factor IXa molecules for the activated platelet surface. (A) Rates of factor Xa formation were measured against varying concentrations of plasma-derived (●), wild type (○), and EGF2 chimeric (×) factor IXa molecules. The reactions were carried out as described under Methods using 5×10^7 activated platelets/mL in HEPES−Tyrode's buffer, pH 7.4 with 5 mM $CaCl_2$, 15 units/mL factor VIIIa, and factor X at a concentration $\sim 20 \times K_m$. Each point represents the mean of three independent experiments, each performed in duplicate. Error bars represent the standard error of the mean. (B) Rates of factor Xa formation were measured against higher concentrations of factor IXa_{Xegf2} (×) to achieve saturation. Each point represents the mean of duplicate determinations. Curves were drawn by a linear least-squares fit of the data to eq 1 using Kaleidagraph.

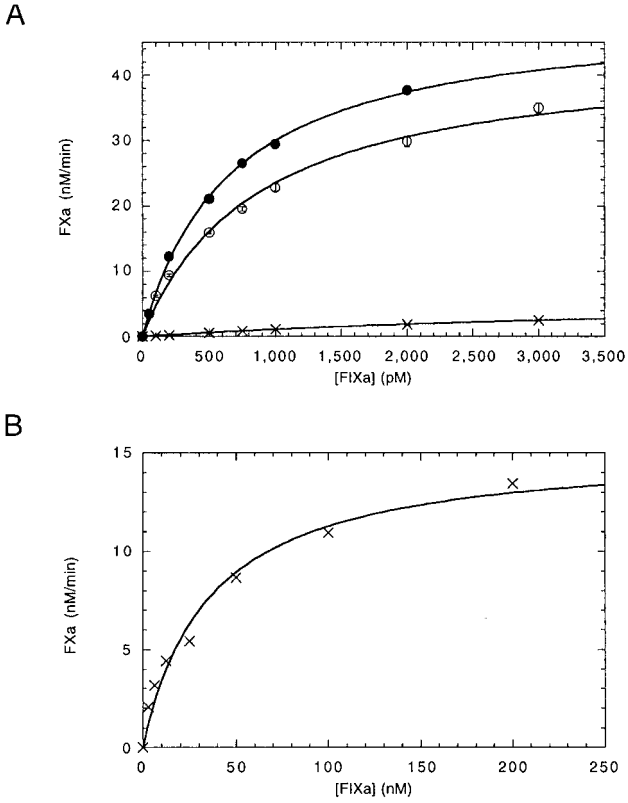

FIGURE 8: Determination of K_d^{app} of various factor IXa molecules for phospholipid vesicles. (A) Rates of factor Xa formation were measured against varying concentrations of plasma-derived (●), wild-type (○), and EGF2 chimeric (×) factor IXa molecules. The reactions were carried out as described under Methods using 200 pM PS/PC vesicles in HEPES−Tyrode's buffer, pH 7.4 with 5 mM $CaCl_2$, 15 units/mL factor VIIIa, and factor X at a concentration $\sim 20 \times K_m$ (250 nM). Each point represents the mean of three independent experiments, each performed in duplicate. Error bars represent the standard error of the mean. (B) Rates of factor Xa formation were measured against higher concentrations of factor IXa_{Xegf2} (×) to achieve saturation at a factor X concentration ~50 × K_m (700 nM). Each point represents the mean of duplicate determinations. Curves were drawn by a linear least-squares fit of the data to eq 1 using Kaleidagraph.

distribution value between the k_{cat} values of the chimera and wild-type factor IXa was 4.413 ($p < 0.01$), and the *t*-distribution value between the k_{cat} values of the chimera and plasma-derived factor IXa was 4.973 ($p < 0.01$).

The relative k_{cat} values for factor IXa_{Xegf2}, factor IXa_{wt}, and factor IXa_{PD} were also determined in the presence of phospholipid vesicles as described under the Methods. Actual k_{cat} values could not be determined due to the unknown number of factor IXa binding sites per phospholipid vesicle. The relative k_{cat} values are presented in Table 1. Compared with k_{cat} values of 34.0 and 27.3 min^{-1} obtained for factor IXa_{PD} and factor IXa_{wt}, respectively, factor IXa_{Xegf2} yielded a relative k_{cat} of 19.6 min^{-1}. The relatively normal k_{cat} values for factor IXa_{Xegf2} obtained in the presence of either activated platelets or phospholipid vesicles imply that the decreased V_{max} values obtained with the chimera are not a consequence of defective catalytic activity of the bound chimera but rather are due to the decreased affinity of the chimera for the surface. Furthermore, since k_{cat} values are cofactor dependent, relatively normal k_{cat} values suggest a normal cofactor interaction.

DISCUSSION

Using equilibrium binding studies to examine the specific interaction of the factor IXa_{Xegf2} chimera with activated platelets, we have shown that the intact EGF2 domain of factor IXa plays a role in the platelet receptor mediated binding that is essential for the assembly of the functional factor X activating complex. The binding studies demonstrate that factor IXa_{Xegf2} binds to ~200−300 sites per platelet, that the number of sites recognized by the chimeric zymogen and the chimeric enzyme are approximately the same, that the site to which factor IXa_{Xegf2} binds is the shared factor IX/IXa binding site, and that factor IXa_{Xegf2} does not bind normally to the specific factor IXa binding site (Figure 1). It follows from this analysis (a) that the intact EGF2 domain promotes the interaction of factor IXa with its specific binding site on activated platelets and (b) that the factor IXa_{Xegf2} chimera interacts normally with the shared factor IX/IXa binding site which is mediated by residues G_4−Q_{11} within the Gla domain. This region of factor IX is relatively unaffected by activation of factor IXa (and therefore is conformationally similar in factor IX and factor IXa), as demonstrated by Astermark and Stenflo (*37*) using confor-

mationally specific antibodies. The conclusion that the Gla domain (G_4-Q_{11}) mediates binding to the shared factor IX/IXa binding site is consistent with data from several laboratories on the interaction of other vitamin K-dependent proteins, such as factor X and prothrombin, with phospholipid surfaces (*27, 38*−*40*). Both the zymogen factor IX and a conformationally constrained synthetic peptide (G_4-Q_{11}) within the so-called "omega loop" of the amino terminus of the Gla domain compete with factor IX/IXa binding to the shared site with a K_i of ∼3 nM (*22*). However, a 55 000-fold higher concentration above the K_i of the synthetic peptide is required to cause significant inhibition of platelet-mediated factor X activation by factor IXa (K_i ∼165 μM). These observations suggest that the Gla domain (G_4-Q_{11}) site mediates binding of factor IXa and factor IX to a shared platelet binding site but not to a specific factor IXa binding site occupancy which is essential for the assembly of the functional factor X activating complex on the platelet surface (*22*). The hypothesis that the intact EGF2 domain is required to mediate this interaction, either directly or indirectly, is supported by the kinetic studies which show that factor IXa_{Xegf2}, which binds with normal affinity to the shared factor IX/IXa site (Figures 1 and 2), binds with significantly reduced affinity (K_d = 65 ± 3.9 nM) to the functional, specific factor IXa binding site (Figures 7 and 8 and Table 1). However, once bound to this site, albeit with reduced affinity, the chimeric enzyme is catalytically normal or possibly even supranormal (Table 1). This contention is confirmed by the data (Figure 7) demonstrating that the maximal rates of factor Xa generation achieved at saturating concentrations of factor IXa_{Xegf2} (∼40 nM/min) are close to those achieved at saturating concentrations of factor IXa_{PD} or factor IXa_{wt} (∼25−30 nM/min). Although factor IXa_{Xegf2} has a decreased affinity for the platelet surface, the normal velocity observed at saturating factor IXa concentrations indicates that once the mutant is bound, it behaves normally. That is, the finite number of factor IXa binding sites available for factor X activating complex formation on the platelet surface can be saturated by factor IXa_{Xegf2}, although the concentration required to achieve this effect is higher than for plasma-derived or wild-type factor IXa, indicating a binding defect.

The data implicating the Gla domain of factor IXa in the high-affinity binding of the enzyme to the shared factor IX/IXa binding site on the activated platelet surface are consistent with data obtained by other investigators from this laboratory and elsewhere (*20*−*22, 27, 38, 41*−*44*). Investigators from these laboratories have shown that the Gla domains of various vitamin K-dependent coagulation proteins are involved in their surface binding properties. Specifically Schwalbe et al. (*44*) and Scandura et al. (*27, 45*) have shown that the Gla domains of several other vitamin K-dependent clotting factors bind to phospholipid surfaces. These interactions have been shown to be of relatively low affinity and low specificity. For example, factor X and prothrombin compete with one another for low-affinity (K_d = 320 + 40 nM) binding to a single high-capacity (16 000 ± 4000 sites/platelet) site on activated platelets (presumably phospholipid) (*27*). This generalized binding property of the Gla domain to the same site is consistent with our observations that the Gla domain of factor IXa binds to a shared high-affinity receptor for either factor IX zymogen or active factor IXa. The observation that residues G_4-Q_{11} mediate binding to the shared factor IX/IXa binding site is consistent with NMR studies which identify residues Leu 6 and Phe 9 as comprising portions of a hydrophobic patch that interacts with artificial lipid membranes (*46*). Interestingly, Ryan et al. (*43*) have shown that the Gla and EGF-like domains are involved in the binding of factor IX to endothelial cells but not to phospholipids, which suggests a protein receptor interaction of either the Gla or the EGF-like domains of factor IXa.

Our data show that it is unlikely that the EGF2 domain is involved in cofactor binding since the kinetic defects characteristic of factor IXa_{Xegf2} were observed both in the presence and in the absence of factor VIIIa and were not significantly exacerbated by the addition of factor VIIIa. Typically, the addition of cofactor results in a 10 000-fold increase in k_{cat}, or a 100-fold increase in V_{max} under the experimental conditions employed here (*18*). If the EGF2 domain were involved solely in cofactor interaction, the chimeric mutant would be expected to display a defective rate enhancement upon the addition of factor VIIIa, whereas in the absence of cofactor, the rate of factor Xa formation should be normal. Such results were obtained by Larson and co-workers (*47*) in studies of a novel, mutant, human factor IX protein from a patient with hemophilia B, demonstrating that structural integrity of the Gla domain of factor IXa is required for its binding to cofactor VIIIa. In the present studies, factor IXa_{Xegf2} was shown to be defective in catalyzing factor X activation in the absence of factor VIII, and the 93-fold increase in V_{max} observed with the EGF2 chimera upon the addition of factor VIIIa to the reaction (an increase from 2.9 ± 0.37 to 270 ± 23 pM/min, see Table 1) was quantitatively similar to that observed with factor IXa_{wt} (130-fold) and factor IXa_{PD} (128-fold). Furthermore, since k_{cat} is predominantly affected by factor VIIIa interaction and the platelet-bound mutant appears to possess normal values for k_{cat}, the factor VIIIa binding site responsible for enhanced catalysis is intact in the mutant. We therefore conclude that in surface-mediated catalysis, factor IXa_{Xegf2} interacts relatively normally with cofactor.

In seeking to explain the complex kinetic abnormalities observed with factor IXa_{Xegf2} (decreased V_{max}, increased K_m), we postulate that defective binding of the chimera to a functionally important platelet or phospholipid binding site might reveal itself as diminished maximal rates of catalysis as a consequence of a decreased concentration of bound (i.e., functionally active) enzyme. The apparent decrease in substrate affinity (i.e., increased K_m) could also result from diminished colocalization of factor IXa_{Xegf2} and factor X within the functional enzyme−substrate complex on the platelet surface. Studies from our laboratory and others have demonstrated the validity of a three-receptor complex in which coordinate occupancy of binding sites for factor IXa, factor VIII, and factor X is required for optimal rates of factor X activation on the platelet surface (*16, 17, 19, 27, 45, 48*). In the absence of factor IXa and factor VIII, factor X binds to a high-capacity (16 000 ± 2000 sites per platelet), low-affinity (K_d = 320 ± 40 nM) site, shared with prothrombin, on the activated platelet surface (*27*). Factor X bound to this site is preferentially activated by platelet-bound factor IXa (*45*). An additional high-affinity (K_d = 10−30 nM), low-capacity (1000−1500 sites per platelet) site, absolutely specific for factor X, is generated in the presence of the

enzyme (factor IXa) and the cofactor (factor VIII) (*49, 50*). This high-affinity factor X binding site probably represents binding of substrate to the functional factor X activating complex (*27*). Thus, the binary complex of factor IXa and factor VIIIa on the activated platelet surface may stabilize factor X binding to the platelet surface by formation of the ternary factor X activating complex consisting of factor IXa, factor VIIIa, and factor X. These facts could explain the increased K_m observed with factor IXa_{Xegf2} if the sole defect of the chimera were its capacity to bind to the functional platelet receptor and generate (with factor VIII) a high-affinity substrate (factor X) binding site.

Alternatively, the increased K_m of the mutant may simply reflect the influence of the fluid phase K_m of a molecule (factor IXa_{Xegf2},) which does not bind surface properly. Thus, factor X activation by factor IXa in the absence of a surface is characterized by a very high K_m ($\sim$80 μM), and the predominant kinetic effect of the presence of activated platelets or phospholipids is a large decrease in K_m (*18*). Consequently, the increased K_m characteristic of the chimera may reflect a composite of fluid-phase and surface-dependent catalysis by factor IXa_{Xegf2}, which binds with decreased affinity to the surface.

The rationale for carrying out kinetic studies of factor X activation by factor IXa_{Xegf2} on phospholipids compared with the activated platelet surface was to determine whether platelet membrane proteins are involved in the specific, high-affinity binding of factor IXa that apparently requires an intact EGF2 domain. Although the exact mechanism of rate enhancement due to a phospholipid surface in factor X activation is not known, presumably it is protein−receptor−independent since phospholipids contain no protein. It follows that a mutant chimeric factor IXa molecule, which is only defective in its protein−receptor binding interaction, should manifest entirely normal kinetics on a phospholipid surface since the rate-enhancing properties of phospholipid are independent of this receptor binding. In contrast, if phospholipids comprise the surface agent in platelet membranes that mediate the EGF2 domain-dependent binding of factor IXa, then identical defects would be seen with the EGF2 chimera on phospholipid vesicles when compared to activated platelets. Although there are some quantitative differences in the kinetic constants (V_{max} and K_m) observed with phospholipids compared with activated platelets, the values of catalytic efficiency (V_{max}/K_m) for factor IXa_{Xegf2} expressed as percentages of normal (Table 1) are generally similar for reactions carried out in the presence of activated platelets or phospholipids either in the presence or in the absence of factor VIII. Similarly, the values of K_d^{app} for factor IXa_{Xegf2} are $\sim$60-fold higher than those for normal factor IXa (either plasma-derived or wild type) for both the phospholipid and the activated platelet surfaces. These data provide no support for the conclusion that a specific protein receptor, present in platelet membranes but absent from phospholipid vesicles, mediates the EGF2-dependent binding of factor IXa to activated platelets. However, the present studies do not directly address the biochemical nature of the platelet receptor and do not exclude the possibility of a phospholipid-like protein receptor for factor IXa in platelet membranes. Moreover, previous studies from our laboratory on the role of electrostatic interactions (*51*) and the effects of annexin V(*52*) on factor IXa catalyzed factor X activation suggest that different mechanisms, possibly involving protein receptors in platelets, mediate the assembly of the factor X activating complex on surfaces consisting of activated platelet membranes and phospholipid vesicles.

The hypothesis that the intact EGF2 domain is required for binding to the specific functional factor IXa binding site on the platelet surface is consistent with the observations of Ryan et al. (*43*), Nishimura et al. (*53*), and Murphy and McGregor (*54*) confirming the importance of EGF-like domains in protein/protein interactions. Murphy and McGregor (*54*) have shown that the lectin and EGF-like domains of p-selectin mediate the binding of monocytes to thrombin-activated endothelial cells. Since EGF primarily functions as a ligand for its membrane-bound receptor, the EGF receptor (*55*), it seems plausible to postulate that through the mechanisms of divergent evolution, the EGF-like modules of factor IX have retained the membrane receptor binding properties of EGF from which it may have evolved.

Several additional studies have focused on the functions of the EGF-like domains of factor IX/IXa (*23, 24, 33, 37, 56*−*61*). Hughes et al. have shown that Tyr 69 in the EGF1 domain of factor IXa is critical for factor VIIIa dependent factor X activation in site-directed mutagenesis studies (*60*). The possibility that the EGF1 domain of factor IXa contains a cofactor binding site has also been suggested by O'Brien et al. (*61*) in their studies of the binding of the light chain of factor IXa to factor VIII. Pemberton et al. (*62*) have also suggested a similar function based on molecular modeling techniques of factor VIII. Zhong et al. (*33*) have shown that the EGF1 domain plays a role in factor IX activation by factor VIIa/tissue factor, whereas Astermark, Stenflo, and their colleagues have presented evidence suggesting that it may be involved in substrate (factor X) binding (*11, 33, 37, 58, 59, 63*). Ambrosini et al. (*56*) have shown that the inter-EGF sequence of factor X binds to effector cell protease receptor-1, a protein receptor on human endothelial cells. Finally, the EGF domains of factor IX may be intimately involved in interdomain interactions with one another and with the Gla domain and in conformational stabilization of the factor IX (IXa) molecule (*64, 65*). Thus, the many roles of the EGF-like domains of factor IX are complex and multifunctional, suggesting both direct and indirect interactions with either substrate, cofactor, and/or receptor by direct binding or conformational stabilization (*66, 67*).

The present functional studies utilizing a chimera (factor IXa_{Xegf2}) consisting of a normal factor IXa molecule with the entire EGF2 domain replaced by homologous amino acid sequences from factor X were based on the premise that, since the backbone structures of many serine proteases are highly conserved whereas surface-exposed amino acids that mediate ligand interactions are often not conserved and are unique to each specific protein (*23, 68*), replacement of the EGF2 domain of factor IXa with the homologous region from factor X should allow the protein to retain its general conformation, whereas any amino acid residues involved in ligand interactions or interdomain contacts should be lost. In the chimera we have prepared, Arg 94 has been changed to an Asp in the native factor X sequence. Brandstetter et al. (*67*) have shown the existence of a salt bridge between Glu 78 in the EGF1 domain and Arg 94 in the EGF2 domain of human factor IX. These two amino acids, Glu 78 and Arg 94, are strictly conserved among factor IX sequences,

suggesting an important interaction. Subsequently, Christophe et al. (*69*) have shown the importance of this interdomain interaction for factor VIIIa binding on a phospholipid surface. A molecule with a Glu 78 Lys mutation was shown to have normal amidolytic activity and normal catalytic activity on a phospholipid surface (*69*). This mutant, however, only possessed 10% of the normal catalytic activity in the presence of cofactor on the phospholipid surface, suggesting that the interaction of Glu 78 with Arg 94 may play a role in cofactor binding on the PS/PC surface. Interestingly, the activity of the mutant was restored when Arg 94 was also mutated to Asp, suggesting that the electrostatic interaction between EGF1 and EGF2 through residues 78 and 94 was critical for factor VIIIa binding. Thus, it is entirely plausible for changes in one domain of factor IXa to affect interdomain interactions within the same molecule. This idea is supported by structural and mutational studies by other investigators (*64*, *67*) demonstrating the importance of interdomain interactions of factor IXa in its function within the factor X activating complex. The importance of this interaction is also suggested by hemophilic mutations of these residues which result in detectable antigen levels but minimal activity (*70*, *71*). Moreover, Hertzberg et al. (*72*) have shown that a hemophilic mutation in Arg 94 resulted in a protein with near-normal antigen levels and minimal clotting activity (1−2%). This mutant possessed an intact Gla domain based on its normal calcium binding properties and normal amidolytic activity. On a phospholipid surface, the factor X activating activity of the mutant was markedly reduced with little effect on K_m. These results (*72*) are consistent with the present data suggesting an important role of Arg 94 in assembly of the factor X activating complex.

The functional roles of the two surface binding sites defined herein, i.e., the shared factor IX/IXa (i.e., zymogen) binding site, mediated by the Gla domain, and the specific factor IXa (i.e., enzyme) binding site, mediated by the EGF2 domain, are subjects for future investigation. The binding of factor IXa to its high-affinity receptor may possibly function to orient the complex to promote optimal spatial orientation of the active site and substrate cleavage site. Although the importance of the shared factor IX/IXa site is not yet understood since it is not required for normal catalysis, it may function to promote catalysis of factor IX activation by colocalizing factor IX zymogen with its activating enzyme factor XIa on the surface of activated platelets (*73*, *74*). Another possibility is that the zymogen binding site may serve to provide a surface-bound pool of activated factor IXa which can then be 'passed' to the functional site receptor in a kinetically efficient two-dimensional, surface-bound system. From the data presented here, we conclude that the intact EGF2 domain of factor IXa is critical for the binding of the enzyme to a specific phospholipid-like platelet binding site which mediates the assembly of the factor X activating complex whereas the Gla domain interacts with a shared factor IX/IXa binding site on the platelet surface.

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