# High-Affinity, Specific Factor IXa Binding to Platelets Is Mediated in Part by Residues 3–11[†]

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*Received March 9, 1994; Revised Manuscript Received June 20, 1994*[⊗]

ABSTRACT: To identify the amino acids in the Gla domain that mediate factor IXa binding to human platelets, we have used chimeric molecules and point mutations in the Gla domain of recombinant factor IX, based on molecular modeling using the coordinates of the Gla domain of bovine prothrombin, which reveals two surface structures whose sequences differ among factor IX, factor X, and factor VII. Binding to thrombin-activated platelets of factor IXa in the presence of factor VIIIa (2 units/mL) and factor X (1.5 $\mu$M) revealed a stoichiometry of ~550 sites per platelet with a $K_d$ of ~0.65 nM compared with a $K_d$ of ~2.5 nM in the absence of factor VIIIa and factor X. In contrast, mutations of factor IX to factor X residues at positions 4 and 5 or at positions 9, 10, and 11 resulted in decreases in the number of sites and affinity of factor IXa binding in the presence or absence of factor VIIIa and factor X. A chimera consisting of the Gla domain of factor VII with factor IX residues at positions 33, 34, 35, 39, and 40 displayed abnormal factor IXa binding and a decreased $V_{max}$ and a normal $K_m$ for factor X activation, and the replacement of amino acid residues 3–10 with those of factor IX restored normal binding and factor X activation kinetics to this chimeric protein. Our data indicate that the high-affinity, specific binding of factor IXa to activated platelets in the presence or absence of factor VIIIa and factor X is the major determinant of rates of factor X activation and that both factor IXa binding and factor X activation are mediated at least in part by amino acids exposed on the surface of the Gla domain within positions 3–11, possibly by residues 4, 5, 9, 10, and 11.

The interaction between blood platelets and coagulation factors is essential for normal coagulation and hemostasis. In our previous studies we have examined the mechanism by which platelets can promote factor X activation by factor IXa (Ahmad et al., 1989a–c, 1992a; Rawala-Sheikh et al., 1990). Recently, we began an analysis of the structural features of the factor IX molecule that are important for assembly of the factor X activating complex on the platelet surface (Ahmad et al., 1990, 1992b; Rawala-Sheikh et al., 1992). These studies have shown that a major determinant of factor IXa binding to its normal human platelet receptor resides within its γ-carboxyglutamic acid (Gla)[1] domain (Rawala-Sheikh et al., 1992). In contrast, the first epidermal growth factor domain of factor IXa does not appear to be involved in factor IXa binding to platelets (Ahmad et al., 1992b). We have used both Gla-modified and Gla-domainless factor IXa molecules and concluded that factor IXa binding to platelets is mediated in part, but not exclusively, by high-affinity $Ca^{2+}$ binding sites in the Gla domain of factor IX (Rawala-Sheikh et al., 1992).

The purpose of the present studies was to identify specific amino acid residues within the Gla domain that mediate the binding of factor IXa to platelets. To accomplish this purpose, we have constructed a computer-generated model based upon the known structure of bovine prothrombin fragment 1 in order to identify amino acid residues exposed on the surface of the protein that might comprise a binding site for the platelet receptor. We reasoned that if such amino acid residues were altered to those present in highly homologous proteins such as factor VII or factor X, the capacity of the resulting mutated or chimeric protein to bind to platelets might be diminished. The assumption underlying this rationale is that the tertiary structure of the Gla domain is highly conserved in the vitamin K-dependent proteins, such as factor IX, factor X, factor VII, and prothrombin, and that surface residues unique to any particular protein mediate specific protein–protein interactions. Recent studies of bovine aortic endothelial cells using several factor IX–factor VII chimeras implicated specific residues within the Gla domain in factor IX binding (Cheung et al., 1991; Toomey et al., 1992). These studies demonstrated, using computer-generated models for the Gla domain of factor IX, that the endothelial cell binding determinant resides on a prominent surface within the first 11 amino acids of factor IX. Recent studies have ruled out any specific binding role for either the first EGF-like domain or the eight amino acid

[†] This study was supported by research grants from the National Institutes of Health (HL45486, HL25661, and HL46213 to P.N.W. and HL38973 to D.W.S.), by the W. W. Smith Charitable Trust (to P.N.W.), and by The Council For Tobacco Research (Grant 3190 to S.S.A.).

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[⊗] Abstract published in *Advance ACS Abstracts,* September 1, 1994.

[1] Abbreviations: EGF, epidermal growth factor; Gla, γ-carboxyglutamic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; PPACK, D-phenylalanylpropyllarginyl chloromethyl ketone.

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hydrophobic stack domain of factor IX in its interaction with its endothelial cell receptor (Cheung et al., 1992). In this paper we have used nine different recombinant factor IX (rFIX) molecules to show that the high-affinity, specific binding of factor IXa to activated platelets in the presence or absence of factor VIIIa and factor X is mediated at least in part by amino acids exposed on the surface of the Gla domain within positions 3–11, possibly by residues 4, 5, 9, 10, and 11.

## EXPERIMENTAL PROCEDURES

*Materials.* The chromogenic substrate, S2337 [Bz-Ile-Glu-(γ-piperidyl)Gly-Arg-*p*-nitroanilide], was purchased from AB Kabi Diagnostica (Stockholm, Sweden). *p*-Aminobenzamidine was obtained from Sigma Chemical Co. (St. Louis, MO). D-Phenylalanylprolylarginyl chloromethyl ketone (PPACK) was purchased from Calbiochem-Behring Corp. (San Diego, CA). Carrier-free $Na^{125}I$ was obtained from Amersham Corp., Arlington Heights, IL. All other reagents and chemicals used were the same as previously reported (Ahmad et al., 1989a) and were obtained from Sigma Chemical Co. (St. Louis, MO), Aldrich Chemical Co. (Milwaukee, WI), and Calbiochem-Behring Corp. (San Diego, CA) and were of the highest grade commercially available.

*Proteins.* Human coagulation proteins, including factor IX, factor IXa, factor VIII, factor X, and α-thrombin, were purified, assayed, and characterized as previously published (Ahmad et al., 1989a). The conditions used for activation of factor VIII with human α-thrombin were identical to those previously published (Ahmad et al., 1989a). All proteins were >98% pure as determined by polyacrylamide slab gel electrophoresis in $NaDodSO_4$ (Laemmli, 1970). Protein concentrations were determined by the Bio-Rad dye binding assay according to instructions provided by the manufacturer (Bio-Rad, Richmond, CA). Mutant proteins were prepared and characterized as previously described (Lin et al., 1990). The proteins, expressed in human embryo kidney cells, were purified from cell supernatants as reported previously (Lin et al., 1990; Cheung et al., 1991). Briefly, these proteins were purified on a monoclonal antibody affinity column (Smith et al., 1987) which binds to factor IX molecules that have undergone a normal conformational alteration in the presence of calcium ions. The purified chimeric factor IX molecules demonstrated normal fluorescence quenching (Cheung et al., 1991) in the presence of increasing concentrations of $CaCl_2$ (data not shown). Analysis of Gla was performed at Merck Sharp and Dohme according to the procedure of Przysiecki et al. (1987). All the normal plasma-derived, the wild-type, and the chimeric factor IX molecules were radiolabeled with $^{125}I$ by the iodogen method as previously described (Ahmad et al., 1989a), and specific radioactivities of all proteins were in the range of (2.0–2.5) × $10^6$ cpm/μg. Activation of both normal and chimeric factor IX molecules by purified factor XIa was carried out as previously described (Ahmad et al., 1989a). We also utilized the *p*-aminobenzamidine fluorescence assay to examine quantitatively the activation of normal, plasma-derived factor IX ($IX_N$) and recombinant factor IX molecules as previously reported (Monroe et al., 1988; Lin et al., 1990). Following gel electrophoresis, autoradiograms of normal and chimeric factor IXa molecules were developed to provide structural characterization of $^{125}I$-labeled proteins. Both recombinant proteins and factor IXa wild type (factor $IXa_{wt}$) migrated under reducing conditions as two polypeptide chains of $M_r$ 27 000 and 17 000 representing the heavy and light chains (Figure 1, lanes 2–11) and were indistinguishable from plasma-derived factor $IXa_N$ (Figure 1, lane 2). Labeled factor $IX_N$, which was a single band at $M_r$ 60 000 under reducing conditions, is shown for comparison (Figure 1, lane 1). Protein-stained gels of all unlabeled proteins used in this study showed entirely similar results (not shown), thus confirming the purity and chain composition of both plasma-derived and recombinant proteins.

*Binding Experiments.* In a typical binding experiment, gel-filtered platelets [(3–4) × $10^8$/mL] in calcium-free 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) Tyrode's buffer, pH 7.4, were incubated at 37 °C in a 1.5-mL Eppendorf plastic centrifuge tube with mixtures of unlabeled and radiolabeled factor IXa (0.1–20 nM), $CaCl_2$ (5 mM), human α-thrombin (0.1 unit/mL) in the presence or absence of factor X (1.5 μM), and thrombin-activated factor VIII (2 units/mL) as detailed previously (Ahmad et al., 1989a). Platelets were separated from unbound proteins as previously described (Ahmad et al., 1989a). The data were analyzed, and the number of binding sites and dissociation constants ($K_d$) were calculated from the mean of three independent determinations, each done in duplicate, as previously described (Ahmad et al., 1989a) using a Macintosh Quadra 900 computer (Apple Computer, Inc., Cupertino, CA) and the LIGAND program was modified by G. A. McPherson (Elsevier Science Publishers BV, The Netherlands, 1985).

*Measurement of Rates of Factor Xa Formation.* Activation of factor X by factor $IXa_N$, factor $IXa_{wt}$, or chimeric or mutant factor IXa molecules was performed at 37 °C in the presence of thrombin-stimulated gel-filtered platelets, factor VIIIa, and $CaCl_2$ as described previously (Rawala-Sheikh et al., 1990). The details of experimental conditions and concentration of reactants are given in the Results section and in the figure legends.

*Calculation of Kinetic Constants.* The kinetic constants for factor X activation by factor IXa were derived on the basis of a one enzyme–one substrate model. The Michaelis constant ($K_m$) and the maximum velocity ($V_{max}$) were calculated from the mean ± SEM of four to six independent experiments each performed in duplicate and analyzed by the Levenberg–Marquardt method (Marquardt, 1963) utilizing Kaleida Graph (Synergy Software, PCS Inc., Reading, PA) run on a Macintosh Quadra 900 computer (Apple Computer, Inc., Cupertino, CA). Values of $K_d$ were obtained from experiments in which rates of factor X activation were determined at different factor IXa concentrations as described previously (Ahmad et al., 1989a–c; Rawala-Sheikh et al., 1990). The turnover number ($k_{cat}$) for platelet-bound enzyme was determined from factor X activation and factor IXa binding performed simultaneously as described previously (Ahmad et al., 1989; Rawala-Sheikh et al., 1992). Briefly, $k_{cat}$ was calculated as $V_{max}$ divided by the amount of factor IXa bound, where the amount bound is $B_{max}[IXa]/(K_d + [IXa])$.

*Molecular Modeling.* The model of the factor IX Gla domain was constructed from residues 1–47 of the coordinates of the bovine prothrombin fragment 1 crystal structure (Soriano-Garcia et al., 1992). Amino acid replacements of factor IX within the prothrombin structure were made according to sequence alignment and were performed using the biopolymer module provided within the SYBYL computational chemistry package (Tripos Associates Inc., St. Louis, MO). The Amber force field, as implemented in SYBYL, was utilized in all the subsequent calculations (Weiner et al., 1984). Atomic parameters describing calcium and the γ-carboxylated glutamic acid residues were added to the force-field tables. Because the atomic properties of calcium (a transition-state metal) are inadequately described within the force field in order to account for the coordination complexes formed with the negative charges of the γ-car-

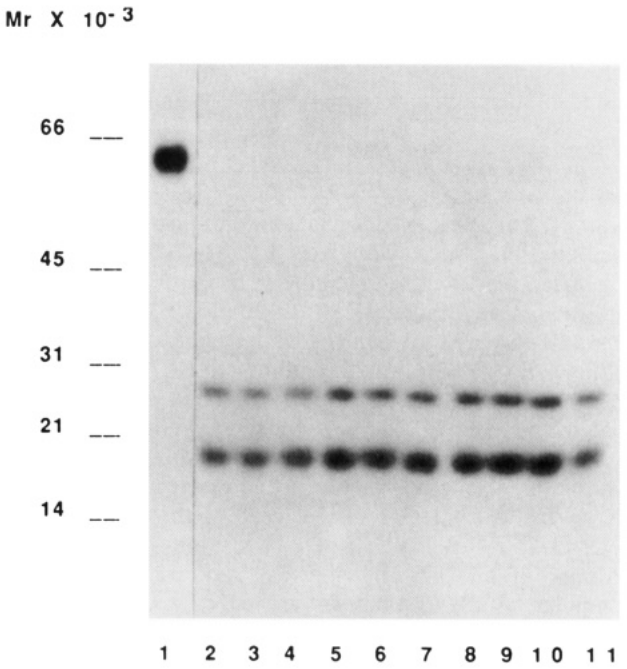


FIGURE 1: Autoradiogram of normal and chimeric $^{125}$I-labeled factor IXa molecules. Autoradiograms are shown of 13% polyacrylamide gel electrophoretograms in NaDodSO$_4$ of $^{125}$I-labeled factor IX$_N$, factor IXa$_N$, and factor IXa$_{wt}$ (lanes 1–3). Lanes 4–11 show various chimeras of factor IXa molecules corresponding to construct numbers 2–9 as shown in Table 1.

boxylated glutamic acids, the distance geometries measured in the prothrombin Gla domain coordinated complexes were held as restraints during the modeling process of creating the factor IX structure.

After all amino acid replacements were completed, the structure was energy minimized to convergence using a conjugate-gradient approach. The newly minimized structure was then solvated with water (two solvent shells were added to ensure that all portions of the surface were adequately solvated) using the Silverware algorithm as implemented in SYBYL. The water–protein complex was again minimized prior to an energy-dependent simulation of molecular motion ($t$ = 100 ps). Reviews of the trajectory files obtained after this dynamic run indicated that a stable low-energy structure was obtained after ~12 ps. The structure shown in Figure 2 represents the low-energy conformer pulled from the trajectory file.

## RESULTS

*Characterization of Labeled Factor IXa Molecules.* We have used one normal and ten recombinant factor IXa molecules in this study. Of the ten recombinant factor IXa molecules one is wild type (factor IXa$_{wt}$) and the rest are chimeric proteins composed of portions of factor VII or factor X in factor IX molecules. These proteins were produced in the human embryo kidney cell line 293, immunopurified, and extensively characterized as previously described (Cheung et al., 1992). The recombinant factor IX molecules were radiolabeled with $^{125}$I and activated to factor IXa by factor XIa as previously described (Ahmad et al., 1989a). The mobilities of these proteins were determined by NaDodSO$_4$—polyacrylamide gel electrophoresis relative to plasma factor IX (Figure 1). The factor IX molecules were further characterized for their Gla content. The analysis revealed that all of the recombinants contained 8.6–9.6 mol of Gla/mol of protein. By comparison, two preparations of purified, plasma-derived factor IX had 8.9 and 9.2 mol of Gal/mol of protein, a value close to that reported as normal for fully carboxylated factor IX (Lin et al., 1990; Derian et al., 1989). Table 1 summarizes the constructs used and the results of the Gla analyses.

*Specific Binding of $^{125}$I-Labeled Factor IXa$_N$ and Various Chimeric Factor IXa Molecules to Thrombin-Activated Normal Human Platelets.* To study the structural requirements for factor IXa binding to platelets, equilibrium binding studies were carried out in the absence and presence of saturating concentrations of factor X and thrombin-activated factor VIII, with nine different forms of chimeric proteins including wild-type recombinant factor IXa molecules (Figure 3 and Table 2) and with purified plasma-derived factor IXa (Table 2).

Scatchard analysis of binding data (Figure 3) gave straight lines, indicating the presence of a single class of binding sites for factor IXa$_N$, factor IXa$_{wt}$, and all chimeric factor IXa molecules. The affinity and stoichiometry of binding for these ligands under equilibrium experimental conditions were determined for each protein in four separate experiments, the means (±SE) of which are given in Table 2. In the absence

Table 1: Summary of the Constructs Used with Corresponding Gla, EGF, and Heavy-Chain (HC) Regions of Each Molecule and Results of Gla Analysis[a]

| Construct No. | Ligand | Gla | EGF + HC | Mol Mol | Gla/ FIX |
|---|---|---|---|---|---|
| 1 | FIXa$_{wt}$ Y N S G K L E E F V Q G N L E R E C M E E K C S F E E A R E V F E N T E R T T E F W | FIX | FIX | 8.6 | |
| 2 | 45 Y N S (f)(-) L E E F V Q G N L E R E C M E E K C S F E E A R E V F E N T E R T T E F W | FIX | FIX | 9.3 | |
| 3 | 9-11 Y N S G K L E E (m)(k)(k) G N L E R E C M E E K C S F E E A R E V F E N T E R T T E F W | FIX | FIX | 9.5 | |
| 4 | Y1A (a) N S G K L E E F V Q G N L E R E C M E E K C S F E E A R E V F E N T E R T T E F W | FIX | FIX | 9.2 | |
| 5 | K5R Y N S G (r) L E E F V Q G N L E R E C M E E K C S F E E A R E V F E N T E R T T E F W | FIX | FIX | 9.2 | |
| 6 | K5A Y N S G (a) L E E F V Q G N L E R E C M E E K C S F E E A R E V F E N T E R T T E F W | FIX | FIX | 9.6 | |
| 7 | V10K Y N S G K L E E F (k) Q G N L E R E C M E E K C S F E E A R E V F E N T E R T T E F W | FIX | FIX | 9.2 | |
| 8 | ---- a n a f - l e e l r p g s l e r e c k e e q c s f e e a r e i f (E)(N)(T) e r t (T)(E) f w | FVII | FIX | 8.9 | |
| 9 | ---- a n a f - l e e l r p g s l e r e c k e e q c s f e e a r e i f (E)(N)(T) e r t (T)(E) f w | FVII | FVII | 8.9 | |
| 10 | ---- (Y) n (S)(G)(K) l e e (F)(V)(Q) g s l e r e c k e e q c s f e e a r e i f (E)(N)(T) e r t (T)(E) f w | FVII | FIX | 9.2 | |

[a] All capital letters depict factor IX sequences. All lower case letters depict factor VII sequences. All italics depict corresponding residues from factor X. All point mutations are circled. Line 1 is factor IX$_{wt}$. Surface residues at 4 and 5 (line 2) and 9, 10, and 11 (Line 3) were changed to comparable residues in human factor X. Line 4 shows Tyr 1 changed to Ala 1 (Y1A) whereas lines 5–7 show point mutations K5R, K5A, and V10K, respectively. Line 8 shows addition of factor IX residues 33–35 and 39–40 to the Gla domain of factor VII attached to factor IX. Line 9 shows the same mutations as in line 8 in a Gla domain of factor VII attached to factor VII molecule. Line 10 shows the addition of factor IX residues 1, 3–5, and 9–11 in addition to the changed residues shown in line 8.

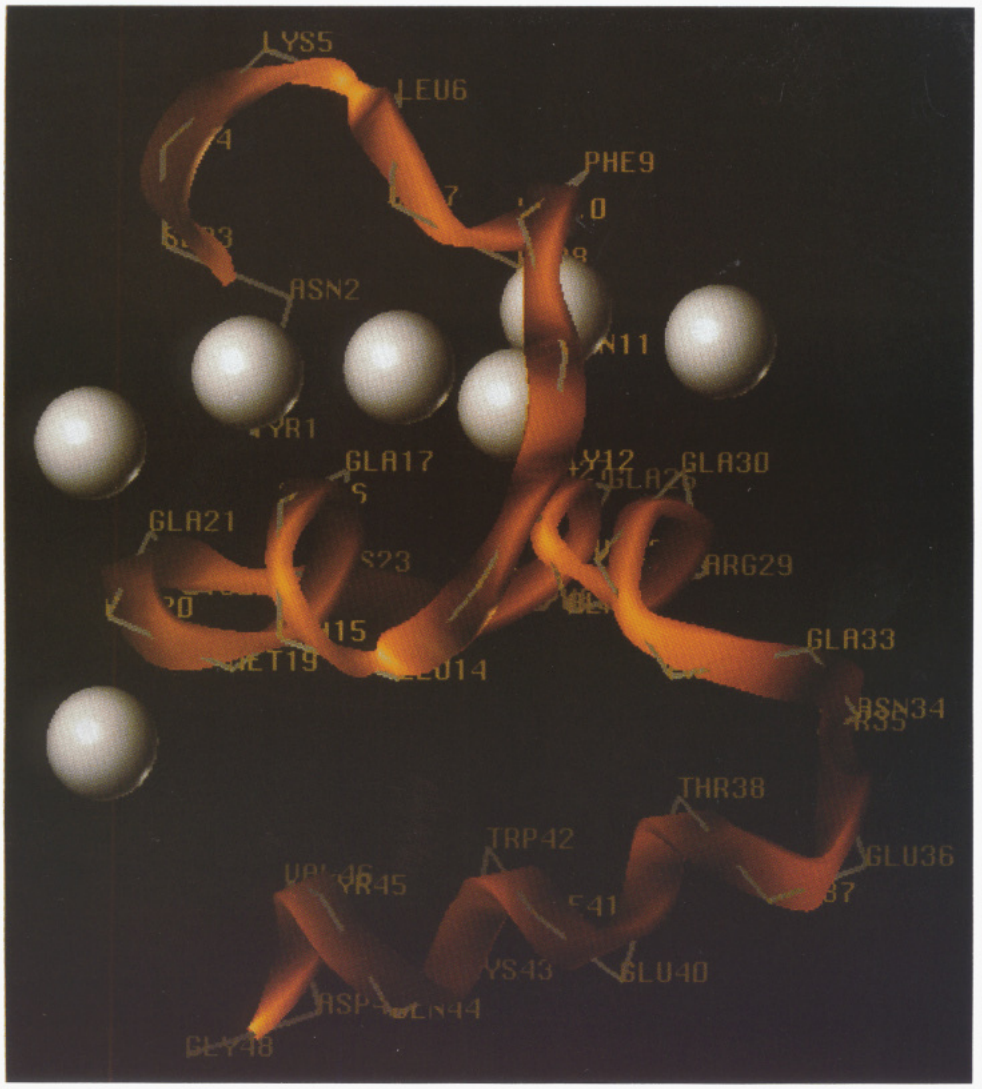


FIGURE 2: Model of the factor IX Gla domain with the inclusion of the calcium ions (white spheres). The model shows a surface-exposed loop structure in profile consisting of the following amino acids: Gly 4, Lys 5, Leu 6, Phe 9, Val 10, and Gln 11.

of factor VIIIa and factor X, there were 610 ± 62 sites per platelet ($K_d$ = 2.70 ± 0.30 nM) for factor $IXa_N$, and a similar number of sites and $K_d$ were obtained for factor $IXa_{wt}$ (570 ± 45 sites per platelet; $K_d$ = 2.9 + 0.25 nM). The presence of factor VIIIa and factor X, both at saturating concentrations, had no effect on the number of binding sites for factor $IXa_N$ or factor $IXa_{wt}$ molecules and resulted in a decrease in the $K_d$ for these proteins to 0.65 and 0.80 nM, respectively. Figure 3B shows the specific binding of chimeric factor IXa molecules 4, 5 and 9–11. In these chimeric factor IXa molecules, surface residues at positions 4 and 5 (construct 2) and surface residues at positions 9, 10, and 11 (construct 3) were changed to comparable residues in human factor X (Table 1). It is apparent that the number of binding sites for factor $IXa_{wt}$ (570 ± 45) was significantly greater than for either factor $IXa_{4,5}$ (300 ± 42) or factor $IXa_{9-11}$ (260 ± 28). These studies also demonstrated that the presence of factor VIIIa and factor X, both at saturating concentrations, had no effect on either the number of binding sites or affinity of binding of factor $IXa_{4,5}$ or factor $IXa_{9-11}$. In addition, the affinity of binding for both factor $IXa_{4,5}$ ($K_d$ = 5.9 ± 1.0 nM) and factor $IXa_{9-11}$ ($K_d$ = 9.10 ± 2.0 nM) was significantly reduced as compared with factor $IXa_{wt}$.

Figure 3C represents specific binding of two mutant factor IXa molecules, Y1A (Tyr 1 changed to Ala) and K5R (Lys 5 changed to Arg), compared with factor $IXa_{wt}$. In the absence of factor VIIIa and factor X, there were 560 ± 40 sites per platelet ($K_d$ = 2.85 ± 0.33 nM) for the factor IXa Y1A mutation and 690 ± 74 sites per platelet ($K_d$ = 3.20 ± 0.28 nM) for the factor IXa K5A mutation which were similar to the number of sites (570 ± 45) and $K_d$ (2.90 ± 0.25 nM) obtained for factor $IXa_{wt}$ under similar experimental conditions. The presence of factor VIIIa and factor X, both at saturating concentrations, had no effect on the number of binding sites for factor $IXa_{wt}$ or recombinant factor IXa mutant Y1A or K5R molecules and resulted in a decrease in the $K_d$ for these proteins to 0.80, 0.70, and 0.90 nM, respectively. Two additional point mutations, K5A (Lys 5 changed to Ala) and V10K (Val 10 changed to Lys), were studied. With K5A and V10K we obtained 400–500 sites per platelet with a $K_d$ of 3.5–3.7 nM in the absence of factor VIIIa and factor X and 400–550 sites per platelet with a $K_d$ of 2.0–3.5 nM in the presence of factor VIIIa and factor X. Thus, the major difference observed between recombinant factor IXa K5A and V10K mutant molecules and factor $IXa_{wt}$ molecules was the absence of enhanced affinity of binding of K5A and V10K to platelets in the presence of factor VIIIa and factor X (Figure 3D and Table 2).

To further characterize the residues within factor IXa responsible for its binding to platelet receptors, we have prepared a number of mutations in the Gla domain and aromatic stack region of factor VII attached to the EGF-like domains, activation peptide, and heavy chain of factor IX. We selected three constructs for this study (Table 1), namely,

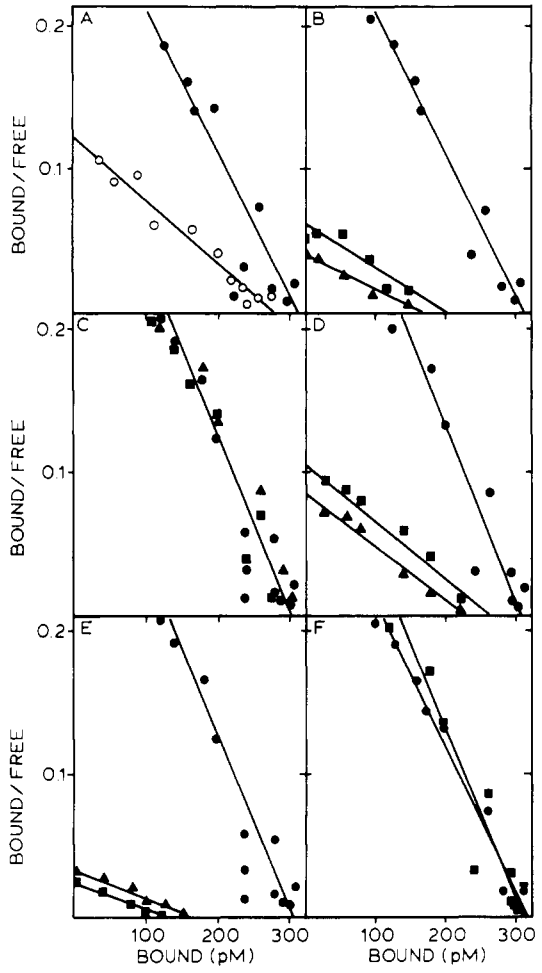


FIGURE 3: Equilibrium binding of normal chimeric factor IXa molecules to platelets. Gel-filtered platelets ($3.5 \times 10^8$/mL) were incubated for 20 min at 37 °C with human $\alpha$-thrombin (0.1 unit/mL), $CaCl_2$ (5 mM), and $^{125}$I-labeled factor $IX_N$, $^{125}$I-labeled factor $IXa_{wt}$, or chimeric $^{125}$I-labeled factor IXa molecules in the presence and absence of thrombin-activated factor VIII (2 units/mL) and factor X (1.5 $\mu$M). Binding was determined as detailed under Experimental Procedures. Nonspecific binding was determined in the presence of excess unlabeled factor IXa (0.44 $\mu$M; 25 $\mu$g/mL) and was subtracted from total binding to obtain specific binding. The results shown represent Scatchard plots of the specific binding of various chimeric factor IXa molecules compared with factor $IXa_{wt}$ in the presence of factor VIIIa and factor X. Panel A represents specific binding of factor $IXa_{wt}$ in the presence (●) and absence (○) of factor VIIIa and factor X which was identical to the binding of factor $IXa_N$ (not shown) under similar conditions. Panel B shows specific binding of chimeric factor IXa molecules 4, 5 (■) and 9–11 (▲) compared to factor $IXa_{wt}$ (●). Panel C represents the specific binding of factor IXa Y1A (■) and factor IXa K5R (▲) compared to factor $IXa_{wt}$ (●). Panel D represents the specific binding of factor IXa K5A (■) and factor IXa V10K (▲) compared to factor $IXa_{wt}$ (●). Panel E shows Scatchard plots of chimeric factor IXa (construct 8) (■) and factor IXa (construct 9) (▲) compared with factor $IXa_{wt}$ (●). Panel F shows Scatchard plots of factor IXa (construct 10) (■) compared to factor $IXa_{wt}$ (●). The plotted results represent mean values from four experiments, each done in duplicate. The data indicate a single class of binding sites for factor $IXa_{wt}$ and chimeric factor IXa molecules. The results are summarized in Table 2.

construct 8 (addition of factor IX residues 33–35 and 39–40 to the Gla domain of factor VII attached to factor IX), construct 9 (the same mutation as construct 8 with the Gla domain of factor VII attached to the factor VII molecule), and construct 10 (the addition of factor IX residues 1, 3–5, and 9–11 in addition to the changed residues shown in construct 8). The rationale for preparing these chimeric proteins is based on the molecular model depicted in Figure 2, which predicts the presence of a solvent-accessible loop structure consisting of the following amino acids: Gly 4, Lys 5, Leu 6, Phe 9, Val 10, and Gln 11. Both construct 8 and 9 chimeric factor IXa molecules failed to bind normally to platelets (Figure 3E) since it is apparent that the number of binding sites and the binding affinity for either construct 8 (222 sites per platelet, $K_d$ = 13 nM) or construct 9 (200 sites per platelets, $K_d$ = 14 nM) were significantly reduced compared with factor $IXa_N$ or factor $IXa_{wt}$. These studies also demonstrated that the presence of factor VIIIa and factor X, both at saturating concentrations, had no effect on either the number of binding sites or affinity of binding of construct 8 or 9 chimeric factor IXa molecules (Table 2). In contrast, replacement of amino acid residues 3–11 (construct 10) to a chimera consisting of the Gla domain of factor VII with factor IX residues at positions 33–35, 39, and 40 restored normal binding (Figure 3F). In the absence of factor VIIIa and factor X, there were 565 binding sites per platelet ($K_d$ = 2.75 nM) for construct 10 chimeric factor IXa molecules, a result which was indistinguishable from those obtained for factor $IXa_{wt}$ (570 sites per platelet; $K_d$ = 2.9 nM). The addition of factor VIIIa and factor X had no effect on the number of binding sites for either factor $IXa_{wt}$ or construct 10 and resulted in a decrease in $K_d$ for construct 10 to 0.68 nM and factor $IXa_{wt}$ to 0.80 nM (Table 2).

Table 2: Binding Constants for Normal and Chimeric Factor IXa Molecules[a]

| construct no. | ligand | factor VIII | no. of sites per platelet | apparent $K_d$ (nM) |
|---|---|---|---|---|
| 1 | $FIXa_{wt}$ | absent | 570 ± 45 | 2.90 ± 0.25 |
| | | present | 570 ± 65 | 0.80 ± 0.05 |
| 2 | 4, 5 | absent | 300 ± 42 | 6.10 ± 0.95 |
| | | present | 310 ± 39 | 5.90 ± 1.00 |
| 3 | 9–11 | absent | 260 ± 28 | 7.80 ± 1.10 |
| | | present | 280 ± 30 | 9.10 ± 2.00 |
| 4 | Ala 1 (Y1A) | absent | 560 ± 40 | 2.85 ± 0.33 |
| | | present | 590 ± 60 | 0.70 ± 0.08 |
| 5 | Arg 5 (K5R) | absent | 690 ± 74 | 3.20 ± 0.28 |
| | | present | 585 ± 55 | 0.90 ± 0.07 |
| 6 | Ala 5 (K5A) | absent | 497 ± 36 | 3.50 ± 0.59 |
| | | present | 550 ± 44 | 2.00 ± 0.25 |
| 7 | Lys 10 (V10K) | absent | 432 ± 50 | 3.70 ± 0.57 |
| | | present | 400 ± 41 | 3.50 ± 0.10 |
| 8 | | absent | 222 ± 27 | 13.00 ± 2.00 |
| | | present | 245 ± 31 | 12.50 ± 2.50 |
| 9 | | absent | 200 ± 32 | 14.00 ± 3.50 |
| | | present | 188 ± 20 | 16.00 ± 4.00 |
| 10 | | absent | 565 ± 35 | 2.75 ± 0.28 |
| | | present | 542 ± 50 | 0.68 ± 0.06 |
| 11 | $FIXa_N$ | absent | 610 ± 62 | 2.70 ± 0.30 |
| | | present | 600 ± 57 | 0.65 ± 0.06 |

[a] Results presented represent the mean (±SEM) of four separate determinations.

*Kinetic Studies of Factor X Activation.* Our previous studies have demonstrated a close correspondence between factor IXa receptor occupancy on activated platelets and enhanced rates of factor X activation (Ahmad et al., 1989b). Therefore, it was important to examine the kinetic consequences in factor X activation studies of amino acid alterations that result in defective factor IXa binding to platelets. Consequently, we selected two chimeric proteins for detailed kinetic studies in comparison with normal factor IXa (either plasma-derived or wild type): one with the Gla domain of factor VII connected to factor IX (construct 8, Table 2) and the other "corrected" chimera in which factor IX like residues replace those within the factor VII Gla domain at residues 1, 3–5, and 9–11 (construct 10, Table 2). The apparent $K_d$ ($K_{d\,app}$) values for binding of factor $IXa_N$, factor $IXa_{wt}$, and the two chimeric constructs were obtained from factor Xa generation studies in the presence of saturating concentrations of factor X and factor VIIIa (Figure 4B and Table 3). The

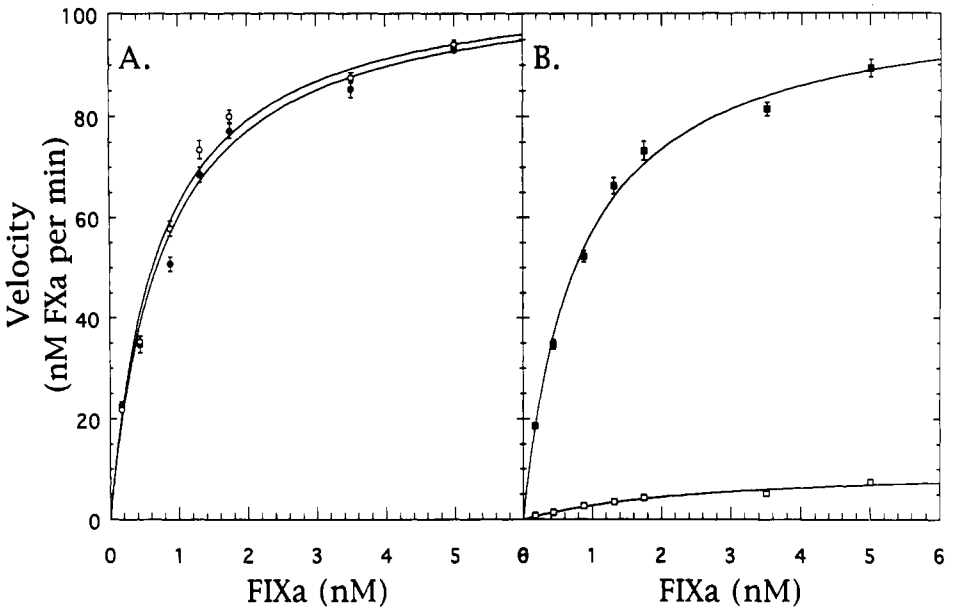


FIGURE 4: Rates of factor Xa formation by various concentrations of factor $IXa_N$, factor $IXa_{wt}$, and chimeric factor IXa molecules in the presence of thrombin-activated platelets and factor VIIIa. The rates of factor X activation were determined at various concentrations (0.17–5.0 nmol/L) of factor $IXa_N$, factor $IXa_{wt}$, construct 8, and construct 10 in the presence of $5 \times 10^7$/mL thrombin-activated platelets at 37 °C in a reaction volume of 100 μL containing 50 mmol/L Tris, 175 mmol/L NaCl, 0.5 mg/mL human serum albumin (pH 7.9), 5 mmol/L $CaCl_2$, 1.5 μmol/L factor X, and 5 units/mL factor VIIIa. Platelets were preincubated for 10 min at 37 °C with 0.1 unit/mL thrombin in the presence of $CaCl_2$ (5 mmol/L) and factor IXa. Excess thrombin was neutralized with 50 mmol/L PPACK before addition of factor VIIIa and performance of assay. For experimental details, see Experimental Procedures. The plotted results are shown in panel A for factor $IXa_N$ (●) and factor $IXa_{wt}$ (○) and in panel B for construct 8 (□) and construct 10 (■). The results represent the mean ± SEM of duplicate observations from four to six separate experiments.

Table 3: Factor X Activation Catalyzed by Factor $IXa_N$, Factor $IXa_{wt}$, and Chimeric Factor IXa Molecules[a]

| | $K_d$ (nM) | $K_m$ (μM) | $V_{max}$ (nM·min$^{-1}$) | $k_{cat}$[b] (min$^{-1}$) | $k_{cat}$[b]/$K_m$ (μM$^{-1}$·min$^{-1}$) |
|---|---|---|---|---|---|
| $FIXa_N$ | 0.78 ± 0.03 | 0.11 ± 0.02 | 15.8 ± 1.2 | 5194 | 47 218 |
| $FIXa_{wt}$ | 0.72 ± 0.02 | 0.09 ± 0.02 | 15.3 ± 1.7 | 5369 | 59 656 |
| construct 8[c] | 2.80 ± 0.80 | 0.12 ± 0.06 | 4.3 ± 0.6 | 6020 | 50 166 |
| construct 10[d] | 0.81 ± 0.03 | 0.10 ± 0.01 | 15.6 ± 0.8 | 5898 | 58 980 |

[a] Results recorded are the mean ± SEM of six identical experiments each performed in duplicate. [b] $k_{cat}$ expressed as moles of factor Xa formed per minute per mole of total platelet bound factor IXa. [c] Addition of factor IX residues 33–35 and 39–40 to the Gla domain of factor VII attached to factor IX. [d] Addition of factor IX residues 1, 3–5, and 9–11 in addition to the changed residues shown in construct 8.

use of this approach to confirm the apparent binding constant is justified in our previously published studies (Ahmad et al., 1989b,c; Rawala-Sheikh et al., 1990).

The kinetic parameters for factor X titration by factor $IXa_N$, factor $IXa_{wt}$, and the chimeric molecules (constructs 8 and 10) were determined in the presence of thrombin-stimulated platelets and factor VIIIa (Figure 4 and Table 3). These studies were carried out at a factor IXa concentration of 10 pM, well below the apparent $K_d$ for binding of these molecules to activated platelets. The $K_m$ and $V_{max}$ values as well as $k_{cat}$ and $k_{cat}/K_m$ values, calculated as specified under Experimental Procedures and as previously reported (Ahmad et al., 1989b), are presented in Table 3.

## DISCUSSION

The assembly of the factor X activating complex on the surface of thrombin-activated human platelets involves the specific, saturable, high-affinity binding of factor IXa to a receptor, occupancy of which is closely correlated with rates of factor X activation (Ahmad et al., 1989a–c; Rawala-Sheikh et al., 1990). Factor IX and factor IXa appear to share a common site on activated platelets (Ahmad et al., 1989a). However, factor IXa can occupy about twice the number of sites (500–600 per platelet) than factor IX (250–300 per platelet). Furthermore, the presence of the substrate (factor X) and the cofactor (factor VIII), while not affecting the number of binding sites for either factor IX or factor IXa, enhances the affinity of factor IXa binding ($K_d$ = ~2.5 nM in the absence of factor VIII and factor X; $K_d$ ~ 0.5 nM in the presence of factor VIII and factor X) but does not affect the affinity of factor IX binding ($K_d$ = ~2.5 nM). Moreover, although factor IX can displace about one-half the bound factor IXa molecules, and factor IXa can displace all the bound factor IX molecules (Ahmad et al., 1989a), the presence of factor IX has no effect whatever on rates of factor X activation by platelet-bound factor IXa (J. M. Scandura, S. S. Ahmad, and P. N. Walsh, unpublished data), which indicates that only one-half (250–300 sites) of the total number (500–600 sites) of factor IXa binding sites are functionally active in the factor X activating complex. This fact may be important in the interpretation of the present results, as discussed below.

The rationale for the present study was based upon our earlier observation that both Gla-domainless (in which the Gla domain was removed by enzymatic cleavage) and Gla-modified (in which the Gla residues were chemically modified) factor IXa molecules were capable of binding to only about one-half (275–350 sites per platelet) of the total number (500–600 per platelet) of platelet binding sites, both with decreased affinity ($K_d$ = 4.5–5.0 nM), compared with normal factor IXa ($K_d$ = 0.61 nM) (Rawala-Sheikh et al., 1992). Therefore, it was important first to determine whether amino acid residues within the Gla domain are those which mediate the binding of factor IXa to platelets and second to identify those specific

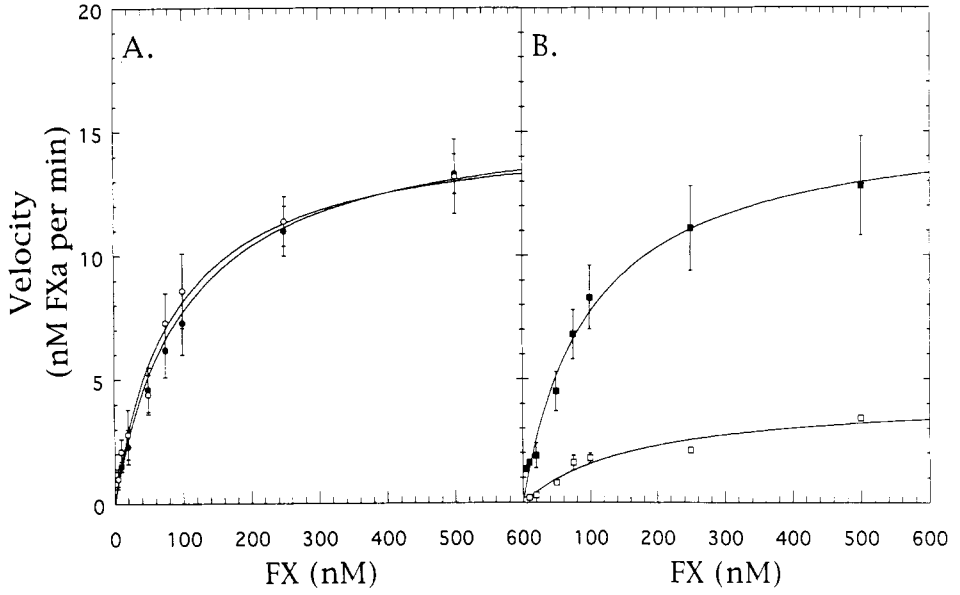


FIGURE 5: Factor X activation by factor $IXa_N$ and factor $IXa_{wt}$ and by factor IXa chimeric proteins in the presence of thrombin-activated platelets, factor VIIIa, and various concentrations of factor X. The experiment was performed essentially the same as described in the legend to Figure 3, except the concentration of factor X was varied and the concentration of factor IXa was kept constant to 10 pmol/L. The plotted results are shown in panel A for factor $IXa_N$ (●) and factor $IXa_{wt}$ (○) and in panel B for construct 8 (□) and construct 10 (■). The results represent the mean ± SEM of duplicate observations from four to six separate experiments.

residues. To ascertain which amino acids might form putative solvent-accessible binding sites, we constructed a computer model of the Gla domain of factor IX based on the known structure of the highly homologous protein domain, prothrombin fragment 1 (Soriano-Garcia et al., 1992). This model (Figure 2) was consistent with the possibility that two surface structures, defined by residues 3–11 and 33–40, might form the platelet binding site. We, therefore, chose to examine in detail the possible role of surface residues 3–5 and 9–11 in binding of factor IXa to platelets, utilizing a series of mutant and chimeric factor IXa molecules as shown in Table 1. The results of equilibrium binding studies with these constructs (presented in Figure 3 and Table 2) demonstrated the following facts: (1) When factor IX residues were mutated to factor X residues at positions 4 and 5 or 9–11 (construct 2 or 3), a decreased number of binding sites (260–310 per platelet) with a decreased affinity ($K_d$ = 6.1–7.8 nM) was observed, and the presence of factor VIII and factor X had no effect on $K_d$. (2) Similar findings were observed with mutations of single residues such as K5A (construct 6) and V10K (construct 7) but not with the conservative mutation K5R (construct 5), which retains basic charge at position 5. (3) Chimeric proteins (constructs 9 and 10) consisting of factor VII residues in the Gla domain (except at positions 33–35 and 39–40) also demonstrated a triad of abnormalities, i.e., a decreased number of molecules bound, a descreased affinity of binding, and the absence of any effect of factors VIII and X on binding affinity. (4) However, when residues 1, 3–5, and 9–11 were restored in the factor VII–IX chimera (construct 8) to the normal factor IX residues (construct 10), entirely normal binding parameters were restored. We conclude from these studies that a solvent-accessible protein surface consisting of Gly 4, Lys 5, Leu 6, Phe 9, Val 10, and Gln 11 is exposed near the amino terminus of the Gla domain of factor IXa that is utilized for the binding of factor IXa to its platelet receptor.

Since the binding of factor IXa to thrombin-activated platelets is closely correlated with and is essential for enhanced rates of factor X activation (Ahmad et al., 1989b) and the catalytic efficiency of factor X activation is enhanced by ($\sim 20 \times 10^6$)-fold when factor IXa is bound to its platelet receptor, the two peptide loop structures identified in these studies would appear to be functionally important in the assembly of the factor X activating complex. Data in support of this hypothesis are presented in Figures 4 and 5 and in Table 3. For these kinetic studies of factor X activation we limited our observations to those carried out with the entire factor X activating complex and with four factor IXa molecules, two normal (factor $IXa_N$ and factor $IXa_{wt}$) and two chimeric (constructs 8 and 10) in which residues 3–5 and 9–11 are either those of factor VII and of factor IX. The results from these kinetic studies confirm the observation from equilibrium binding studies that the affinity of factor IXa binding in the presence of factor VIIIa and factor X is decreased when residues 3–5 and 9–11 are those from factor VII and is restored to normal when these residues are those from factor IX.

The defective factor IXa binding observed with Gla domain mutants and chimeras in the present study is very similar to the abnormalities of binding observed in our previous study with Gla-domainless and Gla-modified factor IXa molecules (Rawala-Sheikh et al., 1992). The abnormalities are multiple and complex, including (1) a decrease in the affinity of factor IXa binding in the absence of factor VIII and factor X, (2) a complete lack of response to the presence of factor VIII and factor X which results in a 5-fold increase in the affinity of binding of normal or wild-type factor IXa, and (3) a decrease in the number of binding sites by about 50% compared with normal. Moreover, the present studies confirm the previous finding with Gla-domainless or Gla-modified factor IXa (Rawala-Sheikh et al., 1992) that although the defective binding of the chimeric or mutant factor IXa molecules reflects itself as expected in a decrease in the $V_{max}$ for factor X activation (Table 3), the catalytic efficiency of the *bound* molecules is entirely normal. Thus, the only detectable defects evident when residues 3–11 are altered are those related to factor IXa binding to platelets and not to factor IXa catalytic activity.

The interpretation of the present results is not entirely straightforward. However, both the present studies and those with Gla-domainless and Gla-deficient molecules (Rawala-Sheikh et al., 1992) indicate that normal factor IXa binding to activated platelets required determinants both within and outside of the Gla domain. The surface within the Gla domain

that mediates factor IXa binding is localized to a conformationally constrained surface within residues 3–11. A separate and distinct site located outside the Gla domain must be postulated to account for the residual (low affinity, low capacity) binding of factor IXa observed when residues 3–11 are altered (present study) or when the Gla domain is absent or altered (Rawala-Sheikh et al., 1992). The precise location of this second site is unknown, but we have presented evidence that it is not localized within the EGF-1 domain since a chimeric factor IX molecule with the EGF-1 domain replaced by that of factor X interacted with the platelet receptor normally both in the presence and in the absence factor VIII and factor X (Ahmad et al., 1992b). On the other hand, a similar chimeric construct in which the EGF-2 domain of factor X replaced the native sequence within the factor IXa molecule was shown to bind to a reduced number of platelet receptors with a decreased affinity in the presence of factor VIII and factor X (Ahmad et al., 1991). Thus, it is possible that two platelet receptor recognition sites exist within factor IXa, one comprising residues 3–11 in the Gla domain and the other within the EGF-2 domain.

What is the significance of the fact that the number of sites as well as the affinity of factor IXa binding is reduced when the Gla domain is removed or modified (Rawala-Sheikh et al., 1992) or when residues 3–11 are altered (present study)? Relevant to this observation is the fact that the zymogen, factor IX, binds to about half the number of sites to which factor IXa binds with reduced affinity in the presence of factor VIII and factor X (Ahmad et al., 1989a). Although factor IX is capable of displacing about half the factor IXa molecules bound in equilibrium binding studies, it has no effect on factor X activation kinetics in the presence of factor VIII and factor X (J. M. Scandura, S. S. Ahmad, and P. N. Walsh, unpublished observations). It follows that the number of *functional* factor IXa binding sites is ~250–300 per platelet and that the remainder are spare receptors (~250–300 per platelet). The fact that the chimeric and mutant proteins of the present study and the Gla-domainless and Gla-deficient factor IX molecules previously studied (Rawala-Sheikh et al., 1992) are catalytically normal once bound to activated platelets means that these defective proteins are bound, albeit with reduced affinity, to functional receptors. Evidence to this effect is presented in Table 3 and demonstrates that the $k_{cat}$, expressed as moles of factor Xa formed per minute per mole of platelet-bound factor IXa, and the catalytic efficiency ($k_{cat}/K_m$) for the factor VIII–IX chimera (construct 8) are entirely normal.

We have presented a working hypothesis which could explain the assembly of the factor X activating complex on the platelet surface (Ahmad et al., 1992a). This hypothesis suggests that, to account for the stoichiometry and affinity of binding of both factor IX and factor IXa and the effects of factor VIII and factor X, there are both a protein and a phospholipid component to the receptor that interact with two sites on factor IXa. Our laboratory has also presented preliminary evidence for the presence of a factor IXa binding protein on the platelet surface that is a candidate for a factor IXa receptor (London & Walsh, 1992). Whatever the nature of the factor IXa receptor, it would appear from the present study that one component of the factor IXa molecule that interacts with it is a solvent–accessible protein surface consisting of the amino acids Gly 4, Lys 5, Leu 6, Phe 9, Val 10, and Gln 11 within the Gla domain.

## ACKNOWLEDGMENT

We are grateful to Yan Zhang for technical assistance and to Patricia Pileggi for her expert assistance in manuscript preparation.

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