Identification of Functionally Important Residues of the Epidermal Growth Factor-2 Domain of Factor IX by Alanine-scanning Mutagenesis

RESIDUES ASN^89–GLY^93 ARE CRITICAL FOR BINDING FACTOR VIIIa*

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** Yu-Jia Chang‡, Hua-Lin Wu‡, Nobuko Hamaguchi‡, Ya-Chu Hsu‡, and Shu-Wha Lin¶**

From the ‡Department of Biochemistry, College of Medicine, National Cheng Kung University, Tainan 701, Taiwan, §Rosenstein Basic Medical Research Center, Brandeis University, Waltham, Massachusetts 02119, and ¶Graduate Institute of Medical Technology, College of Medicine, and the Department of Laboratory Medicine, National Taiwan University Hospital, Taipei 100, Taiwan

This paper describes the consequences of alanine-scanning mutagenesis on 28 positions of the second epidermal growth factor (EGF-2) domain of factor IX. We identified four positions of Gln^97, Phe^98, Tyr^115, and Leu^117 that are critical for secretion of factor IX. Of the remaining mutations, 4 mutants (V86A, E113A, K122A, and S123A) are as active as wild-type factor IX (IXwt); 16 (D85A, K100A, N101A, D104A, N105A, R116A, E119A, T87A, I90A, K91A, R94A, E96A, S102A, K106A, T112A, and N120A) retain reduced but detectable activity, and 4 (N89A, N92A, G93A, and V107A) are nearly inert in the clotting assay. Both factor Xla and the factor VIIa-tissue factor complex effectively catalyzed the activation of these mutants except N89A. The mutant V107A failed to form the factor tenase complex with factor VIIIa because of a 35-fold increase in K_i. The mutants N89A and N92A did not compete with factor IXwt for factor VIIIa binding, and G93A exhibited a 6-fold increase in K_i values in the competitive binding assay. It appears that mutations at these positions have significantly affected the interaction between factor IX and factor VIIIa, although other mutations had little effect on the binding of factor IX to factor VIIIa. Mutations in two regions, Thr^87–Gly^93 and Asn^103–Val^107, significantly increased the K_i value of factor IXa (2–10-fold) in cleavage of factor X in the absence of factor VIIIa. In the presence of factor VIIIa, the catalytic efficiency of each mutant toward factor X paralleled its clotting activity. Briefly, we propose two relatively distinctive functions of factor IX for two adjacent regions in the EGF-2 domain; the first loop region (residues 89–94) is involved with the binding of its cofactor, factor VIIIa, and the third loop with connected ^b^-sheets (residues 102–108) is involved with the proper binding to the substrate, factor X.

Blood coagulation factor IX, a vitamin K-dependent serine protease precursor, plays a critical role in regulation and amplification of the blood coagulation in the intrinsic clotting cascade. The clotting cascade consists of series of serine proteases, in which each protease activates its downstream zymogen in highly specific manners (1, 2). The activation of factor IX by proteolytic cleavages of two peptide bonds by factor Xla or by the factor VIIa-tissue factor (VIIa-TF)^3 complex results in a catalytically active two-chain molecule of factor IXa (3–5). Factor IXa converts zymogen factor X into factor Xa, which is responsible for the activation of prothrombin to thrombin. Generation of factor Xa by factor IXa requires factor VIIIa, the only macromolecular protein cofactor identified for factor IXa (6, 7). The interaction between factor IXa and factor VIIIa requires both factors to be activated. Defects in both proteins are linked to hemophilia.

The zymogen form of factor IX has five structural domains as follows: the Gla domain, two EGF-like domains, the activation peptide, and the catalytic domain (8). Upon activation, the activation peptide is released from factor IXa. A number of experiments have indicated that a few regions in the catalytic domain (residues 301–303 and 333–339) may confer factor VIIIa binding (9–11). Furthermore, two EGF domains have long been proposed to be critical for the interactions between factor IX and factor VIIIa (12–15). The EGF-2 domain of factor IX contains 43 residues (Asp^85–Ala^127, factor IX numbering) and is highly conserved among different organisms, with 80–85% amino acid sequence identity. Furthermore, the EGF-2 domains among factors VII, IX, X, and protein C share 30–50% sequence homology (16). Domain swapping experiments showed that the chimeric recombinant factor IX, in which the EGF-1 domain was replaced by that of factor X or factor VII, retained full factor IXa activity, indicating that the EGF-1 domain does not contribute to factor VIIIa binding in a factor IX-specific manner (13, 17). However, substituting two EGF domains with that of factor X (factor IX_{XeGF^{1+2}}) the chimeric factor IX lost almost all of its clotting activity (13). This strongly suggests that the EGF-2 domain is critical for factor IX-specific function. In other studies using the chimeric recombinant factor IX_{XeGF^{1+2}} (factor IX with the EGF-2 domain from...
factor X), it is suggested that the EGF-2 domain is required for the assembly of the functional intrinsic factor IXa-factor VIIIa (factor tenase) complex on the surfaces of the activated platelets but is not directly involved in the interaction between factor IXa and factor VIIIa (18, 19).

The EGF-2 domain is composed of five β-strands and four β-hairpin loops based on the crystal structure of porcine factor IXa (14). Mutations at residues located along the concave surface of factor IXa (such as residues Ile30, Asn92, and Arg104) appear to be associated with hemophilia, indicating functional significance of this region (14, 20). Arg94 is involved in the formation of a salt bridge with residue Glu78 of the EGF-1 domain, which may be critical for the interaction with factor VIIIa (21). Glu78 is conserved in the EGF-1 domain of factors IX and X, and therefore, replacing only the EGF-1 of factor IX with that of factor X might not influence the stability of the salt bridge. In contrast, changing both EGF domains between factor IX and factor X would eliminate the salt bridge and perturb the integrity between these two EGF domains, and consequently affect the activity of factor IX. These facts are consistent with the aforementioned domain swapping results. In addition, a hydrophobic loop of EGF-1 (formed by Val758–Phe774) protrudes into the hydrophobic concavity formed by residues Ala86, Arg84, Val107, Cys109, and Lys122 in the EGF-2 domain (14). When the hydrophobic region of residues Lys104–Val108 was replaced by the corresponding site of factor VII, the mutation caused a dramatic decrease in factor IXa activity toward factor X both in the absence and the presence of factor VIIIa (22). This suggests that the decrease of the factor IX activity can be attributed to a defect in the interaction of factor IX and its substrate, factor X (22). The study suggested that the proper orientation and interaction of the EGF-1 and -2 domains are crucial for substrate binding. Whether and how the EGF-1 and -2 interdomain interactions affect the bindings of factor VIIIa and factor X remains to be determined.

In this study, we conducted an extensive alanine-scanning mutagenesis of the EGF-2 domain of factor IXa spanning residues 85 and 125 in order to identify the EGF-2 region that interacts with the cofactor and substrates of factor IX. We provide an analysis of the functional roles of the aforementioned salt bridge and the hydrophobic interactions that link the two EGF domains of factor IX.

EXPERIMENTAL PROCEDURES

Materials—All the restriction endonucleases and polymerases were obtained from New England Biolabs, Inc. (Beverly, MA). Genetin (G418) was from Invitrogen. Factor IX-deficient plasma, phosphorylcholine (PC), and phosphatidylserine (PS) were purchased from Sigma. Human factor Xa, factor Xa, and factor X were purchased from Enzyme Research Laboratory (South Bend, IN). Preparations of tissue factor, factor VII, factor VIIa, and phospholipids were described previously (23). Polyclonal anti-factor IX antibodies were prepared by immunization of rabbits with plasma-derived factor IX as described previously (23, 28). Purified wild-type and mutant factor IX proteins (1.8 μM) were incubated at 37 °C with factor IXa (9 μM) and VIIa-TF complex (36 μM). The reaction products of both experiments were analyzed at timed intervals by SDS-PAGE followed by silver staining. To determine the rate of factor IX hydrolysis by the VIIa-TF complex, a two-step kinetic assay was performed in that IXwt and N89A (1.8 μM) were incubated with VIIa-TF (18 μM) in 50 μl of factor serum albumin (BSA) Tris–HCl buffer (5 μM CaCl2, 0.01% bovine serum albumin) at 37 °C. The reaction mixtures were withdrawn at different time points (5–60 min) and stopped by addition of 10 μl of 12 mM EDTA. The factor Xa activity generated by the VIIa-TF complex at each time point was measured by the addition of 40 μl of Spectrozyme FIXa at a final concentration of 4 nM. Hydrolysis was monitored at 405 nm and 37 °C for 1 h, and the initial rate (max/min) for the sample (max/min) was determined. The specific activity of the purified recombinant wild-type factor IXa was comparable with that of plasma-derived factor IX (26).

Activation of Factor Xa by Factor VIIa-TF Complex and the Preparation of Factor IXa—Activation of factor Xa by factor Xa and VIIa-TF was performed as described previously (24, 26). The VIIa-TF complex was prepared as described previously (23, 24). Purified wild-type and mutant factor IX proteins (1.8 μM) were incubated at 37 °C with factor IXa (9 μM) and VIIa-TF complex (36 μM). The reaction products of both experiments were analyzed at timed intervals by SDS-PAGE followed by silver staining. To determine the rate of factor IX hydrolysis by the VIIa-TF complex, a two-step kinetic assay was performed in that IXwt and N89A (1.8 μM) were incubated with VIIa-TF (18 μM) in 50 μl of factor serum albumin (BSA) Tris–HCl buffer (5 μM CaCl2, 0.01% bovine serum albumin) at 37 °C. The reaction mixtures were withdrawn at different time points (5–60 min) and stopped by addition of 10 μl of 12 mM EDTA. The factor Xa activity generated by the VIIa-TF complex at each time point was measured by the addition of 40 μl of Spectrozyme FIXa at a final concentration of 4 nM. Hydrolysis was monitored at 405 nm and 37 °C for 1 h, and the initial rate (max/min) for the sample (max/min) was determined. The specific activity of the purified recombinant wild-type factor IXa was comparable with that of plasma-derived factor IX (26).

Activation of Factor Xa by Factor IXa in the Absence and Presence of Factor VIIa—The activity of wild-type and mutant factor IXa toward factor X was monitored kinetically through the hydrolysis of Spectrozyme FXa by factor Xa in the absence and presence of factor VIIa as described previously (24, 26). Final concentrations were factor IXa, 10 nM, PCPS, 100 μM, Spectrozyme FXa, 0.5 nM, and factor X, 0.025–1 μM. The platelet-rich protein (PRP) generated was computed from a standard curve derived by the hydrolysis of Spectrozyme FXa with factor Xa (Enzyme Research Laboratories) prepared by immobilized Russell’s viper venom. The factor Xa preparation was fully active as analyzed by an active site-specific fluorescent substrate (31, 4-methylumbelliferyl p-guanidinobenzoate (Fluka), as described before (32), in a Hitachi 850 fluorescent spectrophotometer. The factor Xa activity was then calu...
lated according to an active site as described previously (24, 30) by the following equation: $A_{\text{rel}} = aX^2 + bX + c$ (30). Factor VIIIa was prepared freshly by incubation with thrombin as described previously (24, 33). To analyze the interaction of factor IXa with factor X in the presence of factor VIIIa, 25 μl of the factor VIIIa preparation was mixed with an equal volume of factor IXa preincubated with PCPS in TBS/bovine serum albumin. The mixture was used to form the intrinsic factor tenase complex, and its activity toward factor X was subsequently analyzed kinetically by adding 50 μl of a reaction mixture containing PCPS and Spectrozyme FXa. The final concentrations are as follows: for wild-type or mutant factor IXa, 0.25 nM; factor VIIIa, 0.4 nM; PCPS, 100 μM; factor X, 0–200 nM; and Spectrozyme FXa, 0.5 μM. The factor IXa activity was calculated as described above.

**Binding of Factor IXa to Factor VIIIa**—Binding experiments were performed by monitoring the intrinsic factor tenase activity at limiting concentrations of factor VIIIa as described previously (26, 33). Freshly prepared factor VIIIa (1.6 nM, 25 μl) was incubated with 25 μl of different concentrations of wild-type or mutant factor IXa at room temperature for 5 min to form a factor tenase complex. Activity of the resultant complex was then measured as described above. The total volume of the reaction mixtures was 100 μl, and the final concentrations are as follows: factor VIIIa, 0.5 nM; PCPS, 100 μM; factor X, 100 nM; Spectrozyme FXa, 0.5 μM; and factor IXa, 0–40 nM. The reaction was measured at 37 °C kinetically on a microtiter plate reader. Experiments were performed in duplicate for 3–5 independent reactions, and curves were fitted using all data points. The $K_d$ values were derived as previously described (26, 34, 35).

**Preparation of the Active Site-modified Wild-type and Mutant Factor IXa**—Wild-type and mutant factor IXa (N89A, N92A, and G93A) were inactivated with DEGR-CK as described previously (33, 36, 37). Recombinant factor IXa, at a concentration of 1 μM in 50 μl of TBS, was incubated with a 3-fold molar excess of DEGR-CK for 7 h at room temperature and then for 17 h at 4 °C. Then wild-type and mutant factor IXa (DEGR-IXa) were separated from excessive DEGR-CK by extensive dialysis against TBS at 4 °C. The remaining enzymatic activity of the wild-type factor IXa was measured by aPTT clotting test and found to contain less than 0.05% of untreated wild-type factor IXa. The specific clotting activities of the mutant factor IXa (N89A, N92A, and G93A) was measured to determine whether their amidolytic activities toward Spectrozyme FIXa were <0.1% of their uninhibited counterparts.

**Inhibition of Factor X Activation by the Intrinsic Factor Tenase Complex with DEGR-IXa**—Competition experiments were carried out by incubating wild-type factor IXa and factor VIIIa with different concentrations of wild-type and mutant DEGR-IXa as competitors at room temperature for 5 min. The residual factor tenase complex activity was measured for the activity toward factor X as described above. Final concentrations are as follows: factor VIIIa, 0.55 nM; PCPS, 100 μM; factor X, 100 nM; Spectrozyme FXa, 0.5 μM; wild-type factor IXa, 0.05 nM; and wild-type or mutant DEGR-IXa, 0–50 nM. The relative rate of factor Xa generation in the presence of each DEGR-IXa was used to calculate $K_i$ according to Equation 1 as described by Larson et al. (33).

$$\text{relative rate} = \frac{K_i + [\text{VIII}\text{IX}]_a}{2K_i + [\text{VIII}\text{IX}]_a - [\text{IX}]_a + (K_i + K)^{-1} [\text{VIII}\text{IX}]_a [\text{IX}]_a}$$

(Eq. 1)

**RESULTS**

**Expression and Specific Clotting Activities of Factor IX Variants**—In order to preserve disulfide bonds, cysteine residues (positions 88, 95, 99, 109, 111, and 124) were not substituted. Residues 110 and 114 were also omitted from this study because the missense mutations at these positions were reported to cause hemophilia B with little antigen levels. Residues 103 and 118 are alanine in factor IX; thus they were not investigated. Recombinant factor IX mutants were designated as D85A, V86A, and L117A, secreted at levels comparable with wild-type factor IX (IXwt). The secreted factor IX mutants reacted with a polyclonal antibody and three different monoclonal anti-factor IX antibodies. At least one of the antibodies is known to be conformational specific (25) indicating that the alanine replacement at these positions did not alter the global structure of factor IX. Immunostaining showed that Q97A, F98A, Y115A, and D85A were accumulated inside the cells (data not shown), suggesting that these substitutions disrupted the secretion of factor IX. These four mutants were not characterized further in subsequent experiments.

The specific clotting activities of the 24 secreted alanine mutants are summarized in Fig. 1. One mutant, V86A, was found two times more active than factor IXwt. Three (E113A, K122A, and S123A) were as active as factor IXwt. In contrast, N89A, N92A, G93A, and K107A had minimal clotting activities. Of the remaining 16 mutants, 7 (D85A, K100A, N101A, D104A, N105A, R116A, and E119A) had 46–75% IXwt activity, and the other 9 (T87A, I90A, K91A, R94A, E96A, S102A, K106A, T112A, and N120A) showed decreased activity to 7–37%.

**Activation of Factor IX Variants by Factor Xa and the Factor VIIa-TF Complex**—All variants, with the exception of N89A, were activated efficiently by factor Xa and the factor VIIa-TF complex. The cleavage of N89A by factor Xa was as efficient as factor IXwt, but its cleavage by the factor VIIa-TF complex was

**FIG. 1. Specific clotting activities of the alanine-scanned EGF-2 mutants.** The specific clotting activity of each factor IX mutant was determined by ELISA using three monoclonal anti-factor IX antibodies (see “Experimental Procedures”). Their clotting activities were determined by aPTT assays using factor IX-deficient plasma (Parthromtin, Behring Diagnostics, Inc., Somerville, NJ). For both ELISA and aPTT assays, standard curves were derived using serial dilutions of pooled normal plasma in parallel experiments. Specific activities are factor IX levels measured by aPTT divided by the factor IX levels measured by ELISA, and expressed as percentage of pooled normal plasma factor IX. Shown here are mean values of duplicates in multiple experiments. The specific activity of factor IXwt is ~100 ± 3.1%. The structure is taken from Protein Data Bank code 1px (www.rcsb.org). Buried residues (GETAREA (www.scsb.umbc.edu/getarea)) with water-accessible areas less than 30 Å² are marked by asterisks. Other buried residues not shown in the figure are Gln27, Phe83, and Tyr111. Residue Tyr111 has a water-accessible area of 55 Å².
Mutagenesis of Factor IX EGF-2 Module

Activation of Factor X in the Absence and Presence of Factor VIIIα—The proteolytic activities of the mutated factor IXa toward macromolecular substrate, factor X, were evaluated in the absence and the presence of its cofactor, factor VIIIα. In the absence of factor VIIIα, the alanine mutations in two clustered regions resulted in decreased activity for factor X. One region contains mutants T87A-G93A, and the other contains S102A-V107A, and both had nearly 2–10-fold elevation of $K_\text{d}$, whereas their $k_{\text{cat}}$ values were similar to the IXwt (Table I). Interestingly, four mutants with little clotting activities, Asn$^{89}$, Asn$^{92}$, Gly$^{93}$, and Val$^{107}$, exhibited reduced but significant catalytic efficiencies, demonstrating additional defects other than its substrate binding.

In the presence of factor VIIIα, the catalytic efficiency of each mutant was measured as a complete intrinsic factor tenase. As shown in Table II, most of the mutants exhibited catalytic efficiencies ($k_{\text{cat}}/K_\text{m}$) that parallel their clotting activities (Fig. 1). Factor VIIIα failed to enhance the catalytic rates of the mutants N89A, N92A, and G93A. In contrast, there were eight mutants (V86A, T87A, K100A, N105A, E113A, E119A, K122A, and S123A) with significant increase of $k_{\text{cat}}$ (Table I). The increased clotting activity with V86A is due to the increase in catalytic efficiency upon factor VIIIα binding. The slightly decreased clotting activity found with K100A, N105A, and E119A is due to increased $K_\text{d}$ (Table I).

Factor IXa Binding to Factor VIIIα—To investigate the difference in the enhancement of factor IXa activity by factor VIIIα, the binding of the mutant factors IXas to factor VIIIα was examined. Based on the assumption that the intrinsic factor tenase activity is proportional to the concentration of the factor IXa-factor VIIIα complex, the binding affinity was determined by monitoring factor X activation. Table II shows the apparent dissociation constant ($K_\alpha$) of each mutant factor IXα for factor VIIIα. We found that the $K_\alpha$ values for most of the mutants were comparable with that of the activated factor IXwt ($K_\alpha = 1.1 \text{nM}$), suggesting that these mutants bound factor VIIIα as efficiently as factor IXwt. T87A and T112A have slightly increased $K_\alpha$ values (3–4.5-fold) and V107A has a 35-fold increased $K_\alpha$ value, indicating that the decreased clotting activities of these mutants may be caused by the decreased affinities for factor VIIIα.

Because N89A, N92A, and G93A exhibited extremely low activities in the presence of factor VIIIα, competition experiments were designed to determine the affinity for factor VIIIα for these variants. In this assay, increasing concentrations of inactivated factor IXα variants were added to compete with activated factor IXwt for factor VIIIα binding. First, activated factor IXwt was tested to evaluate the assay. As shown in Fig. 3, wild-type DEGR-IXα inhibited the binding of normal factor IXα with a $K_\alpha$ of 0.95 nM that is very consistent with the $K_\alpha$ value estimated from the kinetic assay in the previous section. DEGR-IXαG93A was partially effective in competing with normal factor IXα for binding factor VIIIα ($K_\alpha = 5.7 \text{nM}$), and neither DEGR-IXαN89A nor DEGR-IXαN92A showed effective competition. It appears that alanine substitutions at positions 89, 92, and 93 significantly altered the interaction between factor IXα and factor VIIIα. In addition, the estimated factor VIIIα binding for G93A is considerably tighter than that of V107A ($K_\alpha = 35.8 \text{nM}$); however, the enhancement of factor IXα activity by factor VIIIα is undetectable with G93A. This may indicate that the residue is not only significant for factor VIIIα binding but is also important for the conformational change that occurs upon factor VIIIα binding and normally leads to increased $k_{\text{cat}}$.
Mutagenesis of Factor IX EGF-2 Module

Kinetic parameters of the factor IX EGF-2 mutants

The reaction conditions and calculations are described under "Experimental Procedures." Data represent the mean ± S.D. of five experiments.

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<tr>
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<th>VIIIa (-)</th>
<th>VIIIa (+)</th>
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<tr>
<td></td>
<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
<td>k&lt;sub&gt;cat&lt;/sub&gt;</td>
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<tr>
<td></td>
<td>(nM)</td>
<td>(× 10&lt;sup&gt;-6&lt;/sup&gt;)</td>
</tr>
<tr>
<td>IXWT</td>
<td>389 ± 52.3</td>
<td>18.7 ± 0.32</td>
</tr>
<tr>
<td>D58A</td>
<td>407 ± 10.3</td>
<td>22.39 ± 0.28</td>
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<tr>
<td>V86A</td>
<td>445 ± 26.6</td>
<td>12.87 ± 0.05</td>
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<tr>
<td>T87A</td>
<td>2287 ± 351</td>
<td>21.28 ± 0.32</td>
</tr>
<tr>
<td>N89A</td>
<td>2825 ± 193</td>
<td>13.09 ± 0.17</td>
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<tr>
<td>R90A</td>
<td>1003 ± 51.1</td>
<td>4.82 ± 0.03</td>
</tr>
<tr>
<td>K91A</td>
<td>1208 ± 209</td>
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<tr>
<td>N92A</td>
<td>4375 ± 665</td>
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<tr>
<td>G93A</td>
<td>1940 ± 0.1</td>
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<tr>
<td>R94A</td>
<td>442 ± 30.6</td>
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<tr>
<td>E96A</td>
<td>342 ± 36.1</td>
<td>3.05 ± 0.03</td>
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<tr>
<td>K100A</td>
<td>483 ± 26.1</td>
<td>16.51 ± 0.43</td>
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<tr>
<td>N101A</td>
<td>637 ± 10.2</td>
<td>8.17 ± 0.14</td>
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<tr>
<td>S102A</td>
<td>2541 ± 80.3</td>
<td>23.05 ± 0.08</td>
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<tr>
<td>D104A</td>
<td>3622 ± 259</td>
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<tr>
<td>N105A</td>
<td>805 ± 52.2</td>
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<tr>
<td>K106A</td>
<td>748 ± 20.3</td>
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<tr>
<td>V107A</td>
<td>842 ± 38.8</td>
<td>21.99 ± 0.22</td>
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<tr>
<td>T112A</td>
<td>659 ± 134</td>
<td>5.83 ± 0.09</td>
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<tr>
<td>E113A</td>
<td>412 ± 30.6</td>
<td>23.28 ± 0.26</td>
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<tr>
<td>R116A</td>
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<tr>
<td>E119A</td>
<td>263 ± 20.6</td>
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<td>N120A</td>
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<tr>
<td>K122A</td>
<td>580 ± 24.9</td>
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<tr>
<td>S123A</td>
<td>866 ± 18.1</td>
<td>10.02 ± 0.12</td>
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<sup>a</sup> Units are μ FXa (factor Xα), μ<sup>-1</sup> factor IXa, s<sup>-1</sup>.
<sup>b</sup> ND, not detected.

**DISCUSSION**

In this study, the functional roles of 28 residues in the EGF-2 domain of factor IX were characterized by alanine-scanning mutagenesis. To our knowledge, this is one of the most extensive studies ever performed on the EGF-like domains. We have shown that mutations at four positions, Asn 89, Asn 92, Gly 93, and Val 107, eliminate the clotting function of factor IX. Through competition experiments, we further demonstrated that the loss of the clotting activity was probably due to defects in factor VIIIa binding. As shown in Fig. 4, Arg<sup>94</sup> forms a salt bridge to Glu<sup>78</sup> of the EGF-1 domain. It has been proposed that the salt bridge linking the two EGF domains is required for factor VIIIa binding (21). Our kinetic and competition experiments (Table I, Table II, and Fig. 3) indicated that the mutations at Arg<sup>89</sup>, Asn<sup>92</sup>, and Gly<sup>93</sup> had much greater effects on factor VIIIa binding than that at Arg<sup>94</sup>. Thus we suggest that residues Asn<sup>89</sup>-Gly<sup>93</sup> play a much greater and significant role than Arg<sup>94</sup> in factor VIIIa binding. The side chains of Asn<sup>89</sup>-Gly<sup>93</sup> are on the same side of the molecule as the side chains of residues Lys<sup>301</sup>-Gly<sup>303</sup> and Arg<sup>333</sup>-Ser<sup>339</sup> forms a factor VIIIa binding surface as shown in Fig. 4. The crystal structure of the factor VIIa-TF complex revealed that the TF-binding sites are distributed among the multiple domains of factor VII (39), and the relative locations of these sites are very similar to those of factor VIIIa-binding sites in factor IXα (Asn<sup>89</sup>, Lys<sup>301</sup>-Gly<sup>303</sup> and Arg<sup>333</sup>-Ser<sup>339</sup>). In fact, Val<sup>92</sup>-Asn<sup>93</sup> of factor VII (Asn<sup>89</sup>-Lys<sup>90</sup> of factor IX) makes a hydrophobic interaction with residue Thr<sup>50</sup> of TF. Alanine-scanning mutagenesis on factor VIIIa showed that the EGF-2 domain had a minor contribution to the binding of factor VIIIa to TF (39). Thus, the locations of the cofactor-binding sites in the EGF-2 domains in factor VII and factor IX are similar. However, the significant contribution of the interaction between the EGF-2 domain and cofactor to the overall binding energy appears to be unique to factor IX. The side chain of Asn<sup>93</sup> is exposed to solvent and does not interact with any residue within factor IX (Fig. 1 and see Ref. 14). Asn<sup>93</sup> may directly provide a hydrogen bond to factor VIIIa and support...
factor IX–specific cofactor interaction of the EGF-2 domain. On the other hand, Asn89 and Gly50 are highly conserved among related coagulation factors. The side chain of Asn89 makes hydrogen bonds to Glu78 and Tyr295 in the catalytic domain and is likely integral to the structure of its vicinity. The substitution at Gly78 resulted in moderately increased $K_v$ values for factor VIIIa and loss of the proper conformational change upon factor VIIIa binding. Gly89 is buried under the surface loop containing Asn89. The introduction of an alanine side chain at this position is likely to distort the loop structure, thus affecting factor VIIIa binding through the orientation of Asn89.

The salt bridge formed by Arg94 and Glu78 was proposed as particularly important for factor IX function. In contrast to reports from other groups (21), we observed only a moderate decrease in factor VIIIa affinity (2-fold) when Glu78–Arg94 salt bridge was disrupted. This difference can probably be attributed to the different substitutions studied. Previous work (21) substituted lysine for Glu78, creating an unstable interface with Arg94 that is likely to disrupt the structure. Our substitution with alanine for Arg94 is much less likely to disrupt the structure, and therefore, the decreased factor VIIIa binding affinities are much less prominent when compared with the previous study. Moreover, because the salt bridge could be rebuilt by exchanging the charged residues between positions Glu78 and Arg94 as shown by other groups studying the factor IX E78K/R94D double mutations (21), it is less likely that these residues are actively involved in factor VIIIa binding.

Alanine substitution at residue Asn89 also affects the activation of factor IX by the VIIa-TF complex. Several mutations of factor IX, such as G48R (24) and Q50P (40), have also been shown to affect the activation of factor IX by VIIa-TF. Residues Gly48, Glu50, and Asn89 are on the same side of the molecule in the crystal structure of porcine factor IXa (14). Because the activation of factor IX takes place prior to factor VIIIa binding, it is possible for Asn89 to be involved in both factor VIIIa binding and the factor VIIa-TF complex binding.

Residues 106–108 in the EGF-2 domain are suggested to contribute to the enzymatic activity of factor IXa rather than its cofactor binding (22). Mutant FIX106–108/VII (factor IX with residues Lys106–Val107–Val108 replaced by Lys106–Arg107–Ser108 as in factor VII) displayed dramatically reduced catalytic activity toward factor X both in the presence and the absence of factor VIIIa (22). There was no measurable affinity to factor VIIIa light chain. In the same study, mutant FIX106–108/X (factor IX with residues Lys106–Val107–Val108 replaced by Ser106–Val107–Val108 as in factor X) displayed no significant loss in factor IXa activity. In this study, each position was investigated by alanine substitution individually. We showed that K106A had reduced factor IX activity without any significant effect on factor VIIIa binding. On the other hand with V107A, we observed significantly reduced factor VIIIa binding. Residues 102–106 make the third loop in the EGF-2 domain, and Val107 is buried (Fig. 1 and Fig. 4). We observed that the mutations in this loop (S102A, N105A, and K106A) affect the catalytic efficiency of factor IXa toward factor X more profoundly in the absence of factor VIIa than in its presence with no significant effect on factor VIIIa binding. Thus, we propose that the loop may be required for correct binding of factor X rather than factor VIIIa binding (Fig. 4). The effect of the substitution at Val107 on the catalytic efficiency in the absence of factor VIIa is moderate, and a greater decrease in factor...
VIIIa affinity was observed with V107A, indicating the different functional role of Val107 relative to residues 102–106. Val107 is located near the hydrophobic interface of the EGF-1 and EGF-2 domains. It is buried under the Asn89 loop and between residues Val86–Cys88 and residues Val75–Val77. Thus the primary defect caused by the mutation that truncated the side chain of Val107 may be the decreased factor VIIIa binding by disrupting the Asn89 loop structure and by affecting the concave hydrophobic socket indirectly.

Mutations at four positions, Gln97, Phe98, Tyr115, and Leu117, caused intracellular accumulation of defective factor IX molecules. This observation can explain the extremely low factor X binding. We also proposed the involvement of the third loop of the EGF-2 domain of factor IXa in factor X binding.

In summary, we demonstrated the significant role of the first loop of the EGF-2 domain, especially Asn89, in factor VIIIa binding. We also proposed the involvement of the third loop of the EGF-2 domain of factor IXa in factor X binding.

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