Identification of Residues Asn$^{89}$, Ile$^{90}$, and Val$^{107}$ of the Factor IXa Second Epidermal Growth Factor Domain That Are Essential for the Assembly of the Factor X-activating Complex on Activated Platelets*

Received for publication, June 11, 2004, and in revised form, August 6, 2004
Published, JBC Papers in Press, August 24, 2004, DOI 10.1074/jbc.M406552200

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Activated platelets promote intrinsic factor X-activating complex assembly by presenting high affinity, saturable binding sites for factor IXa mediated by two disulfide-constrained loop structures (loop 1, Cys$^{88}$-Cys$^{93}$; loop 2, Cys$^{95}$-Cys$^{108}$) within the second epidermal growth factor (EGF2) domain. To identify amino acids essential for factor X activation complex assembly, recombinant factor IXa point mutants in loop 1 (N89A, I90A, K91A, and R94A) and loop 2 (D104A, N105A, and V107A) were prepared. All seven mutants were similar to the native factor IXa by SDS-PAGE, active site titration, and content of γ-carboxyglutamic acid residues. Kinetic constants obtained by either titrating factor X or factor VIIa on SFLLRN-activated platelets or phospholipid vesicles revealed near normal values of $K_{\text{m(app)}}$ and $K_{\text{d(app)}}$ for all mutants, indicating normal substrate and cofactor binding. In a factor Xa generation assay in the presence of activated platelets and cofactor factor VIIIa, compared with native factor IXa ($K_{\text{d(app)}}$FIXa $\sim 1.1$ nM, $V_{\text{max}}$ $\sim 12$ nM min$^{-1}$), N89A displayed an increase of $20$-fold in $K_{\text{d(app)}}$FIXa and a decrease of $20$-fold in $V_{\text{max}}$; I90A had an increase of $5$-fold in $K_{\text{d(app)}}$FIXa and $10$-fold decrease in $V_{\text{max}}$; and V107A had an increase of $3$-fold in $K_{\text{d(app)}}$FIXa and $4$-fold decrease in $V_{\text{max}}$. We conclude that residues Asn$^{89}$, Ile$^{90}$, and Val$^{107}$ within loops 1 and 2 (Cys$^{88}$-Cys$^{109}$) of the EGF2 domain of factor IXa are essential for normal interactions with the platelet surface and for the assembly of the factor X-activating complex on activated platelets.

Coagulation factor IX (FIX) is a vitamin-K-dependent serine protease zymogen that plays a pivotal role in blood coagulation. Deficiency of FIX or defective FIX activity results in hemophilia B. FIX circulates in plasma before being proteolytically activated by either FXIa or tissue factor/FVIIa (TF/FVIIa) to form FIXa, a heterodimer consisting of a catalytic-domain-containing heavy chain (−28 kDa) and light chain (−18 kDa). FIXa exhibits negligible activity toward its physiological substrate FX unless it is in complex with the surface (activated platelets or negatively charged phospholipids) and a non-enzymatic cofactor FVIIIa via a mechanism that remains to be fully understood. The FIXa heavy chain contains the serine protease active site that catalyzes the activation of the macromolecular substrate FX in a cofactor-dependent manner (1). The light chain of FIXa, which consists of a γ-carboxylated glutamic acid (Gla)-containing module and two modules that are highly homologous to epidermal growth factor (EGF), also participates in the assembly of the FX-activating complex by interacting with both the surface and the cofactor.

Previous studies from our laboratory have demonstrated that the zymogen, FIX, binds to a discrete number (n $\sim$ 250 sites per platelet) of receptors on the surface of activated platelets ($K_{d}$ $\sim$ 2.5 nM) that can also be occupied by the enzyme, FIXa, and are mediated by residues Gly4-Gln11 within the Gla domain (2–6). In addition, FIXa, but not FIX, can bind to a site (n $\sim$ 250 sites per platelet, $K_{d}$ $\sim$ 0.5 nM) on activated platelets, mediated by residues 88–109 (disulfide constrained loops 1 and 2) but not by residues 110–124 (loop 3) within the EGF2 domain (2–6). Occupancy of these binding sites is closely correlated with optimal rate enhancements of FX activation (>2 $\times$ 10$^{5}$-fold) in the presence of FVIIa, emphasizing the physiological significance of platelet-receptor-mediated coagulation complex assembly (2, 6, 7). Recently, however, alanine-scanning mutagenesis studies of residues within the EGF2 domain of FIX have identified residues Asn$^{89}$-Gly$^{93}$ that were implicated as critical for binding of FVIIa (8). Because it is highly unlikely that the same subdomain of the FIXa EGF2 domain could be important for interactions with both platelet receptors and with FVIIa, we have conducted the present studies, which are designed to identify specific amino acids essential either for binding to activated platelet receptors or to the cofactor, FVIIa. "Candidate" residues, most likely to be involved in mediating FX activation on the surface of activated platelets, were selected for alanine-scanning mutagenesis on the basis of: 1) surface exposure of amino acid side chains by x-ray crystallography; 2) conservation among species; and 3) dissimilarity with those in a homologous protein FVII, which does not participate in the assembly of the intrinsic FX-activating complex and does not bind to activated platelets with high affinity (9). Based on these criteria, we selected and prepared recombinant FIXa point mutants in loop 1 (N89A, I90A, K91A, and R94A) and loop 2 (D104A, N105A, and V107A) of the EGF2 domain to
study the contributions of these residues to FX activation complex assembly on activated platelets (Fig. 1). The present results support the conclusion that residues Asn89, Ile90, and Val107 within loops 1 and 2 (Cys88-Cys109) of the EGFP domain of FIXa are essential for normal interactions with the platelet surface and for the assembly of the FX-activating complex on activated platelets.

MATERIALS AND METHODS

Dubelco's modification of Eagle's medium was obtained from Mediatech (Herndon, VA). Vitamin K₁ (2-methyl-3-phytyl-1,4-naphthoquinone) was from Abbott Laboratories (Chicago, IL), Q-Sepharose anion exchange resin was from Sigma, and Centricon Plus-20 concentration units were from Millipore (Bedford, MA).

HEPES, Tris, fatty acid-free bovine serum albumin (BSA), heparin from porcine intestinal mucosa, benzamidine, and other reagents were purchased from Sigma. Human FIXa, human FX, and human antithrombin III (ATIII) were obtained from Enzyme Research Laboratories (South Bend, IN). The FX preparation, obtained as a lyophilized powder, was dissolved in sterile water and dialyzed against HEPES-Tris buffer (HT) before being used in experiments. Human FXIa was derived, was dissolved in sterile water and dialyzed against HEPES-Tris buffer (HT) before being used in experiments. Human FXIa was concentrated to ~3 ml using Centricon Plus-20 (10,000 molecular weight cut-off, Millipore, Bedford, MA) and dialyzed in TBS buffer.

Protein Concentrations—The concentration of FIX proteins were initially determined using the BCA assay (11) and then were corrected utilizing the results of active site titration as described below.

Carboxyglutamate Analysis—Carboxyglutamate analysis was generously performed by Dr. Rodney Camire (Children's Hospital of Philadelphia, Philadelphia, PA) as described (12).

Activation of FIX Proteins by FXIa—FIX proteins were activated to their active enzymatic forms as follows: FIX proteins were diluted in HT supplemented with 5 mM CaCl₂. FXIa (5 nM) was added at a 1/200 molar ratio, and the reactions were incubated at 37 °C for 90 min. Zymogen activation rates of wild type and mutant proteins were similar. Complete activation was confirmed by SDS-PAGE/silver staining and by active site titration with ATIII.

Active Site Titration of FIXa Proteins—To assay the active-site concentration of mutant and wild type proteins, 10 μM of FIXa protein (100 nM) was incubated with 10 μM of ATIII dilutions (0–100 nM) in HT (15 mM HEPES, 126 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 375 μM NaH₂PO₄, and 5.6 mM glucose, pH 7.2) supplemented with BSA (1 mg/ml), heparin (20 μg/ml), and CaCl₂ (5 mM) at 37 °C for 15 min, and the reaction was diluted by adding HT buffer to 200 μl. Residual FIXa activity was examined by assaying FXa generation activity in the presence of FVIIIa (5 units/ml), FX (400 μM), and α-dioleoyl-PC-PS vesicles (20 μM). The FXa generation reaction was allowed to proceed at 37 °C for 2 min and was stopped by addition of 50 μl of 50 mM EDTA (pH 8.1, 175 mM NaCl, and 20 mM EDTA). The amount of FXa generated was determined by using its chromogenic substrate S2765 as described before (3).

Determination of K_d(app)FIXa and V_max in the Presence of Saturating FVIII—An FX activation vessel containing 1 nM FIXa, 5 units/ml FVIIIa, and SFLLRN-activated platelets (5 × 10⁷ platelets/ml) or extruded PC:PS (mol: mol = 3:1, total concentration = 2 μM) vesicles in HT buffer supplemented with BSA (2 mg/ml) and 5 mM CaCl₂ was used. The reaction was initiated by the addition ofFX to the indicated concentration and allowed to proceed for 2 min at 37 °C. Then 10 μM EDTA was added to stop the reaction, and the FXa generation was measured as indicated above.

Determination of K_m(app)FVIII and FX Activation Velocity in the Presence of Saturating FVIIa (V_max)FVIIa—FVIIa at indicated concentrations was titrated into the FX activation vessel containing 0.5 nM FIXa and SFLLRN-activated platelets (5 × 10⁷ platelets/ml) or extruded PC:PS (mol: mol = 3:1, total concentration = 500 μM) vesicles in HT buffer supplemented with BSA (2 mg/ml) and 5 mM CaCl₂. The reaction was initiated by the addition of 250 nM FX and allowed to proceed for 2 min at 37 °C. Then 10 μM EDTA was added to stop the reaction. The amount of FXa generated was determined as above. V_max was defined as the maximum velocity of FXa generation at a saturating concentration of FVIIa under the experimental conditions described. K_m(app)FVIIa was defined as the concentration of FVIIa required to achieve half-maximal rates of FX activation.

Determination of K_d(app)FVIII and FX Activation Velocity in the Presence of Saturating FIXa (V_max)FVIIa at indicated concentrations was titrated into the FX activation vessel containing 5 units/ml FVIIIa and SFLLRN-activated platelets (5 × 10⁷ platelets/ml) or extruded PC:PS (mol: mol = 3:1, total concentration = 500 μM) vesicles in HT buffer supplemented with BSA (2 mg/ml) and 5 mM CaCl₂. The reaction was initiated by the addition of 250 nM FX and allowed to proceed for 2 min at 37 °C. Then 10 μM EDTA was added to stop the reaction. The amount of FXa generated was determined as above. V_max was defined as the maximum velocity of FXa generation at a saturating concentration of FVIIa under the experimental conditions described. K_d(app)FVIIa was equivalent to the concentration of FVIIa required to achieve half-maximal rates of FXa generation under the experimental conditions described.
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The results indicated that enzyme incorporation was severely impaired for the mutants rFIXa89A, rFIXa90A, and rFIXa107A compared with native FIXa. However, the effective concentration at half-maximal velocity (K_{d(app)}^VIIIa) remained the same for all the FIXa proteins in the presence of either activated platelets or phospholipid vesicles. No significant change in K_{d(app)}^VIIIa values was observed for any of the Ala mutants (Table III), indicating normal incorporation of the cofactor into the enzymatic complex on surfaces. FIXa Titrations—Examination of platelet-mediated FIX activation at increasing concentrations of FX or FVIIIa suggested that the deficiency in FIX activation complex assembly on platelets observed with the Ala mutant proteins was not due to defective substrate or cofactor incorporation. Enzyme titrations were carried out to assess the platelet binding capacities of the mutants. The values of maximal velocity at saturating concentrations of the enzyme were significantly reduced for rFIXa89A (~4.7% V_{max}), rFIXa90A (~11.8% V_{max}), and rFIXa107A (~25% V_{max}) compared with native FIXa (titration curves not shown), and the binding affinities indicated by K_{d(app)}^VIIIa values were severely impaired for these proteins (Table IV). Specifically, the K_{d(app)}^VIIIa values for rFIXa89A were ~50-fold increased, whereas rIXa90A displayed ~12-fold increase and rFIXa107A displayed ~8-fold increase in K_{d(app)}^VIIIa, for activated platelets. Results similar to those obtained with activated platelets (Table IV) were observed when the FIXa mutants were titrated on phospholipid vesicles (data not shown). The results indicated that enzyme incorporation was severely defective for the mutants rFIXa89A, rFIXa90A, and rFIXa107A, which have consistently exhibited severely impaired FX-activating velocities in all the experiments carried out.

**RESULTS**

Characterization of Proteins—FIX EGF2 alanine-scanning “candidate” mutants expressed in HEK293 cells and secreted in the serum-free medium were purified using Q-Sepharose-dependent pseudoaffinity chromatography as described under “Materials and Methods.” Co-migration of each of the recombinant FIX proteins with normal plasma-derived FIX (FIXNP) by SDS-PAGE (data not shown) suggested normal translation and post-translational modification. Additionally, each of the recombinant FIX proteins was found to have the expected number of γ-carboxyglutamate (Gla) residues (10.9–13.2 mol of Gla/mol of protein) (Table I).

Earlier time-course solution-phase FX activation results have shown that FIXa activates all the seven FIX alanine mutants at normal rates compared with native FIX (8), and each of the FIX proteins was fully activated by FIXa in solution. Geodele Blue-stained gels of SDS-PAGE displayed complete disappearance of thezymogen band (~70 kDa) and appearance of heavy (~28 kDa) and light chains (~18 kDa) of FIXa for all the recombinant FIX proteins (data not shown). Active site titration with ATIII also showed complete activation of all FIX proteins. The FIXa concentrations of wild type and mutant proteins used in active site titration were ~100 nM, determined from A_{280} and BCA assays. The active site concentrations exhibited for the recombinant FIXa proteins were 84–120 nM (Table I).

F-X Titrations—In preliminary screening studies, all the seven “candidate” alanine mutants displayed decreased FXa generation activities on phospholipids. In the present studies, FX activation activities of these FIXa mutants were thoroughly examined on both SFLLRN-activated platelets and PC:PS (3:1) vesicles, respectively (Fig. 2). In these assays, kinetic parameters of the plasma-derived FIXa (FIXa) were used as positive controls, although recombinant FIXa (rFIXa) was also included in experiments and showed indistinguishable activities compared with native FIXa. Presented in Table II are the apparent Michaelis-Menten kinetic parameters for the FIXa proteins on both surfaces. Regarding activated platelets on the physiological cell membrane, all seven Ala mutants exhibited reduced V_{max} values in FIXa generation when compared with native FIXa (V_{max} ~ 4.05 nM FXa/min) (Table II and Fig. 2). In particular, rFIXa89A (~1% V_{max}), rFIXa90A (~3% V_{max}), and rFIXa107A (~10% V_{max}) displayed drastically impaired FX activation activities (rescaled in Fig. 2B). However, no significant increase in K_{m(app)} values was observed for most of the chimeras, with the exception of an increase of ~3-fold in the case of rFIXa89A. This suggests that the deficiency in FIXa generation activity does not result from defective FX association with the enzymatic complex. Substrate titrations on artificial surface, extruded phospholipids (l-a-dioleoylPC:PS = 3:1) (regression curves not shown), revealed that rFIXa91A, rFIXa94A, rFIXa104A, and rFIXa105A displayed normal V_{max} values compared with native FIXa (V_{max} ~ 25.1 nM FXa/min) although the K_{m(app)} values were slightly increased (Table II). The three other Ala mutants, rFIXa89A, rFIXa90A, and rFIXa107A displayed significantly decreased V_{max} values, whereas K_{m(app)} values for rFIXa90A and rFIXa107A were not increased. Kinetic values for rFIXa89A on phospholipids were not determined because saturable enzyme kinetic curves were not observed (Table II).

FVIIIa Titrations—FVIIIa was titrated in the presence of either activated platelets or phospholipids to examine FVIIIa interactions with the FIXa chimeras (Fig. 3 and Table III). For SFLLRN-activated platelets, the maximal velocity of FX activation at saturating FVIIIa concentration was drastically decreased for rFIXa89A (~1% V_{max}), rFIXa90A (~10% V_{max}), and rFIXa107A (~20% V_{max}) compared with native FIXa. However, the effective concentration at half-maximal velocity (K_{d(app)}^VIIIa) rescaled in Fig. 4B, and the binding affinities indicated by K_{d(app)}^VIIIa values were severely impaired for these proteins (Table IV). Specifically, the K_{d(app)}^VIIIa values for rFIXa89A were ~50-fold increased, whereas rIXa90A displayed ~12-fold increase and rFIXa107A displayed ~8-fold increase in K_{d(app)}^VIIIa, for activated platelets. Results similar to those obtained with activated platelets (Table IV) were observed when the FIXa mutants were titrated on phospholipid vesicles (data not shown). The results indicated that enzyme incorporation was severely defective for the mutants rFIXa89A, rFIXa90A, and rFIXa107A, which have consistently exhibited severely impaired FX-activating velocities in all the experiments carried out.

**TABLE I**

Characterization of recombinant FIX mutants

| Gla-containing protein | Ave. mol Gla/mol protein | Active site conc. per 100 protein |
|-----------------------|-------------------------|----------------------------------|
| FIXa                  | 13.0 ± 0.1              | 102                              |
| rFIXa                 | 10.9 ± 0.1              | 102                              |
| rFIXa89A              | 12.4 ± 0.05             | 117                              |
| rFIXa90A              | 12.8 ± 0.1              | 110                              |
| rFIXa91A              | 13.2 ± 0.03             | 84                               |
| rFIXa94A              | 13.2 ± 0.03             | 102                              |
| rFIXa104A             | 13.1 ± 0.03             | 120                              |
| rFIXa105A             | 13.1 ± 0.06             | 87                               |
| rFIXa107A             | 13.1 ± 0.06             | 120                              |

* Values are mean ± S.E. of three independent measurements kindly performed by Dr. Rodney Camire (University of Pennsylvania).
Fig. 2. Determination of $K_{\text{m(app)}}$ and $V_{\text{max}}$ on SFLLRN-activated platelets. FIXa proteins were diluted to 1 nM in HT buffer containing SFLLRN (5 μM)-activated platelets (5 x 10^7/ml). FVIIIa was added to 5 units/ml, and FX was added to the indicated concentration. After 2 min, the reactions were terminated by addition of EDTA to 10 mM. Regression curves for FIXaN, rFIXa90A, rFIXa91A, rFIXa94A, rFIXa95A, and rFIXa107A are rescaled in B for clearer demonstration.

Table II

| FIXa            | Platelets | Phospholipids |
|-----------------|-----------|---------------|
|                 | $K_{\text{m(app)}}$ | $V_{\text{max}}$ | $k_{\text{cat}}$ | $K_{\text{m(app)}}$ | $V_{\text{max}}$ |
| FIXaN           | 42.8 ± 2.2 | 4.1 ± 0.1     | 3.4             | 39.6 ± 12.3       | 25.1 ± 2.6 |
| rFIXa90A        | 154.2 ± 35.4 | 0.04 ± 0.005c | 16.6           | ND              | ND         |
| rFIXa91A        | 6.8 ± 1.4c | 0.1 ± 0.005c  | 4.1            | 18.1 ± 2.3c      | 10.3 ± 0.3c |
| rFIXa94A        | 29.5 ± 6.5c | 1.9 ± 0.11c   | 5.2            | 62.9 ± 5.6c      | 30.5 ± 1.0c |
| rFIXa95A        | 22.4 ± 5.6c | 1.7 ± 0.1bc   | 2.9            | 64 ± 4.8c        | 31.7 ± 0.9c |
| rFIXa104A       | 20.0 ± 3.1c | 1.7 ± 0.01c   | 2.9            | 78.7 ± 2.6c      | 35.6 ± 0.5c |
| rFIXa105A       | 25.1 ± 6.6c | 2.3 ± 0.04c   | 3.8            | 86.9 ± 1.9c      | 37.3 ± 0.3c |
| rFIXa107A       | 8.9 ± 1.3c  | 0.4 ± 0.01c   | 3.3            | 34.0 ± 1.6c      | 14.0 ± 0.2c |

Values are mean ± S.E. of independent measurements ($n = 2$). $K_{\text{m(app)}}$ values are calculated as described under "Materials and Methods." $a$ Plasma-derived FIXa (FIXaN). When compared to FIXaN, symbols denote $p$ values as indicated (b $p < 0.05$; c $p < 0.001$; *not significant). $d$ ND, not determined.

Fig. 3. Determination of $K_{\text{m(app)}}$FVIIIa and $V_{\text{max}}$ for FVIIIa stimulation of FXa generation on SFLLRN-activated platelets. FIXa proteins were diluted to 0.5 nM in HT buffer containing SFLLRN-activated platelets (5 x 10^7/ml). FVIII was added to the indicated final concentration and activated to FVIIIa by thrombin (0.1 unit/ml). Reactions were initiated by addition of FX to 250 nM. After 2 min, the reactions were terminated by addition of EDTA to 10 mM. Regression curves for FIXa mutants are indicated in B for clearer demonstration.

Coagulation Assays—Activated partial thromboplastin time assays for the seven "candidate" alanine mutants were previously reported (8) and are listed in Table IV, which confirms the physiological function of the interrupted surface binding property of the FIX mutants in mediating clotting. The three mutants N89A (0.5%), I90A (7%), and V107A (0.5%) exhibited the lowest clotting activities.

Discussion

To identify essential residues in the disulfide-constrained loops 1 and 2 of the EGF2 domain (Cys$^{88}$-Cys$^{109}$) that are important for platelet-mediated FIXa/FVIIIa complex assembly, we utilized the primary amino acid sequence alignment within the disulfide-constrained loops 1 and 2 of the EGF2 domain (Fig. 1) to identify "candidate" residues that are highly conserved among species and different from those in FVII, because residues in FVIII molecules have been shown to be ineffective in mediating platelet surface-mediated FX activation complex assembly (2, 6). Recombinant FIXa point mutants in loop 1 (N89A, I90A, K91A, and R94A) and loop 2 (D104A, N105A, and V107A) were prepared to study the contributions of these residues to FX activation complex assembly on activated platelets. All seven mutants were similar to native FIXa by SDS-PAGE (both reduced and non-reduced); active site titration, which confirmed the presence of one intact active site per FIXa mutant molecule; and, content of γ-carboxyglumatic acid residues. Kinetic constants obtained by titrating FX on SFLLRN-activated platelets in the presence of FVIIIa revealed near normal values of $K_{\text{m(app)}}$ for all mutants, indicating normal substrate binding. It should be emphasized that, whereas the observed $K_{\text{m}}$ values in substrate (FX) titration experiments are indicative of the affinity of substrate incorporation, $K_{s}$ is not the true substrate dissociation constant ($K_{d}$ or $K_{a}$). Rather, the reaction rate contributes to $K_{\text{cat}} (K_{d} + k_{\text{cat}}/k_{\text{cat}})$, which may explain why when the reaction velocity is extremely slow for the FIXa mutants, the $K_{\text{m(app)}}$ values were generally decreased (Table II). However, because decreased $K_{\text{m(app)}}$ values suggest even tighter substrate association to the enzyme complex, we conclude that the substrate incorporation was not interrupted by any of the mutations with the possible exception of the mutant N89A. Even though the Asn$^{89}$ alanine mutant displayed a slightly interrupted substrate incorporation (Table II), the severely impaired velocity ($V_{\text{max}}$) in substrate, cofactor, and enzyme titrations (Tables II–IV) for this mutant coincided with an increase of —50-fold in $K_{\text{m(app)}}$ for FIXa, and a similar $K_{\text{d(app)}}$ value compared with native FIXa, suggesting that enzyme binding to activated platelets or to phospholipids was
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**TABLE III**

*Kₐ_{dissapp}FIXa* and  V_{max} values for stimulation of FXa generation on SFLLRN-activated platelets and phospholipid vesicles (PC:PS = 3:1).

| FIXa          | Platelets                              | Phospholipids                      |
|---------------|----------------------------------------|------------------------------------|
|               | *Kₐ_{dissapp}FIXa* | *V_{max} | *Kₐ_{dissapp}FIXa* | *V_{max} |
|               | unit/mM⁻¹       | nM/min   | unit/mM⁻¹       | nM/min   |
| FIXaN         | 3.4 ± 0.4       | 3.1 ± 0.2 | 1.8 ± 0.2       | 21.9 ± 0.5 |
| rFIXa89A      | 1.9 ± 0.6a      | 0.05 ± 0.006c | 2.6 ± 0.5b     | 1.0 ± 0.1c  |
| rFIXa90A      | 3.6 ± 0.7b      | 0.3 ± 0.02c  | 2.6 ± 0.2b     | 3.0 ± 0.1c  |
| rFIXa91A      | 2.3 ± 0.2b      | 2.0 ± 0.1c   | 1.2 ± 0.2b     | 9.4 ± 0.4d  |
| rFIXa94A      | 3.3 ± 0.5b      | 5.8 ± 0.04c  | 1.2 ± 0.1b     | 18.3 ± 0.6c |
| rFIXa104A     | 3.2 ± 0.3c      | 5.2 ± 0.2c   | 1.4 ± 0.1b     | 16.8 ± 0.3c |
| rFIXa105A     | 3.1 ± 0.3c      | 5.2 ± 0.2c   | 1.9 ± 0.2b     | 17.9 ± 0.8c |
| rFIXa107A     | 5.0 ± 0.6c      | 1.1 ± 0.1c   | 2.4 ± 0.2b     | 5.3 ± 0.1c  |

* a Plasma-derived FIXa (FIXaN). When compared to FIXaN, symbols denote p values as indicated ("not significant; "p < 0.0001; "p < 0.01).  

**TABLE IV**

*Kₐ_{dissapp}FIXa* and  V_{max} values for FXa generation by FIXa on SFLLRN-activated platelets and zymogen activated partial thromboplastin time clotting activities.

| FIXa          | Platelets                              | Clotting activities (zymogen) |
|---------------|----------------------------------------|-------------------------------|
|               | *Kₐ_{dissapp}FIXa* | *V_{max} | %                         |
|               | unit/mM⁻¹       | nM/min   |                           |
| FIXaN         | 1.1 ± 0.2       | 11.6 ± 0.7 | 0.5%                      |
| rFIXa89A      | 53.8 ± 10.1b    | 2.0 ± 0.1c | 7%                        |
| rFIXa90A      | 13.6 ± 0.5b     | 1.4 ± 0.03c | 31%                      |
| rFIXa91A      | 4.9 ± 0.7b      | 6.8 ± 0.05c | 16%                      |
| rFIXa94A      | 2.7 ± 0.1b      | 10.6 ± 0.02c | 68%                      |
| rFIXa104A     | 3.7 ± 0.3b      | 10.8 ± 0.04c | 57%                      |
| rFIXa105A     | 3.3 ± 0.1b      | 10.5 ± 0.02c | 57%                      |
| rFIXa107A     | 9.2 ± 1.3c      | 2.9 ± 0.2c | 0.5%                      |

* a Activated partial thromboplastin time clotting activities of FIX al-anine mutants were assessed as described previously, and published values (8) are included in this table. Symbols denote p values as above by reference to normal pooled plasma as 100% clotting activity.  

Extensive studies have been carried out to identify regions in FIXa that are essential in surface (14–21) or cofactor interaction (1, 22–31). Regions 301–303 and 333–339 in the FIXa light chain (containing the Gla and two EGF domains) of FIXa crystal structure (PDB: 1RFN, not shown) they form a planar surface, which the present studies suggest forms a site utilized for binding to activated platelets, consisting of two hydrophobic residues (Ile and Val) and an uncharged polar residue (Asn) that can participate in hydrogen bonding.

Normal interactions with FVIIIa for all FIXa mutants. In a FXa generation assay in the presence of activated platelets and cofactor FVIIIa, compared with native FIXa (K_{dissapp}FIXa ~ 1.1 nM, V_{max} ~ 12 nM min⁻¹), N89A displayed a ~20-fold increase in K_{dissapp}, ~20-fold decrease in V_{max}; I90A displayed a ~5-fold decrease in K_{dissapp}FIXa and ~10-fold decrease in V_{max}; and V107A displayed a ~3-fold increase in K_{dissapp}FIXa and ~4-fold decrease in V_{max}. It should be noted from the results recorded in Table II that when k_{cat} values were calculated as V_{max} (derived from values recorded in Table II) divided by the concentration of bound enzyme for all the mutant proteins (derived from values recorded in Table IV), these “corrected” k_{cat} values were all either normal (or slightly increased in the case of the rFIXa89A mutant). This result can be interpreted as demonstrating that the only defect arising from these mutations is a consequence of a platelet (or phospholipid)-binding defect and that the catalytic activity of the mutant enzymes is normal. We conclude that residues Asn^{89}, Ile^{90}, and Val^{107} within loops 1 and 2 (Cys^{88–Cys^{109}}) of the EGF2 domain of FIXa are essential for normal interactions with the platelet surface and for the assembly of the FX activating complex on activated platelets. When the locations of these three essential amino acids were displayed using the human FIXa crystal structure (PDB: 1RFN, not shown) they form a planar surface, which the present studies suggest forms a site utilized for binding to activated platelets, consisting of two hydrophobic residues (Ile and Val) and an uncharged polar residue (Asn) that can participate in hydrogen bonding.
EGF2 Domain of FIXa in FX Activation on Platelets

Fixa, however, this contribution is considered to be structural rather than direct, because the segment Fixa variants showed normal FXIIIa binding in surface plasmon resonance experiments (36). Kinetic studies using recombinant FIXa chimeras have consistently shown that residues in the EGFl domain do not mediate FIXa association suggested by both normal kinetic stimulation by FXIIIa and normal Km values (2–4, 6). Thus, the contribution of the non-catalytic domain of FIXa to its interaction with FXIIIa appears to be mainly structural, i.e. to promote optimal positioning of the catalytic domain of FIXa, in order to interact with FXIIIa (31).

On the other hand, extensive platelet binding combined with FIXa generation studies have proven that the light chain of FIXa mediates platelet binding, which leads to FX-activating complex assembly (2, 3, 5–7, 14–18, 20, 37–41). These studies demonstrate that the platelet-bound (not fluid-phase) FIXa is the physiologically functional enzyme and that surface binding is the driving force for FIXa/FXIIIa complex assembly. It is hypothesized that once assembled on the activated platelet or phospholipid membrane, the non-catalytic domain contributes structurally to bring the FIXa catalytic domain in contact with FXIIIa, which alters the local conformation of the FIXa active site (42) and increases its capacity to activate FX (26). The regions in the light chain implicated in mediating platelet binding are the ω loop (Gly4–Gln11) within the Gla domain (15, 20) and the disulfide-constrained loops 1 and 2 within the EGFl domain (Cys88–Cys105) (2–4). The residues contained within these regions appear to be both necessary and sufficient for mediating the incorporation of the enzyme, FIXa, into the FIXa-FXIIIa complex on the platelet surface (6), because the impaired FIXa generation rates resulted exclusively from increases in FIXa Km values rather than Kd values.

Acknowledgments—We are grateful to Rodney M. Camire, Children’s Hospital of Philadelphia and the University of Pennsylvania, Philadelphia, PA for carrying out the reported Gla analyses. We thank Patricia Pileggi for help in preparing the manuscript.

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\textit{J. Biol. Chem.} 2004, 279:46400-46405.
doi: 10.1074/jbc.M406552200 originally published online August 24, 2004

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