Residues 88–109 of Factor IXa Are Important for Assembly of the Factor X Activating Complex*

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Activated platelets and phospholipid vesicles promote assembly of the intrinsic factor X (FX) activating complex by presenting high-affinity binding sites for blood coagulation FIXa, FVIIla, and FX. Previous reports suggest that the second epidermal growth factor (EGF)-like domain of FIXa mediates assembly of the FX activating complex (Ahmad, S. S., Rawala, R., Cheung, W. F., Stafford, D. W., and Walsh, P. N. (1995) Biochem. J. 310, 427–431; Wong, M. Y., Gurr, J. A., and Walsh, P. N. (1999) Biochemistry 38, 8948–8960). To identify important residues, we prepared several chimeric FIXa proteins using homologous sequences from FVII: FIXaEGF2 (FIXa88–124, FVII91–127), FIXaEGF2 (FIXa88–99, FVII91–102), FIXaEGF1 (FIXa95–109, FVII98–112), FIXaEGGF2 (FIXa111–124, FVII114–127), and point mutants (FIXaR94D and FIXaG94R). In the presence and absence of FVIIla, a 2- to 10-fold reduced $V_{\text{max}}$ of FX activation (nm FIXa min$^{-1}$) was observed for FIXaEGF2, FIXaEGF2, and FIXaEGF1, whereas FIXaEGF2 and FIXaR94D were normal. For all of the FIXa proteins, $K_{m(app)}$ values were normal as were EC50 values for interactions with FVIIla. However, $K_{d(app)}$ (in nm) for the FX activating complex assembled on phospholipid vesicles was increased for FIXaEGF2 (43.3 ± 2.70), FIXaEGF2 (10.9 ± 2.8), FIXaEGF2 (70.5 ± 1.60), and FIXaEGF1 (2.90 ± 0.11), FIXaEGF1 (4.6 ± 0.17), FIXaEGF2 (4.5 ± 0.20), and FIXaEGF2 (2.2 ± 0.09) suggesting that reduced $V_{\text{max}}$ is a result of impaired complex assembly. These data indicate that residues 88–109 (but not Arg94) are important for normal assembly of the FX activating complex on phospholipid vesicles.

* This study was supported by the National Institutes of Health Research Grants HL56914, HL56153, and HL46213. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement." This article must therefore be hereby marked "advertisement."

** Factor IXa (FIXa) is a plasma glycoprotein required for normal hemostasis (1, 2). Individuals with a deficiency or a dysfunction of FIX exhibit a bleeding tendency—hemophilia B (3). The mature form of FIX contains 415 amino acids and 18% carbohydrate by mass. FIX circulates as a single chain zymogen until activation by either FIXa or FVIIa/tissue factor (FVIIa/TF) (4, 5). FIX is activated by sequential cleavage at Arg145 and Arg180 to generate FIXa. The light chain of FIXa contains the γ-carboxyglutamate-rich domain (Gla domain) and two consecutive domains with homology to epidermal growth factor (EGF1 and EGF2), whereas the heavy chain is the trypsin-like serine protease domain. Residues 146–180 comprise the carboxydrate-rich activation peptide (6, 7).

FIXa is a serine protease that participates in the intrinsic pathway of blood coagulation. FIXa activates FX as part of the intrinsic FX activating complex. The FX activating complex consists of FIXa, FVIIa (a nonenzymatic cofactor), and FX (the normal macromolecular substrate) assembled on a procoagulant surface (8, 9). Under physiological conditions, thrombin-activated platelets or endothelial cells can provide this surface (10–13). Additionally, synthetic phospholipid vesicles containing phosphatidylserine can also support assembly of the FX activating complex and activation of FX (8, 14). Assembly of the surface-bound FX activating complex results in a dramatic increase (<20 million-fold) in the catalytic efficiency ($k_{cat}/K_m$) of FX activation versus that of FIXa alone in solution (9, 15).

The surface localization of FIXa is a requisite step in the catalytic enhancement of FIXa when assembled in the FX activating complex (9, 14).

Recent investigations have focused on the contributions of the EGF-like domains to FIXa biochemistry. Analysis of patient data suggests that residues contained within EGF1 and EGF2 are important for FIXa functions. Several point mutations within the EGF-like domains result in dysfunctional FIXa activity and a bleeding tendency (3, 16). Investigations of the EGF-like domains have found several important functions for these domains. EGF1 is important for interactions with FVIIa/TF (17), FVIIla (18), and FX (19, 20). EGF1 does not appear to be important for binding to surfaces such as phospholipids (21), platelets, (22), or endothelial cells (23). While EGF2 has not been extensively characterized, experiments have indicated its importance for activation of FX. A chimeric FIXa protein in which both EGF1 and EGF2 domains were replaced by those of FX possessed about 4% clotting activity (24). In contrast, a chimeric protein with the FX EGF1 domain but the wild type FIX EGF2 domain was entirely normal (24), suggesting an essential function for EGF2. More recent experiments from our laboratory have indicated that EGF2 is likely involved in mediating surface binding to both platelets and phospholipids (25, 26).

To further define the contribution of the EGF2 domain to surface binding, we have prepared several chimeric FIXa proteins (see Fig. 1) using homologous sequences from FVII. These FIXa proteins include FIXaEGF2 (FIXa88–124, FVII91–127), FIXaEGF2 (FIXa88–99, FVII91–102), FIXaEGF1 (FIXa95–109, FVII98–112), and FIXaEGF2 (FIXa111–124, FVII114–

The notation Δ denotes a deletion of FIX residues; Ψ represents an insertion of FVII residues.
The mammalian expression vector pCMV5 was obtained as a generous gift from Dr. David Russel from the Texas Southwest Medical Center (Dallas, TX). The cDNA encoding human FIX and the cDNA encoding the human vitamin K-dependent γ-glutamyl carboxylase (pCMV.hgc) were obtained as generous gifts from Dr. Darrel Stafford from the University of North Carolina (Chapel Hill, NC). The cDNA encoding human FVII in the vector pUC19-FVII was supplied from American Type Culture Collection (59790, Manassas, VA). Cloned DNA polymerase from *Pyrococcus furius* and reaction buffer were purchased from Stratagene (La Jolla, CA) in a final reaction volume of 100 µl and overlaid with 100 µl of mineral oil (Sigma). cDNA fragments for each of the chimeric and mutant FIX proteins were subcloned into the expression vector pCMV5 and sequenced at the DNA Sequencing Facility at the University of Pennsylvania.

**Expression and Purification of FIX Proteins**—Stable cell lines expressing the various FIX chimeras and mutants were prepared by transfection of HEK293 cells using the calcium phosphate precipitation technique with commercially available reagents (Promega, Madison, WI) and the manufacturer’s protocol. Transfection experiments included the appropriate FIX expression vector, pCMV.hgc, and a neomycin resistance vector (pSV2Neo). G418-resistant cell lines were subcultured with the aid of sterile polyester swabs (Hardwood Products Company, Guilford, ME). Expression of FIX proteins in the medium was determined by enzyme-linked immunosorbent assay as previously described (26). Cell lines exhibiting maximal expression of FIX proteins were expanded for preparative-scale protein expression. FIX proteins were purified from serum-free conditioned medium using Q-Sepharose chromatography as described previously (26, 30).

Q-Sepharose was used as a “pseudoaffinity” strategy for the preparation of recombinant FIX proteins from serum-free conditioned medium. Serum-free conditioned medium was adjusted to contain 5 mM benzamidine and 5 mM EDTA, centrifuged (6000 × *g*), and filtered through cellulose acetate membranes (0.22-µm pore size) to remove cell debris. Q-Sepharose was pre-equilibrated with TBS supplemented with 2 mM benzamidine and 2 mM EDTA. The medium was chromatographed, and the resin was washed with the equilibration buffer. EDTA was removed from the resin by washing with TBS supplemented with 2 mM benzamidine. FIX proteins were eluted from the column with TBS supplemented with 2 mM benzamidine and 5 mM CaCl2. The FIX proteins recovered from the Q-Sepharose column were dialyzed against HT and concentrated using Centri-prep 3 concentrators. FIX proteins were stored in small aliquots under liquid nitrogen.

**Rat Carboxyglutamate Analysis**—Carboxyglutamate analysis was performed as described by Praszycki (31). It was generously performed by Dr. Rodney Camire in the laboratory of Dr. Katherine High (Children’s Hospital of Philadelphia, Philadelphia, PA).

**Preparation of Proteinase K/Phosphatidylcholine (PSPC) Vesicles**—Extruded phospholipid vesicles composed of (mol/mol) 25% phosphatidylserine and 75% phosphatidylcholine were prepared according to the following protocol: phosphatidylcholine and porcine brain phosphatidylserine were mixed in a 3:1 molar ratio and dried under nitrogen. The lipids were redissolved in benzene, frozen on dry ice, and lyophilized until dry. The lipids were resuspended in HT and incubated on ice for 30 min with intermittent mixing. The lipids were subjected to a freeze-thaw cycle (dry ice, 37 °C) five times and extruded by passage through the stacked polycarbonate membrane filters (100-nm pore size) a total of six times. The final lipid concentration was ~2 mM.

**Activation of FIX Proteins by FXa**—FIX proteins were activated to their FXa forms as follows: FIX proteins were diluted in HT supplemented with 10 mM CaCl2. FXa was added to a 1:2000 molar ratio, and the reactions were incubated at 37 °C for 90 min. Complete activation was judged by SDS-PAGE/ silver staining and by active site titration with ATIII. FIXa proteins were stored in small aliquots at ~80 °C.

**Western Immunoblotting**—Activated FIX proteins were separated on a 8–16% polyacrylamide gradient gel. The gel was placed in transfer buffer (48 mM Tris, 30 mM glycine, 0.037% w/v SDS, 20% v/v methanol) for 20 min with gentle agitation. Immobilon-P PVDF membrane (Millipore) and filter paper were cut to fit the gel. Filter paper was wetted with transfer buffer and transferred with membrane. The transfer of proteins from the gel to the PVDF was performed in a Transblot SD semi-dry transfer cell (Bio-Rad) at constant current (0.8 mA per cm2) for 20 min. The PVDF was agitated in blocking buffer (10% Irish cream in TBS) overnight while shaking. The PVDF was incubated for 1 h with 1:1000 dilution (0.5 mg/ml stock) of affinity-purified goat anti-human FIX polyclonal antibody conjugated to horse-
radish peroxidase in TBS-Tween (TBS supplemented with 0.1% v/v Tween 20) at 4°C. After incubation with the horseradish peroxidase-conjugated antibody, the PVDF was washed two times for 5 min each in TBS-Tween. A commercially available substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was used for visualization according to the following protocol: 3,3’5,5’-tetramethylbenzidine (0.4 g/L) solution was mixed with an equimolar amount of hydrogen peroxide (0.02% in citric acid buffer) and 0.1 volume of membrane-enhancing reagent. The PVDF was incubated at room temperature with the substrate solution until the desired intensity of the bands was achieved. The reaction was terminated by rinsing the membrane with distilled water, and the PVDF membrane was allowed to dry at room temperature.

**Active Site Titration of FIXa Proteins**—FOXa was active site-titrated by ATIII according to the following method (32): ATIII dilutions (0–100 nM) and FIXa proteins (∼100 nM) were prepared in HT supplemented with BSA (0.5 mg/ml), heparin (20 μg/ml), and 5 mM CaCl2. FXa dilution was added to a tube containing the ATIII dilution, incubated at 37°C for 20 min, then diluted 10-fold using HT supplemented with BSA (0.5 mg/ml). Residual FIXa activity was determined by assaying FIXa generation activity in the following assay conditions: 5 mM CaCl2, 400 nM FX, FVIII (5 units/ml), and 20 μM PSPC vesicles. The reactions were initiated by addition of thrombin to 1 unit/ml. After 2 min, the reactions were terminated by the addition of stopping buffer (50 mM HEPES, pH 8.1, 1 mM EDTA, and 20 mM EDTA). FIXa generation was determined by hydrolysis of the chromogenic substrate S-2765. S-2765 dissolved to 0.025 M in 50 mM HEPES, pH 8.1, and 175 mM NaCl was mixed with the sample in micrometer wells, and change in absorbance at 405 nm was monitored immediately.

**Assay of FXa**—FXa was assayed by hydrolysis of the FXa-specific chromogenic substrate S-2765. FXa (50 μl) was added to wells of a microtiter plate, and S-2765 (50 μl) was added to a final concentration of 350 μM. Change in absorbance at 405 nm was measured immediately. Unknown FXa concentrations were determined by comparison to a standard curve prepared with known dilutions of FXa.

**Determination of K_{d,app} and V_{max} in the Absence of FVIIIa**—FIXa proteins were added to 10 nM in reaction vessels containing 20 μM extruded PSPC vesicles in HT supplemented with BSA (0.5 mg/ml) and 5 mM CaCl2. FIXa proteins were incubated with the PSPC vesicles at 37°C for 2 min. The reactions were initiated by addition of FX to the indicated final concentrations. After 20 min at 37°C, the reactions were stopped by addition of EDTA to 10 mM. FXa was determined as described above. Velocity of FXa generation (nm FXa/min) was plotted as a function of input FIXa concentration (nm FXa). K_{d,app} was determined as described below.

**Assay of FXa Generation**—FXa generation was determined as described above. Velocity of FXa generation (nm FXa/min) was plotted as a function of input FIXa concentration (nm FXa). K_{d,app} was determined as described below.

**FX Titration in the Absence of a Surface**—FX proteins were added to 50 nM in reaction vessels containing HT supplemented with BSA (0.5 mg/ml) and 5 mM CaCl2. FXa proteins were incubated at 37°C for 2 min. The reactions were initiated by addition of FX to the indicated final concentrations. After 1 h at 37°C the reactions were stopped by addition of EDTA to 10 mM. FXa was determined as described above. Velocity of FXa generation (nm FXa/min) was plotted as a function of input FXa concentration (nm FXa). Data were analyzed as described below.

**Data Analysis**—Rates of FXa generation from all reactions were fitted to a rectangular hyperbola curve using a non-linear least squares fit. Constants for K_{m}, V_{max}, C_{50}, V_{max}, K_{d,app} and V_{max} were derived using the same Kalaidegraph Software. Between-group differences were tested for statistical significance using analysis of variance followed by pair-wise comparisons with the Bonferroni adjustment procedure for multiple comparisons maintaining an experiment-wise Type I error level of 0.05 (33). For the solution-phase activation of FX, data analysis was performed by least squares regression. A separate regression analysis was performed for each protein. Slopes and intercepts were tested for significance along with linearity of regression. Ninety-five percent confidence limits were generated for each slope and intercept. Differences between slopes were tested using an independent t test with a Bonferroni adjustment to correct for multiple comparisons (34). Differences were considered significant if the probability of chance occurrence was less than or equal to 0.05.

**Prediction of V_{max} Values Derived from Estimated Complex Assembly**—Determined from K_{d,app} and V_{max} Values—Predicted V_{max} values (see Table II) are shown for comparison with experimentally determined V_{max} values. The predicted V_{max} values were determined from the following equation: V_{max} (estimated) = V_{max} × (FXa/[K_{d,app} + (FIXa)]). Where V_{max} is the velocity in the presence of saturating FIXa concentration and K_{d,app} is the apparent dissociation constant for the binding of FIXa to the factor X activating complex and the input FIXa concentration used in the experiment (15 nM).

**RESULTS**

All of the chimeric and mutant FIX proteins were expressed in HEK293 cells and purified from conditioned serum-free medium (30). The amino acid sequences of FIX and FVII within the EGF2 domain that were exchanged to prepare the chimeric proteins utilized in this study are shown in Fig. 1. All of the recombinant FIX proteins were found to have >95% purity as judged by SDS-PAGE and staining with Coomassie Brilliant Blue (Fig. 2A). Each of the FIX proteins was found to comigrate with plasma-derived FIX (FIX\_x) suggesting normal post-translational modifications including removal of the pre- and propeptides and addition of carbohydrate. Furthermore, each of the recombinant FIX proteins was found to have the expected amount of γ-carboxyglutamate (Gla) modification (10–12 mol Gla/mol protein) (Table I). Each of the FIX proteins was activated with FXIa. Both silver-stained gels and Western blotting of SDS-PAGE showed disappearance of thezymogen band and appearance of heavy and light chains of FIXa. All of the FIX proteins were entirely converted to the FIXa form as judged by SDS-PAGE and silver staining (Fig. 2B). A band corresponding to the molecular mass of the FIXa heavy chain (28 kDa) can be observed for all of the FIXa proteins. The FIXa light chain stains poorly as has been observed by others (17, 35). All FIXa proteins were also examined by Western immunoblotting, which confirmed complete conversion to the FIXa form (data not shown). Bands corresponding to the molecular mass of the heavy chain (28 kDa) and light chain (∼18 kDa) and the Gla-containing peptide (10–12 kDa) were observed with equal intensity for each of the eight proteins. No bands at ∼70 kDa were detectable either by silver staining or Western blotting indicating complete activation of the FIXa proteins. Complete activation of all eight FIX proteins was also supported by active site titration with ATIII (FIXa) = ~100 nM as estimated from A280, Fig. 3, Table I). Starting

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activities of the proteins at ATIII = 0 reflect differences in their enzymatic activity in the second stage of this assay. Complete ATIII neutralization of each of their activities occurs at 90–110 nM.

Since effective FX activation involves assembly of the enzymatic complex and flux of FX to FXa, the contribution of each of the loops of the EGF2 domain, as well as that of Arg94, to the assembly of the FX activating complex was determined from FX titrations on phospholipid vesicles (Fig. 4). Kinetic constants determined from the isotherms are reported in Table II. When compared with FIXaN and FIXaWT, the proteins FIXaloop1, FIXaloop2, and FIXaloop1G94R were found to have a reduced $V_{\text{max}}$ (2– to 4-fold), whereas FIXaloop3 and FIXaR94D were entirely normal. These observations confirm previously published observations indicating the importance of the FIXaloop1 mutant. These data suggest that Arg 94 itself is not essential for assembly of the FX activating complex and activation of FX (25, 26).

Interestingly, the R94D mutant showed no reduction in $V_{\text{max}}$. This observation was in contrast to our expectations given that Arg94 has been implicated to be important for interaction with FVIIIa (36). Consistent with this observation, the FIXaloop1G94R protein showed no restoration of activity versus the FIXaloop1 mutant. These data suggest that Arg94 itself is not essential for assembly of the FX activating complex and activation of FX. A discussion of the contrast between our observations and those of other investigators (16, 35–37) is presented below. While Arg94 is present within the linear sequence determined to be important for FX activation (residues 88–109), it does not appear to be essential for this activity.

Since there is no increase in $K_m$ for FIXaloop1G94R, FIXaloop1G94R, and FIXaloop3G94R, the reduced $V_{\text{max}}$ is not likely to be a result of deficient interactions with FX. $K_m$ values for each of these proteins (actually $2\times$– 3-fold lower than those of the FIXaloop1 and FIXaWT) show no evidence of impaired interaction with FX. Therefore, we considered the possibility that the reduced $V_{\text{max}}$ may be a result of impaired ability to interact with either FVIIIa or the phospholipid surface, which would result in a reduced $k_{\text{cat}}$ or a reduced effective enzyme concentration, respectively. In the presence of activated platelets or phospholipids, FVIIa is known to contribute to a several thousand-fold increase in $k_{\text{cat}}$ (9, 14, 15). The reduced $V_{\text{max}}$ values could result from a defective interaction with FVIIa such that the full increase in $k_{\text{cat}}$ is not achieved by the mutants. This hypothesis was tested in two ways. FX titrations were carried out in the

| FIX protein | Mol of Gla · mol of protein $^{-1}$ | Active site concentration $^{ab}$ |
|-------------|-----------------------------------|----------------------------------|
| N           | 11.9 ± 0.04                       | 90                               |
| WT          | 10.5 ± 0.40                       | 91                               |
| Loop 3      | 11.2 ± 0.06                       | 90                               |
| R94D        | 11.2 ± 0.30                       | 90                               |
| FVIIEGF2    | 10.9 ± 0.50                       | 110                              |
| Loop 1      | 11.0 ± 0.10                       | 130                              |
| Loop 2      | 10.4 ± 0.05                       | 101                              |
| Loop 1 G94R | 11.6 ± 0.10                       | 106                              |

$^{ab}$ Mol of γ-carboxyglutamate per mol of FIX protein. Values are mean ± standard deviation of three independent measurements.

$^{b}$ Active site concentration in nM ($10^{-9}$) determined by active site titration with ATIII where the expected active site concentration based on $X_{\text{ATIII}}$ was 100 nM for each protein assayed. Plasma-derived (N), FIXaloop1G94R (wild type), FIXaloop1 (loop 1), FIXaR94D (R94D), FIXaloop3G94R (FVIIEGF2), FIXaloop1 (loop 1), FIXaloop2 (loop 2), FIXaloop3G94R (loop 1 G94R).
presence of phospholipid vesicles but in the absence of FVIIIa as shown in Fig. 4B and kinetic constants determined from the isotherms are reported in Table II. The proteins FIXaFVIIEGF2, FIXaloop1, FIXaloop2, and FIXaloop1G94R were again found to have a reduced $V_{\text{max}}$ (2- to 3-fold) in the absence of FVIIIa suggesting that an impaired interaction with FVIIIa cannot explain the reduced $V_{\text{max}}$ of FIXaFVIIEGF2, FIXaloop1, FIXaloop2, and FIXaloop1G94R. The magnitude of the reduction in $V_{\text{max}}$ (2- to 3-fold, Table II) is similar in both the presence and absence of FVIIIa. Since the $K_{\text{m(app)}}$ values are entirely normal (Table II), in the absence of FVIIIa just as in its presence, the reduced $V_{\text{max}}$ values of FIXaFVIIEGF2, FIXaloop1, FIXaloop2, and FIXaloop1G94R are not the result of impaired ability to interact with FX.

To confirm that the interactions of all FIXa proteins with FVIIIa were indeed normal, EC$_{50}$ values and $V_{\text{max}}^{8}$ (maximum velocity observed in the presence of saturating concentrations of FVIIIa) for FVIIIa stimulation of FXa generation were determined. The EC$_{50}$ values for FVIIIa stimulation of FXa generation were entirely normal for all of the FIXa proteins (Figs. 5, A and B, Table III). A reduced $V_{\text{max}}^{8}$ (5- to 10-fold) for FIXaFVIIEGF2, FIXaloop1, FIXaloop2, and FIXaloop1G94R was also observed in this assay (Fig. 5B). Deficient interactions with FVIIIa would have been expected to result in an increased EC$_{50}$ as well as a reduced $V_{\text{max}}^{8}$. We therefore conclude that a deficiency of functional interactions with FVIIIa does not account for the reduced $V_{\text{max}}$ of FXa generation for FIXaFVIIEGF2, FIXaloop1, FIXaloop2, and FIXaloop1G94R.

Previous investigations suggested that the FIXa EGF2 domain promotes surface assembly of the FX activating complex (25, 26). The reduced $V_{\text{max}}$ of FIXaFVIIEGF2, FIXaloop1, FIXaloop2, and FIXaloop1G94R could be attributed to deficient surface complex assembly. It is predicted by this hypothesis that the activities of all FIXa proteins in the solution phase should be indistinguishable, although in the absence of a surface, an increased $K_{\text{m(app)}}$ would be expected (9). When FX titrations were carried out in the absence of a surface (Fig. 6), these data suggested that the FX activating activities of all FIXa proteins are equivalent in the absence of a reaction surface and confirm that interactions with FX are normal.

Since FX activation by the FIXa proteins is nearly equivalent in the absence of phospholipids, the EGF2 domain may be important for complex assembly only in their presence. To determine the affinity of the FIXa proteins for the FX activating complex in the presence of phospholipid, FIXa titrations were carried out, and the $K_{\text{d(app)}}$ determined from the isotherms shown in Figs. 7, A and B. The $K_{\text{d(app)}}$ (the affinity for the complex) and $V_{\text{max}}^{9}$ (velocity of FXa generation observed in the presence of saturating [FIXa]) are reported in Table IV.
Kinetic properties of the various FIXa EGF2 chimeras and mutants on phospholipid vesicles

Values are mean ± S.E.M. (standard error of the mean) of independent measurements (n = 7). Apparent \( K_e \) values are in nM, \( V_{max} \) values are in nM FXa · min⁻¹. Plasma-derived FIXa (N), FIXaWT (wild type), FIXaloop3 (loop 3), FIXaR94D (R94D), FIXaFVIIEGF2 (FVIIEGF2), FIXaloop1 (loop 1), FIXaloop2 (loop 2), FIXaloop3 G94R (loop 1 G94R). When compared to FIXaN, symbols denote \( p \) values as indicated (*, \( p < 0.0001; \), \( p = 0.0004; \), ‡, not significant). \( V_{max} \) of 15 nM FIXa estimated from \( K_{d(app)} \) FIXa and \( V_{max} \) (Figure 7 and Table IV).

### Table II

| FIXa               | \( K_e \) (nM) | \( V_{max} \) (nM FXa · min⁻¹) | Predicted \( V_{max} \) | \( K_e \) (nM) | \( V_{max} \) (nM FXa · min⁻¹) |
|-------------------|----------------|-------------------------------|-------------------------|----------------|--------------------------------|
| N                 | 53.5 ± 5.8     | 38.5 ± 1.6                    | 36.0                    | 78.6 ± 4.7     | 0.040 ± 0.001                 |
| WT                | 55.8 ± 6.2‡    | 39.6 ± 1.7                    | 35.0                    | 82.8 ± 3.9‡    | 0.048 ± 0.001‡                |
| Loop 3            | 52.8 ± 5.8‡    | 36.6 ± 1.5                    | 35.0                    | 82.2 ± 6.4‡    | 0.034 ± 0.001‡                |
| R94D              | 71.0 ± 6.4*    | 46.4 ± 1.7                    | 42.8                    | 80.3 ± 6.1‡    | 0.032 ± 0.001‡                |
| FVIIEGF2          | 10.6 ± 3.4*    | 8.7 ± 0.7*                    | 6.4                     | 69.6 ± 9.2*    | 0.016 ± 0.001*                |
| Loop 1            | 17.5 ± 3.9*    | 12.3 ± 0.8                    | 13.6                    | 60.1 ± 5.9*    | 0.015 ± 0.001*                |
| Loop 2            | 13.7 ± 3.7*    | 10.5 ± 0.7                    | 5.4                     | 75.6 ± 9.1‡    | 0.024 ± 0.001§                |
| Loop 1 G94R       | 15.6 ± 5.9*    | 13.2 ± 1.3*                   | 11.8                    | 88.8 ± 12.9‡   | 0.011 ± 0.001*                |

### Table III

\( EC_{50} \) and \( V_{max} \) values for stimulation of FXa generation

Values are mean ± standard deviation of independent measurements (n = 3). \( EC_{50} \) values are in units · ml⁻¹, \( V_{max} \) values are in nM FXa · min⁻¹. Plasma-derived FIXa (N), FIXaWT (wild type), FIXaloop3 (loop 3), FIXaR94D (R94D), FIXaFVIIEGF2 (FVIIEGF2), FIXaloop1 (loop 1), FIXaloop2 (loop 2), FIXaloop3 G94R (loop 1 G94R).

| FIXa               | \( EC_{50} \) | \( V_{max} \) |
|-------------------|--------------|--------------|
|                   | units · ml⁻¹ | nM FXa · min⁻¹ |
| N                 | 1.00 ± 0.19  | 28.12 ± 1.33 |
| WT                | 1.46 ± 0.26  | 36.73 ± 1.80 |
| Loop 3            | 1.62 ± 0.25  | 32.20 ± 1.43 |
| R94D              | 1.34 ± 0.22  | 39.09 ± 1.76 |
| FVIIEGF2          | 1.57 ± 0.41  | 3.12 ± 0.27  |
| Loop 1            | 1.99 ± 0.39  | 7.39 ± 0.52  |
| Loop 2            | 1.99 ± 0.35  | 6.83 ± 0.43  |
| Loop 1 G94R       | 1.93 ± 0.36  | 6.44 ± 0.44  |

Fig. 5. Determination of \( EC_{50} \) and \( V_{max} \) for FVIIIa stimulation of FXa generation. FIXa proteins were diluted to 5 nM in HT buffer containing 500 nM PSPC vesicles. FVIIIa was added to the indicated final concentration and activated to FVIIIa by thrombin. Reactions were initiated by addition of FX to 250 nM. After 2 min, the reactions were terminated by addition of EDTA to 10 mM. A, filled circle (FIXaWT), filled square (FIXaloop3), filled triangle (FIXaR94D), open square (FIXaFVIIEGF2), open diamond (FIXaloop1), open diamond (FIXaloop2), open triangle (FIXaloop3 G94R).

Clearly, FIXaFVIIEGF2, FIXaloop1, FIXaloop2, and FIXaloop3 G94R show a reduced affinity for the FX activating complex as manifested by an increased \( K_{d(app)} \).

Using these data, we were able to predict a \( V_{max} \) (in addition to the experimentally determined \( V_{max} \)) for each of the FIXa proteins under the experimental conditions used for our FX titrations in the presence of FVIIIa (Fig. 4A, Table II). The predicted \( V_{max} \) is calculated from the occupancy of FX activating complexes as a function of the input FIXa concentration and the \( K_{d(app)} \) (fractional occupancy = [FIXa]/\( K_{d(app)} \) + [FIXa]). This fractional occupancy determines the expected activity (as a fraction of \( V_{max} \)) since FX activating activity shows a strong dependence on FIXa complex association. The excellent agreement between the determined \( V_{max} \) values and those predicted (Table II) is evidence that the reduction in \( V_{max} \) is a consequence of reduced complex assembly on the part of those predicted (Table II) is evidence that the reduction in \( V_{max} \) is a consequence of reduced complex assembly on the part of FIXEFG2 domains of FIXa in medi-
FIXa loop1, FIXa loop2, FIXa loop1G94R. When compared to FIXa WT, FIXaloop1, and FIXaloop3 to contribute to this interaction. Four chimeric proteins have been developed: FIXaEGF2, FIXaloop1, FIXaloop2, and FIXaloop3 to determine the contribution of each of the loops of the EGF2 domain to assembly of the FX activating complex. The loops are defined by the conserved disulfide-pairing characteristic of EGF-like domains. Loop 1 comprises residues 88–99; loop 2 includes residues 95–109; and loop 3 includes residues 111–124 (Fig. 1).

Structural analysis of FIXa (37) suggests that Arg94 may form a salt bridge with Glu78 in EGF1. The Glu78–Arg94 salt bridge could potentially be important for stabilization of the tertiary structure of the FIXa light chain. Hemophilia B patient data (16) and functional studies (36) have suggested that both Glu78 and Arg94 may be important residues for activation of FX mainly by promoting interaction with FVIIIa. To determine the contribution of Arg94 to assembly of the FX activating complex, two point mutations, FIXR94D and FIXloop1G94R, were prepared.

Optimal rates of FXa generation are achieved only upon assembly of the FX activating complex, which consists of FIXa, FVIIIa, and the substrate, FX, bound to a reaction surface such as phospholipid vesicles or thrombin-activated platelets (2, 9, 14). Understanding of the interactions of FIXa with the reaction surface is key to understanding assembly of the FX activating complex.

The importance of the Gla domain for the binding of FIXa to phospholipid surfaces, thrombin-activated platelets, and endothelial cells has long been recognized (23, 38–40). However, several observations suggest that domains in addition to the Gla domain can mediate surface binding. A peptide corresponding to residues Gly4–Gln11 of the Gla domain was relatively ineffective as an inhibitor of platelet-mediated FX activation (Kd = 165 nM) while able to displace ~50% of the surface-bound FIXa molecules (Kd = 3 nM) (41). FIXa molecules in which the Gla domain was removed by proteolysis, or in which the Gla domain was chemically modified, retained both platelet binding activity (with reduced affinity, Kd = 5 nM) and FX activation activity (42). Moreover, chimeric molecules with FVIII or FX residues inserted into the Gly4–Gln11 sequence of FIX(a) bind to a reduced number of sites (250 sites/platelet) with decreased affinity (Kd = 10–15 nM) and promote FX activation at a reduced rate, a defect which is entirely explained by the decreased binding affinity (40). It is therefore likely that domains other than the Gla domain can mediate surface binding as well.

The interaction of EGF with the EGF-receptor has been well characterized (43). By virtue of their structural homology with EGF, it was thought that the EGF-like domains may contribute to surface binding (37, 44, 45). EGF-like domains are thought to be mediators of protein-protein interactions by conservation of this function from EGF. A physiological contribution of the EGF-like domains is suggested by the finding that mutations occurring within these domains are associated with a bleeding tendency (3, 16). There has been a recent interest in further characterizing these domains and determining their contribution to FIXa procoagulant functions.

The contribution of the EGF-like domains of FIXa to assembly of the FX activating complex is poorly understood. Several investigations have indicated that the EGF1 domain of FIXa does not contribute to surface-binding properties of FIXa. Chimeric proteins in which the EGF1 domain of FIXa was substituted for that of FX were entirely normal for all functional properties measured including rate of FX activation, platelet-binding affinity, and platelet-binding stoichiometry (22, 24). A chimeric FIXa protein with the EGF1 domain of FVII was reported to have no functional impairments but actually showed an ~3-fold increase in catalytic activity (21). In contrast, a FIX chimera in which the EGF1 domain was substituted for that of protein C was found to be impaired in its activation by FVII/TF and impaired (as the enzyme) in its
interactions with FVIIIa in the presence of phospholipids (17, 18). However, these functional deficiencies were found to result from structural abnormalities of the chimeric protein rather than from loss of EGF1 residues involved in functional contacts. Thus the primary contribution of the EGF1 domain to assembly of the FX activating complex appears to be a structural role.

Recent investigations from our laboratory have indicated that the EGF2 domain may mediate binding to the reaction surface. Chimeric FIXa proteins in which the EGF2 domain of FX was substituted for that of FIX were found to have a reduced rate of FX activation (25). Further characterization of the FIXaEGF2 Protein showed that it had a reduced platelet binding affinity and stoichiometry (26). The contribution of the FIXa EGF2 domain was further investigated in this study by preparing several FIXa EGF2 chimeras and mutant proteins. In this paper we have presented the purification, characterization, and evaluation of the kinetic properties of these FIXa proteins.

Our results reported here define a subset of residues within the EGF2 domain (88–109, excluding Arg94) that are primarily responsible for optimal binding of FIXa to the FX activating complex. However, residues 111–124 did not appear to be important since the FIXaEGF2 protein (where residues 111–124 were converted to the FVII sequence) was entirely normal in all assays. It is possible that the FVII residues can effectively substitute for the FIX residues. The consequence of this phenomenon would be to mask any functional contribution of these residues that would have otherwise been detected. However, it is clear from our investigation that residues present within loop1 and loop2 of EGF2 (namely 88–109, excluding Arg94) are important for FX activating complex assembly.

The observation that Arg94 is not essential for FX activation complex assembly is surprising. Mutation of either residue (E87K or R94S) has been linked to hemophilia B with near normal antigen concentration (16). Observations by Christophe, et al. (36) have indicated that a salt-bridge between Glu78 of the EGF1 domain and Arg94 of the EGF2 domain is important for normal interactions with FVIIIa. While an E78K point mutation resulted in an impaired interaction with FVIIIa, an R94D point mutation did not result in a functional deficiency (36). Subsequent investigations have determined that the R94S substitution introduces a carbohydrate modification to this site and accounts for the functional impairment of the R94S substitution (35) in the hemophilia B patient. The normal behavior of FIXaR94D suggests a compensation mechanism that allows the R94D protein to function normally while the E78K protein is impaired. Collectively, these studies indicate that Arg94 is not essential for any functional activity studied thus far.

Kinetic analysis of the FIXa proteins has identified a deficiency of FX activation for FIXaEGF2, FIXaEGF2R, and FIXaEGF2G94R characterized by a reduced Vmax of FX activation in the presence of phospholipids (Tables II and III, Figs. 4–6). Our data support the conclusion that this kinetic defect results from impaired complex assembly in the presence of phospholipids and does not result from impaired interactions with either FVIIIa or FX. First, we excluded the possibility that these mutants were not completely activated or that there was a failure of some active sites to develop after cleavage of the FIXzymogen. Protein stains, Western immunoblot analysis and active site titration with ATIII of FIXa-activated FIX proteins indicates that all of the proteins were completely activated. Western immunoblot analysis shows complete disappearance of the ~70 kDa zymogen band and appearance of bands corresponding to the heavy chain (~28 kDa), light chain (~18 kDa), and activation peptide of FIXa (~10 kDa). Active site titration with ATIII indicated that the expected number of active sites were formed as predicted from protein concentration (Fig. 3 and Table I). Therefore, the reduced Vmax of FX activation for FIXaEGF2, FIXaEGF2R, FIXaEGF2G94R and FIXaEGF2G94R is not due to a reduced concentration of active sites. Finally, fluid phase activation of FX was identical for all forms of FIXa, indicating a similar concentration of active sites.

An additional consequence of the mutations could be impaired interactions with FVIIIa, FX, or both. The physiological function of FVIIIa is a large (1000-fold) increase in kcat for FX activation (9, 15). The reduced Vmax of FX activation for FIXaEGF2, FIXaEGF2R, and FIXaEGF2G94R could result from deficient interaction of these proteins with FVIIIa. However, two lines of evidence indicate that the interaction of all FIXa proteins with FVIIIa is normal. First, the Vmax defect of FIXaEGF2, FIXaEGF2R, and FIXaEGF2G94R is manifested in both the presence and absence of FVIIIa (Fig. 4, A and B, Table II). The reduced Vmax is of the same magnitude (2- to 4-fold) in both the presence and absence of FVIIIa, and the relative increase in Vmax for all of the FIXa proteins attributed to the presence of FVIIIa was similar suggesting that functional consequences as a result of FVIIIa binding were entirely normal. EC50 values for the stimulation of FXa generation by FVIIIa are entirely normal for all of the FIXa proteins (Fig. 5, Table III). The EC50 values suggest that the affinity of each of the FIXa proteins for FVIIIa is normal. Therefore it is highly unlikely that reduced or deficient interaction of FIXa proteins with FVIIIa would result in the reduced Vmax of FX activation.

Finally, the reduced Vmax of FX activation cannot be shown to be a result of impaired interactions of the FIXa mutants with FX. Kd(app) values for all of the FIXa proteins are normal both in the presence and absence of FVIIIa (Fig. 4, A and B, Table II). While we have observed a Vmax deficiency, this property is observed only in the presence of a reaction surface (Figs. 4–5). The solution-phase activation of FX by all of the FIXa proteins suggested that the rates of FX activation (kcat) and thus the affinities for FX are normal (Fig. 6).

The hypothesis that the Vmax deficiencies of FIXaEGF2, FIXaEGF2G94R and FIXaEGF2G94R are a result of reduced surface complex assembly is consistent with all of our experimental results and is confirmed by the FIXa titrations in the presence of phospholipid vesicles presented in Fig. 7, A and B and Table IV. The Kd(app) of the affinities of the FIXa proteins for the surface and the proteins (FVIIIa and FX) complexed with it. The increased Kd(app) values for FIXaEGF2, FIXaEGF2R, and FIXaEGF2G94R indicate a reduction in the affinities of these proteins for the surface-bound complex. In strong support of this argument is the agreement of determined Vmax values with those predicted based on the Kd(app) of each of the FIXa proteins (Table II). The predicted Vmax values were determined by calculating the fraction of functional complexes based on the input FIXa concentration and the Kd(app) as described in the experimental procedures. It has been shown that the concentration of functional active sites, and therefore the activity (Vmax), show a strong correlation with surface occupancy of the FIXa protein (11). The reduced ability of these proteins to assemble into complexes on phospholipid vesicles would result in a reduced number of functional active sites. The reduced effective active site concentration would be expected to result in a reduced Vmax while other kinetic constants (Kcat for FX, EC50 for FVIIIa) would be unaffected.

Unexpectedly, we observed a reduction in Vmax for
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FIXαEGF2, FIXαloop1, FIXαloop2, and FIXαloop1G94R. This observation could be interpreted as a reduction in \( k_{\text{cat}} \) and hence as evidence that the catalytic properties of these proteins have been perturbed. However, data presented in our accompanying paper (46) offer an alternative explanation. \( V_{\text{max}} \) depends on both \( k_{\text{cat}} \) as well as the number of available complexes for the FIXα proteins to bind. The reduced \( V_{\text{max}} \) that we observe could be a consequence of reduced complex stoichiometry (lower number of available binding sites). It is not immediately clear how to reconcile this observation with the current working model for assembly of the FX activating complex in the presence of phospholipids. These observations clearly indicate the need for further investigation.

The aim of this study has been to determine which residues in the FIXα EGF2 domain are important for assembly of the FX activating complex. We have shown here that residues 88–109 (excluding Arg94) are important for normal FIXα activation of FX. These residues appear to be primarily responsible for efficient assembly of the FX activating complex in the presence of phospholipids. Moreover, the failure of FIXα EGF2 mutations to promote normal clotting in hemophilia B patients may be explained by the reduced complex assembly properties of these proteins. Thus, an important function of the EGF2 domain of FIXα is to confer normal FX activating complex assembly in the presence of a reaction surface.

Acknowledgments—We are grateful to John Gaugham (Temple University School of Medicine) for help with the performance of statistical analysis and to Patricia Pileggi for her expertise in manuscript preparation.

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J. Biol. Chem. 2002, 277:5725-5733. doi: 10.1074/jbc.M107027200 originally published online November 28, 2001