The N-terminal Epidermal Growth Factor-like Domain in Factor IX and Factor X Represents an Important Recognition Motif for Binding to Tissue Factor*

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Factors VII, IX, and X play key roles in blood coagulation. Each protein contains an N-terminal γ-carboxyglutamic acid domain, followed by EGF1 and EGF2 domains, and the C-terminal serine protease domain. Protein C has similar domain structure and functions as an anticoagulant. During physiologic clotting, the factor VIIa-tissue factor (FVIIa/TF) complex activates both factor IX (FIX) and factor X (FX). FIXA represents the enzyme, and TF represents the membrane-bound cofactor for this reaction. The substrates FIX and FX may utilize multiple domains in binding to the FVIIa/TF complex. To investigate the role of the EGF1 domain in this context, we expressed wild type FIX (FIXWT), FIXQ50P, FIXPCEGF1 (EGF1 domain replaced with that of protein C), FIXQEGF1 (EGF1 domain deleted), FXWT, and FXPCEGF1. Complexes of FVIIa with TF as well as with soluble TF (sTF) lacking the transmembrane region were prepared, and activations of WT and mutant proteins were monitored by SDS-PAGE and by enzyme assays. FVIIa/TF or FVIIa/sTF activated each mutant significantly more slowly than the FIXWT or FXWT. Importantly, in ligand blot assays, FIXWT and FXWT bound to sTF, whereas mutants did not; however, all mutants and WT proteins bound to FVIIa. Further experiments revealed that the affinity of the mutants for sTF was reduced 3–10-fold and that the synthetic EGF1 domain (of FIX) inhibited FIX binding to sTF with a Ki of ~60 μM. Notably, each FIXa or FXa mutant activated FIXVII and bound to antithrombin, normally indicating correct folding of each protein. In additional experiments, FIXa with or without FIXIla activated FIXWT and FXPCEGF1 normally, which is interpreted to mean that the EGF1 domain of FX does not play a significant role in its interaction with FIXIla. Cumulatively, our data reveal that substrates FIX and FX in addition to interacting with FIXIla (enzyme) interact with TF (cofactor) using, in part, the EGF1 domain.

Human factor IX (FIX)3 and factor X (FX) are vitamin K-dependent glycoproteins with M, of 57,000 and 58,800, respectively (1, 2). Factor VIIa-tissue factor (FVIIa/TF) complex activates FIX to FIXa and FX to FXa by cleaving Arg145–Ala146 and Arg180–Val181 peptide bonds in FIX (3) and the Arg194–Ile195 peptide bond in FX (2). The resulting FIXa or FXa molecule consists of an N-terminal light chain and a C-terminal heavy chain linked by a disulfide bond. The light chain in each case contains a γ-carboxyglutamic acid (Gla) domain and two epidermal growth factor-like domains (EGF1 and EGF2), whereas the heavy chain contains the serine protease domain. In the blood coagulation cascade, FIXa also activates FX to FXa in a reaction that requires factor VIIIa (FVIIIa), phospholipid (PL), and calcium. FXa formed by either pathway then activates prothrombin to thrombin in a reaction that requires factor Va, PL, and calcium (4). In addition, both FIXa and FXa activate FVIII to FVIIla (5–7) and are inhibited by antithrombin (AT) (8, 9).

The conversion of single chain zymogen FVII to enzyme FVIIa involves the cleavage of a single peptide bond between Arg152 and Ile153. The FVIIa formed consists of a light chain of 152 amino acids and a heavy chain of 254 amino acids held together by a disulfide bond (10). Like FIXa and FXa, the N-terminal light chain of FVIIa contains the Gla domain and two EGF-like domains, whereas the heavy chain contains the serine protease domain (10). TF, the cellular cofactor for FVIIa, is composed of two fibronectin type III β-sandwich domains (11, 12). Recently, high resolution x-ray structure of the complex of soluble tissue factor (sTF) and FVIIa has been reported (13). In this structure, the Gla and EGF1 domains make contact with the C-terminal domain of TF and the EGF2 and the protease domains make contact with the N-terminal domain of TF (13). Thus, FVIIa uses all of its four domains in binding to the N- and C-terminal domains of TF (13).

Efforts have been directed to understanding the regions in FVIIa/TF that interact with the substrates FIX and FX. By studying the effect of mutations in the C-terminal domain of TF, it has been proposed that this domain may interact with the Gla domains of FIX and FX (14). Similarly, by mutagenesis and docking experiments, it has been proposed that the Gla

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domain of FVIIa interacts with the Gla domain of FX (15). Further, we reported earlier that the EGF1 domain of FIX is required for its activation by the FVIIa-TF complex (16). However, the role of the EGF1 domain of FX in this context has not been investigated. Moreover, it is not known whether FVIIa or TF in the FVIIa-TF complex interacts with the EGF1 domains of FIX and FX. Thus, the precise function of EGF1 domain of FIX or FX in its interaction with the FVIIa-TF complex is not known.

Protein C is a serine protease with an anticoagulant function whose domain organization is similar to that of FVIIa, FIXa, or FXa (17, 18). Further, activated protein C is not involved in the TF-induced coagulation, and its EGF1 domain near the N terminus has an eight-residue insertion (18). Therefore, substituting the EGF1 domain of FIX (or FX) with the EGF1 domain of protein C should replace the unique determinants present in the EGF1 domain of FIX (or FX) that provides specificity for its interaction with the FVIIIa-TF complex. In this report, in addition to the above two replacement mutants (FIXPC_EGF1 and FXPC_EGF1), we used a point mutant (FIXQ50P) and an EGF1 deletion mutant (FIXEGF1) of FIX to understand the function of this domain in TF-induced coagulation. Data are provided, which strongly indicate that TF interacts with the EGF1 domain in FIX and FX. Our findings represent the first report that assigns a specific function to the EGF1 domain in these proteins.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Carrier-free Na<sup>125</sup>I was obtained from ICN Biomedicals, Inc. Benzoyl-Ile-Glu-Gly-Arg-p-nitroanilide (S-2222) was obtained from Diapharm Inc. Biotinylated Glu-Gly-Arg-chloromethylketone (biotin-EGR-CR) was purchased from Hemalogic Technologies, Inc. Nitrocellulose membrane, polyethylene glycol 8000 (PEG), p-nitrophenyl phosphate, bovine serum albumin (BSA), bovine brain phosphatidylincholine, and phosphatidylserine were purchased from Sigma. Horseradish peroxidase-antimouse IgG and enhanced chemiluminescence (ECL) detection reagents were purchased from Amersham Biosciences. FVIII-depleted plasma and Neoplatin were obtained from Amersham Biosciences. Normal plasma FIX (FIX<sub>NP</sub>) plasma FX (FX<sub>NP</sub>), FXa, Russell's viper venom (RVV), and AT were obtained from Enzyme Research Laboratory. Low molecular weight heparin was purchased from Rhone-Poulenc Rorer Pharmaceuticals Inc. A monoclonal antibody-purified human FVIII concentrate was obtained from Dr. Leon Hoyser (American Red Cross, Rockville, MD). It was activated with 1 μM thrombin in the presence of 0.1% BSA and 5 mM CaCl<sub>2</sub> in Tris/NaCl at 37 °C for 2 min as described earlier (20). The formed FIXa was used in the activation of FIX by FVIIIa-FVIIIA-PL.

For ligand blot experiments, a Ca<sup>2+</sup>-dependent FIX monoclonal antibody (mAb) cell line was provided by Dr. Shirly Miekka of the American Red Cross, and the IgG was purified as described (21). A Ca<sup>2+</sup>-dependent mAb to the heavy chain of FX used for the ligand blot experiments was purchased from American Diagnostics, Inc. PL vesicles (75% phosphatidylcholine, 25% phosphatidylserine) were prepared by the method of Husten et al. (22). TF containing the transmembrane region (residues 1–243) was a gift from Genethic, Inc. The purification of the TF was performed as described (16). sTF that lacks the transmembrane region (residues 1–219) was a gift from Tom Gerard of Pharmacia Corp., St. Louis, MO.

For studies of AT binding and FVII activation, FIXa and FXa were prepared by activating FIX with FXa (16) and activating FIX with RVV (23) in 50 mM Tris, 0.15 mM NaCl (Tris/NaCl), pH 7.4, containing 5 mM CaCl<sub>2</sub> and 0.1% PEG at 37 °C for 2 h. Complete activation of FIX or FX was confirmed by SDS-PAGE (24).

**SDS-Gel Electrophoresis**—SDS-gel electrophoresis was performed using the Laemmli buffer system (24). The acrylamide concentration was 12%, and the gels were stained with Coomassie Brilliant Blue dye. All proteins used in the present study were >98% pure.

**Amino Acid Sequencing and Gla Analysis**—Gla and amino acid sequence analysis were performed by Commonwealth Biotechnologies, Inc. (Richmond, VA). Automated degradation of each protein (<0.5 nmol) was performed using an Applied Biosystems gas phase sequencer. Gla analysis of each protein was performed by alkaline hydrolysis followed by HPLC analysis. The amount of Gla was quantitated based upon A<sub><sup>280</sup></sub> and A<sub><sup>280</sup></sub> present per mol of each protein.

**Expression and Purification of Recombinant Factors IX and VII**—Recombinant FIX<sub>WT</sub>, FIX<sub>Q50P</sub>, FIX<sub>PC_EGF1</sub>, and FIX<sub>EGF1</sub> were expressed in human embryonic kidney 293 cells and purified by using the IX A-7 mAb column as described (16, 25). Each FIX protein had ~12 Gla residues/mol (16). To express FIX<sub>Q50P</sub>, the restriction sites AII and XhoI were introduced at the 5'- and 3'-ends of VII cDNA for ligation into pMol3506b expression vector (26) that was modified to contain AII and XhoI sites. A stable cell line that expressed FIX<sub>Q50P</sub> was established as described in detail by Hippenmeyer and Highkin (26). Medium was collected in the presence of vitamin K as outlined earlier for FIX (16, 25). FIX<sub>Q50P</sub> was purified by using a Ca<sup>2+</sup>-dependent mAb as described (27). It contained 9–10 Gla residues/mol and had ANAFL as the N-terminal sequence. FIX<sub>Q50P</sub> was purposed as earlier, except insoluble FXa (Staphylo-Fxa) was used instead of the soluble FXa as the activator (6). The resin was removed by centrifugation, and the supernatant was passed over a small Chelex-100 column to remove Ca<sup>2+</sup>. Aliquots were kept frozen at −80 °C until used.

**Expression and Purification of Recombinant FX**—An expression vector for FX<sub>WT</sub> was constructed in which the prepro-sequence of FIX was replaced with that of prothrombin as described by Camire et al. (28). The prepro-sequence of prothrombin was amplified by PCR using primers A and B (Table I) and a human liver cDNA library. The prepro-sequence of prothrombin was then linked to the FX cDNA sequence by the overlap extension method using primers A and C (25). The resulting chimeric DNA, containing the prepro-sequence of prothrombin followed by the FX sequence, was digested with AII and XhoI and ligated into pMol3506b expression vector. A stable cell line that expressed FX<sub>WT</sub> was established as described (26). Medium was collected in the presence of vitamin K as outlined earlier for FIX (16, 25). FX<sub>WT</sub> was purified using a Ca<sup>2+</sup>-dependent mAb to the Gla domain of FX (25) followed by FPLC Mono Q column. The conditions for the FPLC Mono Q column were the same as described previously for FIX purification (16, 25). Construction of FIX<sub>PC_EGF1</sub> was performed by the overlap extension method as described (25), and primers D and E (Table I) were used to amplify the protein C EGF1 domain. The establishment of a stable cell line and the purification of FIX<sub>PC_EGF1</sub> were the same as for FIX<sub>WT</sub>.

**Measurement of Rates of Activation of FIX by FVIIa**—For activation of FIX by FVIIa-TF/PL, 2 μM FIX was activated with 8 nM VIIa and 0.5 nM TF in the presence of 1 mM PL, 5 mM CaCl<sub>2</sub>. 0.1% PEG in Tris/NaCl buffer. At different times, 10 μL of the reaction mixture was removed and diluted 10-fold with 20 mM EDTA, pH 7.4. Biotin-EGR-CR was added to the diluted mixture to a final concentration of 20 μM, and the sample was incubated at 37 °C for 2 h and then at 4 °C overnight. To measure the amount of biotin-EGR-Ixa, a 96-well microtiter plate was coated with 100 μL (10 μg/mL) of 1.1 s of NaHCO<sub>3</sub> of the Ca<sup>2+</sup>-dependent FIX mAb at 4 °C overnight. The wells were blocked with 200 μL of 1% BSA and 0.1% Tween 20 in Tris/NaCl for 2 h at 37 °C. At this point,
each biotin-EGF1-IXa sample was further diluted 50-fold in Tris/NaCl containing 1% BSA and 0.1% Tween 20. 100 μl of the diluted sample was added to each well, and the plate was incubated at 37 °C for 2 h for capture of the EGF1-IXa by the FIX mAb. The plate was washed three times with Tris/NaCl containing 0.1% Tween 20 and 5 mM CaCl₂. Each well received 100 μl of a 50-fold concentrated SDS-reducing buffer. Samples were placed in boiling water for 5 min and analyzed by SDS-PAGE (24).

Measurements of Rates of Activation of FIX by FXVIIa—For activation of FX by FXVIIa—TP/PL, 2 μM FX was activated with 8 nM FXVIIa and 0.5 mM TF in the presence of 1 μM PL, 5 μM GL, 0.1% PEG in Tris/NaCl, pH 9.5. The amount of p-nitrophenol generated was measured in a microplate reader at 405 nm. Thrombin (TF=100 nM TF in the presence of 1 mM PL, 5 mM CaCl₂, 0.1% PEG in Tris/NaCl) was used for comparison with known amounts of fully activated FXa.

For activation of FIX with FXVIIa—TF, 4 μM FXVIIa was activated with 0.16 μM FXVIIa—TF in the absence of PL. All other assay conditions were the same as those for the activation of FIX with FXVIIa—TP/PL outlined above. For SDS-PAGE, 12 μl of the reaction mixture was removed at different times and added to 2 μl of 0.5 mM EDTA and 5 μl of 5-fold concentrated SDS-reducing buffer. Samples were placed in boiling water for 5 min and analyzed by SDS-PAGE (24).

Measurements of Rates of Activation of FX by FIX—For activation of FX by FIX—TP/PL: 2 μM FX was activated with 8 nM FIX and 0.5 mM TF in the presence of 1 μM PL, 5 μM GL, 0.1% PEG in Tris/NaCl, pH 9.5. The amount of p-nitrophenol generated was measured in a microplate reader at 405 nm. Thrombin (TF=100 nM TF in the presence of 1 mM PL, 5 mM CaCl₂, 0.1% PEG in Tris/NaCl) was used for comparison with known amounts of fully activated FXa.

Measurements of Rates of Activation of FIX by FXVIIa—TP/PL in the Absence of TF—For activation of FXVIIa—TP/PL, 2 μM FX was activated with 8 nM FXVIIa—TP/PL, 0.5 mM TF in the presence of 1 μM PL, 5 μM GL, 0.1% PEG in Tris/NaCl, pH 9.5. The amount of p-nitrophenol generated was measured in a microplate reader at 405 nm. Thrombin (TF=100 nM TF in the presence of 1 mM PL, 5 mM CaCl₂, 0.1% PEG in Tris/NaCl) was used for comparison with known amounts of fully activated FXa.

Measurements of Rates of Activation of FIX by FXVIIa—TP/PL in the Absence of TF—For activation of FXVIIa—TP/PL, 2 μM FX was activated with 8 nM FXVIIa—TP/PL, 0.5 mM TF in the presence of 1 μM PL, 5 μM GL, 0.1% PEG in Tris/NaCl, pH 9.5. The amount of p-nitrophenol generated was measured in a microplate reader at 405 nm. Thrombin (TF=100 nM TF in the presence of 1 mM PL, 5 mM CaCl₂, 0.1% PEG in Tris/NaCl) was used for comparison with known amounts of fully activated FXa.

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Measurements of Rates of Activation of FIX by FXVIIa—TP/PL in the Absence of TF—For activation of FIX—TP/PL, 2 μM FX was activated with 8 nM FXVIIa—TP/PL, 0.5 mM TF in the presence of 1 μM PL, 5 μM GL, 0.1% PEG in Tris/NaCl, pH 9.5. The amount of p-nitrophenol generated was measured in a microplate reader at 405 nm. Thrombin (TF=100 nM TF in the presence of 1 mM PL, 5 mM CaCl₂, 0.1% PEG in Tris/NaCl) was used for comparison with known amounts of fully activated FXa.
 ranged from 22 to 33% of the experimental cpm. The specific cpm data were plotted against 125I-FIX or 125I-FX to obtain approximate $K_d$ values for the binding of FIX or FX to sTF. The data were fitted to a hyperbolic curve using the program GraFit from Erithacus Software.

In further experiments, the affinity of each protein (WT, mutants, FIX-EGF1 domain) was determined by equilibrium competition assays. In these experiments, a fixed final concentration of 125I-FIX (10 nM) or 125I-FX (30 nM) was used with varying concentrations of the competitor. A 100-μl aliquot of each mixture was added to the sTF or BSA wells to generate the specific binding data as outlined above. The data were analyzed using the nonlinear regression analysis program (GraFit) to obtain the $K_d$ values (concentration of the competitor yielding 50% inhibition) using the $IC_{50}$, four-parameter logistic equation of Halfman (38). To obtain the $K_d(app)$ values for the interaction of competitor proteins with sTF, the following equation described by Craig was used (39).

$$K_{d(app)} = \frac{IC_{50}}{1 + (A/K_{d(WT)})} \quad (Eq. 1)$$

where $A$ is the concentration of 125I-FIX or 125I-FX, and $K_d(WT)$ represents the dissociation constant of WT protein for sTF.

**RESULTS**

Expression of Recombinant FVII and FX—Purified recombinant FVII had normal Gla content and the expected N-terminal sequence corresponding to zymogen FVII. It had 2000 ± 200 units/mg as measured in a one-stage clotting assay (31). SDS-PAGE analysis is shown in subsequent figures.

To obtain fully γ-carboxylated FX, we replaced the preproleader sequence of FX with that of prothrombin as described by Camire et al. (28). FX WT and FX PCEGF1 were expressed in BHK cells and purified using a Ca$^{2+}$-dependent mAb whose epitope is in the Gla domain of FX (29). The purified proteins had normal Gla content (11 ± 1) and revealed two N-terminal sequences in equimolar amounts: one corresponding to the heavy chain (SVQAQ) and a second corresponding to the light chain (ANSLF). This indicates that the preproleader sequences of FX WT and FX PCEGF1 were completely removed. The specific activity was 92 ± 10 units/mg for FX WT, which is similar to that of plasma FX (85 ± 10 units/mg), and it was 12 ± 2 units/mg for FX PCEGF1 in the two-stage clotting assay (31). SDS-PAGE analysis is presented in subsequent figures.

Activation of FIX WT, FIX Q50P, FIX PCEGF1, and FIX PCEGF1 by FVIIa-TF-PL—The activation rates of FIX mutants by FVIIa-TF-PL obtained by measuring the amount of biotin-EGR-IXa formed are presented in Fig. 1A. Examination of the data in Fig. 1A reveals that the initial rates of activation of FIX PCEGF1 and FIX Q50P are ~7 and ~30% of FIX WT, respectively. Under these conditions, FIX PCEGF1 was activated only minimally, and FIX WT was activated at a rate similar to that obtained with FIX WT (data not shown).

Activation of FIX WT and FX PCEGF1 with FVIIa-TF-PL—The rates of activation of FIX WT and FX PCEGF1 by FVIIa-TF-PL are presented in Fig. 1B. The initial rate of activation of FIX PCEGF1 was ~5% of FIX WT. It should be noted that the rate of activation of FIX WT was similar to the rate obtained with FIX WT (data not shown).

Activation of FIX and FX Proteins by FVIIa-sTF Complex—Studies presented above reveal that the activation rates of FIX and FX EGFl domain mutants with FVIIa-TF-PL are impaired. Gla domains of FIX and FX bind to the PL vesicles, and the membrane surface provides a platform for their assembly for maximal activation. Thus, it is possible that the mutations in the EGFl domain alter the distance between the cleavage site of FIX or FX and the PL surface, which results in the misalignment of the substrate and the enzyme FVIIa. This possibility was tested by using sTF (transmembrane region deleted TF) under reaction conditions that do not require assembly of FVIIa-TF and substrates on the PL surface.

The activation of each FIX mutant by FVIIa-sTF in this system where PL is absent is presented in Fig. 2A. Similar to the PL-containing system (Fig. 1A), activations of FIX PCEGF1 and FIX Q50P were impaired. However, in contrast to the PL-containing system, the rate of activation of FIX PCEGF1 in the absence of PL was similar to that of FIX PCEGF1. The initial activation rate was ~33% for FIX Q50P, ~12% for FIX PCEGF1, and ~9% for FIX EGFl when compared with the rate obtained with FIX WT (Fig. 2A). Again, it should be noted that FIX WT activated at a rate similar to FIX WT in this system (data not shown). Activation rates of FIX WT and FX PCEGF1 with FVIIa-sTF were also determined. As is the case with the FIX PCEGF1 mutant, the FIX PCEGF1 with FVIIa-sTF was activated at an initial rate that is ~10% of FIX WT (Fig. 2B).

**SDS-PAGE Analysis of Fix and FX Activation Products**—Next, we performed SDS-PAGE of various FIX and FX proteins activated by FVIIa-sTF. The data obtained for FIX WT are presented in Fig. 3A; data for FIX Q50P are in Fig. 3B; data for FIX PCEGF1 are in Fig. 3C; data for FIX EGFl are in Fig. 3D; data for FIX PCEGF1 are in Fig. 3E; and data for FIX PCEGF1 are in Fig. 3F. As is the case with the enzyme assays, the rate of activation in decreasing order for each FIX protein was FIX Q50P (not shown) ~ FIX WT > FIX Q50P ~ FIX PCEGF1 ~ FIX EGFl. Further, the rate of activation of FIX PCEGF1 as analyzed by SDS-PAGE was also considerably slower than the FIX WT.

Rates of Activation of FIX Proteins by FVIIa-TF in the Absence of TF—The activation of FIX or FX mutants by FVIIa-TF or FVIIa-sTF with or without PL is impaired (Figs. 1–3). Next we measured the activation of FX mutants by FVIIa-TF without TF or sTF as outlined earlier (57, 58). These results are depicted in Fig. 4. The activation rate was 0.19 ± 0.02 nm/min for FX WT and 0.18 ± 0.02 nm/min for FX PCEGF1. Thus, the interaction between FX PCEGF1 and FVIIa-TF is normal in the absence of TF (or sTF). We were unable to measure the rates of activation of FIX under these conditions.

Rate of FVII Activation by FIXa and FXa—FIXa and FXa are the enzymes that convert FVII to FVIIa (5–7). This activation requires only Ca$^{2+}$ and PL without the need for protein cofactors. Thus, this analysis should provide important data regarding whether or not FIXa and FXa mutants can function as
enzymes in activating FVII to FVIIa. Such experiments are presented in Fig. 5. An examination of the results reveals that the activation rate of FVII by each FIXa or FXa protein is similar. The activation of FVII was also confirmed by SDS-PAGE (Fig. 5, inset). These data provide evidence that the mutant proteins are folded correctly and that the interaction of mutant enzymes (FIXa mutants and FXaPCEGF1) with their substrate FVIIa is normal. It also indicates that the EGF1 domain in FIXa or FXa does not play an important role in the activation of FVII.

Binding of FIXa and FXa Proteins to AT—AT binds to the heavy chain of FIXa or FXa. It forms a covalent bond with the active site serine of FIXa as well as that of FXa (8, 9). Whether or not the mutations in the EGF1 domain in FIXa or FXa influence AT binding was examined by studying the tight complex formation of FIXa or FXa with AT. The rate of tight complex formation between AT and FIXa or FXa mutants appeared to be similar to that of FIXaWT and FXaWT as analyzed by SDS-PAGE. In each case, the FIXaAT or FXaAT tight complex formed at 0.15 min was ~20%, at 0.5 min it was ~50%, at 1.5 min it was ~80%, and at 4 min it was essentially complete. These initial data indicate that the EGF1 domains of FIXa and FXa are most likely not involved in AT binding and imply that the protease domains of the mutant FIXa and FXa are correctly folded to bind to their natural inhibitor. These data are consistent with the previous observation that the protease domain in these enzymes plays an important role in binding to AT (8, 9).

**Activation of FX**<sub>WT</sub> **and FX**<sub>PCEGF1</sub> **by FXa-PL and FIXr-PL-FVIIa**—In the coagulation cascade, FX can be activated either by the extrinsic pathway or by the intrinsic pathway. Studies presented above reveal that the activation of FXPCEGF1 by the extrinsic pathway is impaired. Here, we investigated whether or not the activation of FXPCEGF1 by the intrinsic pathway is normal. When the activation was carried out in absence of FVIIa, the initial rate of activation for FX**<sub>WT</sub> was 0.096 nM/min, and for FXPCEGF1 it was 0.073 nM/min.

These data are presented in Fig. 6A. When the activation was carried out in the presence of FVIIa, the rates of activation for FX**<sub>WT</sub>** and FXPCEGF1 were the same (2.2 nM/min). These data are presented in Fig. 6B. It should be noted that FXPCEGF1 was activated by FIXa-PL with or without FVIIa at the same rate as obtained with FX**<sub>WT</sub>**. These results indicate that the EGF1 domain of FX plays virtually no role or a very minor role in the activation of FX by the intrinsic pathway.

**Ligand Blotting of FIX or FX Proteins to sTF or FVIIa on Nitrocellulose Membrane**—FXa and FXa mutants function normally as enzymes in activating FVII and in binding to the serpin inhibitor AT. Thus, our results indicate that the defect in FIX and FX mutants is in their binding either to FVIIa or to TF (or both) in the FVIIaTF complex. To distinguish between these possibilities, we performed SDS-PAGE and transferred the FVIIa and sTF to the nitrocellulose membrane. Each FIX mutant was then used as a ligand to probe FVIIa or sTF on the membrane. When we probed the membrane with FIX**<sub>WT</sub>**, it bound to FVIIa as well as to sTF (Fig. 7A). In these experiments, FIXQ50P, FIXPCEGF1, and FIXQCEGF1 also bound to FVIIa but not to sTF (Fig. 7, B–D). These results indicate that the EGF1 domain of FIX is important for its binding to TF. We also performed similar ligand blotting experiments using FX as a ligand. Both FX**<sub>WT</sub>** and FXPCEGF1 bound to FVIIa, whereas only FX**<sub>WT</sub>** bound to sTF (Fig. 7, F and G). These results indicate that the EGF1 domain of FX, like that of FIX, is important in its binding to TF.

**Affinity of sTF for Various Proteins**—The Western ligand blot assays described above provide qualitative data for binding of sTF to various FIX and FX mutants. To obtain quantitative information, we studied binding of 125I-FIX and 125I-FX to immobilized sTF. A direct binding plot for FIX is presented in Fig. 8A and the K<sub>d</sub>(app) obtained from this plot for binding of 125I-FIX to sTF was 130 ± 20 nM. The affinity of WT and of each mutant FIX was then obtained by its ability to compete with 125I-FIX in binding to sTF. These data are presented in Fig. 8B. Analysis of these data reveal that sTF interacts with FIX**<sub>WT</sub>** with K<sub>d</sub>(app) of ~150 nM, FIXQ50P with K<sub>d</sub>(app) of ~500 nM, FIXPCEGF1 with K<sub>d</sub>(app) of ~1500 nM, and FIXQCEGF1 with K<sub>d</sub>(app) of ~1600 nM. Thus, as compared with FIX**<sub>WT</sub>**, the point mutant (FIXQ50P) has ~3-fold reduced affinity, whereas the replacement (FIXPCEGF1) or the deletion (FIXQCEGF1) mutant has ~10-fold reduced affinity for binding to sTF.

A direct binding plot for binding of 125I-FX to sTF is presented in Fig. 9A and the K<sub>d</sub>(app) obtained from this plot for binding of FX to sTF was 500 ± 100 nM. The affinity of WT and PCEGF1 mutant was then determined by competition with 125I-FX for binding to sTF. These data are shown in Fig. 9B. The K<sub>d</sub>(app) for WT was ~900 nM, and the K<sub>d</sub>(app) for FXPCEGF1 was ~6 μM in binding to sTF. Thus, compared with WT, the FXPCEGF1 has significantly reduced affinity for sTF.

**Studies with FIX-EGF1 Synthetic Domain**—Data presented thus far indicate that mutations in the EGF1 domain of FIX and FX impair their abilities to be activated by the FVIIaTF complex, and this property may be related to diminished binding of the mutants to sTF in the FVIIaTF complex. To examine whether isolated EGF1 domain binds to sTF, we synthesized the 45–87-residue segment of FIX representing its EGF1 domain. The fully reduced peptide had the correct mass (4755 Da) indicating no error in synthetic steps. The peptide was then oxidized and purified as outlined under “Experimental Procedures.” The mass spectrometric analysis of the oxidized purified peptide is shown in Fig. 10. A peak corresponding to the correct mass of 4749 Da represented ~90% of the total molecular species. This analysis also indicated that six cysteines had been oxidized to yield three disulfide pairs. Since only properly

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**Fig. 2.** Activation of FIX and FX proteins with FVIIa-sTF. Four μM of each protein was mixed with 180 nM FVIIa-sTF in Tris/NaCl containing 0.1% PEG and 5 mM CaCl<sub>2</sub>. The biotin-EGF-IXa or FXa formed at each time point was measured as described under “Experimental Procedures.” A, activation of FIX. The proteins used were FIX<sub>WT</sub> (open circles), FIXQ50P (closed circles), FIXPCEGF1 (open squares), and FIXQCEGF1 (closed squares). B, activation of FX. The proteins used were FX<sub>WT</sub> (open circles) and FXPCEGF1 (closed squares).
disulfide-paired and correctly folded EGF1 domain binds Ca\(^{2+}\) (35), we studied the binding of Ca\(^{2+}\) to the folded peptide. These data are presented in Fig. 10 (inset) using a Ca\(^{2+}\)-specific electrode. This peptide contained a single Ca\(^{2+}\) binding site (0.95/mol) with a \(K_d\) of \(-0.6\) mM, which is consistent with correct folding of the domain. Importantly, the folded peptide inhibited the binding of FIX to sTF with a \(K_d\) of \(-60\) \(\mu\)M (Fig. 8B). Moreover, reduced and carboxymethylated peptide prepared essentially by the method of Sodetz and Castellino (40) neither bound Ca\(^{2+}\) (data not shown) nor inhibited FIX binding to sTF (Fig. 8B).

**DISCUSSION**

The FVIIa-TF complex has high specificity and affinity for its substrates FIX and FX, which quite possibly involve regions that are remote from the cleavage sites. The peptides that do not bind to the active site of FVIIa specifically inhibit the activation of FX by FVIIa-TF as well as the TF-initiated clotting (41). Further, when the active site of FVIIa is blocked with peptidyl substrates, the resulting FVIIa-TF complex has nearly the same affinity toward its substrate FX as the uninhibited FVIIa-TF (42). Thus, exosites exist in FX that are responsible for this specific recognition of FX by the FVIIa-TF complex. Moreover, a FVIIa molecule with a point mutation in the Gla domain (Arg\(^{36}\) → Ala) activates FX at a reduced rate in the presence but not in the absence of TF (15). These results indicate that TF facilitates an optimal interaction between the Gla domains of FVIIa and FX (15). Further, TF residues Lys\(^{165}\) and Lys\(^{536}\) that are exposed in the crystal structure of the FVIIa-TF complex (13) are thought to interact with the Gla domain of FX or FIX (43, 44). These TF mutants were deficient in supporting FVIIa activation of normal FX (or FIX) but not of the Gla domainless FX (45). In support of this, when Kirchhofer et al. (14) made a panel of additional TF mutants involving surface-exposed residues 157–185, they found that the mutant TF molecules had reduced affinity for FIX and FX. Based upon
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Experimental Procedures.

sTF and FVIIa was detected by FIX mAb (or FX mAb) as outlined under instead of the ligand FIX or FX, respectively. The FIX (or FX) bound to buffer control, 1% milk solution was used to probe the membranes.

The proteins used were FIXWT (open squares) and FXPCEGF1 (open triangles). The proteins used were FIX WT (closed circles) and FX PCEGF1 (open squares).

FIG. 8. Binding of FIXWT and EGF1 domain mutants to sTF. The data in A represent direct specific binding of 125I-FIX to immobilized sTF. An approximate $K_d$ value for FIXWT and sTF interaction was calculated to be $\sim$120 nM. The data in B depict the abilities of various EGF1 domain mutants and synthetic FIX-EGF1 domain to inhibit the binding of 125I-FIX to sTF. Competitors were FIXWT (solid circles), FIXQ50P (open circles), FIXPCEGF1 (open triangles), FIXMCEGF1 (solid triangles), folded EGF1 domain (open squares), and reduced and carboxymethylated EGF1 domain peptide (closed squares). The concentration of 125I-FIX used was 10 nM with increasing amounts of the competitors. The curves represent best fit to the IC$_{50}$ four-parameter logistic equation of Halfman.

FIG. 7. Ligand blotting of FIX or FX proteins to FVIIa and sTF. FVIIa (0.4 μg), sTF (0.2 μg), FIX (20 ng), or FX (20 ng) was electrophoresed on a 12% SDS-PAGE gel. The proteins were then transferred to nitrocellulose membranes. Each protein that was electrophoresed and blotted onto the nitrocellulose membrane is labeled at the top of each panel. The FIX proteins that were used as ligands to probe the membranes were FIXWT (A), FIXQ50P (B), FIXPCEGF1 (C), and FIXMCEGF1 (D). The FX proteins that were used as ligands to probe the membranes were FXWT (E) and FXPCEGF1 (G). In panels E and H, each labeled as buffer control, 1% milk solution was used to probe the membranes instead of the ligand FIX or FX, respectively. The FIX (or FX) bound to sTF and FVIIa was detected by FIX mAb (or FX mAb) as outlined under “Experimental Procedures.”

The topology of FVIIasTF complex (13), these residues in the C-terminal domain of TF would appear to interact with the Gla domain of substrates FIX and FX.

The EGF1 domain of FIX (or FX) also appears to play an important role in its activation by FVIIa-TF. In an earlier study when FVIIa-TF-PL was used as the activator, it appeared that in this system FIXMCEGF1 could not be activated. Further, FIXPCEGF1 was activated at a slower rate, whereas FIXQ50P was activated at a nearly normal rate in this system (16). The data in these studies were analyzed by SDS-PAGE of the reaction mixtures drawn at different times and thus were only qualitative.

Further, the failure of activation of FIXQ50P in this system could stem from the altered spatial alignment of domains in this mutant. To circumvent this problem, we have now developed an assay to quantitatively measure the formation of FIXa and have conducted studies using the FVIIa-sTF system, where anchoring of the Gla domain on the PL surface is not required. Additionally, we have performed studies using FIXPCEGF1 and compared its activation properties with FIXWT. Our data indicate that the activation rates of FIXQ50P, FIXPCEGF1, FIXMCEGF1, and FIXQ50P are impaired both by the FVIIa-sTF (Figs. 2 and 3) and by the FVIIa-sTF (Figs. 2 and 3). A recent report revealed that mutations at residue 48 in FIX result in delayed activation by FVIIa-sTF (45). This observation is consistent with the extensive kinetic data presented in this paper.

In ligand blot assays, all EGF1 FIX and FX mutants bound to FVIIa but not to TF (Fig. 7). However, results obtained by Western ligand blot assays are at best qualitative in nature. Further, such data do not allow estimation of the binding energy involved in complex formation. For these reasons, we measured the affinity of WT and mutant FIX and FX proteins for binding to immobilized sTF. These data indicate that FIXWT binds to sTF with $K_d$ (app) of $\sim$150 nM. The affinity of the point mutant (FIXQ50P) for sTF was reduced 3-fold, whereas the affinity of the replacement (FIXPCEGF1) or the deletion mutant (FIXMCEGF1) was reduced $\sim$10-fold (Fig. 8). Similarly, the affin-
ity for sTF of FX\texttextsubscript{PCEGF1} mutant was reduced ~6–10-fold as compared with FX\textsubscript{WT} (Fig. 9). Of interest is the observation that FIX\textsubscript{WT} bound to sTF with ~3-fold higher affinity than FX\textsubscript{WT}. The reason(s) for this observation is not known. Extensive additional data are needed to understand whether there are real differences in the affinities of FIX\textsubscript{WT} and FX\textsubscript{WT} in binding to sTF or if they simply reflect experimental difficulties inherent to the technique employed. However, the conclusions drawn from this paper do not depend upon resolution of this issue.

The studies conducted with the EGF1 domain mutants yield data pertaining to loss of function. Direct evidence that the EGF1 domain is involved in binding to sTF comes from the ability of synthetic FIX-EGF1 domain to inhibit the interaction of FIX\textsubscript{WT} with sTF with a $K_i$ 60 $\mu$M. An interesting point emerges from such studies. FIX protein lacking the EGF1 domain or having the EGF1 domain of protein C only has a 10-fold reduced affinity for sTF (Fig. 8B). This information coupled with the basic thermodynamic principles involving equilibrium constants predicts that the isolated FIX-EGF1 domain should bind to sTF with a $K_d$ in the mM range. However, such is not the case, and the isolated properly folded FIX-EGF1 domain binds sTF with 60 $\mu$M $K_d$. Two explanations may be forwarded to explain this phenomenon. First, the binding of FIX to sTF involves two regions (Gla and EGF1 domains) and conformational strain occurs upon binding to the second site either in FIX or sTF. This would yield lower $K_d$ values for the isolated two fragments (FIX lacking the EGF1 domain and the EGF1 domain alone) due to the absence of steric constraints inherent in the full-length molecule. An alternative explanation might be that the synthetic EGF1 domain we have used is devoid of glycosylation, which might interfere with binding to sTF. Further studies are needed to address this issue. However, we currently prefer the first explanation.

These impaired interactions of mutants with FVIIa-TF (or sTF) are not due to defective folding of the proteins. All activated mutants (FIX by FXIa, and FX by RVV) bound AT and activated FVII to FVIIa normally. Furthermore, FXPCEGF1 could be activated by the FIXa-FVIIIa-PL complex at the same rate as FX\textsubscript{WT} (Fig. 6). These data are consistent with the observation of Lapan and Fay (46), who found that the protease domain of FX interacts with FVIIIa in the FIXa-FVIIIa complex. Importantly, our data indicate that the EGF1 domain of FIX or FX contains an exosite(s) that appears to directly interact with TF.

The EGF1 domain in FIXa or FXa also contributes to the assembly of FIXa-FVIIIa or FXa-FVa on the PL surface. Data exist that suggest that the EGF1 domain in FIXa or FXa may

![Figure 9](http://www.jbc.org/)

**FIG. 9.** Binding of FX\textsubscript{WT} and FX\textsubscript{PCEGF1} to sTF. The data in A represent direct specific binding of $^{125}$I-FX to immobilized sTF. An approximate $K_d$ value for FX\textsubscript{WT} and sTF interaction was calculated to be ~500 nm. The data in B depict the abilities of FX\textsubscript{WT} and FX\textsubscript{PCEGF1} to inhibit the binding of $^{125}$I-FX to sTF. Competitors were FX\textsubscript{WT} (open circles) and FX\textsubscript{PCEGF1} (closed circles). The concentration of $^{125}$I-FX used was 30 nM with increasing amounts of the competitors. The curves represent best fit to the IC\textsubscript{50} four-parameter logistic equation of Halfman (38).

![Figure 10](http://www.jbc.org/)

**FIG. 10.** Mass spectrometry and Ca$^{2+}$ binding analyses of the folded FIX-EGF1 domain. The deconvoluted spectrum of the peptide obtained using a Finnigan LCQ Iontrap Electrospray mass spectrometer is depicted. The inset shows binding of Ca$^{2+}$ to the folded peptide as determined by a Ca$^{2+}$-specific electrode. The concentration of the peptide used was 400 $\mu$M, and the free Ca$^{2+}$ concentration is plotted against $r$ (mol of Ca$^{2+}$ bound/mol of peptide).
were from FIX was constructed. This mutant was activated by thrombomodulin-thrombin complex at ~70% of the rate obtained with the wild type protein C (52). The activated protein C mutant was also defective in inactivating FVas and FVIII/ FVIIa in a PL-containing system (52). The decreased activity of activated protein C mutant was attributed to direct protein-protein interaction and/or to misalignment of domains/recognition sites with its physiological substrates. Thus, the EGF1 domain in each vitamin K-dependent protease may be involved in direct binding as well as in specific alignments of recognition motifs with other proteins involved in the assembly. We are currently examining the role of EGF1 domain of FXa in the activation of prothrombin in the presence and absence of FVa and PL using our FXPC_{EGF1} mutant.

Last, we have made attempts to define an exosite in the EGF1 domain of FIX or FX that may interact with TF. Three important considerations were given in search for such an exosite. First, since FVIIa-TF can activate FVII-TF efficiently (53), we opted to select regions that are not involved in binding of FVIIa EGF1 domain to TF. This excluded the interface region involving residues Glu64, Ile69, Phe71, Glu77, and Arg79 of FVIIa (54) and by inference of FIX and FX. Further, this interface is not structurally conserved in FIXa and FXa. Second, we excluded side chains of those residues (Asn47, Gln50, and Asp64) that are involved in binding to Ca^{2+} (54). Third, our data indicate that FIX1_{G350P} mutant in which the Ca^{2+}-binding site is impaired is defective in binding to TF. Thus, we examined the region surrounding the Ca^{2+} site that might be perturbed and therefore a likely candidate for binding to TF. Using these three criteria, we postulate an extended exosite region in the EGF1 domain of FIX as well as in FX that could be involved in binding to TF. This is shown in Fig. 11. Residues Asp49, Gly48, and Asp64, Phe77, and Glu83 are located on the surface and may be the key determinants for binding to TF. Numerous point mutations in these residues cause hemophilia B (55). Studies are in progress to mutate these residues to examine if they are indeed involved in TF binding.

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