Role of the Gla and First Epidermal Growth Factor-like Domains of Factor X in the Prothrombinase and Tissue Factor-Factor VIIa Complexes*

Factor X (FX) has high structure homology with other proteins of blood coagulation such as factor IX (FIX) and factor VII (FVII). These proteins present at their amino-terminal extremity a γ-carboxyglutamic acid containing domain (Gla domain), followed by two epidermal growth factor-like (EGF1 and EGF2) domains, an activation peptide, and a serine protease domain. After vascular damage, the tissue factor-FVIIa (TF-FVIIa) complex activates both FX and FIX. FIXa interacts stoichiometrically with tissue pathway inhibitor (TFPI), regulating TF-FVIIa activity by forming the TF-FVIIa-TFPI-FXa quaternary complex. Conversely, FXa boosts coagulation by its association with its cofactor, factor Va (FVa). To investigate the contribution of the Gla and the EGF1 domains of FX in these complexes, FX chimeras were produced in which FIX Gla and EGF1 domains substituted the corresponding domains of FX. The affinity of the two chimeras, FX/FIX(Gla) and FX/FIX(EGF1), for the TF-FVIIa complex was markedly reduced compared with that of wild-type FX (wt-FX) independently of the presence of phospholipids. Furthermore, the association rate constants of preformed FX/FIX(Gla)-TFPI and FX/FIX(EGF1)-TFPI complexes with TF-FVIIa were, respectively, 10- and 5-fold slower than that of wt-FXa-TFPI complex. Finally, the apparent affinity of FVa was 2-fold higher for the chimeras than for wt-FX in the presence of phospholipids and equal in their absence. These data demonstrate that FX Gla and EGF1 domains contain residues, which interact with TF-FVIIa exosites contributing to the formation of the TF-FVIIa-FX and TF-FVIIa-TFPI-FXa complexes. On the opposite, FXa Gla and EGF1 domains are not directly involved in FVa binding.

The blood coagulation cascade consists of a series of enzymatic conversions driven by the formation of complexes between serine proteases and cell membrane-bound cofactors. Human factor X (FX) is one of the serine protease zymogens playing a central role in coagulation processes leading to the formation of a fibrin clot. This is illustrated by the behavior of FX as a substrate or as an enzyme in three essential blood coagulation complexes. First, FX is a natural substrate, as well as factor IX (FIX), of the tissue factor-factor VIIa (TF-FVIIa) complex (1) considered as the initial enzyme complex in the cascade following vascular damage. FX activation by TF-FVIIa results from specific cleavage and release of a 52-residue activation peptide. Activated FX (FXa) can generate a tiny amount of thrombin from prothrombin in an extremely inefficient reaction (2). Tissue factor pathway inhibitor (TFPI) binds to TF-FVIIa-FXa to limit the production of FXa and FIXa by TF-FVIIa (3, 4). Nevertheless, once produced, thrombin and the initially formed FXa activate small quantities of factor V (FV) to FVa and factor VIII (FVIII) to FVIIIa (5–8). The activation of these two cofactors leads to the formation of two other essential coagulant complexes, both involving FX, at the surface of coagulant phospholipids in the presence of calcium ions (9), FIXa-FVIIa and FXa-FVa, which convert FX to FXa and prothrombin to thrombin, respectively. These complexes are 105–106-fold more active than the serine proteases devoid of their respective cofactors. FVa and FIXa are both specific cofactors by binding to thrombin and promote the formation of a fibrin clot.

FX circulates in blood as a two-chain molecule and has the same modular structure as other vitamin K-dependent blood coagulation proteins such as FVII and FIX (13). The light chain has 11 amino-terminal glutamyl residues that are post-translationally modified in a vitamin K-dependent reaction to form a γ-carboxyglutamyl acid-containing domain or “Gla domain” critical for the binding of calcium ions and phospholipids (14). The Gla domain is followed by two domains homologous to the epidermal growth factor (EGF) precursor, considered important for protein-protein interactions (15). The heavy chain is joined to the light chain by a single disulfide bond and contains a 52-amino acid peptide and a trypsin-like serine protease domain (16), which forms the carboxyl-terminal end of the molecule. Although FXa has substantial sequence similarities and high homologous three-dimensional structure with FVIIa and FIXa (17–19), it displays significant differences in substrate specificity and catalytic activity. Furthermore, each protease requires specific cofactor to express enhanced catalytic activity within the procoagulant complexes of blood coagulation.

Received for publication, December 1, 2002, and in revised form, January 13, 2003
Published, JBC Papers in Press, January 15, 2003, DOI 10.1074/jbc.M212144200

From INSERM U143, Hôpital Bicêtre, 94276 Le Kremlin-Bicêtre Cedex, France

Fabrice Thiece, Ghislaine Cherel, and Olivier D. Christophe

‡ To whom correspondence should be addressed: INSERM U143, Bâtiment Claude Bernard, Hôpital de Bicêtre, 94 Rue du Général Leclere, 94276 Le Kremlin-Bicêtre Cedex, France. Tel.: 33-1-49-59-56-26; Fax: 33-1-46-71-94-72; E-mail: olivier@kb.inserm.fr.

† The abbreviations used are: FX, factor X; FV, factor V; FVII, factor VII; FVIII, factor VIII; FIX, factor IX; TF, tissue factor; TFPI, tissue factor pathway inhibitor; RVV-X purified FX-activating enzyme from Russell’s viper venom; FX/FIX(Gla), FX in which the Gla domain has been replaced with that of FIX; FX/FIX(EGF1), FX in which the EGF1 domain has been replaced with that of FIX; EGF, epidermal growth factor; PS, phosphatidylserine; FC, phosphatidylcholine; BSA, bovine serum albumin; p-NA, p-nitroaniline; S-2238, S-2765, and S-2765, S-2238, and S-2765, respectively; Arg-P, L-arginyl-p-nitroaniline dihydrochloride; H-Phe-Pip-Arg-p-NA, H-Phe-Pip-Arg-p-NA, and H-Phe-Pip-Arg-p-NA, respectively; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]1-propanesulfonic acid; wt, wild type; ELISA, enzyme-linked immunosorbent assay.

This paper is available on line at http://www.jbc.org

10393
It is clear that the catalytic specificities of blood coagulation proteases are supported by the carboxyl-terminal half of the enzymes, a trypsin-like serine protease domain (20). The prime role of the serine protease domain has also been demonstrated in the interaction of the blood coagulation proteases with their cofactors. For instance, site-directed mutagenesis revealed that the serine protease domain is the most important part of FXa and FX in the FXa-FVIIa and FXa-FVs interactions, respectively (21–23). However, there is also evidence that Gla- and EGF-like domains may also mediate protein-protein interactions and may consequently be implicated in the assembly of the protein complexes of blood coagulation. For instance, FVIIa multiple residues, distributed over the entire light chain, are in contact with TF (19). The identification of the FXa light chain residues directly involved in the interaction with FVIIa has to be performed, but three different approaches have suggested that the region around residues 85–90 in the linker area between EG1F and EG2F might contact residues 1804–1818 of FVIIa (24–26). Concerning FX, there is evidence that the light chain of FXa contributes to the interaction of the enzyme with its cofactor (27, 28). However, it is unknown whether the Gla and the first EGF domains interact directly with FVs in the prothrombin activation complex or whether they position the catalytic domain at a correct distance above the phospholipid membrane. Several investigations have suggested by studying the effect of mutations in TF that TF-FVIIa complex binds to the Gla domain of FX (29, 30), and there is evidence that the first EGF-like domain of FX is required for the activation of the substrate by the TF-FVIIa complex (31). From all these analyses it has been suggested that the same residues from FX and FIX within the Gla and EG1F domains are involved in these interactions.

In the present study, the contribution of the Gla and first EGF domains in FX-specific recognition by the TF-FVIIa complex and in the prothrombin activation complex was addressed using chimeric recombinant FX containing either the Gla domain or the first EGF domain of FIX. There are two main reasons for the choice of FIX for exchanging FX homologous domains, the structural similarities between the two molecules and their respective cofactors, as well as the high similitude of the blood coagulation reactions in which they participate. The purified recombinant proteins were then compared with normal FX with regard to their activation by TF-FVIIa complex and to their properties as activated serine proteases in the TF-FVIIa-TFPI-FXa and FXa-FVa complexes.

EXPERIMENTAL PROCEDURES

Materials—H-\(\beta\)-Phenylalanyl-t-pipecolyl-t-arginine-p-nitroaniline dihydrochloride (H-\(\beta\)-Phe-Pip-Arg-p-NA2HCl), product name S-2238, and N-\(\beta\)-benzoxycarbonyl-t-arginyl-t-glycyl-t-arginine-p-nitroaniline dihydrochloride (N-\(\beta\)-Z-Arg-Gly-Arg-p-NA), product name S-2766, were from Chromogenix (Molndal, Sweden). Bovine serum albumin (BSA), sodium pyruvate, vitamin K\(\alpha\), penicillin, streptomycin, and fungizone (American Diagnostica, Andresy, France). Human FVa was purchased from Stago (Asnieres, France). Factor X immunodepleted plasma, Owren-Koller buffer, and rabbit brain thromboplastin-C reagent were purchased from Diagnostica Stago (Asnieres, France).

Proteins—Mouse monoclonal anti-FX antibody KB-FX008 was prepared in our laboratory as described previously (32, 33). KB-FX008 was found as being directed against FX protease domain by Western blot. Polyclonal antibodies against FX conjugated or not with horseradish peroxidase were obtained from Dako (DakoCytomation, Glostrup, Denmark). Purified human plasma-derived FX (pd-FX), FX-activating enzyme from Russell’s viper venom (RVV-X), bovine antithrombin III, human prothrombin, human thrombin, and recombinant human TF were obtained from Kodia (Leiden, The Netherlands). TFPI was obtained from American Diagnostics (Andresy, France). Human FVs was purchased from BioMerieux (Marcy l’Etoile, France). Immobilon polyvinylidene difluoride membranes for Western blotting applications, Sepharose 4 Fast Flow, HiTrapTM Q column, benzamidine-Sepharose, benzamidine. The protein solution was immediately neutralized with 2 \(\mu\)l 5 M \(\text{NH}_4\)OH for elution. The eluate was dialyzed extensively against 50 mM Tris (pH 7.4) and 100 mM NaCl, and stored at \(-80^\circ\text{C}\). Modified Eagle’s medium supplemented with 2.5% fetal calf serum, 100 units/ml penicillin, 100 \(\mu\)g/ml streptomycin, 5 \(\mu\)g/ml vitamin \(\text{K}\), and 1 \(\mu\)g/ml amphotericin B/0.8 \(\mu\)g/ml deoxycholate. FX containing medium was harvested every 48 h. Benazamide was added to a final concentration of 10 mM, and medium was centrifuged (6,000 \(\times\) g) over cellulose acetate membranes (0.22 \(\mu\)m) to eliminate cell debris, and stored at \(-80^\circ\text{C}\). Conditioned medium was thawed at 37 \(^\circ\text{C}\). EDTA was added to a concentration of 5 mM. The medium was diluted to bring the final NaCl concentration to 60 mM. The mixture was then stirred at room temperature for 30 min with QAE-Sephadex A-50 beads to achieve a final concentration of 0.25% (w/v). Beads were washed before eluting with 50 mM Tris (pH 7.4), 500 mM NaCl, and 10 mM benzamidine. Recombinant FX (ELISA) contained in eluted fraction was then concentrated by precipitation with two successive steps of 40% and 70% saturated ammonium sulfate. Proteins obtained after the second precipitation step were diluted in 50 mM Tris (pH 7.4) and 100 mM NaCl and immediately dialyzed against 50 mM Tris (pH 7.4), 100 mM NaCl, containing 10 mM benzamidine, and 10% glycerol. Recombinant FX was purified from the soluble proteins by immunofinity chromatography using monoclonal anti-human FX antibody KB-FX008. After adsorption, the antibody column was washed with 50 mM Tris (pH 7.4), 100 mM NaCl, containing 10 mM benzamidine followed by elution of bound recombinant FX with 0.1 M glycine (pH 2.5), and 10 mM benazamide. The protein solution was immediately neutralized with 2 \(\mu\)l 5 M \(\text{NH}_4\)OH for elution. Recombinant FX containing fractions were combined and dialyzed against 50 mM Tris (pH 7.4), 100 mM NaCl, containing 10 mM benzamidine. If present, residual contaminants were removed by Q-Sepharose Fast Flow chromatography in 50 mM Tris (pH 7.4), 100 mM NaCl, containing 10 mM benzamidine, and a contaminant (0.05–1 \(\mu\)M) for elution. The eluate was dialyzed extensively against 50 mM Tris (pH 7.4), 100 mM NaCl and stored at \(-80^\circ\text{C}\). Before any analysis, a final pass over a benzamidine-Sepharose column equilibrated with 50 mM Tris (pH 7.4) and 100 mM NaCl was used to eliminate trace contaminants of FXa that may have been generated during production or purification of recombinant protein.

Amino Acid Sequence Analysis—Purified recombinant FX derivatives were reduced and loaded onto a 15% SDS-polyacrylamide gel. The resolved proteins were transferred to an Immobilon membrane and stained with Ponceau S. The light chains were excised and sequenced using an Applied Biosystem Procise model 404 sequencer in the service of the Instituts de Biologie et Chimie des Proteines (Lyon, France).

Activation of Recombinant FX Derivatives by RVV-X—Recombinant FX derivatives and wt-FX were activated by RVV-X (37). RVV-X (1 ng) was coupled to 1 \(\mu\)l of CNBr-activated Sepharose 4B according to
manufacturer’s instructions. Recombinant FX derivatives (1 μM) were incubated with coupled RVV-X (30 nM) in 50 mM Tris (pH 7.4), 100 mM NaCl containing 10 mM CaCl₂. After 2 h, the reaction was stopped by addition of 15 mM EDTA. Activated recombinant FX derivatives were dialyzed against 50 mM Tris (pH 7.4) and then with 50 mM Tris (pH 7.4), 100 mM NaCl and loaded on a benzamidine-Sepharose column equilibrated in the same buffer. After washing, bound activated FX was eluted with 50 mM Tris (pH 7.4), 100 mM NaCl containing 5 mM benzamidine. Fractions containing FXa were pooled and precipitated by the addition of solid ammonium sulfate to 80% saturation. The precipitated proteins were diluted in 50 mM Tris (pH 7.4) and 100 mM NaCl, and the protein solution was immediately dialyzed against the same buffer containing 50% glycerol (v/v) and stored at −20 °C until use. The active site concentrations of activated recombinant FX derivatives were determined by titration with known concentration of antithrombin in the presence of heparin, and an active site-specific assay using bovine F-glycine-
aminocaproic acid glutamic acid glycylarginine chloromethyl ketone as described previously (28, 38, 39). Concentrations of activated recombinant FX derivatives and activated wt-FX were found to correlate between the two methods.

Plasma-based Coagulant Activities—The activity of non-activated derivatives of FX and wt-FX were functionally characterized in a prothrombin time assay as described previously (40) with slight modifications. Recombinant FX derivatives or wt-FX were preincubated 5 min at 37 °C in a fluorimeter with FX immunodepleted human plasma. Clotting was initiated by the addition of rabbit brain thromboplastin-C reagent and calculated from a standard curve generated with the clotting times versus the dilutions of pooled normal plasma. Recombinant FX derivatives and wt-FX clotting activities were expressed as a percentage of normal activity.

Relipidation of TF Alopeproteins—Recombinant human TF (2.5 μg) in 100 μl of 50 mM Tris (pH 7.4), 100 mM NaCl containing 10 mM CHAPS was mixed with an equal volume of phospholipid vesicles preparation (PC/PS, 3:1, 2 mM). Phospholipid vesicles (PC/PS, 3:1) of nominal 200 nm diameter were synthesized by the method of membrane extrusion (41) in 10 mM Hepes (pH 7.5), 100 mM NaCl, 5 mM CaCl₂. Phospholipid concentrations were determined by phosphate analysis. After a 1-h incubation at 30 °C, the relipidated TF was analyzed at 4°C for 1 h by gel filtration in a Mono Q column (5 mM Tris (pH 7.4), 100 mM NaCl). The preparation was kept under N₂ at 4°C and used within 2 weeks. The functional concentration of the TF preparation in these vesicles was considered to be 300 mM, half of its total concentration (42). Throughout this paper, unless specified, TF refers to this reconstituted preparation.

Amidolytic Activity—The steady-state kinetics of hydrolysis of S-2765 by pd-FXa, wt-FXa, and recombinant FXa derivatives were assayed in 50 mM Tris (pH 7.4), 100 mM NaCl containing 2 mg/ml BSA, and 5 mM CaCl₂. Kinetic parameters of substrate hydrolysis were determined employing an enzyme concentration of 2 nM and various substrate concentrations ranging from 0 to 5 mM. The release of para-nitrophenol was monitored at 405 nm in a kinetic microplate reader (Bio-Tek Instruments, Winooski, VT). The apparent Kₘ and kₐ values for substrate hydrolysis were calculated from the Michaelis-Menten equation, and the catalytic efficiencies were expressed as the ratio of Kₘ/kₐ.

Thrombin FXa—The rate at which activated recombinant FX derivatives, wt-FXa, or pd-FXa can activate prothrombin to thrombin in the presence of phospholipids as a function of FVa concentrations was compared as described previously with slight modifications (23). Briefly, phospholipid vesicles (PC/PS, 3:1, 30 μM), and 20 nm FVa were incubated for 5 min with various concentrations of FVa (0–1 nm). The reaction was started by the addition of 1 μM prothrombin. The assay was performed in 50 mM Tris (pH 7.4), 100 mM NaCl, 5 mM CaCl₂, and 0.2% (w/v) BSA at 37 °C. Aliquots were taken at specified times, and the reaction was stopped in EDTA (10 mM final). Thrombin formation in each was then determined by measuring the amidolytic activity of the samples toward the synthetic substrate S-2238 (250 μM), in the presence of 15 μg/ml soybean trypsin inhibitor to inhibit amidase activity of FXa. Incubating the assay, less than 5% of prothrombin was converted to thrombin, and thrombin formation was linear. Conversion of substrate was monitored at 405 nm. Concentrations of thrombin generated in the activation reactions were determined from a standard curve prepared from the cleavage rate of S-2238 by known concentrations of thrombin under the same conditions.

The initial rates of prothrombin activation by FX derivatives in the presence of sub-saturating FVa concentration was performed as follow. In the absence of phospholipids, thrombin formation was initiated by the addition of 10 μM prothrombin to a reaction mixture containing 0.5 mM FVa and 75 mM FVa in 50 mM Tris (pH 7.4), 100 mM NaCl, containing 5 mM CaCl₂, and 0.2% (w/v) BSA at 37 °C. In all experiments, aliquots were removed from the reaction mixtures at specified times and diluted into 10 mM EDTA buffer to stop the reaction. Thrombin concentration was calculated as described above.

Activation of Recombinant FX Derivatives by FVIIa and Human TF—Activation of recombinant FX derivatives by FVIIa were measured and compared directly to wt-FX activation. The assay was performed in 50 mM Tris (pH 7.4), 100 mM NaCl, containing 0.2% (w/v) BSA, and 5 mM CaCl₂ at 37 °C. Initial rates of activation by FVIIa and reconstituted TF-FVIIa were determined as described previously with little modification (43). Briefly, 60 pm FVIIa was added to 60 pm relipidated TF in the presence of 30 μM phospholipid vesicles (PC/PS, 3:1). The mixture was incubated at 37 °C for 20 min in order to establish a FVIIa-TF complex, and the reaction was started by the addition of various concentrations of FX (0–3 μM). After diverse incubation times, aliquots were taken, diluted in stop solution containing EDTA (10 mM final), and assayed for FXa formation employing the synthetic substrate S-2765 (250 μM). Conversion of substrate was monitored at 405 nm. The concentration of FXa generated in the activation reaction was determined from a standard curve prepared from the cleavage rate of S-2765 by known concentrations of active site titrated FXa under the exact same conditions.

Comparison of the initial rates of FXa derivatives activation by TF-FVIIa was performed as follow. In the absence of phospholipids, the reaction was initiated by the addition of 250 nm FXa to a reaction mixture containing 50 mM FVIIa and 250 mM TF solubilized at 37 °C with 5 mM CHAPS in 50 mM Tris (pH 7.4), 100 mM NaCl, containing 5 mM CaCl₂ and 0.2% (w/v) BSA. The mixture was incubated at 37 °C for 5 min prior to the addition of the substrate to establish a FVIIa-TF complex. In the presence of phospholipids, the reaction was initiated at 37 °C by addition of 1 μM FXa to a mixture containing 8 nm FVIIa and 0.5 mM TF in the presence of 1 mM phospholipids in 50 mM Tris (pH 7.4), 100 mM NaCl, containing 5 mM CaCl₂ and 0.2% (w/v) BSA. As in the reaction mixture was incubated at 4°C for 5 min prior to the addition of the substrate to establish a FVIIa-TF complex. In all experiments, aliquots were removed from the reaction mixture at specified times and diluted into 10 mM EDTA buffer to stop the reaction. FXa concentration was calculated as described above.

Inhibition of FX Activation by TFPI-FXa—Inhibition of pd-FX activation by TFPI-recombinant FXa derivatives was measured as described previously (44, 45) with minor modifications. Briefly, 25 pM relipidated TF was incubated with 8 nm FXa for 5 min. FXa generation was started by the addition of 400 nm pd-FXa followed by immediate addition of preformed TFPI-recombinant FXa derivatives (25, 50, and 100 pm). After various incubation times, aliquots were removed, diluted in 100 μM FVa, and 30 mM CaCl₂, and 0.2% (w/v) BSA. The mixture was incubated at 37 °C for 5 min until use. The active site concentrations of activated recombinant FXa under the exact same conditions.

The comparison of the initial rates of FXa derivatives activation by TF-FVIIa were estimated by fitting experimental data on FX activation by TF-FVIIa to Equation 1,

\[ [\text{FXa}]_{\text{obs}} = [\text{FXa}]_{\text{max}} (1 - e^{-k_{\text{obs}} t}) \]  

where \([\text{FXa}]_{\text{max}}\) is the maximal concentration of FXa, \([\text{FXa}]_{\text{obs}}\) is FXa concentration at a given time point \(t\), and \(k_{\text{obs}}\) is the observed first order rate constant. The apparent second order association rate constant for inhibition of pd-FXa activation by TFPI-recombinant FXa derivatives at 250 μM was determined by fitting experimental data on FX activation by TF-FVIIa to Equation 1,
analysis disclosed that the average yield for the two glutamic acid residues at the NH₂ terminus was less than 5% of the average yield of the two subsequent residues. Because γ-carboxylation reduces the yield of Glu residues, these data demonstrate that the glutamic residues were appropriately modified. All recombinant FX proteins were activated by RVV-X under conditions similar to those of pd-FX. All FX derivatives could be completely converted into active form, and the final activated preparations were more than 90% active as determined by active site titration.

**Plasma-based Assay of Function**—The clotting activity of wt-FX, FX/FIX(Gla), and FX/FIX(EGF1) was estimated in a prothrombin time assay. Recombinant FX derivatives were incubated with FX immunodepleted human plasma and clotting initiated by addition of rabbit brain thromboplastin. Clotting activity of wt-FX was 75 ± 10% of the activity of pooled normal plasma. In contrast, FX/FIX(Gla) and FX/FIX(EGF1) clotting activities were less than 1 and 6 ± 2% of the activity of pooled normal plasma, respectively. Because this global measurement of the dysfunction of the chimera could be due to abnormal activation and/or catalytic activity, these enzymatic steps were studied separately using purified components.

**Amidolytic Activity**—To explore whether substitutions of the Gla and EGF1 domains of FX by the corresponding domains of FIX affect the amidolytic activity of FXa, hydrolysis of various concentrations of synthetic substrate S-2765 was monitored at an enzyme concentration of 2 nM. Mean values ± S.D. for three experiments are presented.

**Prothrombin Activation**—As the chimeric FX variants displayed a normal reactivity of the catalytic triad, it was of interest to investigate the influence of the Gla and EGF1 domain substitutions in prothrombin activation by the FXa-FVa complex. Therefore, FXa chimeras were compared with wt- and pd-FXa in their ability to activate prothrombin. The rate of thrombin generation was studied as a function of increasing concentrations of FVa and in the presence of an excess of phospholipids. For all FXa tested, thrombin formation was dependent on FVa concentrations and was saturable (Fig. 1). The apparent Kᵥ values were 420 ± 38, 405 ± 26, 230 ± 11, and 225 ± 11 pm for pd-FXa, wt-FXa, FX/FIX(Gla), and FX/FIX(EGF1), respectively, whereas the apparent k_cat values were between 15.5 and 19.9 s⁻¹. These experiments show that activated chimeras have full catalytic activity toward prothrombin in the prothrombinase complex. In addition, substitution of the Gla or EGF1 domains by the corresponding domains of FIX even has a positive effect on the apparent affinity of FVa. To evaluate whether the slight increased affinity of FVa for the chimeras was related to the presence of phospholipids, the initial rates of thrombin formation by the chimeras was compared with that of wt-FXa in the presence of subsaturating concentrations of FVa with phospholipids (Fig. 2A) or without phospholipids (Fig. 2B). The same initial rates of prothrombin activation were obtained for the chimeras and wt-FXa in the absence of phospholipids (Fig. 2B). A similar rate of activation was obtained with pd-FXa (data not shown). Therefore, the increased affinity of FVa observed in the experiment depicted in Fig. 1 was due to the presence of phospholipids. In conclusion, substitution of the Gla or EGF1 domains by the corresponding domains of FIX has no effect on the apparent affinity of FVa for FXa, and even a slight increase of affinity was observed in the presence of phospholipids.

**FX Activation by TF-FVIIa Complex**—FX is one natural substrate of the TF-FVIIa complex, which is considered as the initial enzyme complex in the cascade following vascular damage. Because several studies have implicated the Gla domain (28, 48) and the first EGF domain (31) of FX in the interaction with TF-FVIIa, FX chimeras were compared with wt-FX for their ability to serve as substrates for the complex in the presence of phospholipid vesicles and calcium ions. The apparent k_cat of the TF-FVIIa complex toward all chimeras was slightly decreased compared with that of wt-FX (Table II). Conversely, the apparent affinity of FX/FIX(Gla) and FX/FIX(EGF1) for the enzymatic complex was greatly decreased (Table II). These data indicate that substitution of the Gla or the EGF1 domains by the corresponding domains of FIX impairs the binding capacity of FX to the TF-FVIIa. Because Gla domains of FIX and FX bind to phospholipids which provide a surface for the assembly of the FVIIa-TF-substrate complex, it is possible that the substitutions of the Gla and EGF1 domains alter the alignment between the substrate and TF-FVIIa complex. This possibility was tested by using detergent-solubilized TF in an assay comparing the initial rates of chimeras and wt-FX activation by TF-FVIIa. Similarly to the phospholipid-containing system (Fig. 3A), activation rates of the chimeras were impaired in the presence of solubilized TF (Fig. 3B). The initial rate was 3.5 ± 1.1% for FX/FIX(Gla) and 10.8 ± 3.2% for FX/FIX(EGF1) when compared with the rate obtained for wt-FX (Fig. 3). Wt-FX was activated at a similar rate of pd-FX (data not shown).

**Inhibition of the TF-FVIIa Complex by TFPI-FXa**—Because the chimeric variants FX/FIX(Gla) and FX/FIX(EGF1) displayed a reduced affinity for TF-FVIIa, kinetics of TF-FVIIa inhibition by variable concentrations of preformed TFPI-FXa chimeras was investigated. Conditions of a limited amount of

---

**TABLE I**

| Reaction                             | k_cat/K_m (nM⁻¹ s⁻¹) |
|--------------------------------------|----------------------|
| pd-FXa                               | 558 ± 32             |
| wt-FXa                               | 457 ± 11             |
| FX/FIX(Gla)                          | 520 ± 27             |
| FX/FIX(EGF1)                         | 471 ± 25             |

The apparent dependent on FVa concentrations and was saturable (Fig. 1). Phospholipids. For all FXa tested, thrombin formation was studied as a function of increasing concentrations of FVa with phospholipids (Fig. 2). A similar rate of activation was obtained with pd-FXa (data not shown). Therefore, the increased affinity of FVa observed in the experiment depicted in Fig. 1 was due to the presence of phospholipids. In conclusion, substitution of the Gla or EGF1 domains by the corresponding domains of FIX has no effect on the apparent affinity of FVa for FXa, and even a slight increase of affinity was observed in the presence of phospholipids.
Kinetic parameters of FX proteins activation by the TF-FVIIa complex

Varying concentrations of recombinant FX proteins (0–3 μM) are mixed at 37 °C with 60 pM FVIIa and 60 pM relipidated TF, preincubated for 20 min to establish a TF-FVIIa complex, in presence of 5 mM CaCl₂. FXa formed at each time point was determined as described under “Experimental Procedures.” Data represent the mean values of three experiments.

| FX Protein | Apparent Kd (nM) | k_cat (s⁻¹) |
|------------|------------------|-------------|
| wt-FX      | 285 ± 25         | 46 ± 11     |
| FX/FIX(Gla)| 800 ± 95         | 33 ± 12     |
| FX/FIX(EGF1) | 1400 ± 165   | 32 ± 12     |

FIG. 2. Activation of prothrombin by FVas-FXa complexes in the presence or absence of phospholipids. A, in the presence of 20 μM phospholipid vesicles and 250 pM FVas, thrombin formation from prothrombin (1 μM) activation by 20 pM wt-FXa (open circles), FX/FIX(Gla) (open squares), or FX/FIX(EGF1) (open triangles) was measured at 37 °C in presence of 5 mM CaCl₂. B, in the absence of phospholipids, thrombin formation from prothrombin (10 μM) activation by 0.5 mM wt-FXa (open circles), FX/FIX(Gla) (open squares), or FX/FIX(EGF1) (open triangles) was measured at 37 °C in presence of 75 nM FVas and 5 mM CaCl₂. Thrombin formed at each time point was quantified as described under “Experimental Procedures.” Data represent the mean values of three experiments.

FIG. 3. Activation of recombinant FX by the FT-FVIIa complex in the presence or absence of phospholipids. A, activation of 1 μM wt-FX (open circles), FX/FIX(Gla) (open squares), or FX/FIX(EGF1) (open triangles) was proceeded at 37 °C by 8 nM FVIIa and 500 pM relipidated TF in presence of 5 mM CaCl₂. B, in the absence of phospholipids vesicles, activation of 250 nM wt-FX (open circles), FX/FIX(Gla) (open squares), or FX/FIX(EGF1) (open triangles) was assayed at 37 °C with 50 nM FVIIa and 250 nM detergent-solubilized full-length TF in the presence of 5 mM CaCl₂. FXa formation was determined as described under “Experimental Procedures.” Data represent the mean values of three experiments.

FIG. 4. Kinetics of inhibition of the TF-FVIIa complex by variable concentrations of TFPI-FXa complexes. Varying concentrations of preformed TFPI-FXa complexes are incubated at 37 °C with 25 pM TF-FVIIa complex (made with 25 pM relipidated TF and 8 nM FVIIa) in the presence of 400 nM pd-FX. The final concentrations of TFPI-FXa complexes are 25 (open squares), 50 (closed circles), and 100 pM (open triangles). Activated pd-FX formed at each time point is determined, and data were fitted as described under “Experimental Procedures.” A typical experiment for TFPI-pd-FXa (A), TFPI-wt-FXa (B), TFPI-FXa/FIX(Gla) (C), and TFPI-FXa/FIX(EGF1) (D) is shown.

DISCUSSION

In this study, the role of the Gla and the first EGF-like domains of FX has been explored. To this end, FX chimeras containing the Gla or the EGF1 domains of FIX substituting the corresponding domains of FX were produced. Their properties, regarding different functions of FX, were compared with those of normal FX.

In the prothrombinase complex FVas displays an apparent affinity for FX/FIX-Gla and FX/FIX-EGF1 chimeras ~2-fold lower than that of normal FX.
higher than for normal FXa (Fig. 1). One possibility is that Gla and EGF1 domains of FXa interact with FVa. Therefore, the apparent increased affinity of FVa for the FX chimeras is due to the presence of appropriate binding sites for the cofactor on the Gla and EGF1 domains of FIX. So far, no interactions have been described between FVa and FIXa. A second possibility is that FVa does not interact with the Gla and EGF1 domains of FXa. Hence, in FX the Gla and EGF1 domains would act as spacers between phospholipids and the rest of the molecule to match the cofactor binding sites. Therefore, the presence of the Gla or EGF1 domains of FIX modifies the inclination toward the phospholipid surface of the EGF2 and protease domains of the chimeras compared with that of normal FXa. This mechanism is supported by experimental data showing that in the absence of phospholipids the initial rate of thrombin formation by the chimeras associated to FVa is identical to those observed with normal FXa (Fig. 2B). A similar spacer role has been described previously for FIX in studies using FIX mutated in the EGF1 domain or in which the corresponding domain of protein C substituted the EGF1 domain. The mutated FIX molecule displays a reduced interaction with FVIIIa in the presence of phospholipids but not in its absence (21). Observation of the FX chimeras displayed a normal catalytic activity in the presence of FVa (Fig. 2B) demonstrates that the FVa-binding sites on the chimeras are identical to those of normal FXa. Thus, because no interactions have been described between FVa and FIXa, these data imply that the Gla and the EGF1 domains of FXa do not contain FVa-binding sites and are only involved in the interaction with the cofactor in the presence of phospholipids to conform the enzyme toward FVa.

The FX chimeras containing the Gla or the EGF1 domains of FIX are different from normal FXa as substrate for the TF-FVIIa complex in the presence of phospholipids (Table II). The results indicate that substitution of one of the two amino-terminal regions of FX by the corresponding region of FIX affects the apparent affinity of the substrate for the enzymatic complex. Therefore, these two regions are involved in the formation of the TF-FVIIa-FX complex, and it can be suggested that within this ternary complex both domains of FX are directly or indirectly implicated in the binding to TF-FVIIa. The Gla domain involvement in the interaction with TF-FVIIa exosites is supported by several studies. For instance, by studying the effect of mutations in the carboxyl-terminal domain of TF, an interaction of this membrane-proximal region with the Gla domain of the substrates FIX and FX has been proposed (29, 30, 49). Recently, it has been revealed (31) that FIX and FX interact with TF through, in part, their EGF1 domains. It is noteworthy that the decreased affinity of the FX/FIXI(Gla) and FX/FIXI-(EGF1) compared with normal FX for the TF-FVIIa complex in the presence of phospholipids is associated with a normal $k_{cat}$ (Table II). These data can be integrated to the kinetic model for FX activation by TF-FVIIa proposed by Krishnaswamy and co-workers (50). In their model, substrate recognition by the TF-FVIIa complex is achieved through two sequential steps. Initial interactions between TF-FVIIa exosites and complementary sites on the substrate remote from structures surrounding the scissile bond are followed by an intramolecular binding step that allows the cleavage of adjacent structures to dock with the active site of the enzyme prior to bond cleavage. In the present study, it is observed that in the absence of phospholipids, the initial rates of activation of the chimeras were markedly decreased compared with the activation of wt-FX (Fig. 3). As with the previous data in presence of phospholipids, the defective activation in their absence is due to defective interaction of the chimeras with exosites of the TF-FVIIa complex. Taken together, these results suggest a direct role of the Gla and EGF1 domains in the interaction with the TF-FVIIa. Thus, despite sequence and structural homologies of the Gla and the EGF1 domains of FIX and FX and the probable involvement of the same residues to interact with TF-FVIIa, the severe defective interactions of the chimeras with TF-FVIIa, in the presence or absence of phospholipids (Figs. 2 and 3), demonstrate that these domains cannot be exchanged without altering the substrate affinity for the enzymatic complex. Two possibilities arise from these observations. First, the defective interaction of the chimeras with the enzymatic complex could be due to the involvement of different residues between FIX and FX domains with TF-FVIIa. However, a docking approach that proposed a model for the ternary complex TF-VIIa-FIX has revealed that residues of FIX interacting with TF-FVIIa are rather conserved in FX (51). A second possibility is that the substitutions of Gla or EGF1 domains introduce an alteration of the favorable orientation of the substrate toward the enzymatic complex. The same structural modification is likely the cause of a slight increase of the apparent affinity of FVa for the chimeras in the presence of phospholipids (Fig. 1).

Kinetics of TF-FVIIa inhibition by variable concentrations of preformed TFPI-FXa show that the substitution of the Gla or the EGF1 domains markedly reduces the association constants of the FT-FVIIa-TFPI-FXa quaternary complexes (Table III). This suggests that the defective interaction of TFPI associated with the activated chimeras with TF-FVIIa is probably due to an alteration of the favorable orientation of the inhibitor complex toward the enzymatic complex. These data indicate that similar interactions contribute to the assembly of FX and FXa, after complex formation with TFPI, to the TF-FVIIa complex. This notion is in agreement with a previous study (52) showing that TF residues, which are important for the activation of FX by the TF-FVIIa complex, are required for the accelerated inhibition of the TF-FVIIa complex by TFPI mediated by FXa. These TF residues have been proposed to interact with the Gla domain of the substrates FIX and FX (29, 30, 49). It has been shown previously (53) that FXa devoid of the Gla domain is not able to support the TFPI-mediated inhibition of TF-FVIIa, suggesting the importance of FXa Gla domain in the formation of the TF-FVIIa-TFPI-FXa complex. However, proteolytic fragments of FX-containing multiple domains are not always reliable for the identification of binding sites because it has been observed that EGF domains must be covalently attached to the Gla domain of FX to maintain their properties (53). Therefore, the approach using FX/FIX chimeras carrying FIX regions is more appropriate to identify the role of the Gla and EGF1 domains.

In conclusion, this study demonstrates that the Gla and first EGF-like domains of FX are not directly involved in the interaction of FIXa with FVa. Experimental data indicate that the FX Gla domain interacts with the FT-FVIIa complex. Moreover, this domain, together with the first EGF-like domain, is directly involved in the association of FIXa for complex for-
mution with TFPI to the TF-FVIIa complex. This study also reveals that in a blood coagulation protein a loss of function can be counterbalanced by the gain of another one. Thus, a mutation within a FX molecule, which is responsible for a reduced rate of activation by the TF-FVIIa complex, could be compensated for by its beneficial effect on the activated molecule in the presence of its cofactor. There are other models in nature confirming this paradox. Therefore, it can be suggested that an individual with no bleeding disorders could possess such mutations, which would only be detected during coagulation investigation.

Acknowledgments—We thank the following members of INSERM U143: Jean-Marie Freysinet and Jean-Maurice Lavergne for critical reading of the manuscript, Valérie Proule for helpful discussions, and Dominique Meyer for support and useful suggestions.

REFERENCES

1. Nemerson, Y., and Gentry, R. (1986) Biochemistry 25, 4020–4033
2. Rosing, J., Tans, G., Govers-Riemslag, J. W., Zwaal, R. F., and Hemker, H. C. (1980) J. Biol. Chem. 255, 274–283
3. Broe, G. J., Jr., and Miletich, J. P. (1987) Blood 69, 150–155
4. Sanders, N. L., Bajaj, S. P., Zivelin, A., and Rapaport, S. I. (1985) Blood 66, 204–212
5. Smith, C. M., and Hanahan, D. J. (1976) Biochemistry 15, 1830–1838
6. Eaton, D., Rodriguez, H., and Vehar, G. A. (1986) Biochemistry 25, 505–512
7. Suzuki, K., Dahlback, B., and Stenflo, J. (1982) J. Biol. Chem. 257, 6556–6564
8. Vehar, G. A., and Davie, E. W. (1980) Biochemistry 19, 401–410
9. Mann, K. G., Nesheim, M. E., Church, W. R., Haley, P., and Kranshawamy, S. (1990) Blood 76, 1–16
10. Mertens, K., van Wijngaarden, A., and Bertina, R. M. (1985) J. Biol. Chem. 260, 10952–10962
11. Rezaie, A. R., Neuenschwander, P. F., Rezaie, R. A., and Morrissey, J. H. (1996) J. Biol. Chem. 271, 21752–21757
12. Zhong, D., Bajaj, M. S., Schmidt, A. E., and Bajaj, S. P. (2002) J. Biol. Chem. 277, 3622–3631
13. Meyer, D., Baumgartner, H. R., and Edgington, T. S. (1984) Br. J. Haematol. 57, 609–620
14. Stenflo, J., and Suttie, J. W. (1977) FEBS Lett. 81, 289–292
15. Brandstetter, H., Bauer, M., Huber, R., Lollar, P., and Bode, W. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9796–9800
16. Kalafatis, M., Egan, J. O., van’t Veer, C., Cowern, K. M., and Mann, K. G. (1997) Crit. Rev. Eukaryotic Gene Expr. 7, 241–280
17. Mathur, A., and Bajaj, S. P. (1999) J. Biol. Chem. 274, 18477–18486
18. Colman, J. A., Lenting, P. J., and Mertens, K. (1999) Biochem. J. 339, 217–221
19. Rezaie, A. R. (1996) J. Biol. Chem. 271, 23887–23894
20. Kalafatis, M., Egan, J. O., van’t Veer, C., Cowern, K. M., and Mann, K. G. (1997) Crit. Rev. Eukaryotic Gene Expr. 7, 241–280
21. Mathur, A., and Bajaj, S. P. (1999) J. Biol. Chem. 274, 18477–18486
22. Kollman, J. A., Lenting, P. J., and Mertens, K. (1999) Biochem. J. 339, 217–221
23. Rudolph, A. E., Porche-Sorbet, R., and Miletich, J. P. (2000) Biochemistry 39, 2861–2867
24. Persson, K. E., Viliotreix, B. O., Thamhitz, A. M., Knob, K. E., and Stenflo, J. (2002) J. Biol. Chem. 277, 35616–35621
25. Celis, P. H., Van Stempvoort, G., Frigoli, C., Schurgers, L. J., Lenting, P. J., and Mertens, K. (2002) J. Biol. Chem. 277, 20214–20220
26. Olsen, E. H. N., Roberts, H. R., and Monroe D. M. (2001) Thromb. Haemostasis 86, 10952–10962
27. Hertzberg, M. S., Neve, F. C., and Brownlee, G. G. (1988) J. Biol. Chem. 263, 14159–14165
28. Rezaie, A. R., Neuenschwander, P. F., Rezaie, R. A., and Morrissey, J. H. (1996) J. Biol. Chem. 271, 21752–21757
29. Ruf, W., Miles, D. J., Rehemtulla, A., and Edgington, T. S. (1992) J. Biol. Chem. 267, 6374–6381
Role of the Gla and First Epidermal Growth Factor-like Domains of Factor X in the Prothrombinase and Tissue Factor-Factor VIIa Complexes
Fabrice Thiec, Ghislaine Cherel and Olivier D. Christophe

J. Biol. Chem. 2003, 278:10393-10399.
doi: 10.1074/jbc.M212144200 originally published online January 15, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M212144200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 52 references, 23 of which can be accessed free at http://www.jbc.org/content/278/12/10393.full.html#ref-list-1