The Interface between the EGF2 Domain and the Protease Domain in Blood Coagulation Factor IX Contributes to Factor VIII Binding and Factor X Activation

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ABSTRACT: The light chain of activated factor IX (FIXa) is involved in a number of functional properties, including FIXa enzymatic activity. This suggests the existence of a functional link between the FIXa light chain and the catalytic domain. The FIXa structure includes a few putative interactions between EGF2 and the protease domain. The role thereof has been addressed in this study. Recombinant FIX variants FIX-N92A, FIX-N92H, FIX-Y295A, and FIX-F299A were produced in 293 cells. After activation, the purified mutants were analyzed for a variety of functional parameters. None of these substitutions had a major effect on the interaction with antithrombin or the cleavage of the chromogenic substrate CH_3-SO_2-D-CHG-Gly-Arg-*p*-nitroanilide. All FIXa mutants, however, exhibited a reduced level of factor X (FX) activation. Defective proteolytic activity occurred both in the absence and in the presence of activated factor VIII (FVIIIa). All mutants also exhibited a reduced level of FX activation in the absence of phospholipids. This suggests that putative interdomain contacts involving residues Asn^{92}, Tyr^{295}, and Phe^{299} affect reactivity toward FX. Detailed kinetic studies in the presence of phospholipids and FVIIIa revealed substrate inhibition, particularly for mutants FIXa-N92A and FIXa-N92H. Surface plasmon resonance demonstrated that the same replacements weaken the association with the isolated factor VIII (FVIII) A2 domain and the FVIII light chain. This implies a defect in the formation of the FX-activating complex that is membrane-independent. We conclude that contacts between EGF2 and the protease domain of FIXa are crucial for FIXa enzymatic activity and for the assembly of the FX-activating complex.

Factor IX (FIX)[1] is a vitamin K-dependent plasma protein which plays an important role in the blood coagulation cascade (*1*−*3*). Its importance is apparent from the notion that a deficiency or a functional defect in FIX causes a bleeding tendency known as hemophilia B. FIX is converted into active enzyme factor IXa (FIXa) by proteolytic cleavages of two peptide bonds by either activated factor XI (FXIa) or by the activated factor FVII (FVIIa)−tissue factor (TF) complex (*1*, *4*, *5*). Upon activation of FIX, the activation peptide is released and a 45 kDa enzyme, FIXa, composed of two chains linked by a disulfide bridge is formed. The light chain contains a γ-carboxyglutamic acid (Gla)-containing domain, a short hydrophobic stack, and two EGF-like domains which are connected by linker residues 84−87. The heavy chain comprises the serine protease domain with the catalytic center (*6*).

The function of FIXa in the blood coagulation cascade is to activate factor X (FX) in a process that requires the presence of the phospholipid surface, Ca^{2+} ions, and a cofactor, activated factor VIII (FVIIIa) (*2*). FIXa alone is a poor protease but becomes a potent FX activator upon formation of the complex with FVIIIa (*7*, *8*). FVIIIa is a heterotrimer composed of A1, A2, and A3−C1−C2 subunits. FIXa and FVIIIa assemble into the FX-activating complex through a number of interactive sites. It has been shown that the protease domain of FIXa interacts with the FVIII A2 subunit and that the FIXa light chain binds to the FVIII A3 subunit (*9*, *10*). The EGF1 domain of FIXa is involved in Ca^{2+} binding and may play a structural role in properly positioning the EGF2 and protease domain for binding to FVIIIa (*9*−*11*).

While FIXa requires cleavage of the activation peptide from its protease domain, other domain modules also contribute to its catalytic potential. This has become apparent from previous reports demonstrating that mutations beyond the FIXa catalytic domain can affect FIXa catalytic activity in the absence of FVIIIa (*11*−*15*). One example is the hydrophobic contact between the two EGF-like domains of FIXa that contributes to FIXa enzymatic activity (*12*). This suggests that the FIXa catalytic domain is under the control of the FIXa light chain by an as-yet-unresolved mechanism. A number of putative contacts between the EGF2 domain and the heavy chain can be identified within the three-dimensional structure of the FIXa molecule (*16*−*18*). These include the hydrophobic contacts between Phe^{98} and residues Tyr^{295} and Phe^{299} and a potential hydrogen bond between

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[1] Abbreviations: FIX, factor IX; FIXa, activated factor IX; FVIII, factor VIII; FVIIIa, activated factor VIII; FX, factor X; FXa, activated factor X; pd-FIXa, plasma-derived FIXa; wt-FIXa, wild-type recombinant FIXa; FXIa, activated factor XI; HSA, human serum albumin; Gla, γ-carboxyglutamic acid; EGF2, second epidermal growth factor; *p*NA, *p*-nitroanilide; SPR, surface plasmon resonance.

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Asn[92] and Tyr[295] (*19*). The functional implications, if any, of these putative contacts remain poorly understood.

In this study, we investigated the role of the interface between the EGF2 domain and the protease domain of FIXa with a particular focus on the putative interdomain contacts in the FIXa structure. To this end, recombinant FIX variants with mutations at residue Asn[92], Phe[98], Tyr[295], or Phe[299] were analyzed for a variety of functional parameters, including the interaction with FX, antithrombin, and FVIII.

MATERIALS AND METHODS

Materials. Pfu polymerase and pBluescript vector were obtained from Stratagene (Cambridge, U.K.). Oligonucleotide primers, restriction enzymes, pcDNA3.1(−) vector, DMRIE-C reagent, and Geneticin G-418 were supplied by Invitrogen (Breda, The Netherlands). Penicillin/streptomycin, DMEM/F12 medium, and fetal calf serum were from BioWhittaker (Verviers, Belgium). Cell factories (6320 cm^2) and culture flasks were purchased from Nunc A/S (Roskilde, Denmark). CNBr-Sepharose 4B, Source 15S Sepharose, Q-Sepharose FF, and Protein A-Sepharose were obtained from Amersham Pharmacia Biotech Nederland (Rosendaal, The Netherlands). L-α-Phosphatidyl-L-serine (from bovine brain), L-α-phosphatidylcholine type I EH (from egg yolk), vitamin K_1, and cloning cylinders were from Sigma Chemical Co. (St. Louis, MO). CH_3SO_2-D-CHG-Gly-Arg-*p*-nitroanilide (product name Pefachrome FIXa Pefa 3107) and Pefachrome Xa were from Pentapharm AG (Basel, Switzerland). CH_3SO_2-D-leucyl-L-glycyl-L-arginyl-*p*-nitroanilide (CH_3SO_2-LGR-*p*NA; product name CBS 31.39) was obtained from Diagnostica Stago (Asnières, France). Microtiter plates were from Corning (Badhoevedorp, The Netherlands). The BIAcore3000 biosensor system and reagents (amino coupling kit, CM5 chips, certified grade) were supplied by Biacore AB (Uppsala, Sweden).

Proteins. Normal human plasma FIX was obtained by immunoaffinity chromatography as previously described (*20*). Human plasma FX and prothrombin were purified by conventional chromatography techniques (*21*), and prothrombin was converted to α-thrombin as described previously (*8*). Human FVIII was purified from plasma by anti-light chain affinity chromatography as outlined previously (*22*). The FVIII A2 domain was isolated from thrombin-activated human plasma FVIII as described previously (*23*) with some modifications. Briefly, purification of the FVIII A2 domain was performed using Source 15S chromatography in a buffer containing 5 mM $CaCl_2$, 0.01% (v/v) Tween 20, and 30 mM histidine (pH 5.5). A gradient elution (from 100 to 750 mM NaCl) was used to separate the A2 domain from the A1/A3−C1−C2 dimer. FXIa was purchased from Enzyme Research Laboratories (South Bend, IN). Anti-FIX monoclonal antibody CLB-FIX 11 has been described previously (*20*) and was purified from culture medium using Protein A-Sepharose as recommended by the manufacturer. Purified antithrombin and human serum albumin (HSA) were obtained from Sanquin Plasma Products (Amsterdam, The Netherlands).

Mutagenesis and Construction of Expression Vectors. Plasmid pBluescript containing human FIX cDNA was used as a template to construct cDNAs encoding FIX-F299A, FIX-Y295A, FIX-N92A, FIX-N92H, and FIX-F98A. Site-directed mutagenesis was performed by the QuickChange method using oligonucleotide primers 5′-TAC ACG AAC ATC GCC CTC AAA TTT GGA-3′ (sense) and 5′-TCC AAA TTT GAG GGC GAT GTT CGT GTA-3′ (antisense) for FIX-F299A, 5′-GCT GAC AAG GAA GCC ACG AAC ATC TTC-3′ (sense) and 5′-GAA GAT GTT CGT GGC TTC CTT GTC AGC-3′ (antisense) for FIX-Y295A, 5′-TGT AAC ATT AAG GCT GGC AGA TGC GAG-3′ (sense) and 5′-CTC GCA TCT GCC AGC CTT AAT GTT ACA-3′ (antisense) for FIX-N92A, 5′-TGT AAC ATT AAG CAT GGC AGA TGC GAG-3′ (sense) and 5′-CTC GCA TCT GCC ATG CTT AAT GTT ACA-3′ (antisense) for FIX-N92H, and 5′-AGA TGC GAG CAG GCT TGT AAA AAT AGT-3′ (sense) and 5′-ACT ATT TTT ACA AGC CTG CTC GCA TCT-3′ (antisense) for FIX-F98A. The mutant FIX constructs were digested with restriction enzymes *Bam*HI and *Hin*dIII and subsequently ligated into the corresponding restriction sites in mammalian expression vector pcDNA3.1(−). Final FIX constructs were sequenced to confirm the mutations and to rule out any PCR errors.

Recombinant FIX. 293 kidney cells were grown in DMEM/F12 medium supplemented with 10% fetal calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. The pcDNA3.1(−) expression plasmids containing mutant FIX cDNA were transfected into the cells by lipofection using DMRIE-C reagent. After selection of transfected cells with medium containing Geneticin at a concentration of 500 μg/mL, single clones were picked using cloning cylinders and grown in 12-well dishes in selective medium to produce stable cell lines. Secretion of FIX antigen into the medium was assessed by an enzyme-linked immunosorbent assay (see below). Cell lines producing the appropriate amount of FIX antigen were selected for large-scale production in 1 L cell factories (DMEM/F12 medium supplemented with 4% fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 5 μg/mL vitamin K_1). Recombinant FIX was purified from concentrated medium by immunoaffinity chromatography employing Ca^{2+}-dependent anti-FIX monoclonal antibody CLB-FIX 11 (*20*), which is directed against the FIX Gla domain (*24*). After two extensive washing steps with 10 mM benzamidine, 5% (v/v) glycerol, 5 mM $CaCl_2$, 50 mM Tris (pH 7.4) supplemented with 150 mM NaCl, and 1 M NaCl, FIX was eluted with 20 mM EDTA in the same buffer. FIX-containing fractions were pooled and stored at −20 °C in 5% (v/v) glycerol, 100 mM NaCl, and 50 mM Tris (pH 7.4). All purification steps were performed at 4 °C. Analysis by SDS−PAGE and Coomassie Brilliant Blue staining revealed that all recombinant proteins migrated with an M_r similar to that of purified human pd-FIX. Activation of FIX by FXIa was performed as described previously (*20*). FIXa was purified from the activation mixture employing anion exchange chromatography as outlined previously (*20*). In agreement with previous reports (*25*, *26*), we found similar specific activities for recombinant wt-FIXa and pd-FIXa. This is consistent with the notion that γ-carboxylation of FIX is normal in this expression system (*25*−*27*).

Protein Concentrations. FIX antigen levels were determined by an enzyme-linked immunosorbent assay employing a previously described method (*20*). Proteins were quantified by the method of Bradford, using human albumin as a standard (*28*). FIXa concentrations were measured by active site titration with antithrombin in the presence of heparin

(*20*). FVIII activity was assayed by a spectrophotometric method, employing bovine coagulation factors and a chromogenic substrate specific for FXa (Coatest FVIII, Chromogenix, Mölndal, Sweden). The amount of FVIII present in 1 mL of human plasma (1 unit/mL) was assumed to correspond to 0.35 nM.

Hydrolysis of CH_3SO_2-D-CHG-Gly-Arg-pNA. Cleavage of CH_3SO_2-D-CHG-Gly-Arg-*p*NA was assayed in 33% (v/v) ethylene glycol, 100 mM NaCl, 5 mM $CaCl_2$, 0.2% (w/v) HSA, and 50 mM Tris (pH 7.4) at 37 °C. Briefly, 150 nM wild-type or mutant FIXa was incubated in a 96-well plate with chromogenic substrate CH_3SO_2-D-CHG-Gly-Arg-*p*NA at concentrations varying between 0.5 and 5 mM. The effect of Ca^{2+} ions on amidolytic activity was assayed at a substrate concentration of 1.5 mM in the presence of 10 mM EDTA or 5 mM $CaCl_2$ as outlined previously (*11*). Initial rates of substrate hydrolysis were measured at 37 °C by monitoring the absorbance at 405 nm over time. The experimental data were fitted in the Michaelis−Menten equation to obtain K_m and k_{cat} values.

FX Activation. FXa formation was assessed essentially as described previously (*21*). Phospholipid vesicles (100 μM, 1:1 phosphatidylserine:phosphatidylcholine molar ratio) and Ca^{2+} ions (5 mM) were preincubated for 10 min at 37 °C. For experiments in the absence of FVIII, FIXa (30 nM) was then added and the reaction was started by the addition of FX (20−500 nM). At various time points, subsamples were withdrawn and FXa formation was stopped by the addition of EDTA (10 mM). FXa then was quantified as previously described (*21*) except that Pefachrome Xa was used as a chromogenic substrate. In experiments in the presence of FVIIIa, FVIII (0.1−2 nM) and thrombin (5 nM) were added to the mixture of phospholipids, Ca^{2+} ions, and FIXa (0.1−0.3 nM). After incubation for 1 min, the reaction was started by the addition of FX at various concentrations between 2.5 and 200 nM. Subsamples then were withdrawn at 30 s intervals during the initial 3 min and added to buffer containing EDTA (10 mM) and hirudin (1 unit/mL) to stop further FXa generation and to inhibit thrombin activity. Subsequently, the amount of FXa generated was quantified spectrophotometrically using Pefachrome Xa. When FXa formation was studied in the absence of phospholipids, FX (1.7 μM) was activated in the presence of 20 nM FIXa, 10 nM FVIII, and 5 nM thrombin, and FXa was quantified as described above. Apparent K_m and k_{cat} values were obtained by fitting experimental data to the Michaelis−Menten equation.

Slow-Binding Kinetics. Inhibition of FIXa variants by antithrombin was assessed using the slow-binding kinetic approach, as outlined previously (*29*, *30*). In short, a series of inhibition progress curves was generated at 37 °C using 10 nM FIXa, 2.5 mM CH_3SO_2-LGR-*p*NA, and various concentrations of antithrombin (0.35−2.5 μM). The apparent first-order rate constant (k') was obtained for each antithrombin concentration by fitting the data from the progress curves to the integrated rate equation for slow binding (*30*). A plot of k' versus the inhibitor concentration yields the association and dissociation rate constants (k_{ass} and k_{dis}, respectively), according to eq 2 in ref *29*.

Analysis of FIXa−Antithrombin Complexes by SDS−PAGE. Formation of a complex of FIXa with antithrombin was analyzed by SDS−PAGE as described previously (*20*). Briefly, FIXa (1 μM) was incubated with antithrombin (2 μM) at 37 °C for 1 h in the presence of heparin (2 mg/mL) or overnight in the absence of heparin. The reaction mixtures were analyzed by SDS−PAGE, and proteins were visualized by Coomassie Brilliant Blue staining.

Binding of FIXa Variants to the Isolated FVIII A2 Domain or FVIII Light Chain. The interaction between the FIXa mutants and the FVIII A2 domain or FVIII light chain was assessed by surface plasmon resonance (SPR) analysis employing a BIAcore 3000 biosensor system. Isolated FVIII A2 domain (178 fmol/mm^2) or FIXa variants (153 fmol/mm^2) were immobilized on a CM5 sensor chip using the amine coupling method according to the manufacturer's instructions. In both cases, a control channel was activated and blocked without the addition of proteins. Binding to coated channels was corrected for binding to the noncoated channel. FIXa variants (50−400 nM) were passed over the immobilized A2 domain in a buffer containing 150 mM NaCl, 2 mM $CaCl_2$, 0.005% (v/v) Tween 20, and 20 mM Hepes (pH 7.4) at 25 °C with a flow rate of 20 μL/min. The interaction between FIXa mutants and the FVIII light chain was performed in 50 mM NaCl, 5 mM $CaCl_2$, 0.005% (v/v) Tween 20, and 20 mM Hepes (pH 7.4) at 37 °C with a flow rate of 40 μL/min. Varying concentrations of the FVIII light chain (10−100 nM) were incubated with FIXa variants coupled to a sensor chip. In both cases, the sensor chip surface was regenerated by incubating it with 1 M NaCl, 20 mM EDTA, and 20 mM Hepes (pH 7.4). The data were evaluated using BIAevaluation version 3.1 (Biacore AB).

RESULTS

Recombinant FIX. In this study, we address the role of the interface between the protease domain and the EGF2 domain of FIXa. As shown in Figure 1, FIXa assembles with FVIIIa into a complex that is thought to involve interaction of the light chain of FIXa (domains Gla, EGF1, and EGF2) with that of FVIIIa (domains A3, C1, and C2) and of the FIXa heavy chain (the protease domain) with the FVIIIa A2 domain (Figure 1A). In the FIXa structure, the light and heavy chain of FIXa are in close contact (Figure 1B), and this contact seems to involve amino acid residues 92, 98, 295, and 299 (Figure 1C). To investigate the functional role of these putative contacts, we used site-directed mutagenesis to construct FIX variants FIX-F299A, FIX-Y295A, FIX-N92A, FIX-N92H, and FIX-F98A. For all variants, the amino acid of interest was replaced with a small and neutral Ala residue, except for the FIX-N92H variant in which the Asn at position 92 was exchanged with His. This point mutation (FIX Fukuoka) is known to be associated with moderate hemophilia B (*31*). FIX variants were stably expressed in 293 cells. All mutants except FIX-F98A were secreted into the cultured supernatant at a level equivalent to that of wt-FIX. The level of FIX-F98A expression was too low to allow further characterization. FIX mutants were purified by immunoaffinity chromatography as described in Materials and Methods. All purified mutants could be completely converted to the FIXaβ form using FXIa under the same conditions that were used for wt-FIX. The final preparations of activated FIX mutants and wt-FIXa were more than 90% active as assessed by active site titration with antithrombin.

Amidolytic Activity. The role of the interface between the EGF2 and the protease domain in FIXa activity was

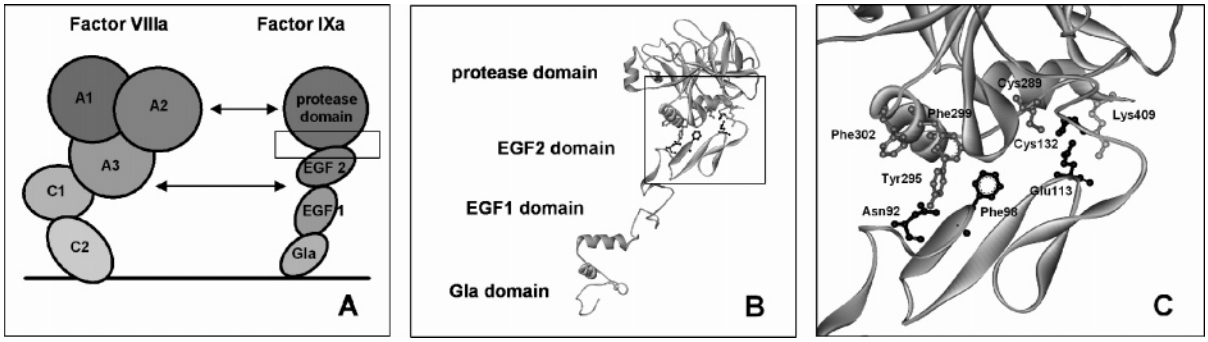

FIGURE 1: Interface between the protease domain and the EGF2 domain in human FIXa. (A) Schematic representation of the FVIIIa·FIXa complex (modified from ref *9*). Complex assembly involves interaction of FIXa with the FVIIIa light chain (domains A3, C1, and C2) and with the A2 domain of the FVIIIa heavy chain (see arrows). (B) Model of full-length human FIXa (*18*), indicating the FIXa light chain (domains Gla, EGF1, and EGF2) and the heavy chain (protease domain). The boxed section refers to the domain interface that is depicted in more detail in the right panel. (C) Detailed view of the interface between the EGF2 domain and part of the catalytic domain as derived from PDB entry 1RFN (*17*). The backbone is represented in solid ribbon format. Residues Asn[92], Phe[98], Glu[113], and Cys[132] are part of the EGF2 domain, and the side chains thereof are depicted in black ball-and-stick format. Within the catalytic domain, the side chains of residues Cys[289], Tyr[295], Phe[299], Phe[302], and Lys[409] are colored gray. The FIXa structure further displays a potential salt bridge between residues Glu[113] and Lys[409], which is located in the proximity of the disulfide bridge between Cys[132] and Cys[289].

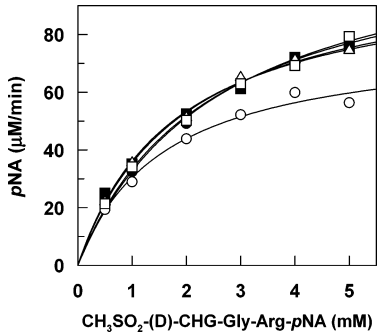

FIGURE 2: Kinetics of CH_3SO_2-D-CHG-Gly-Arg-*p*NA hydrolysis by FIXa variants. Hydrolysis of CH_3SO_2-D-CHG-Gly-Arg-*p*NA by 150 nM wt-FIXa (●), FIXa-Y295A (□), FIXa-F299A (○), FIXa-N92H (△), and FIXa-N92A (■) was assayed in the presence of various concentrations of CH_3SO_2-D-CHG-Gly-Arg-*p*NA (0.5−5 mM) as described in Materials and Methods. Data refer to experiments performed in the presence of 5 mM $CaCl_2$ and represent the mean of three independent experiments. Values for k_{cat} and apparent K_m are given in the text.

examined first by measuring the activity of FIXa variants toward the chromogenic substrate CH_3SO_2-D-CHG-Gly-Arg-*p*NA. As shown in Figure 2, the hydrolysis of CH_3SO_2-D-CHG-Gly-Arg-*p*NA by FIXa-F299A, FIXa-Y295A, FIXa-N92A, and FIXa-N92H was similar to that by wt-FIXa. In the presence of 5 mM Ca^{2+}, kinetic parameters for all mutants proved to be virtually identical to those of wt-FIXa (k_{cat} value of 13.1 ± 1.1 s^{-1} and K_m value of 2.6 ± 0.1 mM) except for FIXa-F299A which displayed a slightly lower k_{cat} value. These data show that the substitutions do not have a major effect on the ability of the mutated proteins to hydrolyze the synthetic substrate in the presence of Ca^{2+}. Moreover, amidolytic activity was also determined in the absence of Ca^{2+} ions at a critical substrate concentration (1.5 mM). Under these conditions, the amidolytic activity of all FIXa variants was approximately 3-fold lower than in the presence of Ca^{2+} (see Table 1). This suggests that all recombinant FIXa variants are similar in Ca^{2+} binding to sites involved in substrate hydrolysis.

FX Activation in the Absence of FVIIIa. To further investigate the role of the interface between the FIXa protease domain and the EGF2 domain, we examined the proteolytic activity of FIXa-F299A, FIXa-Y295A, FIXa-N92A, and FIXa-N92H variants toward the physiological substrate FX. To this end, FXa formation by these FIXa variants was assessed in the presence of phospholipids, Ca^{2+} ions, and various FX concentrations, but in the absence of FVIIIa. Surprisingly, the level of FX activation by all mutants was reduced compared to that of wt-FIXa (Figure 3). The activity of FIXa-Y295A toward FX was 38% of that of wt-FIXa. FIXa-F299A, FIXa-N92A, and FIXa-N92H variants displayed only 17, 7, and 13% of wt-FIXa activity, respectively (Table 1). Data from Figure 3 were used to derive k_{cat} values. In comparison with wt-FIXa, FIXa-Y295A displayed a k_{cat} of FX activation that was 2-fold reduced. In contrast, the k_{cat} was 4-, 7-, and 6-fold decreased for FIXa-F299A, FIXa-N92A, and FIXa-N92H, respectively. However, apparent K_m values for all mutants were similar to that of wt-FIXa (0.1−0.3 μM; see Table 1). It should be noted that under these conditions K_m predominantly represents the assembly of FX with phospholipids and, therefore, only partially reflects the affinity of FIXa for its substrate FX (*32*). These results indicate that residues Phe[299], Tyr[295], and Asn[92] play a role in the FIXa-dependent activation of FX, while residue Tyr[295] contributes to a limited extent.

Inhibition by Antithrombin. Because all FIXa mutants exhibited a decreased reactivity toward FX, it was of interest to study the interaction of the mutants with the physiological inhibitor antithrombin. To this end, inhibition experiments under slow-binding conditions were performed as described in Materials and Methods. In Figure 4, the apparent first-order rate constant (k') obtained at each antithrombin concentration is plotted versus the inhibitor concentration. For all variants, k_{ass} values were between 6×10^3 and 9×10^3 M^{-1} min^{-1} and k_{dis} values were between 1×10^{-3} and 2×10^{-3} min^{-1}. Thus, all mutants that were defective in FX activation displayed normal inhibition by the pseudosubstrate antithrombin. Moreover, complex formation with antithrombin was examined by SDS−PAGE. Both wt-FIXa and the FIXa variants formed the typical SDS-resistant complexes with antithrombin in the presence (not shown) and absence of heparin (see Figure 4, inset). Taken together, these results show that mutations at the interface between the protease domain and the EGF2 domain have a limited effect on the inhibition of FIXa by antithrombin. In contrast,

Table 1: Enzymatic Activity of FIXa Variants[a]

	amidolytic activity (μM *p*NA/min)		FX activation in the absence of FVIIIa		FX activation in the presence of FVIIIa	
	with EDTA	with Ca^{2+}	k_{cat} (min^{-1})	apparent K_m (μM)	k_{cat} (min^{-1})	apparent K_m (μM)
wt-FIXa	12.7 ± 0.6	38.1 ± 3.4	0.0255 ± 0.0007	0.11 ± 0.01	78.8 ± 2.3	0.050 ± 0.003
FIXa-F299A	8.6 ± 1.1	35.1 ± 1.5	0.0063 ± 0.0011	0.22 ± 0.09	17.3 ± 0.7	0.019 ± 0.002
FIXa-Y295A	14.4 ± 1.5	41.5 ± 1.8	0.0142 ± 0.0007	0.25 ± 0.03	39.2 ± 1.3	0.030 ± 0.002
FIXa-N92A	13.3 ± 1.0	38.1 ± 1.2	0.0034 ± 0.0001	0.35 ± 0.03	2.5 ± 0.2	ND[b]
FIXa-N92H	14.0 ± 1.0	41.7 ± 3.1	0.0043 ± 0.0005	0.22 ± 0.06	2.7 ± 0.1	ND[b]

[a] Amidolytic activity was determined at single concentration of enzyme (150 nM) and substrate CH_3SO_2-D-CHG-Gly-Arg-*p*NA (1.5 mM), in a buffer containing either EDTA (10 mM) or $CaCl_2$ (5 mM) as described in Materials and Methods. Values represent means (±standard deviation) from triplicate experiments. Kinetic parameters for FX activation in the absence of FVIIIa were derived from Figure 2. The values of k_{cat} and apparent K_m for FX activation in the presence of FVIIIa were calculated from data shown in Figure 6 and relate to FIXa and FVIIIa concentrations of 0.3 and 2.0 nM, respectively. Values represent means (±standard deviation) calculated from at least three independent experiments. [b] The apparent K_m could not be determined due to substrate inhibition.

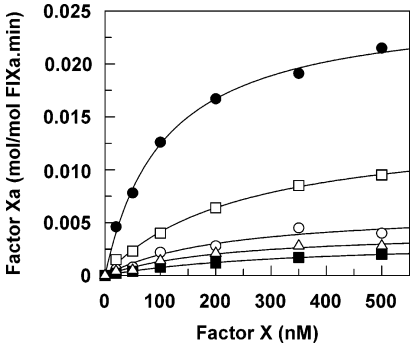

FIGURE 3: FX activation by FIXa variants in the absence of FVIIIa. FX (20−500 nM) was incubated in 5 mM $CaCl_2$, 0.2 mg/mL HSA, 100 mM NaCl, and 50 mM Tris (pH 7.4) with 30 nM wt-FIXa (●), FIXa-Y295A (□), FIXa-F299A (○), FIXa-N92H (△), and FIXa-N92A (■) in the presence of 100 μM phospholipids. FXa formation was quantified as described in Materials and Methods. Mean values of three experiments are presented.

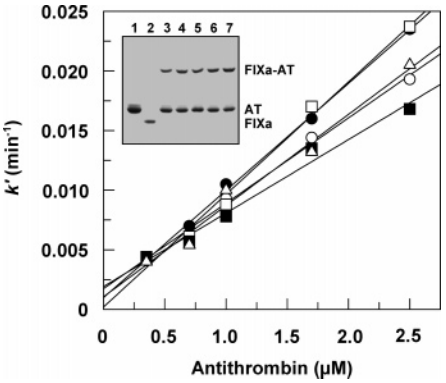

FIGURE 4: Inhibition of FIXa variants by antithrombin. Plot of k' vs the antithrombin concentration for wt-FIXa (●), FIXa-Y295A (□), FIXa-F299A (○), FIXa-N92H (△), and FIXa-N92A (■). Data from slow-binding inhibition curves were fitted to the integrated rate equation for slow binding (*30*), and values for k' were obtained for each antithrombin concentration. The slope of each line represents the k_{ass}, while k_{dis} is equal to the *y* intercept. Data represent the mean value of three experiments. The inset shows SDS−PAGE analysis under nonreducing conditions demonstrating that all mutants formed the typical SDS resistant complexes with antithrombin (AT) in the absence of heparin (see Materials and Methods): antithrombin (lane 1), wt-FIXa (lane 2), and mixtures of antithrombin with FIXa-F299A, FIXa-Y295A, FIXa-N92A, FIXa-N92H, and wt-FIXa (lanes 3−7, respectively).

however, residues Phe^{299}, Tyr^{295}, and Asn^{92} are important for proteolytic activity toward FX.

FX Activation in the Presence of FVIIIa. To further characterize these FIXa mutants, we studied FXa formation

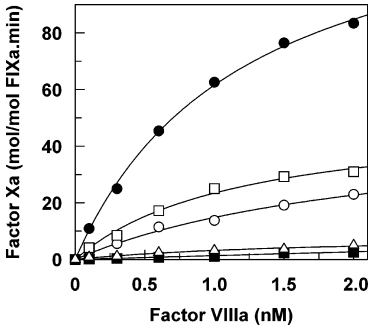

FIGURE 5: FVIIIa dependence of FXa formation by FIXa variants. FX (200 nM) was incubated with 0.1 nM wt-FIXa (●), FIXa-Y295A (□), FIXa-F299A (○), FIXa-N92H (△), and FIXa-N92A (■) in the presence of 100 μM phospholipids and various concentrations of FVIIIa (0.1−2 nM). FX activation was assessed in buffer containing 5 mM $CaCl_2$, 0.2 mg/mL HSA, 100 mM NaCl, and 50 mM Tris (pH 7.4), and FXa formation was quantified as described in Materials and Methods. Data represent the mean of at least three independent experiments.

in the presence of various concentrations of the cofactor FVIIIa. As seen in Figure 5, FXa generation by wt-FIXa was enhanced by FVIIIa in a dose-dependent and saturable manner. The rate of FX activation by all mutants was also increased by the presence of FVIIIa, but the level of FXa formation was markedly reduced compared to that of wt-FIXa (Figure 5). Extrapolation toward saturating FVIIIa concentrations indicated that the activity of FIXa-Y295A and FIXa-F299A toward FX in the presence of FVIIIa was 34 and 22% of that of wt-FIXa, respectively, whereas FIXa-N92A and FIXa-N92H displayed less than 5% of the activity of wt-FIXa.

While the data presented in Figure 5 were obtained at a single FX concentration, substrate dependence was explored at various FVIIIa concentrations (Figure 6). Experiments were performed at a single FIXa concentration (0.3 nM) and varying FVIIIa concentrations (between 0.1 and 2 nM), thus ranging from a 3-fold excess of FIXa over FVIIIa to a 7-fold excess of FVIIIa over FIXa. As shown in Figure 6A, wt-FIXa exhibited the typical substrate dependence at all FVIIIa concentrations that were tested. As expected (see also Figure 5), a tendency toward saturation with FVIIIa occurred at increasing FVIIIa concentrations (Figure 6A). The same was observed for the mutants FIXa-Y295A (Figure 6B) and FIXa-F299A (Figure 6C), although rates of FXa formation were lower than that for wt-FIXa. At the lowest FVIIIa concentration that was tested, FIXa-Y295A proved to be different from wt-FIXa and FIXa-F299A in that some substrate inhibition

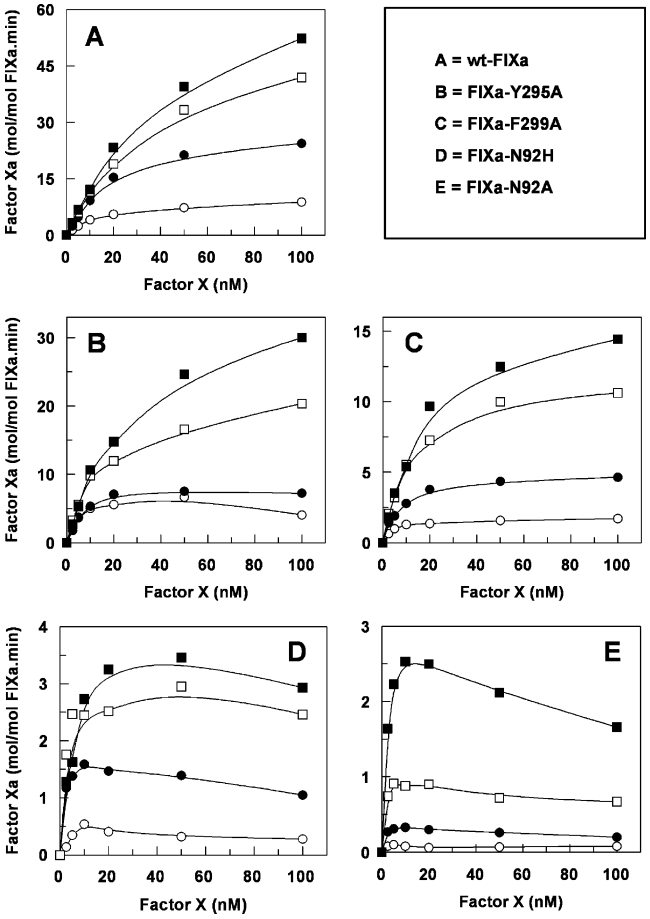

FIGURE 6: Substrate dependence of FXa formation at varying FVIIIa concentrations. FX (2.5−100 nM) was activated by FIXa (0.3 nM) in the presence of 100 μM phospholipids and varying concentrations of FVIIIa, in a buffer containing 5 mM $CaCl_2$, 0.2 mg/mL HSA, 100 mM NaCl, and 50 mM Tris (pH 7.4), and FXa formation was quantified as described in Materials and Methods. The FIXa variants were wt-FIXa (A), FIXa-Y295A (B), FIXa-F299A (C), FIXa-N92H (D), and FIXa-N92A (E). FVIIIa concentrations were 0.1 (○), 0.3 (●), 1.0 (□), and 2.0 nM (■). Data represent the mean of at least three independent experiments. Values for k_{cat} and apparent K_m are given in Table 1.

became apparent [Figure 6B (○)]. However, inhibition by an excess of substrate was much more pronounced for the substitution variants FIXa-N92H (Figure 6D) and FIXa-N92A (Figure 6E) and occurred at all FVIIIa concentrations that were tested. It was further striking that the variant FIXa-N92A differed from all other mutants in that saturation with FVIIIa did not occur, suggesting that the affinity for FVIIIa was significantly reduced. The data from Figure 6 were further used to calculate the k_{cat} and apparent K_m at 2 nM FVIIIa. These values are listed in Table 1. Comparison with k_{cat} values obtained in the absence of FVIIIa demonstrates that, while wt-FIXa is stimulated by FVIIIa by 3000-fold, the most defective variants, FIXa-N92H and FIXa-N92A, still are stimulated by some 600−700-fold. This demonstrates that the substitution of Asn^{92} reduces but does not abolish the response of FIXa to FVIIIa. The variant FIXa-F299A displayed FVIIIa stimulation similar to that of wt-FIXa, whereas FIXa-Y295A exhibited 5000-fold stimulation (Table 1). In the latter mutant, FVIIIa thus partially corrects the defect of FIXa-Y295A that is apparent in the absence of FVIIIa. These findings suggest that residues Asn^{92} and, to a lesser extent, Tyr^{295} contribute to assembly of the FX-activating complex, or to the activity thereof, once formed.

FX Activation in the Absence of Phospholipids. To distinguish between a perturbation of FIXa binding sites for FVIIIa and/or FX and an improper positioning of the FIXa protease domain above the phospholipid surface due to the mutations, we studied the effect of FVIIIa on FX activation in the absence of phospholipids. All FIXa mutants exhibited a lower level of FXa generation than wt-FIXa, with FIXa-N92A and FIXa-N92H showing again the most prominent defect. The very low FXa generation rate did not allow the determination of precise kinetic parameters. Under conditions described in Materials and Methods, rates of FX activation were 0.83, 0.20, 0.31, 0.04, and 0.05 nM/min for wt-FIXa, FIXa-F299A, FIXa-Y295A, FIXa-N92A, and FIXa-N92H, respectively (data not shown). These results are similar to those described above for FXa formation in the presence of phospholipids and FVIIIa. These data suggest that mutations at the interface between the FIXa protease domain and the EGF2 domain lead to a complex assembly defect which is largely phospholipid-independent.

Association of FIXa Variants with the Isolated FVIII A2 Domain or FVIII Light Chain. A defective complex assembly with the FVIII A2 domain and/or FVIII light chain might contribute to the lack of a response to FVIIIa seen for some of the FIXa variants. The association of the FIXa variants with the isolated FVIII A2 domain or FVIII light chain was monitored by employing surface plasmon resonance technology. Various concentrations of FIXa variants were incubated with the FVIII A2 domain coupled to a CM5 sensor chip. Individual association and dissociation curves displayed complex kinetics that precluded the calculation of rate constants using a homogeneous one-site model (data not shown). Therefore, data were expressed in terms of association relative to wt-FIXa. Consistent with our previous observations (*19*), wt-FIXa assembled efficiently with the isolated A2 domain whereas nonactivated FIX displayed only minor association with A2 domain. As seen in Figure 7A, all FIXa variants did assemble with the FVIII A2 domain, with FIXa-F299A being even more efficient than wt-FIXa. In contrast, FIXa-N92A and FIXa-N92H associated with the A2 domain somewhat less efficiently than wt-FIXa and FIXa-Y295A (Figure 7A). The stronger association of the FIXa-F299A mutant with the A2 domain might explain the partial restoration of the proteolytic activity by FVIIIa (Figure 7A). For the interaction between FIXa variants and the FVIII light chain, FIXa variants were immobilized onto a CM5 sensor chip and the FVIII light chain was passed over it at various concentrations. In agreement with our previous results (*20*), nonactivated FIX bound to the FVIII light chain to a lower extent than wt-FIXa (Figure 7B). All mutants displayed clearly impaired FVIII light chain assembly compared to wt-FIXa in particular mutants with substitution at position 92 or 299 (Figure 7B). Altogether, these results indicate that the weakened response to FVIIIa for FIXa-N92A and FIXa-N92H might be due to a combined defect in FVIII A2 domain and FVIII light chain binding.

DISCUSSION

The catalytic domain of serine proteases is a structural motif that occurs in a variety of biological systems, including the coagulation pathway. Blood coagulation proteases differ

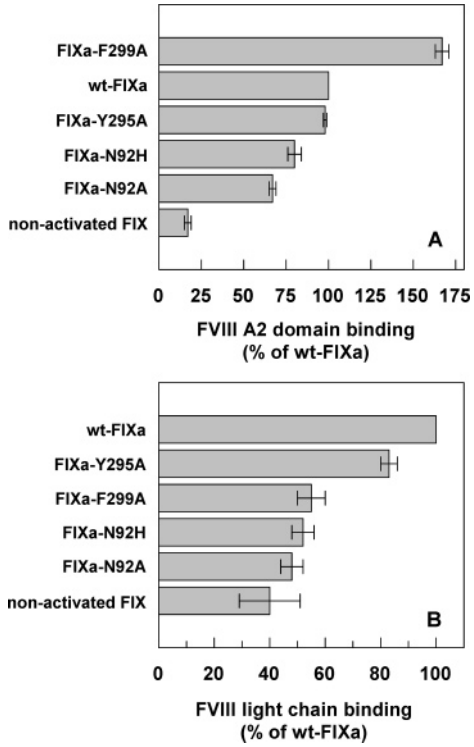

FIGURE 7: Binding of FIXa variants to the isolated FVIII A2 domain or FVIII light chain. (A) FIXa variants (50−400 nM) were passed over the immobilized A2 domain on a CM5 sensor chip at a density of 178 fmol/mm^2. (B) Various concentrations of the FVIII light chain (10−100 nM) were incubated with FIXa variants coupled to a CM5 sensor chip at a density of 153 fmol/mm^2. In both cases, the experiments were performed employing a BIAcore 3000 biosensor system as described in Materials and Methods. Association was followed for 120 s, and the amount of FIXa variants or FVIII light chain bound was expressed as the percentage of wt-FIXa (100%). The data are based on at least five different ligand concentrations. Mean values ± the standard deviation are presented.

from the simple digestive proteases by the fact that they have large noncatalytic segments attached to the amino-terminal end of the serine protease domain (*3*). During evolution, the noncatalytic regions have been acquired by assembly with various modules that originate from autonomous miniproteins (*33*). Among the coagulation enzymes, four members share the same domain arrangement (Gla−EGF1−EGF2−serine protease), i.e., factors VII, IX, and X and protein C (*34*). Individual domains serve distinct functions, which they exert in the various proteins of which they are constituents. For instance, the Gla domain invariably introduces Ca^{2+} and lipid binding, while EGF domains generally contribute to protein−protein interaction (*35*−*37*). For FIX, evidence that the catalytic domain does not function autonomously but also involves some contribution from the noncatalytic modules, in particular the EGF domains (*11*, *12*, *15*), has been obtained.

Our data demonstrate that putative contacts between EGF2 and the protease domain of FIXa have a major impact on its catalytic activity. For all mutants, the level of FX activation was markedly reduced in the absence of FVIIIa (Figure 3 and Table 1), suggesting a FVIIIa-independent FX activation defect. This seems to be specific for the substrate FX, because the same amino acid substitutions do not affect interaction with a small synthetic substrate (Figure 2) or with the macromolecular inhibitor antithrombin (Figure 4). One possibility is that substitutions at the interface between EGF2 and the protease domain affect assembly with FX at a site other than its activation peptide region within the FIXa substrate binding cleft. Alternatively, FX activation may involve intramolecular signaling from the FIXa light chain toward the catalytic center. Crystallographic studies using a truncated human FIX molecule consisting of EGF2 and the catalytic domain have identified multiple contacts between these domains (*17*). Also, in porcine FIX, these domains seem to represent a single structural unit due to extensive noncovalent interactions (*38*). The functional role of these putative contacts, however, can only be established in functional studies, using the complete FIXa protein, including the Gla and EGF1 domains, and its physiological substrate and cofactors. This is the aim of this paper.

As outlined in Figure 1, we hypothesized that the hydrogen bond between Asn^{92} and Tyr^{295}, as well as some hydrophobic contacts between Phe^{98} and residues Tyr^{295} and Phe^{299}, may link the EGF2 domain to the FIXa catalytic domain (*19*). If this really contributes to interdomain cross-talk, one would expect that mutations at the contact residues would affect biological activity. In this respect, it seems surprising that the effect of substitution of residues Tyr^{295} or Phe^{299} is relatively minor (Table 1 and Figures 5 and 6). Both Tyr^{295} and Phe^{299} may support a prominent hydrophobic contact with Phe^{98} in the EGF2 domain (Figure 1C). It seems conceivable that substitution of either Tyr^{295} or Phe^{299} alone is insufficient to disrupt the hydrophobic interaction with Phe^{98} with the hydrophobic pocket in the protease domain (Figure 1C). Moreover, the contact with the EGF2 domain may also involve additional residues such as Phe^{302} in the same hydrophobic pocket. The fact that Phe^{302} is part of the putative FVIIIa binding region of residues 301−303 (*39*) might explain why the EGF2−catalytic domain interface contributes to assembly with FVIIIa (Figures 1 and 7). As for Asn^{92}, it is striking that its substitution results in a functional defect that is particularly complex. Reduced activity in the absence of FVIIIa (Figure 3) implies a weakened interaction with FX, whereas defective activity in the presence of FVIIIa (Figures 5 and 6) seems compatible with a reduced level of FVIIIa binding. Indeed, the dysfunctional variants FIXa-N92H and FIXa-N92A exhibited weakened assembly with the isolated FVIII A2 domain and with the FVIII light chain (Figure 7A,B).

Interestingly, the N92H replacement in FIX occurs in nature and is known as the FIX Fukuoka variant. Kinetic studies showed that the catalytic efficiency of FIXa Fukuoka was 50-fold reduced compared to that of normal FIXa and that the enhancement of its activity by FVIIIa was reduced (*31*). This is consistent with our results which show that the FIXa-N92H variant displays reduced activity in the presence of FVIIIa and is stimulated by FVIIIa to a lower extent than wt-FIXa (Table 1 and Figure 5). From their kinetic data, Nishimura et al. (*31*) could not distinguish between the FIXa-N92H variant being defective in FVIIIa or FX interaction. Our data, however, demonstrate that mutants with a substitution at position 92 display a combined defect in FX and FVIII recognition (Figures 3, 5, and 7).

With regard to the FIXa-N92A variant, this substitution has been described as being associated with a total lack of FVIIIa-dependent rate enhancement (*13*). This is in contrast with our findings, which show that the N92A and N92H

replacements are similar in this respect (Table 1 and Figure 5). The reason for this discrepancy remains unclear. It seems very possible that experimental conditions contribute to the different findings, in particular because of our observation that the FIXa-N92H and FIXa-N92A variants exhibit prominent substrate inhibition in the presence of FVIIIa and physiological concentrations of FX (Figure 6D,E). This may easily lead to an underestimation of the contribution of FVIIIa to FXa formation. It has been previously described for wt-FIXa that substrate inhibition can occur due to displacement of FIXa from the phospholipid membrane by FX, in particular when the membrane surface is rate-limiting (*8*). Such substrate inhibition has also been observed in other studies (*40*−*42*) and may contribute to variability between kinetic studies. The question of why substitution of Asn^{92} makes FIXa so liable to substrate inhibition remains. We previously suggested that FVIIIa normally protects FIXa from displacement by excess of FX from the lipid membrane (*9*). Because of impaired assembly with FVIII (Figure 7), this mechanism might be less effective for the variants FIXa-N92H and FIXa-N92A. Alternatively, substrate inhibition may be explained by a classical model in which two substrate molecules bind to distinct sites on the same enzyme molecule (*43*). It has been established that, apart from the enzyme FIXa, also the cofactor FVIIIa can independently bind FX (*23*). Normally, the formation of the ternary FVIIIa·FIXa·FX complex may proceed via FX binding to the preformed binary FVIIIa·FIXa complex, or via binding of FIXa to the FVIIIa·FX complex (*44*). It seems possible that in FIXa-N92H and FIXa-N92A, defective interaction with both FVIIIa and FX introduces some misalignment of the extended FX binding exosites in the FVIIIa·FIXa complex. At high substrate concentrations, this might lead to FX binding to both the FIXa and the FVIIIa moiety of the FVIIIa·FIXa complex, which would lead to nonproductive substrate binding. Whether this explains the inhibition seen in panels D and E of Figure 6 remains to be investigated.

Apart from the contacts between EGF2 and the protease domain, other interdomain interfaces play a role in FIXa biological activity. The salt bridge between Glu^{78} and Arg^{94} has been clearly identified in the crystal structure of porcine FIXa as a link between the two EGF domains (*16*). Mutagenesis studies provided evidence that this salt bridge maintains the integrity of the FVIII light chain binding site on the FIXa molecule and therefore plays a role in FIXa function in solution (*18*, *45*). However, the role of this salt bridge, and the role of residue Arg^{94} in particular, has been questioned in other studies (*14*, *46*). The same interdomain contact also contributes to assembly with the isolated FVIII A2 domain (*19*), which is thought to involve the FIXa catalytic domain (*9*, *10*, *47*). Moreover, FVIII A2 domain binding also involves the hydrophobic contact between the two EGF domains (*19*). Finally, binding of Ca^{2+} to the EGF1 domain contributes to a variety of FIXa functions, including enzymatic activity and assembly with FVIIIa (*11*). A similar finding has been reported for activated protein C, in which residue Asp^{71} in the Ca^{2+}-binding site in the EGF1 domain contributes to enzymatic activity toward physiological substrates (*48*). In this regard, it is of interest that the close alignment of EGF2 and the catalytic domain is not limited to FIXa but also has been observed in related hemostatic proteins (*38*). Moreover, FIXa residue Asn^{92} is also conserved in factor VIIa, activated protein C, and FXa. It therefore seems conceivable that the catalytic properties of other serine proteases are also controlled by cross-talk with their noncatalytic domain constituents.

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