Hydrophobic Contact between the Two Epidermal Growth Factor-like Domains of Blood Coagulation Factor IX Contributes to Enzymatic Activity*

Patrick H. N. Celie‡, Peter J. Lenting‡, and Koen Mertens‡§

From the ‡Department of Plasma Protein Technology, CLB, 1066 CX Amsterdam, The Netherlands and the §Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, 3584 CA Utrecht, The Netherlands

The three-dimensional structure of activated factor IX comprises multiple contacts between the two epidermal growth factor (EGF)-like domains. One of these is a salt bridge between Glu78 and Arg104, which is essential for binding of factor IXa to its cofactor factor VIII and for factor VIII-dependent factor X activation (Christophe, O. D., Lenting, P. J., Kolkman, J. A., Brownlee, G. G., and Mertens, K. (1998) J. Biol. Chem. 273, 2222–2227). We now addressed the putative hydrophobic contact at the interface between the EGF-like domains. Recombinant factor IX chimeras were constructed in which hydrophobic regions Phe75-Phe77 and Lys106-Val108 were replaced by the corresponding sites of factor X and factor VII. Activated factor IX/factor X chimeras were distinguishable from normal factor IXa with respect to factor IXa enzymatic activity. In contrast, factor IXa75–77/Factor VII displayed 2-fold increased factor X activation in the presence of factor VIII, suggesting that residues 75–77 contribute to cofactor-dependent factor X activation. Activation of factor X by factor IX106–108/factor VII was strongly decreased, both in the absence and presence of factor VIII. Activity could be restored by simultaneous substitution of the hydrophobic sites in both EGF-like domains for factor VII residues. These data suggest that factor IXa enzymatic activity requires hydrophobic contact between the two EGF-like domains.

Factor IX (FIX)1 is a serine protease precursor that participates in the process of blood coagulation (1, 2). The FIX proenzyme is synthesized in the liver and expressed as a single chain protein (3). FIX is converted into the active enzyme factor IXa (FIXa) by proteolytic cleavage by factor XIa (FXIa) and a factor VIIa-tissue factor complex (1, 4, 5). Cleavage of FIX occurs at two distinct sites resulting in the release of the activation peptide and formation of the 45-kDa enzyme FIXa. FIXa is composed of an N-terminal light chain and a C-terminal heavy chain. The light chain consists of the Gla domain, which is enriched in γ-carboxyglutamic acid residues, followed by a short hydrophobic stack and two epidermal growth factor (EGF)-like domains (denoted as EGF1 and EGF2 domain). The heavy chain comprises the protease domain with the catalytic center (3).

FIXa functions as activator of thezymogen factor X (FX). Activation of FX by FIXa is enhanced by several orders of magnitude in the presence of phospholipid membrane, Ca2+ ions, and the cofactor, activated factor VIII (FVIIIa) (2, 6, 7). Factor VIII (FVIII) is a heterodimer consisting of a light chain and a heavy chain. FVIII is activated upon cleavage of the heavy and light chain by thrombin or activated FX (FXa) (8). The activated forms of FVIII and FIX are known to assemble into the FX-activating complex (for review see Refs. 9–11). In the FIXa protease domain, regions 301–303 and 333–339 have been identified as potential binding sites for FVIIIa (12, 13). Most likely, these sites are involved in the binding of the FVIII heavy chain (10).

The crystal structure of porcine FIXa revealed that the relative orientation of the EGF-like domains is fixed by a variety of contacts between residues located at the interface between the EGF1 and EGF2 domain (14). The EGF-like domains define an angle of 110° (14), and this particular orientation may be of importance for FIXa function. This view is supported by the observation that disruption of a salt bridge between Glu78 in the EGF1 domain and Arg104 in the EGF2 domain is associated with strongly reduced FVIIIa-dependent FX activation by FIXa and decreased affinity of FIXa for FVIII light chain (15). These results provide support to the idea that the specific orientation of both EGF-like domains is a prerequisite for proper interaction between FIXa and FVIIIa and that residues near Glu78 and Arg104 may be involved in a direct interaction with FVIIIa. The structure of the interface between the EGF-like domains of porcine FIXa is further characterized by the presence of an exposed hydrophobic site in the EGF1 domain, comprising residues Val75–Phe77, that is captured in a hydrophobic pocket formed by multiple residues, including Val107 and Cys109 in the EGF2 domain (14).

In this study, we focused on the functional role of the regions Phe75–Gly76–Phe77 and Lys106–Val107–Val108 in human FIX. In our approach, we constructed recombinant FIX mutants in which these regions were replaced for corresponding regions of homologous serine proteases FX and FVII. Our results demonstrate that substitution of the FIX residues predominantly affects FIXa enzymatic activity and the extent of stimulation by its natural cofactor, FVIIIa. Our data support a model in which residues 75, 76, 107, and 108 form a hydrophobic contact between the EGF1 and EGF2 domain that is essential for FIXa function.

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‡To whom correspondence should be addressed: Dept. of Plasma Protein Technology, CLB Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands. Tel.: 31-20-5123120; Fax: 31-20-5123680; E-mail: kmertens@clb.nl.

¶The abbreviations used are: FIX, factor IX; FIXa, activated FIX; CH₂SO₂-LGR-pNA, CH₂SO₂-i-leucyl-i-glycyl-l-arginyl-p-nitroaniline; EGF, epidermal growth factor; FVIII, factor VIII; FVIIIa, activated FVIII; wt-FIX, recombinant wild-type factor IX; FX, factor X; FXa, activated FX; FXIa, activated factor XI.
EXPERIMENTAL PROCEDURES

Materials—CNBr-activated-Sepharose CL-4B, protein A-Sepharose CL-4B, CM-Sepharose, and Q-Sepharose FF were from Amersham Pharmacia Biotech. Pefachrome Xa and r-Hirudin were obtained from Pentapharm AG (Basel, Switzerland). Ovalbumin was obtained from NBS Biologicals (Huntingdon, UK). Oligonucleotides were purchased from Amersham Pharmacia Biotech. t-γ-phosphatidyl-l-serine, l-γ-phosphatidylcholine, type I-EH, heparin grade I-A, and vitamin K1 were obtained from Amersham Pharmacia Biotech. Purified antithrombin III was purchased from Clotingard. DNA polymerase was purchased from Stratagene (Cambridge, UK). Culture flasks and cell factories (6320 cm²) were from Nunc (Roskilde, Denmark). Microtiter plates (Immulon) were purchased from Dynatech (Ploetzingen, Germany) unless stated otherwise. CH₃SO₂-LGR-pNA, product name CBS 31,39, was obtained from Diagnostica Stago (Asnières, France). The Thermo Sequenase cycle sequencing kit was obtained from Amersham Pharmacia Biotech. Purified antithrombin and human albumin serum were obtained from the Division of Products of CLB (Amsterdam, The Netherlands).

Construction of Recombinant FIX—The plasmid encoding wild-type FIX (wt-FIX) has been described previously (16). Site-directed mutagenesis was performed using the plasmid encoding wt-FIX as a template to construct plasmids coding for FIX75–77/FX, FIX106–108/FX, FIX75–77/FXII, FIX75–77/FXIII, and FIX75–77/FXIV. These plasmids were used to obtain CHO cell factories (6320 cm²). Medium (Dulbecco’s modified Eagle’s medium, 0.5% penicillin, 0.5% streptomycin, 0.5% fungizone (amphotericin B/desoxycycline), penicillin-streptomycin, Gentamicin G-418 sulfate, and Dulbecco’s modified Eagle’s medium were from Life Technologies Inc. Ffu polymerase was purchased from Stratagene. Cells were transfected with plasmid DNA employing the calcium phosphate precipitation method as outlined previously (16) and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, 100 μg/ml penicillin, and 100 μg/ml hydrocortisone. After 2 weeks, individual clones were isolated and grown in selective medium to obtain stable cell lines. Lineage of FIX was monitored by measuring FIX activity in conditioned medium by immunoaffinity chromatography.

Proteins—Monoclonal antibody CLB-FIX 14 and polyclonal antibodies against FIX have been described previously (18). Antibodies were purified employing protein A-Sepharose as recommended by the manufacturer. FIX, FX, and thrombin were purified from a concentrate of human prothrombin, FIX, and FX (7, 19) and converted into their active forms (FIXa, FXa, and thrombin, respectively) as described (19, 20). Human FIX was purified as described previously (20). FIX was activated by thrombin (molar ratio 30:1) for 10 min, and activated FIXa was obtained from conditioned medium by immunoaffinity chromatography.

Expression and Culturing of FIX Variants—Recombinant FIX was expressed in Madin-Darby canine kidney cells as described previously (15). Cells were transfected with plasmid DNA employing the calcium precipitation method as outlined previously (16) and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, 100 μg/ml penicillin, and 100 μg/ml hydrocortisone. After 2 weeks, individual clones were isolated and grown in selective medium to obtain stable cell lines. A large scale production of FIX in cell factories (6320 cm²). Medium (Dulbecco’s modified Eagle’s medium, supplemented with 2.5% fetal calf serum, 100 units/ml penicillin, 5 μg/ml vitamin K1, 100 μg/ml streptomycin, 1 μg/ml amphotericin B, and 0.8 μg/ml desoxocholate containing secreted FIX was harvested and stored at −20°C.

Purification of Recombinant FIX Variants—Culture medium was filtered through a 0.22 μm membrane (Biofuge, Bad Homburg, Germany) to remove cell debris. wt-FIX and FIX mutants were purified by immunoaffinity chromatography using monoclonal antibody CLB-FIX 14 according to an established procedure (12, 15, 23). After purification, one single FIX band for all FIX variants was present after SDS-polyacrylamide gel electrophoresis followed by Coomassie Brilliant Blue staining. FIX mutants displayed the same electrophoretic mobility, as purified plasmid FIX. In contrast, FIX containing the propeptide-containing unprocessed FIX was present. Recombinant FIX produced by this expression system is indistinguishable from plasmid-derived FIX with regard to binding to barium citrate and to the Ca²⁺-dependent monoclonal antibody CLB-FIX 2 (23). Furthermore, Ca²⁺-dependent activity of wt-FIXα toward a synthetic substrate and toward FIX is similar to that of plasmid-derived factor IXα (15, 16, 23). FIX was converted into FIXα by incubation of 1.4 μM FIX with 7 nM FIXα in 100 mm NaCl, 5 mm CaCl₂, and 50 mM Tris (pH 7.4) for 2 h. Activation was stopped by the addition of EDTA (10 mM) and benzamidine (10 mM). FIXa was loaded on Q-Sepharose and washed with 150 mM NaCl, 5 mM benzamidine, and 50 mM Tris (pH 7.4) to remove FIXa. FIXα was eluted from the column by the addition of 500 mM NaCl, 5 mM benzamidine, and 50 mM Tris (pH 7.4). FIXα was precipitated in 20% glycerol, 50 mM Tris (pH 7.4), and subsequently against the same buffer containing 50% glycerol. Final FIXα preparations were stored at −20°C.

Protein Concentrations—The amount of protein was quantified by the method of Bradford (24) using human serum albumin as a standard. FVIIIa activity was assayed by a spectrophotometric assay employing the amidation reaction factors FIX and FVII inactivated by heparin-Sepharose CL-4B. FVIII assay was performed using a molar extinction coefficient of 9.65 × 10³ M⁻¹ cm⁻¹ for p-nitroanilide and a path length of 0.25 cm for a 100-μl volume.

FX Activation—In the absence of FVIIIa, FX activation was assayed as described previously (25). Briefly, 50 μl of a 5 mM solution of CH3SO2-LGR-pNA was added to 50 μl of a solution containing 300 mM FIXa and various concentrations of CaCl₂ in a microtiter plate (Corning Costar, Badhoevedorp, The Netherlands). Initial rates of substrate hydrolysis were measured by monitoring the absorbance at 405 nm in time. Absorbance values were converted into molar concentrations using a molar extinction coefficient of 9.65 × 10³ M⁻¹ cm⁻¹ for p-nitroanilide and a path length of 0.25 cm for a 100-μl volume.

RESULTS

Recombinant FIX Variants—In the present study, we have investigated the functional role of hydrophobic residues that are located at the interface between the EGF1 and EGF2 domain in the FIXa light chain. To this end, recombinant FIX variants were constructed in which residues were replaced by corresponding residues of FX and FVII. Substitutions were based upon alignment of the human FIX and human FVII sequences and the position of the hydrophobic residues in a three-dimensional structure of human FIX based on the crystal structure of porcine FIX (14) (Fig. 1). This analysis revealed that a homologous domain and preparations specific for FIXa were hydrophobic residues Val197, Val198, and Leu198 in the EGF2 domain. The FIX chimeras were constructed in which the separate sites in the EGF1 domain and the EGF2 domain were replaced by corresponding residues of FIX, whereas a third chimera comprised a combination of these replacements. The same procedure was used for construction of FIX/FVII chimeras. In addition to these chimeric variants, one FIX variant was expressed in Madin-Darby canine kidney cells and purified from conditioned medium by immunoaffinity chromatography.
Substitution of residues Phe 75, Gly 76, and Lys 106 for corresponding domains in human FIXa. The structure is based on the crystal structure of porcine FIXa (14). Porcine FIX residues Val75 and Leu108 were replaced by human FIX residues Phe75 and Val108, and the human structure was obtained by molecular modeling employing the Swiss-Model Automated Comparative Protein Modeling Server (26, 27). Part of the EGF1 domain is shown at the bottom right of the figure and is followed by the EGF2 domain. The backbone of FIXa is represented in ribbon format. Lys106, Val107, and Val108 in the EGF2 domain are indistinguishable from plasma-derived FIXa with respect to high affinity Ca2+ ions, but FIXa, FX, and various concentrations of FVIIIa. For wt-FIXa, FX activation was enhanced by FVIIIa in a dose-dependent manner (Fig. 3A). FIXa/FX substitution variants were identical to wt-FIXa (Fig. 3B). In contrast, FIXa/FVII chimeras and FIXa-Phe77 → Ser displayed different cofactor-dependent FX activation. One chimera, FIXa75–77/FVII, displayed an increased rate of FX activation compared with wt-FIXa (Fig. 2). Reduced activity was observed for FIXa-Phe77 → Ser and the other two FIXa/FVII chimeras. Like in the absence of FVIIIa (Fig. 2), FIXa75–77/FVII displayed about 2-fold increased activity compared with wt-FIXa (Fig. 3A). The elevation in apparent affinity for FVIIIa. In agreement with the data shown in Fig. 4, Arg and Val108 → Ser in the EGF2 domain is compensated for by simultaneous substitution of Phe75 → Pro and Gly76 → Ala in the EGF1 domain.

FX Activation in the Presence of FVIIIa — The contribution of FVIIIa as cofactor for FIXa to the activation of FX was assessed in a kinetic system containing phospholipids, Ca2+ ions, FIXa, and FX, and various concentrations of FVIIIa. For wt-FIXa, FX activation was enhanced by FVIIIa in a dose-dependent manner (Fig. 3A). FIXa/FX substitution variants were identical to wt-FIXa (Fig. 3B). In contrast, FIXa/FVII chimeras and FIXa-Phe77 → Ser displayed different cofactor-dependent FX activation. One chimera, FIXa75–77/FVII, displayed an increased rate of FX activation compared with wt-FIXa (Fig. 2). Reduced activity was observed for FIXa-Phe77 → Ser and the other two FIXa/FVII chimeras. Like in the absence of FVIIIa (Fig. 2), FIXa75–77/FVII displayed about 2-fold increased activity compared with wt-FIXa (Fig. 3A). The elevation in apparent affinity for FVIIIa. In agreement with the data shown in Fig. 4, Arg and Val108 → Ser in the EGF2 domain is compensated for by simultaneous substitution of Phe75 → Pro and Gly76 → Ala in the EGF1 domain.

To examine catalytic parameters of FX activation in the presence of FVIIIa, various concentrations of FX were incubated with FIXa variants in the presence of phospholipids and Ca2+ ions but in the absence of FVIIIa. Most FIXa chimeras displayed normal (FIXa/FX chimeras, FIXa75–77/FVII) or slightly reduced (FIXa-Phe77 → Ser) FX activation (Fig. 2 and Table I). Apparently, substitution of residues Phe75, Gly76, and Lys106 for corresponding residues in FX and FVII does not affect FXa enzymatic activity toward FX, whereas substitution of Phe77 → Ser has only a minor effect. In contrast, FX activation by FIXa106–108/FVII was strongly reduced. This was mainly because of a decrease in the apparent catalytic rate constant ($k_{cat}$) resulting in a 10-fold reduction in the apparent catalytic efficiency ($k_{cat}/K_m$) compared with that of wt-FIXa (Table I). In view of the severe defect of FIXa106–108/FX, it is remarkable that activation of FX by FIXa75–77, 106–108/FX proved only a minor reduction compared with normal FIXa (Fig. 2 and Table I). Apparently, the deleterious effect caused by substitution of Val107 → Arg and Val108 → Ser in the EGF2 domain is compensated for by simultaneous substitution of Phe75 → Pro and Gly76 → Ala in the EGF1 domain.

FX Activation in the Absence of Phospholipids — Recent data suggest that some FIXa variants with mutations in the EGF1 domain display strongly reduced FVIII-dependent FX activa-
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TABLE I

| FIXa variant     | Fixa activation – FVIIa | Fixa activation + FVIIa |
|------------------|-------------------------|-------------------------|
|                  | $K_m, app$              | $k_{cat, app}$ | $k_{cat, app}/K_m$ | $K_m$  | $k_{cat, app}$ | $k_{cat, app}/K_m$ |
|                  | $\mu M$                 | $s^{-1}$          | $s^{-1}$          | $\mu M$ | $s^{-1}$          | $s^{-1}$          |
| wt-FIXa          | 0.17 ± 0.03             | 7.1 ± 0.5         | 4.2 ± 0.8         | 29.2 ± 2.2 | 6.0 ± 0.2 | 2.1 ± 0.2         |
| FIXa75–77/FVII   | 0.14 ± 0.04             | 6.6 ± 0.6         | 4.6 ± 1.4         | 40.5 ± 6.7 | 12.0 ± 0.9 | 3.0 ± 0.5         |
| FIXa106–108/FVII| 0.29 ± 0.04             | 1.2 ± 0.1         | 0.4 ± 0.1         | 15.1 ± 2.7 | 0.8 ± 0.1 | 0.5 ± 0.1         |
| FIXa75–77, 106–108/FVII | 0.23 ± 0.04 | 6.4 ± 0.5 | 2.8 ± 0.5 | 17.0 ± 2.3 | 3.3 ± 0.1 | 1.9 ± 0.4 | 
| FIXa-Phe77→Ser  | 0.17 ± 0.04             | 7.2 ± 0.7         | 4.2 ± 1.1         | nd        | nd        | nd                |
| FIXa75–77, 106–108/FX | 0.19 ± 0.05 | 7.1 ± 0.9 | 3.7 ± 1.1 | nd        | nd        | nd                |
| FIXa-Phe77→Ser  | 0.16 ± 0.05             | 7.4 ± 0.9         | 4.6 ± 1.5         | nd        | nd        | nd                |
| FIXa-Phe77→Ser  | 0.24 ± 0.04             | 5.4 ± 0.4         | 2.3 ± 0.1         | 22.9 ± 5.1 | 4.0 ± 0.3 | 1.8 ± 0.3         |

FIG. 3. FX activation by Fixa/FVII and Fixa/FX chimeras in the presence of FVIIa. A, FX (0.2 μM) was activated by 0.1 nM of wt-FIXa (open circles), Fixa75–77/FVII (closed circles), Fixa106–108/FVII (closed squares), Fixa75–77, 106–108/FVII (closed triangles), and Fixa-Phe77→Ser (open triangles), in the presence of FVIIa (0–1.5 nM), 0.1 mM phospholipids, and 5 mM CaCl2 as described under “Experimental Procedures.” B, activation of FX by wt-FIXa (open circles), Fixa75–77/FX (closed circles), Fixa106–108/FX (open triangles), and Fixa75–77, 106–108/FX (closed triangles). Rates of FX activation were calculated from at least three measurements within the initial 5 min of activation. For each Fixa variant, data were obtained from two to four individual experiments. Apparent $K_a$ values were determined by fitting the data in the one-site ligand binding equation employing the GrafFit Data Analysis and Graphics Program (Erithacus Software Ltd, Middlesex, UK). Calculated apparent $K_a$ values (± S.D.) for the interaction between Fixa and FVIIa were 1.7 (± 0.3), 0.6 (± 0.1) and 2.5 (± 0.3) nM for wt-FIXa, Fixa75–77/FVII, and Fixa106–108/FVII, respectively. For Fixa-Phe77→Ser and Fixa106–108/FX, no valid apparent $K_a$ values could be derived from these data. Calculated apparent $K_a$ values for Fixa75–77/FX, Fixa106–108/FX, and Fixa75–77, 106–108/FX were within the range of 1.3–2.4 nM.

FIG. 4. Substrate dependence of FX activation by Fixa chimeras in the presence of FVIIa. FX (0–100 nM) was incubated with 0.1 nM of wt-FIXa (open circles), Fixa75–77/FVII (closed circles), Fixa106–108/FVII (closed squares), Fixa75–77, 106–108/FVII (closed triangles), and Fixa-Phe77→Ser (open triangles) in the presence of 0.35 mM FVIIIa, 0.1 mM phospholipids, 5 mM CaCl2, 100 mM NaCl, 0.2 mg/ml ovalbumin, and 50 mM Tris (pH 7.4). At various time points aliquots were removed, and the amount of FXa generated was quantified as described under “Experimental Procedures.” Rates of FX activation were calculated from at least three measurements within the initial 5 min of activation. Data represent the mean of two individual experiments.

The serine proteases of the blood coagulation process display a high level of structural similarity (2, 29). Most of these enzymes comprise a protease domain containing the specific Ser/Asp/His catalytic triad and a light chain containing two or more EGF-like domains. EGF-like domains have been demonstrated to be important for a number of functional properties, including Ca2+ binding and protein-protein interactions (30–32). Structural data derived from NMR and crystallographic studies have shed light on the three-dimensional structure of these domains and the orientation thereof in the complete protein (14, 31–35). The crystal structure of porcine FIXa revealed that multiple interdomain contacts exist between the concentrations of FX, FVIIIa, and FIXa were employed. Under these conditions, Fixa75–77/FVII stimulated FX activation to a higher extent compared with wt-FIXa (Fig. 5). The calculated apparent $K_a$ for the interaction between FVIIa and Fixa75–77/FVII was 2-fold lower than the apparent $K_a$ for the interaction between wt-FIXa. Fixa-Phe77→Ser, Fixa106–108/FVII, and Fixa75–77, 106–108/FVII displayed less effective stimulation by FVIIIa. For all Fixa variants the extent of FX activation compared with wt-FIXa was similar to that observed in the presence of phospholipids. These results demonstrate that alterations in Fixa variants are because of modified enzymatic properties and not to differences in phospholipid-dependent enzyme-cofactor or enzyme-substrate complex assembly.

**DISCUSSION**

The serine proteases of the blood coagulation process display a high level of structural similarity (2, 29). Most of these enzymes comprise a protease domain containing the specific Ser/Asp/His catalytic triad and a light chain containing two or more EGF-like domains. EGF-like domains have been demonstrated to be important for a number of functional properties, including Ca2+ binding and protein-protein interactions (30–32). Structural data derived from NMR and crystallographic studies have shed light on the three-dimensional structure of these domains and the orientation thereof in the complete protein (14, 31–35). The crystal structure of porcine FIXa revealed that multiple interdomain contacts exist between the
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Fig. 5. FX activation by FIXa chimeras in the presence of FVIIa in the absence of phospholipids. FX (1.7 μM) was activated by 0–80 nM of wt-FIXa (open circles), FIXa<sup>75-77</sup>/FVII (closed circles), FXa<sup>106–108</sup>/FVII (closed squares), FXa<sup>75–77</sup>/FVII (closed triangles), and FIXa-Phe<sup>77</sup> → Ser (open triangles) in the presence of 10 nM FVIIIa, but in the absence of phospholipids, in 100 mM NaCl, 5 mM CaCl<sub>2</sub>, 0.2 mg/ml ovalbumin, and 50 mM Tris (pH 7.4) for 30 min at 37 °C. FXa generation was measured as described under “Experimental Procedures.” Data represent the mean of at least two independent experiments. Apparent K<sub>d</sub> values (± S.D.) were determined as described in the legend of Fig. 3 and were found to be 30 (± 2), 15 (± 1), and 64 (± 7) nM for wt-FIXa, FIXa<sup>75–77</sup>/FVII, and FIXa<sup>75–77</sup>, 106–108/FVII, respectively. For FIXa-Phe<sup>77</sup> → Ser and FIXa<sup>106–108</sup>/FVII the apparent K<sub>d</sub> was > 200 nM.

EGF1 and EGF2 domain (14). In the present study, we focused on hydrophobic contacts between the two EGF-like domains employing recombinant FIX/X and FIX/VII chimeras (Fig. 1). All of the FIXa/FX chimeras we analyzed (Table I) displayed normal enzymatic activity compared with wt-FIXa, both in the absence and presence of FVIIa (Figs. 2 and 3B). It seems relevant to note that amino acid replacements in the FIXa/FX chimeras were limited to residues 75 and 106, because residues 76, 77, 107, and 108 are identical in FX (see Fig. 1). The observation that substitution of residues 75 and 106 is not accompanied by major changes in enzymatic activity suggests that these residues are not essential for FIXa function. The fact that in the FIXa/FX chimeras the majority of the hydrophobic residues remain unaffected further implies that residues in position 76, 77, 107, and 108 may be more important in maintaining the hydrophobic contact between the two EGF-like domains.

Studies using other chimeric FIXa variants have demonstrated that the complete EGF1 domain can be replaced by that of FX without apparent effect on FIX function (36). In contrast, introducing the entire EGF1 domain from FXVII leads to increased activity in the presence of FVIIIa, which presumably is because of an increased affinity for this cofactor (37). In our study, we observed a rate enhancement for FIXa<sup>75–77</sup>/FVII similar to that reported for FIXa<sup>75–77</sup>/FVIII (Fig. 3A). This suggests that the effect of EGF1 domain replacement could be fully explained by the introduction of Pro<sup>75</sup> and Ala<sup>76</sup> in the FIX molecule. In this respect, our data are compatible with the suggestion made by Chang et al. (37) that elimination of the Phe<sup>75</sup> side chain may increase flexibility between the EGF1 and EGF2 domains, which might facilitate FVIIIa binding. An alternative explanation would be that the sequence Pro<sup>75</sup>-Ala<sup>76</sup>-Phe<sup>77</sup> in FIXa<sup>75–77</sup>/FVII directly binds to FVIIIa. However, this seems less likely because the presence of the same residues in the FIXa<sup>75–77</sup>, 106–108/FVII chimera is not associated with increased response to FVIIIa (Fig. 3A). Our finding that cofactor stimulation is reduced in FIXa-Phe<sup>77</sup> → Ser further supports the role of hydrophobic residues Phe<sup>75</sup>-Gly<sup>76</sup>-Phe<sup>77</sup> in regulation of FVIIIa-dependent FIXa activity (Fig. 3).

Whereas the functional role of residues 75–77 is relatively minor, mutation of residues 106–108 proved to be of major impact on FIXa function. The chimera FIXa<sup>106–108</sup>/FVII displayed dramatically reduced activity toward FX, both in the absence (Fig. 2) and presence (Fig. 3A) of FVIIIa. Because the defect was not only cofactor-independent but also phospholipid-independent (Fig. 5), it is evident that introduction of FVII residues at positions 107 and 108 in the EGFr2 domain does not affect assembly of the FX-activating complex, but that the defect is because of a decrease in enzymatic activity. This suggests that a functional link exists between the EGF2 domain and the FIXa heavy chain. An explanation for the apparent “cross-talk” between the FIXas heavy and light chains, may be derived from the current three-dimensional FIXa structures and the atomic contacts between the two chains therein. Indeed, these structures reveal that there is an intimate contact between the EGF2 domain and the heavy chain (14, 38). In this respect FIXa is similar to FXa, wherein the EGF2 domain and the protease domain may be regarded as a single operational unit (39). Although residues 107 and 108 are not in direct contact with the FIXa heavy chain, it is conceivable that substitution of these light chain residues may alter the interdomain interaction via an allosteric mechanism. These structural alterations in FIXa light-chain-heavy chain contact may affect the arrangement of substrate recognition and cleavage sites in the heavy chain. Analysis of the interface between light and heavy light chain reveals multiple interdomain interactions including the disulfide bridge between Cys<sup>132</sup> and Cys<sup>289</sup>, a salt bridge between Glu<sup>113</sup> and Lys<sup>409</sup>, hydrophobic contact between Phe<sup>98</sup> and residues Tyr<sup>295</sup>, Phe<sup>299</sup>, and Phe<sup>302</sup> (14, 38), and a potential hydrogen bond between Asn<sup>92</sup> and Tyr<sup>295</sup>. Interestingly, mutation of Asn<sup>92</sup> → His in FIX is associated with reduced FIXa enzymatic activity (40), which also might support our idea that contact between the EGF2 domain and the heavy chain contributes to enzymatic activity.

In view of the assumption that residues 106–108 may regulate enzymatic activity via contact between light and heavy chain, it is particularly striking that the detrimental effect of replacing residues 106–108 in the EGFr2 domain is counteracted by the replacement of the complementary hydrophobic site 75–77. Our finding that the FIXa<sup>75–77</sup>, 106–108/FVII chimera has apparently normal enzymatic activity (Figs. 2 and 3A) suggests that these sites comprise a functional link between the two EGF-like domains in FIXa. As such the hydrophobic contact, which has been described as a “hall-and-socket” joint in the FIXa light chain (14), is the counterpart of the salt bridge between Glu<sup>78</sup> and Arg<sup>84</sup>, which also links the EGF-like domains (15). The difference between these contacts, however, is that the salt bridge supports FVIIa binding, whereas the hydrophobic contact primarily seems to regulate FIXa enzymatic activity.

It is further remarkable that substitution of the FIX sequence Phe<sup>75</sup>-Gly<sup>76</sup>-Phe<sup>77</sup> for the FVII sequence Pro<sup>75</sup>-Ala<sup>76</sup>-Phe<sup>77</sup> had no effect on enzymatic activity in the absence of FVIIa (Fig. 2). This might imply that the EGF1 residues 75–77 may not be involved in regulation of enzymatic activity and that only residues in the EGFr2 domain (e.g. residues 92, 107–108) contribute to FIXa enzymatic activity. However, it should be noted that amino acid replacements in FIXa<sup>75–77</sup>/FVII still maintain the hydrophobic character of this region. It seems conceivable therefore, that these FVII residues also are able to support the hydrophobic interaction with the EGFr2 domain and consequently remain without significant effect on the overall structure of the protease domain of this FIXa chimera. The fact that the FIXa<sup>75–77</sup>, 106–108/FVII chimera displays apparently normal activity further implies that the proteolytic activity is influenced by structure elements in the EGF1 domain.
similar effect has been observed for FIXa variants with mutations in position 64 in the EGF1 domain (23). Mutation at this site eliminates the high affinity Ca\(^{2+}\) binding site in this light chain domain and is associated with a variety of molecular defects including enzymatic activity toward both FX and a small synthetic peptide substrate. The role of the light chain in regulation of FIXa activity is further demonstrated by the observation that FIXa amidolytic activity could be completely eliminated by the monoclonal antibody against the light chain of FIXa (20). Although the proposed effects of the amino acid substitutions on the FIXa structure can only be confirmed by crystallographic data, we propose that the structural integrity of the interface between both EGF-like domains serves as an allosteric hinge, which regulates proper orientation of light and heavy chain within the FIXa molecule.

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