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ORIGINAL ARTICLE

Functional analysis of the EGF-like domain mutations Pro55Ser and Pro55Leu, which cause mild hemophilia B

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Summary. We studied the functional role of two mutations, Pro55Ser and Pro55Leu, located in the N-terminal Epidermal Growth Factor-like domain (EGF1) of coagulation factor (F) IX. Both mutations cause mild hemophilia B with habitual FIX coagulant activities of 10–12% and FIX antigen levels of 50%. We found that activation by FVIIa/TF and FXIa was normal for FIXPro55Ser, but resulted in proteolysis of FIX-Pro55Leu at Arg318-Ser319 with a concomitant loss of amidolytic activity, suggesting intramolecular communication between EGF1 and the serine protease domain in FIX. This was further supported by experiments using an anti-EGF1 monoclonal antibody. Activation of FX by FIXaPro55Ser was impaired in both the presence and the absence of phospholipid or FVIIIa, indicating that Pro55 is not directly involved in binding to FVIIIa. We also studied the effect of the two Pro55 mutations on Ca^{2+} affinity and found only small changes. Thus, the Pro55Ser mutation causes hemophilia primarily through to an impaired ability to activate FX whereas at least *in vitro* the Pro55Leu defect interferes with the activation of FIX.

Keywords: coagulation, EGF domain, factor IX, hemophilia B, mutation.

Introduction

Coagulation factor (F) IX is a vitamin K-dependent serine protease zymogen that circulates in plasma [1]. Defects in FIX cause the X-linked bleeding disorder hemophilia B, which affects approximately one in 30 000 males. FIX is a single-chain glycoprotein comprising 415 amino acids. The N-terminal part contains 12 γ-carboxyglutamyl (Gla) residues, that bind Ca^{2+} and mediate membrane binding, followed by two epidermal growth factor-like (EGF) domains [2,3]. The C-terminal part of FIX contains the heavy chain with the catalytic center. Activation of FIX involves cleavage at Arg145, which yields an intermediate called FIXα that is inactive towards its physiological substrate, factor X (FX). A subsequent cleavage at Arg180 results in the serine protease factor IXaβ (commonly referred to as FIXa), a protein consisting of two polypeptide chains that are linked by a disulfide bond [4]. FIX is activated either by active FXI (FXIa) of the intrinsic pathway or by active FVII/tissue factor (FVIIa/TF) of the extrinsic pathway. Physiologically, the FVIIa/TF pathway is most important, but FXIa amplifies the coagulation cascade *in vivo* [5]. Activation of FX is mediated by a cell-membrane-bound macromolecular complex including FIXa bound to its cofactor, active FVIIIa and Ca^{2+} [6].

The EGF domains in FIX act as spacers that position the active site of FIXa at a distance from the membrane that allows interaction with its cofactor and substrate [7,8]. The N-terminal EGF domain (EGF1) binds one Ca^{2+}, which is necessary for the biological activity of FIX. It was recently suggested that the C-terminal EGF domain (EGF2) of FIX, and in particular the linker region between the EGF domains, interacts directly with FVIIIa [9,10].

Earlier studies have shown that several mutations in EGF1 cause hemophilia B [11]. The Asp64Lys mutant FIX exhibits reduced amidolytic activity, which is interesting since Asp64 is a Ca^{2+} ligand in EGF1 [12]. This indicates that there is a long-range interaction between EGF1 and the serine protease domain. Many of the mutations described in the literature decrease the rate of activation of FX, particularly in the presence of FVIIIa [12–16]. Several of these studies were performed in the presence of phospholipid (with or without FVIIIa) and it is not clear whether the disturbed interaction with FVIIIa is caused by changes in EGF1 representing a direct effect, or if it is instead a secondary effect involving for instance, altered localization of the active site of FIXa. There is controversy in the literature concerning the importance of EGF1 in the FVIIa/TF- or FXIa-mediated activation of FIX, which motivates further studies [7,17–19].

We investigated two patients with mutations that cause hemophilia B; FIX Pro55Ser and FIX Pro55Leu that might shed light on the importance of the amino acid Pro55 in FIX

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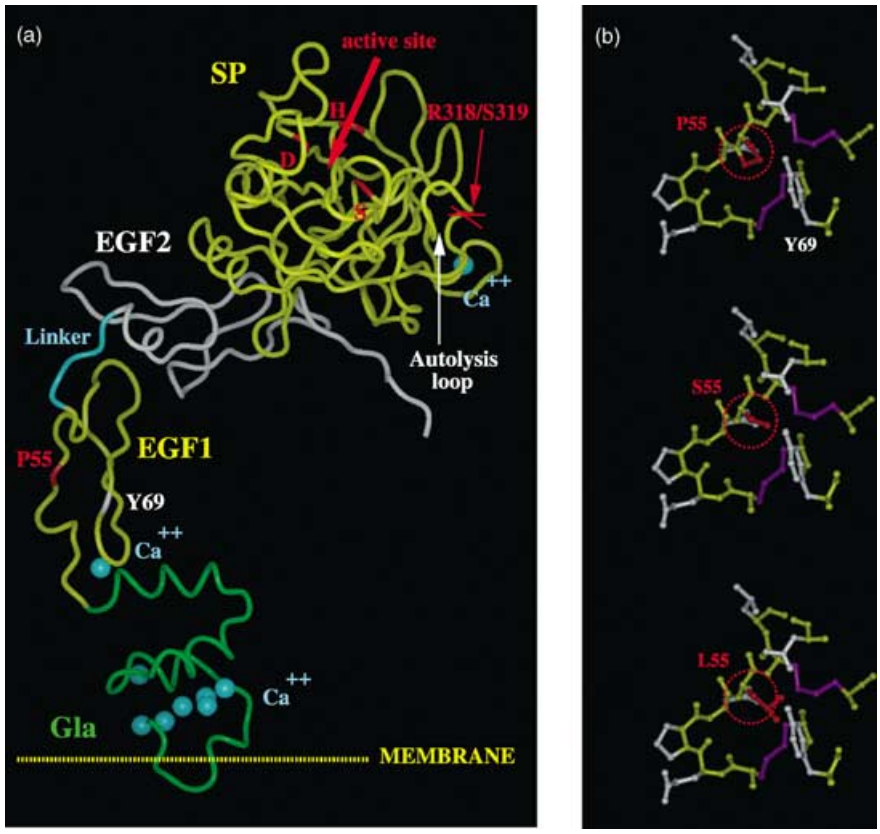

Fig. 1. (a) Overview of the human FIXa model. Residues forming the catalytic triad (H, D, and S) are shown in red, Ca^{2+} ions are shown as blue spheres and the autolysis loop in the serine protease domain is indicated by an arrow. The different regions of FIXa (the Gla, EGF1, linker, EGF2 and serine protease domain) are colored differently to facilitate the interpretation of the figure with Pro55 in red and the nearby Tyr69 in gray (see Discussion). (b) Close-up view of the area around residue 55. A Ser at position 55 (red) might be structurally tolerated (although possibly slightly destabilizing) whereas Leu55 (L55) appears to collide with Tyr69 (Y69, gray). Reorientation of some of the side chains and small movements of backbone atoms might relieve some energy strain. Such structural changes could be propagated in certain areas of the serine protease domain and indeed seem to induce conformational changes in proximity to the autolysis loop, thereby rendering FIXa susceptible to proteolysis.

EGF 1 (Fig. 1). Both patients had habitual FIX coagulant activity of 10–12% of the control level and FIX antigen was 50% below normal, suggesting that these proteins were dysfunctional. We conducted a series of experiments to elucidate the functional defects of this mutant FIX species. Interestingly, we found that the biological activity and other properties of the mutant FIXa differed depending on whether Pro55 was substituted by a Ser or a Leu residue. We also used synthetic EGF domains and recombinant proteins to determine how the two mutations influence the Ca^{2+}-binding of EGF1 as well as their effects in the activations of FIX and FX.

Materials and methods

Proteins

Plasma-derived FIX, FIXa, FX, FXa, FXIa and thrombin were purchased from Kordia (Leiden, the Netherlands), and recombinant FVIII (Recombinate®) was from Baxter Medical AB (Los Angeles, CA, USA). FVIIa was a gift from Novo Nordisk (Copenhagen, Denmark). The mouse monoclonal antibody (mAb) AW is directed against the C-terminal part of EGF1 in human FIX around residues 72 and 80 [20].

Patients

Patient 1 carried the mutation FIX Pro55Ser. He was born in 1947 and diagnosed with mild hemophilia B in 1967, and prior to diagnosis, he had received blood transfusions after tooth extraction and after trauma to a knee. He was treated with FIX concentrate for a short period in 1982 and again during surgical procedures performed in 1985. He did not develop any inhibitors. During the remainder of his life he had no bleeds that required treatment, and he died of multiple myeloma in 1991. The only surviving relative with the disorder is a 2-year-old grandson.

Patient 2 carries the mutation Pro55Leu. He was born in 1945 and was diagnosed with mild hemophilia B in 1967 following three episodes of muscle bleeds in a thigh and calf after playing soccer. He had previously had a prolonged bleeding episode after tooth extraction at the age of 12 years. After being diagnosed he had another bleed in a thigh and one in a knee, both after trauma. On those occasions he was given FIX concentrate, and he did not develop inhibitors. He is the only member of his family known to have hemophilia B.

Mutation analysis of the hemophilia B patients was done by sequencing genomic DNA of the exons a-h, using the modified dideoxi method [21,22].

Construction and expression of recombinant FIX

Recombinant human FIX was produced both as wild-type (wt) protein and with the mutations Pro55Ser and Pro55Leu. The full-length human FIX cDNA was a gift from Dr Darrel W. Stafford (Department of Biology, University of NC, USA). A PCR-fragment of the cDNA, including restriction enzyme sites, was ligated into the vector pRc/CMV 5.5 kb (Invitrogen, Groningen, the Netherlands) with Hind III and Xba I. To induce

the mutation, a Quick-Change™ site-directed mutagenesis kit from Stratagene (CA, USA) was used with the following primers (the mutations are underlined): for Pro55Ser, 5′GGAGATCAGTGTGAGTCCAATTCATGTTTAAATGGC-GGCAG3′ and 5′CTGCCGCCATTTAAACATGAATTGGAC-TCACACTGATCTCC3′, for Pro55Leu, 5′GGAGATCAGTG-TGAGTCCAATCTATGTTTAAATGGCGGCAG3′ and 5′CT-GCCGCCATTTAAACATAGATTGGACTCACACTGATCT-CC3′. Sequence analysis of the entire FIX fragment was then performed as described above to verify the presence of the desired mutations.

Human kidney 293 cells were transfected with the vectors mentioned above, and G418 was used for selection of transfected cells. The cells were grown in Dulbecco's modified Eagle's medium from ICN (Aurora, OH, USA) supplemented with 3.5 mM glutamine, 44 IU penicillin, 44 μM streptomycin, 10 μg mL^{-1} vitamin K (Konakion Novum, Roche, Basel, Switzerland), and 10% fetal calf serum. Three days before harvesting, the medium was changed to Optimem Glutamax (ICN) supplemented with 44 μM Streptomycin and 10 μg mL^{-1} vitamin K (Konakion Novum). Immunoblotting was used to determine which of the colonies of cells that expressed the highest level of FIX (around 10 μg mL^{-1}).

Purification of plasma-derived and recombinant FIX

Plasma from patient 2 (590 mL) was purified through a Q-Sepharose column (Pharmacia, Uppsala, Sweden) and eluted with 5 mM benzamidine, 10 mM $CaCl_2$, 0.18 M NaCl, and 20 mM Tris-HCl (pH 7.5); this step was not necessary for the recombinant proteins. Thereafter, both plasma-derived and recombi-nant proteins were purified on a HiTrap affinity column (5 mL, Amersham Pharmacia Biotech, Uppsala, Sweden) with immobilized mouse monoclonal antibody M43 (directed against human FIX). The medium or plasma was added to the column, and wash buffer (1 mM benzamidine, 2 mM $CaCl_2$, 100 mM NaCl, and 50 mM Tris-HCl, pH 7.5) was supplied until the baseline was reached. The proteins were subsequently eluted with 1 M guanidine hydrochloride, 1 mM benzamidine, 100 mM NaCl, and 50 mM Tris-HCl (pH 7.5) and dialyzed against 100 mM NaCl, and 50 mM Tris-HCl (pH 7.5). The resulting samples were frozen in aliquots.

SDS-PAGE and immunoblotting

Protein samples were analyzed by SDS-PAGE, using 15% (w/v) gels [23] and the gels were subsequently silver stained (Pharmacia, Uppsala, Sweden). For immunoblotting, the proteins were transferred to Immobilon transfer membranes (Millipore, Bedford, MA, USA), which were blocked with 5% dry milk dissolved in quenching buffer (0.05% Tween 20, 0.15 M NaCl, and 10 mM Tris-HCl, pH 8.0). The membranes were then exposed to a primary antibody (10 μg mL^{-1}), either mAb M3B, directed against peptide-bound Gla [24], or the polyclonal rabbit anti human FIX antibody (A0300 DAKO, Glostrup, Denmark), and thereafter washed again and incubated with alkaline phosphatase-conjugated porcine antirabbit IgG (D0306, DAKO). The membranes were subsequently washed and developed with 0.15 mg mL^{-1} 5-bromo-4-chloro-3-indolyl phosphate and 0.3 mg mL^{-1} nitro blue tetrazolium.

FIX coagulant activity

FIX coagulant activity was measured by a one-stage clotting assay performed at 37 °C. In short, 20 μL of test sample, 100 μL of FIX-deficient plasma, and 100 μL of APTT reagent (Diagnostica Stago, Asnières-Sur-Seine, France) were incubated for 5 min. Thereafter, 100 μL of preheated 25 mM $CaCl_2$ was added, and the clotting time at 37 °C was recorded with a KC 10 Amelung coagulometer (Germany). Citrated pooled plasma from healthy blood donors was used as a standard (defined as containing 1 U mL^{-1} of FIX activity).

FIX antigen levels

An ELISA was performed according to the instructions of the manufacturer (DAKO) to measure the levels of FIX in plasma. Briefly, the immobilized polyclonal rabbit anti human FIX antibody (A0300, DAKO) binds FIX, which is then detected by a peroxidase-conjugated antibody (P380, DAKO) against FIX.

Activation of FIX by FXIa

FIX was converted to FIXa by incubating FIX (4 μM) with FXIa (6 nM) in 5 mM $CaCl_2$, 100 mM NaCl, and 50 mM Tris-HCl (pH 7.4) for 4 h at 37 °C. The activation was terminated by adding EDTA to a final concentration of 10 mM. The same procedure but with Ca^{2+} concentrations up to 20 mM was also performed to ascertain whether Ca^{2+} would influence proteolysis of the Pro55Leu protein. The activated samples were analyzed by SDS-PAGE, and aliquots were frozen on dry ice with ethanol and stored at −70 °C pending measurement of amidolytic activity.

Activation of FIX by FVIIa/TF

FIX (2 μM) was activated with FVIIa (10 nM) in 5 mM $CaCl_2$, 100 mM NaCl, and 50 mM Tris-HCl (pH 7.4) for 6 h at 37 °C. Before incubation the mixture was diluted 1 : 2 with Innovine (recombinant Tissue Factor and phospholipid; Dade Behring, Marburg, Germany). The activation was stopped by adding EDTA (final concentration 10 mM). The activated samples were analyzed by SDS-PAGE, and aliquots were frozen on dry ice with ethanol and stored at −70 °C.

Amidolytic activity of FIXa

Hydrolysis of CH_3SO_2-LGR-pNA (CBS 31.39, Diagnostica Stago, Asnières, France) was assayed as previously described [12]. Briefly, 50 μL of a 5-mM solution of CH_3SO_2-LGR-pNA was added to 50 μL of a solution containing 75–600 nM FIXa in a 96-well microtiter plate (Bibby Sterilin Ltd, Stone, Staffs,

UK). Substrate hydrolysis was measured by recording the absorbance at 405 nm for 15 min at 37 °C.

FX activation

FXa formation was determined as previously described, with some minor modifications [25]. Briefly, phospholipid vesicles (50 μM) were prepared as described elsewhere [26], 70% L-α-phosphatidylcholine (Sigma, Stockholm, Sweden) and 30% phosphatidyl-L-serine (ICN). These vesicles were preincubated in 60% of the final volume (300 μL) of the following: 20 mM Hepes, 0.2% (w/v) human serum albumin, 5 mM $CaCl_2$, and 0.15 M NaCl (pH 7.4) for 10 min at 37 °C. Thereafter, FVIII (9.4 nM), thrombin (6.4 nM) and FIXa (0.1 nM) were added sequentially. The mixture was then incubated for 2 min at 37 °C and the reaction was started by adding FX (0–1 μM). Aliquots were drawn at 0, 10 and 15 s (when formation of FXa was linear). The reaction was terminated by adding 20 mM Hepes and 25 mM EDTA (pH 7.4), and the samples assayed for FXa formation using the chromogenic substrate S-2222 (Chromogenix AB) at a final concentration of 0.3 mM. Less than 5% of FX was converted to FXa during the assay period. Conversion of substrate per minute was monitored at 405 nm, and active site-titrated FXa was used to correlate substrate hydrolysis with FXa concentrations. The same procedure was employed to assess FX activation in the absence of phospholipid, except the concentration of FIXa was increased to 10 nM, FVIII/thrombin concentrations were doubled, and aliquots were drawn at 10, 15, and 30 s (when formation of FXa was linear). In the absence of FVIIIa, the concentration of FIXa was 10 nM, and aliquots were drawn at 1, 2, and 5 min (when formation of FXa was linear). Due to the slow rate of activation when the procedure was performed without both FVIIIa and phospholipid, we used 500 nM FIXa and 1 μM FX in this assay.

Active-site titration

FXa was active-site titrated as described by Byrne *et al*. [27] using 2 μM FXa and 50 μM of p-nitrophenyl-p-guanidinobenzoate. The titration was performed in a Cary 4E spectrophotometer (Varian Pty. Ltd. Mulgrave, Australia).

Peptide synthesis

EGF1 (amino acids 46–84) of human FIX was synthesized as a wild-type domain, with the substitutions Pro55Ser and Pro55Leu; this was done using a Milligen 9050 Plus Synthesizer (Applied Biosystems, Foster City, CA, USA) and standard Fmoc chemistry. The peptides were purified by reversed-phase high-pressure liquid chromatography and folded as described [28].

Ca^{2+} titration of EGF domains

Intrinsic protein fluorescence of the EGF domains was measured at 20 °C using a Fluoromax-3 (Jobin Yvon, Cedex, France) and a protein concentration of 4 μM in 0.1 M NaCl, and 50 mM Tris-HCl (pH 7.5). The excitation and emission bandwidths were 2 and 8 nM, respectively. The excitation wavelength was 280 nM, and emission spectra were recorded from 300 to 400 nM. Ca^{2+} titration was performed at the wavelength at which maximum fluorescence emission intensity was recorded, 359 nM for the wt EGF domain, and 356 nM for the pro55Ser- and Pro55Leu-substituted domains. The emission intensity was measured after repeated additions of 0.25–1.0 μL of a 0.77 or 2.5 mM $CaCl_2$ solution, and the Ca^{2+} concentration was determined by atomic absorption spectroscopy. The integration time was 0.1 s. Dissociation constants and maximum increases in fluorescence at Ca^{2+} saturation were determined by non-linear regression to fit the data to the equilibrium binding equation [29]. Each experiment was repeated twice.

Surface plasmon resonance

We used a surface plasmon resonance system to study the interaction of both wt FIX and the Pro55Ser and Pro55Leu mutants, with the mAb AW. The antibody was immobilized (1500 response units) on a CM5 sensor chip using an amine coupling kit according to the instructions of the manufacturer (Biacore AB, Uppsala, Sweden). The rates of association and dissociation between FIX (in solution) and the immobilized mAb AW were determined on a BIACORE 2000 biosensor at 25 °C and a flow rate of 30 μL min^{-1}. The FIX proteins were diluted in 1 mg mL^{-1} BSA, 5 mM $CaCl_2$, 150 mM NaCl, and10 mM Tris-HCl (pH 7.5). FIX (90 μL) was injected during the association phase (180 s), and there was a continuous flow of buffer (the same buffer used to dilute the proteins) during the dissociation phase (600 s). The FIX concentrations were 100 nM in the association phase and were subsequently stepwise diluted 1 : 2–1.5 nM. The sensor chips were regenerated with a 5 μL pulse (5 μL min^{-1}) of 0.1 M glycine and 0.5 M NaCl (pH 2.7). One lane on the chip was always free of coupled protein to obtain values for subtraction of the bulk sample effect. The data were evaluated with BIA 3.0 software, and each experiment was repeated twice.

Other methods

N-terminal sequences were determined using an Applied Biosystems 494 Procise Sequencer (Foster City, CA, USA). Sequences were performed on the full-length protein in solution and to determine the site of proteolysis in the mutant Pro55Leu, the proteins were transferred to an Immobilon membrane (Millipore), and the stained bands of protein were excised and then evacuated from the membrane prior to sequencing [30].

Protein concentrations of the zymogens were determined by amino acid analysis of acid hydrolysate (24 h hydrolysis in 6 M HCl) as described [31]. Recombinant FIXa concentrations were estimated by comparing the amidolytic activity with active-site titrated FIXa (Kordia). The amount of Gla was measured as described [32].

Results

Patient data and characterization of the plasma-derived and recombinant proteins

Patient 1 (deceased) was found to have a single mutation, C10415T, changing Pro55 to Ser; no protein was available for analysis. Patient 2 had a single mutation C10416T that altered Pro55 to Leu; protein from this patient was purified and used as a control in experiments performed with recombinant FIX Pro55Leu. Recombinant and plasma-derived purified proteins were homogenous as judged by SDS-PAGE, and N-terminal sequence analysis showed more than 95% homogeneity with no signs of internal cleavages (Fig. 2). Immunoblotting with the Gla-specific mAb M3B identified the Gla-containing light chains (not shown). Amino acid analysis after alkaline hydrolysis established that the recombinant proteins, both wt and mutated FIX, were virtually fully γ-carboxylated (11.1–12.9 mol Gla/mol FIX for the recombinant proteins compared to 12.4 for the plasma-derived FIX, triplicate samples).

The relative coagulant activity of the recombinant proteins compared to plasma-derived FIX (mean of three assays) was 96% for FIX wt, 12% for FIX Pro55Ser, and 8% for FIX Pro55Leu. This is consistent with the levels measured in the patient plasma samples, since both subjects had mild hemophilia with 10–12% (U dL^{-1}) coagulant activity and the purified plasma FIX from patient 2 (Pro55Leu) exhibited 10% of the coagulant activity observed in normal, purified, plasma-derived FIX. The antigen levels were 50% of normal in both patients.

Conversion of FIX to FIXa

Activation of the recombinant FIX Pro55Ser by both FXIa and FVIIa/TF was similar to that of FIX wt and plasma-derived FIX as analyzed by SDS-PAGE (shown for FXIa in Fig. 2; not illustrated for FVIIa/TF). In contrast, SDS-PAGE of both FXIa- and FVIIa/TF-mediated activation of the recombinant FIX Pro55-Leu resulted in two extra bands in the lower part of the gel. These bands were cut out and subjected to N-terminal sequencing. The smaller band gave the sequence Ser-Ala-Leu-Val-Leu-Gln, beginning at Ser319. The larger band showed the sequence Val-Val-Gly-Gly-Glu-Asp, starting at Val181, the N-terminus of the heavy chain. The size of this fragment suggested that it starts at Val181 and presumably ends at Arg318. The results for the patient-derived and recombinant FIX Pro55Leu were identical (Fig. 2). Hence, the Pro55Leu mutant protein is very susceptible to cleavage in the autolysis loop. Since the EGF1 Pro55Leu has a slightly lower affinity for Ca^{2+} than the wt EGF1 (see below), we performed experiments to determine whether increasing the concentration of Ca^{2+} to 20 mM would influence the proteolysis, but we observed no such effect. Pro55Ser was not cleaved in the autolysis loop during activation and we were able to obtain FIXa Pro55Ser that could be used in further experiments.

Amidolytic activity

To determine whether the amino acid substitutions at position 55 affected the amidolytic activity, we incubated recombinant FIXa Pro55Ser or Pro55Leu (that had been activated by FXIa or FVIIa/TF) with the synthetic substrate CH_3-SO_2-LGR-pNA. The FIX Pro55Ser was indistinguishable from FIXa wt with respect to substrate hydrolysis, whereas FIX Pro55Leu exhibited only 40–50% of that of the amidolytic activity of FIXa wt (Fig. 3). This finding appears to agree with the degradation revealed by SDS-PAGE.

Affinity of mutated FIX proteins for the mAb AW

The epitope recognized by the mAb AW is around residues 72 and 80 in the C-terminal part of EGF1 of human FIX [19,20]. To

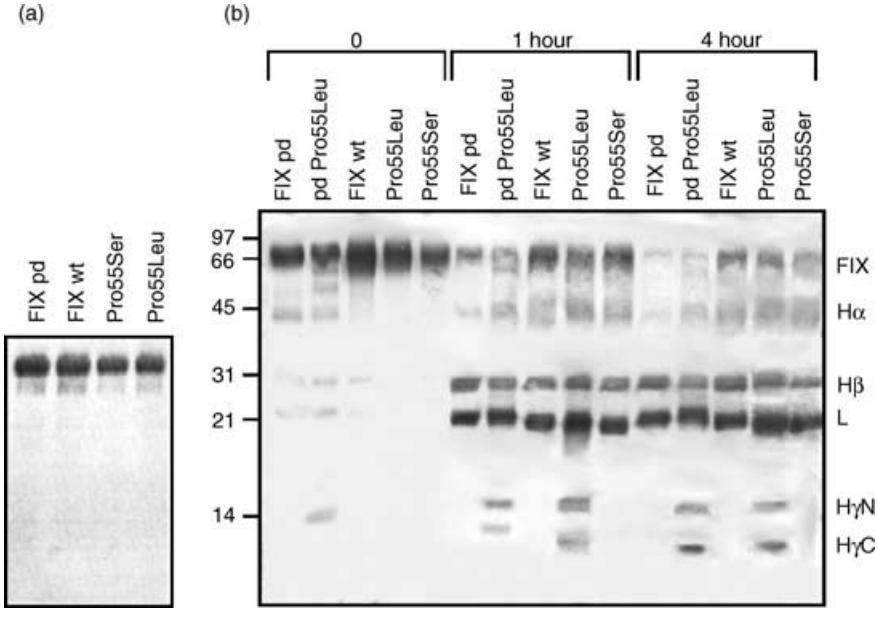

Fig. 2. SDS-PAGE and immunoblotting of FIX/IXa proteins. (a) Silver stained gels from SDS-PAGE (polyacrylamide gel electrophoresis, 15%) of unactivated proteins (0.5 μg of each). (b) Activation of FIX by FXIa. Reduced samples were subjected to Western blotting with a polyclonal antibody against FIX. The samples are designated as follows: Hα, FIX cleaved only at Arg145; Hβ, heavy chain of FIXa; L, light chain of FIXa; HγN, N-terminal part of heavy chain cleaved at Arg318; HγC, C-terminal part of heavy chain cleaved at Arg318.

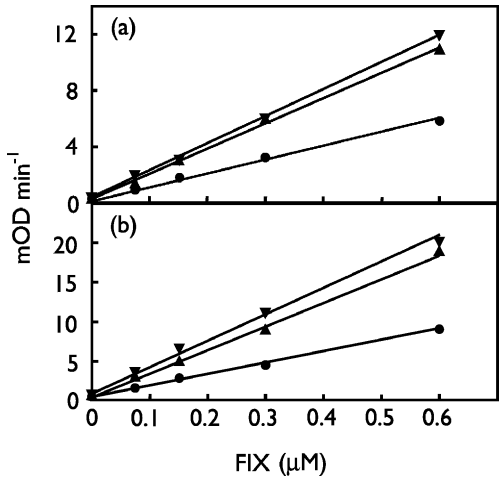

Fig. 3. Amidolytic activity of FIXa. FIXa wt (▼), FIXa Pro55Ser (▲), and FIXa Pro55Leu (●) were activated with FXIa (a) or FVIIa/TF (b) and then analyzed using the synthetic substrate CH_3-SO_2-LGR-pNA.

ascertain whether cleavage in the autolysis loop affects EGF1 of FIX, we investigated the affinity of AW for the mutated FIX proteins. We have previously shown that the K_D for binding of AW to FIX Pro55Ser is 4.5×10^{-8} M, compared to 5.6×10^{-9} M for FIX wt [19], and the present results indicated a K_D of 2.0×10^{-8} M (± 0.2, 1 SD) for binding of AW to Pro55Leu, i.e. similar to that obtained for Pro55Ser. Considering the activated proteins, we found that the K_D for binding to FIXa Pro55Ser was six-fold lower than for binding to the unactivated protein, and this increase in affinity is in the same range as we had previously described for FIX wt [20]. Binding to FIXa Pro55-Leu was too low to allow estimation of the K_D. There was no evidence of protein precipitation.

Activation of FX by FIXa

We studied the FIXa Pro55Ser mediated activation of FX in a system containing phospholipid, $CaCl_2$, and FVIIIa. The apparent k_{cat} ($k_{cat,app}$) value was approximately threefold lower for FIXa Pro55Ser than for FIXa wt (Fig. 4a, Table 1). We also investigated activation of FX by FIXa in the presence of phospholipid but the absence of FVIIIa, and, in this system, $k_{cat,app}$ was about twofold lower for FIXa Pro55Ser than for FIXa wt (Fig. 4b, Table 1). It has been suggested that some FIXa variants with mutations in EGF1 show reduced FVIIIa-dependent FX activation in the presence, but possibly not in the absence, of phospholipid vesicles [8]. Accordingly, we examined the effect of FIXa Pro55Ser on the activation of FX in the absence of phospholipid (but in the presence of FVIIIa), and we found that the $k_{cat,app}$ was fivefold lower for FIXa Pro55Ser than for FIX wt (Fig. 4c, Table 1).

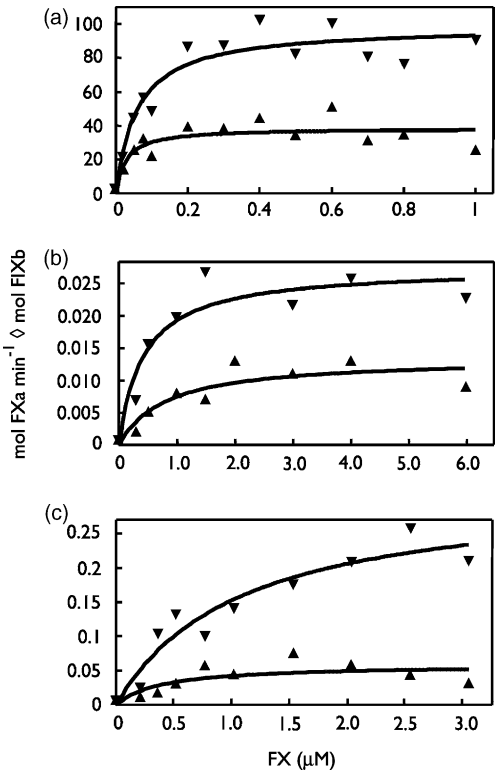

Fig. 4. Activation of FX by FXIa. FX was activated by FIXa wt (▼) or FIXa Pro55Ser (▲). Rates of FX activation were calculated from three measurements when formation of FXa was linear. (a) Activation of FX by FIXa (0.1 nM) in the presence of FVIIIa (9.4 nM) and phospholipid (50 μM). (b) Activation of FX by FIXa (10 nM) in the presence of phospholipid (50 μM) but in the absence of FVIIIa. (c) Activation of FX by FIXa (10 nM) in the presence of FVIIIa (18.8 nM) but in the absence of phospholipid.

We also determined the apparent K_m ($K_{m,app}$) for all the above-mentioned activations of FX. FIXa Pro55Ser did not cause any major changes in $K_{m,app}$, and since some of the absorbance values were low, smaller changes could not be estimated with certainty. Furthermore, we studied activation of FX in the absence of both FVIIIa and phospholipid, and, in this case we found that FIXa Pro55Ser gave a slightly lower rate of FXa formation than FIXa wt. However, the significance of

Table 1 Kinetic parameters of FX activation

	FIXa wt		*FIXa Pro55Ser*	
	$K_{m,app}$ (M)	$k_{cat,app}$ (s^{-1})	$K_{m,app}$ (M)	$k_{cat,app}$ (s^{-1})
+ PL, + FVIIIa	5.9×10^{-8}	1.6	2.8×10^{-8}	0.6
+ PL, − FVIIIa	4.3×10^{-7}	4.6×10^{-4}	8.1×10^{-7}	2.3×10^{-4}
PL, + FVIIIa	1.1×10^{-6}	5.3×10^{-3}	4.0×10^{-7}	9.8×10^{-4}

Activation of FX (0–1 μM) by FIXa (0.1 nM) in the presence of FVIIIa (9.4 nM) and activation of FX (0–8 μM) by FIXa (10 nM) in the absence of FVIIIa was performed with phospholipid (PL, 50 μM) as described in 'Materials and Methods'. In the absence of PL, 18.7 nM FVIIIa and 10 nM FIXa were used. Data points were derived from Fig. 4 and fitted in the Michaelis–Menten equation by use of KaleidaGraph.

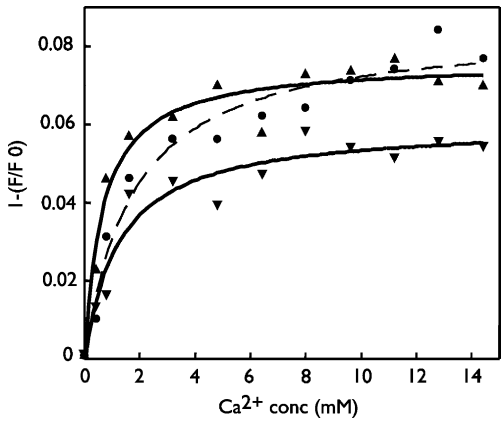

Fig. 5. Ca^{2+} titration of EGF modules. Ca^{2+} titration was monitored by intrinsic protein fluorescence. Examples of titrations for the EGF domains of wt (▲), Pro55Ser (▼), and Pro55Leu (●) are shown. Emission intensity in the presence and absence of Ca^{2+} are, respectively, designated F and F_0.

this finding is not clear due to the low absorbance values (not shown).

Ca^{2+} titration of EGF domains

The synthetic wt and variant (Pro55Ser and Pro55Leu) FIX EGF1 domains all produced a single peak in reversed-phase HPLC performed on a C 8 column. ELISA, using a polyclonal rabbit antihuman FIX antiserum, was positive with the folded EGF1 domains but negative after reduction of the disulfide bonds (the antiserum does not react with reduced FIX). It can therefore be assumed that the proteins were folded to a native conformation. To evaluate the influence of the two different substitutions at position 55 on Ca^{2+}-binding, we titrated the synthetic EGF domains with Ca^{2+}. Addition of Ca^{2+} to the Ca^{2+}-free protein solution quenched the intrinsic fluorescence (EGF1 contains a Trp) of both the wt EGF domain and the substituted domains. Evaluation of the titrations resulted in the following dissociation constants: 1.3 ($\pm$ 0.3) mM for the EGF wt domain (1SD in brackets), 0.7 ($\pm$ 0.1) mM for the Pro55Ser substitution and 2.2 ($\pm$ 0.3) mM for the Pro55Leu substitution (Fig. 5). This means that substitution of Pro for a Ser gave a slightly increased affinity for Ca^{2+}, whereas Pro55 to Leu gave a slightly decreased affinity for Ca^{2+}.

Discussion

We have characterized two mutations affecting position 55, Pro55Ser and Pro55Leu, in the N-terminal EGF domain of FIX. The FIX came from two patients with the same clinical history of mild hemophilia, with bleedings at surgery or after trauma but no spontaneous bleedings. The mutations were reported to the hemophilia B mutation database (http://www.kcl.ac.uk/ip/petergreen/haemBdatabase.html) in 1991. Another substitution at this position (Pro55Ala), which also cause mild hemophilia, has been characterized [11,15]. Most patients with hemophilia B and point mutations have clotting activities in accordance with the antigen levels, and in these cases, the disease is probably caused by a deficiency of FIX due to impaired secretion. Both the Pro55Ser and the Pro55Leu patients had/have habitual FIX coagulant activity of 10–12% but FIX antigen levels of 50%, suggesting that they secrete dysfunctional proteins that will allow studies aimed at elucidating the role of FIX in blood coagulation.

The two EGF-domains of FIX function as spacers that position the active site of FIX at an appropriate distance from the membrane to allow interaction with its cofactors and substrate. The N-terminal EGF domain binds Ca^{2+}, and has been implicated in protein–protein interactions [3,7–10,33,34]. The residues responsible for Ca^{2+}-binding in EGF1 are Asp47, Gly48, Gln50, Asp64 and Asp65 [35].

Pro55 is a well-conserved residue in FIX homologs, such as FX and protein C. This residue is also conserved in coagulation factors of other species, for example bovine FIX and FX, suggesting that it has some important function. Indeed, in the three-dimensional (3D) structure of porcine FIXa and in the subsequent 3D model of human FIXa that we have developed, only a small residue can be tolerated at position 55 [19,36]. Thus, minor conformational changes may occur when Ala or Ser is substituted for Pro, whereas the Pro-to-Leu substitution may cause more severe and destabilizing interference, as Leu seems to collides with Tyr69 (Fig. 1).

Even though Pro55 is not a Ca^{2+} ligand, we wanted to investigate whether the functional defects in two substituted FIX species were due to a decreased affinity for Ca^{2+}. Substitution of a conserved residue at this position might affect the folding of EGF1 in the Ca^{2+}-binding area (Fig. 1). However, we found only minor changes in the affinity for Ca^{2+}, and these alterations presumably do not represent the explanation for the dysfunctional proteins.

We found that both recombinant and plasma derived FIX Pro55Leu were degraded soon after activation, in contrast to the wt protein and FIX Pro55Ser. The proteolysis takes place in the heavy chain (Arg318-Ser319), in the so-called autolysis loop, far from the mutated residue 55 in EGF1. The fact that cleavage occurs in the serine protease domain (at Arg318) when a residue in EGF1 is mutated provides evidence of intramolecular communication between EGF1 and the serine protease domain. Direct contact between EGF1 and the area surrounding residue 318 seems unlikely since analysis of the 3D structure of porcine FIXa has shown that the Gla domain is located at one end of the molecule and the serine protease domain at the other [36]. Other studies have suggested the existence of intramolecular communication in both FIX and FVII [12,19,37]. To further investigate that possibility we used the mAb AW, which is directed against the C-terminal part of EGF1 in FIX. Activated FIX Pro55Ser behaved like the wt protein and showed higher affinity for the antibody than the unactivated protein did, whereas FIXa Pro55Leu had very low affinity for mAb AW. The cleavage that occurs at Arg318 upon activation of FIX may induce a conformational change that is propagated from the serine protease domain to EGF1. Alternatively, the new C-terminus at Arg318 might constitute a steric hindrance to

antibody binding, but this seems unlikely, since the distance from Arg318 to EGF1 is quite large (Fig. 1). Inasmuch as we had found that FIX Pro55Leu had a slightly lower affinity for Ca^{2+}, we examined the possibility that increasing the Ca^{2+} -concentration would have an impact on the cleavage at Arg318 in the autolysis loop, but we found no such effect. Other researchers have observed that mutations in areas involved in Ca^{2+}-binding (e.g. Glu245Val in the serine protease domain) result in the same cleavage at Arg318 in FIX that is activated by FXIa, but not by the FVIIa/TF pathway [38,39]. In our study, FIX Pro55Leu was degraded during activation by both FXIa and FVIIa/TF, whereas no such degradation was seen in Pro55Ala FIX [15]. Thus, compared to other substitutions, introduction of a relatively large hydrophobic residue (Leu) at position 55 (Fig. 1) seems to induce conformational changes that propagate to the serine protease domain, whereas substitution of Pro55, for a residue with a small side chain apparently has no such effect.

Degradation of FIX Pro55Leu led to a concomitant loss of amidolytic activity, another indication of the existence of intramolecular communication between EGF1 and the serine protease domain. Yet, we did not observe this proteolysis in the FIX Pro55Ser mutant, and the amidolytic activity of this protein appeared to be normal. Considering that the two patients we studied had the same clinical symptoms and coagulation activity, the differences we observed may have been the result of the longer FIX activation times needed in our *in vitro* systems compared to the situation *in vivo*. On the other hand, the clinical observations are too limited (i.e. based on only two patients).

In our experiments, FIX Pro55Ser was activated to the same extent as FIX wt by both FVIIa/TF and FXIa. It has been shown that EGF1 of FIX is important for interaction with TF [18], but our results indicate that residue 55 is not involved. Spitzer *et al*. [15] observed that the rate of FX activation induced by FIXa Pro55Ala (all experiments were performed in the presence of phospholipid) showed a larger difference from FIX wt when FVIIIa also was added to the system. Furthermore, Mathur and Bajaj [8] have reported data suggesting that FVIII-dependent activation of FX is markedly reduced by FIXa variants with mutations in EGF1 only in the presence of phospholipid vesicles. Accordingly, we studied the effects of FIX Pro55Ser on activation of FX in the presence and absence of FVIIIa and/or phospholipid, and we found that the $k_{cat,app}$ was reduced to approximately the same extent when either was omitted from the assay. The major increase in $k_{cat,app}$ caused by addition of FVIIIa was essentially preserved in the mutated protein (3500-fold for FIX wt compared to 2600-fold for FIX Pro55Ser), indicating that Pro55 in EGF1 of FIXa does not interact directly with FVIIIa, which is in agreement with our model of the FIXa-FVIIIa complex [19]. If FVIIIa had been in direct contact with the area around residue 55 in EGF1 of FIXa, the decrease in activation of FX caused by the mutation would have been much larger than the approximately three-fold reduction that we observed (Table 1). Nevertheless, Chang *et al*. and Celie *et al*. [9,10] have suggested that the linker region between EGF1 and EGF2 can interact directly with FVIIIa, which is also compatible with our preliminary 3D model of a FIXa-FVIIIa-antibody complex [19]. Any direct or indirect disturbance created by the EGF1 mutation might slightly modify the position of the FIXa active site in relation to the interaction with FX in such a way that the catalytic machinery will not function optimally. In our study, when only FIXa and FX were present in the assay, there was also a reduction in the formation of FXa. However, the degree of inhibition in this case was difficult to estimate due to the low absorbance values.

In conclusion, we found that the mutation Pro55Ser in FIX causes hemophilia primarily by impairing the ability of the protein to activate FX, whereas, at least *in vitro*, Pro55Leu leads to cleavage of FIXa during the activation process. The cleavage of FIXa Pro55Leu occurs in the serine protease domain at Arg318, indicating that intramolecular communication exists between EGF1 and the serine protease domain of FIXa. This finding was further supported by the results of our experiments with the mAb AW. Moreover, we noted that, during the activation of FX, residue 55 does not interact directly with FVIIIa in the Xase complex.

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