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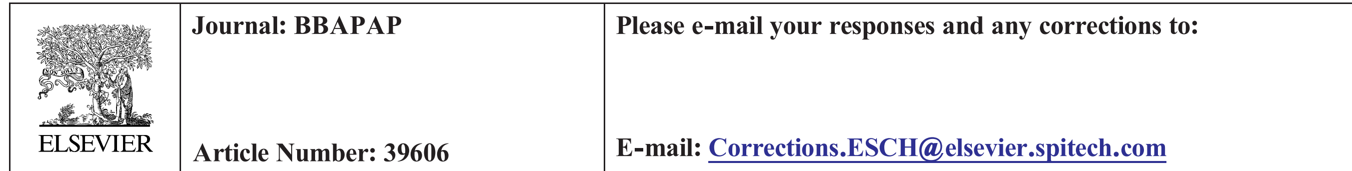

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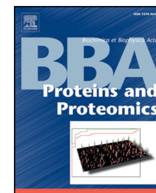

1 # Highlights

Biochimica et Biophysica Acta xxx (2015) xxx–xxx

2 **Asymmetric processing of mutant factor X Arg386Cys reveals differences between**
3 **intrinsic and extrinsic pathway activation**

4 M. Baroni [a,*], G. Pavani [a,b,c,d], M. Pinotti [a], A. Branchini [a], F. Bernardi [a], R.M. Camire [b,c,d]
5
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10 • Mutations at 386 position reduce FX activation by FIXa/VIIIa but not by FVIIa/TF.
11 • 386 substitutions do not affect amidolytic and prothrombinase activity.
12 • The recovery of the rFX386Cys variant in mice is comparable to that of rFXwt.
13 • rFX386Cys variant may be efficiently targeted by thiol-specific ligands.
14

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Supplementary Table 1 Coagulant activity of rFX variants in PT and aPTT based assays.The rFXwt, rFX386Cys, rFX386Ala and rFX379Ala-386Cys (6.6 nM for PT and 8.5 nM for aPTT based assays) were added to FX-deficient plasma and clotting time recorded upon triggering coagulation via the extrinsic or intrinsic pathways. A standard curve of rFXwt was used to evaluate activity of rFX variants, which is expressed as %. Coagulation times are reported as mean ± SD from thee independent assays.

Supplementary Table 2 Parameters of thrombin generation activity of rFX variants in plasma.Evaluation of the thrombin generation curves reported in Fig. 2. Values are reported for each rFX (2.5, 5 and 10 ng/mL) and TF (1 and 5 pM) concentration tested.

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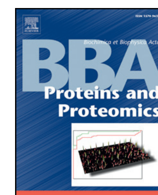

Q1 # Asymmetric processing of mutant factor X Arg386Cys reveals differences between intrinsic and extrinsic pathway activation

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ABSTRACT

Alterations in coagulation factor X (FX) activation, mediated by the extrinsic VIIa/tissue factor (FVIIa/TF) or the intrinsic factor IXa/factor VIIIa (FIXa/FVIIIa) complexes, can result in hemorrhagic/prothrombotic tendencies. However, the molecular determinants involved in substrate recognition by these enzymes are poorly defined. Here, we investigated the role of arginine 386 (chymotrypsin numbering c202), a surface-exposed residue on the FX catalytic domain. The naturally occurring FX386Cys mutant and FX386Ala variant were characterized. Despite the unpaired cysteine, recombinant (r)FX386Cys was efficiently secreted (88.6 ± 21.3% of rFXwt) and possessed normal clearance in mice. rFX386Cys was also normally activated by FVIIa/TF and displayed intact amidolytic activity. In contrast, rFX386Cys activation by the FIXa/FVIIIa complex was 4.5-fold reduced, which was driven by a decrease in the k_{cat} ($1.6 * 10^{-4}\ s^{-1}$ vs $5.8 * 10^{-4}\ s^{-1}$, rFXwt). The virtually unaltered K_m (70.6 nM vs 55.6 nM, rFXwt) suggested no major alterations in the FX substrate exosite. Functional assays in plasma supplemented with rFX386Cys indicated a remarkable reduction in the thrombin generation rate and thus in coagulation efficiency. Consistently, the rFX386Ala variant displayed similar biochemical features suggesting that global changes at position 386 impact the intrinsic pathway activation.
These data indicate that the FXArg386 is involved in FIXa/FVIIIa-mediated FX activation and help in elucidating the bleeding tendency associated with the FX386Cys in a rare FX deficiency case. Taking advantage of the unpaired cysteine, the rFX386Cys mutant may be efficiently targeted by thiol-specific ligands and represent a valuable tool to study FX structure–function relationships both *in vitro* and *in vivo*.
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1. Introduction

Over the past three decades significant progress has been made in characterizing the interactions governing the assembly of coagulation factor macromolecular enzyme complexes. The ideas and models generated indicate that blood coagulation serine proteases and their cofactors have evolved versatile regulatory mechanisms for controlling specificity of protein substrate recognition and activation [1–3]. Factor X (FX) acts at the crossroad of coagulation pathways triggered by blood vessel injury through the activation by factor VIIa/tissue factor (FVIIa/TF, extrinsic tenase complex) or by the amplification loop/contact phase via the factor IXa/factor VIIIa (FIXa/FVIIIa, intrinsic tenase complex). The balance between these activation pathways provides physiologic hemostasis. However, alteration of these pathways could produce bleeding or prothrombotic tendency [4], which has attracted attention for the design of specific anticoagulant therapeutic compounds.

While the interactions of activated FX (FXa) within the prothrombinase complex (FXa, factor Va, anionic membranes and calcium) have been extensively investigated [1,2], less is known about the specific determinants of the tenase complexes with respect to FX substrate recognition. Amino acid substitutions producing discrepancies between FX activation by the extrinsic (prothrombin time, PT) and intrinsic (activated partial thromboplastin time, aPTT) pathways provide potentially valuable models to address these issues and to elucidate structure–function relationships. While rare, there are a limited number of homozygous FX mutations with normal circulating FX levels [5–8], but with altered FX activation mainly by the extrinsic tenase complex [9–11].

The current work stems from the recent characterization of compound heterozygous FX deficient patients with normal or reduced coagulation FX activity levels in PT- or aPTT-based assays, respectively. The biochemical cause of the defect was found to be an Arg386Cys substitution, which is predicted to introduce an unpaired cysteine on the catalytic domain surface [12]. However, the very low levels of the

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recombinant rFX386Cys variant obtained in their experimental system prevented an appropriate and detailed biochemical characterization both in plasma and reconstituted systems. Here, we expressed a panel of FX mutants and show that the rFX386Cys is efficiently secreted and, upon injection in mice, displays normal clearance. Biochemical characterization of the purified variant indicates defective kinetics of activation by the intrinsic FIXa/FVIIIa, leading to a reduced thrombin generation in plasma.

2. Methods

2.1. Materials

The peptidyl substrate methoxycarbonyl-D-cyclohexylglycyl-gylcyl-arginine-para-nitroanilide acetate (Spectrozyme Xa) was from American Diagnostica (Greenwich, CT, USA). Fluorogenic substrates for FXa (SpectroFluor™ Xa, CH_3SO_2-D-CHA-Gly-Arg-AMC.AcOH) and thrombin (Benzoil-Phe-Val-Arg-AMC) were from American Diagnostica and MP Biomedicals (Costa Mesa, CA, USA), respectively.

All tissue culture reagents were from Invitrogen (Carlsbad, CA, USA). Small unilamellar phospholipid vesicles (PCPS) composed of 75% (w/w) hen egg L-phosphatidylcholine and 25% (w/w) porcine brain L-phosphatidylserine (Avanti Polar Lipids, Alabaster, AL, USA) were prepared as previously described [13].

2.2. Proteins

Human thrombin, FX, FXa, FIXa and RVV-X were from Hematologic Technologies (HTI, Vermont, USA). Recombinant factor VIII ReFacto® (FVIII) was from Wyeth Corporation (Collegeville, PA, USA). Hirudin was a kind gift from Dr S. Krishnaswamy (Children's Hospital of Philadelphia).

2.3. rFX molecules expression and purification

Mutations were inserted into the human FX cDNA cloned in pCMV4 [14,15] by the QuikChange site-directed mutagenesis kit (Stratagene, la Jolla, CA, USA) and the forward primers $^{5'}$CGCACGTC ACCTGCTTCAAGGACACC$^{3'}$ (rFX386Cys), $^{5'}$CGTCAC CGCCTTCAAGGA CACC$^{3'}$ (rFX386Ala), $^{5'}$CTCAAGTGGATCGACTGTTCCATGAAAACC$^{3'}$ (rFX424Cys), $^{5'}$GCCAGGGGGACGCCGGGGGCCCGCACGTCACCGG$^{3'}$ (rFX379Ala-386Cys), $^{5'}$CCCGAGTGTGACTGGGCCGAGTCCAC$^{3'}$ (rFX309C) and $^{5'}$GTGTGCTCCTGC GCCTGCGGGTACAC$^{3'}$ (rFX113Cys). The primer $^{5'}$ GCCAGGGGGACGCCGGGGGCCCG CACGTCACCGG$^{3'}$ was used on the pFX386Cys template to create the double mutant 379Ala-386Cys. Reverse primers were perfectly complementary to the forward ones. Direct sequencing validated all vectors.

Human Embryonic Kidney 293 (HEK293) cells were cultured and transiently or stably transfected to express the recombinant FX (rFX) variants as previously described [14–17]. To normalize transfection efficiency cells were co-transfected with the pGL3 vector to allow measurement of the Firefly Luciferase expression [18].

Recombinant FX proteins were purified from conditioned media using three-step chromatographic approach as described [16,17]. Briefly, Q-sepharose ionic exchange, FX immunoaffinity and hydroxyapatite chromatography were used to obtain fully γ-carboxylated FX molecules. rFX variants eluted by hydroxyapatite column were precipitated with ammonium sulfate and stored at −20 °C in 50% glycerol/water.

Protein purity was assessed by SDS-PAGE using 4–12% gels (Invitrogen) under reducing and non-reducing conditions, using the MES buffer system followed by staining with Coomassie Brilliant Blue R-250 (Thermo Fisher Scientific Inc., IL, USA). 5 μg of protein samples was separated for 35 min at 200 V and 13 mA.

2.4. Recovery of rFX in mice

Experiments were conducted on 6 weeks old C57Bl/6J mice (Jackson Laboratories, Bar Harbor, ME). Mice were injected via tail vein with 2.5 μg of rFX variant and blood samples collected by retro-orbital bleeding at 5 min, 1 h, 2 h and 5 h post-injection into capillary tubes containing heparin. FX antigen levels were detected by ELISA exploiting rabbit polyclonal anti-human FX antibodies (Dako, Agilent Technologies, Glostrup, Denmark). FX antigen values at 5 min post-injection were used to evaluate the protein recovery.

2.5. Coagulation assays on recombinant variants

2.5.1. PT-based assays

6.6 nM rFX in 20 mM HEPES, 150 mM NaCl, pH 7.4 (assay buffer) with 0.1% PEG-8000, 2 mM $CaCl_2$, 0.1% BSA, was added to a volume (50 μl) of FX-deficient plasma (George King, Overland Park, KS, USA) and the clotting time was recorded after the addition of thromboplastin (Thromborel, Dade Behring, Marburg, Germany), as source of lipidated recombinant tissue factor (TF).

2.5.2. APTT-based assays

50 μl of FX-deficient plasma were mixed with 50 μl of aPTT reagent, a synthetic mixture of phospholipid vesicles and a surface activator of the intrinsic coagulation system (Dade Behring) and 50 μl of rFX molecules (8.5 nM) diluted in assay buffer with 0.1% PEG-8000, 0.1% BSA, pH 7.4. After 3′ of incubation at 37 °C, 25 μl of 25 mM $CaCl_2$ were added and coagulation times measured.

PT- and aPTT-based assays were standardized using serial dilutions of rFXwt.

2.6. Thrombin generation assays

Thrombin generation assays were performed as previously described [19,20] according to Hemker method [21]. Briefly, the reaction was triggered by 1 or 5 pM TF and the calibrated automated thrombin activity was measured by using a fluorogenic substrate in a FX deficient plasma, reconstituted with 40, 85 or 170 nM rFX. The lag time, time to peak (ttpeak), peak and the endogenous thrombin potential (ETP) were obtained by analysis of the first derivative of relative fluorescence units (RFU) as function of time (min).

2.7. FXa activity

rFX mutants were activated by Russel's viper venom [22,23] (RVV-X) as previously described [24]. FXa amidolytic activity was measured toward increasing concentrations (0–125 μM) of the Spectrofluor™ Xa substrate added immediately before monitoring fluorescence (360 nm excitation, 465 nm emission) as a function of time.

2.8. FX activation

2.8.1. Extrinsic activation

20 pM FVIIa and innovin (1/100) were used to activate 100 nM rFXwt, rFX386Cys and rFX386Ala variants diluted in assay buffer with 0.1% PEG-8000 at 37 °C.

Titration of FX: rFX was activated at increasing concentrations (0–200 nM) and the reaction was stopped after 90 s in quench buffer (assay buffer with 50 mM EDTA).

Time course of activation: aliquots of the reaction were quenched at different time points (0, 30, 60, 90, 120, 180, 300 s) in quench buffer.

The generation of FXa was evaluated toward the synthetic substrate Spectrofluor™ Xa [25,26].

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2.8.2. Intrinsic activation

200 nM FVIII was previously activated by an equimolar concentration of thrombin and blocked after 30 s by adding an excess of 500 nM Hirudin (2.5 fold). The intrinsic tenase complex (0.5 nM FIXa, 5 nM FVIIIa, and 20 μM PCPS 75/25) was assembled in assay buffer with 0.01% (v/v) tween 80, 5 mM $CaCl_2$ at 37 °C. The reaction was initiated by the addition of increasing concentrations of zymogen rFX (2–250 nM) variants. Aliquots of the reaction were quenched at different time points (0, 30, 60, 90, 120, 180 s) in 50 mM EDTA, 20 mM Hepes, 150 mM NaCl, 0.01% (v/v) tween 80, pH 7.4.

Initial rates of rFXa formation were monitored measuring the A_{405} at 22 °C of chromogenic substrate hydrolysis (Spectrozyme Xa, 250 μM). The rFXa generated was quantified using a standard curve of FXa.

2.8.3. Kinetic analysis

Steady-state kinetic constants were determined from measurements of the initial velocity obtained using different substrate concentrations. Data were fitted to the Michaelis–Menten equation, by non-linear regression analysis, using GraphPad Prism software (GraphPad, Inc., California, USA) [27,28].

3. Results

3.1. The rFX386Cys is efficiently secreted

In transient expression experiments, the rFX386Cys (88.6% ± 21.3%) and rFXwt (100% ± 11.8%) levels in conditioned media were comparable. The substitution of other surface-exposed arginine residues with cysteine within the light (rFX113Cys) or heavy (rFX309Cys, rFX424Cys) chains resulted in a remarkable reduction (range 13–27% of rFXwt) of secreted protein (Fig. 1A).

Stable clones were established to purify and characterize rFXwt and the rFX386Cys. To further investigate the role of arginine 386, we also expressed and purified the rFXR386A and, as a negative control, the inactive rFX379Ala–386Cys mutant, carrying the Ser379Ala substitution of the catalytic serine. Similar protein yields were obtained for all purified variants (rFX386Cys 1.83 mg, rFX386Ala 3.83 mg, rFX379Ala–386Cys 3.68 mg). The three-step purification process resulted in fully γ-carboxylated proteins that migrated as a single band on a non-reducing gel with a molecular mass of approximately 59 kDa in SDS-PAGE, and as two bands (heavy and light chain) on a reducing gel (Fig. 1B and C). Recombinant proteins migrated in a similar fashion as plasma-derived FX excluding major differences in post-translational modifications and the presence of disulfide linked homo- or heterodimers.

3.2. The rFX386Cys possesses normal clearance in vivo

To compare the *in vivo* stability, the rFX386Cys or rFXwt proteins were injected in mice (2.5 μg/mouse) and human FX antigen levels monitored over time (Fig. 1D). rFX386Cys recovery in mouse plasma at 5 min post-injection as well as its clearance were not statistically different from those of rFXwt (non-parametric *T* test, P value 0.3836).

3.3. Activity of rFX variants

3.3.1. Reduced rFX386Cys coagulant activity in aPTT-based assay

In PT-based clotting assays the activity of the rFX386Cys (105.7% of rFXwt) as well as that of the rFX386Ala (173.6%) variant was not

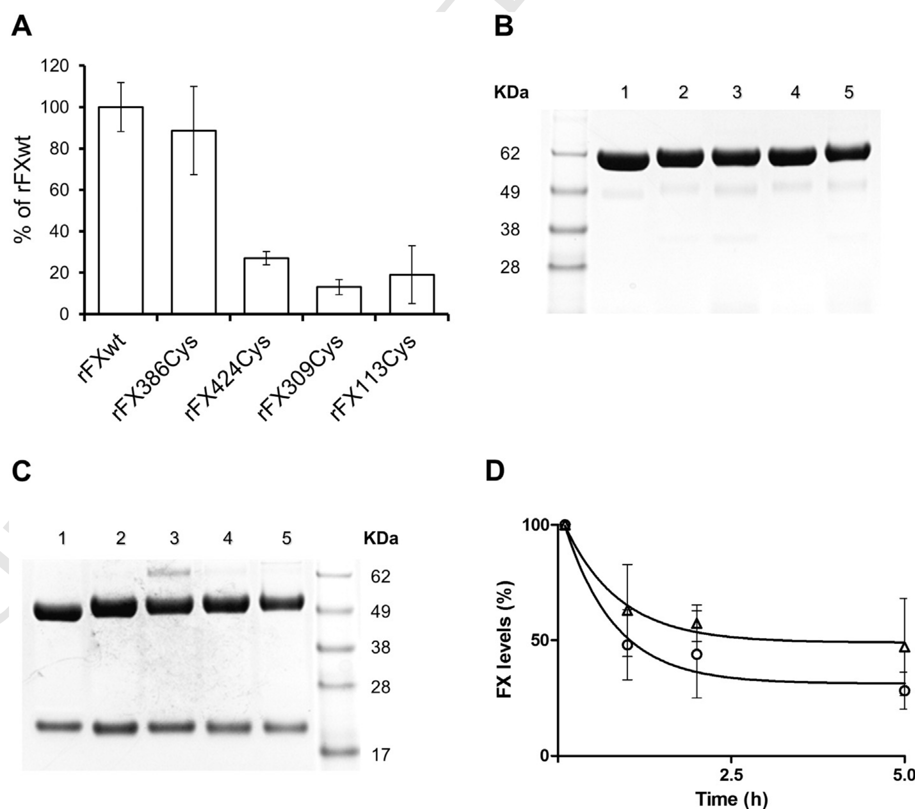

Fig. 1. Evaluation of rFX secretion, protein purification and of *in vivo* clearance. A) Secreted rFX levels from HEK293 cells at 24 h post-transfection. Values are expressed as % of the rFXwt (93.2 ± 11.0 ng/ml) and reported as mean ± standard deviation (SD) from three independent experiments. B, C) Quality control of purified rFX variant. 5 μg of plasma-derived FX (1), rFXwt (2), rFX379Ala–386Cys (3), rFX386Ala (4) and rFX386Cys (5) were separated by SDS-PAGE on 4–12% polyacrylamide gel under non-reducing (B) and reducing (C) conditions in a MES buffer system. rFX variants were revealed by Coomassie Brilliant Blue R-250 staining. D) Clearance of rFXwt and rFX386Cys in mice. Mice (3/group) were injected with 2.5 μg of purified rFXwt (Δ) or rFX386Cys (O) and FX antigen levels monitored at 5 min, 1, 2 and 5 h later. Human FX levels measured at 5′ post-injection were considered as 100%. Values are reported as mean ± SD.

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affected (Supplementary Table 1). However, in aPTT-based assays, the activities of the rFX386Cys (28.7%) and rFX386Ala (52.3%) were significantly reduced compared to rFXwt. As expected, the activity of the rFX379Ala-386Cys protein was undetectable in all conditions. These results indicated that replacement of Arg386 selectively interferes with FX intrinsic activation in plasma.

3.3.2. rFX386Cys has reduced thrombin generation activity

To obtain a more comprehensive picture, thrombin generation assays using FX deficient plasma reconstituted with rFX variants were employed (Fig. 2). The thrombin generation curves of the rFX386Cys differed the most from rFXwt at low TF amount (1 pM), as indicated by the assay parameters (Supplementary Table 2). At physiological concentration of rFX (10 μg/mL) the prolonged lag time (+28%) and time to peak (+22.8%) were associated with a decreased thrombin peak (−31.2%). Intermediate values were detected for the rFX386Ala. When the assay was designed to incorporate a larger contribution from the extrinsic pathway by using higher TF concentrations (5 pM) the differences between the variants and rFXwt were negligible. This again highlights that the Arg386 variants are defective in the intrinsic pathway and not in the extrinsic pathway. To mimic the compound heterozygous condition observed in the previously described patient [12] the assay was performed with lower FX levels (2.5 and 5 μg/mL rFX386Cys). Using these conditions, remarkable differences were detected for all parameters with the mutant compared to rFXwt (Supplementary Table 2) with a peak reduction of 65%.

3.3.3. rFX386Cys has normal amidolytic activity

Preliminary evaluation with the potent non-physiological FX activator RVV-X_{CP} indicated that both the rFXa386Cys and rFXa386Ala variants were activated like rFXwt and exhibited normal amidolytic activity (data not shown). This experiment ruled out an impact of the mutations on the active site conformation and serine protease activity. This provided the rationale for monitoring the rFX to rFXa conversion by the extrinsic and intrinsic tenase complexes using rFXa amidolytic activity as a measure of activation.

3.4. Activation of rFX variants

3.4.1. Normal activation of rFX386Cys by the extrinsic FVIIa/TF tenase complex

The extrinsic tenase activation of rFX386Cys and rFX386Ala was dissected by functional assessments in a reconstituted system with purified proteins. The concentration-dependence of FX activation by TF/FVIIa complex is displayed in Fig. 3A. The kinetic parameters for FVIIa/TF-mediated activation of the rFX386Cys ($K_m = 8.2 \pm 4.6$ nM) and rFX386Ala ($K_m = 16.2 \pm 8.1$ nM) were not significantly different ($P = 0.30$) from those observed for wild-type ($K_m = 18.2 \pm 9.1$ nM), indicating normal interactions among extrinsic Xase complex and rFX molecules. In time course analysis, the rate of rFX386Cys (0.033 ± 0.001 nM s^{-1}) and rFX386Ala (0.043 ± 0.002 nM s^{-1}) activation by FVIIa/TF was similar to that of rFXwt (0.045 ± 0.002 nM s^{-1}, Fig. 3B). These data excluded major alterations in FVIIa/TF-mediated FX activation consistent with TF-mediated functional assays (e.g. PT assay).

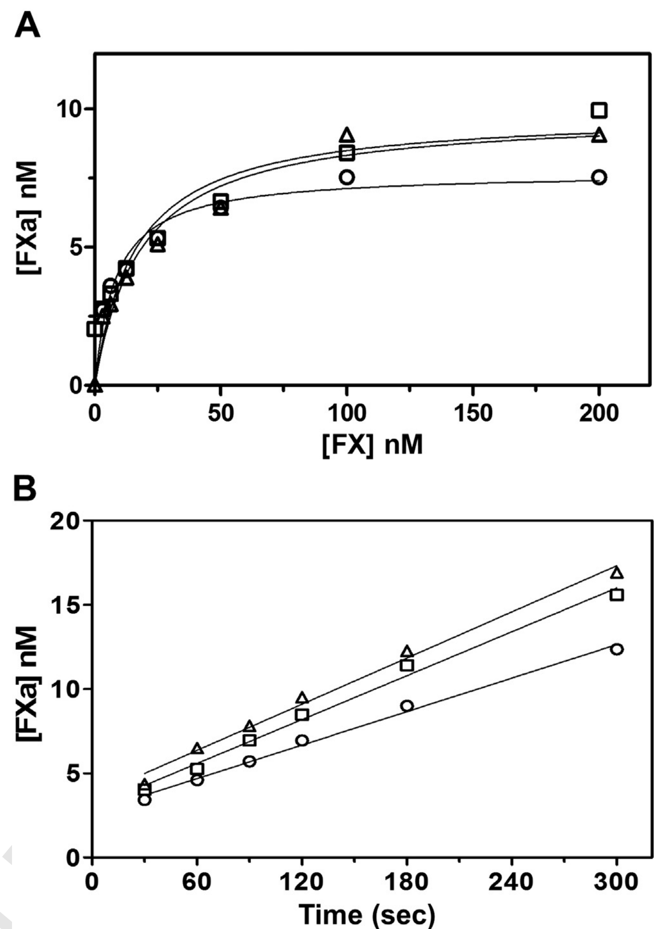

Fig. 3. Activation of rFX variants by the extrinsic FVIIa/TF tenase complex. The rFXwt (Δ), rFX386Cys (O) and rFX386Ala (□) were activated by 20 pM FVIIa in the presence of TF and phospholipids, and the generation of FXa was evaluated as activity toward a specific fluorogenic substrate. The amount of FXa generated was derived from a standard curve of plasma-derived FXa. Results from FX Titration (A) and Time course of FX activation (B) are shown. Graphs A and B are representative of three independent experiments.

3.4.2. Reduced activation of rFX386Cys by the intrinsic FIXa/FVIIIa tenase complex

Kinetics of FX activation by the intrinsic tenase complex (FIXa/FVIIIa) in the presence of anionic phospholipids was assessed using increasing FX concentrations (Fig. 4). Similar K_m values ($P = 0.73$) were obtained for rFXwt (55.6 nM, range: 36.8–74.3 nM), rFX386Cys (70.6 nM, range: 62.4–78.9 nM) and rFX386Ala (64.4 nM, range:

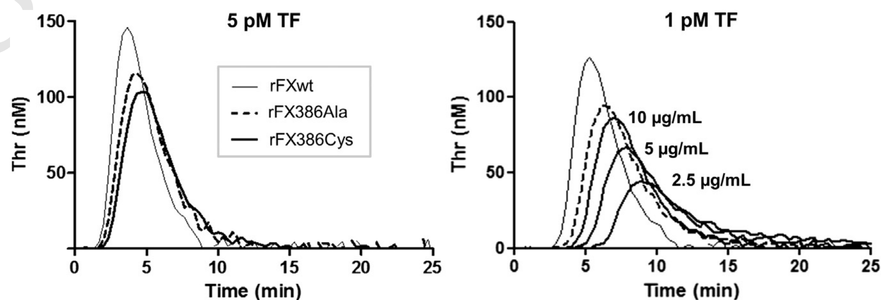

Fig. 2. Thrombin generation activity of rFX variants in plasma. Thrombin generation activity in FX deficient plasma supplemented with 10 μg/mL of rFXwt (thin line), rFX386Cys (thick line) and rFX386Ala (dotted line) upon triggering coagulation with 5 pM or 1 pM of TF. The thrombin generation curve obtained with the rFX386Cys at concentrations of 2.5 and 5 μg/mL is also shown.

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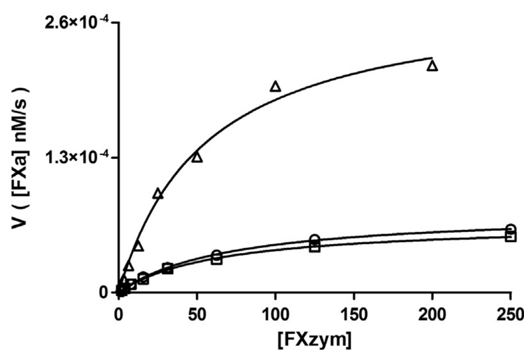

Molecules	K_m (nM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($nM^{-1}*sec^{-1}$)	V_{MAX} ($nM^{*}sec^{-1}$)
rFXwt	55.6	5.8*10^{-4}	1.0*10^{-5}	2.9*10^{-4}
rFX386Cys	70.6	1.6*10^{-4}	2.2*10^{-6}	7.8*10^{-5}
rFX386Ala	64.4	1.3*10^{-4}	2.1*10^{-6}	6.7*10^{-5}
rFX379Ala-386Cys	nd	nd	nd	nd

Fig. 4. Activation of rFX variants by the intrinsic FIXa/FVIIIa tenase complex. rFX variants (2–250 nM) were activated by FIXa (0.5 nM), FVIIIa (5 nM) and PCPS 75/25 liposomes (20 μM) and the reaction quenched at different time points (0, 30, 60, 90, 120, 180 s). The initial rates of rFXa formation were monitored measuring the A_{405} of chromogenic substrate hydrolysis. The K_m, k_{cat} and V_{max} for intrinsic FX activation were determined by fitting data by non-linear regression to the Michaelis–Menten equation. Curves and kinetic values are the average of two independent experiments.

56.0–72.8 nM). However, the initial rates of FX activation were substantially reduced for rFX386Cys and rFX386Ala compared to rFXwt. Data analysis showed that the k_{cat} of rFXwt ($5.8 * 10^{-4}$ s^{-1}, range: $5.0–6.6 * 10^{-4}$) was 4–5 times higher compared to rFX386Cys ($1.6 * 10^{-4}$ s^{-1}, range: $1.5–1.6 * 10^{-4}$) and rFX386Ala ($1.3 * 10^{-4}$ s^{-1}, range: $1.3–1.4 * 10^{-4}$). These findings indicate that the mutation at position 386 has no impact on the ability of FX to engage FIXa/VIIIa but on its ability to be converted into the product. It is likely that this is mediated through allosteric effects that impact the ability of the enzyme to catalyze substrate cleavage.

4. Discussion

Blood coagulation serine proteases and their cofactors have evolved versatile regulatory mechanisms for controlling the specificity of protein substrate activation. The extrinsic and intrinsic enzyme complexes that activate FX are of noticeable importance for physiologic hemostasis and dysregulation of their activity can produce a hemorrhagic or prothrombotic tendency.

We and other have demonstrated that the GLA domain strongly contributes to the activity of the extrinsic [9–11] rather than the intrinsic tenase complex. Here, by taking advantage of the biochemical characterization of the purified rFX386Cys natural variant we have shown arginine 386 (c202) is crucial for optimal FX activation via the intrinsic pathway.

Among human vitamin K-dependent coagulation factors position 386 (c202, chymotrypsin numbering) is occupied by several amino acids (E, H, K, N, R and S). In the crystallographic structure of FXa [29], arginine 386 is surface exposed (Fig. 5) thus potentially underlying a functional exosite, far from the known exosites for FVa in the prothrombinase complex [30–33]. Moreover, R386 does not belong to the activation loops 140s (142–152) and 170s (170–182) that in FVII have been shown to be involved in the allosteric conformational rearrangements following zymogen cleavage and leading to maturation of the active site [34]. In line with this, substitutions at the 386 position did not affect the amidolytic and prothrombinase activity of the resulting FXa as indicated by functional assays upon FVIIa/TF activation.

Our findings are consistent with the hypothesis that mutations at position 386 reduce FX activation by the FIXa/VIIIa complex rather than affecting the assembly in the extrinsic tenase complex. The analysis of individual kinetic constants indicates that these mutations do not destabilize the Michaelis complex, as evidenced by the unchanged K_m values. This observation supports the conclusion that this region of FX marked by residue 386 does not contribute to the substrate binding exosite for the FIXa/FVIIIa complex. On the other hand, these data contribute to understand the poorly known processes underlying the FIXa/FVIIIa-mediated FX activation [35,36] and point toward a mechanism in which the R386 substitution imposes a conformational change in the FX zymogen and affects the allosteric conformational changes leading to the proper FXa conversion.

The overall detrimental effect of the rFX386Cys and rFX386Ala mutations was relatively modest as shown by a 4.5-fold lower k_{cat}/K_m, relative to rFXwt. From this observation, and particularly the limited impact of a large change in the side chain at position 386, we infer that the Arg386 is only one among many interactions participating in the network of contacts leading to zymogen FX recognition and activation by the FIXa/FVIIIa complex. However these data indicate that the FX region at and near position 386 act as a key determinant for the activity and specificity of the intrinsic tenase complex so far identified [37].

In line with the kinetic constants, results from thrombin generation assays in plasma systems displayed a significant effect of the Arg386Cys substitution. These assays offer an appreciable estimate of the functional properties of the rFX386Cys in plasma, particularly at low TF concentration, a condition that decreases the contribution of the extrinsic pathway and better mimics physiological coagulation. Moreover, this experimental system was exploited to simulate the FX deficiency reported in the patient [12], characterized by reduced levels of the

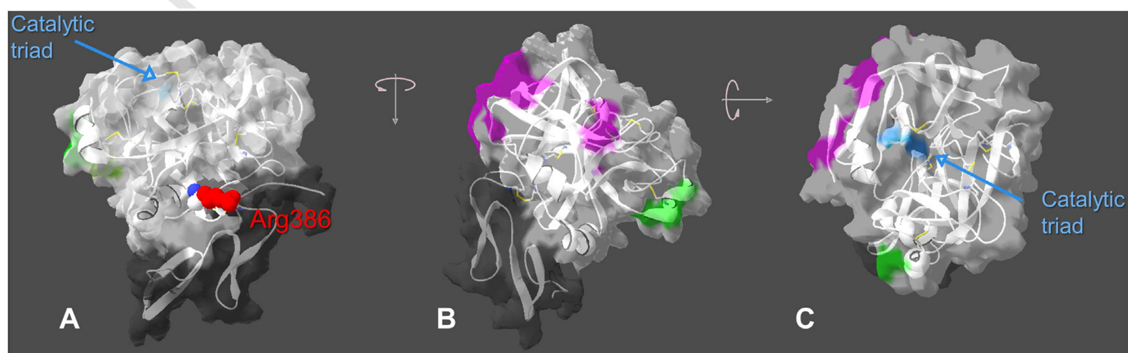

Fig. 5. Model of the three-dimensional structure of the activated FX. Three views of the FXa (PDB ID: 1HCG), two sides (A and B) and the upper view (C). In the model are highlighted the heavy subunit (light gray), the second epidermal growth factor-like domain portion of the light subunit (dark gray), the disulfide bonds (yellow), the catalytic triad (light blue), the Arg386 (red), the residues involved in prothombinase complex assembly (violet) [32], the Arg165 and Lys169 (green) described to play a key roles in factor FXa–FVa recognition [33]. Images were generated by using Swiss-PDBViewer 4.1.

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FX386Cys only. Noticeably, we observed a substantial impact on thrombin generation rate, which is a recognized determinant of coagulation efficiency [38].

The availability of a directly labeled FX variant with features able to dissect the extrinsic and intrinsic activation pathways is of great interest to address the impact of these pathways *in vivo*. Interestingly, the rFX386Cys variant was efficiently secreted in spite of the insertion of an unpaired cysteine, which clearly affects secreted levels of the other Arg to Cys changes on the protein surface that we have investigated. Moreover, studies in mice indicated that the rFX386Cys and rFXwt molecules behaved similarly in terms of recovery. These features make the rFX386Cys variant a potential target for thiol-specific ligands for study in mice using established models such as intravital microscopy [39,40].

5. Conclusions

The definition of the molecular bases of a rare FX deficiency coupled to the recombinant expression and characterization of the variant showed that Arg386 is involved in the specific activation by the intrinsic FIXa/FVIIIa complex. In turn, the rFX386Cys molecule is a valuable tool to study FX structure–function relationships as well as the individual contribution of activation pathways to thrombus formation *in vivo*.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbapap.2015.05.012.

Competing interests

R.M.C. receives licensing fees and research funding from Pfizer for work related to FXa. The other authors declare no competing interests.

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References

[1] S. Krishnaswamy, Exosite-driven substrate specificity and function in coagulation, J. Thromb. Haemost. 3 (2005) 54–67.
[2] P. Kamath, J.A. Huntington, S. Krishnaswamy, Ligand binding shuttles thrombin along a continuum of zymogen- and proteinase-like states, J. Biol. Chem. 285 (2010) 28651–28658.
[3] J.A. Huntington, Natural inhibitors of thrombin, Thromb. Haemost. 111 (2014) 583–589.
[4] D. Gailani, T. Renné, Intrinsic pathway of coagulation and arterial thrombosis, Arterioscler. Thromb. Vasc. Biol. 27 (2007) 2507–2513.
[5] F. Peyvandi, M. Menegatti, E. Santagostino, S. Akhavan, J. Uprichard, D.J. Perry, S.J. Perkins, P.M. Mannucci, Gene mutations and three-dimensional structural analysis in 13 families with severe factor X deficiency, Br. J. Haematol. 117 (2002) 685–692.
[6] D.S. Millar, L. Elliston, P. Deex, M. Krawczak, A.I. Wacey, J. Reynaud, H.K. Nieuwenhuis, P. Bolton-Maggs, P.M. Mannucci, J.C. Reverter, P. Cachia, K.J. Pasi, D.M. Layton, D.N. Cooper, Molecular analysis of the genotype–phenotype relationship in factor X deficiency, Hum. Genet. 106 (2000) 249–257.
[7] F. Peyvandi, P.M. Mannucci, Rare coagulation disorders, Thromb. Haemost. 82 (1999) 1207–1214.
[8] J. Uprichard, D.J. Perry, Factor X deficiency, Blood Rev. 16 (2002) 97–110.
[9] M. Pinotti, G. Marchetti, M. Baroni, F. Cinotti, M. Morfini, F. Bernardi, Reduced activation of the Gla19Ala FX variant via the extrinsic coagulation pathway results in symptomatic CRMred FX deficiency, Thromb. Haemost. 88 (2002) 236–241.
[10] H.H. Watzke, K. Lechner, H.R. Roberts, S.V. Reddy, D.J. Welsch, P. Friedman, G. Mahr, P. Jagadeeswaran, D.M. Monroe, K.A. High, Molecular defect (Gla + 14Lys) and its functional consequences in a hereditary factor X deficiency (Factor X "Voralberg"), J. Biol. Chem. 265 (1990) 11982–11989.
[11] A.E. Rudolph, M.P. Mullane, R. Porche-Sorbet, S. Tsuda, J.P. Miletich, Factor XSt. Louis II. Identification of a glycine substitution at residue 7 and characterization of the recombinant protein, J. Biol. Chem. 271 (1996) 28601–28606.
[12] A.L. Vanden Hoek, K. Talbot, I.S. Carter, L. Vickars, C.J. Carter, S.C. Jackson, R.T. MacGillivray, E.L. Pryzdial, Coagulation factor X Arg386 specifically affects activation by the intrinsic pathway: a novel patient mutation, J. Thromb. Haemost. 10 (2012) 2613–2615.
[13] D.L. Higgins, K.G. Mann, The interaction of bovine factor V and factor V-derived peptides with phospholipid vesicles, J. Biol. Chem. 258 (1983) 6503–6508.
[14] M. Pinotti, R.M. Camire, M. Baroni, A. Rajab, G. Marchetti, F. Bernardi, Impaired prothrombinase activity of factor X Gly381Asp results in severe familial CRM+ FX deficiency, Thromb. Haemost. 89 (2003) 243–248.
[15] R. Toso, H. Zhu, R.M. Camire, The conformational switch from the factor X zymogen to protease state mediates exosite expression and prothrombinase assembly, J. Biol. Chem. 283 (2008) 18627–18635.
[16] P.J. Larson, R.M. Camire, D. Wong, N.C. Fasano, D.M. Monroe, P.B. Tracy, K.A. High, Structure/function analyses of recombinant variants of human factor Xa: factor Xa incorporation into prothrombinase on the thrombin-activated platelet surface is not mimicked by synthetic phospholipid vesicles, Biochemistry 37 (1998) 5029–5038.
[17] R.M. Camire, P.J. Larson, D.W. Stafford, K.A. High, Enhanced gamma-carboxylation of recombinant factor X using a chimeric construct containing the prothrombin propeptide, Biochemistry 39 (2000) 14322–14329.
[18] C. Bertolucci, N. Cavallari, I. Colognesi, J. Aguzzi, Z. Chen, P. Caruso, A. Foá, G. Tosini, F. Bernardi, M. Pinotti, Evidence for an overlapping role of CLOCK and NPAS2 transcription factors in liver circadian oscillators, Mol. Cell. Biol. 28 (2008) 3070–3075.
[19] A. Branchini, L. Rizzotto, G. Mariani, M. Napolitano, M. Lapecorella, M. Giansily-Blaizot, R. Mari, A. Canella, M. Pinotti, F. Bernardi, Natural and engineered carboxy-terminal variants: decreased secretion and gain-of-function result in asymptomatic coagulation factor VII deficiency, Haematologica 97 (2012) 705–709.
[20] N. Cavallari, D. Balestra, A. Branchini, I. Maestri, A. Chuamsunrit, W. Sasanakul, G. Mariani, F. Pagani, F. Bernardi, M. Pinotti, Activation of a cryptic splice site in a potentially lethal coagulation defect accounts for a functional protein variant, Biochim. Biophys. Acta 1822 (2012) 1109–1113.
[21] H.C. Hemker, P. Giesen, R. Al Dieri, V. Regnault, E. de Smedt, R. Wagenvoord, T. Lecompte, S. Béguin, Calibrated automated thrombin generation measurement in clotting plasma, Pathophysiol. Haemost. Thromb. 33 (2003) 4–15.
[22] R.G. Di Scipio, M.A. Hermodson, E.W. Davie, Activation of human factor X (Stuart factor) by a protease from Russell's viper venom, Biochemistry 16 (1977) 5253–5260.
[23] M. Baroni, G. Pavani, D. Marescotti, T. Kaabache, D. Borgel, S. Gandrille, G. Marchetti, C. Legnani, A. D'Angelo, M. Pinotti, F. Bernardi, Membrane binding and anticoagulant properties of protein S natural variants, Thromb. Res. 125 (2010) e33–e39.
[24] S. Krishnaswamy, W.R. Church, M.E. Nesheim, K.G. Mann, Activation of human prothrombin by human prothrombinase. Influence of factor Va on the reaction mechanism, J. Biol. Chem. 262 (1987) 3291–3299.
[25] O. Olivieri, N. Martinelli, M. Baroni, A. Branchini, D. Girelli, S. Friso, F. Pizzolo, F. Bernardi, Factor II activity is similarly increased in patients with elevated apolipoprotein CIII and in carriers of the factor II 20210A allele, J. Am. Heart. Assoc. 2 (2013) e000440.
[26] A. Branchini, M. Baroni, C. Pfeiffer, A. Batorova, M. Giansily-Blaizot, J.F. Schved, G. Mariani, F. Bernardi, M. Pinotti, Coagulation factor VII variants resistant to inhibitory antibodies, Thromb. Haemost. 112 (2014) 972–980.
[27] J.P. Wood, J.R. Silveira, N.M. Maille, L.M. Haynes, P.B. Tracy, Prothrombin activation on the activated platelet surface optimizes expression of procoagulant activity, Blood 117 (2011) 1710–1718.
[28] M. Baroni, C. Pizzirani, M. Pinotti, D. Ferrari, E. Adinolfi, S. Calzavarini, P. Caruso, F. Bernardi, F. Di Virgilio, Stimulation of P2 (P2X7) receptors in human dendritic cells induces the release of tissue factor-bearing microparticles, FASEB J. 21 (2007) 1926–1933.
[29] K. Padmanabhan, K.P. Padmanabhan, A. Tulinsky, C.H. Park, W. Bode, R. Huber, D.T. Blankenship, A.D. Cardin, W. Kisiel, Structure of human des (1–45) factor Xa at 2.2 A resolution, J. Mol. Biol. 232 (1993) 947–966.
[30] A. Chattopadhyay, H.L. James, D.S. Fair, Molecular recognition sites on factor Xa which participate in the prothrombinase complex, J. Biol. Chem. 267 (1992) 12323–12329.
[31] A.E. Rudolph, R. Porche-Sorbet, J.P. Miletich, Definition of a factor Va binding site in factor Xa, J. Biol. Chem. 276 (2001) 5123–5128.
[32] M. Wilkens, S. Krishnaswamy, The contribution of factor Xa to exosite-dependent substrate recognition by prothrombinase, J. Biol. Chem. 277 (2002) 9366–9374.
[33] A.R. Rezaie, Identification of basic residues in the heparin-binding exosite of factor Xa critical for heparin and factor Va binding, J. Biol. Chem. 275 (2000) 3320–3327.
[34] J. Jin, L. Perera, D. Stafford, L. Pedersen, Four loops of the catalytic domain of factor viia mediate the effect of the first EGF-like domain substitution on factor viia catalytic activity, J. Mol. Biol. 307 (2001) 1503–1517.
[35] S.H. Qureshi, L. Yang, A.R. Rezaie, Contribution of the NH2-terminal EGF-domain of factor IXa to the specificity of intrinsic tenase, Thromb. Haemost. 108 (2012) 1154–1164.
[36] M. Takeyama, H. Wakabayashi, P.J. Fay, Factor VIII light chain contains a binding site for factor X that contributes to the catalytic efficiency of factor Xase, Biochemistry 51 (2012) 820–828.
[37] L. Chen, C. Manithody, L.K. Yang, A.R. Rezaie, Zymogenic and enzymatic properties of the 70–80 loop mutants of factor X/Xa, Protein Sci. 13 (2004) 431–442.
[38] Q. Liang, Q. Chen, Q. Ding, F. Wu, X. Wang, X. Xi, H. Wang, Six novel missense mutations causing factor X deficiency and application of thrombin generation est, Thromb. Res. 131 (2013) 554–559.
[39] A.T. Krueger, B. Imperiali, Fluorescent amino acids: modular building blocks for the assembly of new tools for chemical biology, Chembiochem 14 (2013) 788–799.
[40] L. Ivanciu, S. Krishnaswamy, R.M. Camire, New insights into the spatiotemporal localization of prothrombinase in vivo, Blood 124 (2014) 1705–1714.

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