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BRIEF REPORT

The carboxyl-terminal region is NOT essential for secreted and functional levels of coagulation factor X

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Summary. *Background:* The homologous coagulation factor X (FX), VII (FVII), IX (FIX) and protein C (PC) display striking differences in the carboxyl-terminus, with that of FX being the most extended. This region is essential for FVII, FIX and PC secretion. *Objectives:* To provide experimental evidence for the role of the FX carboxyl-terminus. *Methods:* Recombinant FX (rFX) variants were expressed in multiple eukaryotic cell systems. Protein and activity levels were evaluated by ELISA, coagulant and amidolytic assays. *Results and discussion:* Expression of a panel of progressively truncated rFX variants in HEK293 cells revealed that the deletion of up to 21 residues in the carboxyl-terminus did not significantly affect secreted protein levels, as confirmed in HepG2 and BHK21 cells. In contrast, chimeric rFX-FVII variants with swapped terminal residues showed severely reduced levels. The truncated rFX variants revealed normal amidolytic activity, suggesting an intact active site. Intriguingly, these variants, which included that resembling the activated FXβ form once cleaved, also displayed remarkable or normal pro-coagulant capacity in PT- and aPTT-based assays. This supports the hypothesis that subjects with nonsense mutations in the FX carboxyl-terminus, so far never identified, would be asymptomatic. *Conclusions:* For the first time we demonstrate that the FX carboxyl-terminal region downstream of residue K467 is not essential for secretion and provides a modest contribution to pro-coagulant properties. These findings, which might suggest an involvement of the carboxyl-terminal region in the divergence of the homologous FX, FVII, FIX and PC, help to interpret the mutational pattern of FX deficiency.

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Introduction

The unique specificity and regulation of serine proteases acting in the coagulation pathway, which share high structure and sequence similarities both at the gene and protein level, stem from the evolution and divergence of specific protein regions [1]. The identification of determinants underlying the specific features of the homologous pro-coagulant factors VII (FVII), IX (FIX) and X (FX) and the anti-coagulant protein C (PC) [2–4] contributes to understanding the evolutionary pathways that led to their divergence and specialization within the well-orchestrated coagulation cascade [5].

One of the protein regions displaying the most striking differences is represented by the carboxyl-terminus, which varies both in length and amino acid composition. Previous studies conducted by us [6–8] and others [9–12], through the characterization of nonsense and missense as well as frame-shifted variants of FVII, FIX and PC, have demonstrated that this region represents a key determinant for efficient secretion. So far, very little is known about FX, which is characterized by the most extended carboxyl-terminal region.

In this study we addressed this issue by evaluating the secreted protein and pro-coagulant activity levels of an ample panel of recombinant FX (rFX) variants progressively truncated at the carboxyl terminus. The appreciable secretion as well as pro-coagulant and amidolytic activity levels of these FX variants help to interpret the mutational pattern of FX deficiency.

Materials and methods

Expression vectors

Expression vectors were created by site-directed mutagenesis of the human FX cDNA, cloned into the pCMV4 plasmid, and of the human FVII cDNA, cloned into pCDNA3, by using the QuickChange® II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), essentially as previously described [13]. The forward oligonucleotides 5′-GAGGTCATAACG*TGA*TCTCCATTAAAG-3′ (pFX-484X), 5′-GCCCCGGAGGTC*TAG*ACG TCC TCTCC-3′ (pFX-482X), 5′-GAGCCATGCCCCG*TAG*GT CATAACG-3′ (pFX-480X), 5′-GCCAAGAGCCATGCC-TAGGAGGTCATAACG-3′ (pFX-479X), 5′-GCCAAGA GCCAT*TGA* CCGGAGGTCATAACG-3′ (pFX-478X), 5′-GGCTTGCCCAAG*TAG*AAGAGCCATGCC-3′ (pFX-474X), 5′-CCAGGGGCTTG*TAG*AAGGCCAAGAGC-3′ (pFX-472X), 5′-CCATGAAAA CCAGG*TGA*TTGCC-CAAGGCC-3′ (pFX-470X), 5′-GTCCATGAAAACC*TGA* GGCTTGCCC AAG-3′ (pFX-469X), 5′-CAGGTCCATG AAA*TAG*AGGGGCTTGCCC-3′ (pFX-468X), 5′-GA CA GGTCCATG*TAG*ACCAGGGGCTTG-3′ (pFX-467X), 5′-GCCAAGAGCCATGCCCCG TTTCCCTAGGAGGTC ATAACGTCC-3′ (pFX_{FVII}), 5′-CCTCCTGCGAGCCCC AGAGGTCA TAACGTCCTTTCCCTAGCCCAGCAG C-3′ ($pFVII_{FX\text{-}EVITS}$) and 5′-GAGGTCATAACGTCC TCTCCATTAAAGTGATTTCCCTTGCCCAGCAGC-3′ ($pFVII_{FX}$) were used. The modified nucleotides (underlined) and nonsense triplets (italics) are indicated. Reverse oligonucleotides were complementary to the forward ones. All plasmids have been validated by sequencing.

Transient expression of recombinant variants

Human embryonic kidney (HEK) 293, baby hamster kidney (BHK) 21 and hepatocellular carcinoma (HepG2) cells were transiently transfected as previously described [7]. Transfection efficiency was evaluated by cell co-transfection with the pGL3 vector encoding the firefly luciferase followed by luciferase reporter assay in cell lysates (Promega, Madison, WI, USA) by using the GloMax® 20/20 Luminometer (Promega). All transfections were performed at least in triplicate.

Protein levels for rFX variants in conditioned media were evaluated by ELISA with anti-human FX polyclonal antibodies (Cedarlane, Burlington, ON, Canada). Known concentrations of plasma-derived human FX (Haematologic Technologies Inc., Essex Junction, VT, USA) diluted in mock-medium were used as the reference curve. Recombinant FVII (rFVII) protein levels were evaluated by ELISA as previously described [7].

Coagulation assays

Coagulant activity assays were assessed in FX-depleted plasma (HemosIL, Instrumentation Laboratory, Lexington, MA, USA) supplemented with rFX variants in conditioned medium. In prothrombin time (PT)-based assays, RecombiPlasTin 2G (HemosIL) was used as the trigger. Activated partial thromboplastin time (aPTT)-based assays were performed as previously described [8]. Coagulation times from serial dilutions of rFX-wt were used as the reference curve. All assays were conducted in triplicate.

Amidolytic activity toward the FXa-specific fluorogenic substrate

The amidolytic activity of rFX variants in response to the activated FX (FXa) fluorogenic substrate (250 μM, SpectroFluor FXa, American Diagnostica Inc., Greenwich, CT, USA) was assayed by measuring fluorescence emission (Relative Fluorescence Units, RFU) over time on a microplate fluorometer (Fluoroskan Ascent FL, Thermo Fisher Scientific, Helsinki, Finland) after activation with Russell's viper venom (RVV, Haematologic Technologies Inc) for 15 min at 37 °C in the presence of 8 mM $CaCl_2$ [14]. Serial dilutions of rFX-wt were used as reference. All assays were conducted at least in duplicate.

Data analysis

Data were analyzed by the statistic software GRAPHPAD PRISM 5 (GraphPad Software, San Diego, CA, USA). Coagulant assays were analyzed by a two-phase decay non-linear fit of coagulation times. The amidolytic activity was determined by a linear regression analysis of the initial rate of fluorescence emission. Statistical differences in protein levels were evaluated by the *t*-test.

Results and discussion

The study rationale stems from previous observations showing that the natural full-length carboxyl-terminus is essential for the secretion of FVII, FIX and PC [6–12].

We therefore investigated this issue in the homologous FX by expressing a panel of recombinant variants progressively truncated at the carboxyl-terminus through the tailored insertion of premature translation termination codons in the FX cDNA (Fig. 1). In the initial scanning, three rFX variants were designed to mimic the length of FVII (rFX-482X, FVII-like), PC (rFX-480X, PC-like) and FIX (rFX-470X, FIX-like), which is the shortest family member. Beside mimicking the boundaries provided by these homologous proteins, five truncated proteins with intermediate length (rFX-484X, rFX-479X, rFX-478X, rFX-474X, and rFX-472X) were explored. Interestingly, expression studies in HEK293 cells showed that protein levels in the medium of each truncated rFX variant were comparable with those of rFX-wt (2.34 ± 0.27 μg mL^{-1}) (Fig. 2A), which indicates a negligible impact of carboxyl-terminal deletions on FX secretion.

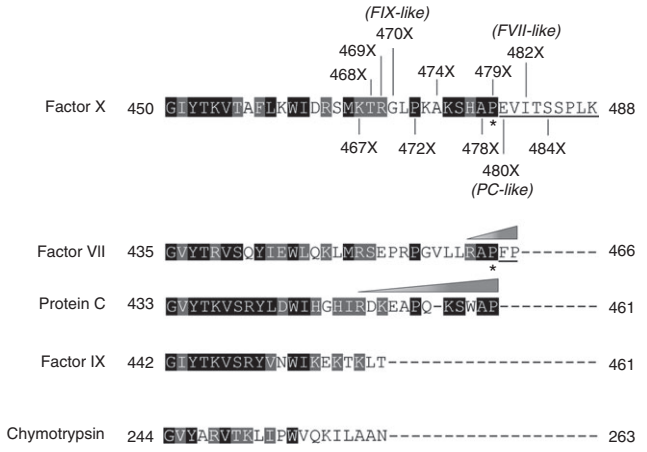

Fig. 1. Recombinant FX variants and alignment of the carboxyl-terminal region of the homologous coagulation FVII, PC and FIX. Schematic representation of the carboxyl-terminal sequence of human FX (NP_000495.1), FVII (NP_000122.1), PC (NP_000303.1) and FIX (NP_000124.1), and of chymotrypsin (NP_001897.4) as reference. Stop codons (indicated by X) indicate the position of the new carboxyl-terminus created by mutagenesis. Bold numbers indicate FX variants corresponding to the position of the FVII, PC and FIX carboxyl-terminus. Asterisks indicate the conserved Ala-Pro (AP) sequence delimiting the swapped FX and FVII residues (underlined letters). The extent of deletions responsible for barely detectable or undetectable secreted FVII and PC protein levels is represented by grey triangles. Numbers on the left and right of primary sequences indicate the first and the final aligned residues in each protein.

This finding prompted us to further shorten the FX protein through the sequential removal of single amino acids upstream of position 469. Expression levels of the rFX-469X (101.4% ± 7.9% of rFX-wt) and rFX-468X (81.8% ± 20.2%) variants were still normal. These observations indicate that the removal of up to 21 residues did not impair rFX secretion and identify the carboxyl-terminal FX sequence, beginning at K467 (c261), as dispensable for normal secretion (Fig. 2A).

In contrast, further deletion (rFX-467X) resulted in a dramatic decrease of rFX levels in medium ($P < 0.0001$) that dropped to 6.1% ± 3.8% (Fig. 2A). These differences were not caused by differential mRNA levels, as demonstrated by RT-PCR on mRNA isolated from cells transfected with the pFX-468X and pFX-467X constructs, which showed mRNA levels comparable to those of rFX-wt (data not shown).

Moreover, to verify whether our observations were dependent on the specific cell type, we validated the expression studies in HepG2 cells, being FX of liver origin, and BHK21, a well-established cell line to produce recombinant coagulation serine proteases (Fig. 2B). Results obtained in both experimental systems, with rFX-467X and rFX-468X, and rFX-478X and rFX-482X, as selected controls, were consistent with findings in HEK293.

Interestingly, a large difference in secretion levels was observed between FX variants truncated at residues 468 and 467, a finding comparable to that obtained for PC [11] and marking the carboxyl-terminal region of chymotrypsin (Fig. 1).

Comparison of the carboxyl-terminus among homologous coagulation factors FX, FVII, PC and FIX (Fig. 1) reveals an Ala-Pro (AP) sequence conserved in FX, FVII and PC that is followed by an amino acid stretch only in FX and FVII, which show the highest degree of sequence identity. Intrigued by this observation, we performed a swap experiment by replacing the FX carboxyl-terminus downstream of the AP sequence with that of FVII (FX_{FVII}) and *vice versa* ($FVII_{FX}$) (Fig. 3A). Expression of the rFX_{FVII} and $rFVII_{FX}$ resulted in significantly reduced ($P < 0.0001$) secreted levels for both chimeras (5.8% ± 1.7% and 2.3% ± 0.6%, respectively) as compared with the corresponding wild-type protein. Interestingly, the $FVII_{FX}$ variant ($rFVII_{FX\text{-}EVITS}$) retaining roughly half of the FX carboxyl-terminus showed intermediate secretion levels (25.4% ± 6.7%) (Fig. 3B). Noticeably, the rFX-482X variant, the other 'FVII-like' molecule for its length, was normally secreted (Fig. 2A).

Taken together these data, and particularly the sharp decline in expression levels, revealed a peculiar feature of the carboxyl-terminus of FX as compared with that of the other family members, in which the relationship between the extension of this region and the secretion levels depicted a sort of gradient (Fig. 1). As a matter of fact, deletion of up to 21 residues in FX had no significant effects whereas a further one residue deletion resulted in remarkably inefficient secretion. On the other hand, data from truncated and swapped variants suggest that alterations of the whole carboxyl-terminal sequence, rather than deletions, have the most detrimental impact. Indeed we observed normal levels for the rFX-482X and rFX-480X variants and poor secretion for the swapped rFX_{FVII} chimera. In contrast, swapping of other domains, producing chimeric FX-FVII [15] or FX-FIX [16] variants, did not affect protein levels in media.

After demonstrating that the carboxyl-terminus can be removed without substantial loss of secretion efficiency, we investigated whether this region was also dispensable for pro-coagulant function. As a matter of fact, even the shortest variant retains the conserved carboxyl-terminal sequence of chymotrypsin (Fig. 1), thus pointing to an intact catalytic activity. Moreover, this region in FX is involved, upon auto-proteolytic cleavage of the peptide bond R469-G470, in the conversion of the activated FXα (FXaα) into the FXaβ form, which is also catalytically competent [17]. We therefore assessed the coagulant activity of the truncated rFX-470X, which interestingly mimics the FXaβ form once cleaved, and of the shortest but normally secreted rFX-468X and rFX-469X variants, and of rFX-478X and rFX-482X as controls.

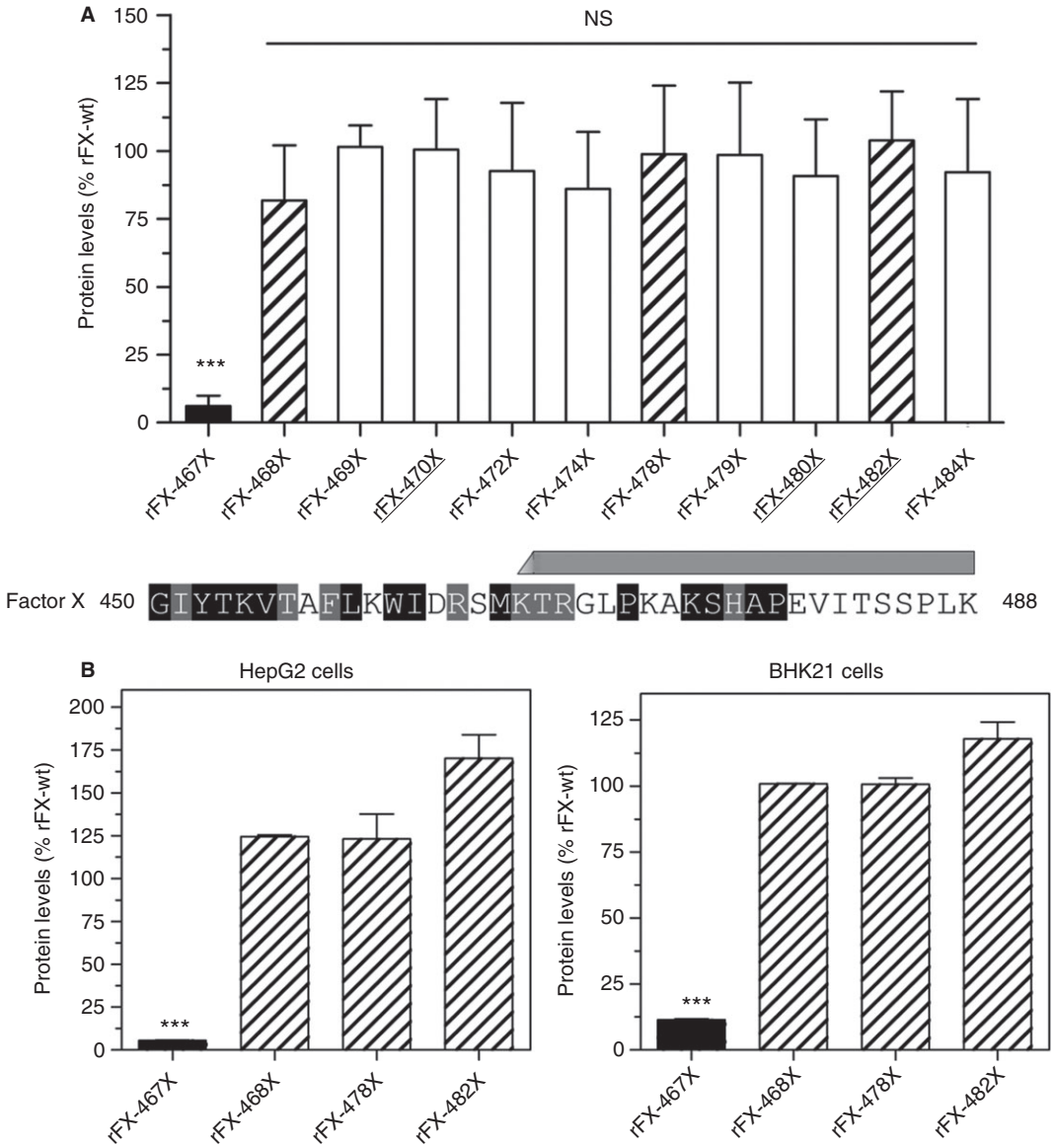

Fig. 2. Expression levels of truncated variants. (A, upper panel) Expression levels of rFX variants after transient transfection of HEK293 cells. The black bar represents the rFX-467X variant with protein levels significantly lower (6.1% ± 3.8%, ***, $P < 0.0001$) than rFX-wt (2.34 ± 0.27 μg mL^{-1}). rFX variants resembling the length of FVII (rFX-482X), PC (rFX-480X) and FIX (rFX-470X) are underlined. (A, lower panel) The grey bar above the primary sequence, represented as in Fig. 1, indicates the FX region dispensable for normal secretion levels and beginning at the K467-T468 sequence. (B) Selection of truncated variants (black and striped columns in A) expressed in HepG2 (left panel) and BHK21 (right panel) cells. Results, consistent in all cell types, indicate the lowest secretion levels for the rFX-467X variant. Results are reported as mean ± standard deviation and expressed as % of rFX-wt. ***, $P < 0.0001$.

In PT-based assays the rFX-468X, rFX-469X and rFX-470X variants displayed a pro-coagulant activity corresponding to 35.4% ± 10%, 62.5% ± 5.0% and 66.2% ± 3.6% of the rFX-wt, respectively (Fig. 4A). The aPTT-based assays produced a similar pattern for the rFX-468X (18.8% ± 4.6% of the rFX-wt), rFX-469X (34.9% ± 8.0%) and rFX-470X (58.9% ± 6.8%) variants (Fig. 4B). In both assays, the rFX-478X (PT-based, 102.3% ± 7.9%; aPTT-based, 96.9% ± 9.3%) and rFX-482X (PT-based, 95.3% ± 5.5%; aPTT-based, 88.8% ± 5.8%) variants showed intact pro-coagulant activity. These results suggest that a slight reduction in pro-coagulant activity is present even with extensive deletion of the carboxyl-terminus, in particular for the rFX variant resembling the full sequence of the FXβ form (rFX-470X). This finding is supported by the comparative analysis of FX across species (human, bovine, chicken, rabbit, mouse and rat), which shows that sequence identity is remarkable up to position G470 and decreases further downstream. Moreover, the data are compatible with previous experimental evidence suggesting that residues 455–469 are involved in the interaction between FXa and

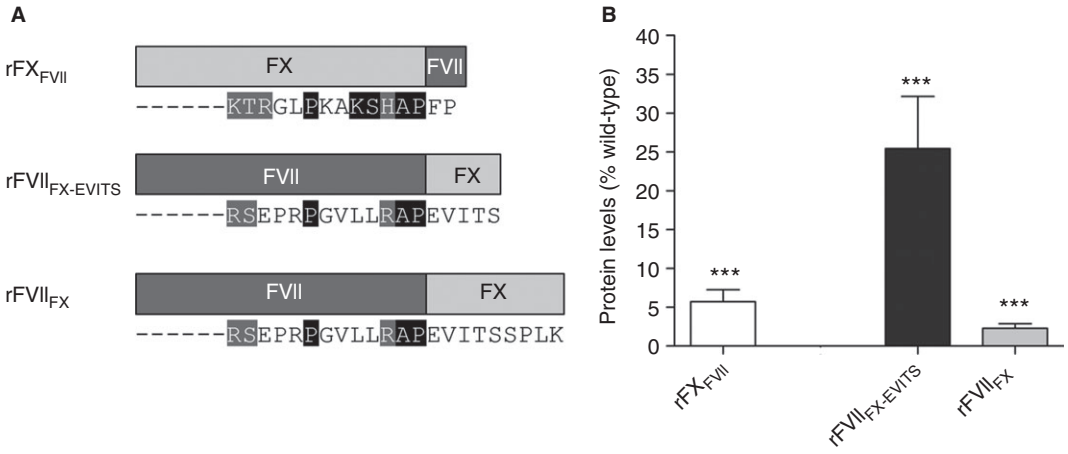

Fig. 3. Expression of FX and FVII variants with replaced terminal residues. Schematic representation of variants with swapped terminal residues downstream of the conserved Ala-Pro (AP) sequence (A) and secretion levels after expression in HEK293 cells (B). Results are reported as mean ± standard deviation and expressed as % of the corresponding wild-type protein (rFX-wt, 2.47 ± 0.2 μg mL^{-1}; rFVII-wt, 276.4 ± 32.2 ng mL^{-1}). ***, $P < 0.0001$.

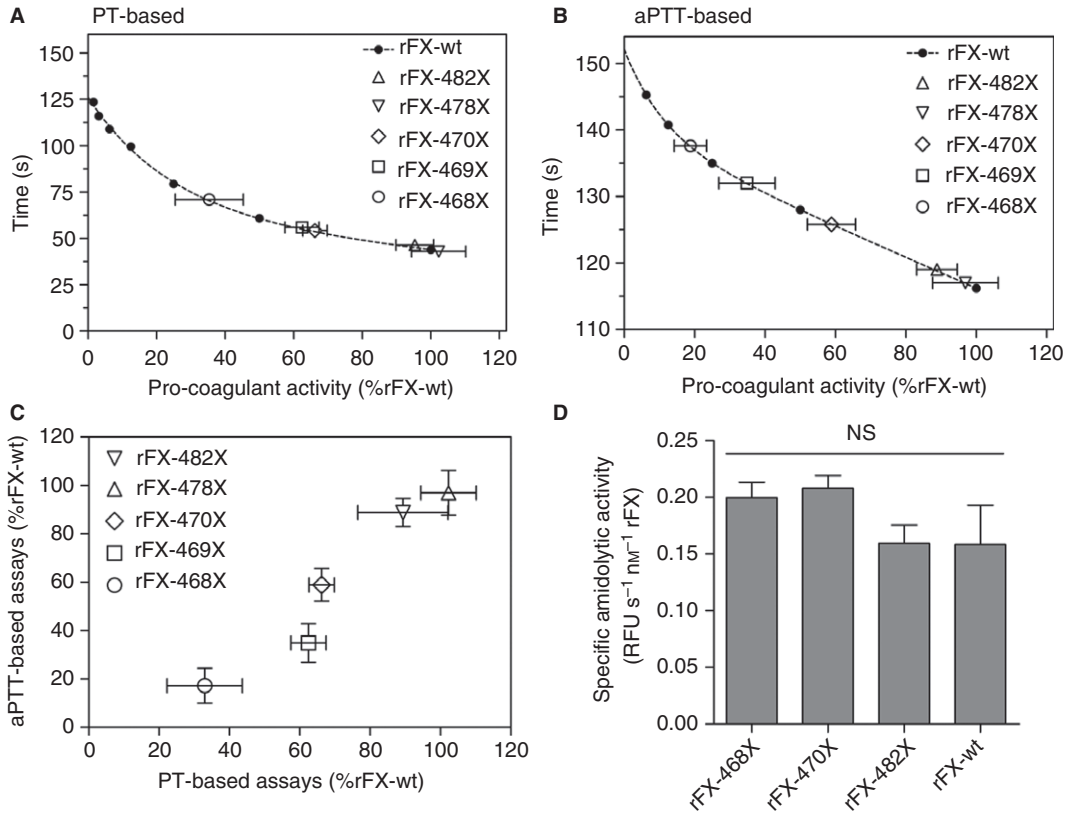

Fig. 4. Pro-coagulant and amidolytic activity of rFX variants. (A–B) Coagulant activity of the truncated rFX variants. The activity of rFX variants in PT- and aPTT-based assays (A and B, respectively) is compared with serial dilutions of rFX-wt (black circles). (C) Correlation between coagulant activity levels in PT- and aPTT-based assays. Results in A, B and C are indicated as mean ± standard deviation and expressed as % of rFX-wt. (D) Specific amidolytic activity of rFX variants after RVV activation. Results are expressed as the ratio between amidolytic activity (RFU/s) and rFX concentration (nM) in media, and reported as mean ± standard deviation.

prothrombin in the prothrombinase complex [18]. Noticeably, the 7- and 11-residue truncated rFX-482X and rFX-478X variants, with a proper 455–469 region, displayed virtually normal activity (Fig. 4C).

Interestingly, we did not observe significant differences in amidolytic activity among the rFX-468X (0.20 ± 0.01 Rfu/s/nM rFX), rFX-470X (0.21 ± 0.01) and rFX-482X (0.16 ± 0.01) variants as compared with the rFX-wt

(0.16 ± 0.04) (Fig. 4D), which supported a properly folded active site.

In conclusion, we provide experimental evidence for a peculiar feature of FX, which tolerates significant deletions in the carboxyl-terminus without apparent effects on secretion and amidolytic activity, and with a minor impact on pro-coagulant properties. These findings encourage further studies aimed at elucidating the role of this region in other FX functions (i.e. PAR activation) and regulation (i.e. TFPI-mediated inhibition), which would contribute to understanding of the mechanisms that have led to the divergence of the homologous FX, FVII, FIX and PC proteins.

Our novel observations, obtained through site-directed mutagenesis, help interpret the mutational pattern of coagulation factor deficiencies. The features of the truncated rFX variants are in fact consistent with the virtual absence of patients with nonsense mutations in the carboxyl-terminus of FX [19,20] as compared with the panel of mutations so far described in this region of FVII [7,21–24], FIX [www.factorix.org] and PC [25,26], which are mostly associated with deficiency. Indeed, the present data strongly support that nonsense mutations in the carboxyl-terminus of FX would result in appreciable FX antigen and activity levels, and thus in asymptomatic phenotypes [27], which would prevent the identification of carriers.

Addendum

A. Branchini created recombinant vectors for truncated variants and performed secretion studies in HEK293 cells. M. Baroni and F. Burini performed expression studies in HepG2 and BHK21 cells as well as amidolytic activity assays and mRNA analysis. F. Puzzo and F. Nicolosi created recombinant plasmids and performed expression studies for swapped variants. R. Mari performed coagulant assays. D. Gemmati carefully revised the manuscript. A. Branchini, F. Bernardi and M. Pinotti conceived the study, designed the research, analyzed and interpreted data and wrote the manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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