Thrombosis Research 173 (2019) 4–11

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Full Length Article

The carboxyl-terminal region of human coagulation factor X as a natural linker for fusion strategies

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ARTICLE INFO

Keywords:
Coagulation factor X
Protein engineering
Linkers
Albumin fusion proteins

ABSTRACT

Fusion with human serum albumin (HSA), which represents a well-established technique to extend half-life of therapeutic proteins, commonly exploits intervening peptide linkers as key components. Here, we explored the human coagulation factor X (FX) carboxyl-terminal region, previously demonstrated by us to be dispensable for secretion and coagulant activity, as a natural linker for fusion purposes. To test our hypothesis, we compared direct FX-HSA fusion with the designed FX-HSA fusion proteins mimicking the recombinant activated factor VII (rFVIIa)-HSA or factor IX (FIX)-HSA chimeras, both strongly dependent from artificial linkers.

Three constructs were produced by direct tandem fusion (FX-HSA) and through flexible (glycine/serine; FX-GS-HSA, mimicking rFVIIa-HSA) or cleavable (incorporating the FX activation site; FX-CL-HSA, mimicking FIX-HSA) linkers. The FX-HSA was efficiently secreted and displayed prolonged plasma persistence in mice. All chimeras possessed remarkable pro-coagulant activity, comparable to FX for FX-HSA (88.7 ± 6.0%) and FX-CL-HSA (98.0 ± 16.4%) or reduced for FX-GS-HSA (55.8 ± 5.4%). Upon incubation with activators, FX-HSA and FX-CL-HSA displayed a correct activation profile while the FX-GS-HSA activation was slightly defective. In fluorogenic-based assays, FX-HSA showed normal activity over time and a specific amidolytic activity (1.0 ± 0.12) comparable to that of FX. Overall, the FX-HSA features indicate that the FX carboxyl-terminal region represents an intrinsic sequence allowing direct tandem fusion.

Our results provide the first experimental evidence for i) a coagulation factor fusion protein with biological properties independent from artificial linkers, ii) the suitability of FX carboxyl-terminal region as a natural linker for fusion purposes.

1. Introduction

Several engineering techniques have been developed to improve the biological properties of the target proteins, with a particular focus on half-life [1]. In this field, fusion with human serum albumin (HSA) currently represents one of the best-established strategies [2–6]. The rationale relies on the acquired capacity of the fused partner to undergo the recycling pathway mediated by the neonatal Fc receptor (FcRn) and responsible for the extremely long HSA half-life [7], even higher than that of the IgG Fc region that exploits a similar mechanism [8].

Coagulation factors have represented preferred models for protein engineering aimed at modulating half-life, with the aim to overcome the limitation of the short half-life of infused proteins [9–12]. The intense research on long-acting factors highlighted the importance of linker sequences, either the glycine/serine flexible amino acid stretch for recombinant activated factor VII (rFVIIa) or the cleavable linker for factor IX (FIX), to guarantee protein secretion and function [13,14]. The requirement for linkers of rFVIIa and FIX might stem from the fact that the carboxyl-terminal region of these proteins, essential for secretion [15–18], does not tolerate potential constraints introduced by the fused partner.

Interestingly, by deletion scanning we demonstrated that the

Abbreviations: CL, cleavable linker; Fc, fragment crystallizable; FcRn, Neonatal Fc receptor; FVII, factor VII; FVIIa, activated factor VII; FIX, factor IX; FIX-HSA, fusion protein between FIX and HSA; FX, factor X; FX-HSA, fusion protein between FX and HSA; FX-GS-HSA, fusion protein between FX and HSA separated by a GS linker; FX-CL-HSA, fusion protein between FX and HSA separated by a CL linker; FXa, activated factor X; GS, glycine-serine linker; HEK293, Human Embryonic Kidney 293; HSA, Human Serum Albumin; IgG, Immunoglobulin G; rFVIIa, recombinant activated factor VII; rFVIIa-HSA, fusion protein between rFVIIa and HSA; pdFX, plasma-derived factor X; PT, prothrombin time; RVV, Russell's Viper Venom

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https://doi.org/10.1016/j.thromres.2018.11.007
Received 19 July 2018; Received in revised form 29 October 2018; Accepted 8 November 2018
Available online 09 November 2018
0049-3848/

carboxyl-terminal region (residues 467–488) of human coagulation factor X (FX), which is more extended than that of FVII and FIX, is dispensable for secretion and pro-coagulant activity [19]. These features prompted us to hypothesize that the FX carboxyl-terminal region may act as a natural linker sequence for fusion purposes.

Here, we designed a FX-HSA direct tandem fusion with preserved activation and pro-coagulant properties even without an artificial linker, thus demonstrating that the virtually unique features of the FX carboxyl-terminal region makes it an ideal linker for fusion purposes.

2. Materials and methods

2.1. Creation of expression vectors

2.1.1. pFX and pFX-HSA direct tandem fusion

The human FX cDNA was PCR amplified from the pCMV4-ss-pro-II-FX construct [20] with the forward primer 5′AAAAAGCTTATGGCGCA CGTCC GAGG3′ (*HindIII* restriction site underlined) and the reverse primers 5′AAACTCGAGTCACTTTAATGGAGAGG ACGTTATG3′ (to create pFX) or the 5′AAAAAGCTTCTTTAATGGAGAGGACGTTATGA CC3′ (to create pFX-HSA), both incorporating the restriction sites (*Xho*I and *Hind*III, respectively; underlined) and with the latter suppressing the FX stop codon. The first amplicon was then cloned into the pCDNA3 vector through the *Hind*III/*Xho*I restriction sites to generate the pFX vector. The human albumin (HSA) cDNA sequence corresponding to mature HSA (aa 25–609) was amplified with primers 5′AAAAAGCTT GATGCACACAAGAGTGAGGTTGC3′ and 5′AAACTCGAGCTATAAGCCT AAGGCAGCTTGAC3′ that introduce the *Hind*III and *Xho*I sites (underlined), respectively. The amplicon was then cloned into the pCDNA3 vector together with the FX devoid of the stop codon through the *Hind*III/*Xho*I sites to generate the pFX-HSA vector. The central *Hind*III site, inserting two additional codons between FX and HSA, was removed by mutagenesis (Agilent Technologies, Santa Clara, CA, USA).

2.1.2. pFX-GS-HSA

The glycine/serine(GS)-encoding linker was inserted into the pCDNA3 vector as described [13]. The FX cDNA was amplified with primers 5′AAAAAGCTTATGGCGCACGTCCGAGG3′ and 5′AAACTCGA GCCCTTTAATGGAGAGGACGTTATGA CC3′ and cloned upstream of the GS-encoding sequence by *Hind*III/*Xho*I restriction sites (underlined). HSA was cloned through *BamH*I restriction sites (underlined) after PCR amplification with primers 5′AAAGGATCCGATGCACACAAGAGTGAG GTTGC3′ and 5′AAAGGATCCCTATAAGCCT AAGGCAGCTTGAC3′.

2.1.3. pFX-CL-HSA

The cleavable linker(CL)-encoding sequence, resembling that recognized by activated factor VII (FVIIa), was created by annealing of the 5′GTGCTCGAGCGGGGGATCTGGCGGGTCTCCT GAACGAGATAAT GCTCTTACTCGT3′ and 5′CACTCTAGATTATCAGGATCCCGACCCT CCAGACCCGCCAGAAACTGAACGAGTAAGGCATTAT3′ oligonucleotides followed by cloning into the pCDNA3 backbone through *Xho*I and *Xba*I restriction sites. The subsequent cloning procedures were as those described for the pFX-GS-HSA construct.

All plasmids have been validated by sequencing.

2.2. Creation of stable clones and evaluation of secreted protein levels

Human embryonic kidney (HEK) 293 cells were stably transfected as previously described [21]. FX levels in media were evaluated by ELISA (Cedarlane, Burlington, ON, Canada), and known concentrations of plasma-derived human FX (pdFX; Haematologic Technologies Inc., Essex Junction, USA) were used as reference.

Detection of fusion proteins in media was performed by ELISA-based assays with a capture polyclonal anti-HSA antibody (Bethyl Laboratories, Montgomery, TX, USA) followed by detection with a polyclonal HRP-conjugated anti-human FX antibody (Cedarlane), as well as Western blotting analysis with polyclonal sheep anti-human FX (Cedarlane) and rabbit anti-sheep HRP-conjugated (DAKO, Agilent) antibodies. Densitometric analysis of blotting images was performed by the Image Laboratory Software version 4.0 (Bio-Rad, Hercules, CA, USA).

2.3. Evaluation of in-vivo persistence in mice

Eight-week-old wild-type C57BL/6 mice (n = 3 per group) were tail vein injected with FX (5 μg) or FX-HSA (10 μg) variants concentrated by centrifugal filter devices from conditioned media and diluted in PBS, as previously described [22]. Blood samples were collected from the tail vein at the selected time points and protein levels evaluated by ELISA, as above described. Human FX levels in mouse plasma at 5 min post-injection were used to evaluate protein recovery. FX values at the following time points (4, 8, 16, 20, 24 and 36 h) were considered to estimate FX persistence in mouse plasma.

All animal procedures were approved and conducted under the guidelines established by the Italian Ministry of Health, in compliance with the ARRIVE guidelines.

2.4. Functional assays and activation of FX proteins

Thrombin generation as well as prothrombin time (PT)-based assays were performed in a FX-deficient plasma system as described [19,21].

The activation profile was evaluated after incubation of FX variants for 15 min at 37 °C with recombinant activated FVII (rFVIIa; NOVO Nordisk, Bagsværd, Denmark) in the presence of Innovin (Dade Innovin, Siemens Healthcare, Marburg, Germany) as a source of tissue factor, calcium and phospholipids, or with Russell's Viper Venom (RVV; Haematologic Technologies Inc.).

Time-course activation was performed in the presence of rFVIIa and Innovin and by quenching of sample aliquots in 50 mM EDTA at the selected time points. Activity was assayed towards a fluorogenic substrate (250 μM, SpectroFluor FXa; Sekisui Diagnostics, Lexington, MA, USA) specific for activated FX (FXa) and by measuring fluorescence emission (Relative Fluorescence Units, RFU) over time on a microplate fluorometer (Fluoroskan Ascent FL, Thermo Fisher Scientific, Helsinki, Finland) [19].

2.5. Data analysis and “in silico” prediction

Data were analyzed by the statistic software GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Coagulant and thrombin generation assays as well as amidolytic activity were analyzed as described [16,19].

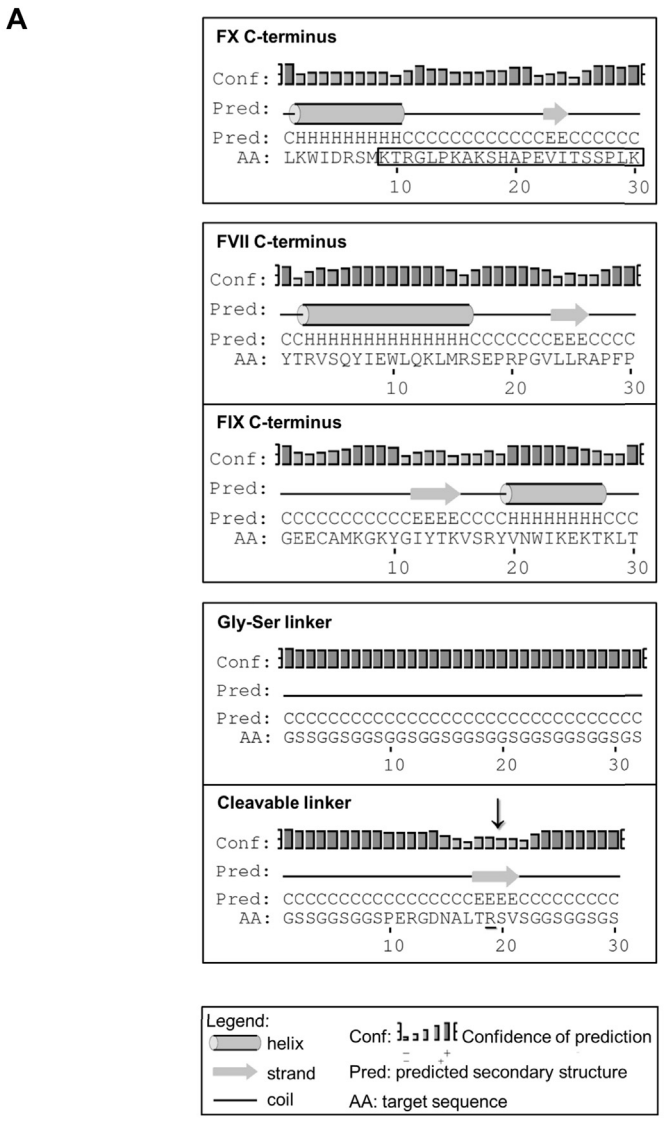
Secondary structure prediction was performed by the PSIPRED Protein Sequence Analysis on-line software (http://bioinf.cs.ucl.ac.uk/psipred/).

3. Results

We explored HSA as a model fusion partner in order to compare, by using FX as common scaffold, the direct tandem fusion strategy with other designed FX fusion proteins mimicking the rFVIIa-HSA or FIX-HSA, whose productive fusion is strongly dependent from artificial linkers.

3.1. Rational design and expression of the fusion protein joining FX and HSA

The factor-specific features of the carboxyl-terminal regions of the homologous coagulation serine proteases (FVII, FIX and FX) [15–19] shape their secretion and functional properties. As an example, the swap of FX and FVII carboxyl-terminal regions causes ample perturbation of secreted but not activity levels (FX) or of both properties



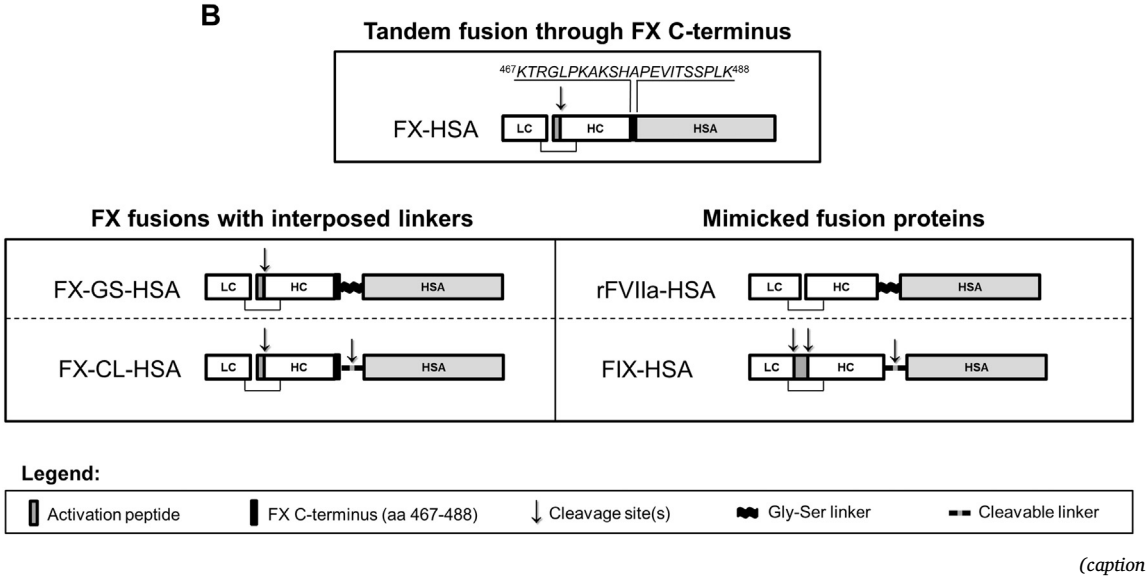


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Fig. 1. Design of fusion variants joining FX and HSA.
A) Comparison among the predicted secondary structure of the carboxyl-terminal region of FX (upper panel) with that of the homologous FVII and FIX (middle panels) and of the two intervening linker sequences exploited for fusion between FX and HSA (lower panels).
The amino acid sequence of the FX carboxyl-terminal region hypothesized to serve as natural fusion linker is highlighted by the black rectangle.
↓, cleavage site at arginine (underlined) within the designed cleavable linker.
B) Schematic representation of fusion variants joining FX with HSA. Fusion proteins were designed as direct tandem fusion (FX-HSA) by exploiting the FX carboxyl-terminal region (aa 467–488, residues on top) as natural linker, or by intervening a flexible 32-residue glycine/serine (GS) linker (FX-GS-HSA, mimicking rFVIIa-HSA) or the designed cleavable (CL) linker, flanked by glycine/serine residues, resembling the natural FVIIa cleavage site within FX activation peptide (FX-CL-HSA, mimicking FIX-HSA).
LC, light chain; HC, heavy chain; HSA, human serum albumin, mature protein (aa 25–609).

(FVII) (Supplementary Fig. 1). Interestingly, secondary structure prediction suggests that the carboxyl-terminal regions of FIX and FVII are more structured than that of FX (Fig. 1A, upper and middle panels). These differences have implications for the requirement of specific intervening linker sequences permitting productive fusion with other partners.

The virtually unique FX properties led us to express and characterize a fusion protein between FX and HSA by direct tandem fusion (FX-HSA). As comparison we investigated the FX-HSA chimera separated through two artificial linkers with flexible or cleavable features and mimicking those required for optimal fusion of HSA with rFVIIa [13] and FIX [14]. In particular, we designed i) a flexible glycine/serine (GS) linker, known to confer efficient separation and preserve biological properties of the fused partners, which resulted in the FX-GS-HSA mimicking rFVIIa-HSA, or ii) a cleavable (CL) linker resembling the FX activation site recognized by FVIIa, which resulted in the FX-CL-HSA variant mimicking FIX-HSA (Fig. 1B). After preliminary evaluation of secretion efficiency in transient transfection studies in HEK293 cells, which was comparable for the three fusion variants (data not shown), we characterized them from stably-expressing clones.

Through Western blotting analysis and ELISA-based assays we clearly detected the fusion variants with the expected molecular weights (~130 kDa; Fig. 2A) and possessing both fusion partners (Fig. 2B). At variance, FX alone was not detected by the combined ELISA. The expression of stable clones produced similar amounts of wild-type FX and fusion variants, as indicated by both Western blotting and ELISA-based assays.

These results indicate that the direct tandem fusion of HSA to FX is compatible with efficient secretion.

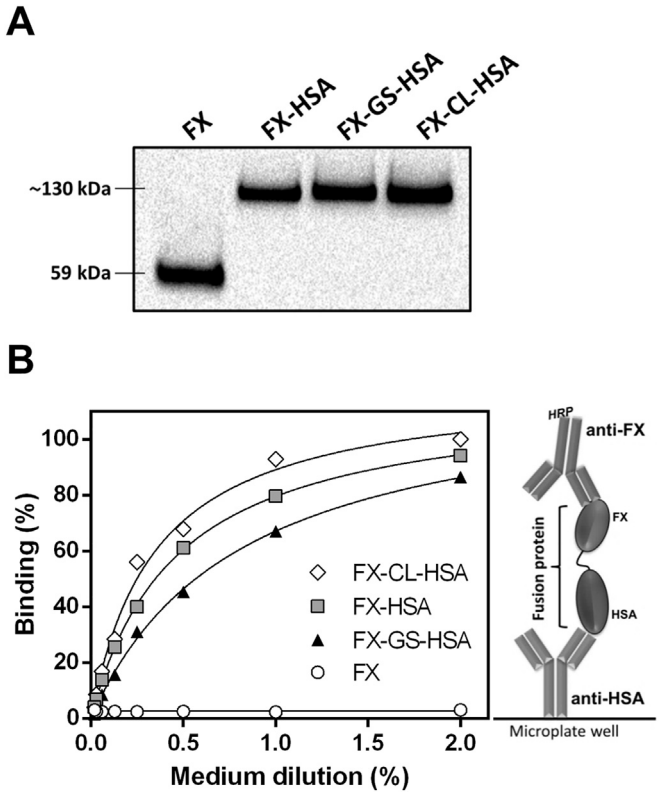

Fig. 2. Expression and validation of fusion variants.
A) Western blotting analysis of FX and of fusion variants with anti-FX polyclonal antibodies. Relative molecular weights for each form are shown.
B) ELISA-based assays with coated anti-HSA and detecting anti-FX antibodies (scheme on the right). Serial dilutions of medium containing fusion proteins (indicated as percentage) were evaluated in comparison with unfused FX exploited as negative control.

3.2. The FX-HSA fusion protein displays prolonged plasma persistence in mice

To validate *in vivo* the fusion strategy between FX and HSA, we preliminarily investigated whether the fusion was compatible with an improved plasma persistence of the FX-HSA variant. Due to the unviability of FX-deficient mice [23,24], we evaluated the FX-HSA levels, and their decay over time, in wild-type mice by an optimized ELISA based on the use of human-specific anti-FX antibodies. FX and FX-HSA were concentrated from conditioned media and intravenously injected at comparable molar concentration in mice to evaluate circulating protein levels over time. Coherent profiles were obtained in all animals and, as shown in Fig. 3, the FX-HSA chimera was clearly detectable by ELISA at 36 h post-injection whereas FX alone disappeared at 16 h.

These results indicate that the direct tandem fusion of HSA to FX is compatible with prolonged persistence *in vivo*, thus providing experimental evidence about the suitability of FX carboxyl-terminal region as fusion linker.

3.3. The FX-HSA fusion protein displays a normal functional profile

To assess the functional features of the FX-HSA variant in comparison with the reference FX-GS-HSA and FX-CL-HSA molecules, we evaluated their ability to restore the efficiency of the coagulation cascade in FX-deficient plasma in terms of thrombin generation (Fig. 4A) and coagulant activity in prothrombin time (PT)-based assays (Fig. 4B).

In these assays, all fusion variants were characterized by remarkable activity, with FX-HSA and FX-CL-HSA displaying comparable functional features in both systems. This was evident by the comparable ability of the two fusion proteins to act on key functional parameters, such as lag time, peak and area under the curve (Table 1). The two chimeras were also able to shorten coagulation times in a dose-dependent manner (Fig. 4B), with virtually normal pro-coagulant activity for FX-HSA (88.7 ± 6.0% of FX) and FX-CL-HSA (98.0 ± 16.2%). Conversely, the thrombin generation parameters, and particularly the prolonged lag time and time-to-peak, pointed towards a reduced activity for the FX-GS-HSA variant, clearly demonstrated by the coagulant activity (55.8 ± 5.4%) that was significantly lower than that of the FX-HSA ($p < 0.0001$) and FX-CL-HSA ($p = 0.0005$) chimeras (Table 1).

Overall, the functional assays indicate that, among the designed fusion proteins, the FX-HSA variant and the control FX-CL-HSA, mimicking FIX-HSA due to the cleavable features of its linker, are characterized by a virtually preserved activity.

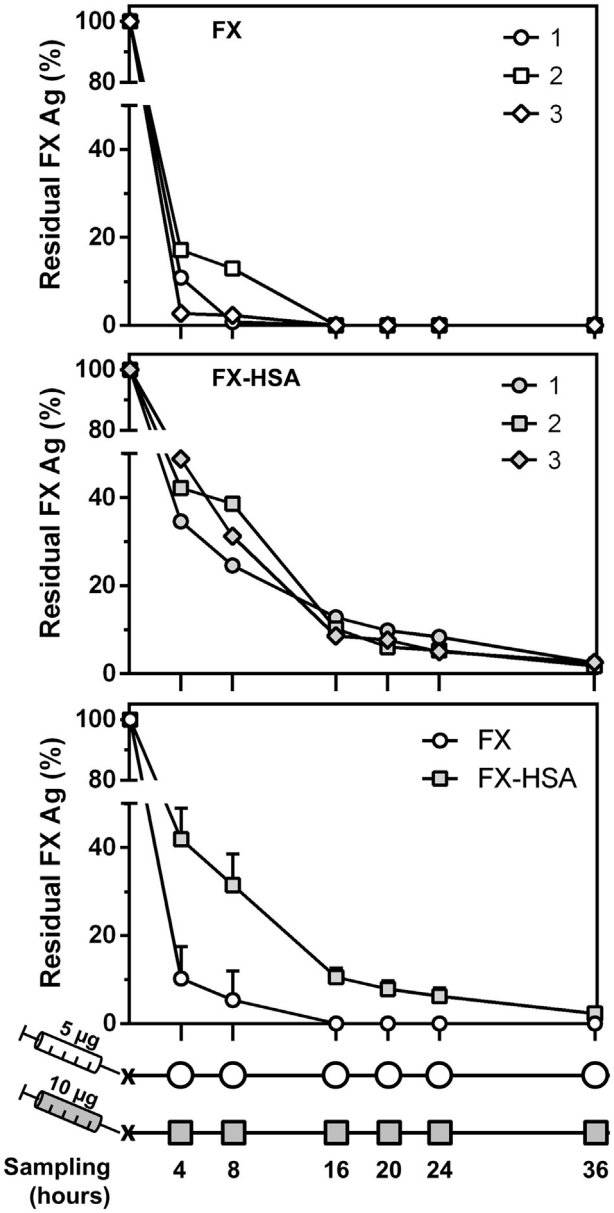

Fig. 3. Plasma persistence of the FX-HSA protein.
Plasma persistence of the FX-HSA protein in wild-type C57BL/6 mice. FX (upper panel) or FX-HSA (middle panel) was injected in three mice. The overall profiles (mean ± standard deviation) of the two proteins are reported in the lower panel.
Blood samples of FX or FX-HSA (injected at 5 or 10 µg/mouse, respectively) were collected at different time points (from 4 to 36 h, sampling scheme in the bottom part) and FX antigen measured by a human-specific ELISA. Residual antigen levels are reported as the percentage of FX amount detected at 5 min (indicated by the x in the sampling scheme) after injection.

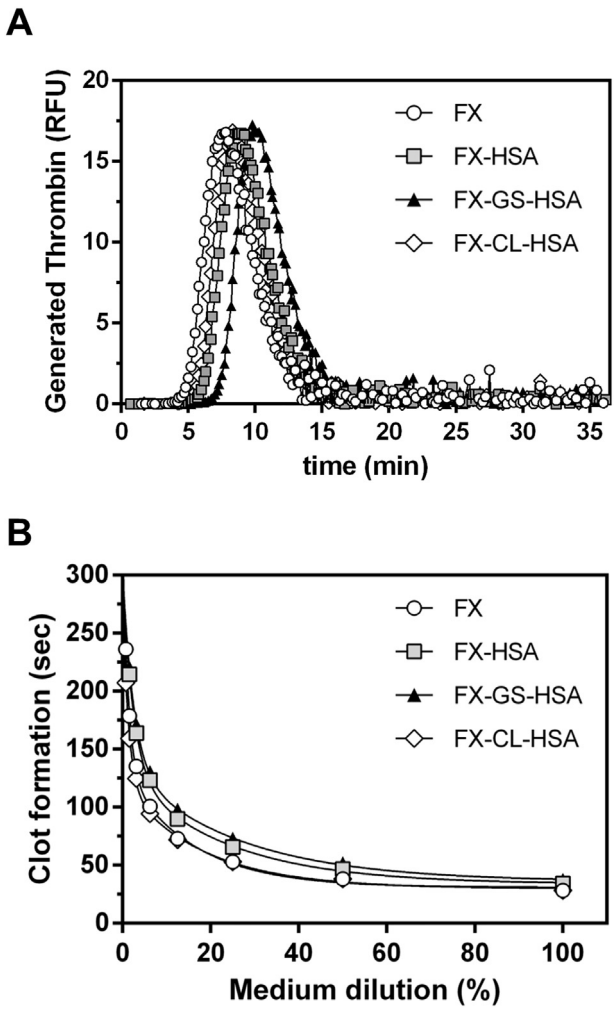

Fig. 4. Activity of fusion proteins in FX-deficient plasma systems.
Thrombin generation (A) and prothrombin time (PT)-based (B) activity after FVIIa-mediated activation in FX-deficient plasma. Fusion variants were compared with FX alone.
RFU, Relative Fluorescence Units; sec, seconds.

3.4. FX-HSA is efficiently activated by physiological and non-physiological activators

To dissect the functional impact of the differentially-fused albumin on FX properties, we investigated the activation profile of the three fusion variants in the presence of the physiological activator rFVIIa and the non-physiological activator RVV in Western blotting analysis with anti-FX antibodies.

Although in the presence of uncleaved FX, a common observation with recombinant FX in conditioned medium [25], all fusion proteins showed the band corresponding to the activated form (~117 kDa) resulting from the zymogens (~130 kDa) that have lost the FX activation peptide (52 residues, ~13 kDa) [26] upon incubation with rFVIIa (Fig. 5A). Moreover, the ratio between bands corresponding to the activated and zymogen forms points towards a defective activation of the FX-GS-HSA (0.45) as compared with FX-HSA (0.79) and FX-CL-HSA (0.72) variants, a finding coherent with its reduced coagulant activity. Noticeably, the ratio between the activated and zymogen forms observed in FX-HSA is higher than that of FX (0.48), pointing out an efficient activation. Interestingly, and in accordance with the designed fusion strategy, only the FX-CL-HSA showed a band of approximately 49 kDa attributable to the activated FX (FXa)α form that, upon the rFVIIa-mediated cleavage at the linker site, has lost HSA. The slightly higher molecular weight of this fragment, as compared to that of FXaα alone (46 kDa), is due to the persistence of the linker portion upstream of the rFVIIa cleavage site (Fig. 1B). Finally, all fusion variants showed traces of the FXaβ form (~44 kDa), arising from auto-proteolytic cleavage and release of the downstream HSA.

The same pattern was observed after incubation with RVV (FX-HSA, 0.78; FX-GS-HSA, 0.60; FX-CL-HSA, 0.77; FX, 0.47) (Fig. 5B), thus confirming the differential activation profile of the three FX fusion proteins.

In this setting, the FX-HSA protein, as well as the FX-CL-HSA comparator, showed a normal activation profile, thus helping to interpret

Table 1
Functional features of FX fusion variants in FX-deficient plasma systems.

Fusion strategy		Thrombin generation				Coagulant
Linker type	Protein name	LT (min)	Peak (RFU)	TTP (min)	AUC (RFU ∗ min)	PT (%FX)
FX C-ter	FX-HSA	5.9 ± 0.5	17.8 ± 0.2	8.9 ± 0.5	67.0 ± 1.0	88.7 ± 6.0
Gly/Ser	FX-GS-HSA	6.9 ± 0.2	17.3 ± 0.3	9.8 ± 0.5	66.7 ± 1.3	55.8 ± 5.4
Cleavable	FX-CL-HSA	5.3 ± 0.2	17.1 ± 0.3	8.1 ± 0.4	67.2 ± 3.0	98.0 ± 16.2
–	FX	4.1 ± 0.6	16.8 ± 0.2	6.9 ± 0.8	67.6 ± 1.0	100

LT, lag time; TTP, time to peak; AUC, area under curve; RFU, Relative Fluorescence Units; PT, coagulant activity measured in prothrombin time (PT)-based assays. Data are represented as mean ± standard deviation.

the functional observations in thrombin generation and PT-based assays.

3.5. The direct tandem fusion with HSA preserves an efficient rFVIIa-mediated activation

To corroborate and integrate our data on the functional/activation profile of the FX-HSA fusion variant, we performed a time-course activation assay in the presence of the physiological activator rFVIIa.

Evaluation of the ability of the FX-HSA protein to cleave the artificial fluorogenic substrate specific for FXa at different time points showed an efficient activation profile over time that was comparable with that of FX alone (Fig. 6), as also indicated by the preserved specific amidolytic activity of FX-HSA (1.0 ± 0.12) (Fig. 6, inset).

Overall, the direct tandem fusion of FX with HSA is compatible with efficient secretion, prolonged plasma persistence and preserved activation/function, thus providing experimental evidence for the virtually-unique suitability of the carboxyl-terminal region of FX as a natural linker for fusion purposes.

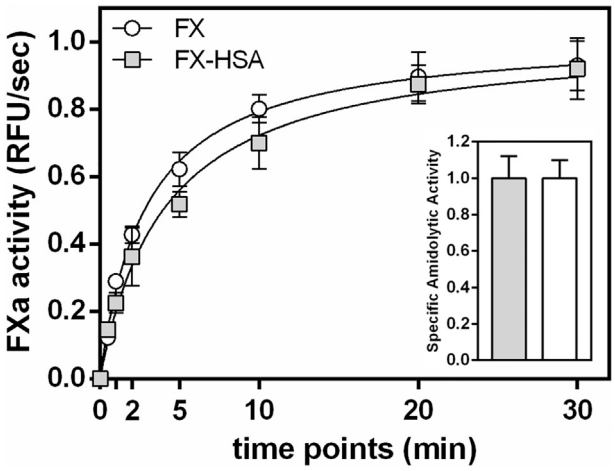

Fig. 6. Time-course activation and amidolytic activity of FX-HSA.
Amidolytic activity after time-course activation of FX and FX-HSA in the presence of rFVIIa. The amidolytic activity towards a FXa-specific fluorogenic substrate was evaluated at 0, 30″, 1′, 2′, 5′, 10′, 20′ and 30′ time points.
Inset. Specific amidolytic activity of FX-HSA and FX towards the FXa fluorogenic substrate.
RFU, Relative Fluorescence Units; sec, seconds.
Data are represented as mean ± standard deviation.

4. Discussion

We have previously demonstrated that, at variance from other homologous coagulation serine proteases such as FVII, FIX and protein C [15–18,27], the carboxyl-terminal region of factor X (residues 467–488) is dispensable for secretion and function and tolerates

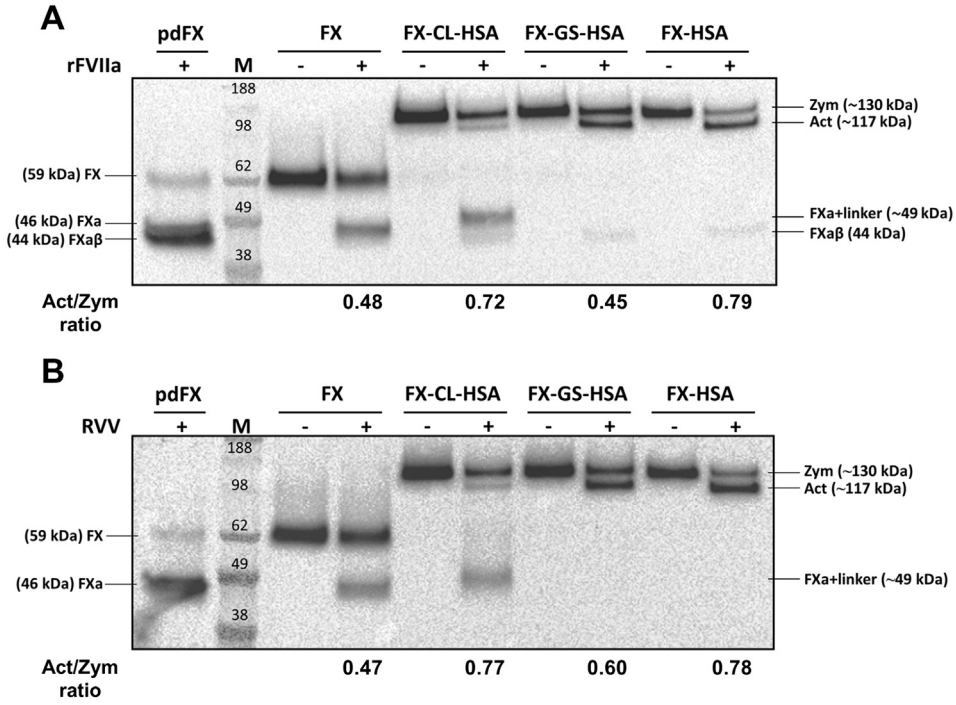

Fig. 5. Activation profile of fusion variants after incubation with physiological and non-physiological activators.
Western blotting analysis of FX and fusion variants in the absence (−) or in the presence (+) of the physiological activator rFVIIa (A) or the non-physiological activator RVV (B). The FXa + linker form is referred to the FXa form disjoined from the FX-CL-HSA fusion protein and retaining part of the linker upstream of the cleavage site (Fig. 1B). The ratio between the activated and zymogen form after activation is shown below images. Relative molecular weights for each form are indicated. Plasma-derived FX (pdFX) was used as external control. Zym, zymogen forms of fusion variants; Act, rFVIIa- or RVV-activated fusion proteins; M, molecular weight marker.

deletions of up to 21 residues [19]. For this reason, we explored this region as a direct linker sequence for fusion with protein partners such as HSA (Fig. 1B). Interestingly, HSA fusion to other pro-coagulant serine proteases [13,14] required the presence of intervening linker sequences, either flexible or cleavable, to maintain biological properties.

In the coagulation field, the same evidence was obtained for other examples of fusion proteins, in which both the N-terminal and C-terminal joining required a spacer sequence to optimize the fusion strategy [28–33]. Indeed, although direct tandem fusion with HSA at the carboxyl-terminal end would represent the simplest method, it may result in impaired expression, folding or biological function [34–36]. Thus, the identification of suitable linker sequences, providing optimal spacing between partners [37] as those occurring in proteins with multiple domains [38,39], is of great relevance when designing a fusion protein [40]. It is worth to note that our analysis predicted that the carboxyl-terminal region of FX is compatible with linker requirements since it is i) composed of amino acids that have high propensity to be found in naturally-occurring protein linkers, ii) unstructured, with a length (21 residues) in line with large natural linkers [41], and iii) displays partly flexible and rigid properties (Fig. 1A, upper panel). Similar features were also predicted for the flexible (GS) or the designed cleavable (CL) linkers exploited in our fusion strategy (Fig. 1A, lower panels).

Here, our hypothesis-driven design of a fusion protein directly joining FX and HSA was compared with two fusion strategies exploiting a flexible (FX-GS-HSA, mimicking rFVIIa-HSA) [13] or a cleavable (FX-CL-HSA, mimicking FIX-HSA) [14] linker (Fig. 1B). In our experimental settings, the direct tandem FX-HSA fusion was compatible with efficient secretion and noticeably prolonged plasma persistence *in vivo* in comparison with wild-type FX, as indicated by the differential profiles observed (Fig. 3), which validated the fusion strategy.

In functional assays, only the FX-HSA and FX-CL-HSA efficiently shortened coagulation times and generated thrombin, as shown by thrombin generation parameters detailing FX activity levels [42]. The virtually normal FX-CL-HSA functional properties are probably attributable to the cleavable features of the linker, which drives the disjoining of the fusion partner and release of the active protease, as previously observed for FIX-HSA [14]. Noticeably, the direct fusion in the FX-HSA variant, albeit not permitting the FVIIa-mediated proteolytic release of HSA, was as efficient as that joining FX and HSA through a cleavable linker, thus ensuring functional features close to normal. On the other hand, our data on the FX-GS-HSA do not parallel those reported for the rFVIIa-HSA protein, in which the GS linker, unfavorable for FX, is compatible with optimal spatial separation and FVIIa activity [13]. The productive FX-HSA functional pattern was further interpreted by evaluating its activation profile through analysis of protein forms after activation with rFVIIa as well as in time-course and amidolytic activity assays.

While the *in-vitro* expression of FX is rather straightforward, as witnessed by the numerous FX variants so far characterized [21,25,43–45], the recombinant technologies have not been exploited with FX deficiency treatment as the main goal. In the coagulation field, where a specific plasma-derived product for the treatment of FX deficiency has only recently been introduced [46], our data provide the proof-of-principle for FX as a versatile platform for protein engineering, with potential therapeutic meaning for FX deficiency. It is worth noting that our chimera could be further engineered in the FX moiety [43] to potentially treat a variety of bleeding conditions.

5. Conclusion

In conclusion, we provide experimental evidence for i) a coagulation factor fusion protein with preserved biological properties independent from artificial linkers, an evidence unseen in the coagulation field, and ii) the suitability of FX carboxyl-terminal region as a natural linker for fusion purposes with protein partners such as HSA, its domains or the IgG Fc region.

Conflicts of interest

All authors declare no competing financial interests.

Financial support

This work was supported by PFIZER EuroAspire projects WI199905 (Alessio Branchini) and WI193137 (Mirko Pinotti).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2018.11.007.

CRediT authorship contribution statement

Mattia Ferrarese: Investigation, Methodology, Data curation, Formal analysis, Writing - review & editing. **Silvia Pignani:** Investigation, Methodology, Validation, Writing - review & editing. **Silvia Lombardi:** Investigation, Methodology, Validation, Writing - review & editing. **Dario Balestra:** Investigation, Methodology, Writing - review & editing. **Francesco Bernardi:** Formal analysis, Writing - original draft, Writing - review & editing. **Mirko Pinotti:** Conceptualization, Funding acquisition, Formal analysis, Writing - original draft, Writing - review & editing. **Alessio Branchini:** Project administration, Supervision, Conceptualization, Funding acquisition, Formal analysis, Writing - original draft, Writing - review & editing.

Acknowledgments

We would like to thank Prof. Rodney Camire (The Children's Hospital of Philadelphia, Philadelphia, PA, USA) for the chimeric pCMV4-ss-pro-II-FX construct and Dr. Rosella Mari for the technical support in performing coagulant assays. This work was supported by PFIZER EuroAspire projects WI199905 (AB) and WI193137 (MP).

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