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ORIGINAL ARTICLE

The TFPIα C-terminal tail is essential for TFPIα-FV-short-protein S complex formation and synergistic enhancement of TFPIα

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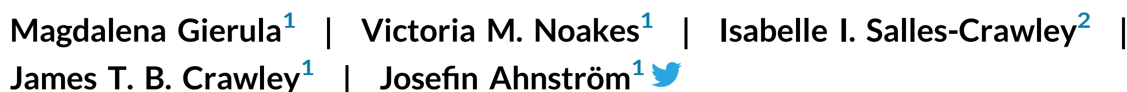

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Abstract

Background: For maximal TFPIα functionality, 2 synergistic cofactors, protein S and FV-short, are required. Both interact with TFPIα, protein S through Kunitz 3 residues Arg199/Glu226 and FV-short with the C-terminus. How these interactions impact the synergistic enhancement remains unclear.

Objectives: To determine the importance of the TFPIα-protein S and TFPIα-FV-short interactions for TFPIα enhancement.

Methods: TFPIα variants unable to bind protein S (K3m [R199Q/E226Q]) or FV-short (ΔCT [aa 1–249]) were generated. TFPIα-FV-short binding was studied by plate-binding and co-immunoprecipitation assays; functional TFPIα enhancement by FXa inhibition and prothrombin activation.

Results: While WT TFPIα and TFPIα K3m bound FV-short with high affinity (K_d~2nM), TFPIα ΔCT did not. K3m, in contrast to WT, did not incorporate protein S in a TFPIα-FV-short-protein S complex while TFPIα ΔCT bound neither FV-short nor protein S. Protein S enhanced WT TFPIα-mediated FXa inhibition, but not K3m, in the absence of FV-short. However, once FV-short was present, protein S efficiently enhanced TFPIα K3m (EC50: 4.7nM vs 2.0nM for WT). FXa inhibition by ΔCT was not enhanced by protein S alone or combined with FV-short. In FXa-catalyzed prothrombin activation assays, FV-short enhanced TFPIα K3m function in the presence of protein S (5.5 vs 10.4-fold enhancement of WT) whereas ΔCT showed reduced or lack of enhancement by FV-short and protein S, respectively.

Conclusion: Full TFPIα function requires the presence of both cofactors. While synergistic enhancement can be achieved in the absence of TFPIα-protein S interaction, only TFPIα with an intact C-terminus can be synergistically enhanced by protein S and FV-short.

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KEYWORDS
anticoagulant, cofactor, factor V-short, protein S, tissue factor pathway inhibitor

1 | INTRODUCTION

Tissue factor pathway inhibitor (TFPI) is the primary inhibitor of initiation of coagulation. It directly inhibits activated factor (F) X (FXa) activity as well as the activation of FIX and FX by the formation of a quaternary complex with tissue factor (TF)-FVIIa-FXa [1]. There are 2 main isoforms of TFPI. TFPIα is the soluble form of the protein, present in plasma as well as in platelets where it is released upon activation [2]. TFPIβ is membrane-bound, specifically present on the endothelium surface. TFPIβ does not require cofactors to achieve anticoagulant function, as it is already bound to the endothelial cell surface where it exerts its actions [3]. In contrast, TFPIα requires cofactors for full functionality. Protein S and various forms of FV have been shown to function as TFPIα cofactors, with protein S and FV often acting in synergy [4–9]. The most efficient form of FV shown to function synergistically together with protein S is FV-short [8]. FV-short is a splice isoform of FV, initially identified in individuals with East Texas Bleeding disorder [10,11]. However, it was also demonstrated to be present in the plasma of healthy individuals, albeit at much lower concentrations [11]. Although the concentration of FV-short in normal plasma has not been formally quantified, it is predicted to correspond to ~1% of the total FV pool in circulation, corresponding to 0.2-0.3nM [8,11]. Importantly, FV-short circulates in a complex with TFPIα and protein S [12]. While not experimentally shown, the 2 cofactors are thought to enhance TFPIα anticoagulant function, at least in part, by augmenting its association to the negatively charged phospholipid surfaces to enhance inhibition of FXa.

TFPIα consists of an acidic amino N-terminal, followed by 3 Kunitz type domains and a basic C-terminus. Kunitz domain 1 (K1) binds and inhibits TF-FVIIa, Kunitz domain 2 (K2) binds and inhibits FXa while Kunitz domain 3 (K3) binds protein S. The TFPIα C-terminus is responsible for binding to FV/FV-short [13–15]. The TFPIα-mediated inhibition of FXa is a two-step process where the formation of a loose encounter complex between FXa and TFPIα is followed by slow isomerization into a tight FXa-TFPI complex [16]. Protein S specifically enhances the FXa-TFPIα encounter complex formation by 4 to 10-fold [4,7,17]. We have previously shown that this enhancement is dependent upon a direct interaction between TFPIα K3 and the laminin G-type domain 1 of protein S, specifically involving residues Arg199 and Glu226 in TFPIα and residues Lys255, Glu257, Asp287, Arg410, Lys423, and Glu424 in protein S [9,17–19].

While the TFPI cofactor function of protein S has been extensively characterized *in vitro*, comparatively little is known of the synergistic cofactor function by protein S together with FV-short. We recently found that, similar to protein S alone, the synergistic enhancement by protein S and FV-short efficiently increases the rate of formation of the initial FXa-TFPIα encounter complex [19]. However, the importance of the interactions between TFPIα and its 2 cofactors is currently not known. FV-short lacks 702 amino acids from the B domain (756-1458) which exposes an acidic region (1493-1537) that binds the basic C-terminal tail of TFPIα with very high affinity (Kd in low nM range) [12,15,20]. Despite the high binding affinity, FV-short alone is a very poor TFPIα cofactor [8,21]. This completely changes in the presence of protein S, where both proteins together enhance TFPIα-mediated FXa inhibition 10-fold more efficiently than protein S alone [8,19,21]. This is also consistent with protein S further enhancing the TFPIα-FV-short interaction [12]. Recent studies showed that FV-short residues 1458-1492 and, more specifically, the FV-short hydrophobic patch (1481-1486), are essential for the formation of the TFPIα-FV-short-protein S complex and for the synergistic enhancement to occur [12,21].

Despite recent advances, the molecular mechanisms involved in the synergistic enhancement of TFPIα by its 2 cofactors are not known. Here, we determined the importance of the specific interactions between TFPIα and protein S and FV-short for the synergistic enhancement of TFPIα-mediated FXa inhibition and prothrombin activation.

Essentials

- Protein S and factor (F) V-short synergistically enhance TFPIα anticoagulant function.
- Cofactor incorporation into TFPIα-FV-short-protein S requires their direct binding to TFPIα.
- FV-short rescues the TFPIα enhancement when the protein S-TFPIα K3 interaction is absent.
- Only TFPIα with an intact C-terminus can be synergistically enhanced by protein S and FV-short.

2 | METHODS

2.1 | Expression and purification of TFPIα and FV-short

Wild-type (WT) TFPIα and its variants, containing an N-terminal 6xHis-tag (pcDNA3.1), were transiently expressed in mammalian HEK293T cells, purified, and quantified as previously described [19]. Briefly, TFPIα was purified using a nickel column, followed by a heparin column. Due to the heparin binding sites present in the TFPIα C-terminus, WT TFPIα and TFPIα K3m bound to the heparin column and were separated from the contaminants via a NaCl gradient

elution, as previously described [17,19]. In contrast, TFPIα ΔCT was separated from the column-bound contaminants by instead passing through the column. Following this purification step, TFPIα ΔCT was isolated as an essentially pure protein from the flow through fraction. WT TFPIα and its variants were quantified by ELISA [17]. WT TFPIα and TFPIα K3m were estimated by ELISA [17,19] to be 80% to 95% full-length whereas, due to the lack of C-terminus, only the total concentration of TFPIα ΔCT was determined. Functional activity of the variants was confirmed by FXa inhibition assays.

FV-short was stably expressed in baby hamster kidney cells, purified, and quantified as previously described (Supplementary Figure S1B) [19]. Briefly, FV-short was purified using nickel purification, followed by anion exchange chromatography on a Q FF column. The concentration of FV-short was determined by absorption at 280 nm using extinction coefficient (E1%, 1 cm) of 15.4 [7,15].

2.2 | Plate binding

The direct interaction between FV-short and TFPIα was studied using plate-binding assays, based on the method published by Dahlbäck and Tran [12,21].

For this, FV-short was immobilized on a microplate in 15mM Na_2CO_3, 35mM $NaHCO_3$, containing 2mM $CaCl_2$ and 10mM benzamidine. The plate was washed 4 times with 50mM Tris, 150mM NaCl, 2mM $CaCl_2$, pH 7.5, 0.05% Tween (TBSCT) buffer and blocked for 2 hours with 1% bovine serum albumin (BSA) in the same buffer. TFPIα (0-10nM) in TBSCT buffer/0.5% BSA/10mM benzamidine was added and bound TFPIα was detected using monoclonal antibodies against TFPIα (AHTFPI-5138; Prolytix), recognizing the TFPIα N-terminus, followed by horseradish-peroxidase-conjugated goat anti-mouse antibodies (DAKO). The apparent dissociation constants ($K_{D\ (app)}$) were determined using nonlinear analysis assuming one site binding.

2.3 | Co-immunoprecipitation of TFPIα-FV-short-protein S

To investigate the ability of TFPIα to interact with FV-short and protein S, tosylactivated magnetic beads (Life Technologies) were coated with polyclonal sheep anti-human TFPI antibodies (AHTFPI-S; Prolytix) according to the manufacturer's instructions. Once coated, the beads were blocked using 0.5% BSA in 50 mM Tris, pH 7.4, 150mM NaCl and 5mM $CaCl_2$ for 2 hours. TFPIα (0.5 or 5nM) was preincubated with FV-short (1 or 5nM, respectively) in the presence or absence of protein S (10nM; Enzyme Research Laboratories) for 2 hours at room temperature. Samples were added to the beads and incubated for 1 hour (1 mg beads for 200μL sample). The supernatant was removed and the beads were washed twice with 2 mL of 50mM Tris, pH = 7.4; 0.15M NaCl; and 5mM $CaCl_2$. All bound proteins were eluted using 35μL LDS buffer and analyzed by western blotting. TFPIα was detected by an in-house mouse monoclonal anti-TFPI K3 antibody, FV-short by AHV-5112 (Prolytix) and protein S by polyclonal anti-protein S antibodies (DAKO). The FV-short and protein S co-immunoprecipitated together with TFPIα were detected using a Chemidoc Touch Imaging system (BioRad) followed by analysis in Image lab software version 6.1. The quantifications were performed using adjusted volume intensity where the FV-short and protein S eluted from beads incubated in the absence of TFPIα was subtracted as background. All quantifications were performed without post-image processing.

2.4 | Phospholipid vesicle preparation

Phospholipids (Avanti Polar Lipids) 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-Dioleoyl-sn-glycero-3-phosphoserine (DOPS), and 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were mixed at a molar ratio 60:20:20 and extruded as previously described [22].

2.5 | FXa inhibition assay

FXa inhibition (0.5nM; Enzyme Research Laboratories) was monitored in real-time by the cleavage of the FXa-specific chromogenic substrate S-2765 (200μM; Chromogenix) at 405nm over 60 minutes. The FXa activity was followed in the presence or absence of 25μM phospholipid vesicles, and 5mM $CaCl_2$, in the presence and absence of TFPIα (0-32nM), protein S (0-160nM) and/or FV-short (0-4nM), as described [7,19].

The initial velocity (V_0) of the S-2765 cleavage was determined using nonlinear regression as previously described [4,17]. To derive the EC_{50} of TFPIα-mediated inhibition of FXa, the V_0 was plotted against TFPIα, protein S, or FV-short concentration, as required. The EC_{50} was determined by one-phase exponential decay nonlinear curve fit [17,18].

2.6 | Prothrombin activation assay

Prothrombin activation by FXa was analyzed similar to previous reports [6,7]. For this, FV-short or FVa (1nM) was incubated with increasing concentrations TFPIα (0-16nM) in the presence or absence of 10nM protein S for 10 minutes at 28 °C in 20mM Tris; pH, 7.4; 0.15M NaCl (TBS) containing 0.5% BSA. Thrombin-specific chromogenic substrate S-2238 (117μM; Chromogenix), 25 μM phospholipid vesicles, 5 mM $CaCl_2$, and 1μM prothrombin (Enzyme Research Laboratories) were preincubated in parallel. Thrombin generation was initiated by addition of prothrombin/phospholipids and substrate to TFPIα/FV-short/protein S, followed by the addition of 0.6pM of FXa. Prothrombin activation was followed at 405 nm for 60 minutes.

All experiments were performed in the presence of the thrombin inhibitor dansylarginine N-(3-ethyl-1,5-pentanediyl)amide (DAPA; 3 μM; Prolytix) and all concentrations are final.

For experiments involving FVa, 50nM of FV-short was activated by thrombin (1nM; Enzyme Research Laboratories) in 0.5% BSA in TBS at 37 °C for 15 minutes. The thrombin required for FV-short activation remained during prothrombinase activation reactions (20pM) but did not influence the prothrombin activation readings (data not shown).

The slopes of the linear portions of these progress curves, representing rates of thrombin formation, were expressed as percentages of the rate obtained in the absence of TFPIα and plotted as a function of the TFPIα concentration. IC_{50} values were determined by one-phase exponential decay nonlinear curve fit.

3 | RESULTS

3.1 | Evaluation of binding of WT TFPIα and TFPIα variants to FV-short and incorporation into a TFPIα-FV-short-protein S tri-molecular complex

TFPIα is functionally enhanced by its 2 cofactors, protein S and FV-short. In this study, we assessed the mechanisms underlying their synergistic cofactor function by investigating the functional importance of their respective interactions with TFPIα using 2 TFPIα variants (Figure 1A). TFPIα K3m, contains 2 K3 amino acid substitutions (Arg199Gln and Glu226Gln) to assess the importance of the TFPIα-protein S interaction [9,17]. The basic region of the TFPIα C-terminal tail contains the interaction site for various forms of FV, including the interaction site for FV-short. For this purpose, we generated TFPIα ΔCT, truncated before the basic region at aa 249 (ie, lacking last 27aa) [7]. WT TFPIα and its variants were successfully expressed and purified to homogeneity (Supplementary Figure S1A).

While the residues substituted in TFPIα K3m have been shown to abolish protein S interaction [9,17], the impact of the truncation of the TFPIα C-terminus (at aa 249) to FV-short cofactor function has not been formally tested. To address this, the binding of WT TFPIα and its variants to FV-short was assessed using plate-binding assays [12,21]. WT TFPIα and TFPIα K3m both bound to FV-short with a $K_{D\ (app)}$ ∼2nM (Figure 1B and Table). As expected, very little binding between TFPIα ΔCT and FV-short was observed, confirming the importance of the basic region of the TFPIα C-terminus for the FV-short interaction.

Since TFPIα circulates as a complex together with FV-short and protein S in plasma, we performed co-immunoprecipitation assays to test whether TFPIα K3m and TFPIα ΔCT could be incorporated in such a complex solution. When TFPIα (5nM) was preincubated together with FV-short (5nM) in the presence and absence of protein S (10nM), FV-short was efficiently co-eluted with both WT TFPIα and TFPIα K3m (Figure 1C). These results agree with the similar TFPIα-FV-short binding affinities determined in the solid-phase binding assays (Figure 1B). As previously shown [12], protein S enhanced the WT TFPIα-FV-short interaction, as seen by the approximately 3.2-fold increase in FV-short binding (Figure 1C–D). In contrast, no such increase was seen for the TFPIα K3m-FV-short binding and very little co-elution of protein S was observed (Figure 1C–E), suggesting that a TFPIα-protein S interaction is required for protein S to bind to TFPIα also in the presence of FV-short. TFPIα ΔCT bound neither FV-short nor protein S. The co-immunoprecipitations were also performed at more physiological concentrations of TFPIα (0.5nM) and FV-short (1nM) where, again, the WT TFPIα-FV-short interaction was enhanced by protein S, whereas no such enhancement could be seen for either TFPIα K3m or TFPIα ΔCT (Figure 1C–E).

3.2 | Inhibition of FXa by WT TFPIα and TFPIα variants in the absence of cofactors

The functionality of WT TFPIα and its variants were assessed in direct FXa inhibition assays in the absence of protein S and FV-short. For this, FXa was inhibited by increasing concentrations of TFPIα (0-32nM). TFPIα-mediated inhibition of FXa was monitored over time and demonstrated that both TFPIα K3m and TFPIα ΔCT inhibited FXa with a comparable efficiency to WT TFPIα (Figure 2A–C). The initial rates of S-2765 hydrolysis were plotted as a function of TFPIα concentration (Figure 2D). The IC_{50} values derived for WT TFPIα and TFPIα K3m were comparable at 8.1 ± 2.3nM and 9.6 ± 2.9nM (Table), respectively. As previously described for the same variant, there was an approximate 2-fold reduction in the IC_{50} obtained for TFPIα ΔCT (15.4 ± 5.9nM) [7]. Although not statistically significant, these results confirm those from previous reports that even though TFPIα K2 is responsible for FXa inhibition, the C-terminus is also required for most efficient FXa regulation [7,9,23].

3.3 | Protein S enhancement of FXa inhibition by WT TFPIα and TFPIα variants

To investigate whether the substitutions in the TFPIα K3 domain and deletion of C-terminus affect enhancement by protein S, the direct FXa inhibition was performed in the presence of increasing concentrations of protein S (0-160nM) (Figures 3A–D). As expected, WT TFPIα was dose-dependently enhanced by protein S with an EC_{50} of 11.3 ± 0.4nM (Figure 3A, Table). In contrast, protein S had no effect on TFPIα K3m mediated inhibition of FXa (Figure 3B). In agreement with previous reports [9,17,24], this finding demonstrates that protein S enhancement depends on a direct TFPIα–protein S interaction. Interestingly, protein S enhancement of TFPIα ΔCT was also severely reduced and too low to derive an EC_{50} value from the initial velocities (Figure 3C–D). Since the TFPIα C-terminus is involved in the FXa-TFPIα encounter complex formation in the absence of protein S [25], we also plotted the endpoint absorbance against protein S concentration to assess the full enhancement by protein S. While TFPIα ΔCT showed a marginally more efficient enhancement by protein S, this was still appreciably reduced compared with WT TFPIα (Figure 3E).

Since the ability of TFPIα ΔCT to inhibit FXa was reduced in the absence of protein S, the titration of protein S was repeated at higher TFPIα concentration (5nM) (Supplementary Figure S2). Under these conditions, protein S was more effective in enhancing both WT TFPIα

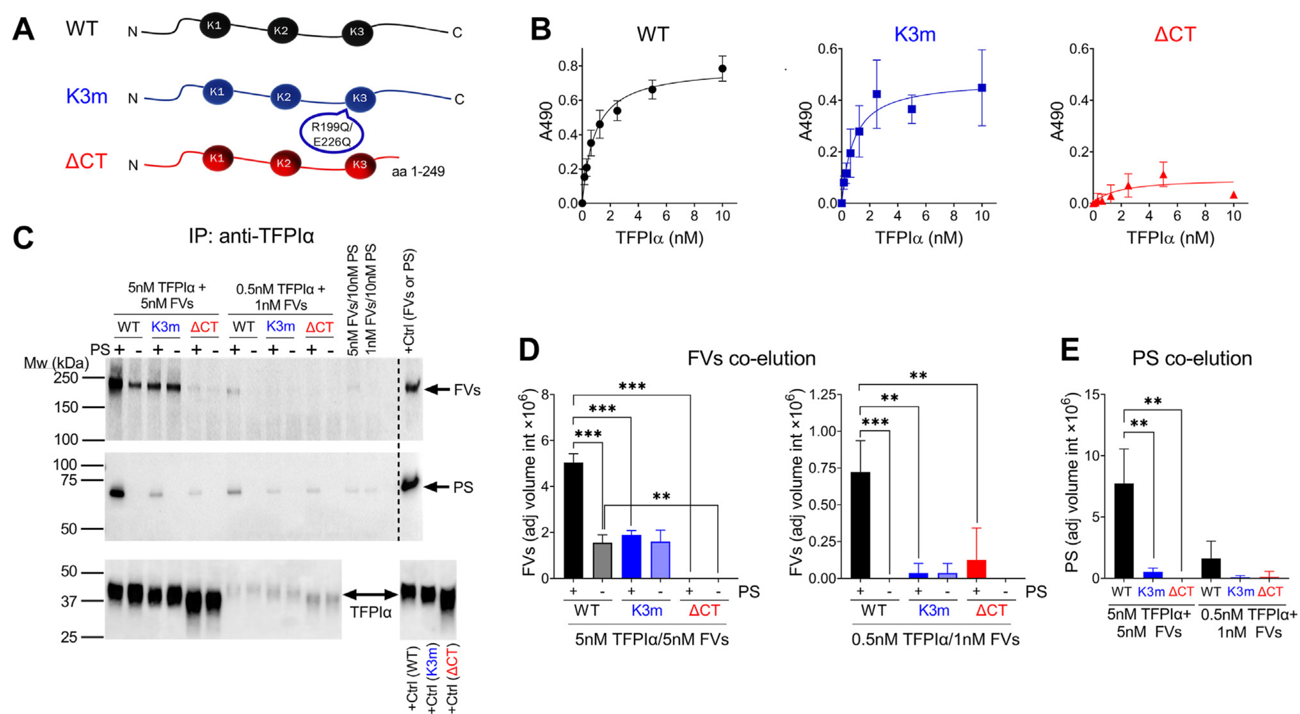

FIGURE 1 Binding of WT TFPIα and its variants to FV-short and the structural requirements for the formation of a TFPIα-FV-short-protein S complex. (A) Schematic diagrams of WT TFPI and its variants used in the study. TFPIα K3m contains 2 amino acid substitutions in the K3 domain; R199Q and E226Q. TFPIα ΔCT has been truncated at amino acid residue 249 at the C-terminus. (B) Binding of WT TFPIα and TFPIα variants to FV-short was assessed using plate-binding assays. WT TFPIα, TFPα K3m, and TFPIα ΔCT were titrated (0-10nM) over immobilized FV-short. Bound TFPIα was detected using monoclonal antibodies against TFPIα (AHTFPI-5138). Results are presented as mean ± SEM (*n* = 3-5) and the affinities given in Table. (C-E) Binding of FV-short and protein S to TFPIα in solution was further assessed using co-immunoprecipitation. Here, TFPIα (5 or 0.5nM) was incubated with FV-short (5 or 1nM, respectively) in the presence or absence of 10nM protein S. The proteins were incubated for 2 hours at room temperature, allowing for interactions to form. Following the incubation, TFPIα was immunoprecipitated using tosylactivated magnetic beads coated with polyclonal anti-TFPI antibodies. Bound proteins were eluted and analyzed by western blotting, detecting FV-short, protein S, or TFPIα. The results from a representative experiment are shown in (C). (D–E) The amount of FV-short (D) and protein S (E) co-immunoprecipitated together with TFPIα was quantified using BioRad Image Lab 6.1. The adjusted volume intensity is presented as mean ± SD (*n* = 3). Statistically significant differences are highlighted as $^{**}P < .01$ and $^{***}P < .001$ according to Student's *t*-test. F, factor; WT, wild-type.

and TFPIα ΔCT-inhibitions of FXa. However, the enhancement of TFPIα ΔCT remained severely reduced as was evident from the minor reduction in V_0 (Supplementary Figure S2C). Due to the increased concentration of TFPIα present in these experiments, an EC_{50} could be determined for TFPIα ΔCT from endpoint absorbance reads but was approximately 10-fold reduced in comparison to WT TFPIα (3.3 ± 0.3nM for WT vs 33.9 ± 2.0nM for ΔCT; Supplementary Figure S2D). These results agree with the previously described importance of the TFPIα C-terminus for the FXa-TFPIα encounter complex formation, both in the presence and absence of protein S [6,25].

3.4 | Inhibition of FXa by WT TFPIα and TFPIα variants in the presence of both cofactors

Since protein S functions in synergy with FV-short, we next evaluated the protein S enhancement of TFPIα-mediated inhibition of FXa in the presence of FV-short. While the inhibition of FXa by TFPIα is largely independent of phospholipid surfaces, the enhancement of TFPIα by protein S in the absence of FV-short is entirely phospholipid dependent. However, since the TFPIα-FV-short-protein S complex is present in plasma and therefore not dependent on a membrane surface for the 3 proteins to interact, we tested whether the complex may enhance inhibition of FXa also in the absence of phospholipids. FXa inhibition assays were therefore initially set up in the absence of phospholipid vesicles. Under these conditions none of the TFPIα variants (0.5 or 2nM), including WT, were enhanced by the cofactors (5nM FV-short and 10nM protein S), suggesting that, despite interacting in the absence of phospholipid membranes, functional enhancement by the 2 cofactors requires a membrane surface (Supplementary Figure S3).

Next, FXa inhibition assays were performed in the presence of phospholipid surfaces. As FXa inhibition is much more efficient in the presence of both cofactors, the TFPIα concentration was reduced to 0.5nM in these assays, which is also within the physiological range of TFPIα in circulation [26]. The assays included increasing concentrations of protein S (0-40nM) and in the presence and absence of saturating concentrations (2nM) of FV-short (Figure 4A–F). The

TABLE FV-short binding and functional enhancement of WT TFPIα and its variants.

Assay conditions	WT TFPIα	TFPIα K3m	TFPIα ΔCT
TFPIα-FV-short binding			
$K_{d\ app}$ (nM)	2.1 ± 0.6	1.9 ± 0.9	N.D
EC_{50} of TFPIα-mediated FXa inhibition (nM)			
TFPIα (0-32nM)	8.1 ± 2.3	9.6 ± 2.9	15.4 ± 5.9
2.5nM TFPIα + protein S (0-160nM)	11.4 ± 0.4	N.D	N.D
5nM TFPIα + protein S (0-160nM)	6.4 ± 1.2	N.D	N.D
0.5nM TFPIα + 2nM FV-short + protein S (0-40nM)	2.0 ± 0.3	4.7 ± 2.4[a]	N.D
0.5nM TFPIα + 5nM protein S + FV-short (0-4nM)	0.47 ± 0.10	0.68 ± 0.04	N.D

The $K_{d\ apparent}$ for TFPIα binding to FV-short was assessed by plate-binding assays. The results are presented mean ± SEM (n = 3-8). The EC_{50} for the functional inhibition of FXa by TFPIα in the presence and absence of protein S and/or FV-short is reported. The EC_{50} is calculated from the V_0 for the titrated protein from the data presented in Figures 2 to 6. The results are presented mean ± SD (n = 3-6).
[a]P < .05 according to the Mann–Whitney U-test, compared to WT TFPIα. EC_{50}, ____; N.D., not determined; WT, wild-type;

results obtained in the absence of FV-short, as expected, reflect those presented in Figure 3, but with less efficient enhancement by protein S (Figure 4A, C, and E). As expected, when protein S was titrated in the presence of FV-short, the enhancement of FXa inhibition by WT TFPIα was much more pronounced (Figure 4B). Here 50% of the maximal enhancement of TFPIα-mediated FXa inhibition was achieved with as little as 2nM of protein S (Figure 4H; Table). To our surprise, when FV-short was added to the assay, protein S dose-dependently enhanced

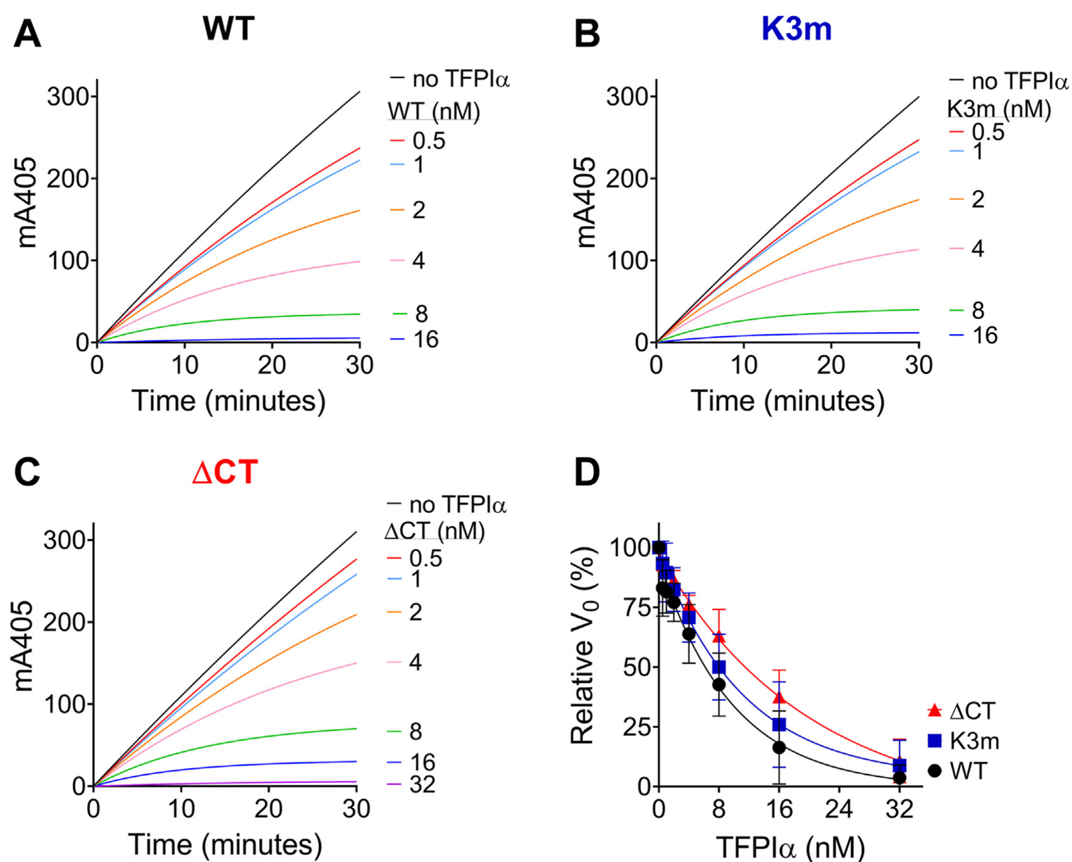

FIGURE 2 The inhibition of FXa by WT TFPIα, TFPIα ΔCT and TFPIα K3m. FXa activity (0.5nM) was followed in real-time through the cleavage of S-2765 (200μM) at 405 nm in the presence of 25μM phospholipids and increasing concentrations (0-32nM) of WT TFPIα (A), TFPIα K3m (B), or TFPIα ΔCT (C). Results from representative experiments are shown (n = 3-5). (D) The initial velocity (V_0) was calculated for each curve and plotted against TFPIα concentration. V_0 is expressed as percentage of the V_0 observed in the absence of TFPIα. Results are given as mean ± SD. The EC_{50} values of TFPIα mediated FXa inhibition were derived and are presented in Table. EC_{50}, half maximal effective concentration; F, factor; WT, wild-type.

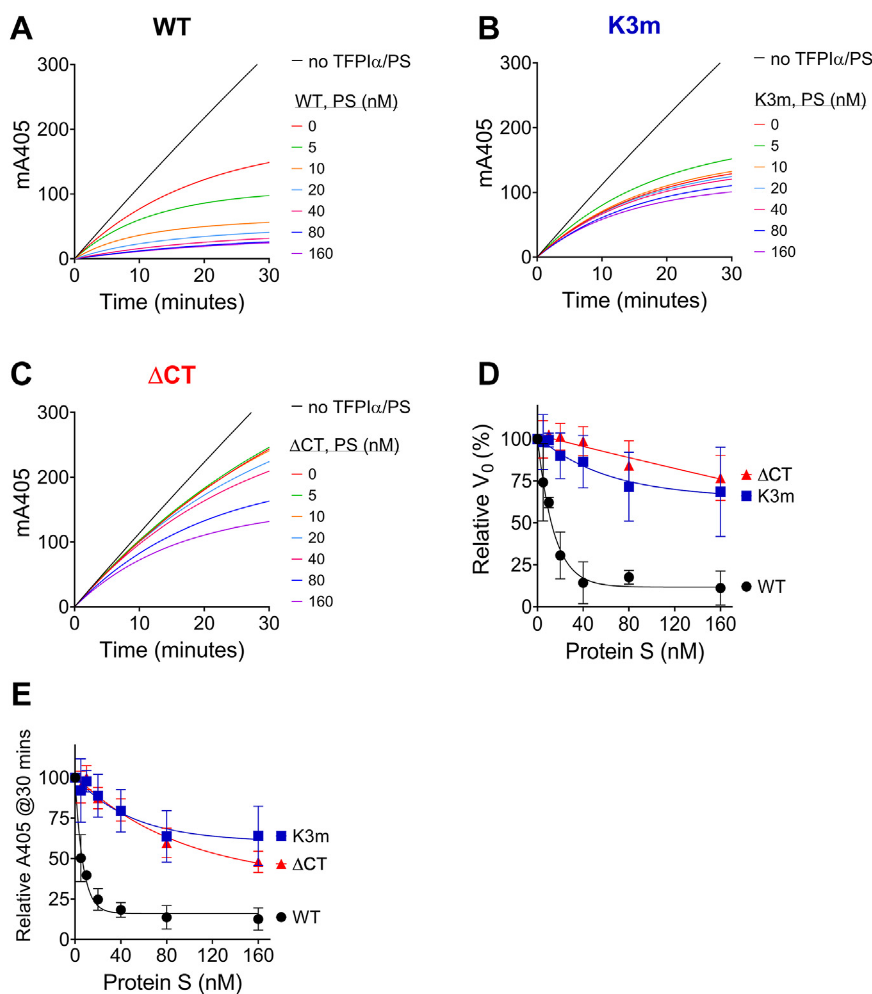

FIGURE 3 Enhancement of 2.5nM TFPIα and its variants by protein S in the inhibition of FXa. FXa activity (0.5nM) was followed in real-time through cleavage of S-2765 (200μM) at 405 nm in the presence of 25μM phospholipids and absence or presence (2.5nM) of WT TFPIα (A), TFPIα K3m (B), or TFPIα ΔCT (C) and increasing concentrations (0-160 nM) of protein S. Results from representative experiments are shown (n = 3-4). (D) V_0 was calculated for each curve and plotted against protein S concentration. Relative V_0 was calculated as percentage of the V_0 obtained for TFPIα alone. (E) A405 measured at 30 minutes of FXa inhibition was plotted against concentration of protein S. Relative A405 was calculated as a percentage of the A405 measured for TFPIα alone. Results are given as mean ± SD. The EC_{50} values of TFPIα mediated FXa inhibition were derived and are presented in Table. EC_{50}, half maximal effective concentration; F, factor; WT, wild-type.

TFPIα K3m (Figure 4D). The EC_{50} for the TFPIα K3m-mediated FXa inhibition was only increased by approximately 3-fold compared with WT TFPIα (Table), suggesting that the presence of FV-short rescues the synergistic enhancement by protein S and FV-short when the TFPIα-protein S interaction has been disrupted.

In contrast, the efficiency of TFPIα ΔCT-mediated inhibition of FXa was not enhanced by protein S regardless of whether FV-short was present or not (Figure 4E–H).

3.5 | Synergistic enhancement of FXa inhibition cannot be achieved without an intact TFPIα C-terminus

To further investigate how FV-short augments TFPIα-mediated inhibition of FXa, inhibition assays were performed using increasing concentrations of FV-short in the absence and presence of protein S. As previously shown [8,19], in the absence of protein S, FV-short was unable to significantly enhance the inhibition of FXa by TFPIα, with little (WT TFPIα and TFPIα K3m) or no (TFPIα ΔCT) enhancement being observed (Figure 5A, C, and E). In contrast, and as reported previously by us and others [8,19], when protein S was present, FV-short showed significant enhancement of FXa inhibition by WT TFPIα (Figure 5B and Table). In agreement with the results from the protein S titrations (Figure 4D–F), the enhancement of K3m by FV-short in the presence of protein S was comparable to that of WT (Figure 5D and Table). In contrast, FV-short did not enhance the inhibition of FXa by TFPIα ΔCT even in the presence of protein S (Figure 5F).

3.6 | Enhancement of TFPIα-mediated inhibition of prothrombinase activation is dependent on the TFPIα C-terminus

A physiological consequence of FXa inhibition by TFPIα is reduced FXa-catalyzed prothrombin activation. Following on from the FXa inhibition assays, the impact on these findings on prothrombin activation was therefore tested. These were performed in the presence of either FVa or FV-short. Increasing concentrations of TFPIα were preincubated with FVa/FV-short (1nM) in the presence or absence of protein S (10nM) to allow for any interactions forming to mimic the

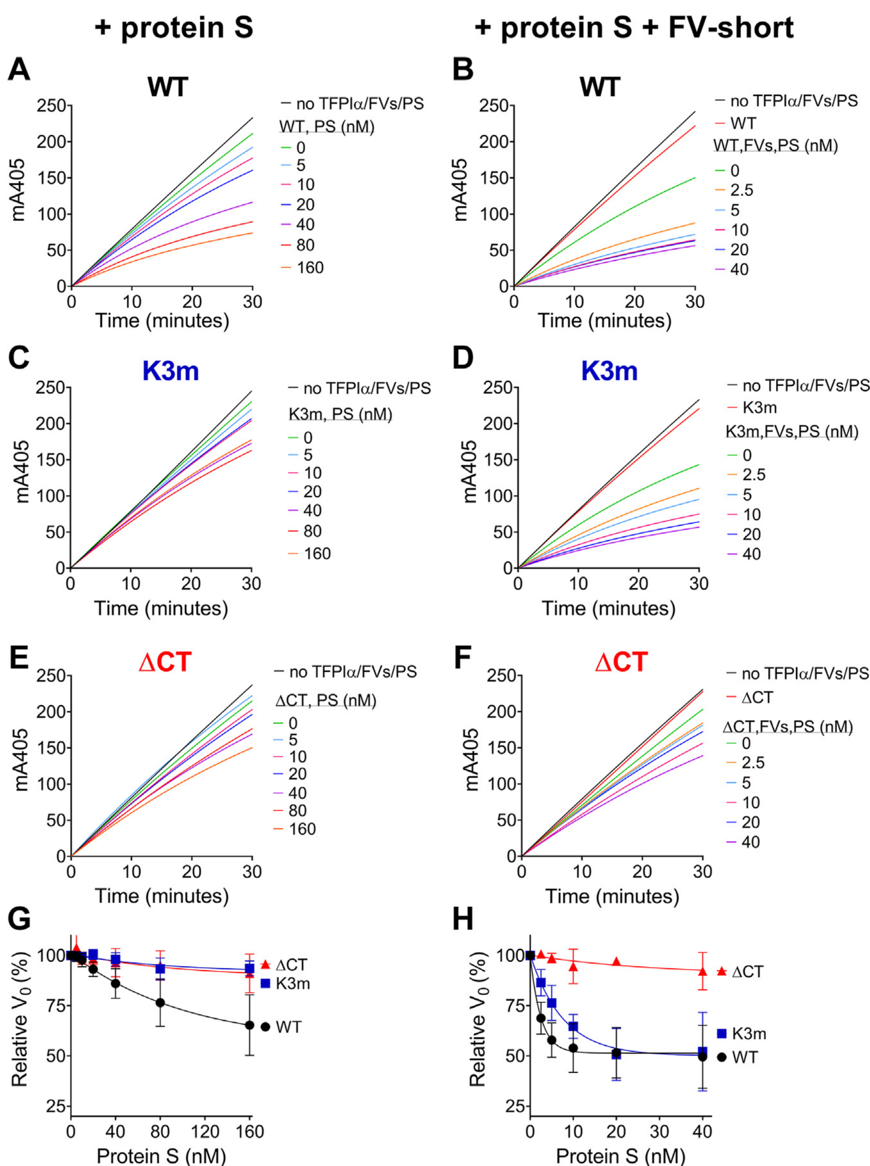

FIGURE 4 Enhancement of WT TFPIα, TFPIα K3m, and TFPIα ΔCT mediated inhibition **of FXa by FV-short and increasing concentrations of protein S in the presence of phospholipids.** FXa activity (0.5nM) was followed in real-time through cleavage of S-2765 (200μM) at 405 nm in the presence of 25μM phospholipids, WT TFPIα or its variants (0.5 nM) and the absence (A, C, E,) or presence of 2nM of FV-short (B, D, F), at increasing concentrations of protein S, 0-160nM (A, C, E,) or 0-40nM (B, D, F). Results from representative experiments are shown (*n* = 3). (G, H) V_0 was calculated for each curve and plotted against protein S concentration. Results are given as mean ± SD and are expressed as percentage of the V_0 for TFPIα alone (A, C, E) or together with FV-short (B, D, F) (*n* = 3). The EC_{50} values of TFPIα mediated FXa inhibition were derived and are presented in Table. EC_{50}, half maximal effective concentration; F, factor; WT, wild-type.

complexes present in circulation. The assays were initiated by addition of prothrombin, followed by FXa (0.6pM). The slopes of the linear portions of these progress curves (Figure 6), representing rates of thrombin formation, were expressed relative to the rate obtained in the absence of TFPIα (Figure 7). As previously described, the prothrombinase complex formed with FVa was resistant to inhibition by TFPIα and protein S and only a modest enhancement of WT TFPIα by protein S was observed (Figure 7A and Supplementary Figure S4). However, no such enhancement of TFPIα K3m or TFPIα ΔCT was observed even when up to 16nM TFPIα and 50nM protein S was added (Figure 7C, E and Supplementary Figure S4). Since FVa, in contrast to FV-short, does not interact with TFPIα, these findings agree with the lack of enhancement of TFPIα K3m and TFPIα ΔCT in the FXa inhibition assays by protein S alone. Next, the inhibition of prothrombin activation by WT TFPIα and its variants were tested in the presence of FV-short. Compared to the assays run in the presence of FVa, WT TFPIα was a much more effective inhibitor of prothrombin activation in the presence of FV-short (IC_{50} 0.32 ± 0.07nM vs N.D. for FVa; Figures 6A, B and 7A, B). The inhibitory function of WT TFPIα was further enhanced by 10.4-fold by protein S (IC_{50} 0.03 ± 0.01nM; Figures 6B and 7B). In comparison, TFPIα K3m showed a moderate, but not statistically significantly, reduced anticoagulant function in these assays, compared with WT TFPIα (Figures 6D and 7D). However, while not enhanced by protein S in the assays performed with FVa, TFPIα K3m was enhanced by approximately 5.5-fold by protein S when performed in the presence of FV-short (IC_{50} 0.20 ± 0.06 vs 1.1 ± 0.40nM; Figure 7D), providing evidence of the synergistic enhancement of TFPIα by FV-short and protein S also in inhibition of prothrombin activation. In contrast, TFPIα ΔCT again showed poor inhibitory function and complete lack of enhancement by protein S even when the assays were run in the presence of FV-short (Figures 6F and 7F).

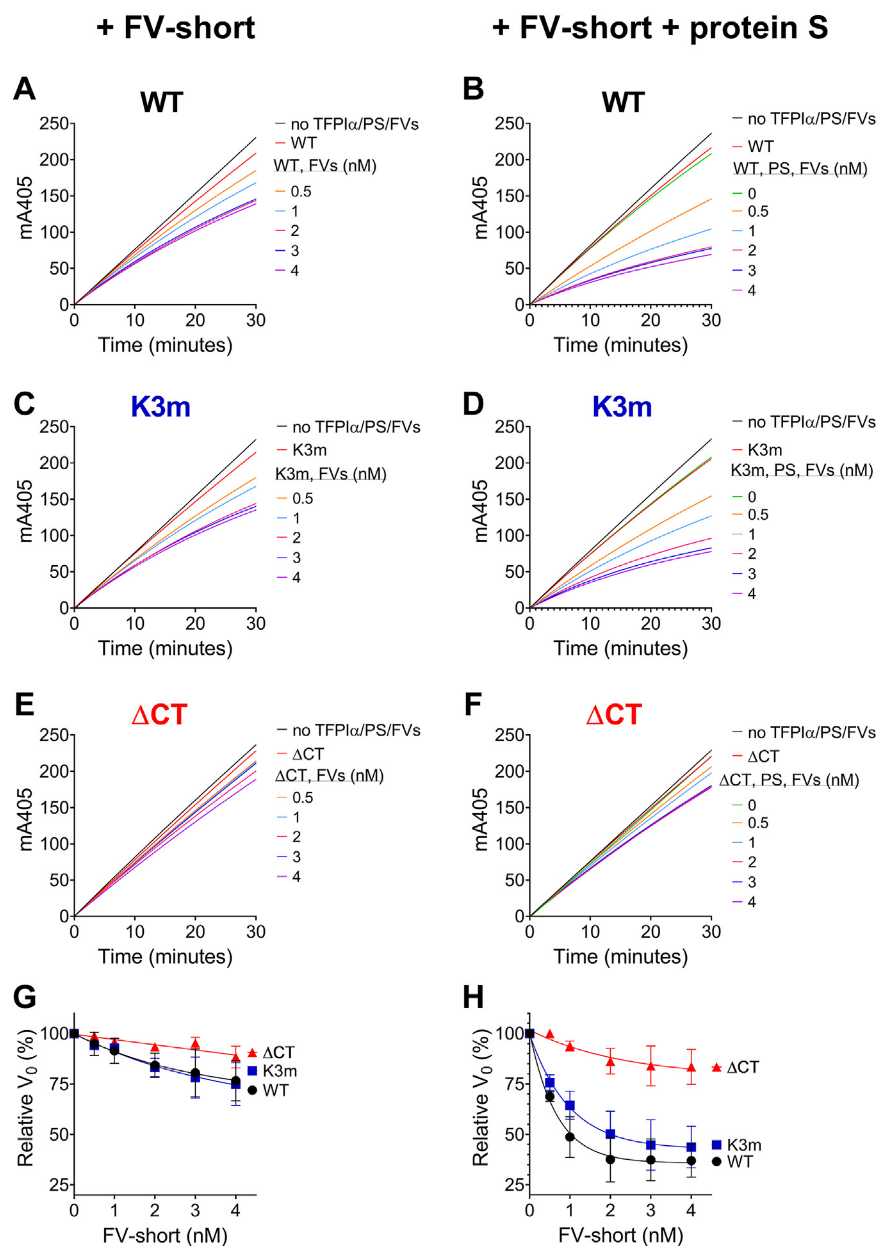

FIGURE 5 Enhancement of WT TFPIα, TFPIα K3m and TFPIα ΔCT mediated inhibition **of FXa by protein S and increasing concentrations of FV short in the presence of phospholipids.** FXa activity (0.5nM) was followed in real-time through cleavage of S-2765 (200μM) at 405 nm in the presence of 25μM phospholipids, WT TFPIα or its variants (0.5nM) and the absence (A, C, E,) or presence of 5nM of protein S (B, D, F), at increasing concentrations of FV-short (0-4nM). Results from representative experiments are shown (*n* = 3). (G, H) The initial velocity (V_0) was calculated for each curve and plotted against FV-short concentration. Results are given as mean ± SD and are expressed as percentage of the V_0 for TFPIα either alone (A, C, E) or together with protein S (B, D, F) (*n* = 3). The EC_{50} values of TFPIα mediated FXa inhibition were derived and are presented in Table. F, factor; WT, wild-type.

4 | DISCUSSION

The recent identification of FV-short and its presence in normal plasma has changed our understanding of the regulation of coagulation by TFPIα [11]. TFPIα interacts directly with both of its cofactors, FV-short and protein S, in circulation and the importance of these interactions are apparent by the positive correlations found between the plasma levels of TFPIα, protein S, and FV [26–29]. All 3 proteins act cooperatively to form a TFPIα-FV-short-protein S complex [12,28] and the results presented here show that this occur at their respective physiological concentrations (Figure 1). To date, it has not been established how the respective interactions between TFPIα and its 2 cofactors influence their ability to form a complex in solution and how this in turn, impacts their synergistic TFPIα enhancement. To address this, we generated 2 TFPIα variants, TFPIα K3m and TFPIα ΔCT that are unable to bind to protein S and FV-short, respectively. As designed, truncation of the TFPIα C-terminus in TFPI ΔCT almost completely abolished the TFPIα-FV-short interaction. In contrast, TFPIα K3m bound to FV-short with high affinity and showed that the TFPIα K3 residues involved in the protein S interaction are not important for the interaction with FV-short (Figure 1B). When testing the TFPIα variants' ability to form a complex with FV-short and protein S using co-immunoprecipitation, we found that an effective TFPIα-protein S interaction must occur for the tri-molecular complex to form as no/very little protein S binding or enhancement of the TFPIα-FV-short interaction was detected in its absence (Figure 1C–E). Furthermore, when the TFPIα-FV-short interaction was absent, no protein S interaction was seen, which is in agreement with the low

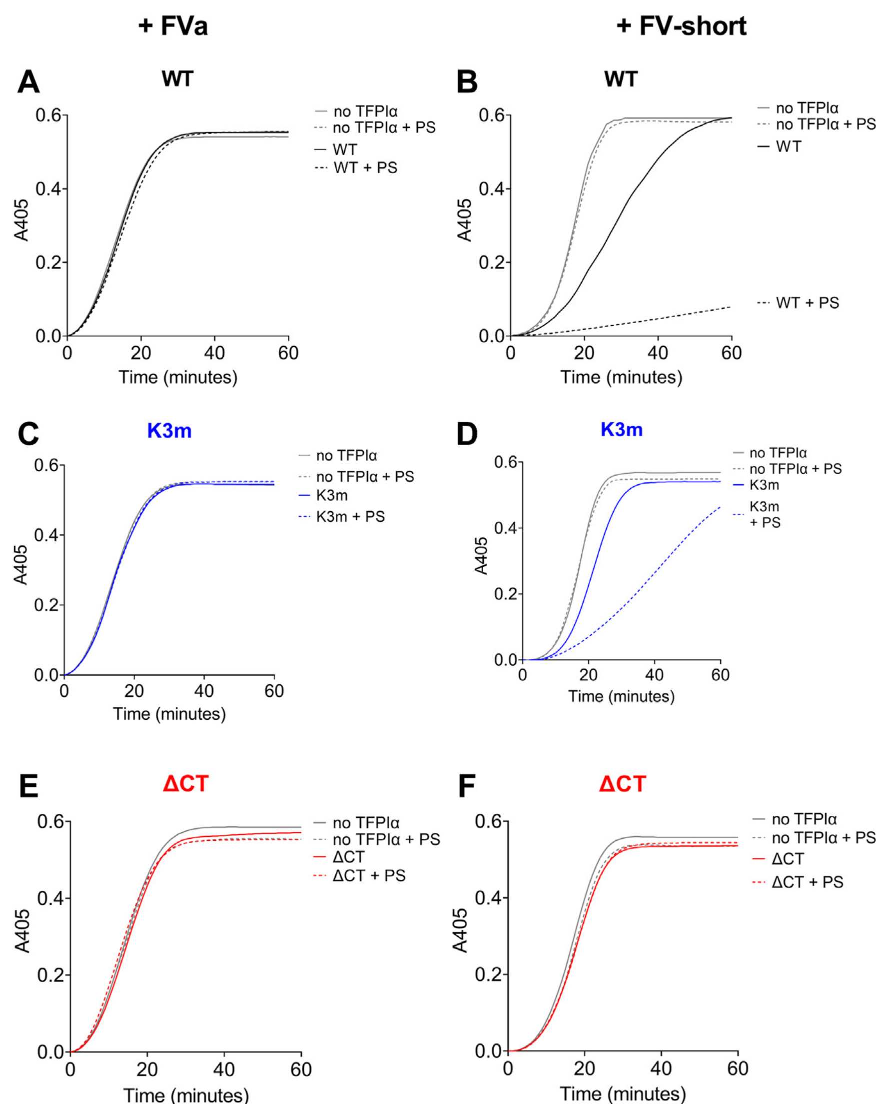

FIGURE 6 The effect of TFPIα and protein S on FXa-catalysed prothrombin activation in the presence of FVa or FV-short. FVa or FV-short (1nM) was incubated with 0.5nM WT TFPIα (A-B), TFPIα K3m (C-D) or TFPIα ΔCT (E-F) in the presence or absence of 10nM protein S. Prothrombin (1μM), phospholipid membranes and 117 μM substrate S-2238 was added to TFPIα/FV-short/protein S, followed by initiation of prothrombin activation by the addition of FXa (0.6pM). The thrombin activity was measured through cleavage of the chromogenic substrate at 405 nm over time. A representative experiment is shown (*n* = 3). PS, protein S; F, factor; WT, wild-type.

affinity previously determined for TFPIα-protein S in the absence of FV-short [9,17,18,28].

While our binding assays provide information on determinants required for the 3 proteins to form a complex solution, it is not clear how this translates into the functional enhancement of TFPIα. To study the TFPIα cofactor function of our TFPIα variants, we initially employed FXa inhibition assays. Experiments run in the absence of cofactors confirmed that both variants were functional (Figure 2). We then tested their ability to be enhanced by the cofactors individually. As previously described, no enhancement of TFPIα by protein S was detected in the absence of a TFPIα-protein S interaction (Figure 3) [9,17]. The protein S enhancement of TFPIα ΔCT was also severely reduced. While the main interaction site for protein S in TFPIα has been located within the K3 domain, truncations of the C-terminus have been shown to moderately reduce its affinity for protein S [9] which could explain these results. However, considering the TFPIα C-terminus is expected to bind to FV-short in circulation, the physiological impact of this interaction for the circulating TFPIα pool is questionable and instead highlights the importance of studying the effect of the cofactors in combination. The experiments described above were performed at supraphysiological concentrations of TFPIα (2.5nM) and were also repeated at physiological levels (0.5nM; Figure 4). At this TFPIα level even 160nM protein S only caused a modest enhancement of WT TFPIα, emphasizing the requirement of a second cofactor for efficient FXa inhibition.

While FV-short alone did not enhance FXa inhibition by either WT TFPIα or its variants, efficient enhancement was seen of WT TFPIα when added in the presence of protein S (Figure 5). These findings are consistent with previous reports and show that both cofactors together augment the FXa inhibition by TFPIα to much greater extent [8,19]. Like protein S, the synergistic enhancement by protein S and FV-short is clearly visible as a reduction in V_0, showing how they together enhance the FXa-TFPIα encounter complex formation. Intriguingly, while the TFPIα-protein S interaction is essential for the enhancement by protein S alone, FV-short could restore the synergistic enhancement of TFPIα even in the absence of an optimal TFPIα-

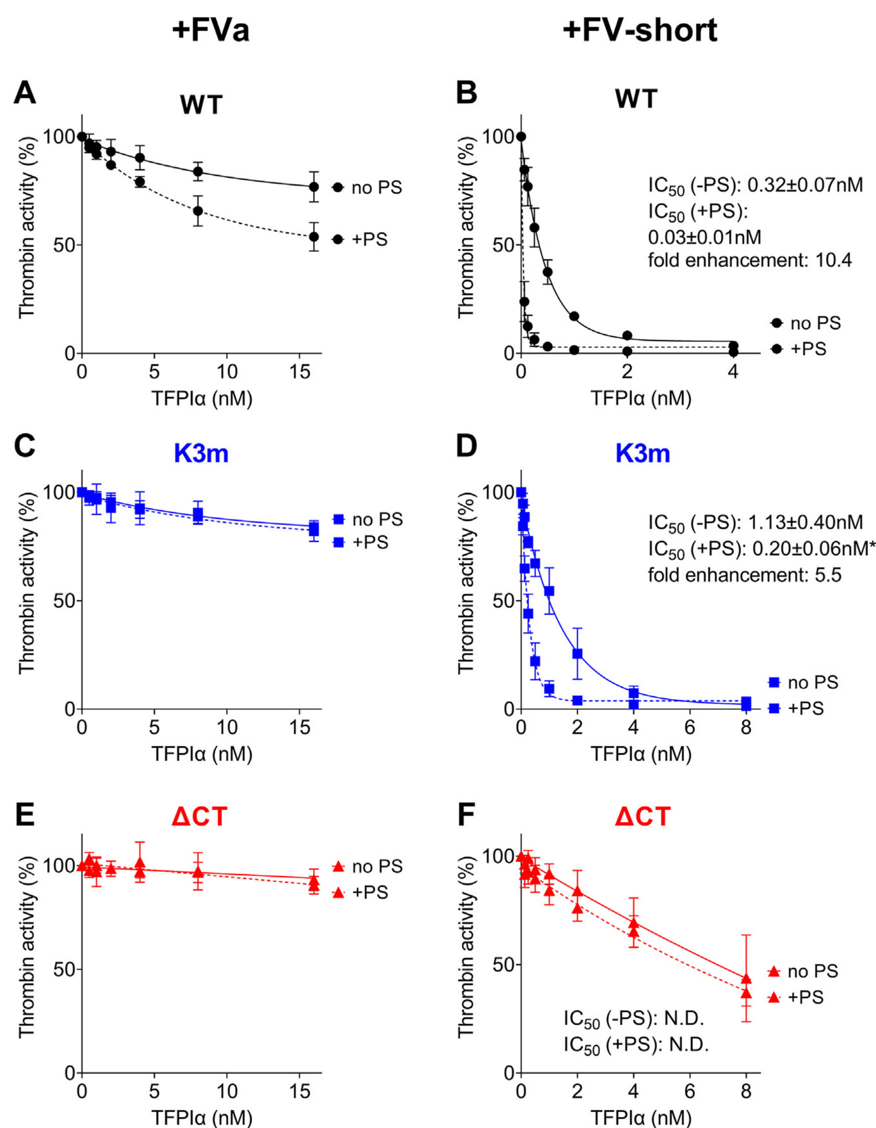

FIGURE 7 Enhancement of TFPIα-mediated inhibition of FXa-catalysed prothrombin activation in the presence of FVa or FV-short and in the presence and absence of protein S. FVa or FV-short (1nM) was incubated with WT TFPIα (A–B), TFPIα K3m (C–D) or TFPIα ΔCT (E–F) in the presence or absence of 10nM protein S. Prothrombin (1μM), phospholipid membranes and 117 μM substrate S-2238 was added to TFPIα/FV-short/protein S, followed by initiation of prothrombin activation by the addition of FXa (0.6pM). The thrombin activity was measured through cleavage of the chromogenic substrate at 405 nm over time. Thrombin formation was expressed as a percentage of the rate obtained in the absence of TFPIα. The results are expressed as means ± SD, (n = 3). Note the x-axis scale change in *B, D, and F; C* compared to *A; C and E* to better show the enhancement by FV-short and protein S at lower TFPIα concentrations. For each curve, only the linear range of curve was used for measuring thrombin activity as stated in the methods section. *P < .05 according to Mann–Whitney U-test, compared to the absence of protein S. F, factor; N.D., not determined; PS, protein S.

protein S interaction. In contrast, the TFPIα C-terminus is critical for FXa inhibition also in the presence of both protein S and FV-short, agreeing with the limited anticoagulant function of truncated TFPI versions in plasma [1].

Following on from our FXa inhibition assays it was essential to determine how our findings ultimately influence FXa-catalyzed prothrombin activation. Importantly, the experiments were set up in such a way that allowed TFPIα to bind to its cofactors before the assay was initiated to mimic the situation in circulation. As previously shown, TFPIα is a poor inhibitor of prothrombin activation when FVa is present [6,7,14], which agrees with TFPIα only being an effective anticoagulant during the initiating stages of coagulation. Despite this, we saw a modest enhancement of WT TFPIα by protein S, whereas protein S was unable to enhance both TFPIα K3m and TFPIα ΔCT (Figures 6 and 7). These results are consistent with neither TFPIα variant being enhanced by protein S in FXa inhibition in the absence of FV-short. WT TFPIα was significantly more efficient in the inhibition of prothrombin activation when performed in the presence of FV-short (Figures 6 and 7). These results agree with those previously reported for FV-short and other FV species interacting with TFPIα, including FXa activated FV [14,15], and highlight the importance of a TFPIα-FV-short interaction for TFPIα-mediated inhibition of prothrombin activation. The inhibition of prothrombin activation by WT TFPIα in the presence of FV-short was further enhanced by protein S (10.4-fold). Importantly, this study is the first to show protein S enhancement of TFPIα-FV-short in the inhibition of prothrombin activation. Similar to the results obtained for inhibition of FXa, the presence of FV-short partially restored the anticoagulant properties of TFPIα in the absence of a TFPIα-protein S interaction also in the inhibition of prothrombin activation. The partial rescuing of TFPIα K3m by FV-short also provide evidence of FV-short and protein S functioning as synergistic cofactors for TFPIα in inhibition of prothrombin activation. Results obtained using TFPIα ΔCT again highlighted the importance of TFPIα C-terminus for TFPIα anticoagulant functions.

Armed with the data obtained from this study, we may need to review our picture of the mechanisms involved in TFPIα anticoagulant functions. Since the main functional effect of protein S is observed in the enhancement of the FXa-TFPIα encounter complex formation, it has long been suggested that the role of protein S is to localize TFPIα on the membrane surface where FXa is present. This may be supported by the enhancement by protein S, alone and together with FV-short, being phospholipid dependent (Supplementary Figure S3). However, with FV-short itself binding to negatively charged surfaces with high affinity, the requirement for protein S in the synergistic enhancement of TFPIα is unclear. Furthermore, the difference in requirement of the direct TFPIα-protein S interaction in the presence and absence of FV-short suggest more complex roles of protein S in the enhancement of TFPIα than simple localization. The synergistic enhancement of TFPIα K3m could potentially be explained by a new protein S exosite or interaction site formed by TFPIα-FV-short, similar to the recently suggested protein S interaction site in the hydrophobic patch in the preAR2 region of FV-short [12]. However, this is not supported by our co-immunoprecipitation experiments where very little protein S bound TFPIα K3m-FV-short, suggesting that the explanation may lay elsewhere. Whether TFPIα-FV-short enhancement by protein S involves direct interactions of the cofactors [30–32] and/or FXa [33] that may or may not result in conformational changes remains to be determined. Interestingly, the structure of FV-short was recently resolved through cryogenic electron microscopy [20]. This structure may facilitate future structural analysis of the TFPIα-protein S-FV-short complex in the presence or absence of FXa which would shed further light on molecular interactions and mechanisms involved in TFPIα mediated inhibition of FXa.

Together, our results demonstrate that synergistic enhancement of TFPIα can be achieved even when the main binding site for protein S is removed. In contrast, the intact basic C-terminus of TFPIα is mandatory for the synergistic enhancement by protein S and FV-short to occur.

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AUTHOR CONTRIBUTIONS

M.G. designed the study, ran the experiments, analyzed the data and wrote the manuscript; V.M.N. ran the experiments, analyzed the data and revised the manuscript; I.I.S.-C. and J.T.B.C. designed the study, interpreted the data, and revised the manuscript; J.A. initiated, designed, and supervised the study and wrote the manuscript.

DECLARATION OF COMPETING INTERESTS

J.A. is a consultant for Silence Therapeutics Ltd. M.G., V.M.N., I.I.S-C., and J.T.B.C. have no competing financial interests to declare.

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SUPPLEMENTARY MATERIAL

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