The LIKT sequence in C-terminus of tissue factor pathway inhibitor (TFPIα) crucially important for the synergistic TFPIα-cofactor activity of protein S and FV-short

Björn Dahlbäck,[1] Sinh Tran,[1] Piotr Draczkowski[2]

[1]Department of Translational Medicine, Lund University, University Hospital, Malmö; [2]Department of Biochemistry and Biophysics, National Bioinformatics Infrastructure Sweden, Science for Life Laboratory, Stockholm University, Solna, Sweden

ABSTRACT

Background: Inhibition of factor Xa (FXa) by tissue factor pathway inhibitor α (TFPIα) is potentiated by FV-Short and protein S. The C-terminus of TFPIα is important for formation of the TFPIα/protein S/FV-Short complex. It contains a hydrophobic sequence (LIKT). The purpose was to investigate the importance of the LIKT sequence.

Methods: Two TFPIα C-terminal peptides were synthesized, one wild-type and the other with LIKT replaced by AAKA. The effect of the peptides on TFPIα function was tested. An AAKA-mutant TFPIα was created and compared with wild-type TFPIα. AlphaFold was used to elucidate a mechanism for the role of the LIKT sequence.

Results: The wild-type peptide efficiently inhibited the FXa-inhibitory activity of TFPIα/protein S/FV-Short complex, whereas the AAKA peptide did not. The LIKT sequence in TFPIα was crucially important for the synergistic TFPIα-cofactor activity between protein S and FV-Short. AlphaFold (DeepMind, London, UK) suggested an interaction between LIKT and FV-Short B-loop 1510-1517 mediated through a network of hydrogen bonds and hydrophobic interactions.

Conclusions: The C-terminal TFPIα-peptide induced complex formation between protein S and FV-Short. The LIKT sequence in TFPIα was crucially important for the ability of protein S and FV-Short to function as synergistic TFPIα cofactors.

Key words: factor V, protein S, tissue factor pathway inhibitor, factor Xa, blood coagulation, AlphaFold.

Corresponding author: Björn Dahlbäck, Department of Translational Medicine, Lund University, Wallenberg Laboratory, 2nd floor, Inga Marie Nilsson's Street 53, 21428 Malmö, Sweden.
E-mail: bjorn.dahlback@med.lu.se

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Introduction

Tissue factor pathway inhibitor (TFPI) is a crucially important regulator of the extrinsic pathway of coagulation.[1-4] It is present in several different isoforms and fulfills its function as regulator of the tissue factor (TF) pathway both on the vascular surface and in fluid phase. The full-length isoform (TFPIα) present in circulation is composed of three Kunitz-type domains and a highly basic C-terminal peptide, whereas the form associated with the vascular surface is a splice variant just comprising the first two Kunitz domains. The TFPI form present on the vascular cell surfaces inhibits freshly activated factor Xa and VIIa (FXa and FVIIa) associated with TF, FVIIa being inhibited by Kunitz 1, whereas Kunitz 2 inhibits FXa.[1-4] The circulating full-length TFPIα form can also inhibit FXa that is not associated with the TF/FVIIa complex. The function of TFPIα is stimulated by the synergistic cofactor activity of protein S and a splice variant of factor V called FV-Short.[4-9] The three proteins form a tri-molecular complex, protein S interacting with Kunitz 3 and FV-Short with the C-terminal tail of TFPIα.[4,8-10] The tri-molecular complex efficiently inhibits FXa in a reaction stimulated by negatively charged phospholipid surfaces.[7-9,11-14]

FV-Short is a naturally occurring splice variant of FV, the splicing resulting in a 702 amino acid residues long deletion

of the large B-domain of FV.[4-6,13,15-17] This results in the exposure of highly acidic region called acid region 2 (AR2) in the C-terminus of the B-domain. The highly basic C-terminus of TFPIα binds to AR2. The TFPIα-binding site in the AR2 region can also be exposed upon partial activation of FV (FVai) that releases the N-terminal part of the B-domain.[5,7,9,13,14] The FXa inhibitory activity of TFPIα bound to AR2 of FV-Short or FVai is synergistically stimulated by protein S. Protein S is a vitamin K-dependent multi-domain protein that in addition to being a TFPIα-cofactor also serves as a cofactor to activated protein C (APC) in degradation of factors Va and VIIIa.[18,19] Protein S comprises a Gla-domain that binds negatively charged phospholipids, four EGF-like domains and two C-terminal Laminin G-type (LG) domains, the TFPIα interactive site of protein S is in the LG1 domain.[20]

The formation of the FXa-inhibitory tri-molecular TFPIα/protein S/FV-Short complex is dependent on multiple protein-protein interactions. Direct binding reactions have been shown between TFPIα and protein S and between TFPIα and FV-Short, whereas no direct binding between protein S and FV-Short has been demonstrated.[5,9,11,12] However, binding of TFPIα to FV-Short generates a high affinity binding site for protein S that is dependent on a hydrophobic patch in the preAR2 region of FV-Short and an AlphaFold (DeepMind, London, UK) generated model suggests that the hydrophobic patch interacts with residues 268-276 and 422-426 of protein S.[12] A hydrophobic patch (LIKT) is also present in TFPIα just N-terminal of the basic C-terminus. The LIKT sequence has been reported to be important for the ability of TFPIα to directly inhibit early formation of prothrombinase complex.[21,22] Those experiments were not performed in the presence of protein S and were thus not focused on the synergistic TFPIα-cofactor activity of FV-Short and protein S. We now report that a synthetic C-terminal TFPIα peptide stimulates binding between protein S and FV-Short and furthermore that the LIKT sequence of TFPIα is crucially important for the synergistic TFPIα-cofactor function of protein S and FV-Short and for the formation of the tri-molecular complex formation between TFPIα, FV-Short and protein S.

Materials and Methods

Materials

A monoclonal antibody (mab) against the light chain of FV (HV1) was obtained from Sigma. Mab HPS54 against the EGF1-domain of protein S is previously described.[23] AHTFPI-5138, a mab against the N-terminus of TFPIα, PAHTFPI-S, a sheep polyclonal TFPIα-antibody and Factor Xa were from Hematologic Technologies (HTI). Goat-anti mouse conjugated with horseradish peroxidase (GaM-HRP) was from Dako and TMB (tetramethylbenzidine) ONE soluble HRP substrate was from Kementec. Protein S was purified from human plasma as previously described.[24] Recombinant FV-Short was prepared as previously described.[6,25] Phospholipids [phosphatidylserine (PS), phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC)] were from Avanti Polar Lipids. Phospholipid vesicles (PS/PE/PC:20/20/60) were prepared using dialysis as described and used within 4 weeks. Synthetic substrate S2765 was from Chromogenix Ltd, Milan, Italy.

Synthetic peptides

Two synthetic peptides P-TFPI (CLIKTKRKRKKQRVKIAYEEIFVKNM) and P-AAKA-TFPI (AAKAKRKRKKQRVKIAYEEIFVKNM) were purchased from JPT Peptide Technologies GmbH Berlin. The sequence of P-TFPI corresponds to residues 252-276 of TFPIα, the N-terminal Cys was added to obtain a free SH group which however was not used in this study; P-AAKA-TFPI contained no added Cys.

Recombinant wild-type and AAKA-TFPIα

Recombinant wild-type TFPIα was prepared and characterized as previously described.[12] A cDNA clone in pcDNA3.1 in which the LIKT sequence was replaced by AAKA was purchased from ThermoFischer Scientific. A stable BHK cell clone expressing the recombinant AAKA-TFPIα was created and the recombinant protein was purified and characterized as described for wild-type TFPIα.[12] The concentration of TFPIα was determined with an ELISA using a polyclonal sheep anti-human TFPIα (PAHTFPI-S) as catcher and the monoclonal anti-TFPI ATFPI-5138 as detector as previously described.[6,12]

Inhibition of FXa by TFPIα-variants, protein S and FV-Short

FV-Short (0-10 nM) was incubated with protein S (0-100 nM), phospholipid (20:20:60 of PS:PE:PC, 25 nM), and TFPIα-variants (0-10 nM) in HNBSACa^{2+} buffer (25 mM Hepes, 0.15 M NaCl, 5 mM $CaCl_2$ pH 7.7, containing 0.5 mg/ml bovine serum albumin) for 10 minutes at 37°C. Addition of S2765 (0.7 mM) and FXa (0.3 nM) initiated the reaction, which was monitored for 900 seconds by reading the absorbance at 405 nm in a Tecan Infinite 200 system. The absorbances reached at the end of the incubations were plotted against the FV-Short/protein S/TFPIα variant concentrations. The concentrations given were the final concentrations.

Binding experiments

The FV-Short, TFPIα-variants, and protein S were individually immobilized in microtiter plates as described.[11,12,25] Various combinations of protein and peptide variants, as indicated in respective figures, were added and incubated. The buffer used was 50 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.5, containing 0.05% Tween, 10 mM benzamidine and 1% BSA. After incubation at 4°C overnight, followed by four times washing, binding of the added proteins was tested by addition of the respective monoclonal antibody, i.e. FV-Short was analyzed with HV1, TFPIα with AHTFPI-5138 and protein S with HPS54. Goat-anti-mouse HRP was added after two hours incubation followed by washing. After 2 hours incubation with goat-anti mouse HRP and washing, the plates were developed with TMB ONE soluble HRP substrate. The absorbance values are presented in the figures. For the binding experiments repeated three times the means±SD was calculated.

Prediction of structure and interaction sites of tri-molecular complex between FV-Short, protein S and TFPIα using AlphaFold

Trimolecular complex structures composed of FV-Short, protein S, and TFPIα and of dimolecular complexes made by

various combinations of the above were predicted using AlphaFold3 Server, as previously described.[12,26,27] Protein S and TFPIα sequences (numbered as in the mature proteins) were retrieved from the UniProt database under the IDs P07225 and P10646, respectively. The sequence of FV-Short used for the structure prediction was the same as the sequence of the recombinant natural FV-Short used for other experiments, and residues were numbered corresponding to their position in full-length FV. In total 10 predictions were run with different starting seeds. The top-ranked predicted models were evaluated in terms of per-residue confidence predictions (pLDDT scores), the overall accuracy of the predicted structures of each complex component, and the quality of the predicted interface between them (reflected in pTM and ipTM scores). The pTM values in the predicted models ranged from 0.59 to 0.63, while the ipTM values ranged from 0.43 to 0.5. Despite overall low ipTM, the interaction between the C-terminus of TFPIα, the protein S LG1 domain, and the FV-Short B domain was repeatedly observed across all the top-ranked models. The average pLDDT score for the region of the discussed TFPIα, protein S, and FV-Short interaction in the AlphaFold model used herein presented structural analysis was 62. To further evaluate the quality of the AlphaFold-generated models, the predicted structure of FV-Short was compared with the available cryo-EM structure of the protein (Protein Data Bank accession code: 8FDG),[28] which showed good agreement between the predicted and experimental structure. UCSF ChimeraX was used for visualization and analysis of the predicted structures.[29]

Statistical analysis

The results were analyzed with Prism 10.0 (Graphpad Software La Jolla, CA, USA) using standard statistical analyses. The means±standard deviations (SD) were calculated.

Results

The C-terminal TFPIα peptide stimulates binding between FV-Short and protein S

We have previously demonstrated TFPIα-dependent binding between FV-Short and protein S using the microtiter plate-based assay.[11,12,25] To investigate whether a C-terminal synthetic peptide (P-TFPI) can play the same role as TFPIα and stimulate the binding, P-TFPI was included in binding experiments using microtiter plates with either immobilized FV-Short (Figure 1A) or protein S (Figure 1B). P-TFPI demonstrated dose-dependent stimulation of the binding of protein S to immobilized FV-Short. The binding of protein S was completely dependent on the presence of P-TFPI and at a fixed concentration of 100 uM P-TFPI protein S bound in a dose-dependent fashion (Figure 1A). Likewise in the experiment with immobilized protein S, the interaction between FV-Short and protein S was dependent on the presence of P-TFPI (Figure 1B).

C-terminal peptide of TFPIα inhibits synergistic TFPIα-cofactor activity of FV-Short and protein S in inhibition of FXa: importance of LIKT-sequence in TFPIα

The binding experiments suggest that a FV-Short/protein S complex is formed in the presence of the P-TFPI peptide. This suggests that the C-terminal peptide could potentially compete with TFPIα in the formation of the tri-molecular complex between TFPIα, FV-Short and protein S and thus serve as an inhibitor of the synergistic TFPIα-cofactor activity between FV-Short and protein S. To investigate this, increasing concentrations of the P-TFPI peptide were added to a FXa-inhibitory assay containing TFPIα cofac-

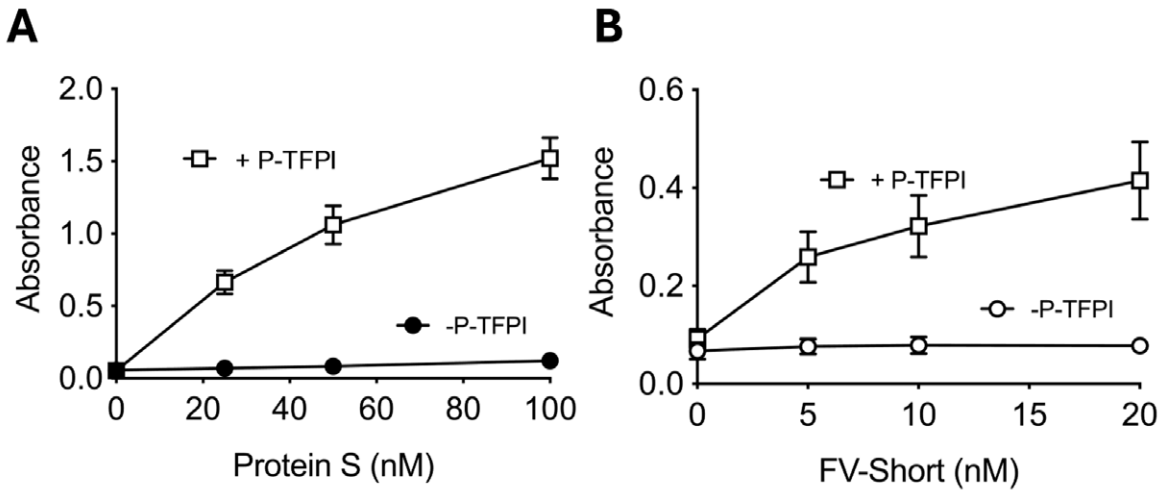

Figure 1. Protein S/FV-Short interaction stimulated by P-TFPI. A) P-TFPI (100 uM) was added to microtiter plates with immobilized FV-Short in the presence of increasing concentrations of protein S and after overnight incubation the amount of bound protein S was determined with a monoclonal antibody against protein S (HPS54). B) increasing concentrations of FV-Short was added to microtiter plates with immobilized protein S in the presence or absence of P-TFPI (100 uM). After overnight incubation the amount of bound FV-Short was determined with a monoclonal antibody against the light chain of FV (HV1). Each experiment was performed three times and the means ± standard deviation were plotted.

tors FV-Short and protein S (Figure 2). In addition, the importance of the hydrophobic LIKT sequence in TFPIα was tested using a modified C-terminal TFPI peptide (P-AAKA-TFPI). The P-TFPI peptide efficiently inhibited the FXa inhibition suggesting that the peptide disrupted the tri-molecular complex formation between TFPIα, FV-Short and protein S, whereas only the highest concentrations of the P-AAKA-TFPI peptide had any inhibitory effect.

Importance of the LIKT-sequence in TFPIα for TFPIα-cofactor activities of protein S and FV-Short

To further investigate the importance of the LIKT sequence in TFPIα, a mutant TFPI was created in which LIKT was mutated to AAKA. The function of TFPI-AAKA as FXa inhibitor was compared with that of TFPIα using three different FXa-inhibition experimental conditions: (A), in the absence of its cofactors; (B), in the presence of 100 nM protein S or (C), in the presence of both protein S (3 nM) and FV-Short (2 nM) (Figure 3). Please note that in the experiment presented in A, the TFPI concentrations varied between 0 and 10 nM, whereas in B and C experiments the TFPI concentrations were between 0 and 1 nM. In the absence of cofactors, TFPIα and TFPI-AAKA were equally efficient with 50% inhibition at approximately 0.7 nM TFPIα or TFPI-AAKA. In the presence of 100 nM protein S, the two TFPI variants were also equally efficient with 50% inhibition at around 0.12 nM TFPIα variant. In contrast, in the presence of both protein S and FV-Short the efficiency of TFPI-AAKA was significantly less efficient than TFPIα.

In the experiment shown in Figure 3B, the protein S concentration was 100 nM which is approximately the concentration of circulating free protein S. This is much higher than that required for full protein S activity in the presence of FV-Short. Therefore, the protein S concentration dependence of TFPI-AAKA and TFPIα were tested in the absence or presence of FV-Short (Figure 3D). In the absence of FV-Short, the two TFPI variants demonstrated equal protein S concentration dependence, whereas in the presence of FV-Short, TFPI-AAKA was significantly less efficient than TFPIα (Figure 3E). These results suggested that the LIKT sequence is important for the TFPIα-FV-Short interaction, whereas it is not involved in the TFPIα-protein S interaction.

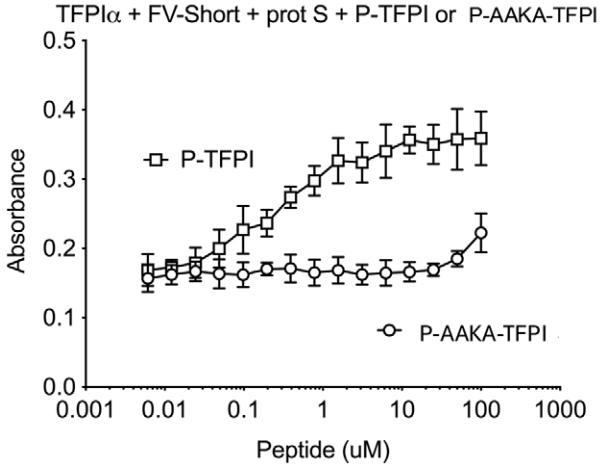

Figure 2. P-TFPI inhibits FXa-inhibition by TFPIα/FV-Short/protein S-complex whereas P-AAKA-TFPI does not. Increasing concentrations of P-TFPI or P-AAKA-TFPI were included in a FXa-inhibition assay containing 0.25 nM TFPIα, 2 nM FV-Short and 3 nM protein S. The absorbances reached at the end of the incubations were plotted against the peptide concentrations. Each experiment was performed three times and the means ± standard deviation were plotted.

Importance of LIKT sequence in TFPIα for formation of trimolecular complex between TFPIα, FV-Short and protein S

We have previously demonstrated that the binding of TFPIα to immobilized FV-Short is stimulated by the presence of protein S.[11,12,25] To investigate the importance of the LIKT sequence for the binding we tested the TFPI-AAKA mutant and compared with that of wild-type TFPIα (Figure 4A-C). We analyzed binding of protein S to immobilized FV-Short in the presence of either TFPIα or TFPI-AAKA (Figure 4A). In the presence of TFPIα strong protein S binding was demonstrated in agreement with results on record.[11,12,25] In contrast, in the presence of TFPI-AAKA no detectable binding could be demonstrated.

We have previously reported strong binding of TFPIα to immobilized FV-Short in the presence of protein S.[11,12,25] In the experiment shown in Figure 4B TFPI-AAKA demonstrated weak binding to immobilized FV-Short and the binding was unaffected by the addition of protein S. The binding of FV-Short in the presence or absence of protein S to the immobilized TFPI-AAKA variants was also tested (Figure 4C). We have reported strong FV-Short binding to immobilized TFPIα in the presence of protein S.[11,12,25] In contrast, we now found no FV-Short binding to TFPI-AAKA neither in the presence nor the absence of protein S.

AlphaFold-prediction of structure and interaction site of between FV-Short and TFPIα LIKT sequence

AlphaFold3 was employed to predict the structural details of the LIKT region in the context of FV-Short, TFPIα, and protein S tri-molecular complex (Figure 5). The predicted structure of the complex showed no direct involvement of the LIKT sequence in the TFPIα-protein S interaction. Instead, residues 252-260 of TFPIα, which include the LIKT sequence, formed a beta-sheet structure with FV-Short residues 1509-1517 through a network of hydrogen bonds between protein backbones (Figure 5B). The interaction appears to be guided by electrostatic interactions between the basic residues following the LIKT-region and the highly acidic region of FV-Short that binds this TFPIα motif (Figure 5C). Additionlly, the C-terminal part of TFPIα downstream to LIKT sequence, residues 265-273, FV-Short residues 1482-1502, and protein S residues 269-275 formed an extended beta-sheet structure (Figure 5D). The FV-Short 1482-1502 comprises of the PLVIVGL (1481-1487) hydrophobic patch in the preAR2 region, which we previously showed to be important for the assembly of the trimolecular FXa-inhibitory complex, and the AR2 region (1493-1537).[12] Both fragments form a beta hairpin motif in which the preAR2 interacts with the hydrophobic pocket in the protein S LG1 domain, while the AR2 interacts with the TFPIα C-terminus, together forming the beta-sheet structure through backbone-backbone hydrogen bonds.

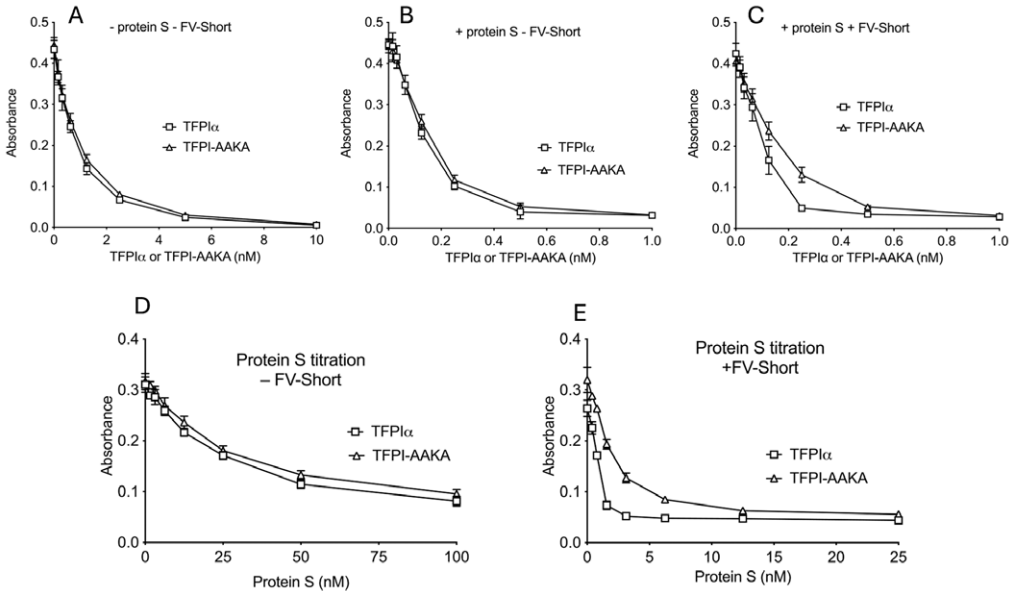

Figure 3. FXa-inhibition by TFPIα or TFPI-AAKA in presence or absence of protein S and FV-Short. A) Increasing concentrations of TFPIα or TFPI-AAKA (0-10 nM) were added to FXa (0.3 nM) in presence of 25 uM PL (PS/PE/PC:20/20/60) and S2756 (0.7 mM). B) Increasing concentrations of TFPIα or TFPI-AAKA (0-1 nM) were added together with protein S (100 nM) to a mixture of FXa, PL and S2756. The reactions were followed and plotted as in A. C) FV-Short (2 nM) and protein S (3 nM) were mixed with increasing concentrations of TFPIα or TFPI-AAKA (0-1 nM) and added to FXa, PL and S2756. D) Increasing concentrations of protein S (0-100 nM) and TFPIα (0.25 nM) or TFPI-AAKA (0.25 nM) were added to FXa, PL and S2756. E) Increasing concentrations of protein S (0-25 nM) were added together with FV-Short (2 nM) and TFPIα (0.25 nM) or TFPI-AAKA (0.25 nM) to FXa, PL and S2756. The inhibition of FXa was monitored for 900 seconds. The absorbances reached at the end of the incubations were plotted against the TFPI-variant concentrations. Each experiment was performed three independent times and the means ± standard deviation are plotted.

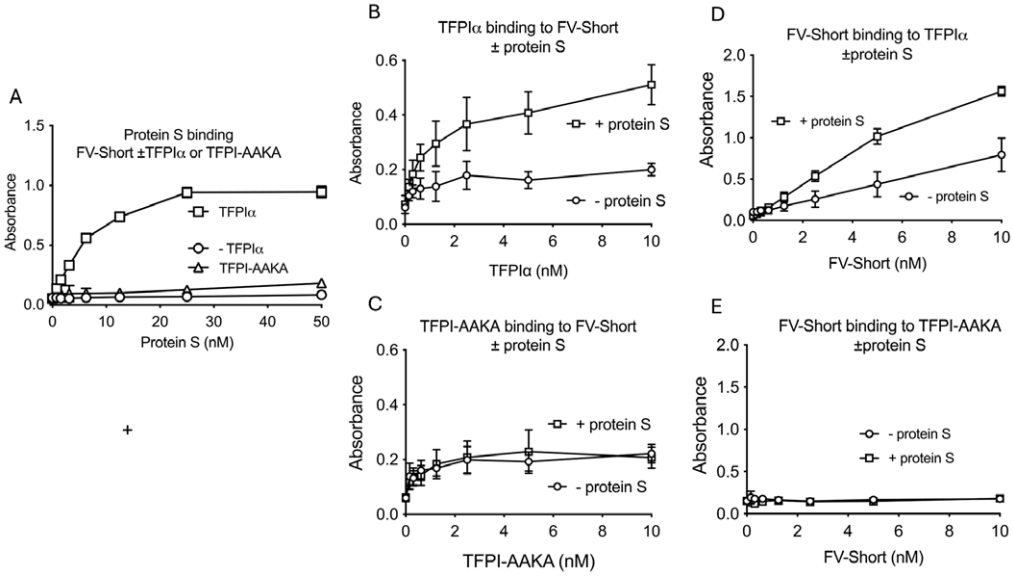

Figure 4. Binding between protein S, FV-Short and TFPIα or TFPI-AAKA. A) Increasing concentrations of protein S (0-50 nM) with or without TFPIα or TFPI-AAKA (10 nM) were added to microtiter wells coated with FV-Short. After overnight incubation and washing, the bound protein S was measured with the protein S monoclonal antibody. B and C) Increasing concentrations of (B) TFPIα (0-10 nM) or (C) TFPI-AAKA (0-10 nM) were added with or without protein S (50 nM) to microtiter wells coated with FV-Short. After overnight incubation and washing, the bound TFPI-variant was measured with the TFPI monoclonal antibody. D and E) Increasing concentrations of FV-Short (0-10 nM) were added with or without protein S (5 nM) to microtiter wells coated with TFPIα (D) or TFPI-AAKA (E). After overnight incubation and washing, the bound FV-Short was measured with the HV1 monoclonal antibody. Each experiment was performed three independent times and the means ± standard deviation are plotted. The results presented in B and D have been published recently and are here shown for reference purposes.[12]

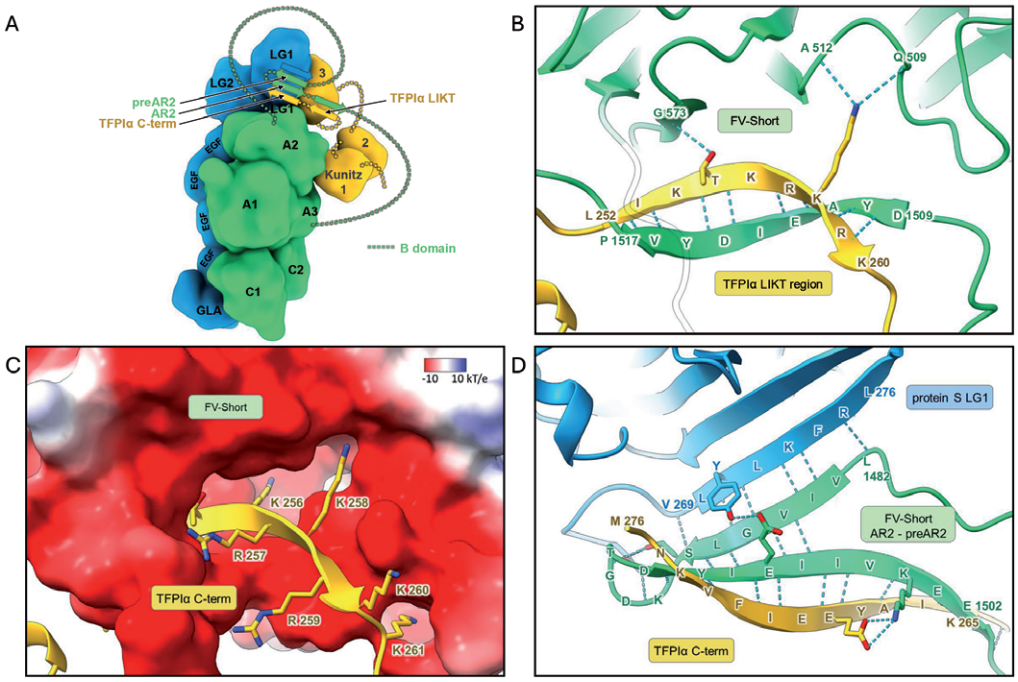

Figure 5. Predicted structural details of LIKT sequence interacting with FV-Short. A) Overview of the proposed structure of the tri-molecular complex formed by the FV-Short (colored green with the unstructured B domain shown as dashed line), protein S (colored blue), and TFPI α (colored yellow). Note that except for the interaction interfaces shown in panels B-D, the relative positions of the FV-Short, protein S, and TFPI α are arbitrary, as AlphaFold could not confidently predict them. B) The TFPIα region containing the LIKT sequence (residues 252-260) formed a beta-sheet structure with FV-Short residues 1509-1517 through hydrogen bonds between protein backbones. C) The interaction involved also electrostatic interactions between the basic residues within the LIKT region and the acidic residues of FV-Short surrounding the binding pocket. Consistent with biochemical data, no direct involvement of the LIKT sequence in the TFPIα-protein S interaction was observed. D) The TFPIα residues at its C-terminus (residues 265-273) formed another extensive beta-sheet structure involving FV-Short and protein S. The FV-Short beta hairpin forming the binding interface comprises AR2 and hydrophobic preAR2 regions, previously showed to be important for the assembly of the trimolecular complex.

Discussion and Conclusions

The full-length TFPIα (40 kDa) protein circulates in plasma in complex with FV-Short and protein S and acts as an inhibitor of FXa.[4,5,11,25] The concentration of the complex is in the sub-nM range and determined by the concentration of FV-Short. FV-Short, protein S and FXa all have binding sites for negatively charged phospholipids and the inhibition of FXa takes place on the phospholipid surface. The assembly of the tri-molecular inhibitory complex is driven by multiple protein-protein interactions. Protein S binds to Kunitz3 and the C-terminus of TFPIα binds to FV-Short and induces binding of protein S to FV-Short.[4-7,11,12,20,30,31] The C-terminus of TFPIα contains a stretch of basic residues that interact with highly acid AR2 region of FV-Short.[3-5,7,9,14] The hydrophobic LIKT sequence of TFPIα is located just N-terminal of the basic region. It has been suggested to be important for inhibition of early prothrombinase by TFPIα.[21,22] However, these studies were done without protein S and FV-Short and before the knowledge of the synergistic TFPIα cofactor activity of FV-Short and protein S. Therefore, we now focused on the importance of the LIKT sequence for the synergistic cofactor activity. In addition, we investigated the effects of a synthetic C-terminal TFPIα-peptide on tri-molecular complex assembly and on FXa-inhibition.

The C-terminal TFPIα-peptide induced complex formation between FV-Short and protein S. The TFPI peptide efficiently inhibited the TFPIα-mediated inhibition of FXa suggesting that the peptide competed with TFPIα for interaction with FV-Short and protein S. The LIKT sequence was important for the inhibition as the AAKA-TFPI peptide was without effect.

The importance of the LIKT sequence for the synergistic cofactor activity was further demonstrated using the recombinant TFPI-AAKA variant. This variant was equally as efficient as TFPIα in the absence of cofactors and when protein S was used as sole cofactor. The results suggested that the LIKT sequence was crucial for the synergistic TFPIα-cofactor activity between FV-Short and protein S. This conclusion was further supported by the inability of the TFPI-AAKA protein to induce binding between FV-Short and protein S.

To elucidate the molecular mechanisms underlying the role of the LIKT sequence, AlphaFold3 was employed to predict the structural details of the LIKT region within the context of the FV-Short, TFPIα, and protein S tri-molecular complex. The predicted structure showed no direct involvement of LIKT in the protein S binding. Instead, the TFPIα region containing the LIKT sequence (residues 252-260) formed a beta-sheet structure with FV-Short residues 1509-1517 through backbone-backbone hydrogen bonds and stabilized by electrostatic interactions (Figure 5). The FV re-

gion involved in this interaction is located within the B-loop domain and is C-terminal to the region previously predicted to interact with both TFPIα and protein S.[12] Considering the predicted structure and the experimental data, it seems likely that binding of the TFPIα LIKT sequence to FV-Short displaces the B-loop. This displacement would facilitate interaction of the FV-Short AR2 and preAR2 regions with the basic C-terminus of TFPI and further enable binding with protein S. This mechanism may account for the enhanced inhibition of FXa by TFPIα and protein S in the presence of FV-Short. Further experimental structural studies of the FV-Short, TFPIα, and protein S tri-molecular complex are required to verify this proposed mechanism and reveal the atomistic details of complex formation.

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