Received: 19 January 2022 | Accepted: 25 February 2022

DOI: 10.1111/jth.15690

ORIGINAL ARTICLE

A hydrophobic patch (PLVIVG; 1481–1486) in the B-domain of factor V-short is crucial for its synergistic TFPIα-cofactor activity with protein S and for the formation of the FXa-inhibitory complex comprising FV-short, TFPIα, and protein S

Björn Dahlbäck | **Sinh Tran**

Department of Translational Medicine, University Hospital, Lund University, Malmö, Sweden

Correspondence
Björn Dahlbäck, Department of Translational Medicine, Lund University, Wallenberg Laboratory, 5th floor, Inga Marie Nilsson's Street 53, 21428 Malmö, Sweden.
Email: bjorn.dahlback@med.lu.se

Funding information
Vetenskapsrådet; Alfred Österlunds Stiftelse; Hjärt-Lungfonden

Abstract

Background: Factor V-short (FV756-1458) is a natural splice variant functioning in synergy with protein S as tissue factor pathway inhibitor alpha (TFPIα)–cofactor in inhibition of factor Xa (FXa). An exposed acid region (AR2; 1493–1537) in the B domain binds TFPIα. The preAR2 (1458–1492) is crucial for the synergistic TFPIα–cofactor activity between FV-short and protein S and for assembly of a trimolecular FXa-inhibitory complex among FV-short, protein S, and TFPIα.

Objective: To identify which part of preAR2 is required for the synergistic TFPIα–cofactor activity between FV-short and protein S.

Methods: A FXa-inhibition assay was used to test the synergistic TFPIα cofactor activity between protein S and new FV-short variants FV709-1476, FV712-1478, FV712-1481, FV712-1484, FV712-1487, and FV712-1490. A microtiter-based assay analyzed binding among FV-short variants, protein S, and TFPIα.

Results: FV709-1476, FV712-1478, and FV712-1481 were fully active as synergistic TFPIα cofactors with protein S; FV712-1484 showed intermediate activity; and FV712-1487 and FV712-1490 were inactive. TFPIα interacted with all variants in the absence of protein S but FV712-1478 and FV712-1481 bound TFPIα with highest affinity. None of the FV-short variants bound directly to protein S in the absence of TFPIα. In the presence of TFPIα, efficient cooperative binding was demonstrated between protein S, TFPIα, and FV709-1476, FV712-1478, or FV712-1481. In contrast, no cooperativity among TFPIα, protein S, and FV712-1484, FV712-1487, or FV712-1490 was seen.

Conclusion: A short hydrophobic patch in preAR2 (PLVIVG, 1481–1486) in FV-short is crucial for the synergistic TFPIα-cofactor activity between FV-short and protein S and

Manuscript handled by: Alan Mast

Final decision: Alan Mast, 25 February 2022

for the assembly of a trimolecular FXa-inhibitory complex among FV-short, protein S, and TFPIα.

KEYWORDS
blood coagulation, factor V, factor Xa, protein S, tissue factor pathway inhibitor

1 | INTRODUCTION

Factor V (FV) plays a central role in the regulation of coagulation having the capacity to express both pro- and anticoagulant functions.[1-4] It circulates as a high molecular weight (330 kDa) single chain procofactor comprising A1, A2, B, A3, C1, and C2 domains. It is activated to a procoagulant by thrombin or factor Xa (FXa), which cleaves at Arg709, Arg1018, and Arg1545 and thus releases the B domain. The active FVa is formed by the heavy (A1 and A2) and the light (A3, C1, and C2) chains, FVa serving as a cofactor to FXa in the activation of prothrombin. The C-terminal part of the B domain contains a highly acid region (AR2—residues 1493–1537), which in intact FV interacts with a very basic region (residues 963–1008), thereby keeping FV as an inactive procofactor.[5-7] The anticoagulant function of intact FV is as a synergistic cofactor to activated protein C (APC) with protein S in the regulation of factor VIIIa (FVIIIa) in the tenase complex (FIXa, FVIIIa, negatively charged phospholipid).[8-12]

The large B domain of FV (residues 710–1545) is encoded by a single exon (exon 13). An alternative splicing event was revealed during the elucidation of an autosomal dominant bleeding disorder affecting a family in East Texas.[13] A single A to G point mutation in the codon for Ser756 activated a natural splice donor site resulting in 702 amino acid residues deletion, the generated FV-short (FV756-1458) reaching high concentrations in circulation (≈5 nM). The alternative splicing also occurs naturally but to a much smaller degree than in cases with the East Texas bleeding and the normal circulating concentration of FV-short is estimated to be around 0.2 nM.[13] The basic region (973–1008) is lost in FV-short and as a consequence the highly acid AR2 is exposed. The exposed AR2 contains a high affinity binding site for tissue factor pathway inhibitor alpha (TFPIα).[13-15] Due to the increased concentrations of FV-short in East Texas cases, the circulating concentration of TFPIα is increased (≈ 5 nM), which causes the bleeding tendency.[13] Recently, a second family with the East Texas exon 13 point mutation was described, affected individuals demonstrating similar clinical symptoms and laboratory findings as the original East Texas family.[16] Two other *F5* gene exon 13 mutations have been reported with increased FV-short and TFPIα. In FV-Amsterdam (*F5 C2588G*), 623 resides are deleted generating FV835-1458.[17] In FV-Atlanta (*F5*: *c.2413-3244del*) a 832 bp deletion in exon 13 activates the East Texas splice donor site via cis-acting regulatory sequences resulting in very high concentrations of the East Texas FV-short variant (FV756-1458).[18]

TFPIα is the full-length form of TFPI (plasma concentration ≈0.2 nM) comprising three Kunitz domains and a highly basic C-terminal tail.[19-21] TFPIα regulates initiation of coagulation by inhibiting factor VIIa (FVIIa) bound to tissue factor (FVIIa/TF) and the generation of FXa. TFPIα is also able to inhibit free FXa in the presence of negatively charged phospholipid membranes. Inhibition of FXa by TFPIα is stimulated by the synergistic cofactor activity of protein S and FV-short.[11,22-28] FV-short, protein S, and TFPIα assemble into a tri-molecular complex, which in the presence of negatively charged phospholipid membranes efficiently inhibits FXa.[22,24] In TFPIα, the first Kunitz domain inhibits FVIIa, the second Kunitz domain inhibits FXa, the third Kunitz domain binds protein S, and the positively charged C-terminal tail binds FV-short.[15,19-21,29,30]

ESSENTIALS

- Factor V (FV)-short and protein S are synergistic cofactors to tissue factor pathway inhibitor alpha (TFPIα) in inhibition of factor Xa (FXa).
- New FV-short mutants were tested for cofactor activity and binding of protein S and TFPIα.
- A hydrophobic patch (PLVIVG, 1481–1486) is crucial for TFPI-α-cofactor synergism and protein S binding.
- Cooperative binding of protein S to TFPIα/FV-short drives assembly of the FXa-inhibitory complex.

We have recently reported that the preAR2 region between residues 1458 (splice junction site) and 1492 (start of AR2) contains elements that are crucially important for the synergistic TFPIα-cofactor activity between FV-short and protein S and for the assembly of TFPIα, protein S, and FV-short into a tri-molecular FXa-inhibitory complex.[24] Thus, an extensively studied artificial FV-short variant denoted FV810 (or FV810-1492), was found to be unable to express synergistic TFPIα–cofactor activity with protein S.[24] Furthermore, although FV810-1492 binds TFPIα with high affinity, the FV810/TFPIα-complex cannot recruit protein S into formation of the FXa-inhibitory tri-molecular complex. We have now created a number of novel FV-short deletion mutants to define which part of preAR2 is required for the synergistic TFPIα-cofactor activity and identified a short hydrophobic patch (residues 1481–1486; PLVIVG) as crucially important.

2 | MATERIALS AND METHODS

2.1 | Materials

FV monoclonal antibody HV1 was obtained from Sigma. Monoclonal antibody HPS54 against protein S against an epitope in the EGF1-domain is previously described.[31] PAHTFPI-S, a sheep polyclonal

antibody against TFPIα; AHTFPI-5138, a monoclonal antibody against the N-terminus of TFPIα; monoclonal antibody AHV5146 against FVa heavy chain; monoclonal antibody AHV5112 against FVa light chain; and FXa were from Haematologic Technologies (HTI). Goat–anti mouse conjugated with horseradish peroxidase (GaM-HRP) was from Dako. OPD (o-phenylenediamine dihydrochloride) tablets and TMB (tetramethylbenzidine) ONE soluble HRP substrate were from Kementec. A pool of outdated human citrated plasma from five anonymous donors was obtained from the blood bank at Växjö Hospital, Sweden. Human plasma protein S was purified as previously described.[32] Recombinant TFPIα was a kind gift from Dr. T. Hamuro at the Chemo-Sero-Therapeutic Research Institute, Japan. 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; 1 mM PL mixed with 200 mM n-octyl-β-D-glucopyranoside (Calbiochem) and then dialyzed against 25 mM Hepes, 0.15 M NaCl, pH 7.5 overnight. The phospholipid vesicles were used within 2 weeks. Synthetic substrate S2765 was kindly provided by Chromogenix Ltd.

2.2 | Recombinant FV-short variants

Recombinant FV756-1458 (original FV-short), FV712-1458, and FV713-1492 are previously described.[13,24] The FV709-1476 mutant was created by Marquette et al. and the cDNA was kindly provided by Dr. Randy Kaufman.[33] This mutant is resistant to thrombin cleavage at residue 709 but can be cleaved by thrombin at Arg1545. To prepare the five new FV-short mutants FV712-1478, FV712-1481, FV712-1484, FV712-1487, and FV712-1490 (amino acid residues between the given numbers are deleted, i.e., residues 713–1477 are deleted in FV712-1478, etc.), the following strategy was used. As recently described, we created a FV756-1458 variant containing four useful restriction enzyme sites; a Mlu1 site (ACGCGT) at codons for amino acid residues 678–679 (ThrArg), a BsiW1 site (CGTACG) at amino acid residues 1527–1528 (ArgThr), a BspE1 site (TCCGGA) at position 1688–1689 (SerGly), and a Xma1 site (CCCGGG) at amino acid residues 1693–1694 (ProGly).[24] None of these nucleotide changes affected the amino acid sequence. Each of the five new deletion mutants was created with three subsequent polymerase chain reactions using (1) a 5'-3' end primer (nucleotides corresponding to amino acids residues 411–419) and a middle 3'-5' hybrid primer containing the respective junctions 712–1478, 712–1481, 712–1484, 712–1487, 712–1490; (2) a 3'-5' end primer (nucleotides corresponding to amino acids residues 1695–1702) with a middle 5'-3' hybrid primer complementary to the junction primers in step (1); (3) mixtures of reactions (1) and (2) with the 5'-3' and 3'-5' end primers. The resulting cDNA bands were cleaved with Mlu1 and BspE1 and ligated into Mlu1 and BspE1 cleaved "four-site mutated" FV756-1458. The nucleotide sequences of the mutant FV-short coding regions were checked using nucleotide sequencing. Stable baby hamster kidney cell clones expressing the recombinant FV-short variants were created and the recombinant proteins purified following previously published protocols.[15,22,24,34] On sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting, the purified proteins migrated in the 200 kDa region having slight molecular weight differences as expected from their sequences. After incubation with thrombin, the FV-short variants were cleaved to the expected heavy (≈105 kDa) and light (≈75 kDa) chains of FVa (Figure S1 in supporting information).

2.3 | Electrophoretic techniques

SDS-PAGE was performed under reducing conditions using 4–20% gradient gels (BioRad) and stained with PageBlue (Thermo Fischer Scientific). Western blotting was performed with standard technique and visualized with monoclonal antibodies AHV5146 (FVa heavy chain), AHV5112 (FVa light chain), HPS54 (protein S), or AHTPI5138 (TFPIα).

2.4 | Immune precipitation of TFPIa/FV-short/protein S complexes from plasma

TFPIα/FV-short/protein S complexes were immune precipitated using biotinylated AHTFPI-S immobilized to streptavidin-coated magnetic beads (80 μg antibodies per ml bead slurry) as previously described.[13] In short, pooled citrated plasma (5 ml) was incubated at 4°C for 2 h with 1 ml beads. The beads were collected, washed in 50 mM Hepes, 0.15 M NaCl, pH 7.5 containing 1% bovine serum albumin (BSA; HNSBSA) and dissolved in 100 μl sample preparation buffer and 10 μl were applied to unreduced SDS-PAGE and western blotting. The starting plasma and the supernatants were diluted 1:100 in 2× sample preparation buffer and 10 μl of each were applied to unreduced SDS-PAGE and western blotting. Three SDS-PAGE were run and the western blots were developed with AHV5146, HPS54, or AHTFPI5138.

2.5 | Inhibition of FXa by TFPIα, protein S, and FV-short variants

FV-short variants (0–2.5 nM) were incubated with protein S (0–25 nM), phospholipid (20:20:60 of PS:PE:PC, 25 μM), and TFPIα (0–1 nM) in HNBSACa^{2+} buffer (25 mM Hepes, 0.15 M NaCl, 5 mM $CaCl_2$ pH 7.7, containing 0.5 mg/ml BSA) 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 concentrations given were the final concentrations.

2.6 | Binding experiments

The FV-short variants, protein S, and TFPIα were individually immobilized in microtiter plates as described.[24] The experiments were done in

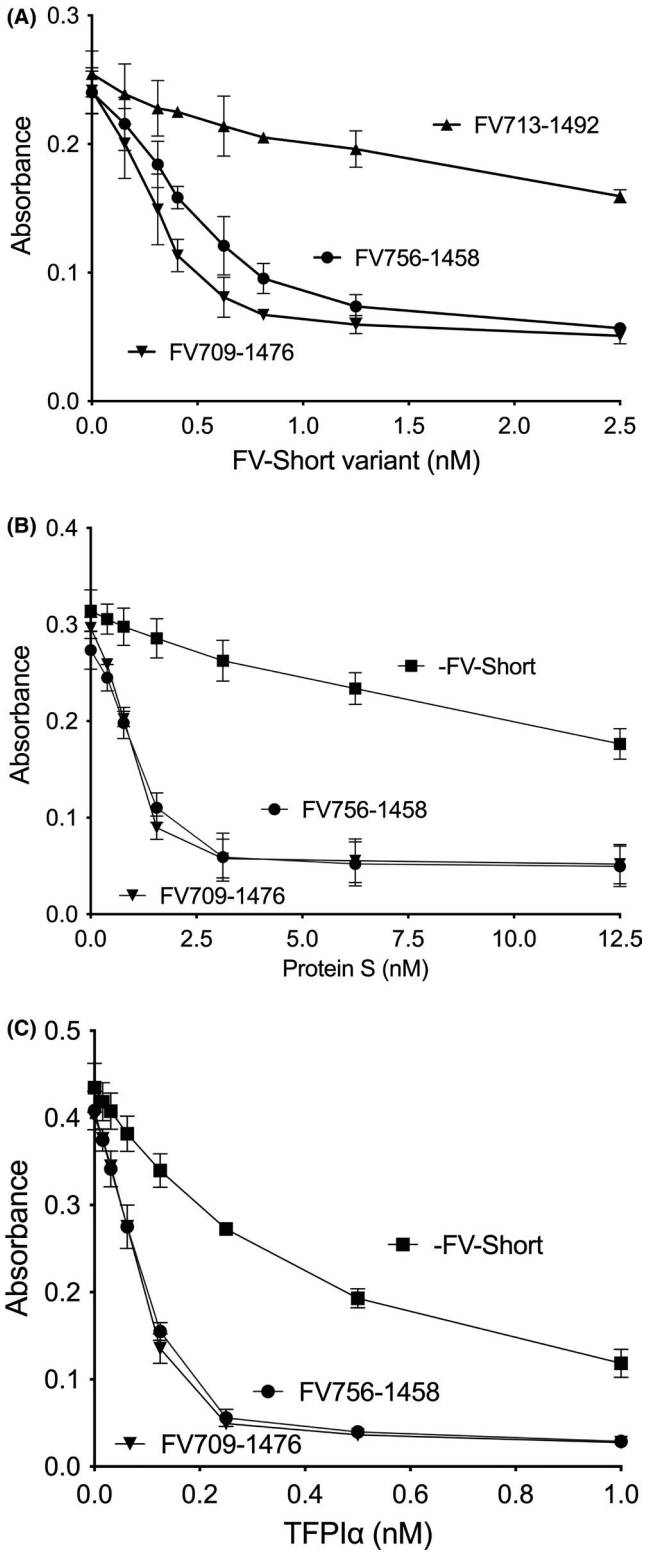

FIGURE 1 FV709-1476 and FV756-1458 expressing similar synergistic tissue factor pathway inhibitor alpha (TFPIα)-cofactor activities. The activity of FV709-1476 as synergistic TFPIα-cofactor with protein S was compared to that of FV756-1458 and FV713-1492. A, Increasing concentrations of the two FV-short variants (FV709-1476 and FV756-1458) were mixed with TFPIα (0.25 nM), protein S (3 nM) ,and 25 uM PL (PS/PE/PC:20/20/60). Factor Xa (FXa; 0.3 nM) and S2756 (0.7 mM) were added and the inhibition of FXa was monitored for 900 s. The absorbance reached at the end of the incubation was plotted against the FV-short variant concentration. B, In this experiment, the concentrations of FV-short variants were 2 nM, TFPIα (0.25 nM) and the protein S concentration varied between 0 and 12.5 nM. A control without FV-short variant is included. C, The concentrations of TFPIα were varied between 0 and 1 nM in this FXa-inhibition experiment, FV-short variants were 2 nM and protein S was 3 nM. A control without FV-short variant is shown. Each experiment was performed three independent times and the means ± standard deviation were plotted. The effects of FV756-1458 (original FV-short) and FV713-1492 in this assay have previously been presented[24] and they are included for comparative purposes

parallel in one or two plates to minimize experimental variation. Thus, to compare the FV-short mutants, they were immobilized in different rows of one or more microtiter plates. Dilution series of TFPIα ± a fixed concentration of protein S or a dilution series of protein S ± a fixed concentration of TFPIα were added to the different rows. 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, that is, FV-short mutants were analyzed with HV1, TFPIα with AHTFPI-5138, and protein S with HPS54. Goat-anti mouse HRP was added after 2 h of incubation followed by washing. After 2 h 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. All binding experiments were repeated three times and the means ± standard deviation (SD) was calculated.

2.7 | Statistical analysis

The results were analyzed with Prism 9.0 (GraphPad Software) using standard statistical analyses. The means ± SD were calculated.

3 | RESULTS

3.1 | Residues 1476–1491 in preAR2 of FV-short sufficient for synergistic TFPIα cofactor activity and assembly of trimolecular FV-short, protein S, and TFPIα complex

We recently reported that B-domain region 1458–1491 (preAR2) is crucial for the synergistic TFPIα-cofactor activity between protein S and FV-short. To elucidate which part of preAR2 is important for the ability of FV-short to function as a synergistic TFPIα-cofactor with protein S we tested FV709-1476, a FV-short variant available in the laboratory. In the FXa-inhibition assay, FV709-1476 was highly active as synergistic TFPIα cofactor (Figure 1). In this test, FV709-1476, protein S, and TFPIα were individually titrated. In the FV-short variant titration (Figure 1A), the FV709-1476 was found to be somewhat more active than FV756-1458, whereas in the protein

S and TFPIα titrations the two FV-short variants were equally active (Figure 1B, C). A recently created FV-short variant lacking synergistic TFPIα-cofactor activity (FV713-1492) was used as negative control (Figure 1A).[24]

FV709-1476 and FV756-1458 were also compared in a binding assay in which the FV-short variants, protein S, or TFPIα were individually immobilized in microtiter plates. The proteins to be tested were added alone or together and the binding analyzed with monoclonal antibodies. In Figure 2A, TFPIα was immobilized and increasing concentrations of FV709-1476 or FV756-1458 were added with or without protein S. In the presence of protein S, both FV709-1476 and FV756-1458 yielded hyperbolic binding curves suggesting strong, saturable binding. FV709-1476 bound with higher affinity than FV756-1458. Without protein S, the FV-short variant binding to TFPIα was weak and non-saturable although FV709-1476 yielded slightly stronger direct binding than FV756-1458. In experiments shown in Figure 2B, protein S was immobilized and binding of the FV-short variants tested in the presence and absence of TFPIα. When TFPIα was included, both FV709-1476 and FV756-1458 bound strongly, yielding saturable binding curves. Just like in Figure 2A, FV709-1476 bound better than FV756-1458. Without added TFPIα, none of the FV-short variants yielded any binding. In Figure 2C, D, FV709-1476 and FV756-1458 were individually immobilized and the binding of either protein S (Figure 2C) or TFPIα (Figure 2D) was tested. In Figure 2C, addition of increasing concentrations of protein S together with a constant concentration of TFPIα yielded saturable binding curves suggesting high-affinity binding. In contrast, no protein S binding was detectable when TFPIα was not included in the incubation mixture. In Figure 2D, TFPIα was added at increasing concentrations with or without a constant concentration of protein S. With protein S present, addition of TFPIα yielded high-affinity saturable binding curves; the highest affinity binding was obtained in wells with immobilized FV709-1476. In the absence of protein S, both FV-short variants demonstrated weak TFPIα binding, the FV709-1476 variant yielding somewhat stronger binding than FV756-1458. Taken together these results demonstrate that in the absence of TFPIα no binding between any of the two FV-short variants and protein S could be detected. In contrast, strong binding was observed when TFPIα was present. Direct weak binding between TFPIα and FV-short variants was observed in the absence of protein S. When protein S was included, strong TFPIα and FV-short variant binding was observed. In all tested conditions, FV709-1476 yielded stronger binding than FV756-1458 suggesting that the N-terminal region of the B-domain that is present in FV756-1458 but not in FV709-1476 inhibits the binding, possibly through steric hindrance.

3.2 | Hydrophobic patch PLVIVG (1481–1486) crucial for synergistic TFPIα-cofactor activity of FV-short

The results obtained with the FV709-1476 demonstrated the 1476–1491 region to contain residues crucial for the cooperative interaction among FV-short, TFPIα, and protein S. To further narrow down which amino acid residues are important, five new FV-short variants were created (Figure 3) in which the preAR2 was gradually shortened. FV712-1478 contained preAR2 residues **EFNPLVIVGLSKDG**, FV712-1481 **PLVIVGLSKDG**, FV712-1484 **IVGLSKDG**, FV712-1487

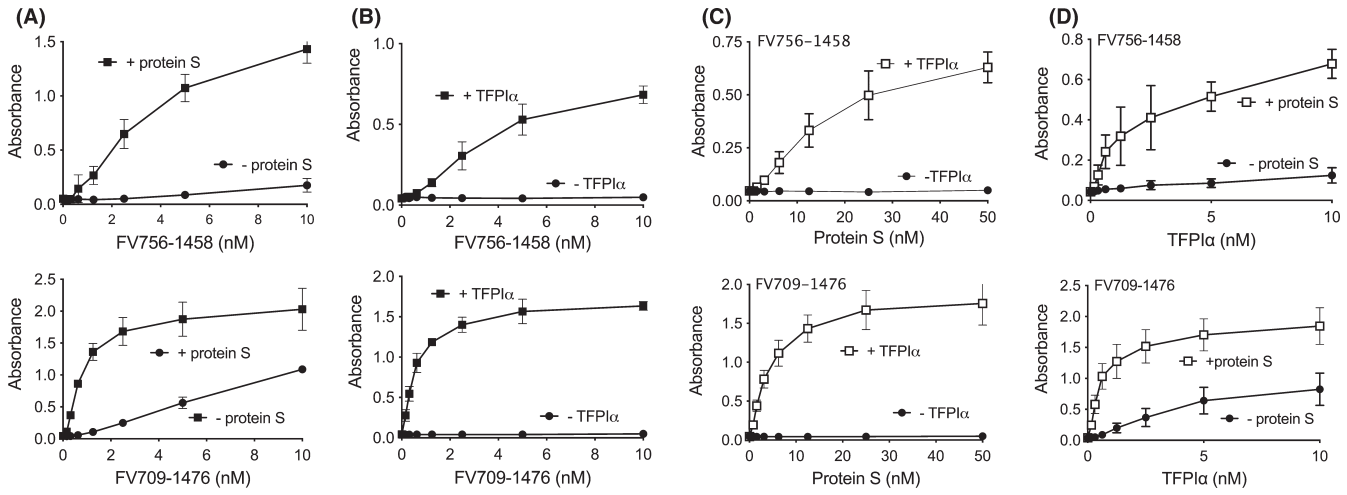

FIGURE 2 Assembly of tri-molecular complexes among FV-short variants, tissue factor pathway inhibitor alpha (TFPIα), and protein S. A, Increasing concentrations of FV756-1458 (upper) or FV709-1476 (lower) were added with or without protein S (50 nM) to microtiter plates with immobilized TFPIα. B, Increasing concentrations of FV756-1458 (upper) or FV709-1476 (lower) were added with or without TFPIα (10 nM) to microtiter plates with immobilized protein S. After overnight incubation, the FV-short binding was determined with a monoclonal antibody against factor V (FV) light chain (HV1). C and D, The microtiter plates contained immobilized FV756-1458 (upper) or FV709-1476 (lower). Increasing concentrations of protein S ± TFPIα (10 nM) (C) or TFPIα ± protein S (50 nM) (D) were added. After overnight incubation, binding of protein S and TFPIα was determined using monoclonal antibodies HPS54 and AHTFPI-5138, respectively. Each experiment was performed three times and the means ± standard deviation were plotted. Results obtained with FV756-1458 have previously been presented and is used as reference for comparison.[24]

LSKDG, and FV712-1490 **DG**. The five mutants were expressed in stable cell lines and purified. On SDS-PAGE Blue and western blotting, they migrated to the expected position slightly below FV756-1458. Incubation with thrombin resulted in generation of FVa heavy and light chains (Figure S1).

The synergistic TFPIα-cofactor activities of the five mutants were tested in the FXa-inhibition assay. In Figure 4A, the concentrations of FV-short variants were titrated, whereas protein S and TFPIα were kept at fixed concentrations. FV712-1478 and FV712-1481 were equally active as FV709-1476 in Figure 1A, whereas FV712-1487 and FV712-1490 were inactive and FV712-1484 yielded intermediate activity. In the protein S titration (Figure 4B), efficient FXa-inhibition was obtained already at a few nM protein S when FV712-1478 and FV712-1481 were used. In contrast, FV712-1487 and FV712-1490 were essentially inactive and FV712-1484 yielded intermediate activity. In the absence of FV-short variant, protein S demonstrated weak TFPIα-cofactor activity and even at 25 nM protein S FXa was not fully inactivated. In the TFPIα titration (Figure 4C), FV712-1478 and FV712-1481 efficiently stimulated the activity of TFPIα. FV712-1487 and FV712-1490 weakly stimulated the FXa inhibition and FV712-1484 yielded intermediate stimulation. Taken together the results of Figure 4A–C demonstrate that preAR2 residues 1481–1486 (**PLVIVG**) were crucial for expression of full synergistic TFPIα-cofactor activity between FV-short variant and protein S.

3.3 | Hydrophobic residues 1481–1486 (PLVIVG) required for assembly of a tri-molecular complex among FV-short, protein S, and TFPIα

The ability of the five FV-short preAR2 mutants to interact with protein S and TFPIα were tested in the microtiter binding assay and compared with the FV712-1458 (Figures 5 and 6). In Figure 5A, TFPIα was immobilized and increasing concentrations of FV-short variants were added with or without protein S. In the absence of protein S, the six tested FV-short variants all bound TFPIα. FV712-1490 appeared to bind slightly less efficiently to TFPIα raising the possibility that the residues of the preAR2 are required for optimal TFPIα binding. The addition of protein S resulted in strong stimulation of binding for three of the FV-short variants (FV712-1458, FV712-1478, and FV712-1481) whereas no stimulation of binding was observed for the other three FV-short variants (FV712-1484, FV712-1487, and FV712-1490). In Figure 5B, the microtiter plates were immobilized with protein S and the six FV-short variants were added at increasing concentrations with or without TFPIα. In the absence of TFPIα, no or very little FV-short variants bound; it is, however, noteworthy that there was a very low binding signal for FV712-1478 and FV712-1481. When TFPIα was included strong binding of FV712-1458, FV712-1478, and FV712-1481 was obtained, whereas the other three FV-short variants did not bind.

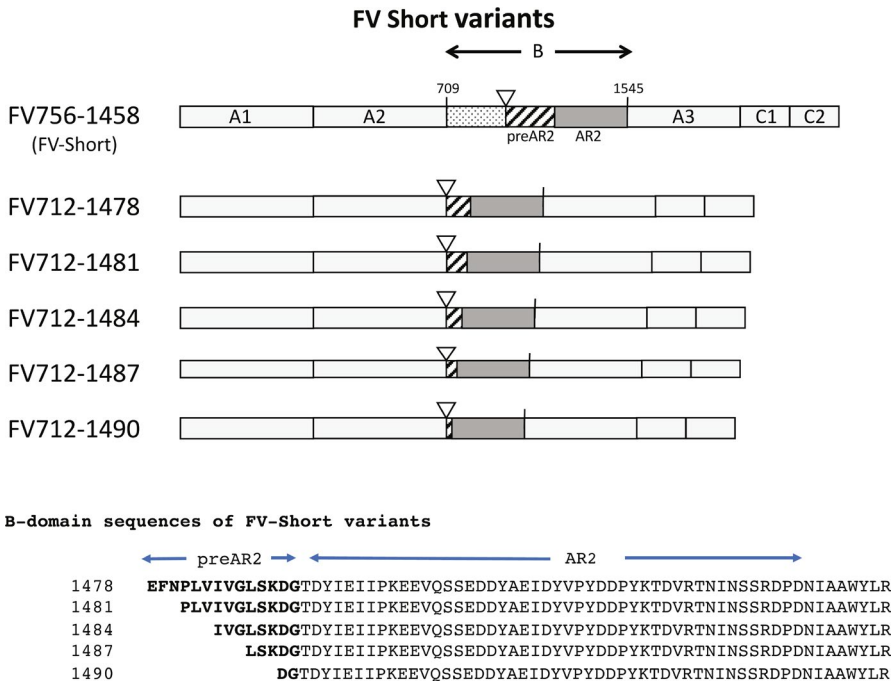

FIGURE 3 Schematic models of the five new FV-short variants used in this study. In the original East Texas FV-short variant (FV756-1458), 702 amino acid residues are deleted from the B domain (triangle). The shortened B domain contains the N-terminal part (710–756), a preAR2 region (1458–1492) and the 1492–1545 sequence containing the AR2 (1493–1537). The thrombin-cleavage sites at 709 and 1545 are indicated. Five new FV-short variants denoted FV712-1478 (Δ713–1477), FV712-1481 (Δ713–1480), FV712-1484 (Δ713–1483), FV712-1487 (Δ713–1486), FV712-1490 (Δ713–1489); deleted (Δ) residues are shown in brackets. The remaining preAR2 and AR2 sequences of the five mutants are shown at the bottom of the figure, the number of the starting residue of the preAR2 of each construct denoted

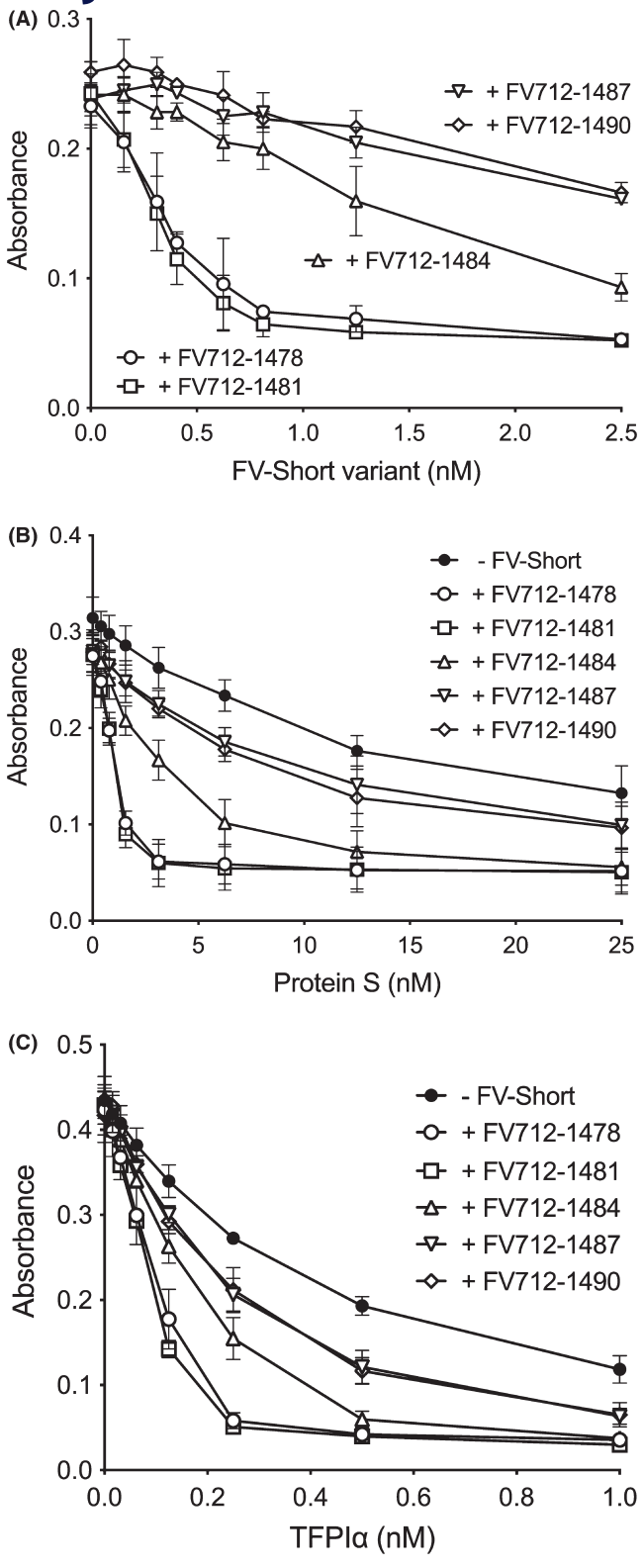

FIGURE 4 FV-short preAR2 variants tested for synergistic tissue factor pathway inhibitor alpha (TFPIα)-cofactor activities. The synergistic TFPIα-cofactor activities of the five new FV-short preAR2 variants were analyzed. A, Increasing concentrations of the five new FV-short variants were mixed with TFPIα (0.25 nM), protein S (3 nM), and 25 uM PL (PS/PE/PC:20/20/60). Factor Xa (FXa; 0.3 nM) and S2756 (0.7 mM) were added and the inhibition of FXa was monitored for 900 s. The absorbance reached at the end of the incubation was plotted against the FV-short variant concentration. B, The protein S concentrations were varied between 0 and 25 nM, the concentrations of FV-short variants were 2 nM, TFPIα (0.25 nM). A control without FV-short variant is included. C, In this experiment, the concentrations of TFPIα were varied between 0 and 1 nM, FV-short variants were 0 or 2 nM and protein S was 3 nM. Each experiment was performed three independent times and the means ± standard deviation were plotted

Figure 6 demonstrates results that were obtained when the six FV-short variants were immobilized and the binding of TFPIα (Figure 6A) or protein S (Figure 6B) was investigated. In Figure 6A, increasing concentrations of TFPIα were added with or without a constant concentration of protein S. Without added protein S, TFPIα bound to all six FV-short variants. Particularly strong binding was obtained with FV712-1478 (K_d ≈ 0.2 nM) and FV712-1481 (K_d ≈ 0.2 nM), whereas FV712-1484 (K_d ≈ 0.6 nM), FV712-1487 (K_d ≈ 0.6 nM), and FV712-1490 (K_d ≈ 1.0 nM) bound less efficiently. Inclusion of protein S stimulated binding of TFPIα to immobilized FV712-1458, FV712-1478, and FV712-1481, whereas no stimulation was observed in wells containing immobilized FV712-1484, FV712-1487, and FV712-1490. In Figure 6B, increasing concentrations of protein S were added with or without TFPIα. No binding of protein S was observed to any of the six FV-short variants, whereas with protein S added, strong binding was obtained in the wells containing FV712-1458, FV712-1478, and FV712-1481. In particular, FV712-1481 yielded binding curves suggesting high affinity saturable protein S binding. In contrast no protein S binding was observed in the wells containing FV712-1484, FV712-1487, and FV712-1490. Taken together, the binding experiments suggest hydrophobic residues 1481–1486 (**PLVIVG**) to be crucial for assembly of a tri-molecular complex among FV-short, protein S, and TFPIα. It is also worth noting that in the absence of protein S, TFPIα bound most strongly to FV-short variants containing the whole preAR2 sequence (PLVIVGLSKDG), which is required for the synergistic TFPIα-cofactor function between FV-short and protein S.

3.4 | Tri-molecular complexes among FV-short, TFPIα, and protein S in plasma

To elucidate whether there is a tri-molecular weight complex among FV-short, TFPIα, and protein S circulating in plasma, pooled plasma was subjected to immune precipitation with a polyclonal antibody against TFPIα (Figure 7). The starting plasma, the supernatant after immune precipitation, and the immune precipitate were analyzed by western blotting using monoclonal antibodies against FV, protein S, and TFPIα. In the immune precipitate there was a strong signal for TFPIα, whereas no TFPIα bands were observed in the starting plasma and supernatant due to the low TFPIα concentration in plasma. In the blot against FV, a strong signal for FV-short was observed in the immune precipitate, whereas no FV-short could be

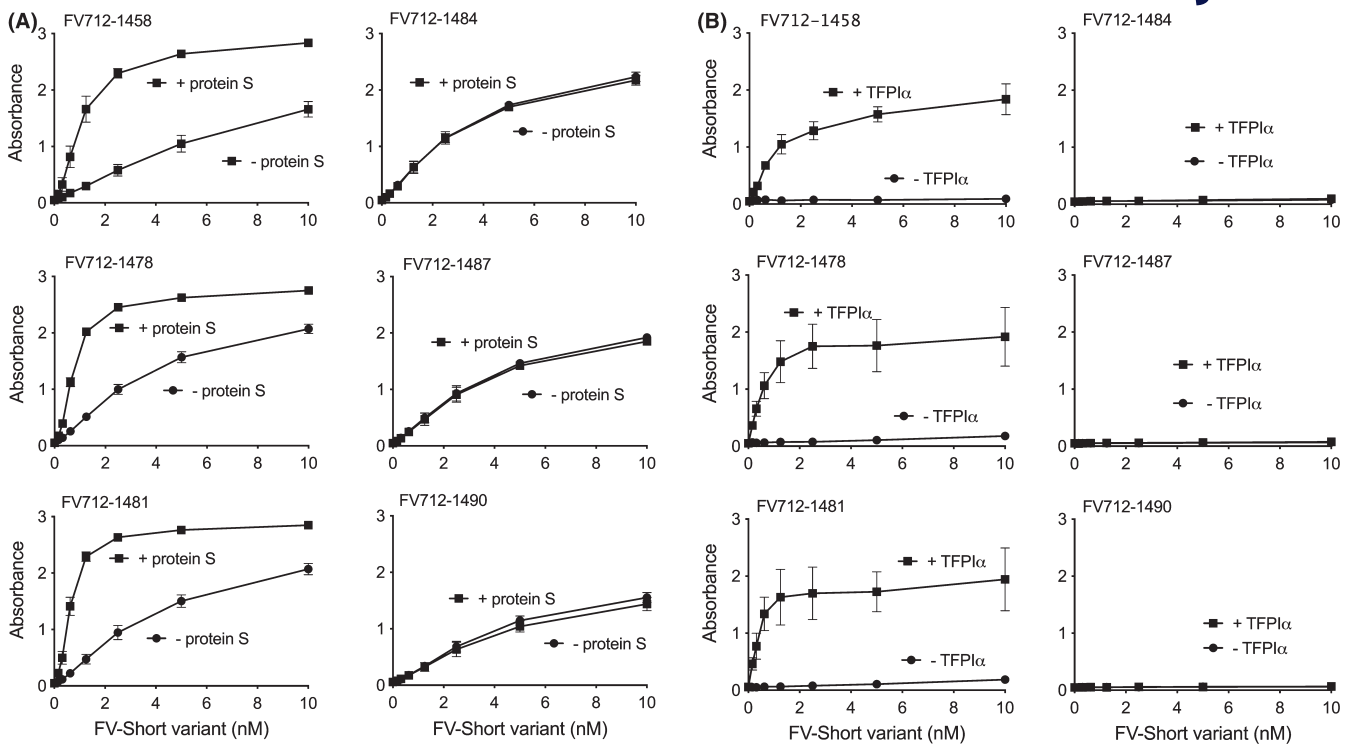

FIGURE 5 Assembly of tri-molecular complexes among FV-short preAR2 variants, tissue factor pathway inhibitor alpha (TFPIα), and protein S. A, Increasing concentrations of FV-short preAR2 variants were added with or without protein S (50 nM) to microtiter plates with immobilized TFPIα. The FV-short variants that are used are indicated in each subsection of the figure. B, Increasing concentrations of FV-short preAR2 variants were added with or without TFPIα (10 nM) to microtiter plates with immobilized protein S. The FV-dhort variants that are used are indicated in each subsection of the figure. In addition to the five new variants, previously characterized FV712-1458 (containing the whole preAR2) is included as reference.[24] After overnight incubation, the FV-short binding was determined with a monoclonal antibody against FV light chain (HV1). Each experiment was performed three times and the means ± standard deviation were plotted

seen in the starting plasma and the supernatant after immune precipitation. This is explained by the very low plasma concentration of FV-short and the enrichment of FV-short in the TFPIα immune-precipitate. Full-length FV and proteolyzed FV fragments were observed in the plasma and supernatant. In the blot developed with HPS54, distinct protein S bands were observed in all three lanes indicating that only a small fraction of protein S was recovered in the immune precipitate. The results are compatible with the hypothesis that a tri-molecular complex among FV-short, TFPIα, and protein S circulates at sub-nanomolar concentration in plasma.

4 | DISCUSSION

A naturally occurring alternative splicing of the *F5* gene creates the FV-short (FV756-1458) variant that exposes a high affinity binding site for TFPIα in the acid C-terminal region (AR2, residues 1493–1537) of its truncated B domain.[1,2,13] Families with autosomal dominant bleeding disorder have been described to carry exon 13 mutations that result in increased alternative splicing yielding high concentrations of FV-short and as a consequence increased concentration of circulating FV-short/TFPIα complexes.[13,16-18] The alternative splicing is inefficient in healthy individuals and FV-short represents only a minor fraction of circulating FV (≈1%), but it is important for its binding of TFPIα.[2,13,15,22,24] The FV-short/TFPIα complex binds protein S and in the presence of negatively charged phospholipid membranes, the FV-short/TFPIα/protein S complex efficiently inhibits FXa.[22-25] We recently demonstrated that formation of the tri-molecular FXa-inhibitory complex is not only dependent on TFPIα binding to the exposed AR2 but also of the preAR2 region (residues 1458–1492) in FV-short.[24] Thus, an artificial FV-short variant lacking the whole preAR2 region (FV810-1492) was demonstrated to be unable to function as synergistic TFPIα-cofactor with protein S even though AR2 in FV810-1492 binds TFPIα. Due to the lack of the preAR2, the FV810-1492/TFPIα complex doesn't have the capacity to interact with protein S. We have now created additional FV-short variants and identified a site in preAR2 located close to the preAR2/AR2 junction that is absolutely required for the synergistic TFPIα-cofactor function between FV-short and protein S. It is also crucial for the binding of protein S to the FV-short/TFPIα-complex, and for the ability of the three proteins to cooperatively assemble into the FXa-inhibitory complex (Figure 8). This site comprises a short stretch of amino acid residues (PLVIVG, 1481–1486) that form a hydrophobic patch.

The FV-short variants that contain the residues 1481–1486 are fully functional as synergistic TFPIα-cofactors with protein S, whereas those FV-short variants that lack these residues have zero synergistic TFPIα-cofactor activity. When tested in binding assays,

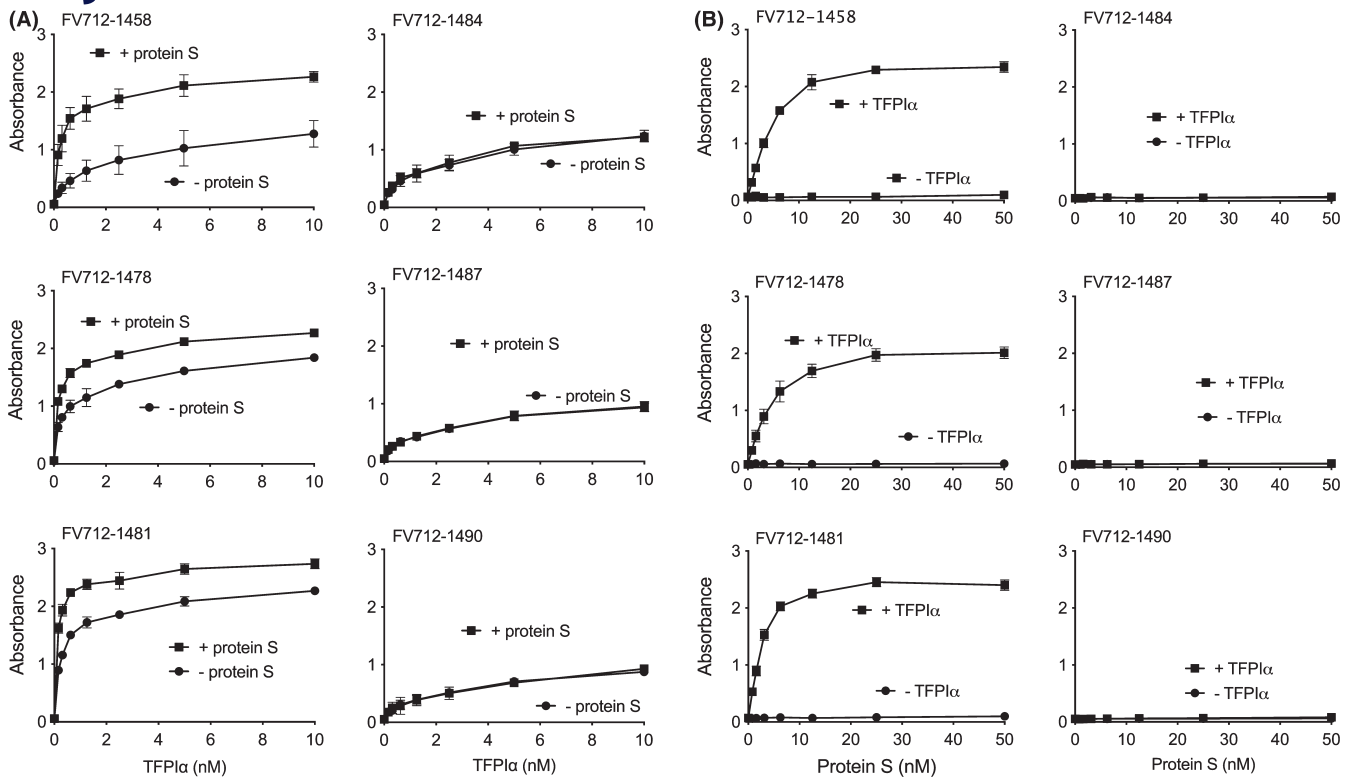

FIGURE 6 Assembly of tri-molecular complexes among FV-short preAR2 variants, tissue factor pathway inhibitor alpha (TFPIα), and protein S. A, Increasing concentrations of TFPIα were added with or without protein S (50 nM) to microtiter plates with immobilized FV-short preAR2 variants as indicated in each subsection. B, Increasing concentrations of protein S were added with or without TFPIα (10 nM) to microtiter plates with immobilized FV-short preAR2 variants as indicated in each subsection. After overnight incubation, binding of TFPIα and protein S was determined using monoclonal antibodies AHTFPI-5138 and HPS54, respectively. In addition to the five new variants, previously characterized FV712-1458 (containing the whole preAR2) is included as reference.[24] Each experiment was performed three times and the means ± standard deviation were plotted

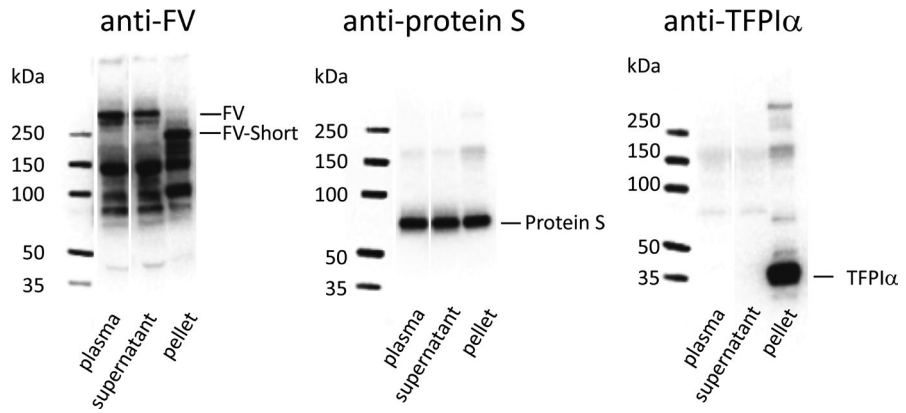

FIGURE 7 Tri-molecular complexes among FV-short, protein S, and tissue factor pathway inhibitor alpha (TFPIα) in plasma. Pooled citrated plasma (5 ml) was incubated with 1 ml streptavidin-coated magnetic beads carrying biotinylated polyclonal antibodies against TFPIα (AHTFPI-S). After 2 h at 4°C, the beads were collected, washed, and dissolved in 100 μl sample preparation buffer and 10 μl applied to the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The starting plasma and the supernatants were diluted 1:100 in 2 × sample preparation buffer and 10 μl of each were applied to the SDS-PAGE. Three SDS-PAGE were run and the western blots were developed with AHV5146, HPS54, and AHTFPI5138 as indicated

the 1481–1486 containing variants demonstrated cooperative binding among the FV-short variants, TFPIα, and protein S, whereas those lacking the 1481–1486 residues did not stimulate such cooperative binding even though they bound TFPIα. It is noteworthy that none of the FV-short variants were able to bind protein S directly, whereas when TFPIα was present, the 1481–1486 containing FV-short

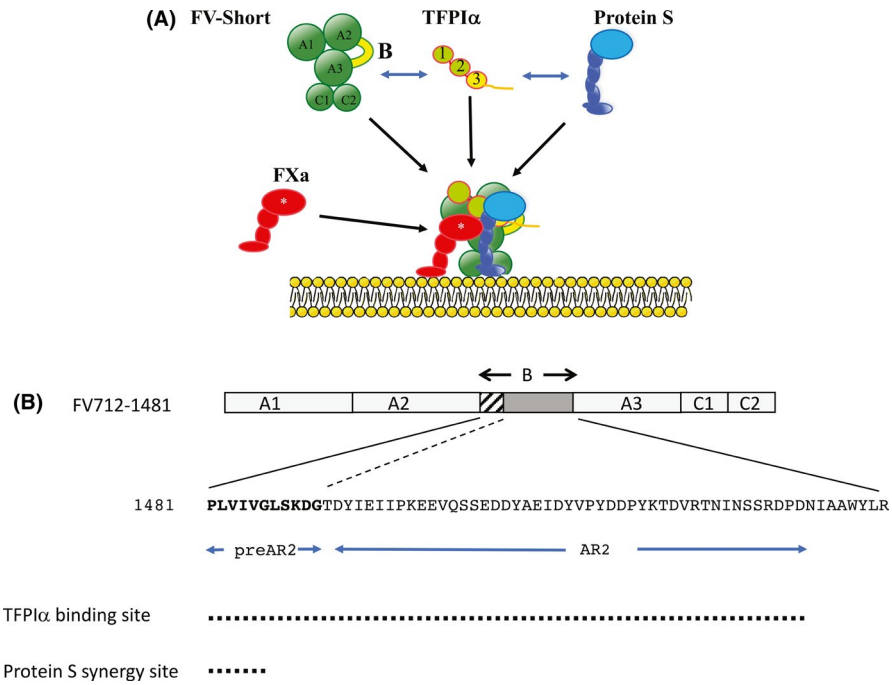

FIGURE 8 Cooperative interaction among FV-short, protein S, and tissue factor pathway inhibitor alpha (TFPIα) as basis for efficient factor Xa (FXa) inhibition. A, In circulation, multiple interactions among FV-short, TFPIα, and protein S result in the formation of an efficient FXa-inhibitory complex. The FXa inhibition occurs on the surface of negatively charged phospholipid membranes to which both FXa and the FV-short/TFPIα/protein S complex binds. The interacting regions in each of the participating proteins are marked, the B-domain of FV-short in yellow, Kunitz 3 and the C-terminus in TFPIα with yellow/orange, and the SHBG-region in protein S with turquoise.[1,2,11,12,15,19,20,22,24-26,29] B, The sequence of the shortest functionally important region of the B domain is shown. The TFPIα binding site in the B-domain involves both AR2 and preAR2. The hydrophobic patch PLVIVG (residues 1481–1486) is crucial for the cooperative interaction among FV-short, TFPIα, and protein S and thus the synergistic TFPIα-cofactor activity among the three proteins

variants efficiently participated in the formation of the tri-molecular complex comprising FV-short, protein S, and TFPIα. The complexes between TFPIα and PLVIVG-containing FV-short variants bind protein S with high affinity demonstrating that the PLVIVG hydrophobic patch is important for protein S interplay with the other proteins in the tri-molecular FXa-inhibitory complex. Whether PLVIVG directly interacts with protein S or if PLVIVG in FV-short and Kunitz 3 in TFPIα jointly create a protein S binding site cannot be judged from the results. Possibly PLVIVG contains a low-affinity protein S binding site which cooperatively interacts with the protein S binding site in the Kunitz 3 domain of TFPIα. The very weak direct binding observed in Figure 5B between FV712-1478 and FV712-1481 and immobilized protein S in the absence of TFPIα could indicate such weak direct interaction between the PLVIVG site and protein S. However, no support for such weak interaction was observed when FV712-1478 or FV712-1481 were immobilized and protein S binding tested (Figure 6B).

The binding site in TFPIα for FV-short is located in the C-terminal tail of TFPIα (LIKTKRKRKKQRVKIAYEEIFVKNM), which contains a stretch of highly basic residues (KTKRKRKKQRVK) that interact with the acidic residues in AR2.[14,15,19,30,35] In TFPIα a hydrophobic patch (LIKT) is located just N-terminal of the basic region and it is tempting to speculate that this patch interacts with the hydrophobic patch (1481–1486, PLVIVG) in preAR2 of FV-short. In support for this hypothesis, we observed much stronger direct TFPIα binding in the absence of protein S to the PLVIVG-containing FV-short variants (FV712-1478 and FV712-1481) than to those without PLVIVG (FV712-1487 and FV712-1490; Figure 6A). Thus, the PLVIVG is both important for optimal binding of TFPIα to FV-short and crucial for the binding of protein S to the TFPIα/FV-short complex and the cooperative assembly of the FXa-inhibitory complex (Figure 8). The hydrophobic patch LIKT in TFPIα has recently been reported to be functionally important for the ability of TFPIα to inhibit prothrombinase activity with FXa-activated FV but not with FV810 (FV810-1492).[30] FXa, which cleaves FV at R709 and R1018 but not at R1545, generates a FVa with retained preAR2 and AR2 regions of the B-domain.[36] It is therefore expected to function like FV-short. In contrast, in FV810, which does not function as synergistic TFPIα-cofactor, the preAR2—and thus the PLVIVG patch—is lacking. The published results obtained with FXa-activated FV and FV810[30] taken together with our now reported results support the hypothesis that PLVIVG in FV-short interacts with LIKT in TFPIα and strengthens the interaction between TFPIα and FV-short. It is noteworthy that the basic region (973–1008) in the B-domain of FV, which interacts with AR2 and thus keeps FV as a procofactor, also contains the LIKT sequence just N-terminal of its basic residues (LIKTRKKKKEKHTHHA).[5-7,15] It is tempting to speculate that in intact FV, the LIKT sequence located just prior to the basic B-domain

regions interacts with the PLVIVG patch in preAR2 thus strengthening the intra B-domain interaction that is important for keeping FV in its procofactor state.

The functional FXa-inhibitory assay is performed in the presence of negatively charged phospholipid vesicles, whereas the binding experiments were done in the absence of negatively charged phospholipids. This may explain why FV712-1484 (only contains IVG of the PLVIVG patch) has intermediate synergistic TFPIα-cofactor activity but is unable to support cooperative binding of the FV-short/TFPIα/protein S complex. The strong cooperative binding between PLVIVG-containing FV-short, protein S, and TFPIα observed in the absence of phospholipids demonstrates that the FXa-inhibitory complex can form in fluid phase. This suggests that a preformed trimolecular FXa inhibitory complex is present at sub-nanomolar concentration in circulation. All FV-short (≈0.2 nM) and TFPIα (≈0.2 nM) are expected to be in complex, whereas only a minor fraction of plasma protein S (100 nM) is complexed. However, the high plasma concentration of protein S would drive the equilibrium into formation of the complex. The immune-precipitation experiments that we performed using a polyclonal anti-TFPIα as catcher lends support to the hypothesis of such a tri-molecular complex existing in plasma (Figure 7). The FV-short/TFPIα/protein S complex efficiently binds to negatively charged phospholipid membranes, for example, exposed on activated or apoptotic cells, and there efficiently inhibits FXa (Figure 8). Both FV-short and protein S have bindings sites for negatively charged phospholipids.[1,2,11,12] FXa also has affinity for the negatively charged phospholipids and in addition contains binding sites for all three proteins in the FXa-inhibitory complex.[1,4,8,11,12] The multiple protein–protein and protein–phospholipid interactions ensure efficient inhibition of FXa. The very low concentration of the highly efficient FV-short/TFPIα/protein S complex in circulation suggests that its function is to regulate low levels of FXa generated at site with low degree of procoagulant stimulation.

In conclusion, we have identified a hydrophobic patch (1481–1486, PLVIVG) in the preAR2 of FV-short that is crucial for the synergistic TFPIα cofactor activity between FV-short and protein S and required for the binding of protein S to the TFPIα/FV-short complex and the cooperativity in interactions among FV-short, protein S, and TFPIα that results in the generation of an efficient FXa-inhibitory complex (Figure 8).

ACKNOWLEDGMENTS

The study is supported by grants from the Swedish Research Council (BD), the Swedish Heart-Lung Foundation, and by Österlund's Foundation. Dr. T. Hamuro at the Chemo-Sero-Therapeutic Research Institute, Japan is acknowledged for the kind gift of TFPIα. Dr. Randy Kaufman at Sanford-Burnham Prebys Medical Discovery Institute, La Jolla, CA, USA is acknowledged for the gift of the FV709-1476 cDNA.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest with the content of the paper.

AUTHOR CONTRIBUTIONS

B Dahlbäck initiated, designed, and supervised the study; analyzed data; and wrote the manuscript. S Tran performed experiments, analyzed data, and participated in writing of the paper.

ORCID

Björn Dahlbäck https://orcid.org/0000-0003-1546-0328
Sinh Tran https://orcid.org/0000-0002-1259-0672

REFERENCES

1. Camire RM. Rethinking events in the haemostatic process: role of factor V and TFPI. *Haemophilia*. 2016;22(Suppl 5):3-8.
2. Dahlback B. Novel insights into the regulation of coagulation by factor V isoforms, tissue factor pathway inhibitoralpha, and protein S. *J Thromb Haemost*. 2017;15:1241-1250.
3. Nicolaes GA, Dahlback B. Factor V and thrombotic disease: description of a janus-faced protein. *Arterioscler Thromb Vasc Biol*. 2002;22:530-538.
4. Dahlback B. Blood coagulation. *Lancet*. 2000;355:1627-1632.
5. Bos MH, Camire RM. A bipartite autoinhibitory region within the B-domain suppresses function in factor V. *J Biol Chem*. 2012;287:26342-26351.
6. Bunce MW, Bos MH, Krishnaswamy S, Camire RM. Restoring the procofactor state of factor Va-like variants by complementation with B-domain peptides. *J Biol Chem*. 2013;288:30151-30160.
7. Camire RM, Bos MH. The molecular basis of factor V and VIII procofactor activation. *J Thromb Haemost*. 2009;7:1951-1961.
8. Dahlback B. Pro- and anticoagulant properties of factor V in pathogenesis of thrombosis and bleeding disorders. *Int J Lab Hematol*. 2016;38(Suppl 1):4-11.
9. Shen L, Dahlback B. Factor V and protein S as synergistic cofactors to activated protein C in degradation of factor VIIIa. *J Biol Chem*. 1994;269:18735-18738.
10. Thorelli E, Kaufman RJ, Dahlback B. The C-terminal region of the factor V B-domain is crucial for the anticoagulant activity of factor V. *J Biol Chem*. 1998;273:16140-16145.
11. Dahlback B. Vitamin K-dependent protein S: beyond the protein C pathway. *Semin Thromb Hemost*. 2018;44:176-184.
12. Gierula M, Ahnstrom J. Anticoagulant protein S-New insights on interactions and functions. *J Thromb Haemost*. 2020;18:2801-2811.
13. Vincent LM, Tran S, Livaja R, Bensend TA, Milewicz DM, Dahlback B. Coagulation factor V(A2440G) causes east Texas bleeding disorder via TFPIalpha. *J Clin Invest*. 2013;123:3777-3787.
14. Wood JP, Bunce MW, Maroney SA, Tracy PB, Camire RM, Mast AE. Tissue factor pathway inhibitor-alpha inhibits prothrombinase during the initiation of blood coagulation. *Proc Natl Acad Sci USA*. 2013;110:17838-17843.
15. Petrillo T, Ayombil F, Van't Veer C, Camire RM. Regulation of factor V and factor V-short by TFPIalpha: Relationship between B-domain proteolysis and binding. *J Biol Chem*. 2021;296:100234.
16. Peterson JA, Gupta S, Martinez ND, Hardesty B, Maroney SA, Mast AE. Factor V east Texas variant causes bleeding in a three-generation family. *J Thromb Haemost*. 2022;20(3):565-573.
17. Cunha ML, Bakhtiari K, Peter J, Marquart JA, Meijers JC, Middeldorp S. A novel mutation in the F5 gene (factor V Amsterdam) associated with bleeding independent of factor V procoagulant function. *Blood*. 2015;125:1822-1825.
18. Zimowski KL, Petrillo T, Ho MD, et al. F5-Atlanta: A novel mutation in F5 associated with enhanced East Texas splicing and FV-short production. *J Thromb Haemost*. 2021;19:1653-1665.
19. Broze GJ Jr, Girard TJ. Tissue factor pathway inhibitor: structure-function. *Front Biosci*. 2012;17:262-280.

20. Mast AE. Tissue factor pathway inhibitor: multiple anticoagulant activities for a single protein. *Arterioscler Thromb Vasc Biol*. 2016;36:9-14.
21. Wood JP, Ellery PE, Maroney SA, Mast AE. Biology of tissue factor pathway inhibitor. *Blood*. 2014;123:2934-2943.
22. Dahlback B, Guo LG, Livaja-Koshiar R, Tran S. Factor V-short and protein S as synergistic tissue factor pathway inhibitor (TFPIa) cofactors. *Res Pract Throm Haemost*. 2017;2:114-124.
23. Dahlback B, Guo LJ, Zoller B, Tran S. New functional test for the TFPIalpha cofactor activity of Protein S working in synergy with FV-Short. *J Thromb Haemost*. 2019;17:585-595.
24. Dahlback B, Tran S. The preAR2 region (1458–1492) in factor V-Short is crucial for the synergistic TFPIalpha-cofactor activity with protein S and the assembly of a trimolecular factor Xa-inhibitory complex comprising FV-Short, protein S, and TFPIalpha. *J Thromb Haemost*. 2022;20:58-68.
25. Teraz-Orosz A, Gierula M, Petri A, et al. Laminin G1 residues of protein S mediate its TFPI cofactor function and are competitively regulated by C4BP. *Blood Adv*. 2022;6(2):704-715.
26. Hackeng TM, Maurissen LF, Castoldi E, Rosing J. Regulation of TFPI function by protein S. *J Thromb Haemost*. 2009;7(Suppl 1):165-168.
27. Hackeng TM, Sere KM, Tans G, Rosing J. Protein S stimulates inhibition of the tissue factor pathway by tissue factor pathway inhibitor. *Proc Natl Acad Sci USA*. 2006;103:3106-3111.
28. Peraramelli S, Thomassen S, Heinzmann A, et al. Role of exosite binding modulators in the inhibition of fxa by TFPI. *Thromb Haemost*. 2016;115:580-590.
29. Ahnstrom J, Andersson HM, Hockey V, et al. Identification of functionally important residues in TFPI Kunitz domain 3 required for the enhancement of its activity by protein S. *Blood*. 2012;120:5059-5062.
30. Wood JP, Petersen HH, Yu B, Wu X, Hilden I, Mast AE. TFPIalpha interacts with FVa and FXa to inhibit prothrombinase during the initiation of coagulation. *Blood Adv*. 2017;1:2692-2702.
31. Dahlback B, Hildebrand B, Malm J. Characterization of functionally important domains in human vitamin K-dependent protein S using monoclonal antibodies. *J Biol Chem*. 1990;265:8127-8135.
32. Dahlback B. Purification of human vitamin K-dependent protein S and its limited proteolysis by thrombin. *Biochem J*. 1983;209:837-846.
33. Marquette KA, Pittman DD, Kaufman RJ. The factor V B-domain provides two functions to facilitate thrombin cleavage and release of the light chain. *Blood*. 1995;86:3026-3034.
34. Toso R, Camire RM. Removal of B-domain sequences from factor V rather than specific proteolysis underlies the mechanism by which cofactor function is realized. *J Biol Chem*. 2004;279:21643-21650.
35. Ndonwi M, Girard TJ, Broze GJ Jr. The C-terminus of tissue factor pathway inhibitor alpha is required for its interaction with factors V and Va. *J Thromb Haemost*. 2012;10:1944-1946.
36. Monkovic DD, Tracy PB. Activation of human factor V by factor Xa and thrombin. *Biochemistry*. 1990;29:1118-1128.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Dahlbäck B, Tran S. A hydrophobic patch (PLVIVG; 1481–1486) in the B-domain of factor V-short is crucial for its synergistic TFPIα-cofactor activity with protein S and for the formation of the FXa-inhibitory complex comprising FV-short, TFPIα, and protein S. *J Thromb Haemost*. 2022;20:1146–1157. doi:10.1111/jth.15690