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Coagulation factor V^{A2440G} causes east Texas bleeding disorder via TFPI{\alpha} 

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The autosomal dominantly inherited east Texas bleeding disorder is linked to an A2440G variant in exon 13 of the F5 gene. Affected individuals have normal levels of coagulation factor V (FV) activity, but demonstrate inhibition of global coagulation tests. We demonstrated that the A2440G mutation causes upregulation of an alternatively spliced F5 transcript that results in an in-frame deletion of 702 amino acids of the large activation fragment, the B domain. The approximately 250-kDa FV isoform (FV-short), which can be fully activated by thrombin, is present in all A2440G carriers’ plasma (n = 16). FV-short inhibits coagulation through an indirect mechanism by forming a complex with tissue factor pathway inhibitor-\(\alpha\) (TFPI{\alpha}), resulting in an approximately 10-fold increase in plasma TFPI{\alpha}, suggesting that the TFPI{\alpha}:FV-short complexes are retained in circulation. The TFPI{\alpha}:FV-short complexes efficiently inhibit thrombin generation of both intrinsic and extrinsic coagulation pathways. These data demonstrate that the east Texas bleeding disorder–associated F5^{A2440G} leads to the formation of the TFPI{\alpha}:FV-short complex, which inhibits activation and propagation of coagulation.

Introduction

Coagulation factor V (FV) is a cofactor protein that can both promote and inhibit coagulation (1, 2). Located on 1q24–25, the F5 gene is composed of 25 exons that transcribe a 6.8-kb mRNA (3–7). The translated 330-kDa glycoprotein precursor contains 2,196 amino acids organized into the domain structure A1-A2-B-A3-C1-C2 and is highly homologous to factor VIII (FVIII), sharing 35%–40% identity in the A and C domains (4, 5, 8). Human FV is primarily produced by hepatocytes and circulates in plasma as an intact 330-kDa precursor at a concentration of about 20 nM (7 pg/ml) (9–12). Approximately 20% of the total human FV found in whole blood is stored in platelet α granules in a partially proteolyzed form in conjunction with the binding protein multimerin. This platelet FV derives from the plasma FV pool and is secreted upon platelet activation to create a high, localized concentration of the cofactor at sites of injury (12–15).

The procoagulant cofactor function of FV is primarily dictated by its interaction and cleavage by thrombin and active factor X (FXa). Its cleavage by thrombin is deemed the most biologically important early event in the blood clot formation process (16–19). As an intact single-chain precursor, FV expresses less than 1% of its potential procoagulant cofactor activity (20). Upon sequential cleavage of Arg-709, Arg-1018, and Arg-1545 by thrombin, the large connecting B domain is removed from the intact FV molecule to produce the heavy chain (A1-A2) and the light chain (A3-C1-C2) that have M{\alpha}s of 105,000 and 74,000, respectively. The heavy chain and the light chain noncovalently associate in the presence of calcium to produce an active procoagulant cofactor (FVα). FVα and FXa assemble on negatively charged phospholipid (PL) membranes to form the prothrombinase (PTase) complex. The presence of FVα in this complex greatly accelerates the activation of prothrombin to α-thrombin by 300,000-fold relative to the activation by FXa alone (20). This activity is downregulated through activated protein C–mediated (APC-mediated) cleavages at Arg-506, Arg-306, and Arg-679 in FV. Cleavage at Arg-306, which occurs 20-fold more rapidly in the presence of protein S, completely inhibits FVα cofactor function (1, 21–23).

Recently, FV in plasma was demonstrated to interact with tissue factor pathway inhibitor-\(\alpha\) (TFPI{\alpha}), and this interaction affects TFPI{\alpha} plasma concentrations, with TFPI{\alpha} levels being approximately 70% lower in plasma from FV-deficient patients relative to normal plasma (24). Moreover, immunodepletion of FV in normal plasma decreased TFPI{\alpha} levels by approximately 60%–90%. Surface plasmon resonance analysis using immobilized TFPI{\alpha} demonstrated half-maximum binding at 13.5 nM FV. TFPI{\alpha} is a multivalent Kunitz-type protease inhibitor that regulates tissue factor–induced (TF-induced) coagulation by inhibiting FXa and FVIIa (25) and exists in multiple alternatively spliced isoforms. The full-length isoform TFPI{\alpha} is a 276-residues long glycoprotein (approximately 40 kDa) that contains 3 tandem Kunitz-type domains followed by a basic C terminus. In plasma, the total TFPI{\alpha} concentration is approximately 70 ng/ml (1.6 nM), with TFPI{\alpha} only constituting approximately 20% of the total TFPI{\alpha} in plasma. TFPI{\alpha} is also present on the surface of endothelium, where it is partly bound to proteoglycans. A truncated TFPI{\alpha}, which lacks the third Kunitz domain and the C-terminal peptide, is the major isoform of TFPI{\alpha} in plasma (approximately 80%). It circulates bound to the lipoproteins, mainly to LDL (25). Another truncated isoform denoted TFPI{\beta} is present on endothelium, where it is bound with a GPI anchor (26). Recently, Ndonwi et al. demonstrated that the positively charged C terminus of TFPI{\alpha} contains the binding site for FV (27). The characteristics of the binding site in FV remain to be elucidated, but it is noteworthy that both FV and FVα were shown to bind TFPI{\alpha}.

In 2001, a novel moderately severe autosomal dominant bleeding disorder was described in a large family from east Texas (OMIM 605913; family pedigree in ref. 28 and Supple-
The A2440G mutation causes a splicing event in exon 13 that creates an in-frame deletion of 2106 base pairs that encodes amino acids 756–24546 deleted (Figure 2). Thus, this variant transcript in affected patients’ RNA was the 840 bp, and the variant is in exon 13, which encodes the B domain that is not part of active FVα (1, 2, 30). We have now elucidated the complicated and indirect mechanisms through which A2440G produces the bleeding disorder. The A2440G mutation, whereas those marked with minus signs represent unaffected family members. The arrows point at full-length FV (FV-FL) and FV-short. The 2 lanes in the upper row that are marked with asterisks represent pools of unaffected (∗+) and affected (±) family members. Note the weak FV-short band in the seventh sample from the left in the upper row, the sample being derived from individual IV:17 in family pedigree (Supplemental Figure 1).

**Results**

Short form of factor V identified in affected family members’ plasma. Plasma from individual family members was analyzed by immunoblotting using a specific antibody against the heavy chain in combination with HRP-conjugated goat anti-mouse antiserum. Blots were developed with SuperSignal West Dura Extended Duration Chemiluminescence Substrate. The SDS-PAGE (4–15%) was run under nonreducing conditions. Lanes marked with plus signs represent affected individuals carrying the A2440G mutation, whereas those marked with minus signs represent unaffected family members. The arrows point at full-length FV (FV-FL) and FV-short. The 2 lanes in the upper row that are marked with asterisks represent pools of unaffected (∗+) and affected (±) family members. Note the weak FV-short band in the seventh sample from the left in the upper row, the sample being derived from individual IV:17 in family pedigree (Supplemental Figure 1).
validate its expression in normal controls and in the liver, which is the primary source of the FV found in the bloodstream (12). Sequencing of this transcript-specific RT-PCRs of individuals with F5 A2440G mutation confirmed that this mutation is expressed (Figure 2C) and appears to be preferentially amplified, as the normal (A2440) allele is not visualized in the sequencing reaction. Figure 3A confirms that this FV-short transcript is present in the RNA from unaffected individuals and in RNA from a control liver. Quantitative real-time PCR confirmed that FV-short transcript expression levels in individuals with A2440G (n = 3) were increased by an average of 22-fold (P < 0.02) compared with controls (n = 3) (Figure 3B), with considerable interindividual variation in expression levels of the FV-short transcript among the 3 affected individuals. Total F5 transcript levels assessed using a commercial assay spanning the exon 12 and 13 splice junction did not significantly differ between patients and controls (data not shown). 

Quantification of FV variants in plasma of family members. The concentrations of total FV and FV-FL were assessed in affected individuals (n = 16) with ELISAs using detecting antibodies against the light chain (HV1) or the B domain (MK30), respectively. The total FV and FV-FL concentrations (mean ± SD) were 25.6 ± 4.9 nM and 20.7 ± 4.1 nM, respectively. Corresponding values of an unaffected pool were 22.7 and 21.3 nM, respectively. The concentration of FV-short in individual affected family members (estimated by subtracting the MK30 ELISA values from those of the HV1 ELISA) demonstrated considerable interindividual variation, 4.8 ± 2.0 nM mean ± SD (range 1.7–8.4 nM).

Presence of coagulation inhibitor in affected patient plasma. A thrombin generation assay (TGA) was used to evaluate the extrinsic coagulation pathway in plasma from individual family members. All affected family members carrying the F5 mutation demonstrated decreased thrombin generation with prolonged lag phase (mean ~12 minutes vs. ~4 minutes in unaffected) as well as decrease in peak heights and area under the curves (Figure 4). Mixing affected and unaffected plasma at increasing ratios demonstrated gradually increased inhibition of thrombin generation with prolonged lag phases and decreased peak heights, suggesting that affected plasma contains a coagulation inhibitor (Figure 4). The delayed thrombin generation correlated to slow activation of both FV-FL and FV-short in affected plasma, as visualized by the slow production of both heavy and light FVa chains (Figure 5, B and C, respectively).

Inhibitory activity associated with FV-short in affected plasma. To elucidate whether the inhibitory activity present in affected plasma was associated with FV-short, the plasma was subjected to immunodepletion using polyclonal FV antibodies and then tested in the TGA after addition of recombinant FV-FL (rFV-FL) or rFV-short. FV depletion completely blunted the thrombin generation in both affected and unaffected plasma. The addition of either rFV-FL or rFV-short to the FV-depleted plasmas resulted in normal thrombin generation in both affected and unaffected plasma (Figure 6). These results demonstrated that the inhibitory activity was directly associated with FV in the affected plasma and was removed by immunodepletion. Selective immunodepletion of the FV-FL with a B domain monoclonal antibody (MK30) demonstrated that the inhibitor was specifically associated with FV-short in affected plasma because the inhibitory activity remained in the FV-FL–depleted plasma (Figure 7).

The FV-short–associated inhibitor in affected patient plasma identified as TFPIα. The results presented above suggested the possibility that the FV-short–associated inhibitory activity was not an intrinsic property of FV-short itself, but caused by an inhibitor that was
associated with FV-short in affected plasma. As TFPIα has been shown to interact with FV in normal plasma (24, 27), individual plasmas from family members were analyzed by immunoblotting using an antibody against the N terminus of TFPI (AHTFPI-S138) (Figure 8). All affected individuals demonstrated a very strong TFPI immunoreactive band at 40 kDa, whereas the corresponding TFPI band in unaffected family members was barely detectable. This observation was further confirmed using a rabbit monoclonal antibody against the C terminus of TFPIα, which yielded a similarly strong band in affected plasma (Supplemental Figure 5).

The rTFPIα migrated at a slightly higher molecular weight position than plasma TFPIα, most likely due to glycosylation differences, as the migration of the 2 was similar after deglycosylation with N-glycosidase F (Supplemental Figure 5). A dilution series of recombinant TFPIα analyzed by immunoblot analysis (not shown) semi-quantitatively estimated the TFPIα concentration to be approximately 5 nM in the affected plasma pool and less than 0.5 nM in the unaffected plasma pool. Three different ELISA variants against TFPI were used to estimate the concentrations of the different isoforms of TFPI. The first ELISA measuring total TFPI, using polyclonal anti-TFPI as catcher and a monoclonal antibody against Kunitz 1 as detector, demonstrated that affected family members’ plasma contained 5.3 ± 1.2 nM (mean ± SD) TFPI, whereas a pool of unaffected plasma contained 1.4 nM TFPI. The second ELISA recognizing TFPIα, using the same polyclonal as catcher and a monoclonal antibody against Kunitz 3 as detector, suggested the TFPIα concentration to be 2.5 ± 0.7 nM in affected family members and 0.36 nM in the unaffected pool. The third ELISA (Asserachrom Free TFPIα; Stago), using a monoclonal antibody detecting all TFPI isoforms as catcher and a monoclonal antibody against the C terminus as detector, measured 1.25 ± 0.7 nM TFPIα (mean ± SD) in affected family members and 0.14 nM in the unaffected plasma pool. To prove that the high concentrations of TFPIα in plasma of affected family members caused the inhibition in the TGA, polyclonal antibodies were added to affected plasma prior to the TGA. This resulted in complete normalization of the TGA (Figure 9A). Furthermore, addition of increasing concentrations of rTFPIα to unaffected plasma resulted in a dose-dependent inhibition of thrombin generation, yielding curves similar to those of plasma from affected individuals (Figure 9B).

Complexes between FV-short and TFPIα in plasma. Our results suggested that TFPIα was specifically associated with FV-short in plasma of affected family members. This was validated when immunodepletion using polyclonal TFPI antibodies removed most of FV-short, while leaving FV-FL, in the TFPI-depleted plasma (Figure 10, A and B). FV-short was recovered in the TFPI immunoprecipitate. The conclusion that TFPIα was specifically associated with FV-short gained additional support from immunodepletion experiments using HiTrap columns coupled with either the B domain monoclonal antibody MK30 or an Ig fraction of the polyclonal FV antiserum #8806 (Figure 10, C and D). MK30 specifically removed FV-FL from plasma, leaving both FV-short and TFPIα in the immune-absorbed plasma. In contrast, the polyclonal FV antibodies immunodepleted FV-FL and FV-short as well as TFPIα.

Figure 4
TF-induced TGAs of plasma from affected and unaffected family members. Individual thrombin generation curves are shown to the left. Affected family members are represented by solid lines, whereas the unaffected individuals are denoted with dotted lines. The affected individual with the highest thrombin generation was individual IV:17, who, in the Western blotting (Figure 1), demonstrated a weak FV-short band. The thrombin generation was monitored using fluorogenic substrate I-1140, the thrombin concentration given as Δ FU/ml (FU, fluorescence units). The right part of the figure demonstrates the thrombin generation using mixtures of affected and unaffected pooled plasma.

Figure 3
Analysis of the expression of FV-short alternative splicing variant in affected patients. (A) Transcript-specific reverse transcription of 1 μg of RNA was completed using Superscript II RNase H Reverse Transcriptase and FVSshortR primer. cDNA was PCR amplified using Ex Taq Polymerase and FVshortF and FVshortR primers. Lane 1: unaffected liver RNA RT-PCR; lane 2: normal peripheral leukocyte RNA; lanes 3–5: unaffected patient RNA RT-PCR; lanes 6–8: affected patient RNA RT-PCR. The lanes were run on the same gel but were noncontiguous. (B) Quantitative real-time PCR of FV-short transcript was completed using a custom TaqMan assay. Fold difference of FV-short expression in unaffected controls (n = 3) compared with affected patients (n = 3). Fold differences are normalized to 18S expression and relative to corresponding target expression in normal peripheral leukocyte RNA. The 1-tailed t value calculated using the Student’s t test was 0.02.
Plasma from unaffected individuals was also found to contain the TFPI\(^{\alpha}\):FV-short complexes but at much lower concentrations. This was evident when TFPI immunoprecipitates from larger volumes of normal versus affected plasma (5 ml vs. 0.3 ml) were analyzed by immunoblotting for FV and TFPI (Figure 10, E and F). It is noteworthy that the immunoprecipitate from normal plasma also contained FV-FL. According to the Image Lab analysis tool, the FV-short and FV-FL band signals were similar in strength, suggesting TFPI\(^{\alpha}\) in normal plasma to be approximately equally distributed between the 2 isoforms of FV.

TFPI\(^{\alpha}\) binds with higher affinity to FV-short than to full-length FV. The results presented above suggest that TFPI\(^{\alpha}\) binds with higher affinity to FV-short than to FV-FL. To verify this hypothesis, rTFPI\(^{\alpha}\) was incubated with mixtures of rFV-short and FV-FL at different concentrations, including conditions chosen to mimic the situation in unaffected and affected plasma. The mixtures were subjected to immunoprecipitation using the AHTFPI-S antiserum bound to magnetic beads and the supernatants and immunoprecipitates were analyzed by immunoblotting using monoclonal antibodies to FV (AHV-5146) and TFPI (AHTFPI-5138) (Figure 11). rTFPI\(^{\alpha}\) was found to preferentially bind to rFV-short even when FV-FL was present in molar excess, demonstrating that the binding affinity of TFPI\(^{\alpha}\) for FV-short is higher than for FV-FL.

Discussion

Patients affected by the east Texas type bleeding disorder exhibit mild prolongations of their APTT and/or PT, but have bleeding episodes indicative of a moderately severe disease (28). Although their FV levels and activities clinically measure as normal, we provide evidence that the A2440G mutation in the B domain exon 13 of the F5 gene causes the bleeding disorder through an indirect gain-of-function mechanism. In affected individuals, the mutation increases the use of an alternative splice donor site in exon 13 and increases the production of an alternative isoform of FV. This isoform encodes a shortened FV with an in-frame 702 amino acid deletion in the B domain, deemed FV-short. FV-short indirectly inhibits coagulation by forming a high-affinity complex with the coagulation inhibitor TFPI\(^{\alpha}\). As a result of the association with FV-short, TFPI\(^{\alpha}\) concentrations in plasma of affected family members are dramatically increased, presumably due to the retention in circulation of the complex of FV-short and TFPI\(^{\alpha}\). This is the first description, to our knowledge, of an autosomal dominant bleeding disorder associated with a gain-of-function mutation in the F5 gene. Additionally, this is the first known bleeding phenotype resulting from increased plasma levels of TFPI\(^{\alpha}\).

Our extensive evidence demonstrates that FV-short is the result of alternative splicing and not due to aberrant proteolytic cleavage. The FV-short isoform reacted with both the monoclonal antibody AHV-5146, which has its epitope between residues Arg-306 and Arg-506 in the heavy chain of FV (31), and the monoclonal antibody AHV-5112 against the light chain. In contrast, the B domain–specific monoclonal antibody MK30 does not recognize FV-short. HPLC-TOF MS/MS analysis of the partially purified FV-short indicated the presence of peptide sequences found in domains A3 and C1 of FV. Given these findings and the fact that the light chain is only approximately 74 kDa, there are no possible proteolytic cleavages that can account for the loss of approximately 80 kDa of protein observed for FV-short. Instead, our results support the conclusion that a splicing event in exon 13 occurs normally to a low degree in controls and the efficiency of this splicing event is increased by the presence of the A2440G mutation. However, the essentially normal levels of circulating
FV-FL in affected individuals suggest that the mutant allele is only partially spliced at this site. The concentration of circulating FV-short in affected family members is consistently elevated, but the variation among individuals in its levels suggests variation in the splicing efficiency at this site also exists among affected individuals. We were unable to obtain RNA samples on all affected individuals to directly correlate this transcript expression to the protein differences observed in their plasma. FV-short was not observed in direct plasma immunoblots of unaffected individuals, but was observed in partially purified FV extracts of plasma (Supplemental Figure 3A), suggesting that low levels of FV-short are present in normal individuals. Moreover, immunoprecipitation of larger volumes of unaffected plasma as compared with affected individuals to directly correlate this transcript expression to the protein differences observed in their plasma. FV-short was not observed in direct plasma immunoblots of unaffected individuals, but was observed in partially purified FV extracts of plasma (Supplemental Figure 3A), suggesting that low levels of FV-short are present in normal individuals.

TFPIα, in normal plasma is reported to interact with FV, and depletion of FV results in an 80% decrease in TFPIα levels (24). The affinity between normal FV and TFPIα was suggested to be relatively low, and half-maximum binding of FV to immobilized TFPIα in surface plasmon resonance analysis was observed at 13.5 nM (24). Under normal conditions, the plasma concentration of FV (≈20 nM) is approximately 50- to 100-fold higher than the plasma concentration of TFPIα (≈0.2 nM), suggesting that only a minor fraction of circulating FV-FL carries a TFPIα molecule. We now demonstrate that low levels of FV-short in normal plasma also bind TFPIα, but the normal distribution of TFPIα between the 2 FV isoforms remains to be elucidated. The situation in affected family members dramatically differs with the presence of much higher concentrations of the FV-short isoform, all of which circulate in complex with TFPIα, as judged by immunoprecipitation. Likewise, most or all of TFPIα in affected plasma is associated with the FV-short form. We currently have no direct, specific method to accurately determine the concentration of FV-short, but we estimated the mean FV-short concentration to be 4.8 nM in affected individuals using 2 ELISAs, one determining the total FV and the other FV-FL, with the difference attributed to the presence of FV-short. The total TFPIα concentration in affected plasma was measured to be 5.3 nM and in unaffected plasma 1.4 nM. The 2 ELISA assays for TFPIα isoforms using detecting monoclonal antibodies either against Kunitz 3 or the C terminus yielded 2.5 nM and 1.25 nM TFPIα in affected plasma, respectively. Corresponding values in unaffected plasma were 0.36 and 0.14 nM. The difference between the 2 TFPIα assays is compatible with the observation that approximately half of TFPIα in plasma is truncated in the C terminus (25). From these numbers it is difficult to accurately estimate the stoichiometry between FV-short and TFPIα in affected plasma, but the results are compatible with a 1:1 stoichiometry. Our results also suggest that the affinity of the TFPIα:FV-short interaction in affected plasma is considerably higher than that between FV-FL and TFPIα and that TFPIα preferentially binds to the FV-short isoform despite a molar excess of FV-FL. This hypothesis gained experimental support when recombinant FV-short and FV-FL were allowed to compete for rTFPIα, and even when FV-FL was present at molar excess, TFPIα preferentially bound FV-short over FV-FL. This taken together with the presence of much higher concentrations of the FV-short isoform, all of which circulate in complex with TFPIα, as judged by immunoprecipitation. Likewise, most or all of TFPIα in affected plasma is associated with the FV-short form.

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Figure 6
TGA after factor V depletion/reconstitution. (A) TGAs of unaffected (solid line) and affected patient samples (dashed line). Unaffected (B) and affected plasma (C) were subjected to FV immunodepletion using a polyclonal antibody against FV (#8806) that was covalently coupled to a 1-ml HiTrap column. Due to the FV depletion, thrombin generation was completely abolished (dashed lines in B and C). Upon the addition of 2 nM recombinant FV-FL or FV-short, both plasmas exhibited similar, normal thrombin generation.

Figure 7
Inhibitor remaining after depletion of FV-FL. Pooled plasma was subjected to immunodepletion using a 1-ml HiTrap column with MK30, which specifically depleted FV-FL, leaving FV-short in the supernatant (see Figure 10). The immunodepleted plasma was subjected to TGA, either alone or after mixing with unaffected plasma.
ence of TFPI:α-FV-short complexes in normal plasma supports the conclusion that FV-short binds with higher affinity than FV-FL, as the concentration of FV-short in normal plasma is approximately 20-fold lower than that of FV-FL.

Ndowmi et al. (27) recently reported that the binding site in TFPI:α to both FV and FVa is located in the last 25 C-terminal amino acids of the protein, a region that contains a highly positively charged cluster of lysines and arginines. This fact taken together with the ionic nature of the interaction suggests that the binding site for TFPI:α in FV/FVa should be a highly negatively charged region. There are several such areas in FV/FVa, including the region upstream of the thrombin cleavage sites at positions 709 and 1545. Further studies will determine the details of the TFPI:α binding to normal FV/FVa and to FV-short and address the question as to why TFPI:α preferentially binds the FV-short isoform rather than the FV-FL. A recent paper by Bos and Camire (32) reported that 2 regions in the B domain are important to keep FV in a procofactor state — one being positively charged (963–1008) and the other, close to the 1545 thrombin-cleavage site (1493–1537), being negatively charged. Deletions in recombinant FV of either the basic or the acidic cluster yielded FV in an active state. According to the hypothesis proposed by Bos and Camire, the 2 regions bind to each other in intact FV and thus maintain FV in its procofactor form. This potentially unbalanced acidic region (1493–1537) in the B domain of FV-short may be involved in creating a high affinity binding site for TFPI:α.

Figure 8
High TFPI concentrations in plasma of affected family members. Immunoblot analysis of plasma (equivalent to 0.5 μl) from affected and unaffected family members, with TFPI being detected using the monoclonal AHTFPI-5138 against Kunitz 1 in combination with HRP-conjugated goat anti-mouse antiserum and developed with chemiluminescence. The SDS-PAGE (4%–15%) was run under nonreducing conditions. Lanes marked with plus signs represent affected individuals carrying the A2440G mutation, whereas those marked with minus signs represent unaffected family members. The arrows point at the approximately 40-kDa TFPI:α band. The 2 lanes in the upper row that are marked with asterisks represent pools of unaffected (−) and affected (+) family members. The 4 right lanes in the upper row were derived from an immunoblot separate from those shown on the 8 lanes to the left. Note the weak TFPI band in the seventh sample from the left in the upper row, the sample being derived from individual IV:17 in family pedigree (Supplemental Figure 1).

TFPI:α is a relatively low molecular weight protein (∼40 kDa) and unless bound to a carrier would be eliminated by filtration in the kidneys. Presumably the binding of TFPI:α to FV-short in affected family members’ plasma results in its retention in circulation, thus causing the higher TFPI:α plasma concentrations. Even though the concentration of TFPI:α in plasma is normally very low, significant amounts are present in the vasculature associated with the endothelium (25, 26). Infusion of heparin releases part of the TFPI:α from the endothelium to the circulation. Possibly, the FV-short

Figure 9
Normalization of thrombin generation in affected plasma by anti-TFPI and effects of adding TFPI:α to unaffected plasma. (A) Polyclonal antibodies against TFPI (40 μl; final concentration of 100 μg/ml) or 40 μl HNBSA buffer control were mixed with 40 μl pooled plasma from affected or unaffected family members, and after 5 minutes incubation at 37°C, the TGA was started by addition of substrate (20 μl) and TF/PL/Ca2+ mixture (20 μl). (B) Increasing concentrations of rTFPI:α (in 8 μl) were added to unaffected plasma (72 μl), yielding the indicated final concentrations, and tested in the TGA. A dose-dependent prolongation of the lag phase was observed, the highest TFPI concentration also decreasing the thrombin peak.
The use of high amounts of TF in the routine tests, which generated plasma made in the assays, and this dilution in combination with complexes. Presumably, this is the result of the dilutions of patient plasma of TFPI α by FV-short, could explain formation competes with the endothelial binding, which together with retention in the circulation of TFPIα by FV-short, could explain the increased plasma concentrations of TFPIα.

It is noteworthy that the clinical levels and activities of FV and the other coagulation factors in our patients measured as normal despite the high concentrations of the inhibitory FV-short–TFPI complexes. Presumably, this is the result of the dilutions of patient plasma made in the assays, and this dilution in combination with the use of high amounts of TF in the routine tests, which generates high concentrations of FVIIa and FXa, overcomes the inhibitory activity of the TFPIα. In contrast, the TGAs were performed at low plasma dilution and in the presence of small amounts of TF and were therefore directly influenced by the TFPIα-FV-short complexes in affected individuals. The prolongation of the APTT observed in affected individuals suggests that the TFPIα-FV-short complexes are also efficient in inhibiting FXa when generated by the intrinsic pathway. In agreement with this, we found that an intrinsic pathway–specific TGA was also pathological when affected family members, control liver (Ambion), or leukocyte RNA (Clontech) was performed using an oligo-dt or F5 gene-specific primer, F5ShortR, (5'-TGGAGAGGTTGATGTGTCGATG-3') with Superscript II RNase H Reverse Transcriptase (Invitrogen) in a total volume of 100 μl according to the manufacturer’s instructions. Exon 13 splice variants were amplified using the primers F5e13F (5'-CATGGGAGGAATCT-GTGACGGT-3') and F5e13R (5'-GTGCTGAGGTTGGTTACTCGC-3') in a 20-μl reaction containing 2 μl of oligo-dt reverse-transcribed RNA, 0.025 U/μl Ex Taq Polymerase (Takara), 1 μl PCR buffer, and 0.15 mM dNTPs (Invitrogen). The PCR was initiated at 94°C for 2 minutes, amplified with 35 cycles of 30 seconds at 94°C, 45 seconds at 55°C, and 7 minutes at 68°C, and contained a final elongation for 10 minutes at 68°C.

The FV-short splice variant was specifically targeted for amplification using 0.25 μM of the aforementioned reverse primer FVshortR and a for-
ward primer, FVShorF, with sequence 5′-CGGAAAATGCATGATCGTT-3′ in the same 20-μl PCR reaction mentioned above, with the exception of the use of 2 μl of F3 gene-specific reverse-transcribed RNA in lieu of the oligo-dT cDNA. The PCR was initiated at 94°C for 1 minute, amplified with 35 cycles of 10 seconds at 94°C, 20 seconds at 55°C, and 20 seconds at 72°C, and contained a final elongation for 2 minutes at 72°C. PCR products were sequenced using BigDye Terminator v3.1 chemistry and ABI 3130xl Genetic Analyzer (Applied Biosystems).

For real-time PCR, reverse transcription of 1 μg of affected (n = 3) and unaffected (n = 3) patients and control peripheral leukocyte RNA in a reaction volume of 100 μl was performed using each transcript-specific primer: total F5′-CAAGAGTATTAGTCTCTAGGC-3′, eukaryotic 18S ribosomal RNA 5′-CCTAGCTGCGGTATCCAGGC-3′, and FV-short 5′-TGAGGAGTTGATGTTTGTCC-3′. Real-time RT-PCR analysis of reverse transcripts was performed with the ABI Prism Sequence Detection System 7700 using TaqMan Reaction Master Mix v1.5 and ABI 3130xl Genetic Analyzer (Applied Biosystems). In addition, a custom TaqMan assay for total human F5, which spans the exon 12/13 splice junction, and His99999901_s1 for eukaryotic 18S ribosomal RNA (Applied Biosystems). In addition, a custom TaqMan assay for FV-short splicing transcript was produced with the following specifica-

**Figure 11**

TFPIα preferentially binds to FV-short. Mixtures of rTFPIα (0.5 or 5 nM) and rFV-short (0.5 or 5 nM) ± plasma-derived FV (5 or 20 nM) were incubated overnight in a total volume of 100 μl and then subjected to immunoprecipitation with Streptavidin-coated magnetic beads (300 μl) carrying biotinylated polyclonal antibodies against TFPI (AHTFPI-S). The bead pellets were eluted with 33 μl sample preparation buffer, 10 μl being loaded to the gel. The pellets, the start mixes, and the supernatants (1 μl each) were analyzed by immunoblotting for (A) FV (AHV-5146) and (B) TFPI (AHTFPI-5138).
NaCl, pH 7.5, containing 1% BSA (HNSBSA), and dissolved in sample preparation buffer, 300 μl for affected and 100 μl for unaffected. The start plasma, the supernatants, and the eluted immunoprecipitates were analyzed by immunoblotting for FV and TFPIα (Figure 10).

Generation of rTFPIα-FV-short complexes. Complexes between rTFPIα and FV-short were formed by incubating the 2 proteins at equimolar concentrations (5 nM) in HNBSA containing 2 mM CaCl2 (HNSBSA-Ca) overnight in the refrigerator. To investigate whether TFPIα preferred FV-short over FV-FL, TFPIα (0.5 or 5 nM) was incubated with mixtures of FV-short (0.5 or 5 nM) and FV-FL (5 or 20 nM) and then subjected to immunoprecipitation using AHTFPIα beads, as described above. The start mix, the supernatants, and the immunoprecipitates were analyzed by immunoblotting for FV and TFPIα (Figure 11).

Determination of FV and TFPI variants by ELISA. B. domain-containing full-length FV was determined with an ELISA using polyclonal rabbit anti-human FV (#8806) as catcher and the monoclonal domain antibody MK30 as detector (41). To measure total FV (full-length plus FV-short), MK30 was replaced by an antibody against the light chain of FV (HV1; Sigma-Aldrich). The concentration of total FV in plasma was determined with an ELISA in which a polyclonal TFPI antibody (PAHTFPI-S, Haematologic Technologies Inc.) was used as catcher and a monoclonal anti-TFPI antibody against the N terminus (AHTFPI-S138; Haematologic Technologies Inc.) as detector. To measure TFPIα, 2 different ELISAs were used. The first used a polyclonal TFPI antibody (AHTFPI-S) as catcher and a monoclonal antibody against the third Kuniz domain (MBS3325091 clone M105273 from Mybiosource) as detector. The assays were standardized with purified recombinant full-length TFPIα, provided by T. Hamuro (Chemo-Sero-Therapeutic Research Institute, Kaketsukun, Kumamoto, Japan) (42). The second TFPIα ELISA was Asserachrom Free TFPI (Stago) in which a monoclonal antibody (2C6) was used as catcher and second monoclonal antibody against the C terminus (HGS) as detector.

Statistics. To analyze results of the QRT-PCR, the 1-tailed Student’s t test was used. P < 0.05 was considered statistically significant.

Study approval. All participants gave written informed consent. The Institutional Review Board and the Center for Protection of Human Subjects atUTHSC-H approved this study (MS-02-157).

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30. Camire RM, Bix MH. The molecular basis of fac-


