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FUNCTIONAL INTERACTIONS BETWEEN FACTOR V AND TFPIα DURING ONSET OF BLOOD COAGULATION

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ISBN 978-94-6375-318-0

Printed by Ridderprint
Cover design and layout: © evelienjagtman.com

FUNCTIONAL INTERACTIONS BETWEEN FACTOR V AND TFPIα DURING ONSET OF BLOOD COAGULATION

Proefschrift

Ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. Dr. Rianne M. Letschert,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen op
woensdag 1 mei 2019 om 16:00 uur

door

Peter van Doorn
Geboren op 9 december 1987 te Helmond

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The work described in this thesis was supported by the Dutch Thrombosis Foundation (grant nr. 2014-1)

Financial support by Stichting Hart Onderzoek Nederland (www.hartonderzoek.nu), is gratefully acknowledged

Financial support by the Dutch Heart Foundation for the publication of this thesis is gratefully acknowledged

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CHAPTER 1

General introduction

INTRODUCTION

Haemostasis consists of a series of processes that maintain the fluidity of the blood, repair damaged blood vessels and eventually remove blood clots once these vessels have been repaired [1]. A disruption of these processes by genetic or acquired factors can cause thrombosis or bleeding.

Two important events in haemostasis are the formation of a plug by activated platelets (primary haemostasis) and the subsequent stabilisation of this plug by fibrin (secondary haemostasis). Primary haemostasis starts after a blood vessel has been damaged. Platelets adhere to the exposed sub-endothelium, aggregate and secrete coagulation factors and other substances that promote further platelet activation. Activated platelets also provide a surface of negatively charged phospholipids for coagulation reactions. Secondary hemostasis starts with tissue factor (TF), a transmembrane protein which is exposed at the site of injury and triggers the coagulation cascade. Coagulation reactions lead to the formation of thrombin, a serine protease that converts fibrinogen to fibrin. The processes involved in secondary haemostasis are the most relevant to this thesis and are discussed in more detail below.

COAGULATION

Blood coagulation is a protective mechanism which limits blood loss after injury. The process involves proteins, known as coagulation factors, which are produced by hepatocytes and secreted in plasma as inactive precursors. After damage of a blood vessel, a series of intricate reactions is initiated to activate the coagulation enzymes and their non-enzymatic cofactors to eventually form a fibrin clot and stop the bleeding.

Initiation of the coagulation cascade can occur via two pathways, the extrinsic and intrinsic pathway, as schematically shown in **Figure 1**. The extrinsic pathway is initiated when TF becomes exposed after vessel damage. Trace amounts of circulating activated factor VII (FVIIa) form a membrane-bound complex with the exposed TF and activate small amounts of factor X (FX) to FXa and factor IX (FIX) to FIXa. FXa is required for the initial activation of factor V (FV) [2] to activated FV (FVa) (more on this under the heading "FV"). FXa and FVa then form the prothrombinase complex, which efficiently converts prothrombin to thrombin. Thrombin activates additional FV, further amplifying prothrombinase activity. Thrombin also activates coagulation factors belonging to the intrinsic pathway, such as factor XI (FXI) and VIII (FVIII). Activated FXI (FXIa) converts FIX to FIXa, which forms a complex with activated FVIII (FVIIIa) to form the tenase complex. The intrinsic pathway then merges with the extrinsic pathway into the common pathway with the activation of FX to FXa by the tenase complex [1].

Additionally, the intrinsic pathway can also be activated via contact activation. Contact activation occurs when blood is exposed to physiological or artificial surfaces that promote the (auto)activation of factor XII (FXII), such as extracellular nucleic acids, protein aggregates or polyphosphates. FXIIa activates prekallikrein to kallikrein, accelerating the formation of more FXIIa. FXIIa then activates the intrinsic pathway by conversion of FXI to FXIa [3].

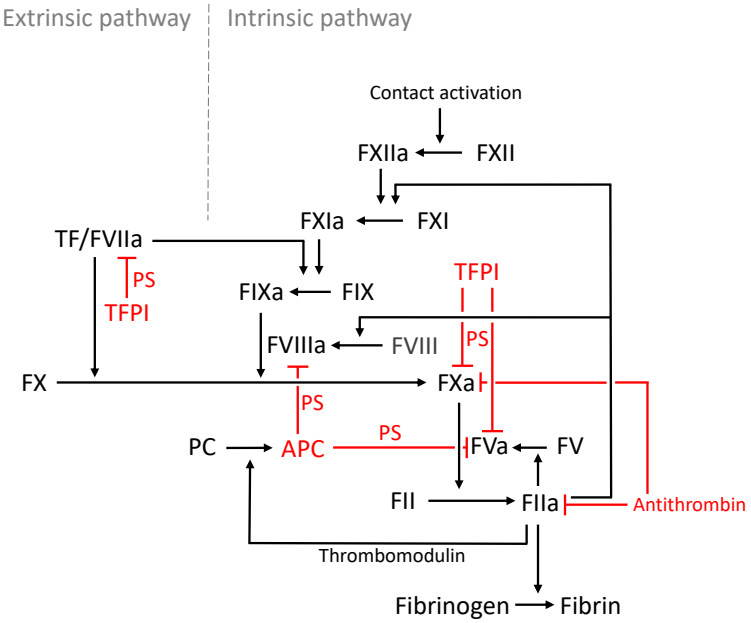

Figure 1. Schematic representation of the coagulation cascade. The intrinsic pathway is shown on the left starting at the TF/FVIIa complex. The intrinsic pathway starting with contact activation is shown on the right side. Both pathways converge at FXa in to the common pathway. Activation of coagulation factors is shown with black arrows and inhibition with red bars. The cofactors FVIIIa, FVa, Protein S (PS) and thrombomodulin are indicated next to the arrow of their corresponding activation/inhibition. FII, prothrombin; FIIa, thrombin; TF, tissue factor; PC, protein C; APC, activated protein C

ANTICOAGULATION

Besides coagulation factors, anticoagulant proteins are equally important in the regulation of blood coagulation. These inhibitors are either constitutively active or become activated during coagulation. They prevent excessive clot formation by inhibiting the coagulation cascade at multiple levels (**Figure 1**). The main anticoagulant factors/mechanisms are antithrombin, the protein C pathway and tissue factor pathway inhibitor (TFPI).

Antithrombin

Antithrombin is a liver-derived serpin that can inhibit several coagulation serine proteases, including FVIIa, FIXa, FXa, FXIa, FXIIa and thrombin. However its predominant inhibitory activity is directed towards thrombin and FXa. This inhibitory activity is greatly increased by heparin [4].

Protein C pathway

Protein C is the zymogen of a serine-protease that is activated by thrombin bound to thrombomodulin on endothelial membranes. Activated protein C (APC) regulates clot formation by proteolytic inactivation of the essential cofactors FVa and FVIIIa. The anticoagulant activity of APC is increased by protein S and FV, which serve as cofactors to APC. In particular, the combination of Protein S and FV is needed for the optimal inactivation of FVIIIa [5].

TFPI

TFPI is a Kunitz-type protease inhibitor that inhibits the early stages of coagulation. Two main splicing isoforms of TFPI exist in humans: TFPIα and TFPIβ. TFPIα, present in/on endothelial cells, plasma and platelets, comprises an acidic N-terminus, 3 Kunitz domains and a basic C-terminus [6]. The first Kunitz domain inhibits the TF/FVIIa complex and the second Kunitz domain inhibits FXa. The third Kunitz domain binds protein S, which serves as a cofactor of TFPIα in the inhibition of FXa and as a carrier of TFPIα in the circulation [7]. Finally, the basic C-terminus of TFPIα binds the acidic region in the B-domain of coagulation FV [8, 9]. TFPIβ has the same Kunitz 1 and 2 domains as TFPIα, whereas the Kunitz 3 domain and C-terminus are replaced by an alternative C-terminus. The C-terminus of TFPIβ consists of a glycosylphosphatidyl inositol (GPI)-anchor that tethers TFPIβ to the surface of endothelial cells [10]. Throughout this thesis only TFPIα is discussed.

TFPIα is expressed by microvascular endothelial cells and megakaryocytes and circulates in plasma at a concentration of ~1.6 nM in normal individuals [11]. The plasma TFPI pool consists of full length TFPIα (~20%) and C-terminally truncated TFPIα (~80%), which lacks a part of the K3 domain and/or the C-terminal region and is bound to plasma lipoproteins [12]. 5-10% of total TFPIα is also stored in platelets, which release it at sites of injury during platelet aggregation [11]. TFPIα inhibits FVIIa and FXa via the formation of a quaternary TF-FVIIa-FXa-TFPIα complex. This complex is formed after a primary, slow, tight-binding interaction of TFPIα with FXa. The secondary interaction with FVIIa-TF is very fast. While the formation of the complex with FXa is slow, the affinity of TFPIα for FXa can be increased 10-fold by protein S in the presence of phospholipids [13, 14]. Therefore, protein S is a cofactor to TFPIα in the inhibition of FXa and acts by promoting the binding of TFPIα to negatively charged phospholipids, thereby bringing TFPIα closer to its target FXa [13, 15-17]. Experiments in model systems have shown that FV also acts as a cofactor to TFPIα in a similar fashion as protein S [18-20].

Besides the inhibition of serine proteases through its Kunitz domains, TFPIα also inhibits FV *via* its basic C-terminus [9]. The interaction of FV with TFPIα and its consequences are discussed in more detail under the heading "FV and TFPI".

COAGULATION FACTOR V

FV, present in plasma (80%) and platelets (20%) [21], plays an important regulatory role in the maintenance of the haemostatic balance due to its pro- and anticoagulant functions. When FV is activated, it serves as the procoagulant cofactor of FXa in the prothrombinase complex, which converts prothrombin to thrombin. Before it is (fully) activated, FV can also serve as an anticoagulant cofactor of activated protein C (APC) in the inhibition of FVIIIa [22] or as a cofactor of TFPIα in the inhibition of FXa [18-20]. Both anticoagulant reactions are also stimulated by protein S [13, 15, 23].

Structure, activation and inactivation

FV is encoded by the *F5* gene, which extends for ~80 kb on the long arm of chromosome 1 [24, 25]. The *F5* mRNA [26] encodes a mature protein of 2196 amino acids, which is heavily modified at the post-translational level. FV is secreted by hepatocytes as a 330-kDa single-chain multi-domain (A1-A2-B-A3-C1-C2) glycoprotein circulating in plasma at a concentration of ~23 nM. The B-domain of FV has a basic region (residues 963-1008) and an acidic region (residues 1493-1537), both of which are evolutionarily highly conserved. These regions engage in a high-affinity interaction, keeping FV in the inactive procofactor state [27-29]. Removal of the B-domain is required for the activation of FV. Activated FV (FVa) consists of a heavy chain (A1-A2) and light chain (A2-C1-C2) which are held together by a Ca^{2+} ion. FVa is a potent procoagulant cofactor that assembles with FXa on a phosphatidylserine-exposing surface to form the prothrombinase complex and accelerates the conversion of prothrombin to thrombin over a 1000-fold [30].

Activation of FV occurs through limited proteolysis at Arg^{709}, Arg^{1018} and Arg^{1545} by FXa [31, 32] or thrombin [33, 34]. These cleavages gradually increase the affinity of FV(a) for FXa and its prothrombinase cofactor activity. Full procoagulant activity is only achieved after cleavage at Arg^{1545}, which drastically increases the affinity of FVa for FXa and simultaneously abolishes all anticoagulant properties of FV [33, 35] (**Figure 2**). This final cleavage is catalysed more efficiently by thrombin than by FXa [31].

FVa activity is regulated by APC, which cleaves the FVa heavy chain at Arg^{306}, Arg^{506} and Arg^{679} [36]. Inactivation of FVa by APC occurs via a biphasic reaction that consists of a rapid and slow phase. The rapid phase consists of cleavage at Arg^{506}, resulting in FVa with 40% residual cofactor activity to FXa [37]. Cofactor activity is completely removed in the slow phase, where FVa is

cleaved at Arg^{306}. The contribution of cleavage at Arg^{679} to FVa inactivation is normally negligible, but plays a role in the absence of phospholipids. Arg^{679} is then cleaved at a higher rate than Arg^{306} and partially inactivates FVa [38]. A common prothrombotic mutation that interferes with the inactivation of FVa is the FV Leiden (FVL) mutation, which replaces Arg^{506} with a Gln [39]. This mutation removes the Arg^{506} APC cleavage site on FVa, making FVaL more resistant to APC-mediated inactivation [38, 40] and producing a characteristic plasma phenotype known as APC resistance [41]. Since cleavage at Arg^{506} is also required for the cofactor activity of FV to APC in the inactivation of FVIIIa [42], FVL lacks APC-cofactor activity, which adds to the prothrombotic phenotype of the mutation [43].

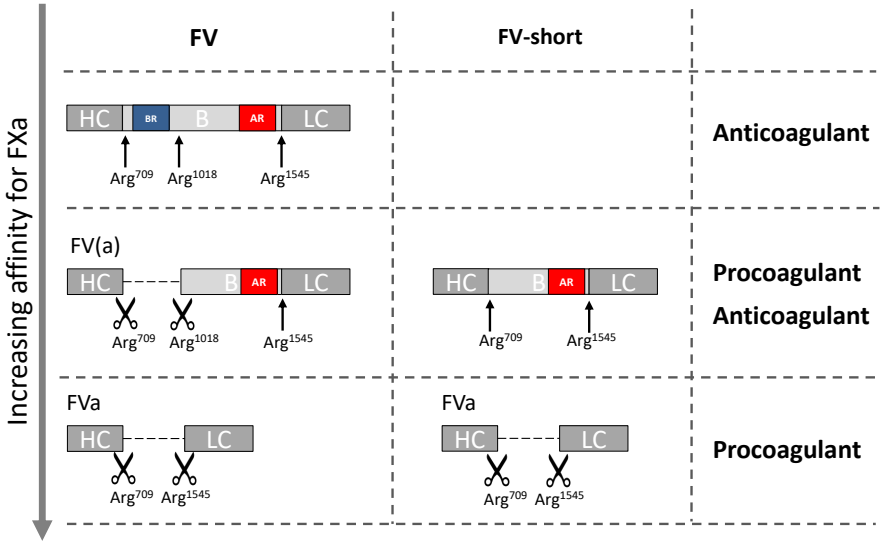

Figure 2. Schematic overview of the activation of FV to FVa. Intact FV consists of a heavy (HC) and light chain (LC) attached to an intact B-domain with a basic (BR) and acidic region (AR). This form of FV lacks all procoagulant activity and only has anticoagulant properties. Procoagulant properties require activation of FV which start at Arg^{709} and Arg^{1018}, gradually removing the B-domain to form FV(a). FV(a) has an increased affinity to FXa but maintains anticoagulant properties due to the presence of the AR. Considerably higher procoagulant activity is reached after the final cleavage at Arg^{1545} which removes the AR and forms FVa.

The plasma FV pool is very heterogeneous, due to alternative splicing of the *F5* pre-mRNA and variable post-translational modification of the protein. Alternative splicing within *F5* exon 13 generates the so-called FV-short isoform [44], which makes up ~5% of plasma FV in normal individuals (calculated from data presented in Vincent *et al.* [44]). FV-short lacks a large portion of the B-domain (residues 756-1458), including the basic region, and hence binds the C-terminus of

TFPIα with higher affinity than full-length FV. FV-short was originally discovered in a family with unexplained bleeding (East Texas bleeding disorder) [44, 45]. Affected members of this family carry a *F5* gene mutation (A2440G) that enhances the FV-short splicing event. Since FV-short is the main carrier of TFPIα in plasma, up-regulation of FV-short leads to a 10-fold increase in plasma TFPIα, in turn resulting in a bleeding phenotype [44]. A similar mutation, FV Amsterdam (C2678G), has been identified in a Dutch family [46]. Like the FV East Texas mutation, FV Amsterdam causes the up-regulation of a similar splicing isoform of FV, FV-short$_{Amsterdam}$, lacking part of the B-domain (residues 834-1458) and binding TFPIα with increased affinity. However, unlike FV-short$_{East\text{-}Texas}$, the FV-short$_{Amsterdam}$ variant does not occur in normal individuals [46].

FV is also polymorphic with respect to glycosylation at Asn^{2181} in the phospholipid-binding C2 domain: 33% of plasma FV is glycosylated at this position (FV1 isoform), whereas 67% is not (FV2 isoform) [47, 48]. Glycosylation at Asn^{2181} reduces the affinity of FV1 for negatively charged surfaces compared to FV2. Therefore, the activation of FV by FXa, the formation of the prothrombinase complex [47] and the inactivation of FVa by APC [49], all of which are phospholipid-dependent reactions, occur more readily with FV2 than with FV1. Overall, the net result is that FV1 is more thrombogenic than FV2 in the presence of APC [49]. The pathophysiological relevance of glycosylation at Asn^{2181} is illustrated by the common FV R2 haplotype, which predicts several amino acid substitutions in FV. FV R2 carriers have mildly reduced FV levels [50] and an increased FV1/FV2 ratio [51, 52], which results in a mild hypercoagulable state [53].

FV and TFPIα

TFPIα circulates in blood as a complex with FV (and particularly FV-short), which stabilises TFPIα *in vivo*, protecting it from truncation and/or clearance [54]. This interaction is mediated by a basic region in the C-terminus of TFPIα, which has a high homology to the FV basic region of FV and can therefore bind the acidic region in the B-domain of FV [8, 9, 29]. Since the FV basic region competes with TFPIα for the same binding site in the FV acidic region, the preferred targets of TFPIα are FV variants without a basic region, such as partially activated FV, FV-short and platelet FV [55].

In the last 5 years, it has become increasingly clear that the interaction between FV and TFPIα also affects the function of both proteins. In fact, FV acts as a cofactor to TFPIα to enhance the inhibition of FXa [18-20]. This activity of FV has been reported to be phospholipid-dependent [19] and protein S-dependent [20].

On the other hand, it has been shown that TFPIα inhibits prothrombinase complexes assembled with FV(a) species that retain the acidic region [9]. Besides the primary interaction between the basic region in the C-terminus of TFPIα and the acidic region of FV, prothrombinase inhibition

requires an additional secondary interaction between residues Leu^{252}, Ile^{253} and Thr^{255} in the C-terminus of TFPIα and a FXa binding site on the heavy chain of FV(a) [56]. This secondary interaction has been proposed to be disturbed by the Arg^{506}àGln substitution in FVL, making FVL less susceptible to inhibition by TFPIα, which adds to the prothrombotic phenotype of the mutation [56].

The cofactor activity of FV to TFPIα and the ability of TFPIα to inhibit the prothrombinase complex are both abolished by cleavage of FV at Arg^{1545}, which separates the primary TFPIα binding site from the FV(a) light chain [9, 19].

OUTLINE OF THE THESIS

This thesis focusses on the interaction between FV and TFPIα, with the aim to gain a better understanding of the functional consequences of this interaction.

In **Chapter 2** we performed FV titrations of thrombin generation under various assay conditions to demonstrate that FV expresses TFPIα-cofactor activity in plasma. In addition, we compared the TFPIα-cofactor activities of the two glycosylation isoforms of FV (FV1 and FV2, differing for their phospholipid binding affinities) in plasma and in a model system. Finally, we validated our findings in a population of 122 individuals, including 18 heterozygous carriers of the FV R2 haplotype.

In **Chapter 3** we observed that a peptide mimicking the TFPIα C-terminus (TFPIα C-term) inhibits TF- and FXa-triggered thrombin generation in plasma and we followed this finding up by testing the effect of the peptide on FV activation in a model system. In this setting, we also compared FV and FV-short and replicated the results using full-length TFPIα.

Once it was established that TFPIα inhibits both the activation and the prothrombinase activity of FV(a), we hypothesized that inter-individual differences in the susceptibility of FV(a) to inhibition by TFPIα may correlate with the risk of thrombosis or bleeding. Therefore, in **Chapter 4** we developed and validated a prothrombinase-based assay that measures the susceptibility of FV to inhibition by TFPIα using highly diluted plasma as a source of FV. This assay relies on the pre-activation of FV in the absence or presence of TFPIα C-term and on the continuous measurement of prothrombinase activity by means of a chromogenic substrate for thrombin.
During the validation of the TFPIα susceptibility assay described in Chapter 4 we realized that FV and FV-short can be functionally distinguished based on the property that FV needs to be proteolytically activated in order to express prothrombinase activity, whereas FV-short is intrinsically active as a single-chain molecule [28]. Therefore, in **Chapter 5** we introduce

another assay in which prothrombinase activity is measured in highly diluted plasma without pre-activating the plasma FV. Although this project is still in a very early phase of development, we present preliminary evidence that the prothrombinase rates measured with this assay might correlate with the FV-short levels in the test plasmas, for which no specific quantitative assay is yet available.

Chapter 6 concludes this thesis with a general discussion of all presented results and some considerations for future work.

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CHAPTER 2

Factor V has anticoagulant activity in plasma in the presence of TFPIα: difference between FV1 and FV2

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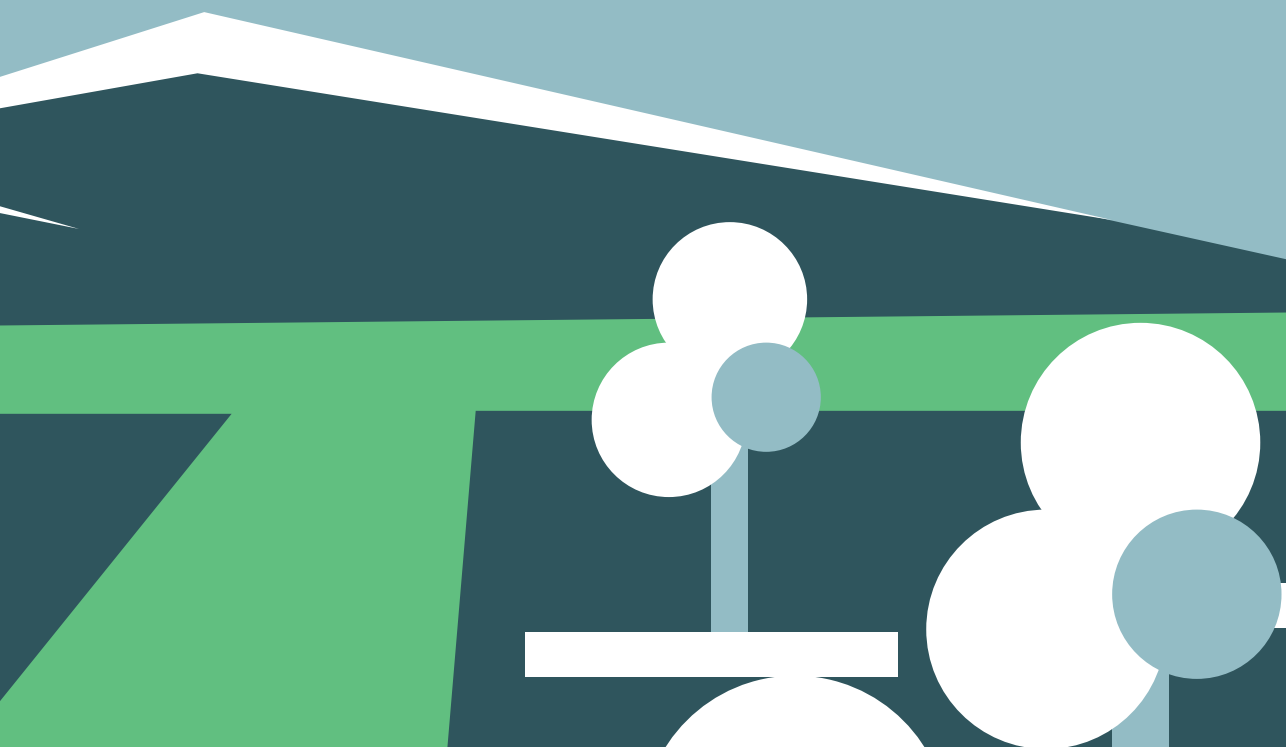
Thromb Haemost. 2018 **118**:1194-1202



ABSTRACT

Background: Activated factor V (FVa) is a potent procoagulant cofactor in the prothrombinase complex, whereas its precursor factor V (FV) stimulates the inhibition of factor Xa (FXa) by tissue factor pathway inhibitor-α (TFPIα), presumably by promoting TFPIα binding to phospholipids. Plasma FV comprises two glycosylation isoforms (FV1 and FV2) with low and high phospholipid binding affinity, respectively. The FV1/FV2 ratio is increased in carriers of the FV R2 haplotype.

Objective: To demonstrate the TFPIα-cofactor function of FV in plasma and to compare FV1 and FV2.

Methods: Thrombin generation at low tissue factor concentration was measured in FV-depleted plasma reconstituted with 0-100% FV, FV1 or FV2, and in 122 individuals genotyped for the R2 haplotype. The TFPIα-cofactor activities of FV1 and FV2 were also investigated in a model system of TFPIα-mediated FXa inhibition.

Results: In the FV titration, thrombin generation first increased (up to 5% FV) and then progressively decreased at higher FV concentrations. This anticoagulant effect of FV, which was also observed with FV2 but not with FV1, was largely abolished by anti-TFPIα antibodies, suggesting that it reflects TFPIα-cofactor activity of FV. In the model system of TFPIα-mediated FXa inhibition, FV2 was a more potent TFPIα-cofactor than FV1, in line with their respective phospholipid affinities. Accordingly, FV R2 carriers had higher thrombin generation than non-carriers, even after correction for demographics and plasma levels of coagulation factors and inhibitors.

Conclusion: FV (and particularly its FV2 isoform) contributes to the TFPIα-dependent down-regulation of thrombin generation in plasma triggered with low TF.

INTRODUCTION

Coagulation factor V (FV) [1] is a liver-derived protein that circulates in plasma as a single-chain multi-domain (A1-A2-B-A3-C1-C2) inactive pro-cofactor. Following limited proteolysis at Arg^{709}, Arg^{1018} and Arg^{1545} by factor Xa (FXa) or thrombin, FV loses the central B-domain and is converted to activated FV (FVa) [2, 3]. FVa is a potent procoagulant cofactor which assembles with FXa on the surface of negatively charged phospholipids to form the prothrombinase complex, thereby increasing the rate of prothrombin activation >1000-fold [4]. In turn, FVa activity is regulated by the anticoagulant enzyme activated protein C (APC) which, together with its cofactor protein S, proteolytically inactivates membrane-bound FVa and the homologous cofactor factor VIIIa (FVIIIa) [5].

While FVa is fully committed to the procoagulant pathway, FV (and/or FV molecular species that retain the C-terminal portion of the B-domain) have anticoagulant properties instead [1, 6]. In fact, (APC-cleaved) FV is known to stimulate the inactivation of FVIIIa by the APC/protein S complex [7, 8]. More recently it has also been reported that FV (and particularly its splicing variant FV-short [9]) acts as a carrier [10] and a cofactor [11-13] of the anticoagulant protein tissue factor pathway inhibitor α (TFPIα) in the inhibition of FXa. These latter functions are mediated by a high-affinity interaction between an acidic region in the B-domain of FV and a basic region in the C-terminus of TFPIα [14, 15].

TFPIα is a multivalent Kunitz-type protease inhibitor that regulates the initiation of coagulation by inhibiting factor VIIa and FXa through its Kunitz-1 and Kunitz-2 domains, respectively [16]. The Kunitz-3 domain of TFPIα binds protein S [17], which has been shown to stimulate the inhibition of FXa by TFPIα both in model systems [18-20] and in plasma [18, 21]. Experiments in model systems have indicated that FV also enhances the TFPIα-mediated inhibition of FXa [11-13], particularly in the presence of protein S [13]. However, the physiological significance of this phenomenon remains unclear, as the cofactor activity of FV for TFPIα has never been demonstrated in plasma. Both protein S and FV are thought to exert their TFPIα-cofactor activities by promoting the binding of TFPIα to negatively charged phospholipids, thereby favouring the encounter between TFPIα and its target enzyme FXa [12].

Plasma FV comprises two isoforms that differ for their glycosylation at Asn^{2181} in the C2 domain: FV1 (~33%) is glycosylated at this position, whereas FV2 (~67%) is not [22, 23]. As a consequence, FV1 has a lower affinity for negatively charged phospholipids than FV2 [22]. This has been shown to interfere with FVa-FXa complex formation [22], FVa inactivation by APC [24] and APC-cofactor activity of FV in FVIIIa inactivation [25], all of which are lipid-dependent reactions. Since the phospholipid requirement is generally higher for anticoagulant than for procoagulant reactions, at suboptimal phospholipids FV1 is 7-fold more thrombogenic than FV2

in model systems incorporating APC [24]. This functional difference between FV1 and FV2 is likely to be physiologically relevant, as carriers of the common FV R2 haplotype [26, 27], who show an increased FV1/FV2 ratio [28, 29] as well as slightly reduced FV levels [26, 30, 31], have a (mild) hypercoagulable phenotype [27, 30].

In this study we observed that thrombin generation at low TF is progressively inhibited at increasing FV concentrations. We demonstrated that this phenomenon reflects the ability of FV to act as cofactor of TFPIα in the inhibition of FXa and further characterized this effect in plasma and in a model system. The effects of FV levels and FV1/FV2 ratio in plasma were also investigated in a group of individuals genotyped for the FV R2 haplotype.

MATERIALS AND METHODS

FV purification

FV, FV1 and FV2 were purified from fresh frozen plasma of healthy donors not carrying the FV Leiden mutation. Purification was performed essentially as described by Nicolaes *et al.* [32] with minor modifications. The full purification protocol is described in the supplementary material.

FV(a)-titrations of thrombin generation

FV-depleted plasma (Siemens Healthcare, Marburg, Germany) was supplemented with 0.4 nM full-length TFPIα (kind gift of Dr. T. Lindhout) and reconstituted with plasma-derived FV, FV1 or FV2 (purified in-house) or FVa (Haematologic Technologies, Essex Junction, VT, USA) to final concentrations ranging between 0% and 100% of the FV concentration in normal pooled plasma (23 nM). As an alternative way of varying FV in plasma, a neutralising anti-FV antibody (AHV-5101, Haematologic Technologies) was titrated in normal plasma. In some experiments reconstituted plasma was incubated for 15 min at 37°C with neutralizing antibodies against protein C (SAPC-IG, 0.32 mg/mL, Affinity Biologicals, Hamilton, Ontario, Canada), protein S (0.5 mg/mL, DAKO, Glostrup, Denmark) or TFPI (equimolar mixture of anti-Kunitz1, anti-Kunitz2, anti-Kunitz3 and anti-C-terminus antibodies, 16 µg/mL Sanquin, Amsterdam, the Netherlands) before activating coagulation.

Thrombin generation was measured by Calibrated Automated Thrombography (CAT) [33], essentially as described before [34]. Briefly, coagulation was initiated with 2 or 10 pM TF (Dade Innovin, Marburg, Germany), 4 µM phospholipid vesicles (DOPS/DOPC/DOPE, 20/60/20, M/M/M) and 16 mM $CaCl_2$, in the presence of 40 µg/mL thermostable inhibitor of contact activation (TICA) [35]. Thrombin activity was followed using fluorogenic substrate Z-Gly-Gly-Arg-AMC (I-1140; Bachem, Bubendorf, Switzerland), which was added to the plasma together with the $CaCl_2$ solution. Fluorescence was read in a Fluoroskan Ascent reader (Thermo

Labsystems, Helsinki, Finland) and thrombin generation curves were calculated with the Thrombinoscope software (Thrombinoscope, Maastricht, the Netherlands). Lag time and peak height were used as the main read-out parameters.

FXa inhibition by TFPIα and its cofactors

The inhibition of FXa by TFPIα and its cofactors protein S and/or FV was studied in a previously established model system [12], in which chromogenic substrate conversion by FXa is monitored in the absence and presence of TFPIα, protein S and/or FV. Reaction mixtures were prepared in HNBSA/Ca^{2+} buffer (25 mM Hepes (pH 7.7 at room temperature), 175 mM NaCl, 3 mM $CaCl_2$ and 5 mg/mL bovine serum albumin) containing 125 μM of the FXa chromogenic substrate CS-11(65) (N-α-Benzyloxycarbonyl-D-arginyl-glycyl-L-arginine-p-nitroaniline-dyhydrochloride) (Hyphen BioMed, Neuville-sur-Oise, France) and 30 μM phospholipid vesicles (DOPS/DOPC/DOPE, 20/60/20, M/M/M) in the absence and presence of 0.5 nM TFPIα, 1 nM protein S (Hyphen) and/or 0-2.5 nM FV1 or FV2. These mixtures were incubated at 37°C for 7 minutes before the addition of 0.1 nM FXa (Enzyme Research Laboratories, South Bend, IN, USA). Substrate conversion was followed in time by reading the absorbance at 405 nm in an Ultra Microplate reader (Bio-Tek, Burlington, VT, USA).

Population study

The study population was selected from a previously described collection of families undergoing screening for the FV Leiden mutation at Padua Academic Hospital (Padua, Italy) [36]. All individuals carrying the FV Leiden mutation and/or using oral contraceptives, hormone-replacement therapy or oral anticoagulant treatment were excluded, leaving 122 individuals without FV Leiden and free of medication available for study. All participants provided informed consent according to the Helsinki Declaration. Carriership of the FV R2 haplotype was ascertained by genotyping haplotype-tagging SNP rs6027 (6755 A/G in exon 25 [28]) using a 5' nuclease (TaqMan®) assay (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). Thrombin generation was measured in platelet-poor plasma triggered with 1.5 pM TF and 4 μM phospholipid vesicles as described above. Plasma levels of FV were available from a previous study [36]. The levels of prothrombin, FX, antithrombin, full-length TFPIα, total protein S and free protein S were measured as previously described [37]. All factor levels were expressed as percentage of the normal plasma concentration. Demographic variables, factor levels and thrombin generation parameters were compared between FV R2 carriers and non-carriers using Student's t-test. The effects of age, sex, venous thromboembolism (VTE), R2 genotype as well as the levels of coagulation factors and inhibitors (independent variables) on the peak height of thrombin generation (dependent variable) were assessed by multiple linear regression analysis. Effects were expressed as unstandardised regression coefficients (B). Statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS, version 24).

RESULTS

Effect of the FV concentration on thrombin generation at low TF

To study the effect of the FV concentration on thrombin generation, purified FV was titrated in FV-depleted plasma from 0% to 100% of the normal plasma concentration (23 nM) and thrombin generation was measured at 2 pM TF. To compensate for the low level of TFPIα in FV-depleted plasma [10], TFPIα was also added to a final concentration of 0.4 nM, which made thrombin generation in reconstituted FV-depleted plasma similar to thrombin generation in normal plasma. No thrombin generation was observed in FV-depleted plasma without added FV. As the FV concentration was increased, the lag time of thrombin generation decreased from 13.5 min at 2.5% FV to 4.17 min at 100% FV (**Figure 1A**). In contrast, the thrombin peak height first increased, reaching a maximum at ~5% of FV, but then gradually decreased at higher FV concentrations (**Figure 1A,B**), suggesting an anticoagulant effect of FV. In fact, the peak height (44.6 nM) at 100% FV was only 33% of the maximal peak height (133.6 nM) observed at 5% FV. A similar pattern was observed when the FV level was varied by titrating a neutralizing anti-FV antibody in normal plasma (**Supplemental Figure 1**). In particular, addition of increasing concentrations of the antibody resulted in an initial increase of thrombin generation, reflecting inhibition of the anticoagulant activity of FV, followed by a progressive decrease of thrombin generation, resulting from inhibition of the procoagulant activity of FV.

To get more insight into the mechanism of this anticoagulant effect of FV, the FV titration was repeated in the presence of neutralizing antibodies against the main anticoagulant proteins that interact with FV: protein C, protein S and TFPIα. Addition of an anti-protein C antibody did not affect thrombin generation at any FV concentration (**Figure 2A**, compare with **Figure 1A**), indicating that no APC is formed in the plasma under these assay conditions. Addition of the anti-protein S (**Figure 2B**) and anti-TFPI antibodies (**Figure 2C**) decreased the lag time and increased the peak height of thrombin generation, indicating that the TFPIα/protein S system does contribute to regulating thrombin formation at this TF concentration. When the peak heights of thrombin generation were expressed as percentage of the maximum peak height (observed at 5% FV) and plotted as a function of FV concentration (**Figure 2D**), the graphs obtained in the absence of antibodies and in the presence of anti-protein C or anti-protein S antibodies were superimposable, showing an initial increase and a later decrease of thrombin generation at increasing FV concentrations. In contrast, the inhibition of thrombin generation at increasing FV concentrations was largely abolished in the presence of anti-TFPIα antibodies, suggesting that the anticoagulant effect of FV observed in the absence of antibodies is TFPIα-dependent, possibly reflecting cofactor activity of FV for TFPIα.

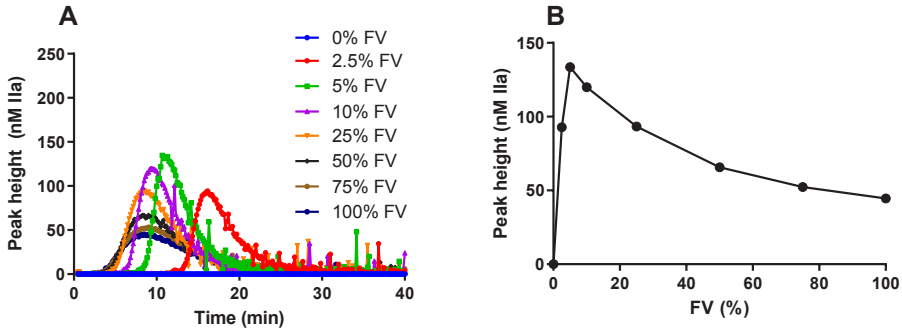


Figure 1. Effect of the FV concentration on thrombin generation at low TF. FV-depleted plasma was supplemented with 0.4 nM TFPIα and reconstituted with 0-100% FV (100% = 23 nM). Thrombin generation was triggered with 2 pM TF (**A**). Peak heights of thrombin generation were plotted as a function of the FV concentration (**B**).

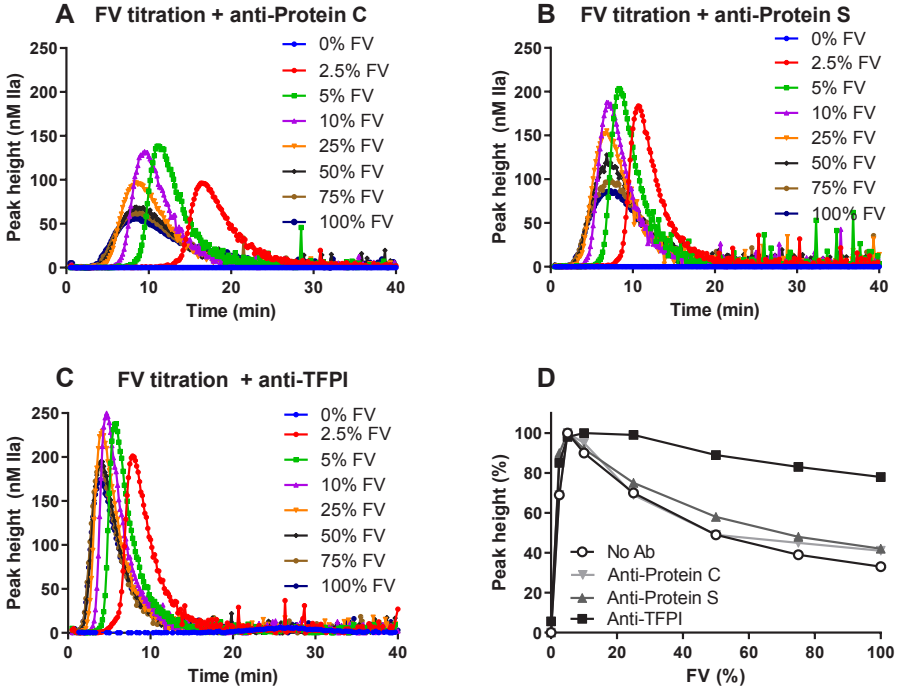


Figure 2. Effect of the FV concentration on thrombin generation at low TF in the presence of different antibodies. FV-depleted plasma was supplemented with 0.4 nM TFPIα and reconstituted with 0-100% FV (100% = 23 nM). Thrombin generation was triggered with 2 pM TF in the presence of antibodies against protein C (**A**), protein S (**B**) or TFPIα (**C**). Peak heights of thrombin generation in the absence of antibodies (taken from Figure 1) and in the presence of the different antibodies were expressed as percentage of the maximal peak height and plotted as a function of the FV concentration (**D**).

To further explore this phenomenon, thrombin generation in FV-depleted plasma reconstituted with different amounts of FV was also measured at higher TF (10 pM), a condition that is much less sensitive to TFPIα action [38]. At this TF concentration, the peak height of thrombin generation reached a maximum (249.6 nM) at 10% FV and progressively decreased at higher FV concentrations, but not as steeply as at low TF (**Figure 3A,C**). In fact, the peak height (174 nM) at 100% FV was still 70% of the maximum peak height, in line with the minor role of TFPIα in regulating thrombin formation under these conditions. Finally, purified FVa was also titrated in thrombin generation at low TF (2 pM). Since FVa lacks the B-domain (and hence the acidic region through which FV binds to TFPIα [15]), it does not function as a cofactor for TFPIα and it even inhibits TFPIα-mediated FXa inhibition in model systems [12, 13]. Titrating FVa between 0% and 100% of the normal FV concentration had little effect on thrombin generation in plasma (**Figure 3B,C**). The peak height reached a maximum (123 nM) at 5% FVa and showed only a mild decrease at higher FVa concentrations up to 100% FV, where the peak height (103 nM) was still 83% of the maximum.

Based on these findings, we concluded that the decrease in the peak height of thrombin generation at increasing FV concentration observed at low TF (**Figure 1**) was largely attributable to the TFPIα-cofactor activity of FV.

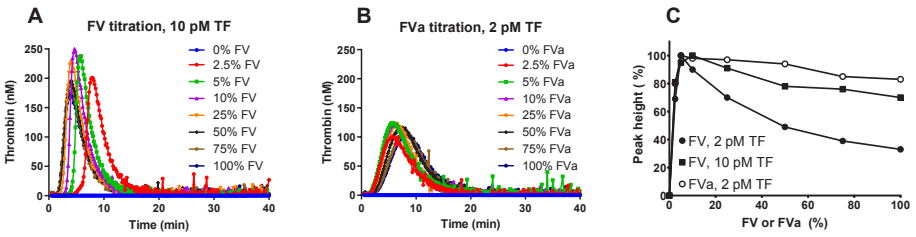


Figure 3. Effect of the FV concentration on thrombin generation at high TF and effect of the FVa concentration on thrombin generation at low TF. FV-depleted plasma was supplemented with 0.4 nM TFPIα and reconstituted with 0-100% FV (**A**) or FVa (**B**). Thrombin generation was triggered with 10 pM TF (**A**) or 2 pM TF (**B**). Peak heights of thrombin generation were expressed as percentage of the maximal peak height and plotted as a function of the FV or FVa concentration (**C**). The peak heights of the FV titration at 2 pM TF (taken from Figure 1) are shown for comparison.

TFPIα-cofactor activities of FV1 and FV2 in plasma

FV has been proposed to stimulate TFPIα by promoting its binding to negatively charged phospholipids [12]. Since FV has two glycosylation isoforms (FV1 and FV2) that differ in their affinities for phospholipids [22], we tested these isoforms for TFPIα-cofactor activity in our plasma-based assay. To this end, FV-depleted plasma was reconstituted with different amounts of purified FV1 or FV2 (corresponding to 0-100% of the total FV concentration in normal plasma) and thrombin generation was measured at 2 pM TF in the absence and presence of anti-TFPIα

antibodies. When FV-depleted plasma was supplemented with FV1, the isoform with low affinity for phospholipids, no thrombin generation was observed at FV1 concentrations ≤ 10% (**Figure 4A**). At higher FV1 concentrations thrombin generation increased steadily, eventually reaching a plateau at 75% FV1. In contrast, the FV2 titration was similar to the titration of total FV: thrombin generation was already measurable at 2.5% FV2, reached a maximum at 10% FV2, and progressively decreased at higher FV2 concentrations (**Figure 4B**). Moreover, lag times were shorter with FV2 than at equal concentrations of FV1, possibly reflecting faster lipid-dependent activation of FV2 by FXa. Plotting the peak heights of thrombin generation as a function of FV concentration (**Figure 4C**) showed that, in the low FV range, more FV(a)1 than FV(a)2 is needed to support thrombin generation, in line with the notion that FVa1 is less efficient than FVa2 in prothrombinase complex formation [22]. However, at higher FV concentrations, enough FVa is generated to saturate prothrombinase and further increases in FV concentration affect only the anticoagulant (TFPIα-cofactor) function of FV. The fact that thrombin generation decreases at increasing concentrations of FV2 but not of FV1 suggests that FV2 expresses TFPIα-cofactor activity, while FV1 does not (at least not in this concentration range).

In the presence of anti-TFPI antibodies, a condition that reflects only FV activation and expression of prothrombinase activity, increasing FV concentrations resulted in progressively shorter lag times and higher peak heights of thrombin generation, both for FV1 (**Figure 4D**) and for FV2 (**Figure 4E**). When peak heights were plotted as a function of FV concentration (**Figure 4F**), both FV isoforms showed qualitatively similar profiles of steadily increasing thrombin generation. Again, in the low FV range, FV(a)2 supported more thrombin generation than FV(a)1, but this difference decreased at higher FV concentrations and eventually disappeared at 100% FV, where FV1 and FV2 yielded virtually identical thrombin peak heights (181 nM *vs.* 187 nM). The decrease in thrombin generation observed between 10% and 100% FV2 in the presence of TFPIα (**Figure 4C**) was completely abolished in the presence of anti-TFPI antibodies (**Figure 4F**).

Effects of FV1 and FV2 on the inhibition of FXa by TFPIα and protein S in a model system

The TFPIα-cofactor activities of FV1 and FV2 were also tested in a previously established model system of FXa inhibition by TFPIα and protein S [12, 13, 18], in which FXa activity is monitored continuously by following the conversion of a chromogenic substrate for FXa (**Figure 5**). FXa (0.1 nM) converts the substrate at a constant rate, which was not affected by the addition of FV1 or FV2 (Supplemental **Figure 2**). The addition of TFPIα (0.5 nM) caused a rapid inhibition of FXa activity, which was enhanced by the simultaneous addition of protein S (1 nM). Both FV1 and FV2 (0-2.5 nM) further enhanced the inhibition of FXa by TFPIα/protein S in a dose-dependent manner, but the stimulation was more pronounced for FV2, confirming that FV2 is a more efficient TFPIα-cofactor than FV1. Moreover, the highest concentration of FV1 and FV2 (2.5 nM) also stimulated the inhibition of FXa by TFPIα alone, although the effect was smaller than in the presence of protein S and similar for FV1 and FV2 (**Supplemental Figure 2**).

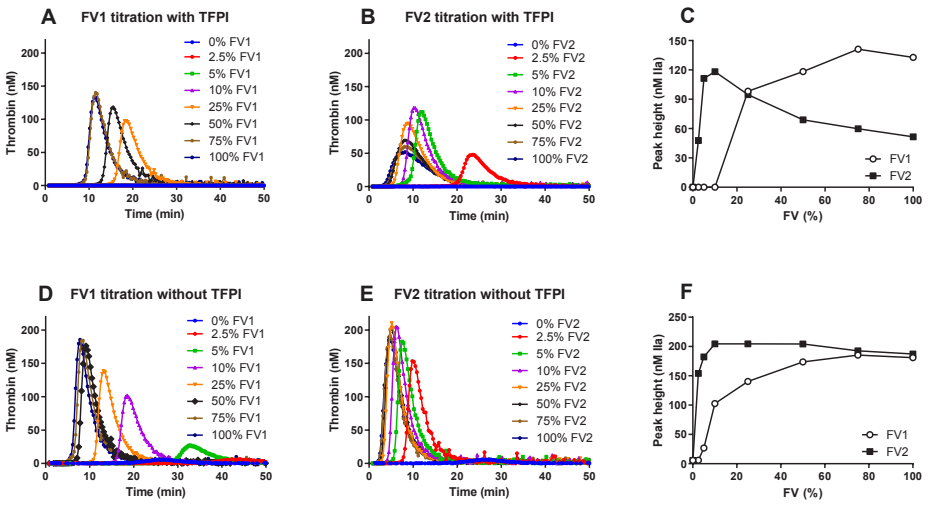


Figure 4. Effects of FV1 and FV2 concentrations on thrombin generation at low TF. FV-depleted plasma was supplemented with 0.4 nM TFPIα and reconstituted with 0-100% FV1 (**A, D**) or FV2 (**B, E**) and thrombin generation was triggered with 2 pM TF in the absence (**A, B**) or presence (**D, E**) of anti-TFPI antibodies. Peak heights were plotted as a function of the FV1 or FV2 concentration in the absence (**C**) and presence (**F**) of anti-TFPI antibodies.

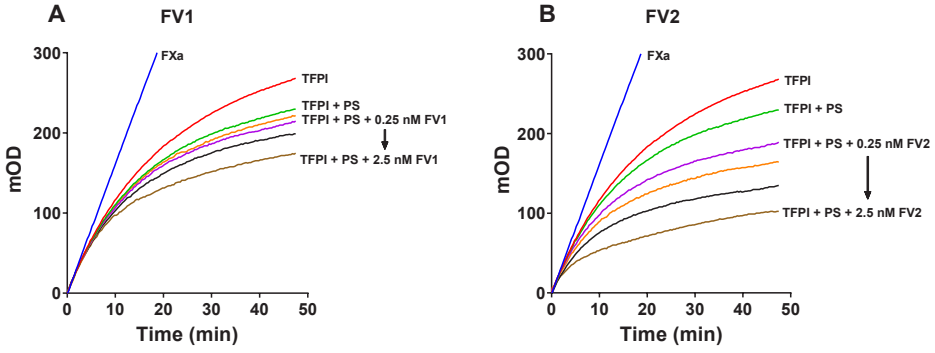


Figure 5. Effects of FV1 and FV2 on the inhibition of FXa by TFPIα and protein S. 0.5 nM TFPIα (or HNBSA/Ca^{2+} buffer, for the FXa only control) was incubated at 37°C with 30 µM phospholipid vesicles (20/60/20 DOPS/DOPC/DOPE) and 125 µM CS-11(65) substrate in the absence and presence of 1 nM PS and 0-2.5 nM FV1 or FV2. Reactions were started with 0.1 nM FXa and the absorbance at 405 nm was followed for 50 min. Averages of quadruplicate (FXa, TFPI and protein S) or duplicate (FV1 and FV2) experiments are shown.

Population study

Carriership of the FV R2 haplotype is associated with mildly reduced FV levels and increased FV1/FV2 ratio [28], both of which predict a reduction of TFPIα-cofactor activity. To verify this, we measured thrombin generation and the plasma levels of relevant coagulation factors and inhibitors (prothrombin, FV, FX, antithrombin, TFPIα and protein S) in a population of 122 individuals, of whom 18 were heterozygous carriers of FV R2 (**Table 1**). Demographic parameters and plasma levels of coagulation proteins were similar between FV R2 carriers and non-carriers, but thrombin generation was higher in FV R2 carriers than in non-carriers (peak height 95.1 nM *vs.* 70.7 nM, p=0.002). In a multiple regression model including age, sex, VTE, R2 genotype and plasma factor levels as independent variables (**Table 2**), both TFPIα levels (B= -3.1 nM IIa/10% TFPIα, p=0.001) and FV levels (B= -3.5 nM IIa/10% FV, p=0.008) were negative determinants of the peak height, whereas FX levels were a positive determinant (B= 7.4 nM IIa/10% FX, p<0.001). However, even after correction for these and all other variables, FV R2 carriership remained a strong positive determinant of peak height (B= 29.7 nM, p<0.001), suggesting that the low FV levels do not fully explain the higher thrombin generation of FV R2 carriers and that the altered FV1/FV2 ratio may also contribute to the hypercoagulable state associated with FV R2.

Table 1. General characteristics of the study population.

	R1R1 (n=104)	**R1R2 (n=18)**	**p-value**
Age (years)	40.2 ± 17.9	38.8 ± 20.2	0.768
Sex (0, 1)	51/53	10/8	0.613
VTE (n)	3	2	0.306
Prothrombin (%)	104.3 ± 14.2	105.3 ± 11.3	0.792
FV (%)	99.2 ± 20.9	91.9 ± 25.4	0.189
FX (%)	105.4 ± 17.7	101.6 ± 16.3	0.401
Antithrombin (%)	101.3 ± 10.7	104.3 ± 8.7	0.276
TFPIα (%)	105.3 ± 32.4	107.3 ± 49.3	0.831
Total protein S (%)	108.4 ± 19.2	108.5 ± 18.8	0.989
Free protein S (%)	107.3 ± 23.5	108.3 ± 21.5	0.859
Peak height (nM IIa)	70.7 ± 29.0	95.1 ± 33.9	0.002

Sex (0 = male, 1 = female); VTE, venous thromboembolism

Table 2. Multiple regression analysis of peak height of thrombin generation at low TF.

	B	p-value
Constant	80.835	0.003
Age (years)	-0.039	0.836
Sex (0, 1)	9.383	0.112
VTE (0 , 1)	-17.925	0.145
R2 genotype (0, 1)	**29.696**	**0.000**
Prothrombin (%)	0.264	0.255
FV (%)	**-0.352**	**0.008**
FX (%)	**0.738**	**0.000**
Antithrombin (%)	-0.415	0.194
TFPIα (%)	**-0.309**	**0.001**
Total protein S (%)	0.137	0.494
Free protein S (%)	-0.218	0.169

Sex (0 = male, 1 = female); VTE, venous thromboembolism (0 = no; 1 = yes); R2 genotype (0 = R1R1; 1 = R1R2)

B, unstandardized regression coefficient. Determinants with a significant p-value are shown in bold.

DISCUSSION

While FV(a) is best known for its procoagulant activity as cofactor of the prothrombinase complex, it also expresses anticoagulant activities as cofactor of the APC/protein S system in the inactivation of FVIIIa [7] and as cofactor of the TFPIα/protein S system in the inhibition of FXa [11-13]. So far, the TFPIα-cofactor activity of FV has been demonstrated only in model systems [11-13] and little is known about its structural requirements, molecular mechanisms and physiological relevance [6]. Here we show for the first time that this anticoagulant activity of FV can be detected in plasma by measuring thrombin generation at low TF concentrations. These assay conditions are known to be particularly sensitive to the anticoagulant action of TFPIα [39] and have been already exploited for the development of plasma-based thrombin generation assays to quantify the activity of the TFPIα/protein S system [21]. When FV-depleted plasma was reconstituted with varying amounts of FV, the peak height of thrombin generation first increased (up to 5% FV), but then progressively decreased to ~33% of its maximum value at 100% FV. The fact that this effect could be reproduced by titrating an anti-FV antibody in normal plasma confirmed that it is a property of FV and not due to a contaminant in the FV preparation used for the FV titration in FV-depleted plasma. The decrease of thrombin generation at increasing FV concentrations could be attributed to (increasing) TFPIα-cofactor activity of FV based on several lines of evidence. First of all, the effect was much more pronounced at low than at high TF, in line with the respective sensitivities of these assay conditions for TFPIα [39]. Moreover,

the effect was abolished by anti-TFPIα antibodies, but not by anti-protein C or anti-protein S antibodies. This ruled out the involvement of the APC-cofactor activity of FV, which also causes thrombin generation to decrease at increasing FV concentrations when APC is present [40]. Finally, the effect was hardly observed with FVa, which lacks the acidic region necessary for interaction with TFPIα [15]. In fact, the minor decrease in thrombin generation at increasing FVa concentrations (which was also observed in the presence of anti-TFPIα antibodies, data not shown) might be attributable to a TFPI-independent anticoagulant effect of FVa described in an earlier report [41].

Our experiments also showed that FV2, the FV glycosylation isoform that has a higher affinity for negatively charged phospholipids [22], is a more efficient TFPIα-cofactor than FV1, both in plasma and in a model system of FXa inhibition. Unless glycosylation at Asn^{2181} interferes with the interaction between FV and TFPIα and/or protein S, this finding corroborates the mechanism that has been proposed to underlie the TFPIα-cofactor activity of FV, *i.e.* increased binding of TFPIα to the phospholipid surface in the presence of FV [12, 18].

Whether or not protein S is required for the expression of the TFPIα-cofactor activity of FV has been a matter of debate. In fact, it has recently been argued that the stimulation of TFPIα by FV alone reported in earlier publications [11, 12] may have been caused by contamination of plasma-derived FV preparations with protein S and/or TFPIα [13]. The in-house purified FV isoforms used in the current study were virtually free of protein S and TFPIα as assessed by ELISA (<0.1% contamination on a molar basis, data not shown), but still expressed some TFPIα-cofactor activity in the absence of protein S. Moreover, the anticoagulant activity (TFPIα-cofactor activity) of FV observed in plasma was not affected by neutralizing antibodies against protein S, suggesting that in plasma FV expresses TFPIα-cofactor activity in the absence of protein S. While these findings suggest that protein S is not necessarily required for the expression of the TFPIα-cofactor activity of FV, we have observed that the protein S requirement varies widely among apparently indistinguishable preparations of plasma-derived FV and is critically dependent on the phospholipids used in the assay. At present, we do not have a good explanation for these differences in protein S requirement for the expression of TFPIα-cofactor activity by FV, but it is possible that reaction conditions (*e.g.* phospholipids) and the presence/generation of partially cleaved FV(a) intermediates with different affinities for TFPIα [6] modulate the protein S requirement of the TFPIα-cofactor activity of FV in model systems and in plasma.

Previous experiments in model systems have shown that, in the presence of APC and suboptimal phospholipids (concentration and composition), FV1 is more thrombogenic than FV2, due to the fact that its lower affinity for phospholipids impairs not only its ability to assemble productive prothrombinase complexes, but also (and even more) its susceptibility to APC-catalysed inactivation [24]. Similarly, the titrations of FV1 and FV2 in FV-depleted plasma reported in the

present study indicate that FV1 supports more thrombin generation than FV2 under conditions that are sensitive to the TFPIα-cofactor activity of FV. In other words, the reduced TFPIα-cofactor activity of FV1 contributes to the higher procoagulant potential of FV1 compared to FV2. This conclusion was also supported by a small population study in which we compared thrombin generation determined at low TF in carriers and non-carriers of the FV R2 haplotype, a common FV allele associated with slightly decreased FV level [26, 30, 31], increased FV1/FV2 ratio [28, 29] and a mild hypercoagulable state [27, 30]. FV R2 carriers had higher thrombin generation than non-carriers, and FV R2 genotype remained a strong (positive) predictor of peak height even after correction for all demographic variables and for the levels of coagulation factors and inhibitors. This suggests that the increased FV1/FV2 ratio (which was not included in the regression model, but for which the FV R2 genotype is a surrogate marker), just as the lower FV level, contributes to increasing thrombin generation in these individuals by decreasing the TFPIα-cofactor activity of FV.

In summary, we have shown that FV contributes to the TFPIα-dependent regulation of thrombin generation in plasma triggered with a low concentration of TF, most likely by stimulating FXa inhibition by TFPIα/protein S [12, 13]. Similar to the APC-cofactor activity of FV [25, 40], the TFPIα-cofactor activity of FV increases throughout the physiological range of FV concentrations, without reaching saturation, and is more pronounced in FV2 than in FV1. Since the procoagulant activity of FV is already saturated at low (~10%) FV, whereas the anticoagulant activities of FV are not, individuals with partial FV deficiency (20-50% FV) tend to have a hypercoagulable state compared to individuals with normal FV levels, which will be further aggravated by the reduction in TFPIα levels that accompanies (partial) FV deficiency [10]. Remarkably, both low FV levels and decreased phospholipid binding capacity of FV (a reflection of the FV2/FV1 ratio) were strongly associated with deep-vein thrombosis in a Japanese study [42]. However, further research is needed to assess the patho-physiological implications of the anticoagulant activities of FV.

ACKNOWLEDGEMENTS

This work was supported by a research grant from the Dutch Thrombosis Foundation (TSN 2014-1 to E. Castoldi). Jan Rosing is Van de Laar Professor of Coagulation Enzymology.

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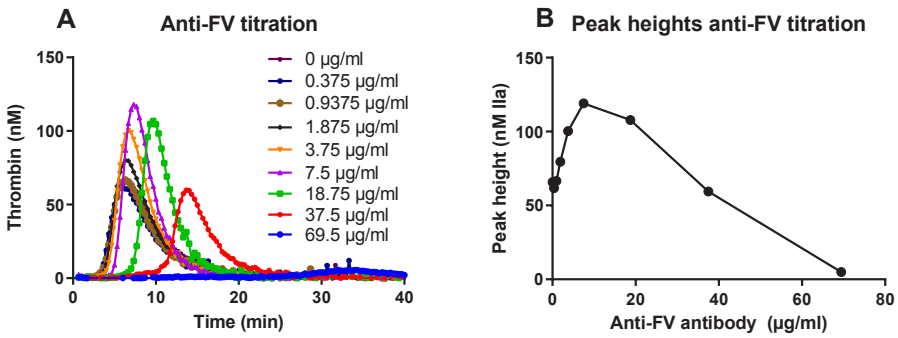
SUPPLEMENT

FV purification

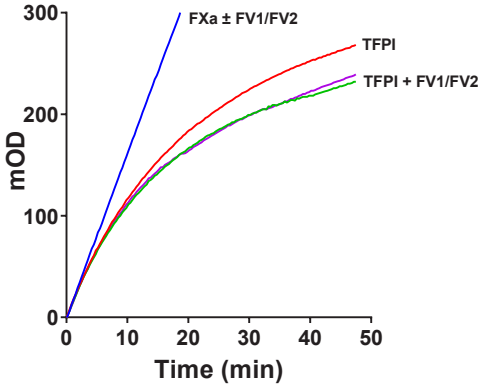
All steps were performed at ~4°C except for the chromatography steps, which were run at room temperature on an AKTA FPLC (GE Healthcare, Uppsala, Sweden). Plasma was thawed and the following inhibitors were added: phenylalanyl-prolyl-arginine chloromethylketone (PPACK) (Calbiochem, La Jolla, Ca, USA) (2 µM), soybean trypsin inhibitor (Sigma-Aldrich, Zwijndrecht, the Netherlands) (50 mg/l) and benzamidine (Sigma-Aldrich) (10 mM). $BaCl_2$ (Acros organics, Geel, Belgium) was added dropwise (80 ml of 1 M solution per liter of plasma). The formed barium citrate pellet was removed by centrifugation at 4500g for 30 minutes. Polyethylene glycol (PEG)-6000 (Merck, Darmstadt, Germany) was added dropwise to a concentration of 7% (w/v) of the remaining supernatant and stirred for 60 minutes. The pellet was removed by centrifugation for 30 minutes at 4500g. PEG-6000 was then added to 13% (w/v) of the supernatant and stirred for 60 minutes. The precipitate was collected by centrifugation at 4500g for 30 minutes. The precipitate was dissolved in 100 ml (per liter plasma starting material) of 50 mM Tris pH 7.5, 10 mM benzamidine and 5 mM $CaCl_2$ (DEAE buffer A). The dissolved precipitate was then centrifuged at 30000g for 15 minutes to remove any debris. The dissolved precipitate was then applied to DEAE-Sepharose (Amersham Pharmacia, Uppsala, Sweden) prepared in a XK-26 Pharmacia column (125 ml resin volume). After application of the sample, fractions were collected by elution with a linear gradient from 100% DEAE buffer A to 100% buffer A plus 1 M NH_4Cl (DEAE buffer B). Elution fractions were tested for FV activity using a prothrombinase based assay (1). Samples containing FV were pooled and precipitated using solid $(NH_4)_2SO_4$ (ammonium sulfate) (Merck) with a 70% saturation. Pooled sample was left to stir overnight. The pellet was collected after centrifugation at 30000g for 15 minutes. The ammonium sulfate pellet was resuspended in as small a volume as possible of 10 mM HEPES pH 7.5, 175 mM NaCl and 5 mM $CaCl_2$ (Superdex buffer). The dissolved pellet was centrifuged again at 10000g for 5 minutes. The resulting supernatant was loaded on a Superdex S200 26/600 column (Pharmacia biotech, Uppsala Sweden) (volume of supernatant was 1% of column volume). Application of the sample and elution was done at a flow rate of 1.3 ml/min. FV activity in the fractions was measured using the prothrombinase-based assay. Bovine serum albumin (BSA) (MP Biomedicals, Illkirch, France) (5 mg/ml) was added to samples containing FV and fractions were pooled. The pooled sample was applied to a Resource S column (GE Healthcare). The column was washed and eluted with a linear gradient from 100% 25 mM Hepes pH 6.5 to 100% 25 mM Hepes pH 6.5 plus 1 M NH_4Cl. Using the A280 measurement two pools were made corresponding to two overlapping peaks (FV1 and FV2). Both pools were run separately on a mono S column (GE Healthcare) to further separate FV1 from FV2. A pH and salt gradient was created using 25 mM MES pH 6.5, 500 mM NH_4Cl and 5 mM $CaCl_2$ (mono S buffer A) and 25 mM Hepes pH 8.0, 1000 mM NH_4Cl and 5 mM $CaCl_2$ (mono S buffer B) to separate FV1 from FV2. FV pools were adjusted to 500 mM NH_4Cl with buffer B before application to the column. After elution 5 mg/ml BSA was added to fractions containing FV1 or FV2. To check that FV1 and FV2 had been

completely separated, both isoforms were activated with thrombin and analysed by Western blots, as described (2), using the polyclonal rabbit anti-FV light chain 90177 antibody (kind gift of Dr. H. Bakker) and a swine anti rabbit-HRP labelled secondary antibody (DAKO, Glostrup, Denmark). In addition, thrombin-activated FV1 and FV2 were run on a mono S column as FVa1 and FVa2 have more distinct elution patterns than their single-chain counterparts.

Total FV was purified according to the same protocol, but without separating the FV1 and FV2 peaks on the Resource S and Mono S columns.



Supplemental Figure 1. Anti-FV titration of thrombin generation in normal plasma. (A) Thrombin generation in pooled normal plasma was triggered with 2 pM TF in the presence of increasing concentrations (0-69.5 µg/mL) of an inhibitory antibody against FV (AHV-5101). (B) Peak heights of thrombin generation were plotted against the antibody concentration.



Supplemental Figure 2. 0.5 nM TFPI or HNBSA/Ca^{2+} buffer was incubated at 37°C with 30 µM phospholipid vesicles (20/60/20 DOPS/DOPC/DOPE) and 125 µM CS-11(65) substrate in the absence and presence of 2.5 nM FV1 or FV2. Reactions were started with 0.1 nM FXa and the absorbance at 405 nm was followed for 50 minutes. Averages of duplicate experiments are shown.

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CHAPTER 3

The C-terminus of TFPIα inhibits factor V activation by protecting the Arg1545 cleavage site

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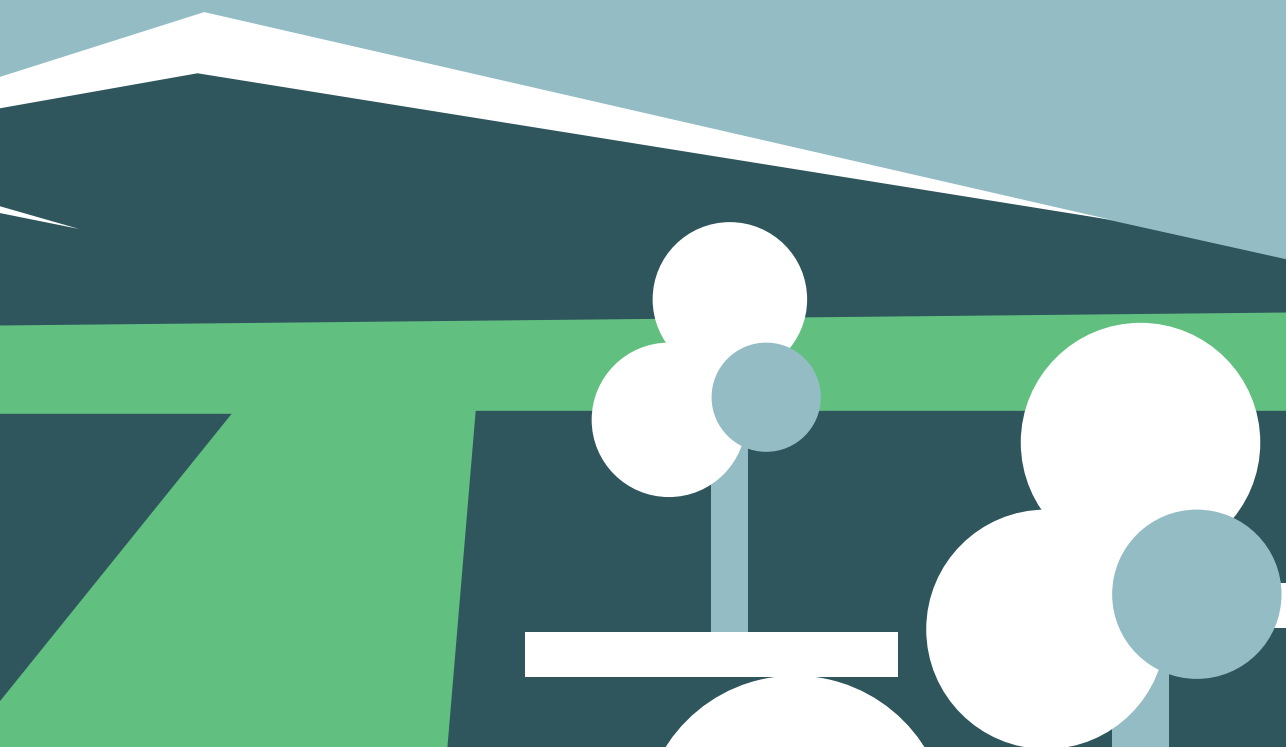
J Thromb Haemost. 2017 **15**:140-149



ABSTRACT

Background: Factor V (FV) is a carrier and a cofactor of the anticoagulant protein tissue factor pathway inhibitor-α (TFPIα), whose basic C-terminus binds to an acidic region in the B-domain of FV. Proteolysis of FV at Arg^{709}, Arg^{1018} and Arg^{1545} by factor Xa (FXa) or thrombin removes the B-domain and converts FV into a procoagulant cofactor (FVa) of FXa in the prothrombinase complex. However, retention of the acidic region in partially activated FV makes prothrombinase activity susceptible to inhibition by TFPIα.

Objective/Methods: We investigated the effect of the TFPIα C-terminal peptide (TFPIα C-term) on thrombin generation in plasma and on FV activation in model systems.

Results: TFPIα C-term inhibited tissue factor- and FXa-initiated thrombin generation in a dose-dependent manner. Failure to inhibit thrombin generation in FV-depleted plasma reconstituted with FVa indicated that the peptide effect was mediated by the acidic region of FV and localised at the level of FV activation and/or prothrombinase. In model systems TFPIα C-term inhibited both FV activation and prothrombinase activity. Western blot analysis showed that the peptide impaired cleavage at Arg^{1545} by both thrombin and FXa. The inhibition was stronger for FV-short, which binds TFPIα with higher affinity. Similar results were obtained with full-length TFPIα.

Conclusions: Cleavage of FV at Arg^{1545}, which abolishes the anticoagulant properties of FV and commits FV to the procoagulant pathway, is inhibited by binding of the TFPIα C-terminus to the FV acidic region. Possible targets of this new anticoagulant function of TFPIα are low-abundance FV(a) species retaining the acidic region.

INTRODUCTION

Coagulation factor V (FV) (reviewed in ref. [1]) is a 330-kDa multi-domain (A1-A2-B-A3-C1-C2) glycoprotein that circulates in plasma as an inactive procofactor. The inactive state is maintained by a high-affinity interaction (K_d ~2 nM) between evolutionary conserved basic and acidic regions (residues 963-1008 and 1493-1537, respectively) in the B-domain [2-4]. Limited proteolysis of FV by factor Xa (FXa) [5, 6] or thrombin [7, 8] releases the B-domain and generates activated FV (FVa), which consists of a heavy chain (A1-A2) and a light chain (A3-C1-C2) held together by a Ca^{2+} ion. FVa binds to FXa on negatively charged phospholipids to form the prothrombinase complex, accelerating prothrombin activation by several orders of magnitude [9].

Both FXa and thrombin cleave FV at Arg^{709}, Arg^{1018} and Arg^{1545} [6, 8]. These proteolytic events disrupt the interaction between the basic and acidic regions [4] and progressively expose the FXa-binding site [10]. Cleavage at Arg^{709} and Arg^{1018}, which are the kinetically favoured sites, removes the basic region, yielding an intermediate with considerable FXa-cofactor activity. However, full FV activation requires cleavage at Arg^{1545} [7], which is proteolysed more efficiently by thrombin than by FXa [5]. An additional FXa-cleavage site has been identified at Arg^{1765} in the light chain [6].

FV also acts as a carrier of plasma full-length tissue factor pathway inhibitor-α (TFPIα) [11], a multivalent Kunitz-type protease inhibitor that inhibits the tissue factor (TF)/factor VIIa complex and FXa (reviewed in [12]). The interaction with FV is mediated by the TFPIα C-terminus [13], which is strikingly homologous to the basic region of FV and can therefore bind to the acidic region of FV [13, 14]. Recent studies in model systems have elucidated two important functional implications of the FV-TFPIα interaction. First of all, it has been observed that TFPIα inhibits prothrombinase complexes containing forms of FVa that retain the acidic region [14]. Moreover, it has been shown that FV stimulates the inhibition of FXa by TFPIα, most likely by promoting TFPIα binding to phospholipids [15]. These anticoagulant effects are abolished by cleavage of FV at Arg^{1545}, which separates the acidic region of the B-domain from the light chain of FVa.

Recently, it has also been reported that FV has a minor splicing isoform which lacks most of the B-domain, including the basic region [16]. This so-called FV-short isoform binds TFPIα with much higher affinity than FV, because its acidic region is free to interact with the C-terminus of TFPIα. Accordingly, it has been estimated that, although FV-short represents only ~5% of all plasma FV, plasma TFPIα is equally distributed between FV and FV-short. An excess of FV-short is associated with increased plasma TFPIα levels and a moderate bleeding tendency (East Texas bleeding disorder) [16].

To get more insight into the functional significance of the FV-TFPIα interaction, we have studied the effect of a peptide with the same sequence as the C-terminus of TFPIα on thrombin generation in plasma and on FV activation in model systems. The most important findings were also validated with full-length TFPIα.

MATERIALS AND METHODS

TFPIα C-terminal peptide

A peptide (TFPIα C-term) with the same amino acid sequence as the C-terminus of human TFPIα (NAc-GFIQRISKGGLIKTKRKRKKQRVKIAYEEIFVKNM-COOH, residues 242-276 of the mature protein) was made by solid-phase peptide synthesis and purified by HPLC, as described [17]. Control experiments with small chromogenic/fluorogenic substrates indicated that this arginine-rich peptide does not act as an alternative substrate for FXa or thrombin.

Plasma samples for thrombin generation

Normal pooled plasma was prepared by pooling citrated plasma from 23 healthy volunteers (13 males and 10 females, mean age 34.7 years), as described [18].

FV-depleted plasma (Siemens Healthcare, Marburg, Germany) was reconstituted with 2.5 nM purified FV or FVa (Haematologic Technologies, Essex Junction, VT, USA) and supplemented with 0.25 nM full-length TFPIα (kindly provided by Dr. Lindhout) to compensate for the low level of TFPIα in FV-depleted plasma [11].

Thrombin generation

Thrombin generation was measured in normal pooled plasma or reconstituted FV-depleted plasma by Calibrated Automated Thrombography (CAT) [19]. Plasma was pre-incubated with 40 μg mL^{-1} thermostable inhibitor of contact activation (TICA, synthesized in house) [20] and different concentrations (0-5 μM) of TFPIα C-term. In experiments requiring neutralization of TFPI, a cocktail of mouse monoclonal antibodies directed against human TFPI Kunitz domains 1, 2 and 3 (Sanquin, Amsterdam, the Netherlands) was added to the plasma and incubated at 37 °C for 15 minutes before measurement. Thrombin generation was initiated with 0.25-10 pM TF (Dade Innovin, Marburg, Germany) or 225 pM human FXa (Enzyme Research Laboratories, South Bend, IN, USA), 16 mM $CaCl_2$ and 30 μM phospholipid vesicles, as previously described [18]. Thrombin activity on fluorogenic substrate Z-Gly-Gly-Arg-AMC (I-1140, BACHEM, Bubendorf, Switzerland) was monitored continuously in a Fluoroskan Ascent reader (Thermo Labsystems, Helsinki, Finland) using the Thrombinoscope software (Maastricht, the Netherlands). All experiments were carried out multiple times and representative results are shown.

Expression of recombinant FV and FV-short

Recombinant FV was expressed using the wild-type pMT2/FV construct [21]. A construct for the expression of recombinant FV-short was prepared by removing nucleotides 2441-4546 (encoding amino acids 756-1458) as described by Vincent et al. [16]. COS-1 cells were grown to 80-90% confluence in Dulbecco's modified Eagle medium (DMEM) (Lonza, Basel, Switzerland) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin, 10% fetal bovine serum and 2 mM L-glutamine. The medium was then changed to OptiMEM (ThermoFisher, Waltham, MA, USA) and the cells were transiently transfected with the pMT2/FV or pMT2/FV-short construct using Lipofectamine 2000 (Invitrogen, Bleiswijk, the Netherlands) according to the manufacturer's instructions. After 48 hours conditioned media were harvested and centrifuged at 1000g for 10 minutes to pellet cell debris. The supernatants were concentrated using Amicon Ultra Centrifugal Filter Units with 100-kDa cut-off (Merck Millipore, Cork, Ireland), snap-frozen and stored at -80 °C. The FV concentration in the media was determined using a prothrombinase-based activity assay [11].

Time-courses of FV activation

Purified plasma FV or recombinant FV was diluted to 2.5 nM in 25 mM Hepes (pH 7.7 at room temperature), 175 mM NaCl, 3 mM $CaCl_2$ and 5 mg mL^{-1} BSA (HNBSA/Ca^{2+}). Phospholipid vesicles (40 µM DOPS/DOPC, 10/90, mol/mol) and TFPIα C-term (0-5 µM) were added to the FV dilution, which was pre-warmed at 37 °C for 5 minutes. FV activation was started by the addition of 2.5 nM human thrombin (Haematologic Technologies) or 2.5 nM bovine FXa (purified in house). In the experiments with recombinant FV, TFPIα C-term was not added to the FV dilution, but pre-mixed with the thrombin used for activation. Timed aliquots were taken from the activation mixture, diluted 10-fold in HNBSA-buffer containing 30 mM Ca^{2+} and 1 µM Pefabloc TH (Pentapharm, Basel, Switzerland) and transferred to a prothrombinase mixture containing 40 µM phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol), 30 pM FXa, 1 µM prothrombin (Haematologic Technologies) and 1 µM Pefabloc TH (final concentrations) in HNBSA (3 mM Ca^{2+} was carried over from the dilution mixture). Prothrombin activation was stopped after 1 minute by subsampling to ice-cold EDTA-buffer (50 mM Tris, pH 7.9 at room temperature, 175 mM NaCl, 20 mM EDTA and 0.5 mg mL^{-1} ovalbumin) and the amount of thrombin formed was quantified with chromogenic substrate S2238 (Pepscan, Lelystad, The Netherlands). The total FV(a) dilution from the activation mixture to the prothrombinase assay was ≥100-fold. Control experiments with pre-activated FVa indicated that the TFPIα C-term carried over to the prothrombinase mixture did not affect prothrombinase activity under our assay conditions.

The activation of purified plasma FV or recombinant FV (2.5 nM) by thrombin (2.5 nM) was also followed in the absence and presence of full-length TFPIα (10 nM). To correct for the inhibition of prothrombinase activity by the TFPIα carried over from the activation mixture, the buffer

used to dilute the FV(a) subsamples taken from the activation mixtures without TFPIα was supplemented with TFPIα, resulting in equal TFPIα carry-over to the prothrombinase mixture. In addition, the prothrombinase assay was performed at 300 pM FXa.

Western blot analysis of FV activation

Purified plasma FV (2.5 nM) was activated with 2.5 nM thrombin or FXa in HNBSA/Ca^{2+} and 40 μM phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol) in the absence or presence of 5 μM TFPIα C-term or 10 nM TFPIα. At set time-points, samples were taken in non-reducing sample buffer (40 mM Tris, pH 6.7, 50% glycerol and 3.3% sodium dodecyl sulfate (SDS)), denatured at 96 °C for 5 minutes and electrophoresed on polyacrylamide gels in Laemmli running buffer (0.025 M Tris, 0.192 M glycine and 0.1% SDS) together with an unstained Precision Plus Protein standard (Bio-Rad). After transferring the proteins to PVDF membranes by semi-dry blotting, bands were visualised using a mouse monoclonal antibody directed against the light chain of human FV (AHV-5112, Haematologic Technologies) and a secondary horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (DAKO, Glostrup, Denmark). Western blots were developed using WesternBright ECL HRP substrate (Advansta, Menlo Park, CA, USA) and scanned with an ImageQuant LAS 4000 (GE Healthcare Life Sciences, Eindhoven, The Netherlands).

RESULTS

Effect of TFPIα C-term on thrombin generation

TFPIα C-term inhibited thrombin generation in normal plasma in a dose-dependent manner. At low TF concentration (2 pM), the lag time was prolonged from 3 minutes in the absence of peptide to 8 minutes in the presence of 5 μM peptide, whereas the thrombin peak height decreased from 68 nM to 13 nM (**Figure 1A**). The IC_{50} of the peptide was ~2.5 μM on peak height and ~5 μM on the endogenous thrombin potential (ETP). At higher TF (10 pM), the effect on the lag time was less pronounced and the effect on the peak height was almost completely abolished (**Figure 1B**).

To investigate whether the effect of TFPIα C-term was mediated by plasma TFPI, thrombin generation at 2 pM TF was also measured in the absence and presence of inhibitory anti-TFPI antibodies (which were not directed against the C-terminus of TFPIα, in order not to interfere with TFPIα C-term). In the absence of anti-TFPI antibodies, TFPIα C-term prolonged the lag time and decreased the peak height of thrombin generation (**Figure 2A**), as already observed in **Figure 1A**. In the presence of anti-TFPI antibodies the inhibitory effect was largely abolished, except for a two-fold prolongation of the lag time (**Figure 2B**). Since failure of the peptide to decrease thrombin generation in the presence of anti-TFPI antibodies could be simply due to

the enhanced thrombin generation associated with TFPI inhibition, the effect of the peptide on thrombin generation in the presence of anti-TFPI antibodies was tested at a lower TF concentration (0.25 pM), chosen to yield a similar thrombin peak as observed at 2 pM TF in the absence of anti-TFPI antibodies. Also under these conditions the peptide affected only the lag time of thrombin generation, causing a similar prolongation of the lag time as was observed at 2 pM TF in the absence of anti-TFPI (**Figure 2C**).

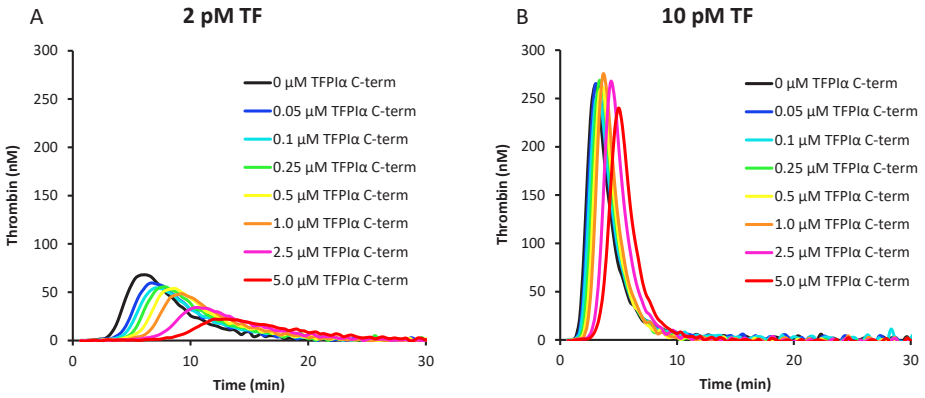


Figure 1. Effect of TFPIα C-term on thrombin generation triggered with TF. Thrombin generation was measured in pooled normal plasma triggered with 2 pM TF (**A**) or 10 pM TF (**B**) in the presence of increasing concentrations (0- 5 µM) of TFPIα C-term.

To further localize the effect of TFPIα C-term in the coagulation cascade, thrombin generation was initiated with FXa instead of TF. The concentration of FXa (225 pM) used to initiate thrombin generation was chosen to yield a similar peak height as with 2 pM TF. Also under these conditions the peptide inhibited thrombin generation in a dose-dependent manner, progressively prolonging the lag time and decreasing the peak height of thrombin generation (**Figure 3A**). The observed effects were even more pronounced than with TF-triggered thrombin generation (the IC_{50} of the peptide was ~0.25 µM on peak height and ~1 µM on the ETP) and persisted in the presence of anti-TFPI antibodies (**Figure 3B**).

The fact that TFPIα C-term inhibits thrombin generation triggered with FXa indicated that the peptide acts downstream of FXa, *i.e.* at the level of FV activation and/or prothrombinase activity. Since the TFPI C-terminus is known to interact with the acidic region of FV [11, 13], we tested the effect of TFPIα C-term on thrombin generation in FV-depleted plasma reconstituted with purified FV or thrombin-activated FVa, which lacks the whole B-domain including the acidic region. The TFPI level, which is typically low in FV-depleted plasma [11], was normalized by supplementing 0.25 nM TFPIα. The peptide potently inhibited thrombin formation in the

presence of FV (**Figure 4A**), but not at all in the presence of FVa (**Figure 4B**), suggesting that the inhibitory action of the peptide is indeed mediated by its interaction with the acidic region of FV and not due to aspecific effects on other coagulation factors. Control experiments also showed that TFPIα C-term up to a concentration of 5 µM does not directly inhibit FXa or thrombin activity (data not shown).

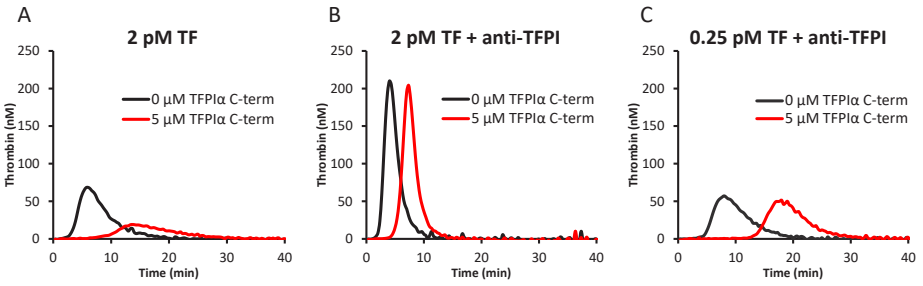


Figure 2. Effect of TFPIα C-term on thrombin generation in the absence and presence of anti-TFPI antibodies. Thrombin generation in the absence and presence of 5 µM TFPIα C-term was measured in pooled normal plasma triggered with 2 pM TF (**A**), 2 pM TF in the presence of neutralising anti-TFPI antibodies (**B**) and 0.25 pM TF in the presence of neutralising anti-TFPI antibodies (**C**).

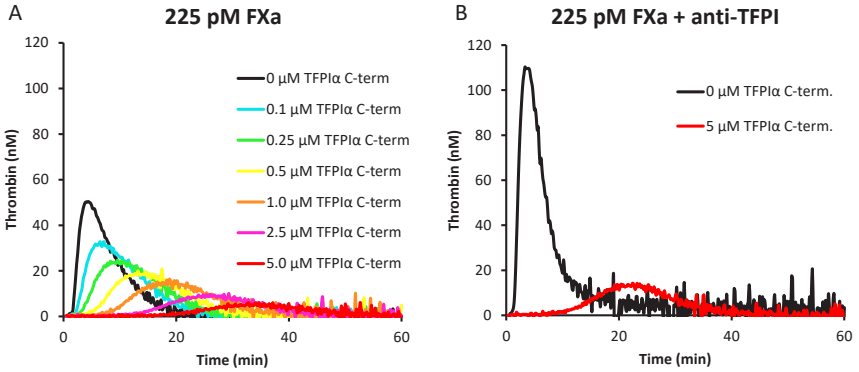


Figure 3. Effect of TFPIα C-term on thrombin generation triggered with FXa in the absence and presence of anti-TFPI antibodies. Thrombin generation was measured in pooled normal plasma triggered by 225 pM FXa in the presence of increasing concentrations (0-5 µM) of TFPIα C-term in the absence (**A**) or presence (**B**) of neutralising anti-TFPI antibodies.

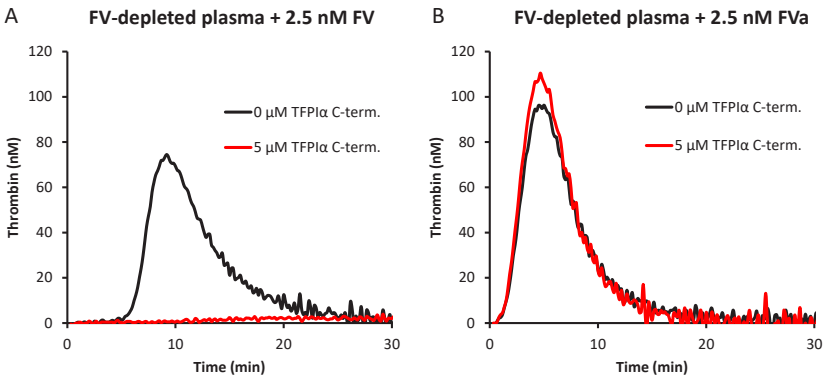


Figure 4. Effect of TFPIα C-term on thrombin generation in FV-deficient plasma reconstituted with FV or FVa. FV-deficient plasma was supplemented with 0.25 nM TFPIα and reconstituted with 2.5 nM purified plasma FV (**A**) or FVa (**B**). Thrombin generation was initiated with 2 pM TF in the absence and presence of 5 µM TFPIα C-term.

Effect of TFPIα C-term on FV activation

Failure of TFPIα C-term to inhibit thrombin generation in the presence of FVa suggested that the peptide interferes with FV activation and/or with prothrombinase activity supported by partially activated FV species. To test the effect of TFPIα C-term on FV activation, we incubated purified FV with thrombin or FXa in the presence of increasing concentrations of peptide and followed the generation of FVa activity by subsampling in time to a prothrombinase assay (**Figure 5**). In the absence of peptide, FV activation by thrombin was faster than FV activation by FXa and reached a higher plateau of FVa activity, possibly because of more efficient cleavage of FV at Arg^{1545} [5]. Inclusion of TFPIα C-term in the activation mixture decreased FV activation/activity in a dose-dependent manner, both with thrombin (**Figure 5A**) and with FXa (**Figure 5B**).

This effect could be due to inhibition of the proteolytic activation of FV or to the previously reported inhibition of prothombinase assembled with partially activated FV(a) [14], or both. To investigate whether TFPIα C-term affects FV proteolysis, the time-courses of thrombin- and FXa-catalysed FV activation in the absence and presence of TFPIα C-term were also followed by Western blotting, in order to monitor the peptide bond cleavage pattern during FV activation (**Figure 6**). This experiment confirmed that cleavage at Arg^{1545}, which generates the light chain of FVa, occurs more efficiently in the presence of thrombin than in the presence of FXa. Moreover, it showed that TFPIα C-term inhibits FV proteolysis by both thrombin and FXa, leading to prolonged persistence of the $FV_{1018\text{-}2196}$ activation intermediate (**Figure 6A**) and/or delayed appearance of the light chain band ($FV_{1545\text{-}2196}$) (**Figure 6A-B**). These findings are consistent with the peptide inhibiting cleavage of FV at Arg^{1545}.

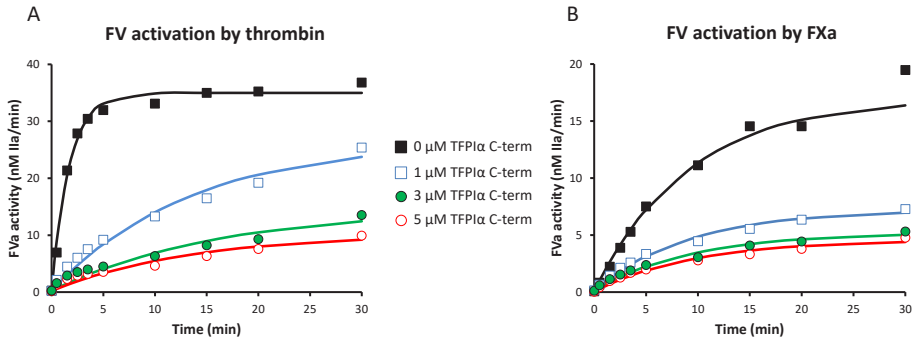


Figure 5. Effect of TFPIα C-term on FV activation by thrombin and FXa. Purified plasma FV (2.5 nM in HNBSA/Ca^{2+}) was incubated with 2.5 nM thrombin (**A**) or 2.5 nM FXa (**B**) in the presence of 40 µM 10/90 DOPS/DOPC phospholipid vesicles and increasing concentrations (0-5 µM) of TFPIα C-term. The generation of FVa activity was followed over time by regular subsampling to a prothrombinase mixture containing 30 pM FXa, 1 µM prothrombin, 40 µM 10/90 DOPS/DOPC phospholipid vesicles, 1 µM Pefabloc-TH and 3 mM Ca^{2+}. To minimise peptide carry-over, FV(a) was diluted 125 times from the activation mixture to the prothrombinase assay. FVa activity is expressed as nM thrombin (IIa)/min formed in prothrombinase.

Effect of TFPIα on FV activation by thrombin

To verify whether full-length TFPIα also inhibits FV activation by thrombin, the time-course of FV activation was followed in the absence and presence of 10 nM TFPIα. As shown in **Figure 7**, TFPIα also inhibited thrombin-mediated cleavage of FV at Arg^{1545} as assessed by Western blotting (**Figure 7A**) and shifted the $t_{1/2}$ of FV activation ~4-fold as determined by a prothrombinase-based FVa assay (**Figure 7B**).

Effect of TFPIα C-term and TFPIα on the activation of FV-short

Since FV-short has a higher affinity than FV for the C-terminus of TFPIα [16], we hypothesised that TFPIα C-term would inhibit the activation of FV-short more strongly than the activation of full-length FV. To test this hypothesis, recombinant FV and FV-short were activated with 2.5 nM thrombin in the absence and presence of 1 µM peptide (**Figure 8A,B**). Since FV-short showed partial (~30%) constitutive activity before proteolytic activation, the FVa activity data were corrected for the respective 0-point and expressed as percentage of the final plateau in order to facilitate comparison between FV and FV-short. Although FV-short was activated by thrombin more rapidly than FV, TFPIα C-term inhibited the activation of FV-short more potently than the activation of FV, 1 µM peptide increasing the $t_{1/2}$ of FV activation ~3.5-fold for wild-type FV (**Figure 8A**) and ~10-fold for FV-short (**Figure 8B**).

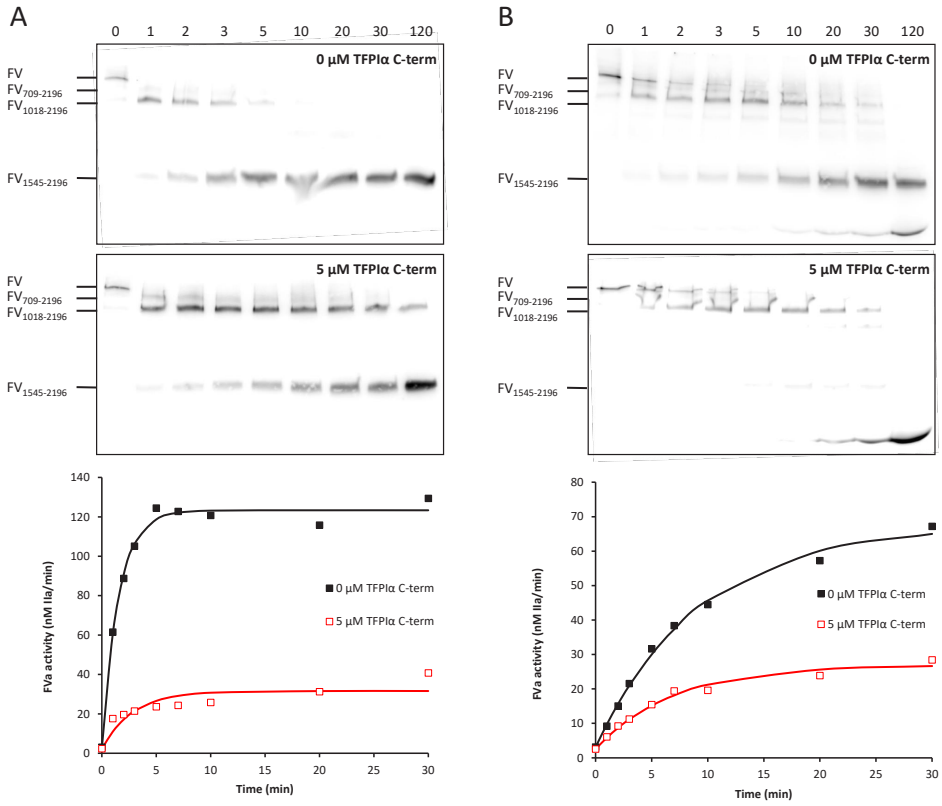


Figure 6. Effect of TFPIα C-term on FV proteolysis by thrombin and FXa. Purified plasma FV (2.5 nM in HNBSA/Ca^{2+}) was incubated with 2.5 nM thrombin (**A**) or 2.5 nM FXa (**B**) and 40 µM 10/90 DOPS/DOPC phospholipid vesicles in the absence and presence of 5 µM TFPIα C-term. Timed aliquots were subjected to SDS-PAGE on pre-cast 4-15% gradient polyacrylamide gels (Bio-Rad Laboratories, Veenendaal, the Netherlands) under non-reducing conditions and analysed by Western blotting using a monoclonal antibody directed against the FV light chain. Time points (in minutes) are indicated above the gel. FV fragments are defined by their respective amino acid residues. The lowest band in the time-course of FV activation by FXa is the product of the cleavage of the FVa light chain at Arg^{1765} [6]. FVa activity was followed over time by subsampling to a prothrombinase mixture containing 300 pM FXa, 1 µM prothrombin, 40 µM 10/90 DOPS/DOPC phospholipid vesicles, 1 µM Pefabloc-TH and 3 mM Ca^{2+}. To minimise peptide carry-over FV(a) was diluted 125 times from the activation mixture to the prothrombinase assay. FVa activity is expressed as nM thrombin (IIa)/min formed in prothrombinase.

The activation of recombinant FV and FV-short by thrombin was also followed in the absence and presence of 10 nM full-length TFPIα (**Figure 8C,D**). While TFPIα hardly affected the activation of FV (**Figure 8C**), it shifted the $t_{1/2}$ of FV-short activation ~2-fold (**Figure 8D**), confirming a more pronounced effect on FV-short than on FV.

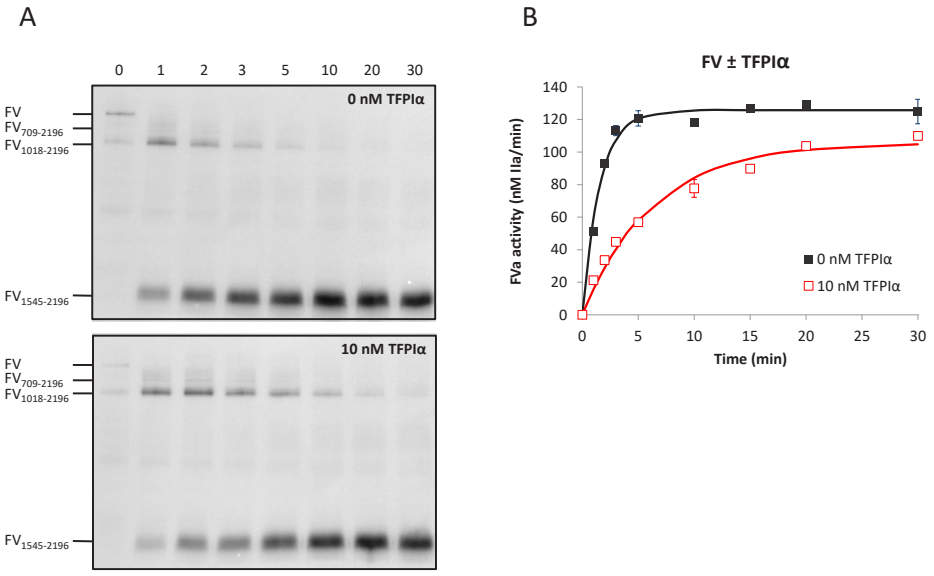


Figure 7. Effect of TFPIα on FV activation by thrombin. Purified plasma FV (2.5 nM in HNBSA/Ca^{2+}) was incubated with 2.5 nM thrombin and 40 μM 10/90 DOPS/DOPC phospholipid vesicles in the absence and presence of 10 nM TFPIα. **A**) Timed aliquots were subjected to SDS-PAGE on home-made 6% polyacrylamide gels under non-reducing conditions and analysed by Western blotting using a monoclonal antibody directed against the FV light chain. Time points (in minutes) are indicated above the gels. FV fragments are defined by their respective amino acid residues. **B**) The generation of FVa activity was followed over time by regular subsampling to a prothrombinase mixture containing 300 pM FXa, 1 μM prothrombin, 40 μM 10/90 DOPS/DOPC phospholipid vesicles, 1 μM Pefabloc-TH and 3 mM Ca^{2+}. FV(a) was diluted 100 times from the activation mixture to the prothrombinase assay. To correct for possible inhibition of the prothrombinase by the TFPIα carried over from the activation mixture, the buffer used to dilute the FV(a) subsamples taken from the activation mixture without TFPIα was supplemented with TFPIα, resulting in equal TFPIα carry-over to the prothrombinase mixture. FVa activity is expressed as nM thrombin (IIa)/min formed in prothrombinase (average of triplicate experiments ± standard deviation).

By comparing **Figure 5A** and **Figure 8A**, as well as **Figure 7B** and **Figure 8C**, we observed that the effects of TFPIα C-term and full-length TFPIα on FV activation/activity were more pronounced for purified plasma FV than for recombinant FV. Control experiments showed that this was due in part (<20%) to the cell medium present in the recombinant FV preparations (data not shown), the rest being probably attributable to intrinsic differences between recombinant FV and plasma-derived FV. Due to the presence of cell medium, the effects of TFPIα (C-terminus) on the activation of FV and FV-short shown in **Figure 8** are likely to be underestimated and should be interpreted with caution.

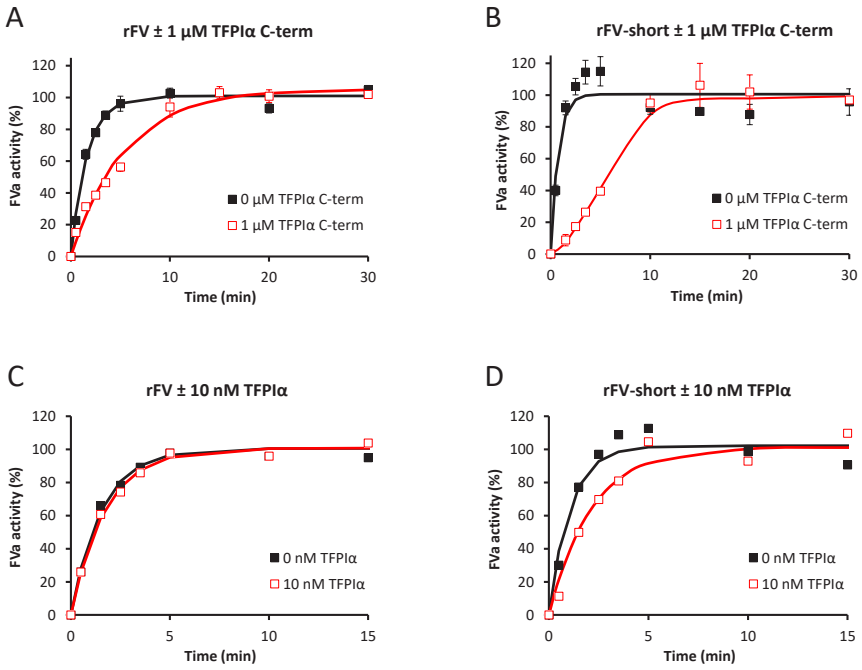


Figure 8. Effect of TFPIα C-term and TFPIα on the activation of FV and FV-short by thrombin. Recombinant FV (**A,C**) and FV-short (**B,D**) (2.5 nM in HNBSA/Ca^{2+}) were incubated with 2.5 nM thrombin and 40 µM 10/90 DOPS/DOPC phospholipid vesicles in the absence and presence of 1 µM TFPIα C-term (**A,B**) or TFPIα (**C,D**). The generation of FVa activity was followed over time by regular subsampling to a prothrombinase mixture containing 30 pM (**A,B**) or 300 pM (**C,D**) FXa, 1 µM prothrombin, 40 µM 10/90 DOPS/DOPC phospholipid vesicles, 1 µM Pefabloc-TH and 3 mM Ca^{2+}. FV(a) was diluted 100 times from the activation mixture to the prothrombinase assay. In panels **C** and **D**, to correct for possible inhibition of the prothrombinase by the TFPIα carried over from the activation mixture, the buffer used to dilute the FV(a) subsamples taken from the activation mixture without TFPIα was supplemented with TFPIα, resulting in equal TFPIα carry-over to the prothrombinase mixture. Since FV-short displayed considerable constitutive activity before proteolysis, FVa activity was expressed as percentage of the final plateau after correction for the FVa activity at t=0. The curves presented in panels **A** and **B** are averages of triplicate experiments ± standard deviation.

DISCUSSION

The discoveries that the C-terminus of TFPIα binds to the acidic region of the B-domain of FV [4, 14, 22] and that a large fraction of plasma TFPIα actually circulates in complex with FV [11] have raised new questions about the functional significance of the interaction between TFPIα and FV [23]. Studies in model systems have shown that TFPIα inhibits prothrombinase complexes containing forms of FVa that retain the acidic region [14] and that FV stimulates the inhibition of FXa by TFPIα [15]. To get more insight into the functional significance of the FV-TFPIα interaction, we have investigated the effect of a peptide with the same amino acid sequence as the C-terminus of TFPIα (TFPIα C-term) on thrombin generation in plasma. By using a peptide instead of full-length TFPIα, we were able to isolate the effects arising from the FV-TFPIα interaction from the inhibition of the TF/FVIIa complex and of FXa by the Kunitz-domains of TFPIα.

TFPIα C-term had an overall anticoagulant action, prolonging the lag time and decreasing the peak height of thrombin generation in a dose-dependent manner. The peptide was more effective at low than at high TF concentration, suggesting that its anticoagulant effect could be mediated by plasma TFPIα, which expresses anticoagulant activity particularly at low procoagulant stimuli [24]. Addition of neutralising anti-TFPI antibodies that were directed against the Kunitz domains of TFPIα but not against its C-terminus, in order not to interfere with the peptide action, showed that the effect of the peptide was indeed less pronounced in the absence of TFPIα. When coagulation was initiated with FXa instead of TF, the peptide inhibited thrombin generation even more potently, localising the effect downstream of FXa in the coagulation cascade. Finally, the peptide did not inhibit thrombin generation in FV-depleted plasma reconstituted with purified FVa, which lacks the acidic region. This indicated that the anticoagulant action of the peptide is mediated by an interaction with the acidic region of FV and further localised the inhibitory effect at the level of FV activation and/or prothrombinase activity.

Since an effect of the C-terminus of TFPIα on prothrombinase activity had been reported earlier [14], we focussed on the possibility that the peptide could inhibit FV activation by blocking access to the Arg^{1545} cleavage site, which is located next to the acidic region. To test this hypothesis, we followed the FXa- and thrombin-catalysed activation of purified plasma FV in the presence of increasing concentrations of TFPIα C-term by subsampling in time to a prothrombinase assay. Inclusion of TFPIα C-term in the activation mixture inhibited both thrombin- and FXa-catalysed FV activation/activity in a dose-dependent manner. Although part of this effect could be attributed to inhibition of prothrombinase complexes containing intermediates of FV activation that retain the acidic region, Western blot analysis indicated that TFPIα C-term also interfered with the proteolysis of FV, specifically delaying cleavage of FV at Arg^{1545}. Remarkably,

a similar finding has been reported for FV-short in a recent abstract [25]. The two effects of the peptide are in fact synergistic, as inhibition of FV proteolysis at Arg^{1545} prolongs the window of opportunity for prothrombinase inhibition by preventing the separation of the acidic region from the light chain of FVa.

Due to the proximity of the acidic region to the Arg^{1545} cleavage site, TFPIα C-term is likely to act by blocking the access to the Arg^{1545} cleavage site of FV. This "steric hindrance" mechanism is supported by the observation that TFPIα, which is much bulkier than the C-terminal peptide, inhibited FV activation by thrombin at a 100-fold lower concentration than the peptide (as also observed for prothrombinase inhibition [14]). Moreover, the activation of FV-short, which has a higher affinity than FV for the TFPIα C-terminus due to the absence of the basic region [16], was more efficiently inhibited by the peptide.

The fact that inhibition of FV cleavage at Arg^{1545} by the TFPIα C-term peptide could be replicated using full-length TFPIα suggests that this anticoagulant mechanism may play a physiological role. The physiological target of this anticoagulant mechanism is presently unclear, but it is unlikely to be intact full-length FV, whose plasma concentration (~25 nM) is 100-fold higher than the concentration of full-length TFPIα (~0.25 nM). A more likely candidate could be FV-short, whose plasma concentration (1-2 nM) is closer to that of TFPIα and whose affinity for TFPIα is at least 10-fold higher than that of normal FV [16]. In fact, although FV-short displays partial constitutive activity before proteolysis, our data show that TFPIα can delay its further activation by inhibiting cleavage at Arg^{1545}. Other possible targets of this anticoagulant activity of TFPIα might be early intermediates of FV activation as well as platelet FV, which retain the acidic region and bind TFPIα with high affinity, and which have already been proposed to be inhibited by TFPIα in the prothrombinase [14, 23]. These FV species are either formed in small amounts during initiation of coagulation (early FV activation intermediates) or released at the site of injury by activated platelets (platelet FV), which also locally release TFPIα. The TFPIα requirement for inhibition of cleavage at Arg^{1545} in these low-abundance high-affinity targets might be lower than the supraphysiological TFPIα concentration used in our experiments.

Taken together, our data point out a novel anticoagulant function of full-length TFPIα, namely the ability of its C-terminus to delay FV activation by inhibiting cleavage of FV at Arg^{1545}. This cleavage is a crucial event that marks the definite transition from FV, an anticoagulant molecule that does not support prothrombinase activity and actually acts as a cofactor of TFPIα in FXa inhibition [15], to FVa, a procoagulant species that binds to FXa with high affinity [10], thereby supporting prothrombinase activity and protecting FXa from inhibition by TFPIα [15]. By delaying cleavage at Arg^{1545}, TFPIα could therefore contribute to the down-regulation of coagulation *via* several mechanisms. First of all, it would slow down (the generation of) prothrombinase activity, because FV activation intermediates cleaved only at Arg^{709} and/or Arg^{1018} have a high

FXa requirement for prothrombinase complex formation [10] and their prothrombinase activity is susceptible to inhibition by TFPIα [14]. Moreover, it would preserve the TFPI-cofactor activity of FV (which relies on the interaction between the TFPIα C-terminus and the acidic region of FV [15]), thereby enhancing and prolonging the ability of TFPIα to inhibit FXa. A third potential effect of delayed cleavage at Arg^{1545} would be the maintenance of the cofactor activity of FV for activated protein C (APC) in the inactivation of factor VIIIa [26]. In fact, it has been reported that this anticoagulant activity of FV requires the C-terminal portion of the B-domain and is lost upon cleavage at Arg^{1545} [27]. Although the molecular basis of the APC-cofactor activity of FV is still largely unknown [28], preliminary experiments from our laboratory suggest that TFPIα C-term may improve the APC-cofactor activity of FV in FVIIIa inactivation as probed by the Immunochrom APC resistance test [29], presumably *via* protection of the Arg^{1545} cleavage site in FV (data not shown).

In conclusion we propose that the TFPIα C-terminus may act as a "gatekeeper" of the Arg^{1545} cleavage site in FV, which controls the transition of FV from an anticoagulant to a procoagulant cofactor. Since FV is much more abundant than TFPIα in plasma, further studies are needed to assess the physiological relevance and the molecular forms of FV that are targeted by this novel anticoagulant function of TFPIα.

ACKNOWLEDGEMENTS

We thank Dr. F. Nuzzo for preparing the FV-short construct and Mr. D.P. Suylen for synthesising the TFPIα C-terminal peptide. This work was supported by a research grant from the Dutch Thrombosis Foundation (TSN 2104-1 to E. Castoldi).

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CHAPTER 4

Development of a plasma-based assay to measure the susceptibility of factor V to inhibition by the TFPIα C-terminus

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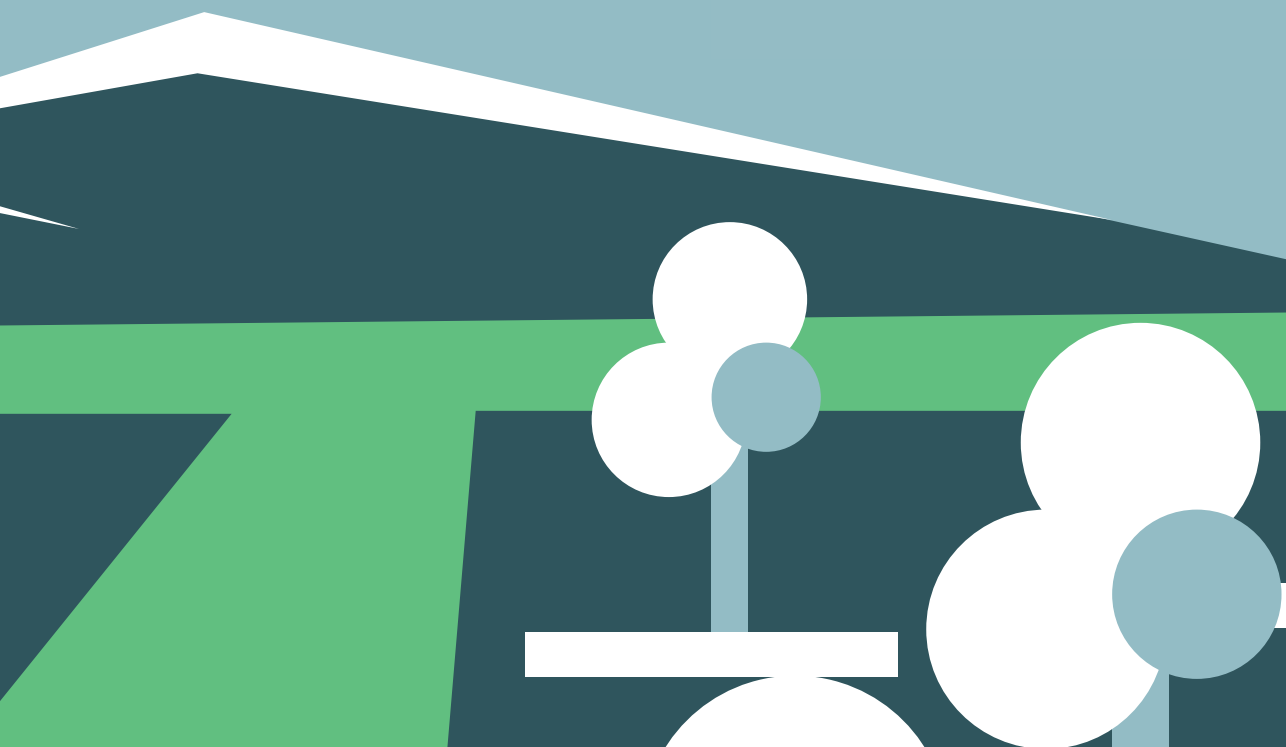
Submitted



ABSTRACT

Background: Factor V (FV) is proteolytically activated to FVa, which assembles with factor Xa (FXa) in the prothrombinase complex. The C-terminus of tissue factor pathway inhibitor-α (TFPIα) inhibits both the activation and the prothrombinase activity of FV(a), but the pathophysiological relevance of this anticoagulant mechanism is unknown. FV Leiden (FVL) is less susceptible to inhibition by TFPIα, while over-expression of FV splicing variants with increased affinity for TFPIα (FV-short) causes bleeding.

Objective: To develop a plasma-based assay that quantifies the susceptibility of FV(a) to inhibition by the TFPIα C-terminus.

Methods: FV in highly diluted plasma was pre-activated with FXa in the absence or presence of the TFPIα C-terminal peptide. After adding prothrombin, thrombin formation was monitored continuously with a chromogenic substrate and prothrombinase rates were obtained from parabolic fits of the absorbance tracings. TFPI-resistance was expressed as the ratio of the prothrombinase rates with and without peptide (TFPIr).

Results: The TFPIr (~0.30 in normal plasma) was independent of the FV level. The TFPIr increased from normal individuals (0.29, 95%CI 0.28-0.31) to FVL heterozygotes (0.35, 95%CI 0.34-0.37) and homozygotes (0.39, 95%CI 0.37-0.40), confirming TFPI-resistance of FVL. Two individuals over-expressing FV-short$_{Amsterdam}$ had markedly lower TFPIr (0.16, 0.18) than a normal relative (0.29), in line with the high affinity of FV-short for TFPIα.

Conclusions: We have developed and validated an assay that measures the susceptibility of plasma FV to the TFPIα C-terminus. This assay can be used to test whether this property of FV(a) correlates with thrombosis or bleeding risk in population studies.

INTRODUCTION

Coagulation factor V (FV) is the inactive precursor of activated factor V (FVa), a potent procoagulant cofactor that assembles with factor Xa (FXa) on negatively charged phospholipids to form the prothrombinase complex [1], thereby accelerating the conversion of prothrombin to thrombin more than 1000-fold [2]. FV circulates in plasma as a single-chain protein with an A1-A2-B-A3-C1-C2 domain structure. Its inactive state is maintained by a high-affinity interaction between a basic region and an acidic region in the B-domain [3-5]. FV is activated by factor Xa (FXa) or thrombin [6, 7] *via* limited proteolysis at Arg^{709}, Arg^{1018} and Arg^{1545}, which dismantles the B-domain and progressively exposes the FXa-binding site [4, 8]. Rapid cleavage at Arg^{709} and/or Arg^{1018} generates FV activation intermediates (denoted here as FV(a)) with low affinities for FXa [8] and poor cofactor activities in prothrombinase [9, 10]. Slower cleavage at Arg^{1545} (mainly by thrombin [7]) releases fully activated FVa, consisting of non-covalently linked heavy (A1-A2) and light (A3-C1-C2) chains. FVa binds FXa with high affinity and expresses full cofactor activity.

FVa is inactivated by activated protein C (APC), a serine-protease that cleaves the FVa heavy chain at Arg^{306}, Arg^{506} and Arg^{679}, thereby abolishing FXa-cofactor activity [11, 12]. The common FV Leiden (FVL) mutation [13], predicting the replacement of Arg^{506} by a Gln, eliminates the kinetically favoured APC-cleavage site at Arg^{506}, making FVaL partially resistant to APC-catalysed inactivation and increasing the risk of venous thrombosis [14].

Recent studies have shown that tissue factor pathway inhibitor α (TFPIα), a Kunitz-type protease inhibitor known to inhibit factor VIIa and FXa [15], also contributes to regulating the procoagulant activity of FV(a), especially in the early phases of coagulation [16]. In fact, TFPIα circulates in complex with FV [17, 18]and hampers both FV activation (by interfering with cleavage at Arg^{1545}) [19] and the incorporation/activity of partially activated FV(a) in the prothrombinase complex [20, 21]. These inhibitory functions are mediated by a basic region in the TFPIα C-terminus, which is highly homologous to the FV basic region [22, 23] and binds with high affinity to the acidic region in the B-domain of FV [5, 20, 21]. As a consequence, TFPIα preferentially targets forms of FV that lack or have lost the endogenous basic region [24], such as FV(a) activation intermediates (cleaved at Arg^{709} and/or Arg^{1018}), platelet FV and the so-called FV-short variants. The latter are FV splicing isoforms with partial B-domain deletions that were originally discovered in two unrelated families with unexplained bleeding from East Texas and Amsterdam, respectively [25, 26]. These families segregate unique *F5* gene mutations that up-regulate distinct but similar FV-short isoforms with high affinity for TFPIα, resulting in strongly elevated TFPIα levels and trauma-related bleeding [25, 26]. Besides the primary interaction between the basic region of TFPIα and the acidic region of FV, a secondary interaction between three neutral residues in the TFPIα C-terminus (Leu^{252}, Ile^{253}

and Thr^{255}) and the FV(a) heavy chain, presumably interfering with the binding of FXa to FV(a), has recently been shown to be essential for efficient prothrombinase inhibition [21]. The Arg^{506}àGln substitution in FVL would weaken this interaction [21], making prothrombinase complexes containing FV(a)L partially resistant to inhibition by TFPIα, which may contribute to the prothrombotic potential of the FVL mutation [27].

Although the pathophysiological relevance of TFPIα-mediated inhibition of FV activation and prothrombinase activity is still largely unexplored, inter-individual differences in the susceptibility of FV to inhibition by TFPIα, (*e.g.* due to genetic variation in the *F5* gene, alternative splicing of the *F5* pre-mRNA and/or post-translational modifications of the FV protein) are likely to affect the risk of venous thrombosis or bleeding. Therefore, we aimed to develop a plasma-based assay that specifically measures the susceptibility of FV(a) to inhibition by the C-terminus of TFPIα.

MATERIALS AND METHODS

Plasma samples

Normal pooled plasma was prepared by pooling citrated plasma from 23 healthy donors (13 males and 10 females, mean age 34.7 years), essentially as described [28]. Individuals with different FVL genotypes were identified at Padua University Hospital through FVL screening for familial thrombophilia, and plasma was obtained as previously described [29]. The present study included 15 non-carriers (7 males and 8 females, mean age 37.0 years), 15 FVL heterozygotes (7 males and 8 females, mean age 37.9 years) and 10 FVL homozygotes (5 males and 5 females, mean age 39.7 years). Three FVL heterozygotes and 5 homozygotes had a personal history of venous thrombosis. Plasma from three members of the FV Amsterdam family was obtained as described [26]. All participants provided informed consent to the study in accordance with the Helsinki declaration. FV-depleted plasma was purchased from Siemens Healthcare (Marburg, Germany).

TFPIα C-terminal peptide

A peptide mimicking the C-terminus of TFPIα (TFPIα C-term) was synthesized in-house as previously described [19]. The peptide consists of residues 242-276 of the mature protein (NAc-GFIQRISKGG**LI**K**T**KRKRKKQRVKIAYEEIFVKNM-COOH) and contains both the basic region that binds to the acidic region of FV and the neutral amino acids (bold) that interact with the FV(a) heavy chain [21]. Control experiments indicated that the TFPIα C-term peptide up to a concentration of 5 μM does not interfere with chromogenic substrate conversion by thrombin.

Assay to measure the susceptibility of plasma FV(a) to inhibition by TFPIα

Plasma was diluted 1/400 in HNBSA (25 mM Hepes (pH 7.7 at room temperature), 175 mM NaCl and 5 mg/mL bovine serum albumin) and pipetted in the wells of a microtiter plate, to which either HNBSA or TFPIα C-term was added. After pre-warming at 37°C for 15 minutes, human FXa (Haematologic Technologies, Essex Junction, VT, USA), phospholipid vesicles (DOPS/DOPC/DOPE, 20/60/20, M/M/M) and Ca^{2+} were added to start FV activation. Concentrations in this mixture were: 1/680-diluted plasma (corresponding to ~37 pM FV), ~29 pM FXa, 30 µM phospholipids, 3 mM Ca^{2+} and (if present) 146 nM peptide. After 3 minutes at 37°C, during which FV is partially activated to FV(a), prothrombin activation was started by the addition of human prothrombin (Haematologic Technologies) and chromogenic substrate for thrombin (H-D-Phe-Pip-Arg-pNA, INterface BIOmaterials BV, Geleen, the Netherlands), supplemented with phospholipids and Ca^{2+} to keep the concentrations of these reagents constant in the final mixture. Final concentrations were: 1/1000-diluted plasma (corresponding to ~25 pM FV(a)), 20 pM FXa, 0.5 µM prothrombin, 30 µM phospholipids, 3 mM Ca^{2+}, 0.5 mM chromogenic substrate and (if present) 100 nM peptide. After addition of the prothrombin/substrate mixture, substrate conversion was monitored continuously for 30 minutes by measuring the absorbance at 405 nm in an Ultra Microplate reader (Bio-Tek, Burlington, VT, USA).

Absorbance tracings were truncated at 300 mOD to minimize deviations from parabolic behaviour due to substrate consumption, and fitted to a second-order polynomial equation of the type:

$$y = a^*x^2 + b^*x + c$$

where y is the absorbance in mOD and x is the time in minutes. In this equation, c (intercept with the y-axis) is a constant representing the offset from 0, b*x is the linear absorbance change due to the thrombin already present at time 0, and a^*x^2 is the quadratic absorbance change due to the thrombin generated in time by the prothrombinase complex. The first derivative of the absorbance tracing ($y = 2^*a^*x + b$) is a straight line describing the formation of thrombin in time. Therefore, the slope of this line (2*a) represents the rate of prothrombin activation by prothrombinase. This rate depends not only on the plasma FV concentration, but also on the FV(a) species formed during the pre-incubation step and their prothrombinase activities, both of which are influenced by the TFPIα C-term peptide. The outcome of the assay was expressed as TFPI-ratio (TFPIr), defined as the ratio of the prothrombinase rates obtained in the presence and absence of TFPIα C-term. The TFPIr is a measure of the TFPI resistance of the FV present in the plasma.

Western blot analysis of FV activation

FV in 1/680-diluted plasma was activated with ~29 pM FXa in the presence and absence of the TFPIα C-term peptide, as described above. At set time points, aliquots from the activation mixture were transferred to non-reducing sample buffer (40 mM Tris, pH 6.7, 50% glycerol and 3.3% SDS, 0.1% bromophenol blue) to stop FV activation. Samples were denatured at 96°C for 5 minutes, run on a 6% polyacrylamide gel and blotted essentially as described before [19]. Briefly, FV bands were visualized using a mouse monoclonal antibody directed against the light chain of human FV (AHV-5112; Haematologic Technologies) and a horseradish peroxidase-conjugated goat anti-mouse antibody (Dako, Glostrup, Denmark). Blots were developed using the WesternBright ECL HRP substrate (Advansta, Menlo Park, CA, USA) and scanned with an ImageQuant LAS 4000 imager (GE Healthcare Life Sciences, Eindhoven, The Netherlands).

Statistics

Prothrombinase rates and TFPIr of individual samples measured multiple times were expressed as mean ± standard error of the mean (SEM). TFPIr of FVL genotype groups were expressed as mean and 95% confidence intervals (95%CI) and compared using Student's t-test.

RESULTS

General set-up of the assay

The assay consists of two steps (**Figure 1**). First, FV in highly diluted plasma is activated with a suboptimal FXa concentration in the absence or presence of the TFPIα C-term peptide for a fixed amount of time (FV activation step). Then, prothrombin and a chromogenic substrate for thrombin are added, and prothrombinase activity is followed continuously by monitoring chromogenic substrate conversion in a plate-reader (prothrombinase step). The obtained absorbance tracings can be adequately fitted with a second-order polynomial equation, from which the prothrombinase rate can be calculated as 2 times the coefficient of the x^2-term. The outcome of the assay is expressed as the ratio of the prothrombinase rates determined in the presence and absence of peptide. This ratio (TFPIr) is inversely correlated with the ability of the TFPIα C-term peptide to inhibit the activation and the prothrombinase activity of the plasma FV(a).

The peptide was used instead of full-length TFPIα as it contains all sequences necessary to bind and inhibit FV(a) [21] and thereby optimally probes the FV- TFPIα interaction, while lacking the Kunitz-2 domain which would otherwise inhibit FXa.

Development of a plasma-based continuous prothrombinase assay

Our first goal was to set up and optimize a continuous prothrombinase assay using (diluted) plasma as the source of FV. To this end, a final plasma dilution of 1/1000 (corresponding to ~25 pM FV) was chosen, in order to minimize the influence of the plasma background on the assay. The FXa concentration used to activate FV was arbitrarily set at ~29 pM, yielding 20 pM FXa in the final (prothrombinase) step of the assay.

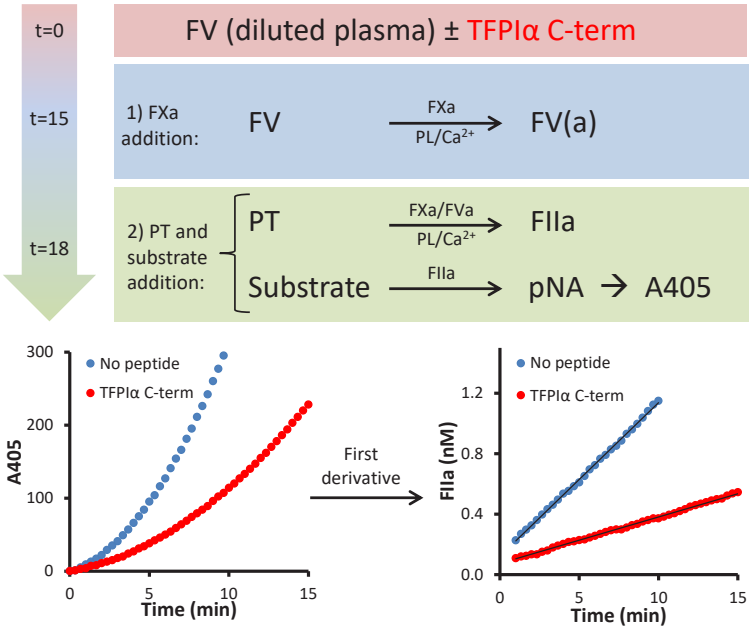


Figure 1. Schematic overview of the experimental set-up. Highly diluted plasma with or without added TFPIα C-term peptide is pre-warmed at 37°C for 15 minutes. Subsequently, a mixture of FXa, phospholipids and Ca2+ is added to partially activate the plasma FV to FV(a) (FV activation step). After 3 minutes, a mixture of prothrombin (PT) and chromogenic substrate for thrombin is added to start prothrombinase, and conversion of the chromogenic substrate by the formed thrombin (FIIa) is followed for 30 minutes by monitoring absorbance at 405 nm (prothrombinase step). This results in parabolic absorbance tracings (blue = without peptide, red = with peptide). The first derivatives of these curves are straight lines representing thrombin formation in time and their slopes are a measure of the rates of prothrombin activation in the absence ad presence of peptide. The peptide inhibits both FV activation and prothrombinase activity, reducing the prothrombinase rate to ~30% of its value in the absence of peptide in normal plasma.

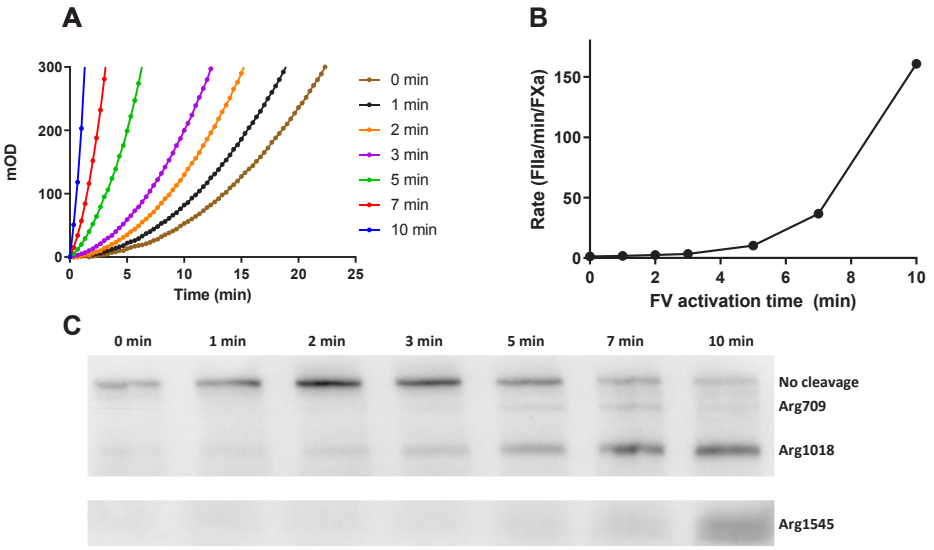


Figure 2. **Time-course of FV activation by FXa**. **(A)** FV in 1/680 diluted plasma was activated with ~29 pM FXa for 0-10 minutes before adding the prothrombin/chromogenic substrate mixture, and absorbance at 405 nm was followed in time. **(B)** The prothrombinase rates obtained from parabolic fits of the absorbance tracings were plotted as a function of the pre-incubation time. **(C)** The FV activation time-course was analysed by Western blot as described under Methods. FV(a) bands were visualized using an antibody against the light chain of FV. Bands arising from FV cleavage at Arg^{709}, Arg^{1018} and Arg^{1545} are indicated.

Time-course of FV activation

To determine the optimal duration of the FV activation step, the pre-incubation time of diluted plasma with FXa was varied between 0 and 10 minutes. Longer pre-incubation times resulted in faster substrate conversion (**Figure 2A**), due to increased activation of FV. The prothrombinase rates corresponding to the different pre-incubation times are shown in **Figure 2B**. Analysis of the FV activation time-course by Western blot showed that the initial slow development of prothrombinase activity correlated with progressive cleavage of FV at Arg^{1018} and to a lesser extent at Arg^{709}, which yield partially activated FV(a) intermediates known to express low prothrombinase activity [8-10]. However, at later time points the FVa light chain became visible (**Figure 2C**), indicating cleavage at Arg^{1545} and generation of fully activated FVa, which was probably responsible for the sharp increase in prothrombinase activity and for feedback-activation of FV by the formed thrombin. The generated thrombin originates from the conversion of plasma prothrombin by early prothrombinase complexes, suggesting that the plasma prothrombin level might affect the assay outcome. To minimize this undesirable phenomenon, *i.e.* to favour the generation of partially activated FV(a) forms that are susceptible to inhibition by the TFPIα C-term peptide, the pre-incubation time was limited to 3 minutes in all subsequent experiments.

The time-course of FV activation was also followed in the presence of 100 nM TFPIα C-term. A comparison of the prothrombinase rates and FV cleavage patterns obtained after different pre-incubation times in the absence and presence of peptide is presented in **Supplemental Figure 1**.

FV dependence

To verify the FV dependence of the measurement, we performed the assay in buffer containing no FV, 25 pM FV (corresponding to the FV concentration in 1/1000-diluted plasma) or 25 pM FVa (**Figure 3A**). The prothrombinase rate obtained with FV (mean ± SEM, 2.63 ± 0.14 nM FIIa/min/nM FXa) was ~12-fold higher than the prothrombinase rate in the absence of FV (0.21 ± 0.01 nM FIIa/min/nM FXa), demonstrating that the assay is indeed dependent on FV. On the other hand, the prothrombinase rate obtained with fully activated FVa (868.5 ± 36.0 nM FIIa/min/nM FXa) was ~330 times higher than that with FV, confirming that under our assay conditions (3 minutes pre-incubation with ~29 pM FXa) the activation of FV is largely incomplete (cf. **Figure 2C**, 3-minute point) and mainly generates FV activation intermediates with low prothrombinase activity. As mentioned above, this is desirable, as these FV activation intermediates retain the acidic region and are therefore susceptible to inhibition by the TFPIα C-term peptide.

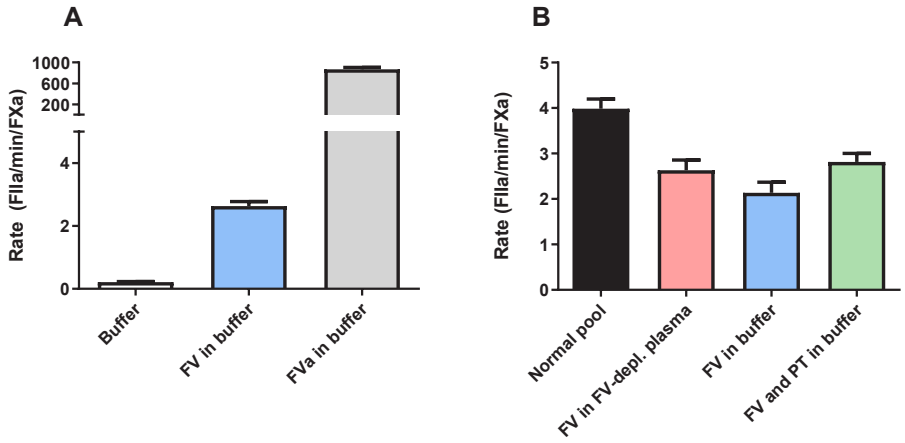


Figure 3. Determinants of the prothrombinase rate. (A) Samples containing no FV, 25 pM purified FV (corresponding to 1/1000-diluted plasma) or 25 pM purified FVa in HNBSA buffer were measured in the continuous prothrombinase assay (in the absence of TFPIα C-term), as described under Methods. Prothrombinase rates obtained from the parabolic fits of the absorbance data were expressed as nM thrombin (FIIa)/minute/nM FXa. Bars represent the mean ± SEM of 2 experiments. **(B)** Purified FV, diluted to 25 pM in 1/1000-diluted FV-depleted plasma, or in HNBSA buffer, or in HNBSA buffer supplemented with 1.5 nM prothrombin (PT), were measured in the continuous prothrombinase assay (in the absence of TFPIα C-term), as described under Methods. A 1/1000 dilution of normal pooled plasma was taken as a reference. Prothrombinase rates obtained from the parabolic fits of the absorbance data were expressed as nM thrombin (FIIa)/minute/nM FXa. Bars represent the mean ± SEM of 6 experiments.

Effect of the plasma background

To test whether the plasma background influences FV activation or prothrombinase activity, we performed the assay on purified FV diluted to 25 pM in either 1/1000 FV-depleted plasma or in HNBSA buffer (**Figure 3B**). The prothrombinase rate obtained in FV-depleted plasma (mean ± SEM, 2.63 ± 0.23 nM FIIa/min/nM FXa) was slightly higher than that obtained in buffer (2.13 ± 0.24 nM FIIa/min/nM FXa), indicating that the plasma background is not completely neutral. However, the difference was small and could be abolished by including 1.5 nM prothrombin (corresponding to the prothrombin concentration of 1/1000-diluted plasma) in the buffer system (2.81 ± 0.19 nM FIIa/min/nM FXa), suggesting that prothrombin is the only plasma component (apart from FV) that influences the prothrombinase rate, most probably by being converted to thrombin and contributing to FV activation during the pre-incubation step (cf. **Figure 2B, C**). The prothrombinase rate of FV-depleted plasma reconstituted with purified FV was consistently lower than that of normal pooled plasma (3.98 ± 0.21 nM FIIa/min/nM FXa), most probably due to the lower prothrombin level of FV-depleted plasma (66%) compared to normal pooled plasma (100%). Control experiments also indicated that 1/1000-diluted plasma does not influence FXa and thrombin activities as assessed by chromogenic substrate conversion.

Effect of the TFPIα C-term peptide

To quantify the susceptibility of FV to inhibition by the TFPIα C-terminus, the continuous prothrombinase assay was carried out in the absence or presence of the TFPIα C-term peptide, which was added to the plasma dilution before starting FV activation with FXa. To determine the optimal peptide concentration to be used in the assay, a peptide titration (0-10 μM) was performed and the prothrombinase rate was determined at each peptide concentration. Increasing peptide concentrations resulted in dose-dependent inhibition of prothrombin activation, reaching maximal inhibition at ~1 μM TFPIα C-term (**Figure 4**). Based on this titration we chose to use 100 nM TFPIα C-term, which reduces the prothrombinase rate in normal plasma to ~30% of its value in the absence of peptide (TFPIr of 0.30). This peptide concentration allows for the detection of both higher and lower susceptibilities of FV(a) to inhibition by TFPIα, resulting in lower and higher TFPIr, respectively.

Effects of plasma FV and prothrombin concentrations on the TFPIr

As mentioned above, the continuous prothrombinase assay was dependent not only on plasma FV, but also on plasma prothrombin, which becomes activated to thrombin during the pre-incubation step and subsequently contributes to FV activation. To test whether differences in FV and prothrombin levels among individual plasmas would influence the susceptibility of FV to inhibition by TFPIα, we prepared samples with different FV concentrations (50-200%, where 100% = 25 nM) and prothrombin concentrations (50-200%, where 100% = 1.5 μM) in HNBSA buffer and performed the continuous prothrombinase assay in the absence or presence of 100 nM TFPIα C-term peptide. As expected, prothrombin activation rates increased at increasing FV and prothrombin concentrations

both in the absence and presence of peptide (**Table 1**), but the TFPIr remained virtually constant throughout the entire range of FV and prothrombin concentrations (**Figure 5**).

Assay reproducibility

To evaluate the assay reproducibility, normal pooled plasma was assayed in quadruplicate on the same plate in 5 separate experiments. The average intra-assay CV (between replicates on the same plate) was 5.3% and 5.6% for the prothrombinase rates without and with TFPIα C-term, respectively, and 6.2% for the TFPIr. The inter-assay variation, estimated by measuring normal pooled plasma on 14 different days (17 plates), was 30.8% and 24.0% for the prothrombinase rates without and with peptide, respectively, and 9.7% for the TFPIr.

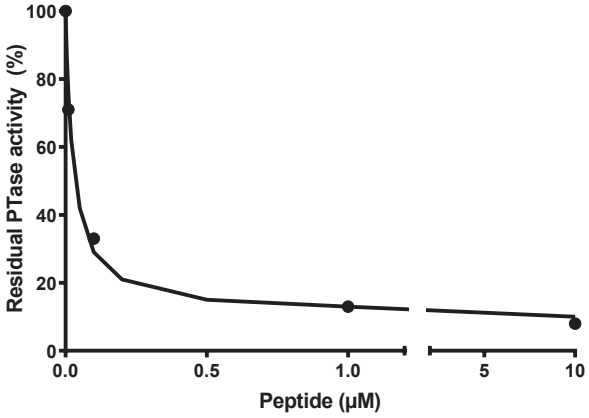


Figure 4. Inhibition of prothrombin activation by the TFPIα C-term peptide. Normal pooled plasma (1/1000 final dilution) was measured in the continuous prothrombinase assay in the presence of 0-10 μM TFPIα C-term peptide, as described under Methods. The residual prothrombinase activity (%) was plotted for each peptide concentration.

Assay validation

The recent report that prothrombinase complexes containing FV(a)L are less susceptible to inhibition by TFPIα than prothrombinase complexes containing normal FV(a) [27] offered us the opportunity to validate our TFPIα susceptibility assay using plasma from FVL carriers. Plasma from 15 non-carriers, 15 FVL heterozygotes and 10 FVL homozygotes was tested in the continuous prothrombinase assay in the absence or presence of 100 nM TFPIα C-term peptide, and prothrombinase rates (**Supplemental Table 1**) and TFPIr were calculated (**Figure 6**). The average TFPIr increased from non-carriers (0.29, 95%CI 0.28-0.31) to FVL heterozygotes (0.35, 95%CI 0.34-0.37, $p<0.001$ *vs.* non-carriers) to FVL homozygotes (0.39, 95%CI 0.37-0.40, $p=0.014$ *vs.* FVL heterozygotes), confirming the ability of our assay to detect the impaired susceptibility of FVL to TFPIα. These results did not change when individuals with a personal history of venous thrombosis were excluded.

Table 1. Effects of FV and prothrombin concentrations on the prothrombinase rate in the absence or presence of TFPIα C-term

	Prothrombinase rate (FIIa/min/FXa)*			
	No peptide			
	50% PT	**100% PT**	**150% PT**	**200% PT**
50% FV	1.72 ± 0.08	1.98 ± 0.09	1.98 ± 0.08	2.10 ± 0.07
100% FV	2.86 ± 0.10	3.01 ± 0.08	3.36 ± 0.10	3.76 ± 0.11
150% FV	3.37 ± 0.12	3.56 ± 0.08	4.11 ± 0.23	4.88 ± 0.27
200% FV	4.39 ± 0.30	4.52 ± 0.09	5.27 ± 0.21	5.60 ± 0.20

* Mean ± SEM of 3 experiments

FIIa, thrombin; FXa, factor Xa; FV, factor V; PT, prothrombin

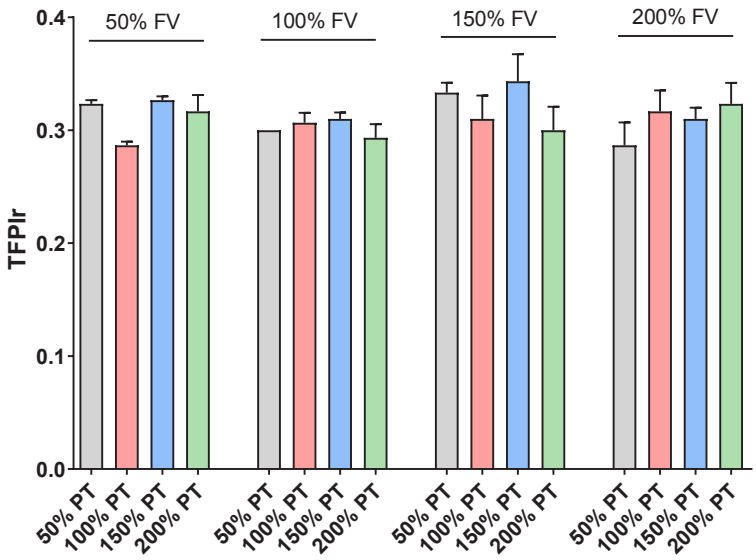


Figure 5. Effects of FV and prothrombin concentrations on the TFPIr. Simulated samples containing 50-200% FV (100% = 25 pM in the final dilution) and 50-200% prothrombin (100% = 1.5 nM in the final dilution) in all possible combinations, obtained by diluting purified FV and prothrombin in HNBSA buffer, were measured in the continuous prothrombinase assay in the absence and presence of 100 nM TFPIα C-term, as described under Methods. Prothrombinase rates with and without peptide were determined from parabolic fits of the absorbance data (see **Table 1**) and TFPIr were calculated. Bars represent the mean ± SEM of 3 experiments.

Prothrombinase rate (FIIa/min/FXa)* 100 nM TFPIα C-term			
50% PT	**100% PT**	**150% PT**	**200% PT**
0.55 ± 0.03	0.56 ± 0.03	0.65 ± 0.03	0.67 ± 0.04
0.85 ± 0.03	0.92 ± 0.04	1.04 ± 0.04	1.10 ± 0.03
1.13 ± 0.06	1.11 ± 0.08	1.40 ± 0.05	1.46 ± 0.04
1.23 ± 0.04	1.44 ± 0.09	1.64 ± 0.11	1.80 ± 0.07

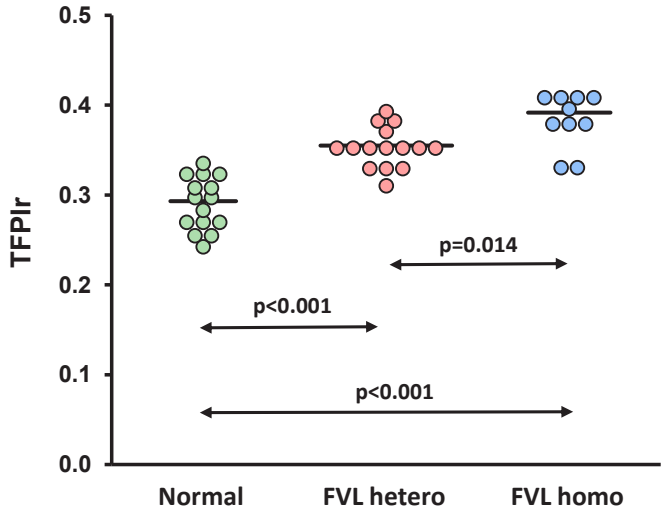


Figure 6. Effect of FV Leiden genotype on the TFPIr. Plasma from 15 non-carriers of the FV Leiden (FVL) mutation, 15 heterozygous carries and 10 homozygous carriers was measured in the continuous prothrombinase assay in the absence or presence of 100 nM TFPIα C-term, as described under Methods. Prothrombinase rates with and without peptide were determined from parabolic fits of the absorbance data (see **Supplemental Table 1**) and TFPIr were calculated. Each sample was measured in duplicate. Results are plotted as column scatter plots of the three genotype groups. Horizontal bars represent means. The TFPIr of the different genotype groups were compared using Student's t-test.

In addition, we also tested plasma from members of the family with the FV Amsterdam bleeding disorder [26], which is caused by a mutation that up-regulates the expression of the FV-short$_{Amsterdam}$ splicing variant. The continuous prothrombinase assay with and without 100 nM TFPIα C-term was performed on plasma from the proband, her unaffected husband and their affected son (**Figure 7A**). As expected from the high affinity of FV-short for TFPIα, both carriers of the FV Amsterdam mutation had markedly reduced TFPIr (mean ± SEM, 0.16 ± 0.03 and 0.18 ± 0.01, respectively) as compared to their normal relative (0.29 ± 0.02) as well as normal pooled plasma (0.29 ± 0.01) (**Figure 7B**). Interestingly, the prothrombinase rates in the absence of peptide were 5-8 times higher in carriers of the FV Amsterdam mutation than in normal pooled plasma (**Supplemental Table 2**), in line with the notion that FV forms lacking the basic or the acidic region express appreciable prothrombinase activity prior to proteolytic activation [4].

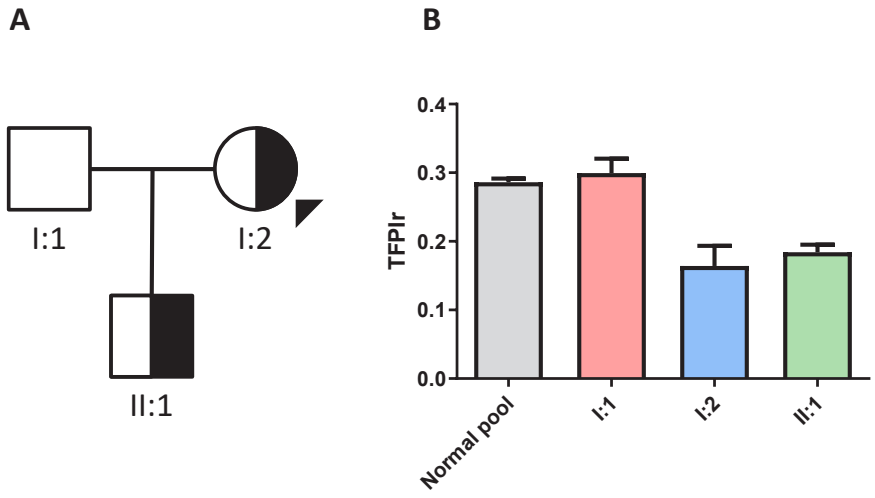


Figure 7. Effect of the FV Amsterdam mutation on the TFPIr. (A) Simplified pedigree of the FV Amsterdam family [26] showing only the three members investigated in this study. Carriers of the FV Amsterdam mutation are marked by half-filled symbols; the proband is indicated by an arrow. **(B)** Normal pooled plasma and plasma samples from the three family members were measured in the continuous prothrombinase assay in the absence or presence of 100 nM TFPIα C-term, as described under Methods. Prothrombinase rates with and without peptide were determined from parabolic fits of the absorbance data (see **Supplemental Table 2**) and TFPIr were calculated. Bars represent the mean ± SEM of 4 determinations.

To better understand at which level the TFPIα C-term peptide exerts its inhibitory action, the peptide was added to plasma before or after the FV pre-activation step. For all tested samples (normal pooled plasma, FVL homozygous plasma pool and both FV Amsterdam carriers), addition of the peptide after the FV pre-activation step resulted in a substantial reduction of the inhibitory effect (**Figure 8**), indicating that the TFPIr obtained in our normal assay set-up reflects peptide-mediated inhibition of both FV activation and prothrombinase. Interestingly, the relative contributions of these two components to total inhibition was different for different samples, as addition of the peptide after the FV pre-activation step increased the TFPIr only 1.7 times (from 0.38 to 0.65) in FVL plasma, but up to 3 times (from 0.17 to 0.51) in FV Amsterdam plasma.

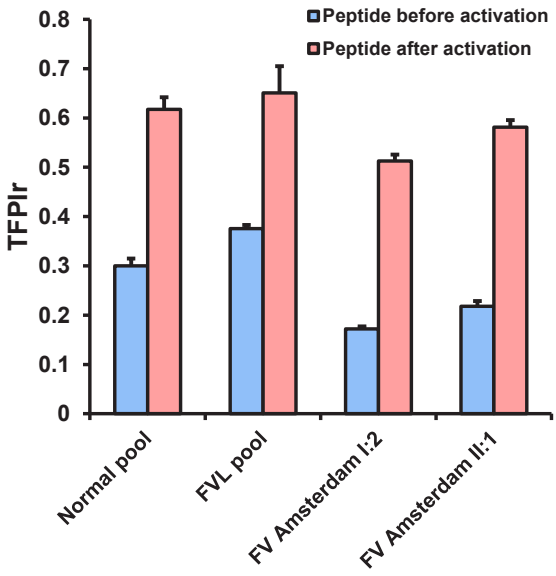


Figure 8. Inhibition of FV activation and prothrombinase activity by the TFPIα C-term peptide. Normal pooled plasma, a FVL homozygous plasma pool and plasma from the two carriers of the FV Amsterdam mutation were measured in the continuous prothrombinase assay in the absence or presence of 100 nM TFPIα C-term. The peptide was added to the plasma either before or after the FV pre-activation step. Prothrombinase rates with and without peptide were determined from parabolic fits of the absorbance data and TFPIr were calculated. Bars represent the mean ± SEM of 4 measurements.

DISCUSSION

The recently discovered ability of TFPIα to inhibit full activation [19] and prothrombinase activity [20] of early FV(a) intermediates represents a new anticoagulant mechanism. Although its pathophysiological significance is still largely unexplored, functional studies in carriers of FV gene mutations associated with coagulation disorders suggest that differences in the susceptibility of FV(a) to inhibition by TFPIα may contribute to the risk of venous thrombosis and/or bleeding [25-27]. To explore this further, we have developed a plasma-based assay that quantifies the susceptibility of FV to inhibition by the TFPIα C-terminus, which contains all known binding sites for FV on TFPIα [21]. This assay relies on FXa-catalysed activation of plasma FV followed by the continuous measurement of prothrombinase activity, both steps performed in the absence or presence of a peptide consisting of the last 35 residues of mature TFPIα.

The main challenge that we faced during the development of this assay was using plasma as the source of FV while preventing other plasma factors from influencing the outcome of the assay. To minimize the effect of the plasma background, we chose a high plasma dilution (1/1000 final, corresponding to ~25 pM FV), exploiting the high sensitivity of the detection system (prothrombinase) to minimal concentrations of FV(a) [30]. Comparison of the prothrombinase activities of FV(a) diluted in buffer or in 1/1000 FV-depleted plasma showed that, even at this high dilution, the plasma background was not completely neutral, but caused a 20-25% increase in prothrombinase rates. This effect could be largely attributed to the activation of the plasma prothrombin by FXa/FV(a) during the FV activation step and consequent feedback activation of FV(a) by the formed thrombin. Since thrombin efficiently cleaves FV(a) at Arg^{1545} [7], thereby removing the acidic region and abrogating the regulation of prothrombinase by TFPIα [19, 20], the pre-incubation time was limited to 3 minutes to minimize thrombin formation during the FV activation step. Although the partially activated FV(a) intermediates generated during this short pre-incubation step express very little prothrombinase activity, they are known to be physiologically relevant and to play a pivotal role in the initiation of coagulation [31].

To probe the susceptibility of FV(a) to TFPIα, the TFPIα C-terminal peptide was used instead of full-length TFPIα to avoid inhibition of FXa by the Kunitz-2 domain [15, 21]. This peptide contains both the basic region that binds with high affinity to the acidic region of FV(a) (close to the Arg^{1545} cleavage site) and the neutral amino acids that compete with FXa for binding to the heavy chain of FV(a) [20, 21]. Therefore, the peptide can inhibit the procoagulant activity of FV(a) at two levels: during the pre-incubation step it interferes with cleavage of FV at Arg^{1545} [19], trapping FV in partially activated forms that retain the acidic region and have a low affinity for FXa [8]; and during the prothrombinase step it inhibits the binding of these FV activation

intermediates to FXa and hence the expression of prothrombinase activity [20]. Actual inhibition of both processes was confirmed by a control experiment in which the peptide was added before or after FV activation, resulting in markedly reduced inhibition when the peptide was present only during the prothrombinase step. In fact, inhibition of FV activation represented a substantial proportion of the overall inhibition by the peptide, especially in the FV Amsterdam samples. Taken together, our data indicate that the prothrombinase rate obtained in the presence of a standard concentration of peptide (100 nM), normalised to the prothrombinase rate in the absence of peptide (*i.e.* the TFPIr), is a good measure of the overall susceptibility of the plasma FV to inhibition by the C-terminus of TFPIα. While the prothrombinase rates with and without peptide were a function of the plasma FV and prothrombin levels, their ratio was independent of variations of these plasma factors within the physiological range.

The TFPIr of normal pooled plasma was ~0.30, with an intra-assay and inter-assay CV of 6.2% and 9.7%, respectively. These values make the TFPIr a relatively robust assay outcome, despite the large inter-assay variation of the prothrombinase rates without and with peptide. The latter was most likely caused by day-to-day differences in the reagents (especially the phospholipid vesicles, which were prepared fresh every day), since the reproducibility of replicates measured on different plates on the same day approached the intra-assay variation. To minimize the effect of the day-to-day variation, it may be worth considering normalizing the TFPIr to the TFPIr of normal pooled plasma taken along on each plate.

The assay was validated using plasma from carriers of the FVL and FV Amsterdam mutations. FVL [13] predicts an amino acid substitution (Arg^{506}àGln) in the heavy chain of FV(a) that has recently been shown to interfere with the inhibition of prothrombinase by TFPIα [27]. Our assay correctly identified this abnormality as a significantly increased TFPIr in FVL carriers *vs.* non-carriers, confirming that FVL confers TFPI resistance as well as APC resistance, both contributing to the risk of venous thrombosis. In contrast, the FV Amsterdam mutation [26] has been associated with a bleeding disorder due to the over-expression of a pathological FV splicing variant (FV-$short_{Amsterdam}$) with high affinity for TFPIα and consequent up-regulation of circulating TFPIα [26]. Our assay showed that carriers of FV Amsterdam have markedly reduced TFPIr, indicating that FV-$short_{Amsterdam}$ is very sensitive to inhibition by TFPIα. The same is likely to apply to the structurally similar FV-$short_{East\text{-}Texas}$ isoform, whose over-expression causes the East Texas bleeding disorder [25]. In fact, although we could not test plasma from patients with this disorder, simulations in FV-depleted plasma reconstituted with recombinant FV and FV-$short_{East\text{-}Texas}$ showed that FV-$short_{East\text{-}Texas}$ also decreases the TFPIr (data not shown). Whether the high susceptibility of the FV-short isoforms to inhibition by TFPIα also contributes to the bleeding tendency associated with the FV East Texas and Amsterdam mutations, besides the high TFPIα levels and the enhanced cofactor activity of FV-short for TFPIα [32], remains to be elucidated.

These illustrative examples demonstrate the ability of our assay to recognize both decreased susceptibility of FV(a) to TFPIα (as in FVL, a thrombophilic mutation) and increased susceptibility (as in FV Amsterdam, a haemorrhagic defect). Moreover, they show that the assay probes not only the primary, high-affinity interaction between the basic region of TFPIα and the acidic region of FV(a) (as in FV-short$_{Amsterdam}$, where this interaction is tighter due to the absence of the FV basic region), but also the more recently identified secondary interaction between neutral residues of the TFPIα C-terminus and a FXa-binding site in the heavy chain of FV(a) (which is disturbed by the Arg^{506}àGln substitution in FVL) [21].

In conclusion, we have developed and validated an assay that measures the susceptibility of plasma FV(a) to inhibition by the C-terminus of TFPIα. This assay will make it possible to test whether (genetically determined) inter-individual differences in TFPIα-mediated inhibition of FV(a) correlate with the risk of venous thrombosis or bleeding in population studies.

ACKNOWLEDGEMENTS

We thank D.P. Suylen for synthesising the TFPIα C-term peptide. This work was supported by grant 2014-1 from the Dutch Thrombosis Foundation. Jan Rosing is Van de Laar Professor of Coagulation Enzymology.

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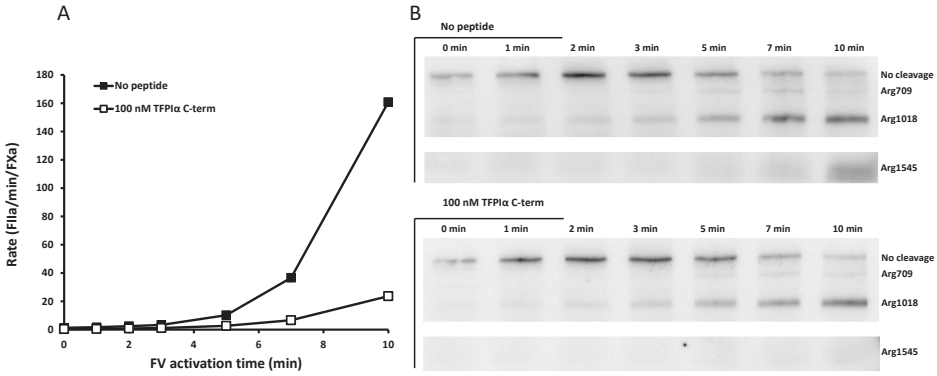
SUPPLEMENT



Supplemental Figure 1. Time-course of FV activation by FXa in the presence and absence of TFPIα C-term. (A) FV in 1/680 diluted plasma was activated with ~29 pM FXa for 0-10 minutes in the absence or presence of the TFPIα C-term peptide before adding the prothrombin/chromogenic substrate mixture. The prothrombinase rates obtained from the parabolic fits of the absorbance tracings were plotted as function of the pre-incubation time. **(B)** The FV activation time-course in the absence and presence of TFPIα C-term was analysed by Western blot as described under Methods. FV(a) bands were visualized using an antibody against the light chain of FV. Bands arising from cleavage at Arg^{709}, Arg^{1018} and Arg^{1545} are indicated. Please note the delay in the appearance of the light chain in the presence of peptide (best visible at the latest time points).

Supplemental Table 1. Prothrombinase rates in the absence and presence of TFPIα C-term and TFPIr in individuals with different FV Leiden (FVL) genotypes

	Sample #	Rate without TFPIα C-term (FIIa/min/FXa)*	Rate with 100 nM TFPIα C-term (FIIa/min/FXa)*	TFPIr
Non carriers	1	2.82	0.94	0.34
	2	2.45	0.72	0.31
	3	2.91	0.91	0.31
	4	2.42	0.68	0.28
	5	2.30	0.69	0.31
	6	3.36	0.91	0.27
	7	6.13	1.37	0.24
	8	3.53	0.96	0.27
	9	2.66	0.74	0.28
	10	2.40	0.62	0.25
	11	3.70	1.14	0.32

Supplemental Table 1. Continued

	Sample #	Rate without TFPIα C-term (FIIa/min/FXa)*	Rate with 100 nM TFPIα C-term (FIIa/min/FXa)*	TFPIr
	12	2.64	0.68	0.26
	13	1.82	0.63	0.33
	14	1.30	0.43	0.33
	15	2.02	0.62	0.30
FVL hetero	16	3.47	1.33	0.38
	17	1.84	0.72	0.39
	18	1.59	0.56	0.35
	19	3.89	1.34	0.35
	20	1.85	0.65	0.36
	21	3.21	1.01	0.31
	22	2.69	0.96	0.36
	23	1.93	0.79	0.39
	24	1.86	0.67	0.36
	25	3.23	1.17	0.37
	26	2.16	0.72	0.33
	27	3.76	1.34	0.36
	28	2.81	0.93	0.33
	29	3.13	1.12	0.35
	30	2.16	0.73	0.33
FVL homo	31	2.94	1.14	0.39
	32	2.47	0.98	0.40
	33	3.22	1.28	0.41
	34	1.78	0.68	0.38
	35	4.01	1.31	0.33
	36	1.37	0.46	0.34
	37	1.36	0.56	0.41
	38	2.13	0.86	0.41
	39	2.77	1.07	0.38
	40	1.97	0.82	0.41

* Average of 2 experiments

Supplemental Table 2. Prothrombinase rates in the absence and presence of TFPIα C-term and TFPIr in members of the family with the FV Amsterdam bleeding disorder [1]

Sample	Status	Rate without TFPIα C-term (FIIa/min/FXa)*	Rate with 100 nM TFPIα C-term (FIIa/min/FXa)*	TFPIr
I:1	Not affected	2.38	0.70	0.29
I:2	Affected	21.14	3.29	0.16
II:1	Affected	14.43	2.63	0.18
Normal pool	-	2.72	0.78	0.29

* Average of 4 experiments

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CHAPTER 5

Development of a functional assay to measure factor V-short in plasma

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Preliminary report

CHAPTER 6

General discussion

GENERAL DISCUSSION

Coagulation factor V (FV) is a key regulator of thrombin formation, as it possesses both pro- and anticoagulant functions [1]. It is well established that activated FV (FVa) expresses procoagulant activity as an essential cofactor to factor Xa (FXa) in the prothrombinase complex, which converts prothrombin to thrombin. However, FV also expresses anticoagulant activity as a cofactor to activated protein C (APC) in the inactivation of factor VIIIa (FVIIIa). More recently it has been shown that FV also binds the anticoagulant protein TFPIα [2, 3]. This interaction, which involves the basic C-terminus of TFPIα and an acidic region in the B-domain of FV, prolongs the half-life of full-length TFPIα in the circulation [2, 4], but also affects the activities of both proteins. In fact, it has been observed in model systems that FV enhances the inhibition of FXa by TFPIα [5-7] (a second anticoagulant function of FV), whereas TFPIα inhibits prothrombinase complexes containing partially activated forms of FV [8, 9]. The pathophysiological relevance of these interactions is underscored by the recent discovery of FV splicing isoforms (FV-short) with increased affinity for TFPIα, which are up-regulated in families with unexplained bleeding [4, 10].

This thesis focuses on the role of functional interactions between FV and TFPIα in the regulation of the early phases of coagulation, which is currently an active area of research. This chapter provides a critical overview and general discussion of our findings.

MAIN FINDINGS OF THIS THESIS

The work presented in this thesis sheds new light on the functional consequences of the FV-TFPIα interaction and describes new assays that measure parameters relevant to the FV-TFPIα interaction in individual plasmas. The main findings can be summarized as follows:

1) The cofactor activity of FV for TFPIα, which so far has been shown only in models systems with purified proteins, is also detectable in plasma, where it manifests itself as lower thrombin generation at higher FV concentrations. This effect is mostly mediated by the FV2 glycosylation isoform, corroborating the hypothesis that FV acts by promoting the binding of TFPIα to phospholipids (**Chapter 2**).

2) TFPIα not only inhibits the prothrombinase activity of partially activated forms of FV, but also inhibits the cleavage of FV at Arg^{1545} by both FXa and thrombin. This is important because cleavage at this position makes FV fully active as a procoagulant cofactor and completely abolishes its anticoagulant functions (**Chapter 3**).

3) Since inter-individual differences in the susceptibility of FV to inhibition by TFPIα are likely to contribute to the risk of venous thrombosis or bleeding, we have developed an assay that measures the susceptibility of FV to inhibition by the C-terminus of TFPIα in plasma from different individuals (**Chapter 4**).

4) In the absence of an ELISA for FV-short, we have devised a functional assay to measure the levels of FV-short in plasma (**Chapter 5**). The development and validation of this assay are still ongoing.

CONSEQUENCES OF THE FV-TFPIα INTERACTION

Anticoagulant activity of FV as cofactor to TFPIα

Experiments in model systems have demonstrated that FV (and particularly FV-short [11]) expresses cofactor activity to TFPIα in the inhibition of FXa [5-7], which is greatly enhanced by protein S [7, 11]. In **Chapter 2** we show that this anticoagulant activity of FV is also observed in plasma. In fact, when plasma-purified FV was titrated in FV-depleted plasma, thrombin generation increased between 0 and 5% FV, but progressively decreased at higher FV concentrations. This anticoagulant effect of FV could be attributed to the cofactor activity of FV to TFPIα based on several observations: 1) The effect was abolished by inhibitory antibodies against TFPIα, but not by anti-protein S or anti-protein C antibodies; 2) The anticoagulant effect was much more pronounced at a low than at high tissue factor (TF) trigger, in line with the different sensitivities of these assay conditions to TFPIα; 3) The effect was virtually lost when FV was replaced by FVa, which does not bind TFPIα. At low FV levels (5-10%) the procoagulant activity of FV is already saturated, but the cofactor activity to TFPIα is not. Therefore, higher FV concentrations only contribute to the anticoagulant activity of FV, leading to a decrease of thrombin generation. This conclusion is supported by recent epidemiological findings in the MEGA study, a large population-based case-control study on venous thrombosis, showing an association between low FV levels (57-63% FV) and an ~30% elevated risk on venous thrombosis [12]. Interestingly a recent paper also describes a patient with a FV deficiency (20% FV) with recurring episodes of thrombosis [13].

By analogy with protein S, it has been proposed that FV stimulates TFPIα by promoting its binding to negatively charged phospholipids, where FXa, the target of the inhibition, localizes. To test this hypothesis, we compared two glycosylation isoforms of FV with different phospholipid binding affinities (FV1 and FV2). FV2, which has a higher affinity for negatively charged phospholipids, proved to be a better cofactor to TFPIα than FV1, both in a model system and in plasma. The physiological relevance of this difference was further supported by a population study with carriers of the FV R2 haplotype, a common FV allele associated with lower FV levels

and an increased FV1/FV2 ratio. Carriers of this haplotype showed higher plasma thrombin generation than non-carriers, which could not be explained by the lower FV levels of FV R2 carriers, suggesting that the increased FV1/FV2 ratio also contributes to increase thrombin generation in FV R2 carriers. This is in line with a Japanese study, where both low FV levels and low phospholipid binding capacity of FV were associated with a >6-fold increased risk of deep-vein thrombosis (<66.5% FV-antigen and <63% Phospholipid bound FV) [14].

Protein S and FV are known to act as synergistic cofactors to TFPIα in the inhibition of FXa [6, 7, 11], but it is still a matter of debate whether or not protein S is essential for the expression of the TFPIα-cofactor activity of FV. In fact, conflicting results have been reported and it has been suggested that protein S may be inadvertently introduced in the reaction mixture as a contaminant of plasma-derived FV preparations [7]. In our hands, FV did express TFPIα-cofactor activity in model systems without protein S, as well as in plasma in the presence of neutralizing antibodies against protein S. However, the protein S requirement in the model system was strongly dependent on the individual FV preparation, the phospholipid batch and also the order of reagent addition. If FV was pre-incubated with FXa and phospholipids before being added to the reaction mixture containing chromogenic substrate and TFPIα, protein S did not seem to be necessary for TFPIα-cofactor activity (unpublished observation), suggesting that protein S is required in the presence of intact FV, while partially activated FV can act as a TFPIα-cofactor on its own. This could also explain the lack of protein S requirement in our plasma experiments, since FV becomes partially activated during initiation of thrombin generation. In contrast, fully activated FVa (which lacks the acidic region needed for interaction with TFPIα) has been shown to be devoid of TFPIα-cofactor activity, both in the absence and presence of protein S, and to protect FXa from inhibition by TFPIα [6, 7, 11].

Taken together, these findings suggest that exposure of the acidic region through proteolytic excision of the basic region may enhance the TFPIα-cofactor activity of FV and make it less dependent on protein S. Interestingly, it has recently been reported that FV-short, which constitutively lacks the basic region, is ~10-fold more potent as a TFPIα-cofactor and has a much lower protein S requirement than full-length FV [11]. However, more studies are needed to unravel the precise structural requirements for FV to function as a cofactor to TFPIα in the absence and presence of protein S.

Inhibition of the Arg^{1545} cleavage site by TFPIα

During the early phases of coagulation, a FV activation intermediate is formed which is already cleaved at Arg^{709} and Arg^{1018}, but not yet at Arg^{1545}. This partially activated FV(a) has some affinity for FXa [15] and can express FXa-cofactor activity in the prothrombinase complex [16, 17]. However, since it retains the acidic region while lacking the basic region, its procoagulant activity is efficiently inhibited by TFPIα [8]. Moreover, this intermediate still retains its anticoagulant

activities as a cofactor to TFPIα (unpublished observation) and APC [18]. Only after cleavage at Arg^{1545} and loss of the acidic region FV(a becomes fully activated, committing itself to the procoagulant pathway. Therefore, the ability of TFPIα to inhibit cleavage at Arg^{1545}, as described in **Chapter 3**, is an important regulatory mechanism.

In order to probe the FV-TFPIα interaction, a peptide resembling the C-terminus of TFPIα (TFPIα C-term) was synthesized. Using this peptide, the effect of the TFPIα C-terminus on FV could be isolated from the inhibitory effects that the TFPIα Kunitz domains have on the TF-Factor VIIa complex and FXa. When the TFPIα C-term peptide was titrated in plasma, a dose-dependent inhibition of thrombin generation was observed, which was dependent on the presence of full-length TFPIα. Although this effect could be explained by the previously reported inhibition of prothrombinase by TFPIα [8], control experiments suggested that FV activation might also be affected by the peptide. In fact, Western blot analysis showed that cleavage of the Arg^{1545} site in FV was delayed in the presence of TFPIα C-term. Inhibition of the Arg^{1545} cleavage has various consequences for the onset of coagulation. Firstly, full prothrombinase activity is not achieved, since FV(a) has a lower affinity for FXa [15] and expresses less FXa-cofactor activity [16, 17] than FVa. Moreover, the prothrombinase activity of FV(a) is susceptible to inhibition by TFPIα [8]. Finally, FV(a) retains its TFPIα- (unpublished observations) and APC-cofactor activities [18] until cleavage at Arg^{1545} occurs.

Although full-length TFPIα was much more effective than the TFPIα C-term peptide, the full length TFPIα concentration (10 nM) required for optimal inhibition of FV activation was still ~10 times higher than the physiological TFPIα concentration. This may be due to the fact that our experiments were done with intact single-chain FV, where the acidic region is bound to the basic region of FV and therefore not readily available for interaction with TFPIα. However, the actual targets of this inhibitory mechanism *in vivo* are likely to be forms of FV that lack the basic region and therefore have higher affinity for TFPIα, such as FV-short, partially cleaved FV intermediates generated at the onset of coagulation and platelet FV,(which is released locally at the site of injury together with TFPIα) [19].

Whether Arg^{1545} is the only FV cleavage site that is affected by the TFPIα C-terminus is still unclear. A recently published abstract [20] proposes that the binding site of TFPIα on FV comprises an acidic region near the C-terminus of the FV heavy chain (AR1, amino acids 659-695 as well as the acidic region in the B-domain of FV (AR2, amino acids 1493-1537 [21]). These acidic regions are thought to form a common platform for the binding of the TFPIα C-terminus. Since the Arg^{709} cleavage site is adjacent to AR1, it is possible that Arg^{709}, like Arg^{1545}, is protected from cleavage when TFPIα binds to the acidic platform. Preliminary experiments in model systems indeed indicate that the Arg^{709} cleavage is inhibited in the presence of TFPIα C-term, especially when FXa rather than thrombin is used to activate FV (unpublished observation). Whether or not this inhibition is physiologically relevant and occurs in plasma with full-length TFPIα still has to be addressed.

ASSAY DEVELOPMENT

Susceptibility of FV to TFPIα

Plasma FV is very heterogeneous due to genetic variation in the *F5* gene, alternative splicing of the *F5* pre-mRNA and different post-translational modifications of the FV protein, all of which may affect the susceptibility of FV to inhibition by TFPIα. For example, prothrombinase complexes containing FV Leiden (FVL) have been reported to be less susceptible to inhibition by TFPIα [22], while the FV-short isoform has increased affinity for TFPIα [4] and may therefore be more susceptible to TFPIα-mediated inhibition [23]. Differences in the susceptibility of FV to inhibition by TFPIα may affect the initiation of coagulation and be of pathophysiological importance. Therefore, an assay was developed to quantify the susceptibility of FV to TFPIα, in view of correlating this property of FV with the individual risk of thrombosis and/or bleeding.

The assay, described in **Chapter 4,** relies on the ability of the C-terminus of TFPIα to inhibit both the activation and the prothrombinase activity of FV(a). Highly diluted plasma is used as the source of FV and incubated with and without the TFPIα C-term peptide. FV is pre-activated with FXa to form FV(a) and the resulting prothrombinase activity is monitored continuously using a chromogenic substrate for thrombin. Both these steps (FV activation and prothrombinase) are inhibited in the presence of TFPIα C-term, yielding a lower prothrombinase rate in the presence of peptide. Plasma FV and prothrombin levels were found to affect the prothrombinase rates without and with peptide. However, expressing the outcome of the assay as the ratio of the prothrombinase rates with and without peptide (TFPI resistance ratio, TFPIr), abolished the variability between plasmas arising from differences in coagulation factor levels. Differences in TFPIr were only dependent on the "quality" of the FV. Validation of the assay showed that plasma from FVL carriers resulted in a higher TFPIr, confirming the reduced susceptibility of FVL to TFPIα [22]. On the other hand, plasma from FV Amsterdam carriers, with high levels of FV-short$_{Amsterdam}$, had lower TFPIr, in line with the increased affinity of FV-short for TFPIα [4, 10].

The FVL mutation induces a hypercoagulable state mainly due to its APC resistance. Whether the added procoagulant effect of the reduced susceptibility to TFPIα is also physiologically relevant remains to be elucidated. Similarly, it is still unclear to what extent the increased susceptibility of FV-short to TFPIα contributes to the bleeding phenotype of carriers of the FV Amsterdam mutation. In fact, the bleeding tendencies of the East Texas and Amsterdam family have been generally attributed to the markedly elevated TFPIα levels that accompany the over-expression of FV-short variants [4, 10]. However, experiments in which FV-depleted plasma was reconstituted with FV or FV-short showed that FV-short supports considerably less thrombin generation than FV, even at equal TFPIα levels (unpublished observation). This may be due to the higher cofactor activity of FV-short to TFPIα [11], but also to the increased susceptibility of FV-short to inhibition by TFPIα observed in our study.

Besides FVL and FV Amsterdam, it would be interesting to screen other FV variants for their TFPIα susceptibility and to test whether the latter correlates with the risk of thrombosis or bleeding. Interesting candidates would be *F5* haplotypes that predict amino acid substitutions or differential post-translational modifications in the FV B-domain, as we have observed that recombinant FV (rFV) expressed in COS-1 cells is less susceptible to TFPIα than plasma-derived FV, which is likely caused by differences in their post-translational modifications.

FV-short assay

FV-short is an alternatively spliced isoform of FV present at low levels (≤1 nM) in all individuals [24]. Due to the absence of a large part of the FV B-domain, including the basic region, FV-short has different properties compared to FV, such as a higher affinity for TFPIα and intrinsic prothrombinase activity [4, 19, 25]. FV-short levels vary widely among individuals [4]. Markedly elevated FV-short levels have been associated with a bleeding phenotype [4], whereas low expression of FV-short might be associated with a hypercoagulable state. Unfortunately, the high similarity between FV and FV-short and the much higher abundance of FV in plasma make it difficult to develop an ELISA assay specific for FV-short, with the result that no (quantitative) FV-short assay is yet available. In fact, a subtractive ELISA that quantifies FV-short as the difference between total FV and full-length FV might not be accurate enough to detect small inter-individual differences in the FV-short concentration [4]. Therefore, we devised a functional assay to measure FV-short levels in plasma.

This assay, described in **Chapter 5,** is based on the measurement of prothrombinase activity in highly diluted plasma without pre-activation of the plasma FV. Since FV-short is intrinsically active, whereas FV is not, the measured prothrombinase rates should reflect the amount of FV-short present in plasma. Although the assay is still under development, the preliminary data available so far indicate that the assay is indeed sensitive to FV-short. In fact, plasma from a carrier of the FV-Amsterdam mutation yielded ~3 times higher prothrombinase rates than a normal plasma with the same total FV level. Moreover, recombinant FV-short$_{\text{East-Texas}}$ and FV-short$_{\text{Amsterdam}}$ yielded 30-60 fold higher prothrombinase rates than recombinant FV. However, it is doubtful whether FV-short is the only determinant of the assay, because recombinant FV showed higher prothrombinase rates in buffer than in plasma, suggesting that an unknown plasma component (whose concentration could vary from plasma to plasma) inhibits the assay (≤2-fold).

A small population study including 45 healthy individuals was also performed to assess the determinants of the FV-short assay. This study showed that the inter-individual variation in prothrombinase rate is 3 times as high as the inter-assay variation, pointing at real differences between individuals. In addition, preliminary evidence was obtained for an effect of age (prothrombinase rate decreasing with age) and sex (slightly higher prothrombinase

rates in males than in females) on the prothrombinase rate. The prothrombinase rate showed a borderline-significant positive correlation with total FV, which might be due to the fact that FV-short is a sub-population of the total plasma FV pool and shares transcriptional regulation with full-length FV. Differently, no correlation with full-length TFPIα was observed, despite the fact that FV-short is considered to be the main carrier of TFPIα in the circulation [4].

In the absence of an independent method to measure FV-short, it remains challenging to prove that our prothrombinase assay reliably quantifies FV-short. Correlation of the prothrombinase rates with the levels of expression of the FV-short transcript in blood cells of the same individuals could provide some indications, although blood cells are not the physiological tissue of expression of FV(-short) and mRNA levels do not necessarily correlate with protein levels.

CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, the work presented in this thesis shows some of the effects that arise from the interaction between FV and TFPIα (summarized in **Figure 1**). We demonstrate that FV increases the inhibitory activity of TFPIα towards FXa in plasma, but TFPIα also affects FV by preventing it from achieving its full procoagulant activity. The ability of the TFPIα C-terminus to inhibit FV activation and prothrombinase activity were used for the development of a FV TFPIα susceptibility assay. The observation that this assay is very sensitive to the FV-short isoform have led to the development of a specific assay to measure FV-short levels in plasma.

Application of the developed assays will provide valuable information on the pathophysiological significance of the FV-TFPIα interaction and FV-short levels. It is not unlikely that decreased affinity of FV for TFPIα will result in poor inhibition of the early phases of coagulation, resulting in a higher thrombotic risk. *Vice versa*, an increased susceptibility to TFPIα, which might also correlate with increased concentrations of FV-short, could have the opposite effect.

Future perspectives

Although in recent years we have learnt a lot about the functional significance of the FV-TFPIα interaction, several questions still remain unanswered.

First of all, the cofactor activity of FV to TFPIα has not been fully explored yet. In particular, it is still unclear what are the exact structural requirements and protein S dependence of the TFPIα cofactor activity of FV. Also, it is not yet known whether FV enhances only the inhibition of FXa or also the inhibition of TF/FVIIa by TFPIα (and protein S).

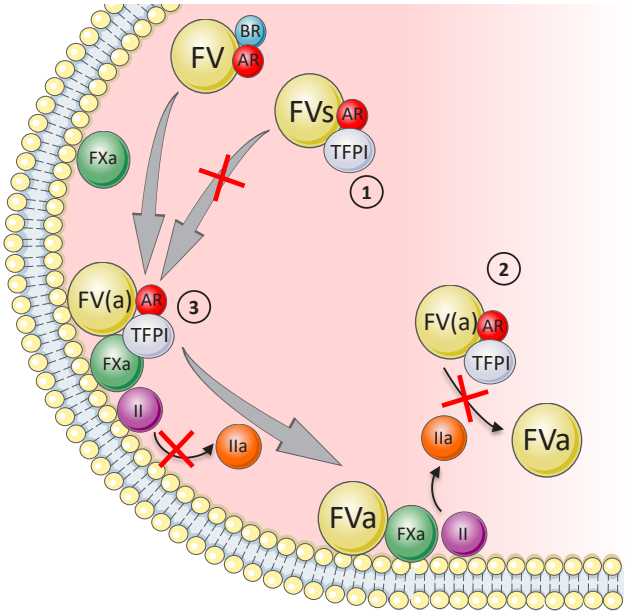

Figure 1. Schematic overview of the functional interactions between FV and TFPIα. TFPIα binds with high affinity to FV variants containing the B-domain acidic region (AR), but not the basic region (BR), such as FV-short (FVs) ① and FV activation intermediates (FV(a)) ②. TFPIα inhibits full activation (cleavage at Arg^{1545}) of FV by both FXa ① and thrombin (IIa) ② (**Chapter 3**), indicated with red crosses. Additionally, TFPIα inhibits complex formation with FXa ①. When FV forms a complex with FXa, it is susceptible to inhibition by TFPIα, due to its exposed AR, resulting in reduced prothrombinase activity ③. Finally, FV acts as a cofactor to TFPIα in the inhibition of FXa ③ by increasing the lipid affinity of TFPIα (**Chapter 2**).

More research is also needed to better characterize the functional properties of FV-short and to understand its role in the regulation of (the early phases of) coagulation. The constitutive prothrombinase activity of FV-short and its TFPIα-dependent anticoagulant properties may have different outcomes depending on the levels of TFPIα. These studies could pave the way to novel therapeutic options for thrombotic and/or bleeding disorders based on the modulation of the FV-short splicing event.

Besides our functional FV-short assay, we are currently working on the development of a FV-short ELISA. This ELISA would facilitate correlating FV-short levels with clinical endpoints in epidemiological studies on venous thrombosis and bleeding.

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CHAPTER 7

Summary

SUMMARY

Coagulation factor V (FV) is a pivotal regulator of thrombin formation, as it has both pro- and anticoagulant properties. The procoagulant activity of FV as an essential cofactor of factor Xa (FXa) in the prothrombinase complex requires activation of FV to FVa by limited proteolysis of the FV B-domain. Activation is initiated by FXa, which cleaves FV at the Arg^{709} and Arg^{1018} sites, removing the B-domain basic region and generating the activation intermediate FV(a). FV(a) has increased affinity for FXa, but its prothrombinase activity is inhibited by tissue factor pathway inhibitor-α (TFPIα), whose basic C-terminus binds to the acidic region in the B-domain of FV(a). Only after the final cleavage of FV(a) at Arg^{1545}, which removes the acidic region, FV becomes fully activated (FVa) and committed to the procoagulant pathway. The anticoagulant properties of FV are largely mediated by TFPIα. FV acts as a carrier of TFPIα, thereby prolonging its half-life in the circulation, and enhances the inhibition of FXa by TFPIα.

TFPIα has particularly high affinity for FV forms that lack the basic region, such as the splicing isoform FV-short. Lack of the basic region makes FV-short constitutively active as a cofactor to FXa in prothrombinase. Moroever, exposure of the acidic region increases the affinity of FV-short to TFPIα, making FV-short a better carrier and cofactor of TFPIα. While FV-short is present in all healthy individuals, it was originally discovered in a family with the so-called East Texas bleeding disorder, which is caused by the genetic up-regulation of FV-short. Later, over-expression of a similar but distinct FV-short isoform was found responsible for the analogous Amsterdam bleeding disorder.

This thesis focusses on the functional interactions between FV and TFPIα and the consequences of this interaction for both TFPIα and FV. Additionally, assays have been developed to quantify the susceptibility of FV to inhibition by TFPIα and to measure the concentration FV-short in plasma.

Chapter 1 provides a general introduction to hemostasis and specifically the coagulation cascade. Extensive information is provided on the structure, function and regulation of the various forms of FV. Finally, the role of TFPIα and its interaction with FV is described in detail.

In **Chapter 2** we demonstrate that FV expresses cofactor activity to TFPIα in plasma. By titrating FV in FV-depleted plasma, we observed that thrombin generation increases between 0 and 5% FV, but progressively decreases at higher FV concentrations. This anticoagulant effect was dependent on the presence of TFPIα. Similar titrations with the glycosylation isoforms FV1 and FV2, which differ in their affinity for phospholipids, showed that this cofactor activity is dependent on phospholipid binding and mainly mediated by the FV2 isoform.

Following the effect of FV on TFPIα described in Chapter 2, in **Chapter 3** we examined how the interaction between FV and TFPIα affects FV. To this end, a peptide resembling the C-terminus of TFPIα (TFPIα C-term) was used to isolate the anticoagulant effects of the FV-TFPIα interaction from the inhibitory functions of the Kunitz domains of TFPIα. The effect of the TFPIα C-term was investigated in thrombin generation experiments and on FV activation in model systems. TFPIα C-term was found to inhibit thrombin generation triggered with tissue factor or FXa. The observation that FV-depleted plasma reconstituted with FVa was not inhibited by the TFPIα C-term pointed at FV activation and/or prothrombinase activity as the target of the inhibition. By following FV activation over time using Western-blot analysis, we observed that cleavage at the Arg^{1545} site was inhibited in the presence of TFPIα C-term. This inhibition was even more pronounced in FV-short. Protection of the Arg^{1545} cleavage site is an important regulatory mechanism of TFPIα, because cleavage at this position abolishes all anticoagulant functions of FV and commits FVa to its procoagulant function.

The inhibition of FV activation and prothrombinase activity by the TFPIα C-terminus may be of physiological importance if different forms of FV differ in their susceptibility to TFPIα. In fact, FV Leiden (FVL), associated with a thrombotic risk, has been reported to be less susceptible to inhibition by TFPIα, while FV-short has increased affinity for TFPIα. In **Chapter 4** we describe the development of an assay that measures the susceptibility of FV for the TFPIα C-terminus. The assay uses highly diluted plasma as a source of FV, which is pre-activated with FXa in the presence and absence of the TFPIα C-terminal peptide. After activation, prothrombinase is started by the addition of a mixture containing prothrombin and chromogenic substrate for thrombin, and absorbance is followed for 30 minutes in a plate reader. The ratio of the prothrombinase rates obtained in the presence and absence of TFPIα C-term (TFPIr) was taken as a measure of TFPI-resistance. The assay was validated using plasmas of heterozygous and homozygous FVL carriers (which showed increased TFPIr) and carriers of the FV Amsterdam mutation (which showed markedly decreased TFPIr). This assay provides a tool to investigate whether the susceptibility of FV to TFPIα is associated with the risk of thrombosis or bleeding in the general population.

During the development of the assay described in Chapter 4, we noticed that the prothrombinase rate in the absence of TFPIα C-term was sensitive to the amount of FV-short. This was likely caused by the intrinsic FXa-cofactor activity of FV-short. This property of FV-short was exploited in **Chapter 5** for the development of an assay that quantifies the FV-short concentration in plasma. In this assay FV is not pre-activated, in an attempt to make prothrombinase activity entirely dependent on FV-short. In this way, the obtained prothrombinase rates would reflect the amount of FV-short present in plasma. Preliminary results suggest that this is indeed the case, although other plasma determinants cannot be excluded. Validation of the assay in a small population of 45 healthy individuals is still ongoing.

Chapter 6 discusses the findings of this thesis and gives and overview of the conclusions. The results are analyzed in light of published literature and unresolved questions that are of interest for future research are discussed.

ADDENDUM

Samenvatting

SAMENVATTING

Stollingsfactor V (FV) vervult een centrale rol in de trombinevorming omdat het zowel stollende als antistollende eigenschappen heeft. FV dient eerst te worden geactiveerd voordat het zijn stollende functie kan uitoefenen als de essentiële cofactor van factor Xa (FXa) in het protrombinase complex. Deze activering bestaat uit de verwijdering van het FV B-domein door middel van proteolyse. Activering van FV begint met het knippen op Arg^{709} en Arg^{1018} door FXa. Hierdoor wordt de basische regio uit het B-domein van FV verwijderd wat zorgt voor de vorming van de FV tussenvorm FV(a). FV(a) heeft een hogere affiniteit voor FXa, maar kan tegelijkertijd worden geremd in het protrombinase complex door tissue factor pathway inhibitor-α (TFPIα), waarvan de C-terminus de acidische regio van FV bindt. Pas na de laatste knip op Arg^{1545}, waardoor de acidische regio van FV wordt verwijderd, wordt FV compleet geactiveerd (FVa) tot een volledig stollend eiwit. De antistollende eigenschappen van FV worden voornamelijk gemedieerd via TFPIα. Zo is FV een drager van TFPIα, waardoor de halfwaarde tijd van TFPIα in de bloedsomloop wordt verlengd. Daarnaast draagt FV bij aan de remming van FXa door TFPIα.

TFPIα heeft een uitzonderlijk hoge affiniteit voor FV varianten die de basische regio missen, zoals de splicing isovorm FV-short. Het ontbreken van de basische regio zorgt ervoor dat FV-short intrinsiek actief is als cofactor voor FXa. Bovendien zorgt de blootgestelde acidische regio ervoor dat FV-short een betere drager en cofactor is voor TFPIα. FV-short komt voor in elk gezond individu, maar is oorspronkelijk ontdekt in een familie met de “East-Texas” genetische mutatie die zorgt voor een verhoging van de FV-short expressie. Kort hierna is in een familie uit Amsterdam een soortgelijke mutatie gevonden die zorgt voor een vergelijkbare, maar net wat andere vorm van FV-short.

In dit proefschrift ligt de focus op de functionele interacties tussen FV en TFPIα en de consequenties van deze interacties voor zowel TFPIα als FV. Verder beschrijft dit werk de ontwikkeling van assays om de gevoeligheid van FV voor TFPIα te meten en om de concentratie van FV-short in plasma te bepalen.

Hoofdstuk 1 geeft een algemene inleiding over de hemostase en spitst zich daarna toe op de stollingscascade. Hierin wordt uitgebreid de structuur, functie en regulatie van de verschillende FV varianten besproken. Tot slot wordt de rol van TFPIα en zijn interactie met FV in detail beschreven.

In **Hoofdstuk 2** laten we zien dat FV cofactor activiteit heeft voor TFPIα in plasma. Door FV te titreren in FV-gedepleteerd plasma zagen we dat de trombinegeneratie omhoog ging tussen 0 en 5% FV maar daarna progressief afnam bij hogere concentraties FV. Dit antistollende effect

was afhankelijk van de aanwezigheid van TFPIα. Wanneer vergelijkbare titraties werden gedaan met de glycosylering isovormen FV1 en FV2, welke verschillen in hun affiniteit voor fosfolipiden, zagen we dat deze cofactor activiteit afhankelijk is van fosfolipidenbinding en voornamelijk wordt gemedieerd door FV2.

Als gevolg van het effect van FV op TFPIα, zoals beschreven in Hoofdstuk 2, wilden we in **Hoofdstuk 3** onderzoeken hoe de interactie tussen FV en TFPIα de werking van FV beïnvloedt. Om dit te bewerkstelligen hebben we een peptide gebruikt dat lijkt op de C-terminus van TFPIα (TFPIα C-term) om geen interferentie te hebben van de remmende eigenschappen van de Kunitz domeinen die aanwezig zijn in het volledige TFPIα molecuul. Het effect van de TFPIα C-term werd onderzocht middels trombinegeneratie experimenten en FV activering in model systemen. TFPIα C-term remde de trombinegeneratie wanneer deze werd gestart met tissue factor of FXa. De waarneming dat FV-gedepleteerd plasma gereconstitueerd met FVa niet geremd werd door het peptide wees erop dat de activering van FV of het protrombinase complex werd geremd. Door de activatie van FV in de tijd te volgen met Western-blot analyse zagen we dat de knip op Arg^{1545} was geremd in de aanwezigheid van TFPIα C-term. Deze remming was zelfs nog sterker in FV-short. Bescherming van de Arg^{1545} knip is een belangrijk regulatoir mechanisme van TFPIα omdat deze knip er voor zorgt dat alle antistollende eigenschappen van FV verloren gaan en FVa alleen nog stollende functies heeft.

De remming van de FV activering en de protrombinase activiteit door de TFPIα C-terminus kunnen van fysiologisch belang zijn wanneer verschillende FV varianten een andere gevoeligheid voor TFPIα hebben. Het is al beschreven dat FV Leiden (FVL), dat is geassocieerd met een verhoogd risico op trombose, minder gevoelig is voor remming door TFPIα terwijl FV-short juist een verhoogde affiniteit heeft voor TFPIα. In **Hoofdstuk 4** wordt de ontwikkeling van een assay beschreven die de gevoeligheid van FV voor de TFPIα C-terminus kan meten. De assay maakt gebruik van sterk verdund plasma als bron van FV dat wordt geactiveerd met FXa in de aan- en afwezigheid van het TFPIα C-term peptide. Daarna wordt de protrombinase gestart middels toevoeging van een reactiemengsel bestaande uit protrombine en chromogeen substraat voor trombine. De absorptie wordt vervolgens gemeten in een plate reader gedurende 30 minuten. De ratio die wordt verkregen uit de trombine vorming in de aan- en afwezigheid van de TFPIα C-term (TFPIr) wordt gebruikt om de TFPI-resistentie uit te drukken. De assay is gevalideerd met plasma van heterozygote en homozygote dragers van de FVL mutatie (welke resulteerde in een verhoogde TFPIr) en dragers van de FV Amsterdam mutatie (welke resulteerde in een aanzienlijk lagere TFPIr). Deze assay voorziet nu in een mogelijkheid om te onderzoeken of de gevoeligheid van FV voor TFPIα is geassocieerd met een risico op trombose of bloedingen in een algemene populatie.

Gedurende de ontwikkeling van de assay die beschreven is in hoofdstuk 4 viel het ons op dat de protrombinase snelheid in de afwezigheid van de TFPIα C-term erg gevoelig was voor de hoeveelheid FV-short. Een verklaring hiervoor is de intrinsieke FXa cofactor activiteit van FV-short. Deze eigenschap van FV-short wordt gebruikt in **Hoofdstuk 5** voor de ontwikkeling van een assay die de hoeveelheid FV-short in plasma kan bepalen. In deze assay vindt geen activering van FV plaats om zo de protrombinase activiteit in zijn geheel afhankelijk te maken van FV-short. Op deze manier zou de protrombinase activiteit een reflectie zijn van de hoeveelheid FV-short in het plasma. De voorlopige resultaten suggereren inderdaad dat dit het geval is, maar andere plasma determinanten kunnen nog niet worden uitgesloten. Validatie van de assay in een kleine populatie van 45 individuen is nog gaande.

Hoofdstuk 6 bediscussieert de bevindingen van dit proefschrift en geeft een overzicht van de conclusies. De resultaten zijn geanalyseerd aan de hand van gepubliceerde literatuur en onbeantwoorde vragen die van interesse zijn voor toekomstig onderzoek worden besproken.

ADDENDUM

Valorisation

VALORISATION

This chapter is intended to show how the knowledge obtained in this thesis is relevant outside a scientific context.

Valorisation - "*The process of value-creation out of knowledge, by making this knowledge suitable and available for economic or societal utilisation and to translate this into high-potential products, services, processes and industrial activity.*" [1]

The societal impact of a research project is often obvious when it produces a product or solution that can readily be utilised for an individual or group. This type of applied research is however not possible without prior knowledge about the basic mechanisms, which is obtained from fundamental research. Fundamental research does not have a commercial objective, as it is driven by a desire to test hypotheses and obtain new knowledge. As a result the societal impact may not be obvious at first, when only a small piece of the puzzle is found. The research can however end up being a center piece that connects other pieces of the puzzle.

The fundamental research on the underlying mechanisms of coagulation is pivotal for the development of novel drugs and treatments of venous thrombosis (VT). There is still much to be achieved in this area as VT is the third most common cardiovascular disease, right after coronary artery disease and stroke [2]. Patients suffering from VT carry an increased morbidity and mortality. A venous thrombotic event may lead to pulmonary embolism with death as a major outcome. These patients are highly dependent on long-term treatment, which currently still carries strong side effects such as an increased bleeding risk. The prevalence of VT rises sharply as a person's age rises [3]. With increasing life expectancy it can be predicted that VT will have an even stronger impact on society in the near future. Therefore much is to be gained with a better understanding of the mechanisms of blood coagulation, which in turn can lead to improved and targeted treatments.

The work in this thesis focusses on gaining a better understanding about the interaction between coagulation factor V (FV) and tissue factor pathway inhibitor-α (TFPIα). While this curiosity-driven research started off as fundamental in design, its results have already lead to the development of an assay for the detection of possible risk factors in VT, as explained below.

In **Chapter 2** we found that FV has anticoagulant properties in the presence of TFPIα. Increasing FV from 0 to 10% of normal FV levels increased thrombin generation, while a further increase up to 100% FV gradually decreased thrombin generation. These results imply that partial FV deficiency increases the risk on thrombosis. In fact, low FV levels have been associated with increased risk of thrombosis and a patient with recurrent thrombotic episodes due to low FV

levels has recently been described [4]. *Vice* versa, besides FV affecting TFPIα, TFPIα also affects FV. **Chapter 3** describes how the interaction between the C-terminus of TFPIα and FV prevents FV from reaching its full procoagulant potential. Insights in these mechanisms will benefit future research as the inhibition of TFPIα is currently of high interest as a possible bypassing agent for hemophilic patients with factor VIII inhibitors [5].

The interaction between FV and TFPIα was found to differ between various forms of FV. Certain FV mutations decreased the affinity for TFPIα, while other FV variants had an increased affinity. We therefore set out to develop a functional assay that could measure the susceptibility of different FV variants to inhibition by TFPIα. This assay, described in **Chapter 4**, provides a valuable tool to measure this newly discovered property of FV. The TFPIα susceptibility assay can be used in population studies to determine if the susceptibility of FV to TFPIα correlates with a risk on thrombosis or bleeding.

The interactions between FV and TFPIα as described in Chapters 3 and 4 are likely caused by the splicing isoform FV-short, which has a much higher affinity to TFPIα than full-length FV. FV-short levels are thought to vary greatly between individuals and are likely to be important determinants of the TFPIα levels. Increased levels of FV-short have already been associated with a bleeding phenotype [6]. It is however unknown if lower FV-short levels are also a risk factor for thrombosis. So far there are no assays for the quantification of FV-short. We therefore set out to develop the first functional assay for the detection of FV-short, as described in **Chapter 5**. This assay will in the future provide a valuable tool for the identification of the determinants of FV-short and for the correlation of FV-short levels with clinical end-points.

We are confident that the information obtained and described in this thesis provides a valuable contribution to the existing knowledge and will benefit future (applied) thrombosis research.

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ADDENDUM

Curriculum vitae

CURRICULUM VITAE

Peter van Doorn was born on the 9th of December 1987 in Helmond and grew up in Mierlo. After obtaining his VWO diploma in 2006 from the Jan van Brabant College, he obtained his bachelor degree in Applied Sciences from the Fontys University of Applied Sciences with distinction in 2011. Afterwards he attended the master molecular and cellular life sciences at Utrecht University and obtained his degree in 2013. During this master he performed internships at the cellular protein chemistry department of Utrecht University and MSD Animal Health in Boxmeer. The master's degree was followed up with a PhD project at Maastricht University under the supervision of Prof. T.M. Hackeng and dr. E. Castoldi. This PhD project focused on the consequences of the interaction between coagulation factor V and TFPIα and on the splicing isoform FV-short, as described in this thesis. During his PhD training he presented his work at several national and international congresses and won the award of scientific excellence from the Dutch Society for Thrombosis and Haemostasis in 2016.

ADDENDUM

List of publications

LIST OF PUBLICATIONS

Full papers

A. de Groof, M. Deijs, L. Guelen, L. van Grinsven, L. van Os–Galdos, W. Vogels, C. Derks, T. Cruijsen, V. Geurts,M. Vrijenhoek, J. Suijskens, **P. van Doorn,** L. van Leengoed, C. Schrier, L. Hoek, Atypical Porcine Pestivirus: A Possible Cause of Congenital Tremor Type A–II in Newborn Piglets. Viruses 2016, 8: 271.

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T.M. Hackeng, **P. van Doorn**, J. Rosing, E. Campello, S. Middeldorp, P. Simioni, J.C.M. Meijers, E. Castoldi, A plasma-based assay to measure the susceptibility of factor V(a) to Inhibition by TFPIα, ASH, San Diego CA, USA, 2018

Awards

NVTH Award of Excellence 2016

ADDENDUM

Dankwoord

DANKWOORD

De vier jaar bij de afdeling Biochemie die tot dit boekje hebben geleid was een geweldige tijd waarvan ik ontzettend heb genoten. Hoewel ik een man van weinig woorden ben, zoals de meesten wel zullen weten, wil ik toch graag een moment nemen om iedereen te bedanken. Zonder jullie was dit niet mogelijk geweest.

Beste Elisabetta, **Betta**; Ik heb ontzettend veel van je geleerd in de afgelopen vier jaar. We hebben vele uren samen gezeten om te brainstormen over het opzetten van experimenten om vervolgens nog een paar uur te sparren over de resultaten. Zowel als wetenschapper als persoon ben ik veel gegroeid onder jouw supervisie. Je toewijding aan de wetenschap, eerlijkheid en positiviteit vormen een bron van inspiratie. Keer op keer heb je me weten te fascineren met je kennis over vrijwel alles. Bedankt voor al je geduld en tijd. Ik hoop dat je net zo tevreden bent over dit boekje als ik, want hoewel alleen mijn naam op de voorkant staat heb jij hier ook veel energie in gestoken.

Beste **Tilman**, bedankt voor alle hulp over de afgelopen jaren. Ik had me geen fijnere promotor kunnen wensen. Ondanks dat je agenda doorgaans vol staat is je deur toch altijd open zodat mensen met vragen en hulp bij je terecht kunnen. Je hebt de afdeling Biochemie altijd als je familie beschouwd, dit was ook te merken. Ondanks de diversiteit aan mensen is het een hechte groep geworden waar iedereen zich welkom voelt. Dit ga ik erg missen. Ik bewonder je enthousiasme en kennis en wil je ontzettend bedanken voor je vertrouwen en steun in de afgelopen jaren.

Beste **Jan**, ondanks je terecht verdiende pensioen ben je door je passie voor de wetenschap nog frequent op de afdeling te vinden (met Sjoukje's toestemming natuurlijk). Ik wil je graag bedanken voor je betrokkenheid bij mijn promotietraject. Dankzij jouw kennis en inzichten hebben we in een relatief korte tijd veel kunnen bereiken. Je hebt een onfeilbaar voorspellend vermogen als het gaat om hypotheses opstellen. Bedankt voor je nuchtere blik en nodige scepsis om ons met beide benen op de grond te houden.

Geachte leden van de leescommissie, ik wil u allen hartelijk bedanken voor de tijd en aandacht die u heeft besteed aan het lezen van mijn manuscript.

Simone, vier jaar geleden was het aan jou de taak om mij wegwijs te maken bij de Biochemie. In de tijd dat we kantoorgenoten waren kon ik bij jou altijd mijn ei kwijt, bedankt voor je luisterend oor. Zelfs nadat je naar een andere groep was overgeplaatst kwam je nog geregeld langs voor een gezellig praatje. **Stella**, zonder jou zou de biochemie niet zijn wat het nu is. Je vrolijke aanwezigheid brengt iedereen energie. Voor een borrel of een feestje organiseren ben je altijd

te vinden. Je weet jezelf en anderen telkens uit te dagen met nieuwe “challenges”. Ik heb erg genoten van de tijd samen bij het ISTH in Berlijn. Bedankt voor al je enthousiasme! **Roy**, bedankt voor het wegwijs maken op wat nu nog steeds “Roy’s lab” is. Je bent een grote aanwinst bij Matisse, je expertise wordt nog steeds gemist bij de Biochemie.

Graag wil ik ook mijn twee paranimfen **Daniëlle** en **Annemiek** hartelijk danken voor alle leuke tijd die we hebben gehad in de afgelopen jaren. Fijn dat jullie tijd vrij willen maken om mijn paranimfen te zijn. Daniëlle, ontzettend leuk dat je zo veel enthousiasme had om regelmatig PhD uitjes te organiseren wat veel heeft bijgedragen aan het vormen van een hechte groep. Annemiek, vier jaar geleden zijn wij (bijna) tegelijk begonnen aan het avontuur dat promoveren heet. Succes met de laatste loodjes! Je was een ontzettend leuk kamergenootje, samen met **Danique**, **Joram** en **Greg** hebben we het gezelligste kantoor gedeeld. Danique, je open en to-the-point houding kon ik erg waarderen. Joram, succes (en sterkte) met je taak als lab-manager. Greg, good luck with your future endeavours.

Trees en **Lidewij**, bedankt dat ik altijd bij jullie mocht aankloppen voor praktische ondersteuning. Als er iets geregeld of gepland moest worden waren jullie altijd behulpzaam.

Rick en **Armand**, de lange afstand tussen onze kantoren weerhield jullie nooit om jullie gezelligheid te delen op de borrels en feestjes aan “onze kant van de gang”. **Boğaç**, your laid-back and open attitude made you a great colleague and friend. Good luck with the remainder of your PhD in Amsterdam. **Angelina, Johanna**, we had a great time during the ECTH in Marseille. **Stan,** de toekomstig FXI-expert, veel geluk met je carrière en een mooi huwelijksfeest gewenst. **Kanin**, good luck with all your travels. **Song**, I enjoyed the candy you brought back from China. **Alice**, best of luck with the continuation of the FV-short research. You’re in good hands with Betta as your supervisor. **Stepan**, thank you for introducing us to Russian rap music. **Rory**, knowledgeable about everything. Thank you for your valuable input during the Thursday morning meetings. **Hans**, altijd leuk om je te horen vertellen over je verschillende hobbies. **Ingrid**, succes met je verdere carrière. To my PhD predecessors **Farida**, **Francesca**, **Nahla**, **Stijn** and **Tanja**: thank you for the good working environment and for sharing your (PhD)-experiences. Good luck with your future careers. **Alexandra, Anouk, Dawid, Dennis, Gerry**, **Hessel, Tom,** bedankt voor jullie toevoeging aan de goede sfeer op de afdeling Biochemie. Aan een ieder die ik mogelijk nog ben vergeten te noemen mijn excuses. Bij dezen alsnog bedankt voor je bijdragen aan de ervaringen die ik in de afgelopen jaren heb opgedaan.

In het bijzonder wil ik graag mijn ouders bedanken voor de steun die ik altijd heb mogen ontvangen. Jullie aanmoediging in mijn keuzes heeft er toe geleid dat ik dit pad tot een voltooid PhD traject heb kunnen bewandelen.

Tot slot wil ik natuurlijk nog mijn liefste **Tiffany** bedanken voor de grote rol die ze in mijn leven is gaan spelen. Heel erg bedankt voor je begrip als ik eens laat thuis was, je hulp en inzichten bij zowel werk als persoonlijke aangelegenheden en al het andere dat je voor me betekent! Ik kijk uit naar wat de toekomst ons zal brengen. Maar met jou aan mijn zijde heb ik er alle vertrouwen in.