

The Haemostatic Role of Tissue Factor Pathway Inhibitor

James T.B. Crawley, David A. Lane

Abstract—Under normal conditions the blood circulates freely within the confines of the vascular system, carrying oxygen, nutrients, and hormonal information around the body and removing metabolic waste. If blood gains access to extravascular sites, or the vasculature becomes pathologically challenged, hemostasis may be activated. This process is finely regulated by positive and negative feedback loops that modulate fibrin clot formation. Blood coagulation revolves around the activation and assembly of the components of the prothrombinase complex, which converts the inactive zymogen, prothrombin, into its active form, thrombin. This serine protease catalyzes the conversion of fibrinogen to fibrin, the structural scaffold that stabilizes platelet aggregates at sites of vascular injury. The extent of the hemostatic response is controlled by the action of inhibitory pathways, which ensure that thrombin activity and the spread of the hemostatic plug is limited to the site of vessel damage. This review article focuses on the major physiological regulator of tissue factor–induced coagulation, tissue factor pathway inhibitor, its expression, anticoagulant function, and its role in normal hemostasis. (*Arterioscler Thromb Vasc Biol.* 2007;28:000-000.)

Key Words: TFPI ■ anticoagulant ■ tissue factor ■ thrombosis ■ hemostasis

Overview of Tissue Factor–Dependent Coagulation

The extrinsic, or tissue factor (TF)-dependent, pathway of coagulation is the major physiological route by which thrombin generation is initiated in response to vessel damage.^{1,2} TF, the primary initiator of blood coagulation, is an ≈45-kDa integral membrane protein³ that is traditionally considered to be normally located at extravascular sites that are not usually exposed to the blood (ie, on adventitial fibroblasts and variably on vascular smooth muscle cells).⁴ Therefore, only at sites of vascular injury or endothelial disruption may the blood encounter TF-presenting cells and in turn activate the extrinsic pathway. TF is also expressed in a cell-specific manner within certain organs (ie, lungs, brain, heart, testis, uterus, and placenta).⁴ TF in these locations may provide further hemostatic protection to vascular injury in these organs.

After mechanical injury to the vasculature, blood (containing plasma clotting factors) gains access to cellular and matrix components that are normally separated from the blood by the endothelial barrier. The inactive zymogen, factor VII (FVII), circulates in plasma at a concentration of ≈10 nmol/L. A fraction (≈1%) of total plasma FVII circulates in its active form, FVIIa.⁵ TF binds both FVII and FVIIa with high affinity. FVII/FVIIa is comprised of a Gla domain, 2 epidermal growth factor–like domains and a serine protease domain.⁶ The FVII Gla domain imparts phospholipid-binding function to the protein, and in turn assists in the localization of FVII/FVIIa to the surface of TF-expressing cells. Although negatively-charged phospholipids enhance the association of

FVIIa with TF, they are not essential for complex formation.⁷ The interaction between TF and FVII/FVIIa occurs across an extensive interface between the 2 proteins.^{8,9} This binding reaction induces an allosteric change in the FVIIa serine protease domain that modulates its activity (rather than its substrate-binding function).^{8,10} It is for this reason that although FVIIa can bind its substrate, factor X (FX) (at least in vitro), activation of FX does not occur.^{11,12} Given its normally low plasma concentration in plasma though, it seems unlikely that any appreciable interaction between circulating FVIIa and FX actually takes place physiologically.

The TF-FVIIa that forms after the exposure of TF-presenting cells to the blood initiates the TF-dependent pathway. TF-FVIIa activates the plasma zymogens factor IX (FIX) and FX.¹³ Additional TF-FVIIa may be generated locally from inactive TF-FVII complexes, either by autoactivation or through positive feedback loops involving FXa or FIXa.

The limited quantities of FXa that are generated catalyze the conversion of trace quantities of prothrombin to thrombin, albeit inefficiently.¹⁴ The low concentrations of thrombin that arise from this enable the feedback activation of factor VIII (FVIII) and factor V (FV), nonenzymatic cofactors in the intrinsic tenase, and prothrombinase complexes, respectively.¹⁵ The tenase complex activates further FX and, as a result, bypasses the reliance on TF-FVIIa as a source of FXa generation. FXa, in conjunction with its cofactor, FVa, activates prothrombin ≈3×10⁵-fold more efficiently than FXa alone.¹⁶ Consequently, this allows the explosive gener-

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From the Department of Hematology, Imperial College London, UK.

Correspondence to James T.B. Crawley, Department of Hematology, Imperial College London, 5th Floor Commonwealth Building, Hammersmith Hospital Campus, Du Cane Road, London W12 0NN, UK. E-mail j.crawley@imperial.ac.uk

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ation of thrombin that enables this serine protease to fulfill its myriad functions, including the deposition of fibrin.^{17,18}

TF-initiated thrombin generation occurs rapidly over the surface of TF-presenting cells. However, because of both platelet adhesion and fibrin deposition these cells are rapidly covered, leading to the masking of the initiating procoagulant stimulus. Despite this, the hemostatic plug develops beyond a single layer of platelets and fibrin. Intriguingly, TF can be detected throughout developing thrombi, and it colocalizes with both fibrin and platelets many layers above the exposed TF-presenting cells.^{19,20} It seems that after vessel damage, TF becomes presented on the surface of the tethered/activated platelets. This may occur through rapid de novo translation of TF mRNA stored within platelets^{21,22} or recruitment of encrypted TF microparticles to the activated platelet surfaces.²³ In addition to TF, these microparticles present P-selectin glycoprotein ligand-1 (PSGL-1) on their surface, which enables their rapid/specific accumulation to the hemostatic plug through its interaction with P-selectin on activated platelet surfaces.²³ On recruitment to sites of vessel injury this TF is decrypted and can function in the propagation of a thrombus. In this way, hemostatic plug development is enhanced by microparticle-associated TF.^{23,24} TF microparticles are shed from leukocytes, endothelial cells, vascular smooth muscle cells, platelets, and atherosclerotic plaques.¹ This TF is encrypted while in circulation and so does not activate coagulation. The procoagulant activity of this TF is modulated by self-association and dissociation (ie, encryption and decryption).^{25–27} En/decryption is, at least in part, dependent on membrane phospholipid composition and may also involve dedimerization of inactive TF, which exposes its FVII/VIIa binding site. A further TF decryption mechanism has been proposed involving reduction of an allosteric disulphide bond between Cys186 and Cys209.^{28–30} In its reduced form, TF has limited coagulant function. Decryption of TF can occur in response to specific stimuli, such as tissue damage or calcium influx, which leads to disulphide bond formation controlled by protein disulphide isomerase.²⁸ Once formed, the disulfide bond maintains the conformation needed for enhanced binding of TF to FVIIa.³⁰

The generation of thrombin and the formation of a fibrin clot occur rapidly at sites of vascular injury via the pathways/mechanisms outlined above. However, these processes do not proceed unhindered. A variety of inhibitory mechanisms exist that control and localize both the generation and activity of thrombin.

The principle inhibitor of TF-initiated coagulation is tissue factor pathway inhibitor (TFPI), which binds and inhibits TF·FVIIa in a FXa-dependent manner.³¹ By targeting the initiating procoagulant stimulus, TFPI ensures that a small procoagulant stimulus does not elicit an uncontrolled burst of generation of thrombin. Consequently, a natural threshold must be passed before TF·FVIIa-induced coagulation is allowed to proceed (ie, the FXa generated exceeds the inhibitory potential of TFPI). During the initial stages of the procoagulant response, only small quantities of FXa are generated, which facilitate the limited production of thrombin. The initiation phase, during which limited quantities of thrombin are formed, arises after TF exposure to blood and is

strongly influenced by the inhibitory actions of TFPI. Thereafter, the effect of the positive feedback loops of thrombin become evident, which provides an alternative TF-independent route for continued/enhanced activation of prothrombin.³² During this propagation period, the thrombin facilitates fibrin deposition that stops the passage of blood to extravascular sites. The inhibitory actions of the protein C and antithrombin pathways become more prevalent in controlling thrombin generation, and eventually turning off the procoagulant response once a hemostatic plug has formed.

Given the potency of TF·FVIIa in initiating coagulation, there is an absolute requirement for a specific regulatory mechanism. The first major characterization of the protein responsible for this inhibition came when its cDNA was cloned and from this, its primary structure could be predicted.³³ Originally referred to as extrinsic pathway inhibitor or lipoprotein-associated coagulation inhibitor, this inhibitor was latterly termed TFPI.

TFPI Expression and Distribution

The human TFPI gene (*TFPI*) consists of 9 exons and 8 introns that span ≈70 kb on the long arm of chromosome 2 (q32).³⁴ The major site of TFPI production is in endothelial cells,³⁵ which constitutively express the protein under normal conditions. TFPI is also normally expressed by vascular smooth muscle cells, megakaryocytes/platelets, monocytes, mesangial cells fibroblasts, and cardiomyocytes.^{4,36–38} In addition, TFPI has been detected in macrophages and T cells in atherosclerotic lesions.³⁹

Transcription of *TFPI* yields 2 major sets of mRNA species of ≈1.4 kb and 4 kb, respectively.⁴⁰ This size difference is primarily attributable to the use of alternative 3'-polyadenylation signals. Further heterogeneity also arises through multiple alternative transcriptional start sites. Differential splicing of the short exon 2, which spans part of the 5'-untranslated region, also contributes to size differences between TFPI transcripts. Despite this variability in mRNA size and sequence, the primary product is the well-characterized TFPI protein that is translated from what is now referred to as the TFPI- α transcripts. However, an alternatively transcribed species exists that was originally identified in mice⁴¹ and arises through differential splicing of the TFPI mRNA toward the 5'-end of the coding region. This mRNA species, referred to as TFPI- β , has recently been demonstrated to code for TFPI with a different carboxy terminus.⁴² The ratio of TFPI- α :TFPI- β mRNA in cultured endothelial cells is reported to be between 5 and 10, compatible with the relative abundance of their respective translated products.⁴³

Upregulators of TFPI expression in vitro that might be of physiological significance include endotoxin, interleukin (IL)-1, tumor necrosis factor (TNF)- α , platelet-derived growth factor, heparin, basic fibroblast growth factor, and elevated shear stress.^{38,44–46} The exposure of different cell types to such agonists or conditions may represent a physiological mechanism by which local anticoagulant activity is up-regulated in response to a given challenge.

TFPI Protein

The translated product of the TFPI- α mRNA is a 304 amino acid protein (Figure 1A). The removal of a classical 28

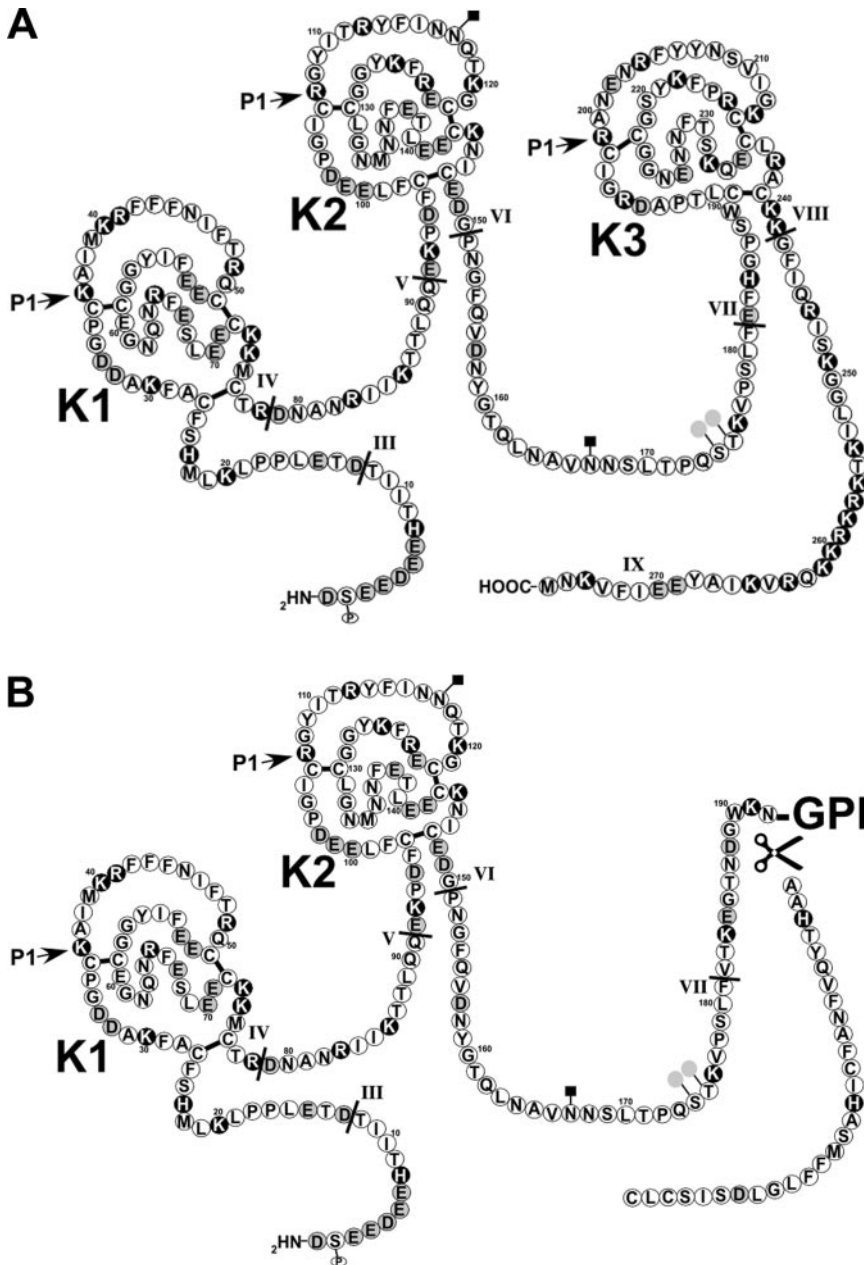


Figure 1. Diagram of TFPI and TFPI β . The primary amino acid sequence of mature TFPI (A) and TFPI β (B) are shown. The exon boundaries are marked and numbered in roman numerals. N-linked glycosylation sites at Asn117 and Asn167 are shown by black squares. O-linked glycosylation sites at Ser174 and Thr175 are denoted by gray circles. Acidic residues are shown in gray, basic residues in black. A, There is a nonuniform charge distribution through TFPI. The amino terminus is negatively charged (gray), the carboxy terminus strongly electropositive (black). Phosphorylation of the amino-terminal Ser2 is also shown. The tandemly arranged Kunitz domains, each containing 3 disulphide bonds, are labeled (K1, K2, K3). In each case, black arrows highlight the P1 residues. In K1, this region of TFPI interacts with FVIIa in the TF-FVIIa complex. The P1 arginine in K2 interacts with FXa. The predicted P1 arginine in K3 has no described inhibitory role. Diagram based on Wun TC et al.³³ B, The tandemly-arranged Kunitz domains, each containing 3 disulphide bonds, are labeled (K1, K2). In each case, black arrows highlight the P1 residues. Unlike TFPI- α , which encodes the TFPI protein, TFPI- β mRNA is alternatively spliced and consequently has an unrelated carboxy terminus after exon VII. The new reading frame encodes a carboxy tail that lacks the third Kunitz domain. This tail can now be processed intracellularly, which removes part of this tail and attaches a GPI-linkage. This causes direct covalent association of TFPI β to the cell surface. Diagram based on Zhang et al.⁴²

residue signal peptide yields the mature 276 amino acid Kunitz-type serine protease inhibitor, TFPI.³³ TFPI contains 3 tandemly arranged Kunitz domains (K1 - Asp13-Arg78, K2 - Glu92-Gly150, K3 - Glu182-Lys241), each containing 3 disulphide bonds that pair in the pattern 1 to 6, 2 to 4, 3 to 5 (Figure 1A). There is a nonuniform charge distribution within the TFPI molecule. The amino-terminal contains several negatively charged acidic residues. The carboxy-terminal sequence includes 15 positively charged basic amino acids.

The predicted molecular mass of TFPI is 32 kDa, but posttranslational modifications result in an observed mass of \approx 43 kDa for the mature secreted protein.³³ TFPI is N-linked glycosylated at Asn117 and Asn167. O-linked carbohydrate attachment occurs at Ser174 and Thr175. Although these posttranslational modifications do not seem to function directly in the inhibitory function of TFPI, they may influence

its cell binding properties and plasma clearance, possibly through their interaction with the basic carboxy terminus. These contentions are based on the seemingly enhanced clearance rate and cell binding properties of recombinant TFPI expressed in bacteria that lacks these carbohydrate structures.⁴⁷

TFPI Structure/Function

The inhibitory activity of TFPI is conferred via a mode that is common to other Kunitz-type inhibitors (eg, aprotinin). This mechanism involves a region of a Kunitz domain mimicking the substrate of the target protease. Once bound, the target enzyme fails to proteolyse the P1-P1' peptide bond, or does so very inefficiently. Therefore, Kunitz-type inhibitors typically confer a tight-binding competitive inhibition. Although the target protease is inactivated, this inhibition is reversible

as dissociation of the Kunitz domain from the active site yields a fully active enzyme. Girard and coworkers demonstrated that the first Kunitz domain of TFPI (K1) interacts directly with TF·FVIIa.⁴⁸ K1 binds ionically to the active site of FVIIa in this complex.⁴⁸ The second Kunitz domain (K2) mediates the binding and inhibition of FXa.⁴⁹ Despite its homology to K1 and K2, the third Kunitz domain (K3) has no described inhibitory function. Although K3 has not been demonstrated to directly interact with either TF·FVIIa or FXa, truncated TFPI variants that lack this domain have reduced inhibitory properties.⁵⁰ By comparison to K2, which has the optimal charge distribution around the P1 residues to make a tight interaction with FXa,⁵¹ K3 does not contain such a charged patch in the vicinity of the predicted P1 residue that might facilitate such binding, potentially explaining its lack of described inhibitory function toward this or similar serine proteases. Recently, a role for K3 has been proposed in mediating, at least in part, the ionic binding of TFPI to cell surfaces.⁵² Interestingly, this interaction involves the predicted P1 residue in this domain (Arg199), as mutation of this residue diminishes the affinity of TFPI for endothelial cell membranes. K3 also contributes to the binding of heparin. The negatively charged sulfate groups of such glycosaminoglycans can interact with a positively charged patch on the surface of this domain formed by Lys213, Lys232, and Lys240—determined by NMR on the structure of K3 in complex with heparin.⁵³ This binding site, in conjunction with a further heparin binding site in the carboxy tail, give TFPI a comparatively high affinity for this glycosaminoglycan.

The carboxy-terminal part of TFPI has an array of structural and functional activities, which are largely attributable to the highly basic nature of this region. Although it remains unclear precisely how this part of TFPI contributes toward its anticoagulant activity, removal of the basic tail reduces the inhibitory potency of TFPI. The cluster of basic amino acids in the carboxy-tail mediate the interaction of TFPI with numerous other charged ligands, including heparin,⁵⁴ cell surface proteoglycans,⁵⁵ plasma lipoproteins,⁵⁶ thrombospondin-1 (TSP-1),⁵⁷ and certain clearance receptors.^{55,58}

TFPI Cell Biology

In addition to the different tissues and cell types in which TFPI is expressed, there are also different pools of cellular TFPI. TFPI is constitutively expressed and secreted by the vascular endothelium, which is its primary site of synthesis. For this reason, the expression and trafficking of TFPI within these cells has been best studied.

It has been reported that a proportion of TFPI is stored close to the membrane surface of cultured endothelial cells.⁵⁹ This cellular fraction can be released in response to different agonists (eg, heparin, thrombin, shear forces).^{45,46,59} It remains contentious whether these are defined intracellular storage granules, or whether they actually represent invaginated glycosphingolipid-rich microdomains, termed caveolae, with which TFPI is known to associate.^{60,61} On secretion from endothelial cells, a small amount of TFPI may associate ionically with the cell surface proteoglycans via K3 and its basic carboxy terminus. Such an ionic interaction might explain the, albeit limited, competitive release of TFPI from

the endothelial surface by heparin.⁴⁶ The mobilization of intracellular/cell surface-associated TFPI into plasma contributes to the anticoagulant effect of heparin administration. Heparin not only induces TFPI secretion into plasma, it also upregulates TFPI gene expression leading to elevation of both cellular and plasma TFPI pools. However, the observation that heparin only competitively releases a fraction of the cell surface-associated TFPI highlights the presence of additional pools of TFPI that are associated with the cell membrane via alternative interactions.

The localization of TFPI within caveolae gave the first suggestion of an alternative mode of membrane attachment. Caveolae are enriched in proteins linked to the surface via glycosylphosphatidylinositol (GPI) anchors. Treatment of endothelial cells with phosphatidylinositol phospholipase C, which cleaves GPI anchors, releases a much larger pool of TFPI than that competitively freed by heparin, demonstrating a GPI-mediated attachment mechanism.⁶⁰ Intriguingly though, the highly basic nature of the TFPI carboxy terminus is not predicted to favor the addition of a direct GPI-linkage, suggesting that attachment is via an indirect GPI-anchoring mechanism.

It seems that TFPI cellular trafficking and surface expression is controlled by an as yet uncharacterised GPI-anchored coreceptor that tightly, but reversibly, binds to TFPI within the ER/Golgi.⁶² The GPI-anchored coreceptor for TFPI not only plays a key role in trafficking TFPI to the cell surface, it also provides the cell surface binding sites for this pool of TFPI. Because of the reversible nature of the binding interaction, not all TFPI binds intracellularly to the coreceptor. This unbound pool of TFPI is consequently secreted by the cell. Once secreted though, TFPI does not appear to be able to bind back its GPI-anchored receptor on the cell surface.⁶²

In recent years, an additional mode of GPI-mediated attachment of TFPI cell surfaces has been elucidated. The translated product of the TFPI- β mRNA gives rise to TFPI that is directly GPI-anchored via the different carboxy terminus that this alternatively transcribed molecule possesses.⁴² The amino terminus, K1 and K2 of TFPI β are identical to those of TFPI (Figure 1B). The alternatively spiced TFPI β lacks K3 and the highly basic carboxyl-region, which are replaced by an unrelated carboxy-terminal sequence (Figure 1B). TFPI β associates with the endothelial cell surface exclusively via this direct GPI anchor.⁴² Despite the lack of K3 and the carboxy terminus which appears to contribute to the anticoagulant function of TFPI, TFPI β exhibits appreciable inhibitory function against TF·FVIIa and FXa.⁴³ It must be considered that at this time much of the published data on TFPI β are derived from *in vitro* studies. Although TFPI- β transcripts have been detected *in vivo* in baboon lungs and in mice,^{41,63} the TFPI β protein has not been specifically detected to date. Consequently, the physiological role or significance of TFPI β remains uncertain at this time.

On the surface of cultured endothelial cells induced to express TF and exposed to FVIIa and FX, inactive TFPI·FXa·TF·FVIIa quaternary complexes have been observed to specifically redistribute into caveolae in a FXa-dependent manner.⁶⁴ This is specific to GPI-linked TFPI and is not a characteristic of proteoglycan-bound TFPI. Although

TFPI anticoagulant function does not depend on the translocation of these complexes into such structures, this movement may serve to sequester cell surface proteolytic activity. As this translocation is reversible, the components of this complex can potentially be released or reexposed to the extracellular environment. Whether other cell types (eg, vascular smooth muscles cells, monocytes, fibroblasts etc) that express TFPI have the same cellular pools that behave in the same way as those in endothelial cells remains uncertain.

Irrespective of its mode of cell surface attachment, though, the precise role that TFPI on endothelial cell surfaces fulfils has been contentious. This is because although endothelial cells can be induced to express TF in vitro, how frequently this occurs in vivo is uncertain. Recently, however, the potential importance of constitutive endothelial TF has been suggested²⁰ that may provide an insight into the reason behind the comparatively large amounts of TFPI attached to the endothelial cell surface.

Plasma TFPI

TFPI secreted by endothelial cells circulates in plasma at a concentration of ≈ 2.5 nmol/L^{65,66} but fluctuates temporally in humans because of their natural circadian rhythms.^{67,68} Plasma levels are increased 1.5- to 3-fold after administration of heparin.⁶⁹ The majority of the plasma pool circulates in association with low-density lipoproteins (LDL),⁷⁰ and as a consequence confer markedly reduced inhibitory potential toward FXa and TF·FVIIa.⁷¹ LDL levels seem to correlate with plasma TFPI concentration. Only about 10% of plasma TFPI circulates in a full-length free 43-kDa form. The other forms of TFPI that exist in plasma have different molecular masses, either because of carboxy-terminal truncation or because of its disulphide bond-mediated association with LDL. The truncated forms of TFPI lack most of their carboxy-terminal and often also most of K3,⁷² and so also exhibit reduced inhibitory activity, and a lower affinity for glycosaminoglycans. Although it remains to be established precisely how the truncated forms of TFPI are generated physiologically, in vitro data have demonstrated that TFPI is cleaved into degraded forms by various proteases that TFPI might encounter physiologically. These include thrombin, plasmin, neutrophil elastase, and certain matrix metalloproteinases.^{73,74} FXa, a natural target for TFPI, will also proteolyse TFPI after the K3 P1 residue (Arg199), but only when in molar excess. Although the cleavage of TFPI in vivo has not been well characterized, the plasmin-dependent reduction of both plasma and monocyte cell surface TFPI has been demonstrated in patients after thrombolytic therapy.⁷⁵ This reduced TFPI, it was speculated, may potentially contribute to thrombotic complications after fibrinolysis in acute myocardial infarction patients. Very recently in a baboon sepsis model, Lupu and coworkers observed a decrease in cellular TFPI in the lung that was accompanied by an increase of plasma levels of TFPI.⁶³ Whereas this suggested enhanced release from the endothelium, this increase in plasma TFPI was not accompanied by a concomitant increase in plasma TFPI function. It seems that enhanced plasmin generation during sepsis proteolytically decreases the amount of cell-associated TFPI giving rise to increased plasma TFPI that

lacks anticoagulant function. Naturally, this may contribute to the pathogenesis of sepsis and potentially other conditions that involve elevated plasmin generation.

Of the total TFPI in blood, between 2% and 5% is present within circulating/resting platelets.⁷⁶ TFPI α (as opposed to the alternatively spliced TFPI β) is expressed by megakaryocytes. It seems that the coreceptor that traffics and GPI links TFPI to the cell surface in endothelial cells is unlikely expressed in megakaryocytes/platelets. Consequently, rather than present on the platelet surface, TFPI is located within intraplatelet stores that are distinct from α -granules. This pool can be released on their full activation.⁷⁶ Interestingly, platelets activated by a single agonist (eg, collagen or thrombin) cause release of α -granule contents, but not intraplatelet TFPI. Only platelets activated simultaneously with both collagen and thrombin exhibit high levels of procoagulant proteins and phosphatidylserine on their surface. This full activation is associated with secretion of both dense and α -granules. It is only these coated-platelets that also present TFPI on their surface. As this TFPI is relatively resistant to removal by PIPLC, it seems that this TFPI is perhaps not GPI-anchored, or if so is linked in a PIPLC-resistant form.⁷⁶

TFPI Clearance

Once the extrinsic pathway is stimulated, the major site of TFPI inhibitory activity is found on the TF-presenting cell surface. On cultured endothelial cells, inactive TFPI·FXa·TF·FVIIa quaternary complexes can be sequestered into caveolae and endocytosed to remove the components from the membrane surface.⁶¹ TFPI complexed with FXa is efficiently internalized by endothelial cells via an uncharacterised pathway that is dependent on proteoglycan binding.⁷⁷ This requires full-length TFPI, as opposed to carboxy-terminal truncated forms. Once internalized the TFPI·FXa complex is targeted for degradation. A separate clearance system is responsible for the removal of TFPI from the circulation. The low-density lipoprotein receptor-related protein (LRP), a multi-functional endocytic receptor normally expressed in the liver and also by vascular smooth muscle cells, facilitates the cellular uptake of TFPI and its clearance from plasma.⁷⁸ A 39-kDa LRP-associated protein acts as a cofactor for this uptake of TFPI. Once internalized by LRP in clathrin-coated vesicles, TFPI is subsequently degraded. It must however be considered that data from clearance studies using bacterially expressed TFPI (ie, lacking glycosylation) like those outlined above,^{47,55,77,79–81} may not reflect precisely the normal physiological clearance of endogenous TFPI.

TFPI Anticoagulant Function

The anticoagulant function of TFPI involves the FXa-dependent inhibition of TF·FVIIa (Figure 2). By targeting these serine proteases, TFPI directly inhibits the initiation phase of coagulation. Although the requirement for FXa is not absolute for the inhibition of TF·FVIIa, it is unlikely that TFPI significantly inactivates this target physiologically in its absence/before its generation. Therefore, TFPI may only impart its anticoagulant function once the initial stages of the pathway have been allowed to proceed.

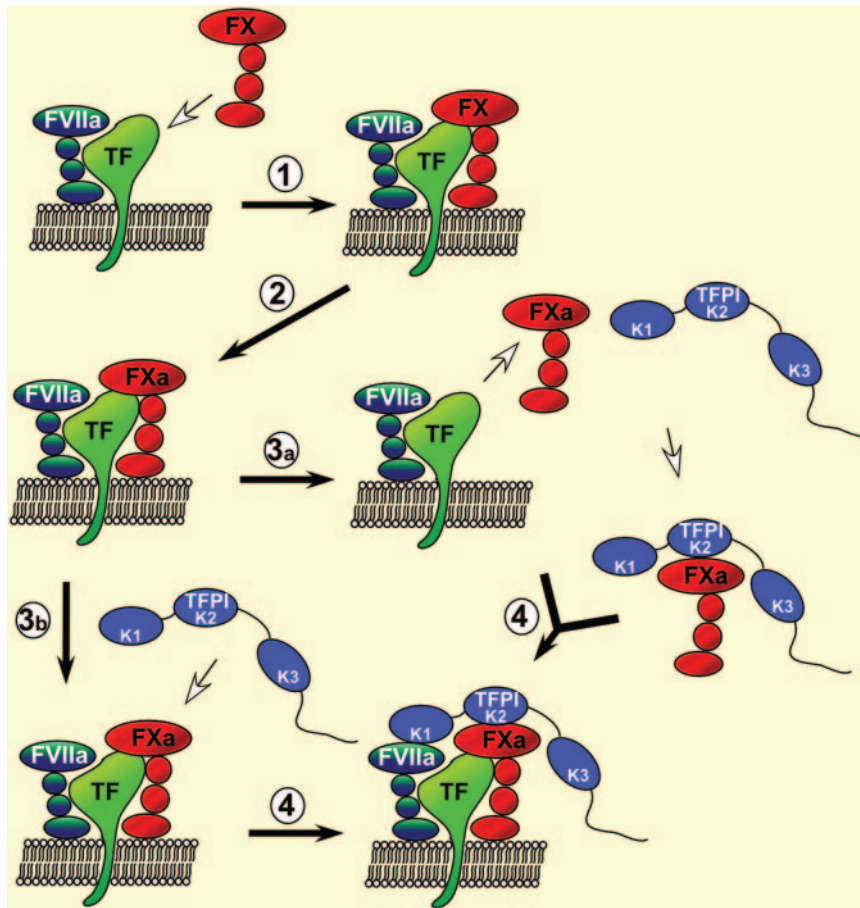


Figure 2. Schematic representation of the inhibition of TF-dependent coagulation by TFPI. After the formation of the TF-FVIIa complex, the circulating zymogen, FX, binds to TF-FVIIa (1) and thereafter gets activated (2). Once activated, FXa may dissociate (3a) from the activating complex. FXa may then elicit its procoagulant function or alternatively, become inactivated by cell-associated or plasma TFPI via its second Kunitz domain. This complex can now reassociate with TF-FVIIa (4) with the first Kunitz domain of TFPI binding to the active site of FVIIa. Although frequently described as a 2-stage process, kinetic studies favor a model whereby TFPI binds and inactivates TF-FVIIa-FXa before FXa release (3b/4). In each case the resulting inactive quaternary complex is the same.

The first stage in the inhibitory function of TFPI involves the reversible inhibition of FXa (Figure 2). This occurs via the ionic binding of FXa to either full-length plasma, or cell-associated TFPI. This interaction involves the P1 residue (Arg 107) in TFPI K2.⁴⁸ As active site-blocked FXa binds TFPI with lower affinity, it seems likely that the major point of contact is at or close to the active site serine of FXa. As truncated forms of TFPI or proteolysed TFPI are less potent inhibitors of FXa, it is likely that other parts of the TFPI molecule are important for the interaction with this serine protease.⁵⁰ Whereas K2 alone will bind FXa, the interaction is significantly enhanced by the presence of K1, K3, and the basic carboxyl-region. However, precisely how these other domains augment this binding is unclear in the absence of an available crystal structure of this complex. Unlike many of the interactions in the coagulation cascade, Ca^{2+} ions are not required for the inhibition of FXa by TFPI. However, Ca^{2+} does augment the potency of FXa inhibition by TFPI in the presence of phospholipid surfaces.⁸² This is dependent on the carboxy-terminal region of TFPI, suggesting that this effect is conferred by the concentrating of the 2 molecules on a membrane surface mediated by the Ca^{2+} -dependent Gla domain FXa and the basic residues in the TFPI tail. Similar effects are also observed in the presence of heparin, which most likely approximates these 2 molecules. Once FXa is assembled in the prothrombinase complex with FVa, it is protected from TFPI inhibition,⁸³ largely due to competition

for the active site of FXa with prothrombin, which is present at much higher concentrations than TFPI.

The second stage in the inhibition of TF-dependent coagulation involves the binding of the TFPI-FXa complex to TF-FVIIa (Figure 2). During this step, the P1 residue in TFPI K1 (Lys36) interacts with the active site of FVIIa.⁴⁸ Unlike the binding/inhibition of FXa, this step is Ca^{2+} -dependent. Interactions between plasma membranes and the TFPI-FXa complex assist in its inhibitory potential toward TF-FVIIa. Consequently, the GPI-anchoring/proteoglycan association of TFPI and the membrane-binding Gla domain of FXa both augment this process.

The FXa-dependent inhibition of TF-FVIIa by TFPI results in the formation of an inactive TFPI-FXa-TF-FVIIa quaternary complex on the plasma membrane. This has no catalytic activity toward either FX or FIX and results in the marked dampening of TF-induced coagulation. Although, as presented here, this process is frequently described as a 2-stage process, first involving the binding of free FXa and thereafter to TF-FVIIa, kinetic data favor a model in which TFPI interacts with FXa that has not yet been released from the activating TF-FVIIa complex.⁸⁴ This explains the rapid nature of FXa inactivation by TFPI. Consequently, the amount of FXa that escapes inhibition is directly related to the concentration/availability of TF. Given the nature of the inhibition of TF-FVIIa and the formation of a quaternary complex, it is unsurprising that the domains/residues important for optimal interaction of FX with TF-FVIIa are also critical to the

potency of TFPI inhibition. These include the Gla domain of FX and Lys165-Lys166 of TF.⁸⁵

TFPI in Hemostasis

It seems highly likely that the different cellular and plasma pools of TFPI influence normal hemostasis in different ways and at different stages of hemostatic plug development. Mechanical injury to the vasculature exposes TF-presenting cells to the blood to initiate coagulation. In this location, the TF procoagulant function is most likely primarily regulated by cell surface TFPI that is also expressed by vascular smooth muscle cells or fibroblasts. However, as previously discussed, this exposed surface is rapidly covered by a layer of fibrin and platelets. Although the cellular TF is no longer exposed to the blood, a new pool of TF now becomes important in the hemostatic response. The tethered/activated platelets may either express *de novo* TF^{21,22} or recruit (and decrypt) microparticle TF to their surfaces.²³ How then might TFPI modulate this procoagulant stimulus? Several mechanisms have been postulated to influence TF function in this location. TFPI is stored within platelets and can be released on their full activation.⁷⁶ However, it seems that by itself TFPI might not readily associate with the activated platelet surface, particularly given the absence of a specific anchoring mechanism. Although the importance of the interaction between TFPI and TSP-1 is unknown, TSP-1 is a major constituent of platelet α -granules.⁵⁷ On the release of their contents, TSP-1 associates transiently with the ECM and several cell surface integrins. Consequently, it has been speculated that in binding TSP-1, TFPI might be efficiently localized to the extravascular space and in turn modulate TF-mediated coagulation in this location.

More recently, a novel mechanism was proposed that might serve to specifically localize plasma TFPI, or newly secreted platelet TFPI, to activated platelet surfaces. This process involves protein S, a vitamin K-dependent protein that is classically considered as the cofactor for activated protein C (APC).⁸⁶ Hackeng and coworkers demonstrated that the inhibitory function of plasma TFPI was enhanced in the presence of protein S, and that this increased the rate of FXa inactivation.⁸⁶ Their model suggests that a specific interaction between TFPI and protein S exists between TFPI K3 or the carboxy terminus that may in turn increase the affinity of TFPI for phospholipid surfaces. Because of its Gla domain, protein S has a high affinity for negatively-charged phospholipid surfaces. Consequently, similar to its cofactor function for APC, protein S may increase the affinity of TFPI for activated platelet surfaces and so increase its local availability for the inhibition of TF-dependent coagulation. Together, the processes that enable the localization of TFPI to activated platelet surfaces may represent important determinants in the control of hemostatic plug growth. Despite this, it has recently been reported that platelet-derived polyphosphates released on activation abrogated TFPI anticoagulant activity.⁸⁷ Precisely how this influences TFPI function over the platelet surface and thus hemostatic plug development *in vivo* remains to be resolved.

What role then does endothelial cell surface TFPI play in this process? This has been the a major question that has

remained largely unanswered. As a major site of TFPI expression, the endothelium provides a continual source of plasma TFPI. TFPI can also be acutely released from these cells on activation to provide a further/localized source of this inhibitor. However, this does not attribute function to the GPI-anchored TFPI on the surface of these cells. One model may be that the endothelium adjacent to a site of vessel damage becomes activated. This can in turn lead to the presentation of P-selectin on the cell surface. Potentially, the presence of P-selectin could enable the fusion of TF-bearing microparticles to the endothelium via specific interactions with PSGL-1. Alternatively, recent findings from Furie and coworkers have revealed intracellular stores of endothelial TF that can be decrypted by protein disulphide isomerase on endothelial stress. In light of this, it might be hypothesized that cell surface TFPI serves to modulate TF-dependent activity over the intact endothelium. Further studies are required to fully characterize the physiological function of this cell surface pool of TFPI.

TFPI in Disease

Given the apparently important role of TFPI in regulating hemostasis, it might be predicted that TFPI deficiency may contribute to the pathogenesis of thrombotic disorders. Indeed, in experimental animal models, TFPI clearly plays a critical regulatory role in controlling the effects of TF. For example, the physiological importance of TFPI is clearly demonstrated by the lethal phenotype exhibited by homozygous TFPI gene deletion in mice.⁸⁸ TFPI-null mouse embryos die *in utero* because of intrauterine coagulopathy and vascular disintegrity.⁸⁸ Furthermore in rabbits, immunodepletion of TFPI dramatically lowers the TF threshold required to initiate coagulation, whereas in other animal models administration of recombinant TFPI can increase this threshold and protect against disseminated intravascular coagulation and venous thrombosis.^{89–92}

In mice, heterozygous TFPI deficiency in mice does not, by itself, appear to enhance susceptibility to thrombosis.⁸⁸ However, the increased thrombogenicity of these mice can be observed when TFPI^{+/-} mice are crossed onto a FV Leiden background. Together these genetic deficiencies become fatal during the perinatal period.⁹³

Despite the key role of TFPI in the regulation of coagulation, few studies have demonstrated a clear-cut association between plasma levels of TFPI and the risk of thrombosis. Certain TFPI polymorphisms have been linked to thrombosis in small groups of patients, but the power of these studies is really too low to provide definitive answers with regard to their role as risk factors. Genetic deficiency in humans appears to be rare though, and consequently its association with thrombotic disorders has not been well studied. There are further difficulties in analyzing the association of TFPI levels/function with thrombotic disease because of (1) the low circulating levels of total plasma TFPI, (2) the very low levels of free full-length TFPI, and (3) the inability to assay cellular TFPI in patients. Despite this, low total plasma TFPI, defined as less than the lowest 10th percentile in control subjects, was found to be a weak risk factor for deep vein thrombosis—even after controlling for hormonal status, the

factor V Leiden mutation, the prothrombin 20210GA gene mutation, antithrombin deficiency, or protein C or S deficiencies.⁹⁴ Furthermore, when the lower cut-off values for TFPI levels were set to below the 5th or the 2nd percentile, a slightly higher risk of deep vein thrombosis was observed, suggesting a threshold effect for the ability of TFPI to protect against thrombosis.⁹⁴

Further studies on the full physiological roles of different TFPI pools and their vascular bed specificity are now needed to give greater insight into the pathophysiological importance of TFPI.

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