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Alternatively Spliced Isoforms of Tissue Factor Pathway Inhibitor

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Abstract

Tissue factor pathway inhibitor (TFPI) is the major regulator of tissue factor (TF)-induced coagulation. It down regulates coagulation by binding to the TF/FVIIa complex in a fXa dependent manner. It is predominantly produced by microvascular endothelial cells, though it is also found in platelets, monocytes, smooth muscle cells, and plasma. Its physiological importance is demonstrated by the embryonic lethality observed in TFPI knockout mice and by the increase in thrombotic burden that occurs when heterozygous TFPI mice are bred with mice carrying genetic risk factors for thrombotic disease, such as factor V Leiden. Multiple TFPI isoforms, termed TFPI α , TFPI β , and TFPI δ in humans and TFPI α , TFPI β , and TFPI γ in mice, have been described, which differ in their domain structure and method for cell surface attachment. A significant functional difference between these isoforms has yet to be described *in vivo*. Both human and mouse tissues produce, on average, approximately 10 times more TFPI α message when compared to that of TFPI β . Consistent with this finding, several lines of evidence suggest that TFPI α is the predominant protein isoform in humans. In contrast, recent work from our laboratory demonstrates that TFPI β is the major protein isoform produced in adult mice, suggesting that TFPI isoform production is translationally regulated.

Keywords

tissue factor pathway inhibitor; blood coagulation; alternative splicing; translational regulation

Blood coagulation is initiated following disruption of intact endothelial surfaces that exposes tissue factor (TF) to FVIIa in flowing blood. The TF/FVIIa complex then activates FIX and FX to initiate the TF dependent pathway of blood coagulation. The primary physiological inhibitor of this pathway is tissue factor pathway inhibitor (TFPI). The discovery and characterization of TFPI has a long history, beginning with the discovery by Carson[1] and Sanders et al.,[2] of a plasma protein that inhibits the activity of the TF/FVIIa complex. This work led to the purification and identification of TFPI from conditioned medium of HepG2 cells by Broze and coworkers.[3]

In order to understand the role TFPI plays in disease, it is imperative to understand the role of TF in disease processes. Tissue factor is constitutively expressed by extra-vascular cells

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surrounding vessel walls and by activated cells within the vasculature.[4–6] Increased TF is present at both intra- and extra-vascular sites in a number of disease processes including infections, inflammation, cancer, and cardiac disease. Additionally, small amounts of circulating TF are present in healthy humans and mice suggesting that a basal low level of coagulation continuously occurs *in vivo*. [7–9] TFPI down regulates this TF activity limiting the formation of intravascular thrombi.

TFPI Structure

TFPI is a 276 amino acid (~43kDa) multivalent Kunitz-type protease inhibitor encoded on chromosome 2.[10] Full length TFPI (also called TFPI α) contains an acidic amino terminal region, three Kunitz protease inhibitor domains and a basic carboxy-terminal region.[11] The first (K1) and second (K2) Kunitz domains inhibit TF/FVIIa and factor Xa, respectively, resulting in the formation of a quaternary TFPI-TF/FVIIa-FXa complex.[12] An inhibitory function for the third (K3) Kunitz domain has not been identified.

Physiological Activity of TFPI

TFPI deficiency has not been described in humans, and mice lacking TFPI activity have an embryonic lethal phenotype demonstrating that TFPI is essential for embryogenesis. Sixty percent of these TFPI knock-out mice die by day E10.5 with the remainder dying at variable times up to day E17.5.[13] Phenotypically, the mice have yolk sac hemorrhage between days E9.5–11.5, vascular abnormalities in the placenta, hemorrhage within the head, spine and tail, and fibrin deposition in the brain and liver. The embryonic lethality is rescued by breeding TFPI deficiency into mice producing low amounts of TF, conclusively demonstrating that TFPI directly counterbalances TF activity *in vivo*. [14]

In contrast to TFPI null mice, TFPI heterozygous mice appear normal without evidence of spontaneous thrombosis.[13] However, several studies have demonstrated that heterozygous TFPI deficiency synergizes with other genetic abnormalities to produce thrombotic disease. Heterozygous TFPI deficiency increases the atherosclerotic burden in the carotid and common iliac arteries in mice lacking apolipoprotein E.[15] When TFPI heterozygosity is bred into mice with FV Leiden, the mice die in the perinatal period with large amounts of fibrin deposition in the liver, lung and kidney.[16] Finally, when TFPI heterozygosity is bred into mice with decreased thrombomodulin function the mice have decreased embryonic survival, a generalized prothrombotic state, increased clot size in an *in vivo* electrolytic injury model, and intravascular fibrin deposition in the liver and in the pia mater vessels of the brain.[17]

Cellular Production of TFPI

TFPI is found in a number of vascular locations. It is predominantly (85%) associated with microvascular endothelial cells.[18;19] Soluble TFPI within human plasma is variably truncated in the C-terminal region and has decreased anticoagulant activity.[20;21] A small amount of TFPI is present on the surface of monocytes[22] and has been detected in the macrophages of atherosclerotic lesions[23] and smooth muscle cells.[24;25] Studies quantifying TFPI in whole blood have found that platelet TFPI accounts for about 8–10% of the total TFPI with plasma accounting for the remainder.[26;27] Interestingly, TFPI is not on the surface of quiescent platelets, but is expressed on the platelet surface following dual stimulation with collagen and thrombin.[27]

Alternatively Spliced Isoforms of TFPI

There are several alternatively spliced isoforms of TFPI designated as α , β , and δ and α , β , and γ in the human and mouse, respectively (Figures 1 and 2). These isoforms differ in their domain

structure and mechanism for cell surface attachment. All alternatively spliced isoforms identified to date contain K1 and K2 and, therefore, can at least theoretically inhibit TF/FVIIa and FXa.

TFPI α

TFPI α is the “full-length” isoform that was originally purified and characterized. It is the only isoform with K3 and the basic C-terminal region. All three Kunitz domains of TFPI α are well conserved from man to zebrafish even though they have not shared a common ancestor for 430Myr.[28] The other alternatively spliced isoforms of TFPI evolved more recently. The evolutionary conservation of K3 and the basic C-terminal region of TFPI suggest that it has an important physiological function. The basic C-terminal region binds to cell surface glycosaminoglycans. Consequently, the human plasma TFPI α concentration increases 2- to 4-fold following heparin infusion.[29;30] However, the major mechanism for the association of TFPI α with cell surfaces is through a glycosyl-phosphatidylinositol (GPI) anchor. GPI-anchored TFPI α is not removed by heparin and is present in approximately 10- to 100-fold higher amounts than heparin releasable TFPI α in human placenta.[19;31;32] Since TFPI does not contain a C-terminal GPI-anchor attachment sequence, it has been hypothesized that it indirectly associates with the cell surface by tightly binding to a, as yet unidentified, TFPI binding protein.[19;31;32]

TFPI β

TFPI β contains K1 and K2 but lacks K3 and the basic C-terminal region of TFPI α . Instead, it has a different C-terminal region that encodes a GPI anchor attachment sequence.[32;33] Thus, TFPI β associates with cell surfaces through direct binding to a GPI-anchor. Mature human TFPI β has 12 C-terminal amino acids not present in TFPI α (Figure 1) while mouse TFPI β has 8 (Figure 2). TFPI β sequence has been identified only in mammals, humans, chimpanzee, long tailed macaque, mice and rats and, therefore, represents a more recent evolutionary adaptation than TFPI α . [28] In studies performed using soluble forms of TFPI, K3 and the basic C-terminal region of TFPI α are required for full inhibitory activity in amidolytic assays measuring inhibition of FXa and in TF initiated plasma clotting assays.[34;35] However, studies of a chimera protein in which K1 and K2 are linked to annexin V to create a protein with high affinity for phosphatidylserine containing vesicles found that its anticoagulant activity increased 250-fold when compared to the same protein lacking annexin V.[36] Thus, the relative anticoagulant activities of the TFPI isoforms are greatly altered by association with cell surfaces. Further studies are needed to compare the anticoagulant activity of cell surface associated TFPI α and TFPI β in order to predict their relative anticoagulant efficacies *in vivo*.

TFPI γ

TFPI γ contains K1 and K2 and has the same 5' splice acceptor site as TFPI β . The 3'-splice acceptor site of TFPI γ is found 276 nucleotides downstream of the TFPI β stop codon within the TFPI β 3' UTR. Alternative splicing for TFPI γ produces a C-terminal region with 18 amino acids not present in TFPI α or TFPI β (Figure 2).[37] This region does not encode a predicted membrane attachment sequence and TFPI γ is secreted when expressed in CHO cells suggesting that it is produced as a soluble protein *in vivo*. [37] TFPI γ is produced exclusively in mice. Homologous sequence is not present in human, chimpanzee, rhesus monkey, rat, dog, cow or horse suggesting that it is a very recent evolutionary adaptation. TFPI γ mRNA is produced in all adult mouse tissues. TFPI γ protein has not been definitively identified in mouse tissues, but TFPI γ inhibits TF/FVIIa procoagulant activity when expressed in CHO cells.[37] Since TFPI γ is a functional anticoagulant, it may partially explain the resistance of mice to coagulopathy in TF-mediated models of disease.

TFPI δ

TFPI δ sequence is present within the NCBI GenBank database. Other than these sequences, no information regarding TFPI δ has been published to date. TFPI δ sequence encodes the K1 and K2 domains. Alternative splicing occurs immediately following K2 and generates a new C-terminal region containing 12 amino acids (Figure 1). TFPI δ sequence has been found in humans and chimpanzees, NCBI protein reference sequences BAD93103.1 and XP_001161803.1, respectively.

Transcription of Alternatively Spliced Forms of TFPI

Real time PCR studies have shown that production of TFPI α message, when compared to a housekeeping message for RPL-19, is greatest in the placenta, and lowest in the brain in both humans and mice. Other tissues produce intermediate amounts of TFPI α mRNA with relatively large amounts made by the heart and lung.[37] In both humans and mice all tissues produce 4- to 50-fold more TFPI α mRNA than TFPI β mRNA depending on the tissue examined.[37] These findings are consistent with studies of human endothelial cell lines in which TFPI α mRNA is approximately 10-fold more abundant than TFPI β mRNA.[38;39]

Translation of Alternatively Spliced Forms of TFPI

Several lines of evidence indicate that TFPI α is the predominant isoform produced by human tissues. These include (i) human placenta produces TFPI α :[19] (ii) human platelets produce exclusively TFPI α :[27] (iii) the transformed human endothelial-like Ea.hy926 cell line produces exclusively TFPI α :[38] and (iv) heparin infusion results in a 2- to 4-fold increase in human plasma TFPI concentration and this heparin-releasable TFPI is exclusively TFPI α . [30] TFPI β and TFPI δ protein have not been identified within human tissue; however, a comprehensive study of alternatively spliced forms of TFPI produced by human tissues has not been reported. In mice, TFPI α and TFPI β are temporally expressed at the level of protein production.[28] The placenta produces predominantly TFPI α along with small amounts of TFPI β while E14.5 embryos produce approximately equal amounts of TFPI α and TFPI β . In contrast to the apparent expression of TFPI α in adult humans, adult mouse tissues produce almost exclusively TFPI β . Consistent with their production of TFPI β , adult mice have a much smaller heparin-releasable pool of TFPI than is present in humans.[28] The evolutionary conservation of TFPI α and its conserved production in mouse placenta and embryonic tissues, but not adult tissues, suggests that unique features of TFPI α , such as K3, the basic C-terminal region and/or its association with a GPI-anchored binding protein, perform specific functions in the regulation of intravascular TF/FVIIa and/or FXa activity during vasculogenesis/angiogenesis that are not performed by TFPI β or TFPI γ .

Conclusion

The past twenty-two years has seen the isolation and basic characterization of TFPI, including identification of multiple isoforms and differences in isoform expression between species. Many unanswered questions of TFPI biology still remain. For instance, are there any *in vivo* functional differences between the different TFPI isoforms? Does TFPI have a role in physiological processes other than coagulation? What are the functions of the different pools of TFPI *in vivo*? How is production of the different isoforms regulated?

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study design, the collection, analysis and interpretation of data, in the writing of the manuscript or in the decision to submit the manuscript for publication.

Abbreviations

TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
K1	first Kunitz domain
K2	second Kunitz domain
K3	third Kunitz domain
GPI	glycosylphosphatidylinositol

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A

B

Figure 1.

(A) Box diagram of the alternatively spliced forms of human TFPI. The amino acid sequence for the C-terminal region of each isoform is indicated. Only the first 12 amino acids of the C-terminal region of TFPI β are predicted to be in the mature protein. The remainder of the C-terminal region encodes a GPI-anchor attachment sequence and is removed in the endoplasmic reticulum. (B) Diagram of the amino acid sequences of TFPI α , TFPI β and TFPI δ showing the Kunitz domain structures and common 5' splice donor site for TFPI α and TFPI β , while the TFPI δ 5' splice site is immediately following K2.

Figure 2.

(A) Box diagram of the alternatively spliced forms of mouse TFPI. The amino acid sequence for the C-terminal region of each isoform is indicated. Only the first 8 amino acids of the C-terminal region of TFPI β are predicted to be in the mature protein. The remainder of the C-terminal region encodes a GPI-anchor attachment sequence and is removed in the endoplasmic reticulum. The coding sequence for the C-terminal region of TFPI γ is located within the 3'UTR of TFPI β . (B) Diagram of the amino acid sequences of TFPI α , TFPI β and TFPI γ showing the Kunitz domain structures and common 5' splice donor site for all three isoforms.