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TFPIβ, a Second Product from the Mouse Tissue Factor Pathway Inhibitor (TFPI) Gene

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Summary

Tissue factor pathway inhibitor (TFPI) contains three Kunitz domains separated by two connecting regions. We have cloned another naturally occurring TFPI gene product from a mouse lung cDNA library which we have called TFPIβ. TFPIβ is derived from alternative splicing of the TFPI gene. Analysis of the cDNA shows that mouse TFPIβ protein is identical to TFPI from the N'-terminus through the second connecting region. However, mouse TFPIβ possesses neither a third Kunitz domain nor an Arg, Lys-rich C'-terminus but instead has a completely different C'-terminal (β-domain) sequence which is not homologous to any known protein. Northern blot analyses show that the tissues for mouse TFPIβ synthesis are heart and lung; in contrast, TFPI appears in Northern blots of heart and spleen. Both TFPIβ and TFPI messages first appear in 7-day-old mouse embryos, but only the TFPI mRNA persists until 17 days. Purified recombinant TFPIβ shows an apparent molecular weight of 38 kDa. Kinetic studies indicate that mouse TFPIβ is a slow-binding enzyme inhibitor for human factor Xa. In addition, heparin does not enhance the inhibition of factor Xa by mouse TFPIβ although it does accelerate factor Xa inhibition by TFPI.

Introduction

Tissue factor pathway inhibitor (TFPI) regulates the initiation of coagulation by interacting with factor Xa, then with the factor VIIa/tissue factor complex to shut down the extrinsic pathway. The cDNA sequences of TFPI from several different species (human, monkey, canine, rabbit, rat, and mouse) have been published (1-6). TFPIs from a variety of species show a high homology in protein sequence as well as domain structure. Basically, TFPI contains a negatively charged N'-terminus, three tandemly arrayed Kunitz type domains which are joined by two connecting regions, and a positively charged C'-terminus rich in Lys and Arg residues. The first Kunitz domain interacts with factor VIIa; the second Kunitz domain binds to factor Xa; and the third Kunitz domain and the C'-terminal region are involved in heparin binding (7-9).

In order to translate genetic information into a biologically active product, genomic DNA must be transcribed into messenger RNA for protein synthesis. Mature mRNA is generated from a larger pre-mRNA by 1) 5'-end modification (CAP); 2) 3'-end cleavage and polyadenylation; and 3) removal of intron sequences from the primary transcript (splicing). Primary pre-mRNA transcripts can be spliced in different ways and result in the synthesis of different proteins with similar or different functions (10-13). For example, by alternative splicing, the human fibronectin gene encodes 20 different proteins which have different characteristics (14).

Recently, we reported the cDNA sequence of mouse TFPI which is homologous to the other reported TFPI sequences (6). Here, we present the identification and characterization of an alternatively spliced mouse TFPI gene product which we have called TFPIβ. This newly observed gene product contains two Kunitz type domains identical to TFPI but a completely different C'-terminus resulting from alternative splicing that has no homology to any known protein.

Material and Methods

A mouse lung cDNA library was purchased from Stratagene (La Jolla, CA). A mammalian cell expression vector, pcDNA3, was purchased from Invitrogen (San Diego, CA). Mouse Multiple Tissue Northern (MTN™) Blot, Mouse Embryo Multiple Tissue Northern (MTN) Blot and ExpressHyb solution were purchased from Clontech (Palo Alto, CA). PCR was performed in a Perkin-Elmer DNA Thermo Cycler (model 480). Enzymes used in the cloning work were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN), Promega (Madison, WI), GibcoBRL Life Technologies (Gaithersburg, MD), and United States Biochemical (Cleveland, Ohio). Oligonucleotides were purchased from Integrated DNA Technologies Inc. (Coralville, IA). α-^{32}P-dATP was purchased from ICN Pharmaceuticals Inc. (Irvine, CA). The matrices for protein chromatographic purification were purchased from Sigma Chemical Co., (St. Louis, MO). Bovine factor Xa and human factor Xa were purchased from Enzyme Research Laboratories Inc. (South Bend, IN). Recombinant human factor VIIa was a gift from Dr. U. Hedner (Novo Nordisk, Copenhagen). Recombinant soluble human tissue factor was a gift from Dr. Y. Nemerson (Mount Sinai School of Medicine, New York). Chromogenic substrate Spectrozyme fXa was purchased from American Diagnostica Inc. (Greenwich, CT). Heparin was purchased from Elkins-Sinn, Inc. (Cherry Hill, NJ).

cDNA cloning. Screening of the lung cDNA library was performed by using two oligonucleotides corresponding to partial sequences in the first Kunitz-type domain and the second connecting domain of mouse TFPI sequence using a PCR technique described previously (6). The probes are indicated by the appropriate arrows in Fig. 1. Clone pools positive for the screened sequences were amplified until the clone of interest was present at a level sufficient to be picked by plating on a 9-cm Petri dish (6). The resulting cDNA clone was sequenced on both strands by the dideoxy-termination technique (15).

Northern blot analyses. A probe specific for the β-domain sequence of mouse TFPIβ was prepared by the PCR technique. As shown in Fig. 2A, primers mb-1 and mb-2 were used to generate a ^{32}P-labeled probe to hybridize with the mouse TFPIβ message, while primers mt-1 and mt-2 were used to make a probe to detect the mouse TFPI message. The PCR reaction was performed in a buffer of 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 0.2 mM of the dNTP, 0.5 mM of each primer, 1.5 mM $MgCl_2$, and

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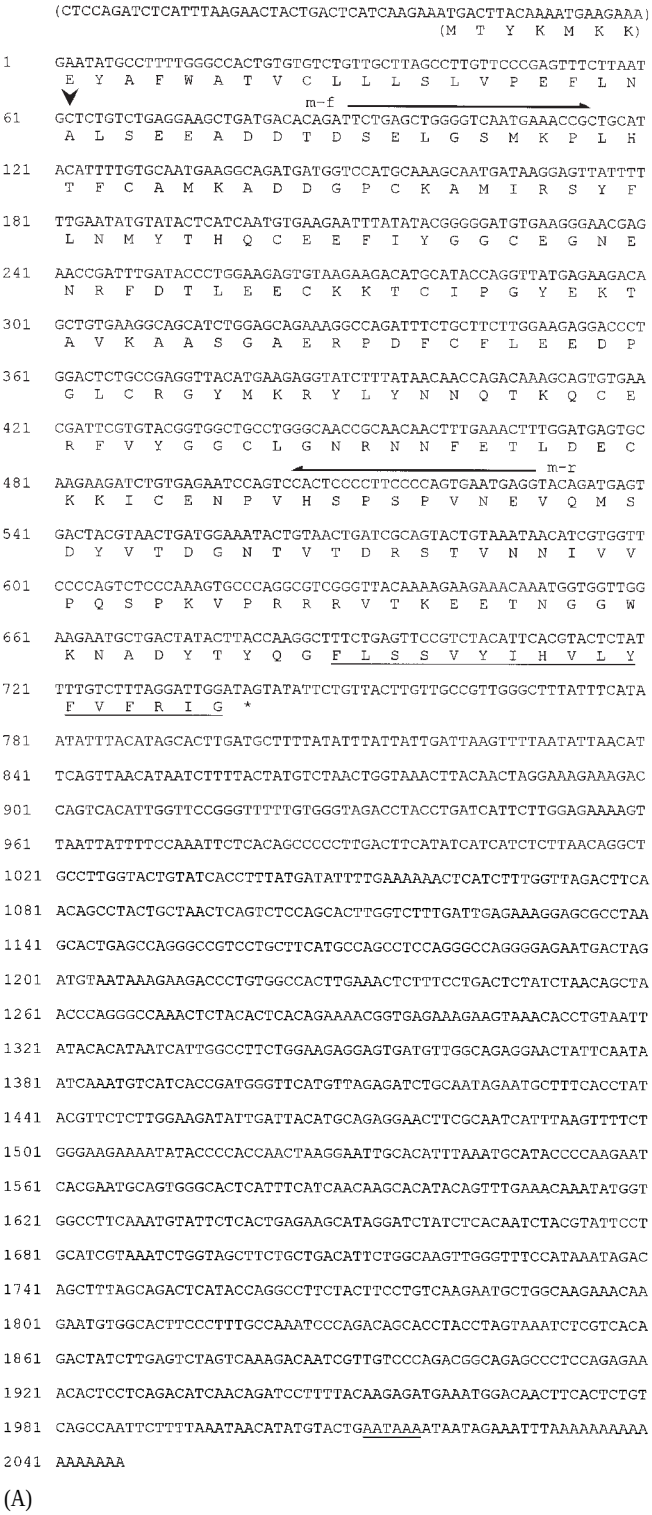
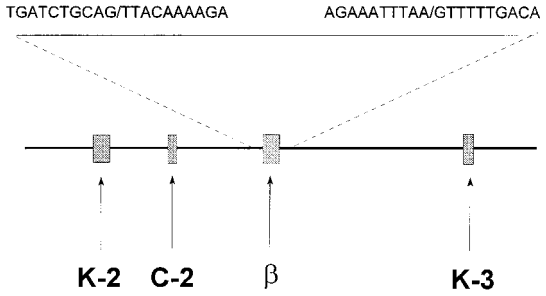


(A)



(B)

2.5 units of Taq DNA polymerase using 30 temperature cycles of 95° C (0.5 min), 55° C (0.5 min), and 72° C (1 min). The multiple tissue blots were pre-hybridized in ExpressHyb solution at 55° C for 30 min. The pre-hybridization solution was then replaced with fresh ExpressHyb containing the labeled probe for another 60 min incubation at 55° C. After hybridization, the membranes were washed twice in 2 × SSC, 0.1% SDS at room temperature and twice in 0.1 × SSC, 0.1% SDS for 15 min at 55° C prior to autoradiography.

Expression and purification of recombinant mouse TFPIβ. Mouse TFPIβ cDNA was subcloned into pcDNA3 and transfected into a human kidney cell line (293) by Lipofectin (Gibco-BRL). The selection and screening of the transfectants and the expression of the recombinant protein were performed as described in a previous report (6). Recombinant mouse TFPIβ was purified from the conditioned medium as follows: the conditioned medium was passed through a heparin-Sepharose column; the flow through was collected and loaded onto a Q Sepharose Fast Flow column; TFPIβ was eluted from the column by a 0 to 1 M NaCl gradient in 20 mM Tris, pH 7.5, 10% glycerol; the fractions that contained TFPIβ were pooled and loaded onto a metal chelate column charged with Cu^{++}; TFPIβ was then eluted from the column by a 0.5 to 0 M NaCl gradient in 20 mM Tris pH 7.5, 10% glycerol; the fractions with TFPIβ were then concentrated by a Q Sepharose column. The concentrated sample was then passed through a phenyl Sepharose column and TFPIβ was collected in the flow through and dialyzed against 20 mM Tris pH 7.5, 10% glycerol buffer. A Q Sepharose column was then used to concentrate TFPIβ in the final step.

Characterization of recombinant mouse TFPIβ. The concentration of purified mouse TFPIβ was determined by the Bradford assay using bovine serum albumin as a standard and bovine factor Xa titration as described before (16). Protein gel analysis was performed by a PhastGel electrophoresis system (Pharmacia Biotech Inc., Piscataway, NJ). Heparin-mediated factor Xa inhibition was analyzed as described by Huang et al. (17).

Other methods. The hydropathy profiles were calculated according to the method of Engelman et al. (18) using the software of MacVector (International Biotechnologies, Inc., New Haven, CT).

Results

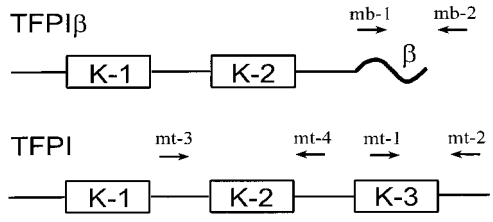
cDNA cloning. We identified a novel TFPI sequence from a mouse lung cDNA library. This clone is about 2 kb in size (Fig. 1A). The cDNA sequence predicts a protein sequence that is identical to TFPI from the N'-terminus to the end of the second connecting domain. However, following the second connecting domain, the C'-terminus is entirely different. A BLAST search of sequences deposited in the GenBank shows that this C'-terminal sequence is not significantly homologous to any submitted protein sequence. Genomic DNA sequencing of partial TFPI gene confirms that this C'-terminal region of TFPIβ is present as an exon in which the polyadenylation signal is included. This exon is located between the exons of the second

Fig. 1 Nucleotide sequence and the predicated amino acid sequence of mouse TFPIβ. (A) The numbers at the left of the lines represent the nucleotide numbers in this cDNA clone. The arrowhead indicates the signal peptide cleavage site. The two oligonucleotide sequences, m-f and m-r, used in the PCR screening of the library are noted by the arrows. The potential polyadenylation signal, AATAAA, in the 3'-untranslated region is underlined. The bracketed sequence was grafted from mouse TFPI cDNA sequence (6). The underlined residues of the predicted protein sequence indicate a hydrophobic region according to the method of Engelman et al. (18). The sequence reported in this paper has been deposited in the GenBank data base (accession no. AF016313). (B) Diagram which shows the intron-exon boundaries around the β-domain

connecting domain and the third Kunitz domain of the TFPI gene and exhibits a type 1 intron-exon boundary (Fig. 1B). The genomic data shows that this cDNA clone is not due to an artifact of the lung cDNA library. Rather, these data indicate that this sequence represents a newly discovered product of the TFPI gene produced by alternative splicing. We have called this alternatively spliced form TFPIβ. One clone out of 10^4 from this mouse lung cDNA library was found to contain this TFPIβ cDNA.

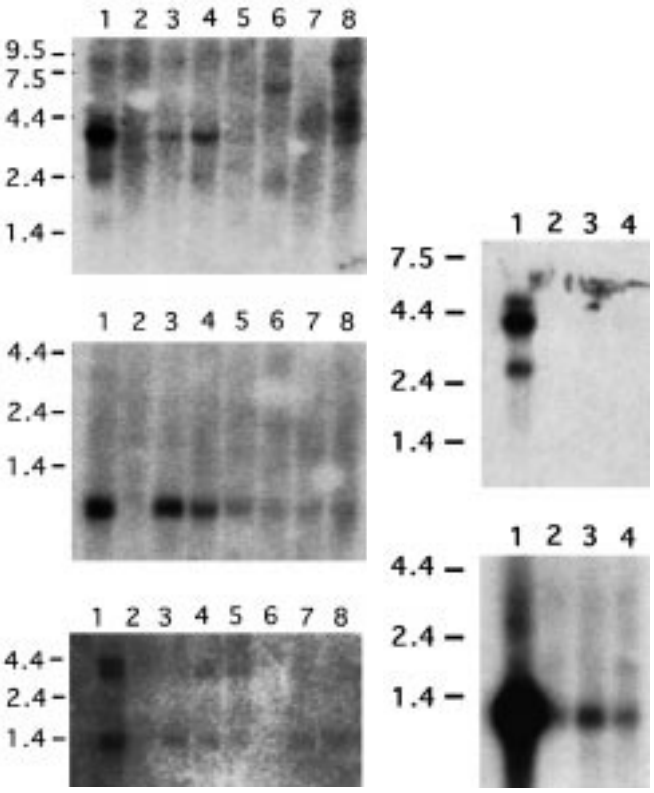
Northern blot analyses. Northern blot analyses give information about sites of expression of TFPIβ and TFPI. We used a PCR technique to generate ^{32}P-labeled probes for the analyses as described in Fig. 2A. Since the two products of the TFPI gene differ from each other in their C'-terminus, the probe for hybridization with TFPIβ contained a sequence that only codes for the β-domain. Therefore this probe would not cross react with TFPI message. Similarly, the probe used to detect the TFPI transcript recognizes only the sequence that codes for the third Kunitz and the C'-terminus of TFPI and thus would not hybridize to TFPIβ. Fig. 2B shows that the heart and lung are the major tissues for synthesis of mouse TFPIβ mRNA. When the same probe was used to investigate TFPIβ in the mouse embryo, the signal only appeared in the 7-day-old embryo (Fig. 2C). The mRNAs detected by the TFPIβ probe were also recognized by a specific TFPI probe that recognized the sequence from the first connecting domain to the second connecting domain (Fig. 2C). This indicates that TFPI and TFPIβ have identical sequences except the C'-termini. It also shows the relative abundance of the messages. Previous studies of Northern blot analyses from both human and rat TFPI have shown two transcripts of different sizes, 4.0 kb and 1.4 kb (1, 5, 19). Similarly the mouse also has two different messenger RNAs that code for TFPIβ. The major message shown on the Northern blot is about 4 kb with another minor message of about 2.7 kb. These two forms of mouse TFPIβ mRNA are probably caused by the presence of two polyadenylation signals, as demonstrated in human TFPI mRNA (19). Our mouse TFPIβ cDNA clone most likely was prepared from the 2.7 kb mRNA. In contrast to TFPIβ, heart and spleen have the greatest amount of mRNA for TFPI synthesis although lung also has a small amount of message. Fig. 2C also shows that although TFPI mRNA is present in all the embryonic stages chosen for the analysis, the 7-day-old embryo has a very significant amount of TFPI message. The probe that specifically recognizes mouse TFPI C'-terminal sequence only hybridized to a mouse TFPI mRNA of 1.3 kb (Figs. 2B, 2C) suggesting that mouse has only one message for TFPI. This is in contrast to the case in humans and rats where there are two different mRNAs for TFPI.

Protein purification and characterization. Recombinant mouse TFPIβ was purified to approximately 90% homogeneity according to the gel analysis (Fig. 3). The concentration of TFPIβ was determined by bovine factor Xa titration which reflects the concentration of active TFPIβ. This concentration agrees well with the concentration determined from a Bradford assay with bovine serum albumin as the standard (data not shown). These results indicate that the protein band shown in the gel (Fig. 3) with the apparent molecular weight of 38 kDa is recombinant mouse TFPIβ. In addition, mouse TFPIβ interacts with human factor Xa in a 1:1 stoichiometry. Fig. 4 shows the inhibition of human factor Xa by mouse TFPIβ. Amino terminal sequencing confirms that the purified mouse TFPIβ has the sequence predicted by the cDNA sequence. The progressive curves indicate that mouse TFPIβ is a slow-binding enzyme inhibitor for human factor Xa and the inhibition is not accelerated by heparin. In addition, we have demonstrated that TFPIβ also inhibits the factor VIIa/tissue factor complex (data not shown).



mb-1: GTTACAAAAGAAGAAACAAATGGTGG
mb-2: TATGAAATAAAGCCCAACGGC
mt-1: CAATTCAGCCACTGGGAAATGCCACC
mt-2: AACTTTCACGAAGGGCGCC
mt-3: ATACCAGGTTATGAGAAGACAGCTGTGAAGGC
mt-4: CTCATTCACTGGGGAAGGGGAGTGGACTGG

(A)



(B) (C)

Fig. 2 Northern blot analyses of mouse TFPIβ. (A) Structure of TFPI and TFPIβ and the strategy to generate probes for the hybridization. The K-1, K-2, K-3 represent each Kunitz domain and β represents the C'-terminal region of TFPIβ. The oligonucleotides used in the PCR are noted by arrows and their sequences are listed. (B) Mouse multiple tissue Northern blot analysis. Each lane contains approximately 2 μg of poly A+RNA. Lanes 1-8, contain, in order, RNA from mouse heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis. The upper panel shows the membrane was analyzed by the probe for TFPIβ. The middle panel shows the membrane detected by the probe for TFPI. The lower panel shows the membrane detected by the probe for both TFPIβ and TFPI. (C) Mouse embryo Northern blot analysis. Each lane contains approximately 2 μg of poly A + RNA. Lanes 1-4 contain, in order, RNA from mouse embryos in the following ages: 7 days, 11 days, 15 days, and 17 days. The upper panel shows the membrane was analyzed by the probe for TFPIβ. The lower panel shows the membrane detected by the probe for TFPI

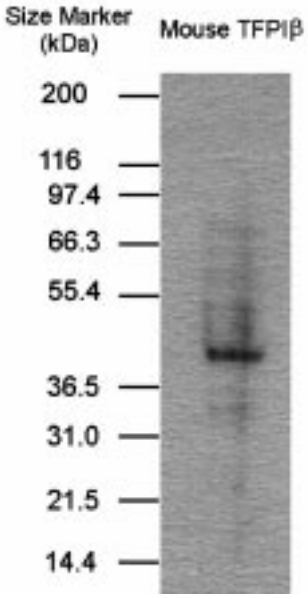


Fig. 3 Protein gel for recombinant mouse TFPIβ. The purified recombinant TFPIβ was loaded and run on a 10-15% gel under reducing conditions. The amount of protein loaded on the gel is 0.96 μg/lane based on the Bradford assay or 1.3 μg/lane based on the result of the bovine factor Xa titration. Protein band was visualized by Coomassie blue staining

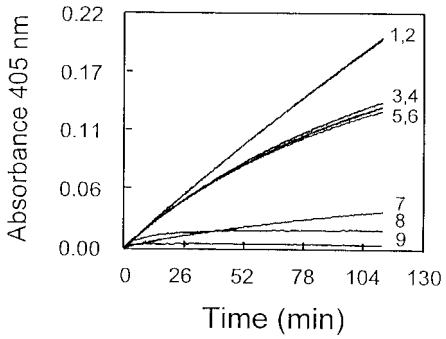


Fig. 4 Effect of heparin on the inhibition of human factor Xa by recombinant mouse TFPIβ. All the reactions contain 0.2 nM of human factor Xa, 5 mM $CaCl_2$ and 0.25 mM of the chromogenic substrate Spectrozyme fXa. Curves 1 and 2 represent the factor Xa cleavage of substrate in the absence and presence of 10 U/ml heparin, respectively. Curves 3, 4, 5, and 6 represent the factor Xa cleavage of substrate in the presence of 2 nM mouse TFPIβ either without heparin (curve 3) or with heparin (1 U/ml, 10 U/ml, 100 U/ml, curve 4, 5, and 6, respectively). Curve 7 represents the factor Xa cleavage of substrate when preincubated with mouse TFPIβ at room temperature for an hour. Curves 8 and 9 show factor Xa cleavage of substrate in the presence of 2 nM mouse TFPI without or with 10 U/ml heparin, respectively

Discussion

Based on the assumption that the endothelial cell is the major source for TFPI synthesis (20), we originally tried to clone mouse TFPI cDNA from a lung cDNA library since lung is rich in endothelial cells. Surprisingly, the cDNA clone we isolated from this lung library contained only two Kunitz domains and a C'-terminal sequence which was not homologous to any known protein sequence. Two possibilities could account for this observation: 1) an artifact resulting from nonspecific ligation when the lung library was prepared, or 2) an alternatively spliced product from the TFPI gene. Nonspecific ligation could be a problem if random primers were used in the reverse transcription. However, the library we used was made by using oligo-dT and ligated into the vector (λ_{ZAP}) in uni-direction. No random primers in the reverse transcription reaction were used. Thus the observation that the 3'-end of this clone differs from that of TFPI could not be attributed to random ligation. In addition, we determined the partial genomic sequence for mouse TFPI gene and confirmed that the exon that encodes for the C'-terminus of mouse TFPIβ is present within the TFPI gene and exihibits a type I intron-exon boundary. These data also indicate that mouse TFPI gene produces more than one gene product as a result of alternative splicing.

Northern blot analysis showed that the expression of mouse TFPIβ is not identical to that of TFPI. Mouse TFPI mRNA is present among all the embryos (7, 11, 15, 17 days old) chosen for the hybridization, suggesting that it may be present during most of the mouse embryonic development period. However, mouse TFPIβ is present only in the 7-day-old embryo in this assay.

A recent publication reported that the disruption of the mouse TFPI gene results in intrauterine lethality in mice (21). The targeting construct used by Huang et al. disrupts the TFPI gene at the first Kunitz domain and thus would affect both TFPIβ and TFPI transcripts. Although 60% of $TFPI_{K1}$ (–/–) mice die between embryonic days 9.5 and 11.5 and none of the mice survive to neonatal period, TFPI is not essential for the development of specific tissues (21). The heart is the organ which expresses significant amounts of both isoforms of TFPI in adult mice (Fig. 2B) and is the first organ to differentiate and to function in the mouse embryo (22). Cardiogenesis occurs at about 7-7.5 days post coitum (p.c.) and the heart starts to contract on day 8 p.c. (22). Since the TFPIβ and TFPI messages are abundant in the 7-day mouse embryo, prior to differentiation of the heart, TFPI gene expression may not be regulated in a tissue (heart) specific manner. It is also possible that the two products of the TFPI gene have functions other than those related to coagulation. Another possible expectation is that TFPIβ and TFPI may each partially compensate for a deficiency of the other. In other words, a mouse that does not express TFPIβ but has normal production of TFPI may survive but may not be healthy.

Since the original mouse TFPIβ cDNA isolated from a mouse lung cDNA library lacked the 5'-end untranslated sequence and 21 bases of the sequence that code for the the first 7 amino acid residues in the signal peptide region, we subcloned this missing fragment from mouse TFPI cDNA (6) to mouse TFPIβ sequence for the purpose of expression. Recombinant mouse TFPIβ was expressed in human kidney 293 cells and purified from serum-free conditioned medium to near homogeneity as shown in Fig. 3. Protein sequencing from the amino terminus of the purified mouse TFPIβ confirmed that the sequence is in agreement with that predicted from the cDNA sequence.

TFPI is a slow-tight inhibitor for factor Xa and heparin is able to accelerate this inhibition (8, 17). The heparin binding sites on TFPI are located in the third Kunitz domain and the positively charged C'-terminus. Since mouse TFPIβ does not contain the heparin binding sites of TFPI, it is reasonable to expect that TFPIβ would not interact with heparin. Our data support this contention in that recombinant TFPIβ does not bind to the heparin-Sepharose column during protein purification. We further demonstrated that the inhibition of human factor Xa is not affected by the presence of various concentrations of heparin, in contrast to that of mouse TFPI, as shown in Fig. 4.

It has been reported that about 45% of TFPI in the circulation is associated with lipoprotein (23, 24), about 10% is carried by platelets (25), and about 45% is free in plasma (23, 26). In addition, the surface glycosaminoglycans of the endothelium are considered to be a TFPI reservoir (27, 28). Many reports have demonstrated that the third

Kunitz domain and the positively charged C'-terminus of TFPI are responsible for binding to heparin (9, 16) as well as for association with lipoprotein (26, 27, 29). This leads us to predict that mouse TFPIβ would not possess the characteristics listed above since this newly discovered molecule does not contain the domains required for such functions. Recombinant mouse TFPIβ does not bind to heparin suggesting that the glycosaminoglycan on the endothelial surface is not a storage place for TFPIβ.

Although TFPIβ and TFPI both inhibit factor Xa and the factor VIIa/tissue factor complex, additional studies are needed to determine whether TFPIβ has functions different from those of TFPI. In addition, further study on the regulation of the expression of each TFPI gene product will contribute our knowledge on the control of blood coagulation and the pathogenesis of thrombosis, and possibly atherosclerosis.

Acknowledgement

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References

1. Wun T-Z, Kretzmer KK, Girard TJ, Miletich JP, Broze GJ Jr. Cloning and characterization of a cDNA coding for the lipoprotein-associated coagulation inhibitor shows that it consists of three tandem Kunitz-type inhibitory domains. J Biol Chem 1988; 263: 6001-4.
2. Kamei S, Kamikubo Y-i, Hamuro T, Fujimoto H, Ishihara M, Yonemura H, Miyamoto S, Funatsu A, Enjyoji K-i, Abumiya T, Miyata T, Kato H. Amino acid sequence and inhibitory activity of rhesus monkey tissue factor pathway inhibitor (TFPI): comparison with human TFPI. J Biochem 1994; 115: 708-14.
3. Girard TJ, Gailani D, Broze GJ, Jr. Complementary DNA sequencing of canine tissue factor pathway inhibitor reveals a unique nanomeric repetitive sequence between the second and third Kunitz domains. Biochem J 1994; 303: 923-8.
4. Wesselschmidt RL, Girard TJ, Broze GJ, Jr. cDNA sequence of rabbit lipoprotein-associated coagulation inhibitor. Nucleic Acids Res 1990; 18: 6440.
5. Enjyoji K-i, Emi M, Mukai T, Kato H. cDNA Cloning and Expression of Rat Tissue Factor Pathway Inhibitor (TFPI). J Biochem 1992; 111: 681-7.
6. Chang J-Y, Monroe DM, Oliver JA, Liles DK, Roberts HR. Cloning, expression, and characterization of mouse tissue factor pathway inhibitor (TFPI). Thromb Haemost 1998; 79: 306-9.
7. Girard TJ, Warren LA, Novotny WF, Likert KM, Brown SG, Miletich JP, Broze GJ, Jr. Functional significance of the Kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor. Nature 1989; 338: 518-20.
8. Wesselschmidt R, Likert K, Huang Z, MacPhail L, Broze GJ, Jr. Structural requirements for tissue factor pathway inhibitor interactions with factor Xa and heparin. Blood Coagul Fibrin 1993; 4: 661-9.
9. Enjyoji K-i, Miyata T, Kamikubo Y-i, Kato H. Effect of heparin on the inhibition of factor Xa by tissue factor pathway inhibitor: a segment, Gly212-Phe243, of the third Kunitz domain is a heparin-binding site. Biochemistry 1995; 34: 5725-35.
10. Leff S, Rosenfeld M. Complex transcriptional units: diversity in gene expression by alternative RNA processing. Ann Rev Biochem 1986; 55: 1091-1117.
11. Maniatis T. Mechanisms of alternative pre-mRNA splicing. Science 1991; 251: 33-4.
12. Rio D. RNA binding proteins, splice site selection, and alternative pre-mRNA splicing. Gene Expression 1992; 2: 1-5.
13. Sharp P. Split genes and RNA splicing. Cell 1994; 77: 805-15.
14. Hynes RO. Fibronectins. Springer-Verlag, New York 1990.
15. Sanger F, Nicklen SL, Coulson AR. DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci U.S.A. 1977; 74: 5463-7.
16. Wesselschmidt RL, Likert K, Girard T, Wun T-C, Broze GJ Jr. Tissue factor pathway inhibitor: the carboxy-terminus is required for optimal inhibition of factor Xa. Blood 1992; 79: 2004-10.
17. Huang Z-F, Wun T-C, Broze GJ, Jr. Kinetics of factor Xa inhibition by tissue factor pathway inhibitor. J Biol Chem 1993; 268: 26950-5.
18. Engelman DM, Steitz TA, Goldman A. Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins. Ann Rev Biophys Biophys Chem 1986; 15: 321-53.
19. Girard TJ, Warren LA, Novonty WF, Bejcek BE, Miletich JP, Broze GJ, Jr. Identification of the 1.4 Kb and 4.0 Kb messages for the lipoprotein associated coagulation inhibitor and expression of the encoded protein. Thromb Res 1989; 55: 37-50.
20. Bajaj M, Kuppuswamy M, Satio H, Spitzer S, Bajaj S. Cultured normal human hepatocytes do not synthesize lipoprotein-associated coagulation inhibitor: evidence that endothelium is the principle site of its synthesis. Proc Natl Acad Sci USA 1990; 87: 8869-73.
21. Huang Z-F, Huguchi D, Lasky N, Broze GJ, Jr. Tissue factor pathway inhibitor gene disruption produces intrauterine lethality in mice. Blood 1997; 90: 944-51.
22. Kaufman MH. The Atlas of Mouse Development, Academic Press, New York 1995.
23. Kokawa T, Abumiya T, Kimura T, Harada-Shiba M, Koh H, Tsushima M, Yamamoto A, Kato H. Tissue factor pathway inhibitor activity in human plasma. Measurement of lipoprotein-associated and free forms in hyperlipidemia. Arterioscler. Thromb Vasc Biol 1995; 15: 504-10.
24. Novotny WF, Girard TJ, Miletich J P, Broze GJ, Jr. Purification and characterization of the lipoprotein-associated coagulation inhibitor from human plasma. J Biol Chem 1989; 264: 18832-7.
25. Novotny WF, Girard TJ, Miletich JP, Broze GJ, Jr. Platelets secrete a coagulation inhibitor functionally and antigenically similar to the lipoprotein associated coagulation inhibitor. Blood 1988; 72: 2020-5.
26. Valentin S, Nordfang O, Bregengard C, Wildgoose P. Evidence that the C-terminus of tissue factor pathway inhibitor (TFPI) is essential for its in vitro and in vivo interaction with lipoproteins. Blood Coag Fibrinol 1993; 4: 713-20.
27. Novotny WF, Palmier M, Wun T-C, Broze GJ, Jr. Miletich JP. Purification and properties of heparin-releasable lipoprotein-associated coagulation inhibitor. Blood 1991; 78: 394-400.
28. Sandset PM, Abildgaard U, Larsen ML. Heparin induces release of extrinsic pathway inhibitor (EPI). Thromb Res 1988; 50: 803-13.
29. Abumiya T, Enjyoji K-I, Kokawa T, Kamikubo Y-I, Kato H. An anti-tissue factor pathway inhibitor (TFPI) monoclonal antibody recognized the third Kunitz domain (K3) of free-form TFPI but not lipoprotein-associated forms in plasma. J. Biochem. 1995; 118: 178-82.
30. Gordon T, Castelli WP, Hjortland MC, Kannel WB, Dawber TR. High density lipoprotein as a protective factor against coronary heart disease. The Framingham study. Am J Med 1977; 62: 707-14.
31. Scanu AM. Lp(a) as a marker for coronary heart disease risk. Clin Cardiol 1991; 14: I-35-9.
32. Austin MA, Breslow JL, Hennekens CH, Buring JE, Willett WC. Krauss RM. Low-density lipoprotein subclass patterns and risk of myocardial infarction. JAMA 1988; 260: 1917-21.

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