

TFPI β , 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 TFPI β . TFPI β is derived from alternative splicing of the TFPI gene. Analysis of the cDNA shows that mouse TFPI β protein is identical to TFPI from the N¹-terminus through the second connecting region. However, mouse TFPI β 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 TFPI β synthesis are heart and lung; in contrast, TFPI appears in Northern blots of heart and spleen. Both TFPI β and TFPI messages first appear in 7-day-old mouse embryos, but only the TFPI mRNA persists until 17 days. Purified recombinant TFPI β shows an apparent molecular weight of 38 kDa. Kinetic studies indicate that mouse TFPI β is a slow-binding enzyme inhibitor for human factor Xa. In addition, heparin does not enhance the inhibition of factor Xa by mouse TFPI β 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 N¹-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 TFPI β . 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 (MTNTM) 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 -³²P-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).

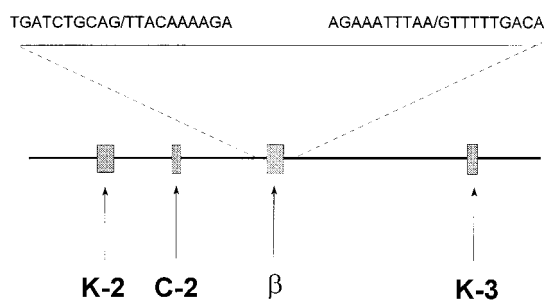
cDNA cloning. Screening of the lung cDNA library was performed by using two oligonucleotides corresponding to partial sequences in the first Kunitz-type domain and the second connecting domain of mouse TFPI sequence using a PCR technique described previously (6). The probes are indicated by the appropriate 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 TFPI β was prepared by the PCR technique. As shown in Fig. 2A, primers mb-1 and mb-2 were used to generate a ³²P-labeled probe to hybridize with the mouse TFPI β 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 MgCl₂, and

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(CTCCAGATCTCATTAAAGAACTACTGACTCATCAAGAAATGACTTACAAAATGAAGAAA)
(M T Y K M K K K)
1 GAATATGCCTTTTGGGCCACTGTGTCTGTGCTTTCGCCGAGTTTCTTAAT
  E Y A F W A T V C L L L S L V P E F L N
61 GCTCTGTCTGAGGAAGCTGATGACACAGATTCTGAGCTGGGGTCAATGAAACCGTGCAT
  A L S E E A D D T D S E L G S M K P L H
121 ACATTTTGTGCAATGAAGGCAGATGATGGTCCATGCAAAGCAATGATAGGAGTATTTT
  T F C A M K A D D G P C K A M I R S Y F
181 TTGAATATGATATCTCATCAATGTGAAGATTTATATACGGGGATGTGAAGGGAACGAG
  L N M Y T H Q C E E F I Y G G C E G N E
241 AACCGATTTGATACCCCTGGAAGAGTGAAGAAGACATGCATACCAGGTTATGAGAAGACA
  N R F D T L E E C K K T C I P G Y E K T
301 GCTGTGAAGGCAGCATCTGGAGCAGAAAGCCAGATTTCTGCTTCTTGAAGAGGACCCCT
  A V K A A S G A E R P D F C F L E E D P
361 GGACTCTGCCGAGTTACATGAAGAGTATCTTTATAACAACAGACAAAGCAGTGTGAA
  G L C R G Y M K R Y L Y N N Q T K Q C E
421 CGATTCTGTACGGTGGCTGCCTGGGCAACCCGCAACTTTGAAACTTTGGATGAGTGC
  R F V Y G G C L G N R N N F E T L D E C
481 AAGAAGATCTGTGAGAATCCAGTCCACTCCCTTCCCCAGTGAATGAGGTACAGATGAGT
  K K I C E N P V H S P S P V N E V Q M S
541 GACTACGTAACATGAAATACTGTAACATGATCGCAGTACTGTAATAACATCGTGGTT
  D Y V T D G N T V T D R S T V N N I V V
601 CCCAGTCTCCCAAAGTGCCAGGCGTGGGTTACAAAAGAAGAACAATGGTGGTGG
  P Q S P K E N P R R R V T K E E T N G G W
661 AAGAATGCTGACTATACTACCAAGGCTTTCTGAGTTCCTGACTACATTCAGTCTACT
  K N A D Y T Y Q G F L S S V Y I H V L Y
721 TTTGCTTTAGGATGGATAGTATATTTCTGTTACTTGTGCGCTGGGCTTTATTTTCATA
  F V F R I G *
781 ATATTACATAGCACTTGATGCTTTTATATTTATTTATGATTAAAGTTTAAATATTAACAT
841 TCAGTTAACAATACTTTTACTATGTCTAACTGGTAAACTTACAACATAGGAAGAAGAC
901 CAGTCACATTTGGTCCGGGTTTTTGTGGGTAGACCTACCTGATCATCTTGGAGAAAAGT
961 TAATTTATTTCCAAATCTCAGAGCCCTTGACTTCATATCATCATCTCTTAACAGGCT
1021 GCCTTGGTACTGTATCACCTTTATGATATTTTGA AAAACTCATCTTTGGTGTAGACTTCA
1081 ACAGCCTACTGCTAARCTCAGTCTCCAGCATTGGTCTTTGATTGAGAAAGGAGCGCCTAA
1141 GCACTGAGCCAGGGCGCTCTGCTTATGCCAGCCTCCAGGGCCAGGGGAGAATGACTAG
1201 ATGTAATAAAGAACCCCTGTGGCCACTTGA AACTCTTCTGACTCTATCTAACAGCTA
1261 ACCCAGGGCCAAACTCTACACTCACAGAAAACGGTGAGAAAGAAGTAAACACCTGTAATT
1321 ATACACATAATCATTTGGCCTTCTGGAAGAGGAGTATGTTGGCAGAGGAACATTTCAATA
1381 ATCAAATGTCATCACCGATGGGTTTATGTTAGAGATCTGCAATAGAATGCTTTCACCTAT
1441 ACGTTCTCTTGGAGATAATTGATTACATGCAGAGGAACCTCGCAATCATTTAAGTTTCT
1501 GGAAGAAAATATACCCCAACAACTAAGGAATTGCACATTTAAATGCATACCCCAAGAAT
1561 CACGAATGCGATGGGCACCTATTTCATCAACAAGCACATACAGTTTGAACAACAATATGGT
1621 GGCCTTCAAATGTATTCTCACTGAGAAGCATAGGATCTATCTCACAATCTCAGTATTCCT
1681 GCATCGTAAATCTGGTAGCTTCTGCTGACATTTCTGCAAGTTGGGTTCCATAAATAGAC
1741 AGCTTAGCAGACTCATAACAGGCTTCTACTTCTGTCRAGAATGCTGGCAAGAACA
1801 GAATGTGGCAGCTTCCCTTTGCCAAATCCAGACAGCACTACCTAGTAAATCTCGTCACA
1861 GACTATCTGAGTCTAGTCAAGACAACTGTTGTCAGAGCGGAGAGCCCTCCAGAGAA
1921 ACACTCTCAGACATCAACAGATCCCTTTTACAAGAGATGAAATGGCAACTCTCACTCTGT
1981 CAGCCAAATCTTTTAAATAACATATGACTGAATAAAATAATAGAAATTTAAAAA
2041 AAAAAA
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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, AATAAA, in the 3'-untranslated region is underlined. The bracketed 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).

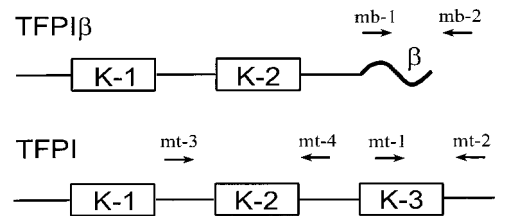
Fig. 1 Nucleotide sequence and the predicted amino acid sequence of mouse TFPIβ. (A) The numbers at the left of the lines represent the nucleotide numbers in this cDNA clone. The arrowhead indicates the signal peptide cleavage site. The two oligonucleotide sequences, m-f and m-r, used in the PCR screening of the library are noted by the arrows. The potential polyadenylation signal, AATAAA, in the 3'-untranslated region is underlined. The bracketed 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

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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 TFPI β . One clone out of 10^4 from this mouse lung cDNA library was found to contain this TFPI β cDNA.

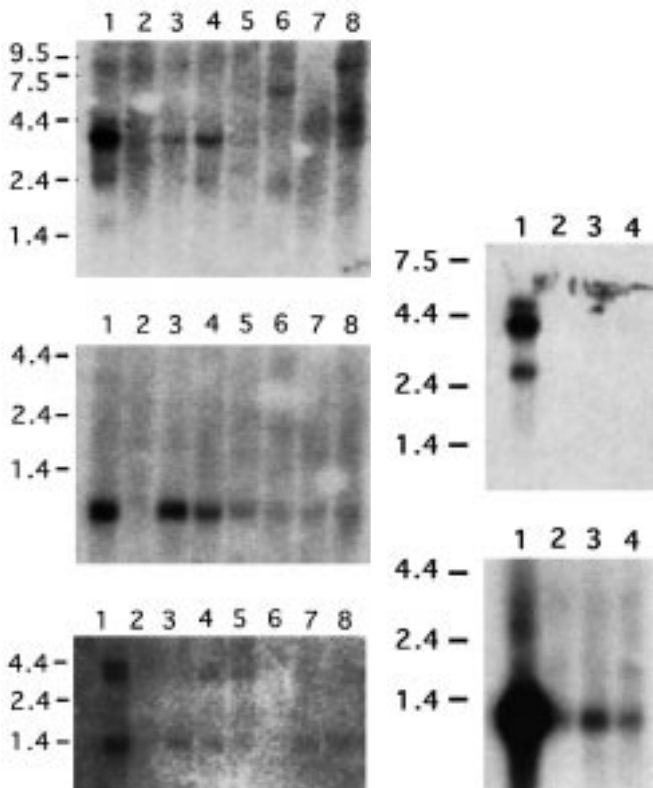
Northern blot analyses. Northern blot analyses give information about sites of expression of TFPI β and TFPI. We used a PCR technique to generate 32 P-labeled probes for the analyses as described in Fig. 2A. Since the two products of the TFPI gene differ from each other in their C'-terminus, the probe for hybridization with TFPI β 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 TFPI β . Fig. 2B shows that the heart and lung are the major tissues for synthesis of mouse TFPI β mRNA. When the same probe was used to investigate TFPI β in the mouse embryo, the signal only appeared in the 7-day-old embryo (Fig. 2C). The mRNAs detected by the TFPI β 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 TFPI β 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 TFPI β . 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 TFPI β mRNA are probably caused by the presence of two polyadenylation signals, as demonstrated in human TFPI mRNA (19). Our mouse TFPI β cDNA clone most likely was prepared from the 2.7 kb mRNA. In contrast to TFPI β , 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 TFPI β was purified to approximately 90% homogeneity according to the gel analysis (Fig. 3). The concentration of TFPI β was determined by bovine factor Xa titration which reflects the concentration of active TFPI β . 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 TFPI β . In addition, mouse TFPI β interacts with human factor Xa in a 1:1 stoichiometry. Fig. 4 shows the inhibition of human factor Xa by mouse TFPI β . Amino terminal sequencing confirms that the purified mouse TFPI β has the sequence predicted by the cDNA sequence. The progressive curves indicate that mouse TFPI β is a slow-binding enzyme inhibitor for human factor Xa and the inhibition is not accelerated by heparin. In addition, we have demonstrated that TFPI β 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: ATACCAGTTATGAGAAGACAGCTGTGAAGGC
 mt-4: CTCATTCACCTGGGGAAGGGGAGTGGACTGG

(A)



(B)

(C)

Fig. 2 Northern blot analyses of mouse TFPI β . (A) Structure of TFPI and TFPI β 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 TFPI β . 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 μ g 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 TFPI β . The middle panel shows the membrane detected by the probe for TFPI. The lower panel shows the membrane detected by the probe for both TFPI β and TFPI. (C) Mouse embryo Northern blot analysis. Each lane contains approximately 2 μ g 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 TFPI β . The lower panel shows the membrane detected by the probe for TFPI

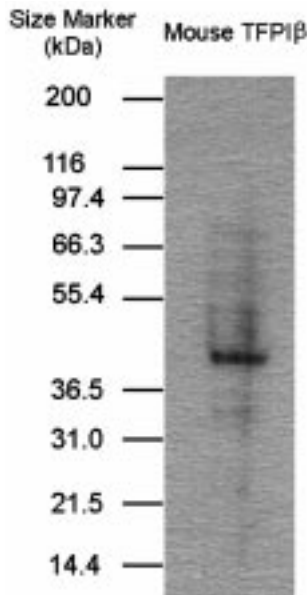


Fig. 3 Protein gel for recombinant mouse TFPI β . The purified recombinant TFPI β was loaded and run on a 10-15% gel under reducing conditions. The amount of protein loaded on the gel is 0.96 μ g/lane based on the Bradford assay or 1.3 μ g/lane based on the result of the bovine factor Xa titration. Protein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

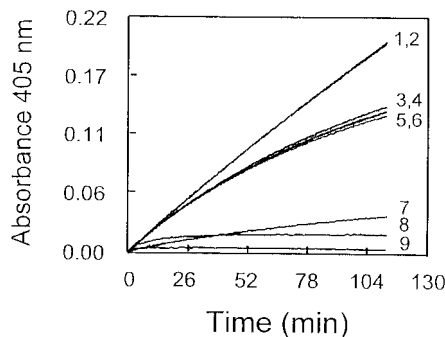


Fig. 4 Effect of heparin on the inhibition of human factor Xa by recombinant mouse TFPI β . All the reactions contain 0.2 nM of human factor Xa, 5 mM CaCl₂ and 0.25 mM of the chromogenic substrate Spectrozyme fXa. Curves 1 and 2 represent the factor Xa cleavage of substrate in the absence and presence 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 TFPI β 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 TFPI β at room temperature for an hour. Curves 8 and 9 show factor Xa cleavage of substrate in the presence of 2 nM mouse TFPI β 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 non-specific 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 TFPI β is present within the TFPI gene and exhibits 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 TFPI β 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 TFPI β 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 TFPI β 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 TFPI β 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 TFPI β and TFPI may each partially compensate for a deficiency of the other. In other words, a mouse that does not express TFPI β but has normal production of TFPI may survive but may not be healthy.

Since the original mouse TFPI β cDNA isolated from a mouse lung cDNA library lacked the 5'-end untranslated sequence and 21 bases of the sequence that code for the first 7 amino acid residues in the signal peptide region, we subcloned this missing fragment from mouse TFPI cDNA (6) to mouse TFPI β sequence for the purpose of expression. Recombinant mouse TFPI β 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 TFPI β 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 TFPI β does not contain the heparin binding sites of TFPI, it is reasonable to expect that TFPI β would not interact with heparin. Our data support this contention in that recombinant TFPI β 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 TFPI β would not possess the characteristics listed above since this newly discovered molecule does not contain the domains required for such functions. Recombinant mouse TFPI β does not bind to heparin suggesting that the glycosaminoglycan on the endothelial surface is not a storage place for TFPI β .

Although TFPI β and TFPI both inhibit factor Xa and the factor VIIa/tissue factor complex, additional studies are needed to determine whether TFPI β 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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