Cloning, Expression, and Characterization of Mouse Tissue Factor Pathway Inhibitor (TFPI)

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Summary

Tissue factor pathway inhibitor (TFPI) acts to regulate the initiation of coagulation by first inhibiting factor Xa. The complex of factor Xa/TFPI then inhibits the factor VIIa/tissue factor factor complex. The cDNA sequences of TFPI from several different species have been previously reported. A high level of similarity is present among TFPIs at the molecular level (DNA and protein sequences) as well as in biochemical function (inhibition of factor Xa, VIIa/tissue factor). In this report, we used a PCR-based screening method to clone cDNA for full length TFPI from a mouse macrophage cDNA library. Both cDNA and predicted protein sequences show significant homology to the other reported TFPI sequences, especially to that of rat. Mouse TFPI has a signal peptide of 28 amino acid residues followed by the mature protein (in which the signal peptide is removed) which has 278 amino acid residues. Mouse TFPI, like that of other species, consists of three tandem Kunitz type domains. Recombinant mouse TFPI was expressed in the human kidney cell line 293 and purified for functional assays. When using human clotting factors to investigate the inhibition spectrum of mouse TFPI, it was shown that, in addition to human factor Xa, mouse TFPI inhibits human factors VIIa, IXa, as well as factor XIa. Cloning and expression of the mouse TFPI gene will offer useful information and material for coagulation studies performed in a mouse model system.

Introduction

Tissue factor pathway inhibitor (TFPI) is a Kunitz-type protease inhibitor found in plasma, which functions as a multivalent protease inhibitor. It contains a negatively charged N-terminus, three tandemly arrayed Kunitz-type domains which are connected by two connecting regions, and a positively charged C-terminus. The first Kunitz domain interacts with factor VIIa; the second binds to factor Xa; and the third Kunitz domain and the C-terminal region are involved in heparin binding. TFPI serves a regulatory role in the coagulation pathway by interacting with the factor Xa and the factor VIIa/tissue factor complex, thereby inhibiting the initiation of coagulation.

The cDNA sequences of TFPI from several different species (human, monkey, dog, rabbit, and rat) have been cloned and reported (5-9). There is a high homology for the TFPI protein sequence and domain structure among different species. In the present study, we used a PCR-based screening method to clone full length mouse TFPI cDNA from the macrophage cDNA library. As expected, mouse TFPI cDNA shows significant homology to the other reported TFPI sequences.

Materials and Methods

The mouse macrophage cDNA library was purchased from Stratagene (La Jolla, 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), United States Biochemical (Cleveland, OH). Oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Human factor Xa was purchased from Enzyme Research Laboratories Inc. (South Bend, IN). Factor VIIa was from Novo Nordisk (Copenhagen, Denmark). Human factor IXa and thrombin were prepared according to previous reports (10, 11). Chromogenic substrates Spectrozyme fIXa, fXa, and fPCa were purchased from American Diagnostica Inc., (Greenwich, CT).

Reverse transcription-polymerase chain reaction. Total RNA from mouse tissues (heart, liver, lung, and kidney from strain 129) were prepared as described previously (12). Reverse transcription was performed by using the anti-sense sequence oligonucleotide (R2, Fig. 1) from the C-terminus of rat TFPI as the primer and 10 μg of total RNA as template for the first strand DNA synthesis. The PCR reaction (50 μl) 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 each dNTP, 0.5 μM of each primer, 2 mM MgCl2, and 2.5 units of Taq DNA polymerase using 40 temperature cycles of 95°C (1.5 min), 25°C (2 min), 72°C (2 min) followed by 20 cycles of 95°C (1.5 min), 40°C (2 min), 72°C (2 min). Primer pairs from the rat cDNA sequence corresponding to the junction of the second Kunitz domain and the second connecting domain (R1) and the carboxyterminus (R2) were used to PCR the template synthesized from reverse transcription described above. This pair of primers gave a fragment of cDNA sequence for mouse TFPI. Another pair of primers (M1 and M2, Fig. 1) was then designed using the mouse TFPI sequence and used to screen the TFPI cDNA from libraries.

Library screening. The screening protocol was modified from Amaravadi and King (13). First, 2 μl of the original cDNA library was 10-fold serially diluted and amplified by incubating with E. coli strain XL-1 Blue and plated onto agar plates. After the plaques were clearly visible, 6 ml SM buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 8 mM MgSO4, 0.01% gelatin) was added to each plate and incubated at 37°C for another 2 h to allow plaque particles to diffuse into the buffer. The phage solution (SM buffer) was centrifuged and the supernatant transferred to a new tube. Two microliters of the phage solution

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from each plate was added to the PCR solution (containing M1 and M2 as primers) and heated at 90°C for 10 min before adding Taq DNA polymerase. The PCR reaction was similar to that described above, except that 30 thermal cycles were run at 95°C (30 s), 55°C (30 s) and 72°C (1 min). Ten microliters of the PCR product from each phage titer was examined by agarose gel electrophoresis. The presence of a 248 bp DNA band indicated that the phage solution used for PCR contained mouse TFPI cDNA. Twenty microliters of the phage solution from the lowest titer plate with a positive PCR result was 10-fold serially diluted. Ten microliters of each of the diluted phage were incubated with XL-1 Blue and plated onto an LB agar plate. Again, the amplified phages were collected and examined by PCR as was done for the original library. Each plate with a positive PCR result was used to prepared 16 plates at a 10-fold lower titer. The phages of each plate were collected and examined as described above. Usually, at least one of these 16 plates showed a positive PCR result so that the targeted clone (mouse TFPI) was effectively concentrated 10-fold. This screening protocol was repeated until one out of 10° plaques theoretically contained the TFPI cDNA. Each individual plaque was then picked and screened by PCR. Positive plaques were treated by helper phage according to the manufacturer’s instructions to excise the phagemid from the Lambda-Zap vector.

Data sequencing. Dideoxy-chain termination sequencing was performed as described (14).

Recombinant TFPI expression, purification and characterization. Full length mouse TFPI cDNA was subcloned into the mammalian expression vector pcDNA3 (Invitrogen) and used to transfect a human kidney cell line (293) by Lipofectin (Gibco-BRL) according to the manufacturer’s instructions. Antibiotic (G418) resistant clones were found to contain full length TFPI cDNA. Each individual plaque was then picked and treated by helper phage according to the manufacturer’s instructions to excise the phagemid from the Lambda-Zap vector.

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Inhibition of activated coagulation factors. The inhibition of activated mouse coagulation factors by recombinant mouse TFPI was tested. Factors Xa, Xla, or thrombin (2 nM) were incubated with 0, 1, 2 or 20 nM mouse TFPI at room temperature for an hour before the appropriate coagulation substrate (Spectrozyme Xa, fIXa, or PCa, respectively) was added. Since the rate of enzyme cleavage of substrate is different for each clotting factor, the pertinent time required for monitoring the absorbance change differs ranging from 20 to 120 min. The absorbance change relative to protease with no inhibitor added at 405 nm was monitored and the enzymatic activity was calculated.

Results
cDNA cloning. Mouse TFPI shows a great deal of homology to the other reported mammalian TFPI sequences. One clone out of 10° from a mouse macrophage cDNA library was found to contain full length TFPI cDNA, which is about 1.3 Kb in length. The complete cDNA sequence and the predicted amino acid sequence are shown in Fig. 2. The mouse sequence shows a high degree of homology to other described TFPI sequences including the same domain arrangement (5-9). The protein sequences between mouse and rat TFPI share 54% identity in the N-terminus, 83% in the first Kunitz domain, 61% in the first connecting domain; 72% in the second Kunitz domain; 55% in the second connecting domain; 75% in the third Kunitz domain and 48% in the C-terminal region.

Expression and characterization. Recombinant mouse TFPI was expressed in the human kidney cell line 293 and purified to homology using a previously described method (16). Recombinant mouse TFPI was loaded and run onto a 8-25% gel under reducing condition. Protein band was visualized by Coomassie blue staining.

Fig. 2 Nucleotide sequence and the predicted amino acid sequence of mouse TFPI. The numbers at the left of the lines represent the nucleotide numbers. The arrow indicates the signal peptide cleavage site. The potential polyadenylation signal, AATAAA, in the 3’ untranslated region is underlined. The numbers at the left of the lines represent the nucleotide numbers.

Fig. 3 Protein gel for the recombinant mouse TFPI. One microgram of recombinant mouse TFPI was loaded and run onto a 8-25% gel under reducing condition. Protein band was visualized by Coomassie blue staining.
has an apparent molecular weight of 42 kDa based on reduced SDS-PAGE (Fig. 3). Protein sequencing confirms that the N-terminal sequence corresponds to the expected protein sequence based on the cDNA sequence (data are not shown).

Inhibition of the activated human clotting factors. To characterize protease inhibitory capacity of mouse TFPI, a 0.5 to 20-fold molar excess of mouse TFPI was incubated with human factors VIIa, IXa, Xa, Xla, thrombin, or activated protein C. These studies were done by incubating TFPI with indicated factor in the absence of any cofactor. As shown in Fig. 4, mouse TFPI inhibits human factor Xa with almost complete inhibition of factor Xa activity observed at 2 nM TFPI (1:1 ratio). Neither human thrombin nor activated protein C was inhibited by mouse TFPI. Interestingly, in addition to inhibiting human factor Xa, mouse TFPI also inhibits the activity of human factors VIIa, IXa and Xla toward the cleavage of appropriate synthetic substrates. While complete inhibition of factor Xla is observed at a 10-fold molar excess of TFPI, inhibition of factors IXa and VIIa was weaker.

Discussion

In the present study, we used PCR to clone mouse TFPI cDNA from a macrophage cDNA library. In this method, neither radioisotopes nor filter membrane blotting were involved in the experimental procedure, making the cloning work relatively safe, easy and quick. TFPI cDNA sequences have been reported for many species. There is a high homology for TFPI protein sequence and domain structure among different species. The mouse TFPI cDNA sequence, protein sequence, and domain structure presented here is also highly homologous to the other reported TFPI sequences especially to that of rat. However, TFPI is not the only protein which contains three Kunitz domains and an Arg, Lys rich C-terminal sequence. Another reported human gene isolated from a placenta cDNA library, TFPI-2 (also called placenta protein 5 [TFPI-2/PP5]), has the same domain structure and is highly homologous to human TFPI (18). The major difference between human TFPI and TFPI-2/PP5 is in the inhibition of factor Xa. Human TFPI-2/PP5 shows no inhibition of the amidolytic activity of factor Xa even in a 20-fold molar excess (18). Our mouse TFPI gene was cloned from a macrophage cDNA library, which suggests that it is not equivalent to TFPI-2/PP5 which would be expected to be found in a mouse placenta library. Furthermore, a 10-fold molar excess of recombinant mouse TFPI inhibits more than 99% of the amidolytic activity of human factor Xa. Taken together, these data support the conclusion that the clone described in this report is mouse TFPI, not mouse TFPI2/PP5.

Similar to human TFPI, mouse TFPI failed to inhibit human thrombin and activated protein C. Interestingly, mouse TFPI inhibits a broader range of human clotting factors than does human TFPI. Previous studies demonstrated that a more than 10-fold molar excess of human TFPI did not inhibit human factors VIIa, IXa, or Xla (19). However, our results show that less than a 10-fold excess of mouse TFPI significantly inhibits human factors VIIa, IXa, and Xla. It suggests that mouse TFPI may function more diversely in mouse plasma than human TFPI does in human plasma, although we can not offer direct proof simply because mouse clotting factors are not easily available.

In summary, although mouse TFPI is not identical to human TFPI, it is significantly homologous in amino acid sequence, domain structure, and function. While it is interesting that mouse TFPI appears to have a somewhat broader spectrum of inhibition than human TFPI, both proteins significantly inhibit factor Xa. Therefore mouse TFPI appears to be sufficiently similar to human TFPI that studies using a mouse model will yield valuable information regarding the role of TFPI in coagulation.

Acknowledgement

These studies were supported by grants HL-06350 and HL-07149 from the National Institutes of Health.

References


Received June 18, 1997 Accepted after resubmission September 10, 1997