Complementary DNA sequencing of canine tissue factor pathway inhibitor reveals a unique nanomeric repetitive sequence between the second and third Kunitz domains

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Tissue factor pathway inhibitor (TFPI) is a factor Xa-dependent inhibitor of the factor VIIa–tissue factor complex of blood coagulation. The primary amino acid sequence of canine TFPI has been deduced from cDNA sequences obtained using the techniques of reverse transcription followed by amplification using PCR and conventional screening of a canine endothelial cell cDNA library. The open reading frame for canine TFPI encodes a signal peptide of 28 amino acids followed by a 40.7 kDa protein of 368 amino acids. Similar to human, rat and rabbit TFPI, canine TFPI contains a negatively-charged cluster of amino acids at its mature amino-terminus, followed by three Kunitz-type proteinase inhibitory domains and a cluster of positively-charged amino acids near its carboxy-terminus. In

contrast to other TFPIs, following its second Kunitz-type proteinase inhibitory domain canine TFPI contains an additional amino acid insert which includes a nanomeric peptide-sequence repeated six times. Recombinant canine TFPI was expressed in both bacterial- and insect cell-expression systems for functional analysis and the generation of antibodies. The recombinant canine TFPI inhibits tissue factor-induced coagulation in an *in vitro* canine system. Immunoprecipitation of TFPI from canine plasma, followed by Western-blot analysis, tentatively identifies canine TFPI as an 80000 kDa protein. Anti-peptide antibodies raised to the nanomeric peptide repeat immunoprecipitate an identical, cross-reactive, 80000 kDa protein.

INTRODUCTION

Upon vascular injury, the plasma proteinase, coagulation factor VIIa, gains access to its essential cofactor, tissue factor, expressed on cells beneath the endothelium. The factor VIIa–tissue factor complex, which forms proteolytically, activates limited quantities of its substrates factors IX and X [1,2]. Activated factor IX (factor IXa) with its cofactor VIIIa can generate additional activated factor X (factor Xa). Factor Xa with its cofactor Va cleaves prothrombin to thrombin leading to the generation of a fibrin clot.

Plasma contains a lipoprotein-associated inhibitor, termed tissue factor pathway inhibitor (TFPI), that binds and inhibits factor Xa directly, and, in a factor Xa-dependent fashion, inhibits factor VIIa–tissue factor activity [3,4]. TFPI contains three Kunitz-type proteinase inhibitory domains. The first Kunitz domain (as it occurs from the amino-terminus) in TFPI binds the active site of factor VIIa while the second Kunitz domain binds the active site of factor Xa forming a factor Xa–TFPI–factor VIIa–tissue factor inhibitory complex. A function for the third Kunitz domain in TFPI is not known [5].

In vivo, TFPI probably functions to limit the initiation of coagulation. The initial factor-VIIa/tissue-factor mediated generation of factor IXa and Xa provides sufficient thrombin to stimulate localized platelet aggregation and activation of co-factors V and VIII. However, with the generation of factor Xa, TFPI produces feedback inhibition of factor VIIa–tissue factor activity. Therefore, any further production of factor Xa must proceed through the action of factor IXa with its cofactor VIIIa. Persistent haemostasis clearly requires continued generation of

factor Xa since haemorrhage, frequently delayed in onset, occurs in haemophiliacs [6].

Studies *in vitro*, using normal and haemophiliac plasma, have shown that depleting plasma of TFPI shortens tissue factor-induced coagulation times [3,7]. Factor IX- and VIII-deficient canine strains provide a model to test whether modulation of TFPI levels *in vivo* effects the bleeding in haemophilia [3]. As steps toward obtaining reagents necessary for such studies, we report herein, the cloning, expression and partial characterization of canine TFPI.

METHODS

Reverse transcription/PCR amplification of TFPI cDNA fragments

Two micrograms of dog spleen- or rabbit lung-derived poly(A +) RNA (Clontech), plus 200 ng of antisense primer B (5′-TTGAA-TTCACATCCACTGTACTTAAATGGGCGGCA-3′), in 9 µl of water was heated to 68 °C for 5 min, cooled on ice, then added to 11 µl of a mixture containing 50 mM Tris/HCl, pH 8.3/50 mM KCl/10 mM MgCl₂/1 mM dithiothreitol/10 µg/ml BSA/2 mM each dNTP, 10 units RNasin (Promega) and 10 units reverse transcriptase (Boehringer Mannheim, Germany). Following a 1 h, 42 °C incubation, 5 µl of reaction volume was used as template for PCR amplification.

PCR amplification reactions were performed in 100 µl reaction volumes containing template, 200 ng each of sense primer A (5′-TTGAATTCAGAATTTATATATGGGIGGATGTGAAGG-3′) and antisense primer B (see above), 2 mM each dNTP, 1 × Taq polymerase buffer and 2.5 units Taq polymerase using 30 temperature cycles of 94 °C (1 min), 42 °C (1 min), 72 °C (1 min).

Abbreviations used: TFPI, tissue factor pathway inhibitor; IPTG, isopropyl β-D-thiogalactopyranoside; SSC, 150 mM HCl/15 mM sodium citrate.

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PCR products were purified using Magic-miniprep resin (Promega) and agarose gels, followed by digestion with *EcoRI* and subcloning into pUC10.

Screening of the canine cDNA library

Three replicate nitrocellulose lifts of 20 plates containing a total of approximately 150 000 plaque forming units of a canine, 4 β -phorbol 12-myristate 13-acetate-stimulated, endothelial cell cDNA library (provided by Dr. Donald Anderson, Baylor University, TX, U.S.A.) were prepared. Individual sets of lifts were screened with ³²P-labelled oligonucleotides C (5'-CCAGATTA-CTGCCATATGAACGAAGAC-3'), D (5'-GATTCTGAGAG-TGGTGGATTACAACAC-3') and E (5'-CCAGCAGACAGA-GGGCTGTGTACGCC-3'), respectively, essentially as previously described [8]. Hybridization conditions were 4 $\times$ 150 mM HCl/15 mM sodium citrate (SSC), 5% Blotto; wash conditions were 1 $\times$ SSC, 55 $^{\circ}$ C. Positive clones were plaque purified and expanded. Phage clone cDNA inserts were excised with *EcoRI* for size analysis on agarose gels and subcloned into pUC19 for sequencing. DNA sequencing was performed using a Sequenase DNA sequencing kit (U.S. Biochemicals).

Expression of canine TFPI in *E. coli*

The reading frame for mature canine TFPI was PCR amplified using sense primer F (5'-CTCTTCGAATTCCATGGCCGT-CCTTGATGAAAGT-3') and antisense primer G (5'-GTGT-GAAGCTTCATTATAGTTTTTAACAAA-3'). Primer F contains *EcoRI* and *NcoI* restriction sites and sequence encoding Met-Ala followed by the predicted N-terminus of mature TFPI. Primer G is complementary to the sequence encoding the C-terminus of TFPI followed by two stop codons and a *HindIII* restriction site. PCR amplification was performed as described above except a clone containing the entire open reading-frame of canine TFPI was used as template. The *NcoI/HindIII* digested PCR product was ligated into pTAC, a spectinomycin-resistant expression vector with an isopropyl β -D-thiogalactopyranoside derepressible trp-lac promoter (Monsanto Corporation, St. Louis, MO, U.S.A.) and transformed into JM101. Positive clones were identified by Coomassie-stained SDS/PAGE analysis of cell lysates.

Purification of *E. coli* expressed recombinant canine TFPI

A transformed JM101 clone was grown at 37 $^{\circ}$ C, with agitation, in 2 $\times$ M9 minimal media (Difco Laboratories, Detroit, MI, U.S.A.) supplemented with 2% case amino acids (Difco)/0.2% glucose/2 mM MgSO₄/0.1 mM CaCl₂. At a culture density of 1 A_{550} /ml, 1 mM IPTG was added to the culture. Four hours after the IPTG induction, cells were harvested by centrifugation, resuspended to 0.1 g/ml in water and lysed by sonication. Greater than 80% of the recombinant TFPI (rTFPI) was found in inclusion bodies. Inclusion bodies were collected by centrifugation (10 000 g, 10 min), washed three times with 20 volumes of 20 mM Tris/HCl, pH 7.5, then solubilized in 40 ml of 8 M urea, 20 mM Tris/HCl, pH 7.5.

Particulate matter was removed by centrifugation at 18 000 g for 20 min. Sulphonation of cystine residues was achieved as follows. 800 mg of sodium sulphate was added to the supernatant followed by rocking at room temperature for 30 min. 400 mg of sodium tetrathionate was added and rocking continued at 4 $^{\circ}$ C for 24 h.

The solution was then dialysed against 20 mM Tris/HCl pH 8.0 with 6 M urea and passed over 25 ml Sepharose fast-flow

anion exchange column. Protein was eluted with a linear 0 to 1.0 M NaCl gradient, fractions containing TFPI were pooled and the pool was diluted with 20 mM Tris/HCl, pH 8.0, with 6 M urea to give a protein concentration of 100 μ g/ml. Solid L-cysteine was added to 2 mM and the solution was stirred slowly at 4 $^{\circ}$ C for 36 h. The solution was diluted 1:1 with dH₂O, additional L-cysteine was added to give a final concentration of 2 mM, and the solution was stirred for an additional 3 days at 4 $^{\circ}$ C.

The pH of the dilute protein solution was adjusted to 6.4 with 1 M HCl chromatography on a 10 ml S-Sepharose fast flow cation exchange column. Protein was eluted with a linear 0 to 1.0 M NaCl gradient in 20 mM Tris/HCl, pH 6.4, with 6 M urea. TFPI containing fractions were pooled and stored at -70 $^{\circ}$ C as a 6 μ M stock.

Expression of canine TFPI in insect cells

An *EcoRI-HindIII* fragment containing the entire coding sequence for canine TFPI was modified at its termini to include an *NheI* restriction sites. The cDNA was ligated into vector pJVP10Z, which undergoes homologous recombination with baculovirus. pJVP10Z contains the sequence for β -galactosidase which allowed colour selection of recombinants. Sf-21 cells (*Spodoptera frugiperda*) were grown in TNMFH media (Sigma, St. Louis, MO, U.S.A.) with 10% fetal bovine serum and 50 μ g/ml gentamicin at 27 $^{\circ}$ C. 2 $\times$ 10⁶ cells were co-transfected with 1 μ g of wild-type baculovirus AcMNPV DNA (*Autographa californica* nuclear polyhedrosis virus) and 2 μ g of canine TFPI/pJVP10Z DNA as previously described [9]. Transfected cells were grown at 27 $^{\circ}$ C until all the cells had lysed (5 days). Serial 1:10 dilution of culture supernatant were used to infect fresh Sf-21 cells which were then overlaid with 1.5% agar containing 75 μ g/ml X-gal. Cultures were grown at 27 $^{\circ}$ C until blue colonies appeared (3-4 days). Blue colonies (containing TFPI/pJVP10Z) were picked with sterile pipettes and were passed through two additional cycles of plaque purification as above. Pure recombinant virus stock was confirmed by the absence of polyhedron inclusions in the nuclei of infected cells. Sf-21 cells were grown to a density of 6 $\times$ 10⁵ cells/ml in 1 l of medium in a spinner flask at 27 $^{\circ}$ C. Cells were pelleted and washed with 200 ml of Sf-900 II SFM medium (Gibco, Grand Island, NY, U.S.A.) in the absence of fetal bovine serum. The cells were resuspended in 100-200 ml of TFPI-baculovirus conditioned media (approx. 5-10 virions/cell) and incubated at 27 $^{\circ}$ C with gentle rocking for 4 h, 500 ml of fresh Sf-900 medium with 50 μ g/ml gentamicin was added and incubation was continued in a spinner flask at 27 $^{\circ}$ C for 4 days. Culture supernatants were stored at -70 $^{\circ}$ C.

Purification of insect cell-expressed recombinant canine TFPI

The purification of canine TFPI from baculovirus supernatants was followed by Western blots using polyclonal anti-peptide 0744 antibody (see below) which recognizes a unique sequence in canine TFPI. Culture supernatant was passed over a 100 ml heparin-agarose column equilibrated with 50 mM Tris/HCl/100 mM NaCl, pH 7.5 (TS) followed by two column volumes of 6 M urea in TS. Under these conditions, TFPI remained bound to the column. Protein was eluted with a 100 ml linear NaCl gradient from 0.1 to 1.0 M NaCl in 6 M urea/50 mM Tris/HCl, pH 7.5. Fractions containing the recombinant protein were pooled and stored frozen at -70 $^{\circ}$ C as a 6.8 μ M stock solution. Immediately prior to use in coagulation assays, the protein was diluted in TS containing 0.1% BSA. The presence

of urea at concentrations used in the TFPI-containing assays did not alter clotting times.

Preparation of anti-canine TFPI IgG

Escherichia coli-produced canine TFPI (500 μ g for goats, or 250 μ g for rabbits) was mixed with an equal volume of Freund's complete adjuvant and administered in multiple subdermal injections. Monthly booster injections were given in the same manner but in incomplete Freund's adjuvant [10]. Synthetic peptides P-0744 (TVSSFLFYGPSWC) and P-0888 (CDSESG-LQHDSSEG) were linked to keyhole limpet haemocyanin using *N*-succinimidyl bromoacetate [11]. Antibodies were raised to the conjugated peptides and purified in an identical manner to those for the whole TFPI protein. IgG was purified from rabbit serum using protein A-affinity chromatography. Goat IgG was obtained by adjusting the goat serum to 35% ammonium sulphate by the slow addition of saturated ammonium sulphate at room temperature. The precipitated protein from 1 l of goat serum was collected by centrifugation, washed once with 35% ammonium sulphate, resuspended in 1 l of TS and dialysed against two 40 l volumes of 75 mM NaCl/20 mM Tris/HCl, pH 7.5 at 4 °C. Remaining precipitate was removed by centrifugation and the supernatant was passed over a 120 ml DEAE Affigel-Blue column (BioRad, Richmond, CA, U.S.A.) equilibrated in the same buffer used for dialysis. Under these conditions, IgG does not bind to the column and it is collected in the flow through. The IgG was stored at -20 °C.

Immunoblot analysis

Western blotting after SDS/PAGE (15% polyacrylamide) [12] was performed as previously described [10] using the primary antibodies described above.

Rabbit or goat anti-(canine TFPI IgG), linked to Affigel-10 (Biorad) at 10 mg/ml was used for immunoprecipitation of TFPI from canine plasma. Briefly, 20 ml peripheral blood samples were collected from dogs into 2 ml of 3.8% sodium citrate either before, or 5 min after, the administration of 10000 units of sodium heparin intravenously. Plasma was separated from cells by centrifugation and was stored at -70 °C. Two ml of plasma was mixed with 100 μ l of anti-canine TFPI IgG-Affigel-10 and rocked at room temperature for 2 h. IgG-Affigel 10 was pelleted by brief centrifugation and washed three times with TS. Protein was eluted by mixing the pellet with 100 μ l SDS non-reducing sample buffer [12] and stored at 4 °C prior to Western-blot analysis.

Coagulation assay

A crude preparation of EDTA-washed tissue factor was prepared from a dog brain using a previously described procedure [13]. Test sample (50 μ l) was added to a fibrometer cup containing 60 μ l of dog tissue factor, diluted 1:1000 in Tris-buffered saline containing 10% rabbit brain cephalin (Sigma) and 60 μ l of dog plasma (Sigma). A 60 μ l volume of 25 mM CaCl₂ was added and the time to clot formation was determined using a fibrometer (BBL, Cockeysville, MD, U.S.A.).

RESULTS AND DISCUSSION

Cloning of canine TFPI

The initial strategy for obtaining canine TFPI cDNA was to generate cDNA from canine RNA using reverse transcription

followed by PCR amplification. Several oligonucleotide primers, designed based on published sequences for human and rabbit TFPI cDNAs [14,15], were synthesized. Multiple attempts to amplify the complete open reading frame of canine TFPI were unsuccessful, probably due to limited sequence homology in 5' and 3' untranslated regions. However, using an antisense primer complementary to highly-conserved sequence encoding within the third Kunitz domains of TFPIs, in combination with a sense primer with sequence corresponding to a highly conserved region encoding within the first Kunitz domains in TFPIs (see Methods), a partial canine TFPI cDNA was generated (Figure 1). Unexpectedly, the amplified product generated from dog-spleen polyA+ RNA was approximately 800 bp in size, while that generated from rabbit-lung polyA+ RNA was slightly over 500 bp as expected. Similar analysis using canine- and rabbit-liver polyA+ RNAs showed that the difference in size of the amplified products is species, rather than organ, specific (data not shown).

Subcloning and sequence analysis identified the canine PCR product as TFPI cDNA, as the sequences juxtaposed to the PCR primers encode the expected regions of a first and a third Kunitz domain and the internal sequence encodes a complete second Kunitz domain. The larger size of the canine PCR-generated fragment is due to an additional, unique 273 bp sequence present in canine TFPI that has not been found in the other TFPI cDNAs from species including humans [14], rabbits [15] and rats [16]. This additional sequence encodes a 71 amino acid domain that follows the second Kunitz domain and includes a nanomeric peptide repeated six times.

To obtain a cDNA encoding the entire open reading frame of canine TFPI, and to confirm that the presence of the nanomeric peptide encoding sequence was not artifactual, a canine cDNA library was screened with three canine-specific oligonucleotides, probes C, D and E (see the Methods section). Probe C hybridizes to a sequence encoding the peptide between the first and second Kunitz domains; probe D hybridizes to a sequence encoding the nanomeric peptide repeat; and probe E hybridizes to a sequence that encodes the region that immediately precedes the third Kunitz domain. Upon screening approximately 150000 recombinants, 104 plaques were found that specifically hybridized to all three probes. No plaques were found that hybridized to probes C and E but not D. A total of 15 canine TFPI cDNA clones were further analysed by sizing appropriate PCR-amplified

Figure 1 Size analysis of PCR-amplified, reverse transcriptase-generated TFPI cDNA fragments

PCR fragments were generated from canine spleen (lane 1) or rabbit lung (lane 2) mRNA using primers encoding conserved sequences in the first and third Kunitz domain of TFPI (see the Methods section). The migration of the PCR products run on a 1% agarose gel stained with ethidium bromide is shown. The exact size of each fragment was determined, retrospectively, from subcloning and sequencing of each cDNA band.

Figure 2 Nucleotide sequence and deduced amino acid sequence of canine TFPI

Nucleotides are numbered above the sequence; amino acids are numbered on the right. The position of the predicted signal peptidase cleavage-site was determined by comparison of canine TFPI sequence with that of human and rat TFPI and is denoted by the upward pointing arrow. Potential sites for *N*-linked glycosylation are marked by asterisks. Sequences of probes used to screen the library (see Methods) are underlined and labelled. Sequences corresponding to the synthetic peptides made to generate antibodies are boxed. Nanomeric amino acid repeats are underlined alternately in solid or dashed lines.

DNA fragments. The size of each PCR fragment was consistent with each clone encoding six complete nanomeric-encoding repeats. Notably, after repeated culturing and subcloning, one plasmid containing a canine TFPI cDNA insert, originally identified as having six copies, was found to have nine copies of this nanomeric repeat. This most likely reflects an artifact of recombination that occurred in the bacteria used to maintain the plasmid (data not shown).

Comparison of the sequences of the TFPIs from different species

The cDNA sequence and the deduced amino acid sequence shows that canine TFPI has a 1188 bp open reading-frame which encodes a predicted 28 amino acid signal peptide followed by a 40.7 kDa protein of 368 amino acids (Figure 2). From this sequence it can be seen that primers A and B (see Methods) used to generate the initial canine TFPI cDNA fragment are identical with the canine sequence with the exception of the one inosine used in primer A and the restriction site linkers.

Similar to human TFPI, both the first and fifth codons of the canine TFPI open reading frame encode methionine [17]. Comparison of the sequences surrounding each of these codons, GAGATGA for position 1 and ACAATGA for position 5, with the proposed optimal eukaryotic translation initiation sequence

ACCATGG [18] suggests position 5 is a more favourable initiation site. Notably, the only possible initiation site for rabbit TFPI corresponds to position 5 of human and canine TFPI, whereas the only possible initiation site in rat TFPI corresponds to position 1.

For human TFPI, two messages, 1.4 and 4.0 kb in size, differing in their 3' untranslated sequences have been identified [17]. Canine TFPI clones with up to 1.8 kb of 3' untranslated sequence were observed, however, no clones analysed were found to contain a 3' polyA tail (data not shown).

Figure 3 is a schematic diagram of the canine TFPI protein showing the predicted signal peptide processing-site and the sulphhydryl-bonding for the three Kunitz type proteinase inhibitory domains. Canine TFPI contains eight potential sites for *N*-linked glycosylation (see Figure 1). Three of these sites, one in the second Kunitz domain, one between the second and third Kunitz domains and one in the third Kunitz domain, are conserved in human TFPI. Canine TFPI contains an additional site in its third Kunitz domain and the peptide domain unique to canine TFPI contains the remaining four sites.

The predicted amino acid sequence of canine TFPI was compared with those of human, rabbit and rat TFPI [14,15,16,19,20]. Each TFPI contains a cluster of acidic residues at its NH₂-terminus. Canine and human TFPI each possess a

Figure 3 Predicted secondary structure of canine TFPI

The predicted signal proteinase processing site is indicated based on the comparison of canine with human and rat TFPI.

Figure 4 Coomassie stain of SDS/PAGE of purified recombinant canine TFPIs expressed from Sf21 insect cells and *E. coli*

The relative migrations of molecular mass protein standards are shown.

serine residue within their cluster of acidic residues and it has been shown with human TFPI that this serine residue can be phosphorylated by casein kinase II [21], resulting in additional localized negative charge.

At its COOH-terminus, each TFPI contains a stretch of basic residues that, in human TFPI, has been shown to mediate TFPI binding to heparin [10]. Infusion of heparin results in increased levels of circulating TFPI [22,23]. It has been postulated that TFPI, via its basic region, binds to glycosaminoglycans on the endothelial cell surface, and that heparin, by competing for this basic region, releases bound TFPI [23].

The TFPI of each species contains three highly-conserved Kunitz-type proteinase inhibitory domains. Determined for hu-

Figure 5 Identification of canine TFPI in plasma by immunoblot

Shown is a Western blot developed using goat anti-(canine TFPI) (raised against *E. coli*-expressed recombinant canine TFPI) as the primary antibody, followed by a rabbit anti-goat IgG alkaline phosphatase conjugate. Samples are purified insect cell-expressed canine TFPI (lane 1); and immunoprecipitates of plasma from a dog prior to (lanes 2, 4 and 6) or 5 min after the dog was infused with heparin (lanes 3, 5 and 7), immunoprecipitated with preimmune IgG (lanes 2 and 3); anti-TFPI IgG (raised in rabbits against *E. coli*-expressed canine TFPI) (lanes 4 and 5); or anti-TFPI-0888 IgG (raised in rabbits against a peptide corresponding to the nanomeric repeat) (lanes 6 and 7).

man TFPI, the first Kunitz domain binds to the active site of factor VIIa/TF while the second Kunitz domain binds to the active site of factor Xa. No role for the third Kunitz domain has been described. The critical P_1 residue in the active site clefts of the first and second Kunitz domains is either lysine or arginine. However, the P_1 residues for the third Kunitz domains are more variable, namely arginine for human and rat, glutamine for rabbit and histidine for canine TFPI. Though no function has been assigned to the third Kunitz domain of the three in TFPI between dog and man.

The outstanding feature of canine TFPI is the presence of an additional ~85 amino acids within the peptide separating its second and third Kunitz domains. In all canine TFPI cDNAs

Figure 6 Effect of recombinant canine TFPI on tissue factor-induced coagulation of canine plasma

Assays were performed as described in the Methods section. The percent apparent TF activity remaining for the concentrations of TFPI shown were determined from a standard TF concentration curve plotting coagulation time versus TF concentration.

examined, this sequence includes a near-identical nanomeric peptide repeated six times (Figure 3).

Expression of recombinant canine TFPI

A pTAC expression vector containing a modified cDNA fragment encoding for mature canine TFPI was used for the production of recombinant canine TFPI in *E. coli*. Bacteria containing this construct expressed an IPTG-inducible protein that co-isolates with inclusion bodies. Following purification and refolding, the recombinant protein inhibits tissue factor-induced coagulation in a canine system (data not shown). As judged by SDS/PAGE analysis (Figure 4), the apparent molecular mass (50 kDa) of this recombinant protein is greater than the 40.7 kDa predicted based on its amino acid composition. However, the size of the purified protein, determined by mass spectroscopy was consistent with that predicted by amino acid composition (data not shown). For the generation of anti-canine TFPI antibodies, this protein was injected into rabbits and goats.

For functional studies, recombinant canine TFPI was expressed by Sf-21 insect cells using a baculovirus expression system. The recombinant protein was purified to apparent homogeneity, as judged by SDS/PAGE, using heparin-agarose affinity chromatography (Figure 4). Western-blot analysis (Figure 5, lane 1) shows that the insect cell-derived recombinant canine TFPI is recognized by antibodies generated using *E. coli*-expressed recombinant canine TFPI.

Using a canine coagulation system, this recombinant canine TFPI inhibits TF induced coagulation in a dose-dependent manner (Figure 6). Whether this apparent TF inhibition is due to the direct inhibition of factor Xa by TFPI, or due to a combination of direct Xa inhibition and Xa-dependent inhibition of VIIa/TF by TFPI, was not determined.

Characterization of canine TFPI in plasma

TFPI in canine plasma was identified on Western blots following

its immunoprecipitation. Previous observations have shown that infusion of heparin into individuals increases circulating TFPI levels several-fold. Canine plasma samples were therefore taken prior to, and 5 min after, infusion of 10000 units of heparin into a dog. Figure 5 shows that both a polyclonal IgG raised to recombinant canine TFPI, and a polyclonal IgG raised to a peptide consisting of the nanomeric repeat unit, immunoprecipitate an 80000 kDa protein that is recognized on Western blots by a second polyclonal IgG raised to the recombinant protein. Consistent with a heparin mediated increase in circulating TFPI levels, the staining on the blot is more intense for the post-heparin samples. The canine TFPI cDNA sequence predicts a 40.7 kDa protein mass; the larger apparent mass observed for TFPI in canine plasma is most likely related to glycosylation.

That the IgG raised to the nanomeric repeat sequence immunoprecipitates this 80000 kDa species demonstrates this repetitive sequence, identified in the cDNA is, in fact, present in the mature protein. Based on sequence similarity to other TFPIs (see above), the additional sequence present in canine TFPI that includes the repetitive unit occurs following the second Kunitz domain exon. In the human TFPI gene, there is a 1 kb intron following the second Kunitz domain [19,20]. Upon sequencing this intron, no significant stretch of sequence homologous to the unique domain in canine TFPI was detected (unpublished observation). A role for this sequence, thus far unique to canine TFPI, is not apparent.

This work was supported by National Institutes of Health Grant HL-34462 and the Monsanto Co.

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