Inhibitory Properties of a Novel Human Kunitz-Type Protease Inhibitor Homologous to Tissue Factor Pathway Inhibitor†

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ABSTRACT: In a previous report, we described the molecular cloning, expression, and partial characterization of a second human tissue factor pathway inhibitor (TFPI), which we designated as TFPI-2 [Sprecher, C. A., et al. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 3353−3357]. Recombinant TFPI-2 inhibited the amidolytic activity of trypsin as well as that of factor VIIa in complex with tissue factor. TFPI-2 recently has been shown to be identical to placental protein 5 (PP5), a glycoprotein originally isolated from placenta that exhibits serine protease inhibitory activity. In the present study, we have examined TFPI-2/PP5 for its ability to inhibit a number of serine proteases involved in blood coagulation and fibrinolysis, inasmuch as TFPI-2/PP5 prolonged the coagulation time of human plasma induced by either tissue factor or contact activation in a dose-dependent manner. In addition to its ability to inhibit the amidolytic and proteolytic activities of the factor VIIa−tissue factor complex, TFPI-2/PP5 strongly inhibited the amidolytic activities of human factor XIa, human plasma kallikrein, and human plasmin with K_i values of 15, 25, and 3 nM, respectively. TFPI-2/PP5 was also a weak inhibitor of the activation of factor X by a complex of human factor IXa and poly(lysine) with an apparent K_i of 410 nM. Heparin markedly enhanced the ability of TFPI-2/PP5 to inhibit factor VIIa−tissue factor both in the solution phase and on cell surfaces. In addition, heparin augmented the inhibition of human factor Xa amidolytic activity at relatively high levels (10−100 nM) of TFPI-2/PP5. No significant inhibition of glandular kallikrein, urinary plasminogen activator, tissue plasminogen activator, human activated protein C, human factor Xa, human thrombin, or leukocyte elastase was observed when these proteases were incubated with TFPI-2 in the absence of heparin.

Tissue factor pathway inhibitor (TFPI)[1] is an important regulator of the extrinsic pathway of blood coagulation (Davie et al., 1991). TFPI (M_r 42 000) is currently thought to be synthesized primarily in endothelial cells and consists of three tandemly arranged Kunitz-type protease inhibitor (KPI) domains. In addition, TFPI contains a negatively charged amino terminal region and a positively charged carboxy terminal region (Wun et al., 1988). In a recent report, we described the molecular cloning and expression of a full-length cDNA that encodes a protein structurally related to human TFPI (Sprecher et al., 1994). Sequence analysis revealed that this encoded novel protein, provisionally designated as TFPI-2, had an overall domain organization similar to that of TFPI, in addition to considerable primary amino acid sequence homology. Following a 22-residue signal peptide, the mature TFPI-2 (M_r = 32 000) was shown to contain 213 amino acids with 18 cysteines and 2 consensus N-linked glycosylation sites. As deduced from the cDNA sequence, mature TFPI-2 contains a short acidic amino terminal region, three tandem KPI domains, and a carboxy terminal tail highly enriched in basic amino acids. When expressed in baby hamster kidney cells and purified to homogeneity, TFPI-2 was shown to inhibit the amidolytic activities of trypsin and a complex of human factor VIIa and tissue factor (Sprecher et al., 1994). TFPI-2, like TFPI, failed to inhibit thrombin. However, in contrast to TFPI, TFPI-2 only weakly inhibited factor Xa aminolytic activity. A specialized role for TFPI-2 in hemostasis was suggested on the basis of (1) its ability to inhibit the factor VIIa−tissue factor complex and (2) its relatively high levels of transcription in umbilical vein endothelial cells, placenta, and liver.

Recent evidence indicates that TFPI-2 is identical to a protein isolated from human placenta over a decade ago and designated as placental protein 5 or PP5 (Miyagi et al., 1994; Kisiel et al., 1994). PP5 is a glycoprotein with a molecular weight of 30 000−36 000 that binds tightly to heparin (Salem et al., 1980), inhibits the amidolytic activity of plasmin (Siiteri et al., 1982) and trypsin (Miyagi et al., 1994), and inhibits the proteolytic activity of thrombin toward fibrinogen (Meisser et al., 1985). While PP5 is found in abundant amounts in the placenta (Seppala et al., 1979; Butzow et al., 1988b), it is also found in endothelial cells (Butzow et al., 1988c), human placenta (Butzow et al., 1988a), seminal plasma (Ranta et al., 1981), seminal vesicles (Wahlstrom et al., 1982), proovulatory follicular fluid (Seppala et al., 1984),

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[1] Abbreviations: TFPI-2, tissue factor pathway inhibitor-2; TF, tissue factor; TF_{1-218}, truncated tissue factor apoprotein consisting of residues 1−218; K_i', apparent inhibition constant; APTT, activated partial thromboplastin time; PP5, placental protein 5; KPI, Kunitz-type protease inhibitor; MCA, 4-methylcoumaryl-7-amide; pNA, *p*-nitroanilide; CHA, cyclohexylalanyl; Cbo, benzyloxycarbonyl; OBzl, γ-benzyl ester.

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and the conditional medium of the human glioblastoma cell line, T98G (Miyagi et al., 1994). Residues 2−19 of the mature form of TFPI-2/PP5 have also recently been shown to share 70−90% sequence homology with the amino terminal sequence of a novel 33 kDa extracellular matrix-associated serine protease inhibitor from human skin fibroblasts (Rao et al., 1995a).

To elucidate a possible physiological role for TFPI-2/PP5 in hemostasis, we have examined the interaction of TFPI-2/PP5 with several serine proteases involved in coagulation and fibrinolysis and demonstrated that TFPI-2/PP5 is a strong inhibitor of factor XIa, plasmin, plasma kallikrein, and chymotrypsin, in addition to its previously described ability to inhibit factor VIIa−tissue factor and trypsin.

MATERIALS AND METHODS

Materials. H-D-Val-Leu-Lys-pNA (S-2251), MeO-Suc-Arg-Pro-Tyr-pNA (S-2586), H-D-Val-Leu-Arg-pNA (S-2266), Glu-Gly-Arg-pNA (S-2444), H-D-Ile-Pro-Arg-pNA (S-2288), Glu-Pro-Arg-pNA (S-2366), H-D-Pro-Phe-Arg-pNA (S-2302), H-D-Phe-Pip-Arg-pNA (S-2238), and *N*-α-Cbo-D-Arg-Gly-Arg-pNA (S-2765) were purchased from Chromogenix (Moelndal, Sweden). MeO-CO-CHA-Gly-Arg-pNA (FXa-1) was obtained from NycoMed (Oslo, Norway). MeO-Suc-Ala-Ala-Pro-Val-pNA and Suc-Ala-Ala-Pro-Phe-pNA were products of Sigma (St. Louis, MO). The fluorogenic peptide Boc-Glu(OBzl)-Ala-Arg-MCA was obtained from Peptide Institute (Osaka, Japan). Heparin (192 USP IU/mg) was from Novo Nordisk.

Proteins. Recombinant human TFPI-2 was prepared as described (Sprecher et al., 1994). Porcine trypsin, porcine chymotrypsin, recombinant human tPA, recombinant human activated protein C, recombinant aprotinin (bovine pancreatic trypsin inhibitor), and recombinant human factor VIIa were obtained from Novo Nordisk (Bagsvaerd, Denmark). Human plasmin and plasma kallikrein were obtained from Kabi (Stockholm, Sweden). The active-site concentrations of trypsin and plasmin samples were determined by titration with aprotinin. The protein concentrations of recombinant aprotinin and TFPI-2 preparations were determined by amino acid analysis. Glandular kallikrein was obtained from Sigma. Urinary plasminogen activator (uPA) was purchased from Serono (Freiburg, Germany). Human leukocyte elastase and cathepsin G were purified according to Baugh and Travis (1976). Human factor Xa (Kondo & Kisiel, 1987), human factor IXaβ (Wildgoose & Kisiel, 1989), and human thrombin (Kisiel et al., 1985) were prepared as described. Human factor XIa and human factor XIIa were generous gifts from Drs. K. Fujikawa (Seattle, WA) and I. Schousboe (Copenhagen, Denmark), respectively. Human fibrinogen was prepared as described (Suenson et al., 1984) and generously provided by Dr. S. Thorsen. Full-length recombinant human TFPI was purified as described (Nordfang et al., 1991) and kindly provided by Dr. O. Nordfang (Novo Nordisk, Gentofte, Denmark).

Cloning, Expression, and Purification of TF_{1-218}. The soluble extracellular domain of human tissue factor was expressed in yeast (strain JG134) and purified through the CM-Sepharose column chromatography step essentially as described by Shigematsu et al. (1992). Tissue factor activity eluted from this column as three peaks: a major peak corresponding to hyperglycosylated sTFα, followed by a smaller peak of sTFβ, and a third, yet smaller, peak designated as sTFγ. The sTFβ peak fractions were pooled, dialyzed against 50 mM Tris-HCl (pH 8)/10 mM $CaCl_2$, and subjected to affinity chromatography at 25 °C on a column (2.6 × 12 cm) of recombinant factor VIIa−AffiGel 15 previously equilibrated with 50 mM Tris-HCl (pH 8.0)/10 mM $CaCl_2$. The sTFβ was eluted from this column in 100 mM Tris-HCl (pH 8.0) containing 30 mM EDTA. The purified sTBβ appeared as a single band in SDS−PAGE and exhibited a molecular mass of 37 kDa in the presence and absence of reducing agent. Amino terminal sequence analysis indicated a sequence of Ser-Gly-Thr-Thr-Asn-Thr-Val-Ala-Ala-Tyr, which is identical to that previously reported for human tissue factor apoprotein (Morrissey et al., 1987; Spicer et al., 1987; Scarpati et al., 1987; Fisher et al., 1987). The sTFγ was also purified by affinity chromatography and exhibited a M_r of 34 000 and a specific activity essentially identical to that observed for sTFβ. Although not studied further, sTFβ and sTBγ presumably represent two forms of the truncated tissue factor apoprotein with different degrees of glycosylation. The protein concentration of sTFβ samples was determined from the absorbance at 280 nm using an $E^{1\%}$ of 14.9 (Waxman et al., 1992).

Coagulation Assays. The inhibitory effects of recombinant TFPI-2/PP5 on a dilute thromboplastin clotting time and an activated partial thromboplastin time was measured at 37 °C as follows. In the dilute thromboplastin clotting assay, 100 μL of pooled human plasma, 100 μL of dilute human brain thromboplastin (Nawroth et al., 1986), 100 μL of TBS/BSA containing various concentrations of TFPI-2/PP5, and 100 μL of 25 mM $CaCl_2$ were added sequentially to a 10 × 75 mm glass culture tube, and the time for clot formation was measured following the addition of $CaCl_2$. In the activated partial thromboplastin time assay, 100 μL of pooled human plasma was incubated at 37 °C for 4.75 min with 100 μL of kaolin (5 mg/mL) in human cephalin (Bell & Alton, 1954), followed by the addition of 100 μL of TBS/BSA containing various concentrations of TFPI-2/PP5 and 100 μL of 25 mM $CaCl_2$.

Inhibition of Factor VIIa Activity on J82 Cells by Recombinant TFPI-2/PP5. Wells of a 24-well plate were seeded with 5×10^5 J82 cells and grown to confluence. Each well was washed with buffer A [10 mM Hepes (pH 7.45) containing 137 mM NaCl, 4 mM KCl, and 11 mM glucose] supplemented with 10 mM EDTA and subsequently washed three times with buffer A. Each well was then incubated with 10 nM recombinant factor VIIa in buffer A supplemented with 0.5% bovine serum albumin and 5 mM $CaCl_2$ (buffer A+) for 2 h at 37 °C with constant oscillation (50 rpm) on an orbit shaker (Lab-Line). At this point, cells were washed six times with buffer A+, and TFPI-2/PP5 (50 nM) or a mixture of TFPI-2/PP5 (50 nM) and heparin (0.5 μg/mL), dissolved in buffer A+, was added to the cells in a volume of 245 μL. Buffer A+ and buffer A+ containing 0.5 μg/mL heparin served as control samples. After a 30 min incubation at 37 °C, 5 μL of human factor X (100 nM final concentration), in buffer A+, was added to each well, and temporal aliquots (150 μL) of the supernatant were transferred to a tube containing 10 μL of 0.5 M EDTA. An aliquot (100 μL) of this incubation mixture was added to a polystyrene cuvette containing 900 μL of 50 mM Tris-HCl (pH 8.3), 150 mM NaCl, and 110 μM S-2765. The absorbance at 405 nm was recorded continuously using a

Beckman DU-65 spectrophotometer. Factor Xa concentrations were interpolated from a standard curve relating ΔA_{405}/min and factor Xa concentrations.

Effect of Heparin on the Inhibition of Human Factor Xa by TFPI and TFPI-2/PP5. Human factor Xa (10 pM) was incubated in a 96-well microtitration plate for 30 min at 25 °C with various concentrations (1 pM to 100 nM) of either TFPI or TFPI-2/PP5 in 50 mM Tris-HCl (pH 7.5)/100 mM NaCl/5 mM $CaCl_2$ in the presence and absence of 10 μg/mL heparin. Substrate (FXa-1, 0.3 mM) was then added and factor Xa amidolytic activity was measured over 18 h.

Factor IXa Inhibition. Factor IXa inhibition was measured essentially as described by Schmaier et al. (1993). Factor IXa (4.5 nM) was incubated for 40 min at 25 °C with various concentrations of TFPI-2/PP5 in 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl, 250 μM $CaCl_2$, 60 nM poly(D-lysine) (M_r = 160 000), and 0.1% PEG 8000. Factor X (400 nM final concentration) was then added and the incubation continued for an additional 40 min at 25 °C. Substrate (S-2765, 0.3 mM) was then added, and the factor Xa activity was measured as the change in absorbance at 405 nm. In this sytem, the factor Xa formed was directly proportional to the factor IXa concentration.

Inhibition Kinetics. Selected proteases were each incubated with various concentrations of recombinant TFPI-2/PP5 for 15 min at 25 °C. Specific chromogenic or fluorogenic substrate was then added, and the residual activity was measured. The reaction was carried out at 25 °C in 96-well microtitration plates in 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl, 5 mM $CaCl_2$, and 0.01% Tween 80 in a total volume of 300 μL. The amidolytic activity was measured as the change in absorbance at 405 nm or as the change in fluorescence emission at 460 nm (excitation at 380 nm). The apparent inhibition constant, K_i', was determined by using the nonlinear regression data analysis program Enzfitter (Biosoft, Cambridge, UK). Where indicated, the data sets on inhibition of proteases were analyzed in terms of the equation for a tight-binding inhibitor:

$$v_i = v_0[\sqrt{(K_i' + [I]_0 + [E]_0)^2 - 4[I]_0[E]_0} - (K_i' + [I]_0 - [E]_0)]/2[E]_0 \quad (1)$$

where v_i and v_0 are the inhibited and uninhibited rates, respectively, and $[I]_0$ and $[E]_0$ are the total concentrations of inhibitor and enzyme, respectively. Other inhibition data ($K_i \gg [E]_0$) were analyzed according to

$$v_i = v_0/(1 + [I]_0/K_i') \quad (2)$$

K_i values were obtained by correcting for the effect of substrate according to (Bieth et al., 1984)

$$K_i = K_i'/(1 + [S]/K_m) \quad (3)$$

RESULTS

Effect of Heparin on the Inhibition of Factor VIIa−Tissue Factor Activity by TFPI-2/PP5. In an earlier study (Sprecher et al., 1994), we reported that TFPI-2 was a relatively weak inhibitor of factor VIIa−tissue factor amidolytic activity. Heparin strongly affected the TFPI-2/PP5 inhibition of factor VIIa−tissue factor amidolytic activity, resulting in a 10−20-fold reduction in the apparent inhibition constant (Sprecher et al., 1994). Preliminary studies indicated that heparin

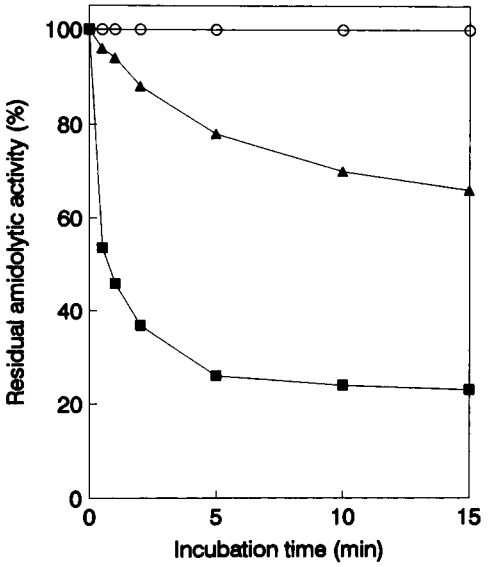

FIGURE 1: Time course for the inhibition of factor VIIa−tissue factor amidolytic activity by recombinant TFPI-2/PP5: effect of heparin. Recombinant human TF_{1-218} (20 nM) and human factor VIIa (20 nM) were coincubated in a 96-well microtitration plate in 50 μL of buffer A+ for 5 min at 37 °C. At this point, either (▲) 50 μL of recombinant TFPI-2/PP5 (100 nM final concentration), (■) 50 μL of recombinant TFPI-2/PP5 (100 nM) containing heparin (1 μg/mL final concentration), or (○) 50 μL of heparin (1 μg/mL) was added to the incubation mixture and incubated further at 37 °C. At selected times, 50 μL of S-2288 (0.67 mM final concentration) was added to the well and the absorbance at 405 nm was determined.

augmented this reaction maximally at 0.2−0.5 unit/mL final concentration (data not shown). To demonstrate that heparin enhanced the rate of factor VIIa−tissue factor inhibition by TFPI-2/PP5, a preformed complex of factor VIIa−TF_{1-218} (20 nM each) was incubated with TFPI-2/PP5 (100 nM) in the presence and absence of 0.2 unit/mL heparin. Temporal analysis of residual factor VIIa−tissue factor amidolytic activity indicated a relatively slow inhibition of factor VIIa−tissue factor activity in the presence of TFPI-2/PP5 alone, while heparin markedly accelerated the initial rate of this reaction (Figure 1). Buffer control with heparin alone revealed that heparin had no measurable effect on factor VIIa−tissue factor amidolytic activity (Figure 1).

The ability of heparin to augment the TFPI-2/PP5-mediated inhibition of factor VIIa−tissue factor proteolytic activity toward factor X was also examined on monolayers of a human bladder carcinoma cell line (J82) that constitutively expresses relatively high levels of cell surface tissue factor (Sakai et al., 1989). In these experiments, J82 cell surface tissue factor was initially saturated with factor VIIa, and the cells were washed and subsequently offered various concentrations (0−200 nM) of TFPI-2/PP5 and/or heparin at 0.2 unit/mL final concentration and incubated at 37 °C for 15 min. Factor X (100 nM) was then added to the system and timed aliquots were removed for factor Xa assay. In the absence of heparin, TFPI-2/PP5 inhibited factor VIIa−tissue factor proteolytic activity in a dose-dependent manner (data not shown). TFPI-2/PP5, at 50 nM final concentration, inhibited this reaction ~40% in the absence of heparin (~1 nM factor Xa formed/min vs 1.6 nM factor Xa formed/min in the absence of TFPI-2/PP5) and ~90% (0.2 nM factor Xa formed/min) in the presence of 0.2 unit/mL heparin. These results indicate that heparin markedly augments the

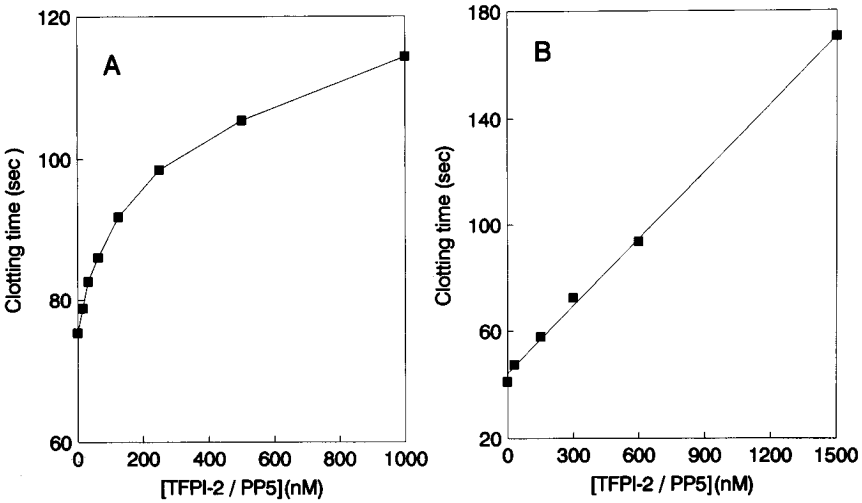

FIGURE 2: Inhibition of plasma coagulation by recombinant TFPI-2/PP5: (A) dilute thromboplastin clotting time; (B) activated partial thromboplastin clotting time. Clotting times were determined as described in Materials and Methods.

ability of TFPI-2/PP5 to inhibit factor VIIa−tissue factor proteolytic activity on cell surfaces and amidolytic activity in the solution phase.

Effect of Heparin on the Inhibition of Human Factor Xa by TFPI-2/PP5. Previous studies (Sprecher et al., 1994) indicated that TFPI-2/PP5 was a relatively weak inhibitor of human factor Xa amidolytic activity using the tripeptide chromogenic substrate S-2222. Our initial studies to assess whether heparin enhanced the inhibition of factor Xa amidolytic activity by TFPI-2/PP5 were complicated by the fact that heparin alone inhibited the amidolytic activity of factor Xa toward S-2222. This effect, however, was not observed when S-2222 was replaced by another factor Xa-specific substrate, FXa-1, which permitted us to evaluate whether heparin affected the inhibition of factor Xa by TFPI-2/PP5 similar to that observed for the inhibition of factor VIIa−tissue factor reported earlier. The results of these studies indicate that high levels of TFPI-2/PP5 (10−100 nM) readily neutralized factor Xa amidolytic activity toward FXa-1 in the presence of 10 μg/mL heparin, with an apparent K_i value of ~50 nM TFPI-2/PP5. When compared to full-length recombinant human TFPI in the presence of 10 μg/mL heparin, TFPI-2/PP5 was approximately 3 orders of magnitude less efficient than TFPI under these conditions (data not shown).

Effect of TFPI-2/PP5 on the Clotting Activity of Normal Plasma. Recombinant TFPI-2/PP5 prolonged the clotting times of normal human plasma in both the dilute thromboplastin clotting time assay (Figure 2A) and the activated partial thromboplastin time (APTT) assay (Figure 2B) in a dose-dependent manner. At 1.5 μM, TFPI-2/PP5 prolonged the clotting time >4-fold in the activated partial thromboplastin time assay. These results suggested that, in addition to its ability to inhibit the factor VIIa−tissue factor proteolytic activity as demonstrated in the dilute thromboplastin clotting time assay, TFPI-2/PP5 was also a potent inhibitor of one or more proteases featured in the intrinsic pathway of blood coagulation.

Effect of TFPI-2/PP5 on Proteases Involved in Intrinsic Coagulation. In these studies, TFPI-2/PP5 was incubated separately with either human factor XIIa, factor XIa, plasma kallikrein, or factor IXa−poly(lysine) to determine which protease(s) was inhibited by TFPI-2/PP5 that could account

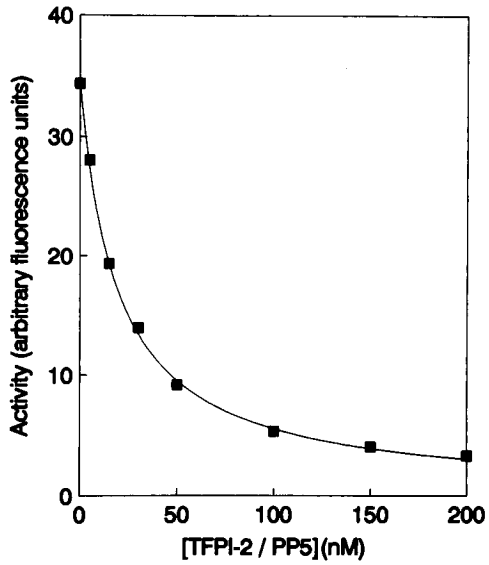

FIGURE 3: Inhibition of human factor XIa amidolytic activity by recombinant TFPI-2/PP5. Factor XIa (3 nM) was incubated at 25 °C with varying concentrations of TFPI-2/PP5 for 15 min. Substrate (0.25 mM Boc-Glu(OBzl)-Ala-Arg-MCA) was then added, and the residual activity was measured by fluorometry (excitation at 380 nm; emission at 460 nm). The activity was measured in arbitrary fluorescence units per minute.

for the prolongation of the APTT. No significant inhibition of human factor XIIa amidolytic activity by TFPI-2/PP5 was observed (data not shown). On the other hand, as shown in Figure 3, TFPI-2/PP5 was a relatively strong inhibitor of human factor XIa amidolytic activity with an apparent inhibition constant of 19 nM. With respect to factor XIa inhibition, this suggests that TFPI-2/PP5 is comparable to another recently discovered Kunitz-type protease inhibitor, designated as human amyloid precursor protein homolog (APPH). The purified KPI derived from this protein (APPH KPI) inhibited factor XIa with a K_i value of 14 nM (Petersen et al., 1994). However, this affinity is still an order of magnitude lower than that of the KPI domain from the amyloid precursor protein (APP KPI), which exhibited a K_i value of 0.7 nM (Petersen et al., 1994). TFPI-2/PP5 also inhibited the amidolytic activity of plasma kallikrein and the proteolytic activity of factor IXa−poly(lysine) toward factor X with apparent inhibition constants of 105 and 410 nM,

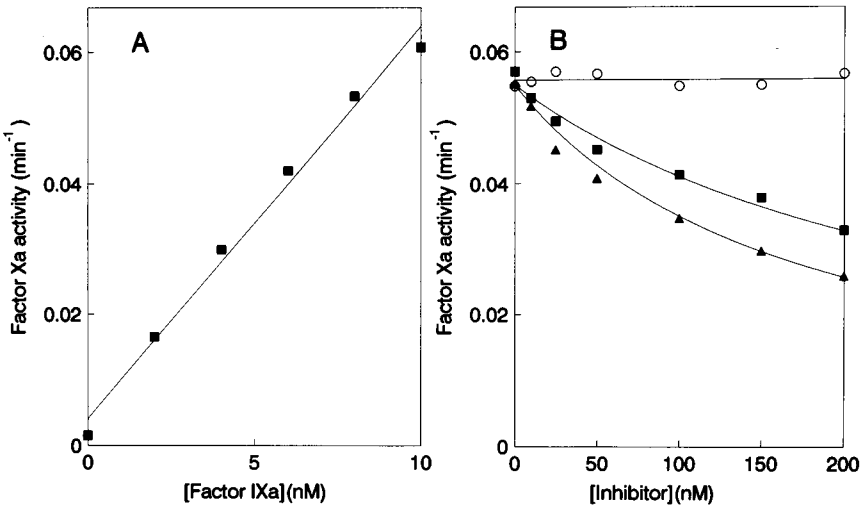

FIGURE 4: Inhibition of factor IXa proteolytic activity by recombinant TFPI-2/PP5. (A) Effect of human factor IXa concentration on the activation of human factor X in the presence of poly(lysine). (B) Factor IXa inhibition by various concentrations of TFPI-2/PP5 (■), APPH KPI (○), and APP KPI (▲). Factor IXa inhibition was assessed as described in Materials and Methods. APPH KPI and APP KPI were purified as described (Petersen et al., 1994).

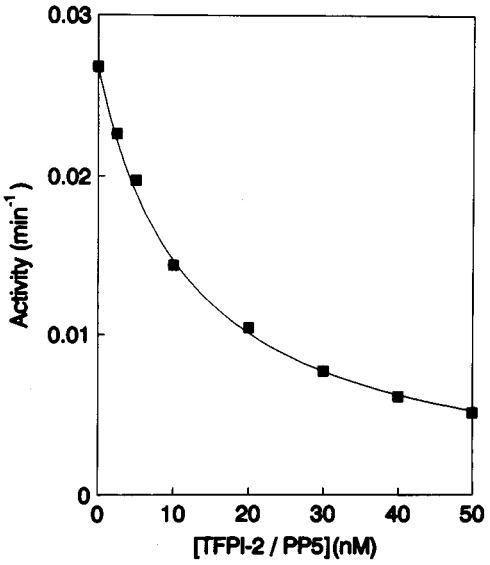

FIGURE 5: Inhibition of human plasmin amidolytic activity by recombinant TFPI-2/PP5. Plasmin (10 nM) was incubated at 25 °C with varying concentrations of TFPI-2/PP5 for 15 min. Substrate (0.6 mM S-2251) was then added, and the residual activity was measured.

respectively. In this system, TFPI-2/PP5 was slightly less effective toward factor IXa−poly(lysine) when compared to APP KPI, whereas purified APPH KPI failed to inhibit factor IXa−poly(lysine) at all concentrations tested (Figure 4). Thus, these data indicate that the prolongation of the APTT by TFPI-2/PP5 is due, in large part, to its ability to inhibit factor XIa proteolytic activity.

Effect of TFPI-2/PP5 on Other Selected Serine Proteases. In addition to its ability to inhibit the amidolytic activity of trypsin, TFPI-2/PP5 also inhibited the amidolytic activities of chymotrypsin, plasmin, and cathepsin G. TFPI-2/PP5 inhibited plasmin amidolytic activity in a dose-dependent manner with an apparent inhibition constant of 12 nM (Figure 5). As shown in Table 1, TFPI-2/PP5 was a relatively strong inhibitor of trypsin, chymotrypsin, plasmin, plasma kallikrein, and factor XIa, with true K_i values in the range of 2−25 nM. TFPI-2/PP5 is a relatively weak inhibitor of factor IXa−poly(lysine), cathepsin G, and factor VIIa−tissue factor in the absence of heparin (Table 1). No significant inhibition of glandular kallikrein, uPA, tPA, activated protein C, thrombin, or leukocyte elastase was observed when these proteases were incubated with 200 nM TFPI-2/PP5 (data not shown). Furthermore, in contrast to previous findings using purified PP5 (Meisser et al., 1985), TFPI-2/PP5 (1 μM final concentration) failed to inhibit the thrombin-mediated conversion of fibrinogen to fibrin (data not shown).

DISCUSSION

The regulation of coagulation is an essential element of hemostasis. In the present study, we demonstrate that a newly described human Kunitz-type protease inhibitor, provisionally designated by us as tissue factor pathway inhibitor-2 (TFPI-2) and shown to be identical to placental protein 5 (PP5), is a prominent inhibitor of the amidolytic activity of trypsin, chymotrypsin, plasmin, plasma kallikrein, factor XIa, factor VIIa−tissue factor, and cathepsin G. TFPI-2/PP5 also inhibited the proteolytic activity of a complex of factor VIIa and cell surface tissue factor toward factor X and inhibited the activation of factor X by a complex of factor IXa and poly(lysine). Consistent with its ability to inhibit factor VIIa−tissue factor and factor IXa, TFPI-2/PP5 also prolonged the clotting time of normal human plasma in a dose-dependent manner in both the dilute thromboplastin clotting time and activated partial thromboplastin clotting time assays.

Heparin markedly accelerated the inhibition of factor VIIa−tissue factor by TFPI-2/PP5 toward a chromogenic substrate in the solution phase, as well as of a protein substrate on the cell surface. A similar effect of heparin has been demonstrated for the inhibition of factor Xa by TFPI (Huang et al., 1993; Jesty et al., 1994). This finding is consistent with our earlier observation that heparin strongly affected the TFPI-2-mediated inhibition of factor VIIa−tissue factor amidolytic activity and induced a 10−20-fold reduction in the apparent inhibition constant (Sprecher et al., 1994). In addition, heparin dramatically enhanced the ability of TFPI-2/PP5 to inhibit human factor Xa amidolytic activity at relatively high concentrations of inhibitor. Systematic efforts to demonstrate that heparin augmented the TFPI-2/

Table 1: K_i Values for the Inhibition of Selected Proteases by TFPI-2/PP5

enzyme	[E] (nM)	substrate	[S] (mM)	K_s (mM)	K_i' (nM)	K_i (nM)
trypsin	8	Val-Leu-Lys-pNA	0.6	0.31	6[a]	2
chymotrypsin	2.5	MS-Arg-Pro-Tyr-pNA	0.6	0.063	190	18
plasmin	10	Val-Leu-Lys-pNA	0.6	0.21	12[a]	3
plasma kallikrein	3	Pro-Phe-Arg-pNA	0.6	0.19	105	25
factor XIa	3	Boc-Glu(OBzl)-Ala-Arg-MCA	0.25	0.9	19	15
factor VIIa/TF	10/16	Ile-Pro-Arg-pNA	0.6	nd[b]	630	nd
factor IXa/poly(lysine)	4.5	factor X	0.1	nd	410	nd
cathepsin G	10	Suc-Ala-Ala-Pro-Phe-pNA	0.6	2.9	241	200

[a] Equation 1 (tight binding inhibition). [b] nd, not determined.

PP5-mediated inhibition of other proteases tested in this study were complicated by the fact that heparin alone inhibited the amidolytic activities of factor XIa, cathepsin G, and leukocyte elastase. Likewise, heparin had a pronounced effect on the activation of factor X by a complex of factor IXa and poly(lysine). In preliminary studies, heparin, at 10 μg/mL, augmented the inhibition of plasmin and activated protein C by TFPI-2/PP5, but had no significant effect on its ability to inhibit either trypsin, chymotrypsin, or plasma kallikrein amidolytic activity.

In a recent study, Rao and co-workers demonstrated that cultured human umbilical vein endothelial cells synthesize three proteins (M_r = 33 000, 31 000, and 27 000), immunologically distinct from inter-α-trypsin inhibitor, that inhibit the degradation of gelatin or casein by trypsin, elastase, plasmin, and α-chymotrypsin, but not by α-thrombin (Rao et al., 1995b). These serine protease inhibitors were found in cell lysates as well as in the extracellular matrix, but were not observed in conditioned medium. Phorbol 12-myristate 13-acetate stimulated the synthesis of all three inhibitors, resulting in increased levels of each inhibitor in cell lysates and extracellular matrix, in addition to their expression in the conditioned medium (Rao et al., 1995b). Furthermore, all three endothelial cell-derived inhibitors bound tightly to heparin−Sepharose as well as to a trypsin affinity column. Aside from differences in elastase inhibition, given the functional and structural similarities between the endothelial cell serine protease inhibitors reported by Rao et al. (1995b) and the inhibitory spectrum of TFPI-2/PP5 reported here, it is reasonable to speculate that one (or all) of the three endothelial cell inhibitors described by Rao and co-workers may in fact be TFPI-2/PP5 or degraded forms of TFPI-2/PP5. Indeed, the results of preliminary immunoblotting studies using a monospecific rabbit anti-human TFPI-2/PP5 IgG preparation indicate that the 33, 31, and 27 kDa proteins obtained from five different batches of human endothelial cell conditioned media are immunochemically identical to TFPI-2/PP5.[2] Whether the 31 and 27 kDa inhibitor bands are true degradation products of TFPI-2/PP5, as noted earlier in PP5 preparations (Butzow et al., 1988b), or represent underglycosylated forms of the mature protein presently is not known and will require further investigation. In this regard, treatment of purified recombinant TFPI-2/PP5 with recombinant PNGase F (New England Biolabs) yielded a 27−28 kDa intermediate, which is eventually converted to a final product that exhibits a M_r of ∼25 000.[3]

The data presented here, together with several reports in the literature regarding the location and activity of PP5, suggest a physiological role for TFPI-2/PP5 in hemostasis. Several lines of evidence indicate that TFPI-2/PP5 is synthesized in endothelial cells by immunohistochemical (Butzow et al., 1988b) and Northern blot analyses (Sprecher et al., 1994) and circulates in blood in normal men and nonpregnant women in extremely low concentrations (0.43−0.49 μg/L) (Butzow et al., 1988a). The serum concentration of TFPI-2/PP5 increases 40−70-fold during pregnancy (Butzow et al., 1988a), and this value increases another 10−40-fold following the injection of small doses of heparin in pregnant women (Menabawey et al., 1985). On the basis of our findings concerning the ability of heparin to augment TFPI-2/PP5 inhibitory activity, and the presence of a heparin-releasable form of TFPI-2/PP5 from endothelium, it is reasonable to assume that TFPI-2/PP5, like TFPI, is synthesized in endothelial cells and binds tightly to either the luminal surface of the endothelial cell via heparin-like glycosaminoglycans or to glycosaminoglycans in the extracellular matrix. Furthermore, it is conceivable that these interactions augment the inhibitory activity of TFPI-2/PP5 on the endothelial cell surface toward several blood-clotting proteases or inhibit degradation of the extracellular matrix by serine proteases. Further studies along these lines of investigation are ongoing in our laboratory.

[2] C. N. Rao and W. Kisiel, unpublished data.
[3] W. Kisiel, unpublished data.

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