

## Matrix Localization of Tissue Factor Pathway Inhibitor-2/ Matrix-Associated Serine Protease Inhibitor (TFPI-2/MSPI) Involves Arginine-Mediated Ionic Interactions with Heparin and Dermatan Sulfate: Heparin Accelerates the Activity of TFPI-2/MSPI toward Plasmin<sup>1</sup>

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**Human tissue factor pathway inhibitor-2 (TFPI-2)/matrix-associated serine protease inhibitor (MSPI), a Kunitz-type serine protease inhibitor, inhibits plasmin, trypsin, chymotrypsin, plasma kallikrein, cathepsin G, and factor VIIa-tissue factor complex. The mature protein has a molecular mass of 32–33 kDa, but exists *in vivo* as two smaller, underglycosylated species of 31 and 27 kDa. TFPI-2/MSPI triplet is synthesized and secreted by a variety of cell types that include epithelial, endothelial, and mesenchymal cells. Because the majority (75–90%) of TFPI-2/MSPI is associated with the extracellular matrix (ECM), we examined which components of the ECM bind TFPI-2/MSPI. We found that TFPI-2/MSPI bound specifically to heparin and dermatan sulfate. Interaction of these two glycosaminoglycans (GAGs) with TFPI-2/MSPI involved one or more common protein domains, as evidenced by cross-competition experiments. However, binding affinity for TFPI-2/MSPI with heparin was**

**250–300 times greater than that for TFPI-2/MSPI with dermatan sulfate. Binding of TFPI-2/MSPI to GAGs was inhibited by NaCl or arginine but not by glucose, mannose, galactose, 6-aminohexanoic acid, or urea, suggesting that arginine-mediated ionic interactions participate in the GAG binding of TFPI-2/MSPI. This supposition was supported by the observation that only NaCl or arginine could elute the TFPI-2/MSPI protein triplet from an ECM derived from human dermal fibroblasts. Reduced TFPI-2/MSPI did not bind to heparin, suggesting that proper disulfide pairings and conformation are essential for matrix binding. To determine whether heparin modulates the activity of TFPI-2/MSPI, we determined the rate of inhibition of plasmin by the inhibitor with and without heparin and found that TFPI-2/MSPI is more active in the presence of heparin. Collectively, our results demonstrate that conformation-dependent arginine-mediated ionic interactions are responsible for the TFPI-2/MSPI triplet binding to fibroblast ECM, heparin, and dermatan sulfate and that heparin augmented the rate of inhibition of plasmin by TFPI-2/MSPI.** © 1999 Academic Press

**Key Words:** tissue factor pathway inhibitor-2; matrix-associated serine protease inhibitor; placental protein-5; extracellular matrix; heparin; dermatan sulfate; glycosaminoglycans.

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Tissue factor pathway inhibitor-2 (TFPI-2,<sup>3</sup> also called matrix-associated serine protease inhibitor (MSPI)), a 32- to 33-kDa Kunitz-type serine protease inhibitor, inhibits trypsin, plasmin, chymotrypsin, cathepsin G, plasma kallikrein, and factor VIIa-tissue factor complex but not urokinase and tissue-type plasminogen activators and thrombin (1–7). On the basis of its amino-terminal amino acid sequence and target protease specificity, TFPI-2 is identical to a partially characterized 30- to 38-kDa serine protease inhibitor from placenta called placental protein-5 (PP-5) (3, 4). A variety of cell types, including epithelial, mesenchymal, and endothelial cells, synthesize and secrete mature TFPI-2/PP-5/MSPI and two other under-glycosylated forms of TFPI-2 with molecular masses of 31 and 27 kDa (5–8). Localization studies of the TFPI-2 triplet in cell-conditioned medium, ECM, and cytoplasmic fractions revealed that 75–90% of the inhibitors associated with the ECM (5–8). More recently, similar observations were reported using human endothelial cells derived from umbilical vein, saphenous vein, aorta, and dermal microvessels (9). Moreover, it was found that both the ECM derived from microvascular endothelial cells and monolayer cultures of these cells bind TFPI-2 in a specific, dose-dependent, and saturable manner (9).

Our interest in TFPI-2 derives from its interaction with ECM and thus its potential effects on tumor invasion and metastasis inasmuch as tumor invasion and metastasis require degradation and remodeling of the ECM. In earlier investigations of TFPI-2's role in matrix degradation and invasion by tumor cells, we found that soluble TFPI-2 effectively inhibited plasmin that was bound to the surface of HT-1080 fibrosarcoma cells and inhibited matrix degradation and Matrigel invasion by these tumor cells (10). ECM-localized TFPI-2 triplet also inhibited plasmin, suggesting that TFPI-2 proteins within the matrix were functional (10). In the present study, we investigated the role of individual matrix components in TFPI-2 binding and found that TFPI-2 specifically bound heparin and dermatan sulfate via arginine-mediated ionic interactions. Similar interactions were also responsible for TFPI-2 triplet binding to a fibroblast ECM. We also provide evidence that proper disulfide pairing/conformation was necessary for binding of TFPI-2 to heparin and

that heparin significantly accelerated the activity of the inhibitor toward plasmin.

## MATERIALS AND METHODS

Materials for this study were obtained as follows. PMA, low-molecular-weight heparin from porcine intestinal mucosa, keratan sulfate from bovine cornea, dermatan sulfate from bovine mucosa, hyaluronic acid from human umbilical cord, fibrinogen from human plasma, and tunicamycin were purchased from Sigma Chemical Co. (St. Louis, MO). Human plasma fibronectin was purchased from New York Blood Center (New York, NY). ECL Western blotting reagents were purchased from Amersham Life Sciences (Buckinghamshire, England). Heparin-Sepharose affinity matrix was purchased from Pharmacia/LKB Biotechnology, Inc. (Uppsala, Sweden). Lysine-plasminogen was a gift from Dr. Bruce Credo (Abbott Research Laboratories, Abbott Park, IL). Human recombinant TFPI-2 and polyclonal anti-TFPI-2 IgG were gifts from Drs. Walter Kisiel (Department of Pathology, University of New Mexico, Albuquerque, NM) and Donald C. Foster (Zymogenetics, Inc., Seattle, WA). The TFPI-2 used in this study contained the 32- to 33-kDa form as the predominant species (>95%) with minor amounts of the 31-kDa species (<5%). Rat laminin-5 was a gift from Dr. Jonathan Jones (Northwestern University, Chicago, IL). Laminin-1, human vitronectin, and collagen type-I and collagen-IV were prepared as described elsewhere (11, 12). Recombinant noncollagenous domain of VII collagen was a gift from Dr. Mei Chen (Northwestern University, Chicago, IL).

**Binding assays using ELISA.** Binding of soluble recombinant TFPI-2 to ECM molecules immobilized on plastic was assayed by colorimetric enzyme-linked antibody reaction as described elsewhere (13). All the steps were performed at room temperature. Ninety-six-well plates (Corning, NY) were coated for 4 h with the following ECM proteins (0.5  $\mu$ g/well) in 15 mM Tris-HCl, pH 7.40: collagen types I and IV, noncollagenous domain of VII collagen, laminins 1 and 5, fibronectin, vitronectin, fibrinogen heparin, chondroitin sulfate A, hyaluronic acid, and keratan sulfate. After removing the unbound proteins with TBST (15 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween 20), the wells were blocked with 1% BSA in TBST for 90 min and incubated with 200–400 ng of recombinant TFPI-2 in binding buffer (TBST containing 1 mg/ml BSA) for 2 h. The binding of recombinant TFPI-2 to ECM proteins was detected with anti-TFPI-2 IgG (1:4000 in TBST with 1% BSA) followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:3000 in TBST with 1% BSA). The development of a colorimetric reaction with *p*-nitrophenyl phosphate as a substrate (Bio-Rad Research Laboratories, Richmond, CA) was measured by reading the absorbance of the product at 405 nm with an ELISA reader (Labsystems Multiskan Multisoft, Finland).

In competition experiments, TFPI-2 was incubated with different concentrations of ECM proteins for 20 min in binding buffer and then added to wells previously coated with ECM proteins and BSA. In blocking experiments, TFPI-2 was suspended in the binding buffer containing NaCl, urea, 6-aminohexanoic acid, glucose, galactose, or mannose and then added to ECM-coated/BSA-blocked wells. To assess the effects of heat and  $\beta$ -mercaptoethanol ( $\beta$ -ME) on the binding of TFPI-2 to ECM proteins, TFPI-2 was boiled for 5 min with or without the  $\beta$ -ME (final concentration, 4%). The reaction mixtures were then cooled on wet ice, diluted with binding buffer, and incubated in the ECM-coated/BSA-blocked wells.

**Binding assays using heparin-Sepharose.** In these assays, 50  $\mu$ l of heparin-Sepharose beads that had been equilibrated with TBS (15 mM Tris-HCl, 0.15 M NaCl, pH 7.4) were incubated with 200 ng of recombinant TFPI-2 for 1 h in 1 ml of binding buffer. The unbound TFPI-2 then was removed by washing the beads three times with TB

<sup>3</sup> Abbreviations used: ECM, extracellular matrix; t12FB, SV-40-transformed human skin fibroblasts; NC-1, noncollagenous domain of type VII collagen; TFPI-2: tissue factor pathway inhibitor-2; PP-5, placental protein-5; MSPI, matrix-associated serine protease inhibitor; uPA, urokinase-type plasminogen activator, Lys-Pg, lysine-plasminogen; TB, 15 mM Tris-HCl, pH 7.40; TBS, TB with 0.15 M NaCl; TBST, TBS with 0.05% Tween 20;  $\beta$ -ME,  $\beta$ -mercaptoethanol; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbant assay.

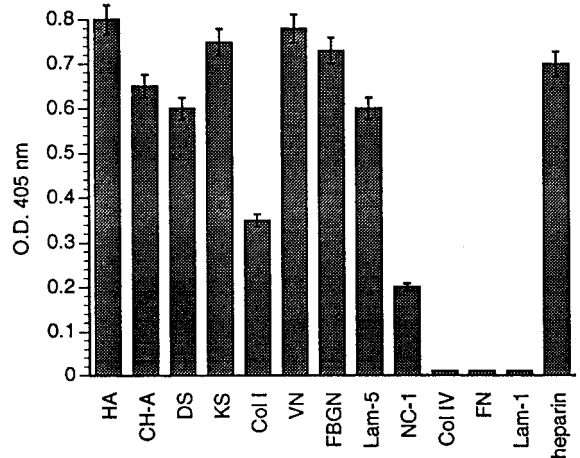
(15 mM Tris-HCl, pH 7.4). Heparin-Sepharose-bound TFPI-2 was then eluted into 200  $\mu$ l of SDS-PAGE sample buffer. To quantify the bound TFPI-2, 25  $\mu$ l of the sample was subjected to SDS-PAGE and Western blotting with anti-TFPI-2 antibody. The heparin-Sepharose binding assay was also used to determine which of the ECM proteins and chemical reagents compete with TFPI-2 for binding to heparin-Sepharose. In blocking experiments, TFPI-2 was suspended in binding buffer containing NaCl, 6-aminohexanoic acid, glucose, galactose, and mannose, and then the TFPI-2 binding to heparin-Sepharose was quantified by Western blotting with antiTFPI-2 antibody. To determine which ECM proteins blocked TFPI-2/MSPI binding to heparin-Sepharose, the inhibitor protein (200 ng) and ECM proteins (1  $\mu$ g–1 mg) were incubated for 20 min in 1 ml of binding buffer and then incubated with heparin-Sepharose for 1 h. The bound inhibitor was then quantified by Western blotting.

Binding of TFPI-2 that had undergone heat-denaturation or disulfide-bond reduction with  $\beta$ -ME to heparin was assayed by heparin-Sepharose binding assay. In this assay, TFPI-2 was boiled for 5 min with or without  $\beta$ -ME (final concentration, 4%) in 100  $\mu$ l of the binding buffer, cooled on wet ice, diluted to 1 ml with the binding buffer, and then assayed for binding to heparin-Sepharose.

**Extraction of the TFPI-2 triplet and the 25-kDa nonglycosylated TFPI-2 from fibroblast ECM.** SV-40-transformed human skin fibroblasts (t12FB) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 50  $\mu$ g/ml penicillin, and 50  $\mu$ g/ml streptomycin. Cells were grown to 70–80% confluence in 24-well tissue culture plates and treated with PMA (50 ng/ml) for 16–20 h to increase the production of the TFPI-2 triplet in the ECM (5–8). Some t12FB cultures were first treated with tunicamycin (1  $\mu$ g/ml) for 16 h to suppress glycosylation and then incubated with PMA, as described above. At the end of the incubations, the medium and cells were discarded and the ECM prepared as described elsewhere (3–5). ECM plates were stored at 4°C until analysis. Before being used, these plates were rinsed with TB, and the ECM was extracted with 600  $\mu$ l of TB containing 0.15–2.0 M NaCl, or TBS containing 2 M urea, 1 M mannose, 1 M galactose, 1 M glucose, 0.1 M 6-aminohexanoic acid, or 10 mg/ml of GAGs heparin, dermatan sulfate, or chondroitin sulfate-A for 2 h at room temperature. The remaining TFPI-2 proteins were then extracted into 300  $\mu$ l of SDS-PAGE sample buffer, and a 25  $\mu$ l aliquot of this extract was used for Western blotting with anti-TFPI-2 antibody.

**Plasmin inhibition assays.** Recombinant uPA (100 IU), 0.4  $\mu$ M lys-plasminogen, 0.4 mM D-Val-Leu-Lys-pNA (S-2251, Sigma Chemicals, St. Louis), and recombinant TFPI-2 (50 nM) were incubated at room temperature with (50 nM) or without heparin for 20 min. Assays were performed in 200  $\mu$ l of a buffer containing 15 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween 80, and 2 mg/ml bovine serum albumin. Free nitrophenolate anion, which is formed from S-2251 by plasmin, was quantitated by monitoring the absorbance value at 405 nm in an ELISA reader at selected times. The absorbance values from S-2251 with Pg alone and uPA alone were considered background (<0.05%) and were subtracted.

**Western blotting.** Proteins were boiled for 3 min, separated by SDS-PAGE with 12% polyacrylamide gels (14) and electroblotted onto nitrocellulose membranes as described elsewhere (15). After electroblotting, the membranes were blocked with 4% nonfat dry milk in 10 mM TBST for 2 h at room temperature. Then, the membranes were incubated either for 2 h at room temperature or overnight at 4°C with normal rabbit serum or anti-TFPI-2 antibody, diluted 1:4000 in TBST containing 1% BSA. After several washes with TBST, the membranes were incubated for 1 h with a peroxidase-conjugated secondary antibody diluted 1:3000 in TBST with 1% BSA. The immunoreactive proteins were identified using the ECL Western blotting system according to the manufacturer's instruc-

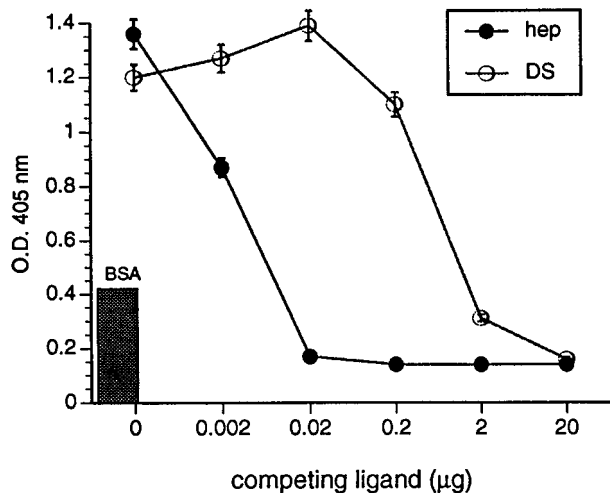


**FIG. 1.** Binding of TFPI-2 to ECM proteins. ELISA wells were coated with the following ECM proteins (500 ng/well): hyaluronic acid (HA), chondroitin sulfate A (CH-A), dermatan sulfate (DS), collagen types I and IV, keratan sulfate (KS), vitronectin (VN), fibrinogen (FBGN), laminins (LAM) 1 and 5, fibronectin (FN), non-collagenous domain of type VII collagen (NC-1), and heparin. The wells were then incubated for 2 h at room temperature with 200 ng TFPI-2. TFPI-2 binding was detected with a polyclonal anti-TFPI-2 IgG. TFPI-2 binding to wells coated with BSA was subtracted from the O.D. values of TFPI-2 binding to ECM proteins. The values shown are means of four independent determinations.

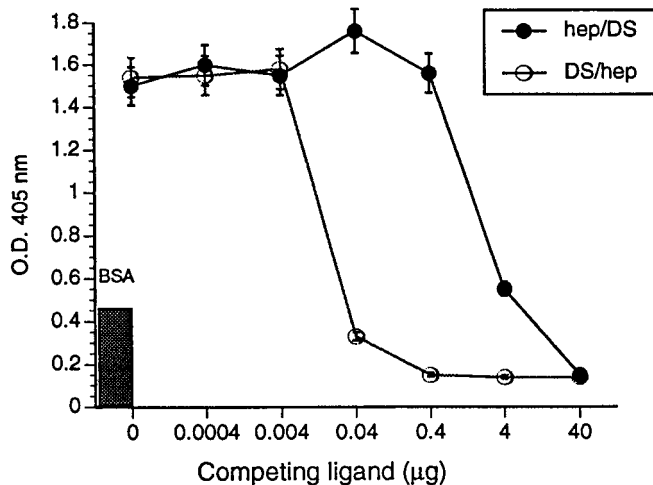
tions. The proteins were quantified by scanning the bands using an imaging densitometer (Model GS 670; Bio-Rad, Richmond, CA).

## RESULTS

**TFPI-2 specifically binds heparin and dermatan sulfate.** Because secreted TFPI-2 is predominantly (75–95%) associated with the ECM (5–9), we used recombinant TFPI-2 in an ELISA system to identify the TFPI-2 binding components of the ECM. We found that TFPI-2 binds hyaluronic acid, chondroitin sulfate-A, dermatan sulfate, keratan sulfate, type I collagen, vitronectin, fibrinogen, laminin-5, NC-1, and heparin but not to type IV collagen, fibronectin, or laminin-1 (Fig. 1). To determine the specificity of the binding between TFPI-2 and ECM proteins, we measured TFPI-2 binding to the immobilized ECM protein in the presence of increasing amounts (2 ng–20  $\mu$ g) of the corresponding soluble ECM protein. Soluble heparin and dermatan sulfate blocked the binding of TFPI-2 to their immobilized ligands in a dose-dependent manner (Fig. 2). The  $IC_{50}$  for heparin was 6 to 8 ng, whereas the  $IC_{50}$  for dermatan sulfate was 1.6 to 1.8  $\mu$ g, which suggests that the binding affinity for TFPI-2 and heparin is 250 to 300 times greater than that for dermatan sulfate. In contrast, TFPI-2 binding to immobilized vitronectin, laminin-5, collagen-I, plasminogen, hyaluronic acid, keratan sulfate, or fibrinogen was not blocked by these soluble proteins but was completely inhibited by 2  $\mu$ g



**FIG. 2.** Binding of TFPI-2 to heparin and dermatan sulfate is specific. ELISA plates were coated with heparin (hep) or dermatan sulfate (DS) (500 ng/well) and incubated with 200 ng of TFPI-2 for 2 h at room temperature. To determine the binding specificity, TFPI-2 was preincubated with hep or DS (2 ng–20 µg) for 20 min and then incubated with hep and DS-coated ELISA plates. The values represent means of four independent determinations.



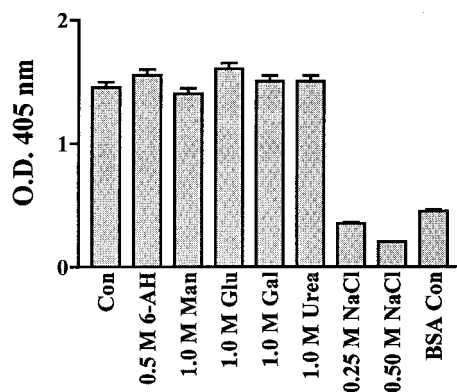
**FIG. 3.** Binding of TFPI-2 to immobilized heparin is inhibited by soluble DS and vice versa. ELISA plates were coated with heparin (hep) or dermatan sulfate (DS) (500 ng/well) and incubated with 400 ng of TFPI-2 for 2 h at room temperature. To determine whether the TFPI-2 binding to immobilized hep or DS could be inhibited by soluble DS or hep (0.4 ng–40 µg), TFPI-2 is preincubated with either GAG for 20 min and then incubated with immobilized GAG. The solid circles indicate soluble DS competing for TFPI-2 binding to immobilized hep, whereas the open circles indicates the soluble hep competing for the TFPI-2 binding to immobilized DS. The values shown are means of four independent determinations.

of heparin (data not shown). Next, we coated 0.125 ng to 500 ng heparin or dermatan sulfate and incubated with 200 ng TFPI-2, and found that TFPI-2 binding to these GAGs was saturable (data not shown).

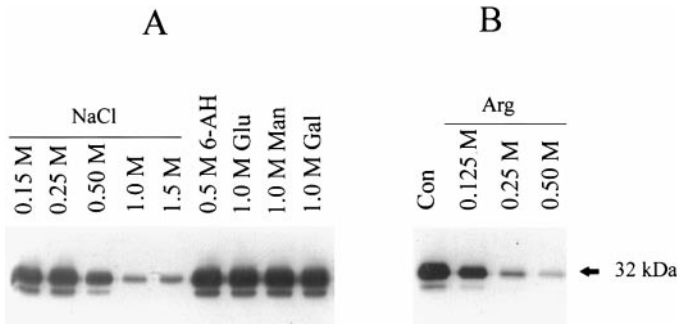
To confirm the greater binding affinity of TFPI-2 for heparin than for dermatan sulfate, we conducted cross-competition experiments. We used 400 ng of recombinant TFPI-2 for binding to 500 ng of heparin or dermatan sulfate-coated wells. Heparin-blocked TFPI-2 binding to dermatan sulfate with an  $IC_{50}$  of 10–12 ng and DS inhibited binding to heparin with an  $IC_{50}$  of 3.0–3.4 µg (Fig. 3). The specificity of the high-affinity interaction between TFPI-2 and heparin was also confirmed by heparin–Sepharose binding assays (data not shown).

*TFPI-2 binding to heparin involves arginine-mediated ionic interactions.* To learn more about the biochemical mechanisms of interaction of TFPI-2 with heparin, we conducted ELISA (Fig. 4) and heparin–Sepharose (Fig. 5) binding assays in the presence of a battery of chemical reagents. Of these reagents, only NaCl and arginine blocked the binding of TFPI-2 to heparin, whereas 1 M urea, 0.5 M 6-aminohexanoic acid, 1 M glucose, 1 M galactose, and 1 M mannose had no effect (Figs. 4, 5A, and 5B). To confirm that NaCl and arginine interfere with the binding of TFPI-2 to heparin, we assessed means of releasing the TFPI-2 bound to heparin–Sepharose. Again, 1 M NaCl and 0.5 M arginine but not the other compounds released TFPI-2 bound to heparin–Sepharose (data not shown).

Collectively, these data suggest that ionic interactions mediated via one or more arginines participate in the binding of TFPI-2 to heparin. Binding of TFPI-2 to dermatan sulfate is also blocked by NaCl and arginine, suggesting that arginine-mediated ionic interactions are also responsible for the binding between TFPI-2 and dermatan sulfate (data not shown).



**FIG. 4.** Arginine and NaCl inhibit TFPI-2 binding to heparin. Binding of TFPI-2 to heparin was determined in the presence or absence of 6-aminohexanoic acid (6-AH), arginine (Arg), mannose (Man), glucose (Glu), galactose (gal), urea, or NaCl as described in the legend to Fig. 1. TFPI-2 binding to heparin without the blocking agent (Con) and to BSA (BSA con) also quantified. The values shown are means of four independent determinations.

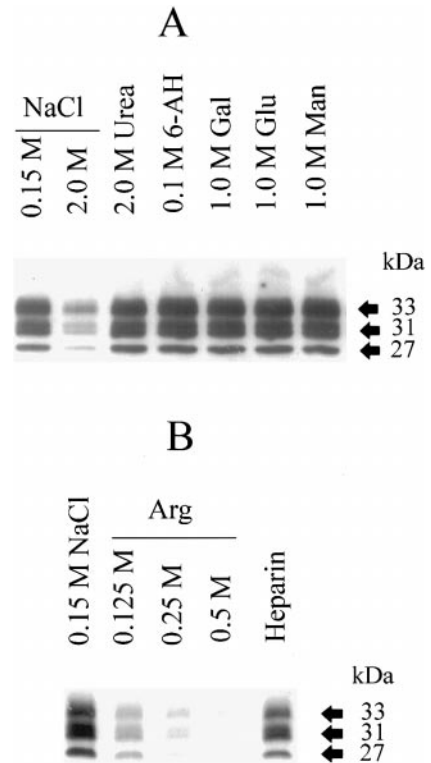


**FIG. 5.** NaCl and arginine inhibit TFPI-2 binding to heparin-Sepharose. TFPI-2 (200 ng) was suspended in TBS (Con) or TBS containing glucose (Glu), galactose (Gal), mannose (Man), 6-aminohexanoic acid (6-AH), and TB containing 0.15–1.5 M NaCl (A) or TBS containing 0.125–0.5 M arginine (Arg) (B) and incubated with heparin-Sepharose for 1 h at room temperature. The bound TFPI-2 was determined by Western blotting, as described under Materials and Methods.

*Arginine-mediated ionic interactions are also responsible for the binding of TFPI-2 triplet to ECM.* To determine whether the arginine-contributed ionic interactions also participate in the binding of TFPI-2 to a native ECM, we evaluated the release of TFPI-2 triplet from the t12FB ECM with NaCl and arginine. NaCl (Fig. 6A) and arginine (Fig. 6B) extracted the TFPI-2 triplet from the fibroblast ECM with 0.5 M arginine producing a >98% decrease in ECM-bound TFPI-2 species. Soluble heparin (10 mg/ml) also released 30 to 35% of the TFPI-2 triplet from the fibroblast ECM (Fig. 6B) but the same amounts of chondroitin sulfate A and dermatan sulfate had no effect (data not shown).

t12FB that had been treated with tunicamycin synthesize and secrete a 25-kDa nonglycosylated TFPI-2 from cells (8). NaCl and arginine, but not 6-aminohexanoic acid, glucose, galactose, mannose, and 2 M urea, eluted the 25-kDa nonglycosylated TFPI-2 from the fibroblast ECM (data not shown).

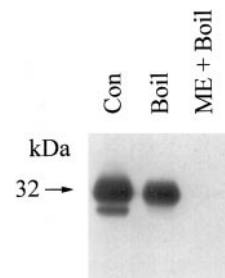
*Proper disulfide bondings/conformation is essential for the binding of TFPI-2 to heparin.* To determine whether a native three-dimensional conformation is necessary for the binding of TFPI-2 to heparin, we analyzed the binding of TFPI-2 that had undergone thermal denaturation or disulfide bond reduction to heparin immobilized on plastic or Sepharose beads. As shown in Fig. 7, TFPI-2 (Con) but not disulfide bond reduced TFPI-2 (ME + Boil) bound heparin-Sepharose. Binding of thermally denatured TFPI-2 (Boil) was approximately 75% that of the native protein, suggesting that the protein is heat-stable. Similar results were obtained using ELISA with immobilized heparin or dermatan sulfate (data not shown). Collectively, these results suggest that TFPI-2 binding to heparin and



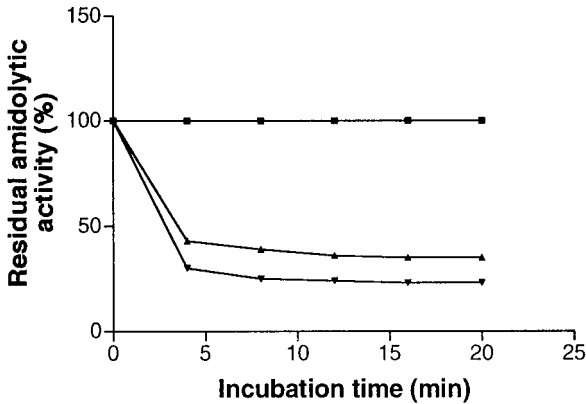
**FIG. 6.** NaCl or heparin extracts TFPI-2 triplet from the fibroblast ECM. t12FB were treated with PMA, ECM prepared (3–5), and extracted with the following: TB (15 mM Tris-HCl, pH 7.4) containing 0.15 M NaCl or 2.0 M NaCl; TBS (15 mM Tris-HCl, 0.15 M NaCl, pH 7.4) containing 2.0 M urea, 0.1 M 6-aminohexanoic acid (6-AH), 1 M glucose (Glu), 1 M galactose (Gal), 1 M mannose (Man) (A), or 10 mg/ml heparin (hep) (B) for 2 h at room temperature. The remaining TFPI-2 proteins in the ECM were quantified by Western blotting.

dermatan sulfate depends on proper disulfide bondings/conformation.

*Heparin enhanced the rate of plasmin inhibition by TFPI-2.* TFPI-2 is a potent inhibitor of plasmin but a relatively weak inhibitor of factor VIIa-tissue factor



**FIG. 7.** Reduction of disulfide bonds destroys binding of TFPI-2 to heparin. TFPI-2 was thermally denatured (Boil) or reduced with  $\beta$ -mercaptoethanol (ME + Boil) and assessed for binding to heparin by heparin-Sepharose binding assays as described under Materials and Methods. The binding of TFPI-2 to heparin was considered 100%.



**FIG. 8.** Heparin accelerated the inhibition of plasmin by TFPI-2. In this assay, lysine-plasminogen (Lys-Pg) ( $0.4 \mu\text{M}$ ) was activated to produce plasmin by recombinant uPA (100 IU). Plasmin activity was assayed by measuring the absorbance of paranitroanilide, a cleavage product of D-Val-Leu-Lys-*p*-nitroanilide (S-2251,  $0.4 \text{ mM}$  final concentration) by plasmin, at 405 nm. Recombinant urokinase-type plasminogen activator (uPA), S-2251, Lys-Pg, and heparin (50 nM) (■); uPA, S-2251, Lys-Pg, and TFPI-2 (50 nM) (▲); and uPA, S-2251, Lys-Pg, TFPI-2 (50 nM), and heparin (50 nM) (▼) were incubated at room temperature for 20 min and the formation of *p*-nitroanilide determined by measuring the absorbance at 405. The values shown are averages of four independent determinations that differed between 1 and 4%.

complex (1, 2). Peterson *et al.* (2) reported that heparin accelerated the inhibition of factor VIIa-tissue factor amidolytic activity as well as the proteolytic activity of factor VIIa-tissue factor toward factor X by monolayers of J82 bladder carcinoma cell line. To demonstrate the effect of heparin on the rate of inactivation of plasmin by TFPI-2, recombinant uPA and lysine-plasminogen were incubated with TFPI-2 (50 nM) in the presence or absence of heparin (50 nM). A time-course analysis of residual plasmin amidolytic activity indicated a relatively strong inhibition of plasmin activity in the presence of TFPI-2 alone, while heparin significantly accelerated the rate of this reaction (Fig. 8). Buffer control with heparin alone had no effect on the plasmin amidolytic activity (Fig. 8).

## DISCUSSION

We report here several lines of evidence that suggest that arginine-mediated ionic interactions mediate the binding of TFPI-2 protein triplet to fibroblast ECM. The first line of evidence comes from the observations that suggest that NaCl and arginine but not 6-aminohexanoic acid, a lysine analogue; urea, an agent that disrupts hydrophobic interactions; and glucose, mannose, and galactose, sugars that disrupt carbohydrate-protein interactions, interfered with the binding between TFPI-2 and heparin or dermatan sulfate. The second line of evidence comes from the selective extraction of TFPI-2 bound to heparin-Sepharose with NaCl

and arginine but not with the sugars, 6-aminohexanoic acid, or urea (data not shown). The third line of evidence comes from the observation that the native TFPI-2 triplet and its 25-kDa nonglycosylated precursor (data not shown) can be released from an intact ECM with NaCl and arginine but not with 6-aminohexanoic acid, urea, galactose, glucose, or mannose.

We also found evidence that the native three-dimensional conformation, but not glycosylation, is required of TFPI-2 triplet for binding to fibroblast ECM, heparin, and dermatan sulfate. First, treatment of TFPI-2 with  $\beta$ -ME abolished binding of the inhibitor to heparin and dermatan sulfate. Second, tunicamycin-treated t12FB synthesizes and secretes a 25-kDa nonglycosylated TFPI-2 that can be released from the ECM with NaCl and arginine but not with sugars, urea, and 6-aminohexanoic acid (data not shown). Third, recombinant TFPI-2 expressed in *Escherichia coli* as a 25-kDa protein does not bind heparin or dermatan sulfate but did bind to these GAGs after a refolding protocol involving a disulfide-interchange reaction (Rao *et al.*, manuscript in preparation).

The role of arginine in matrix binding likely involves interaction of the positively charged arginine(s) on the inhibitor with negatively charged GAGs in the matrix. Computer predictions of the secondary folding structure of TFPI-2 revealed that this inhibitor contains three Kunitz domains that are held together by nine-disulfide bondings, three for each domain (1). The first and third Kunitz domains contain seven arginines, at positions 24, 29, and 34 in domain 1 and four at positions 152, 157, 159, and 184 in domain 3 (1). One or more of these arginines may participate in the TFPI-2:ECM interactions. At present, it is unclear which Kunitz domain in TFPI-2 contains the matrix-binding arginines and whether any other amino acids contribute to the TFPI-2:ECM interactions. Interestingly, the lysine analog, 6-aminohexanoic acid did not interfere with the binding of TFPI-2 to heparin or dermatan sulfate and did not release the TFPI-2 from the ECM. These results exclude the possibility that the basic carboxyl terminal end of TFPI-2, which contains a stretch of five lysines may mediate the TFPI-2:ECM interactions.

The ECM consists of glycoproteins, GAGs, and collagens, many of which mediate the binding of several key regulatory molecules for cell growth, differentiation, and migration (16–18). Of the 13 ECM molecules we tested, TFPI-2 bound heparin specifically and with high affinity, suggesting that heparin and heparin-like GAGs are predominantly responsible for the binding of the TFPI-2 triplet to ECM. This observation is consistent with earlier absorption and competition studies that also suggested interaction between PP-5 and heparin (8, 19–20). However, the inability of heparin to fully extract TFPI-2 triplet from fibroblast ECM sug-

gests that other ECM proteins may also participate in binding or, conversely, that the TFPI-2 triplet is buried within the native fibroblast ECM and is inaccessible to extraction with a high-molecular-weight molecule such as heparin.

Heparin contains a mixture of molecules which differs in size, degree of sulfation, positions of sulfate ester groups, and the composition of sugar subunits within the repeating disaccharide unit of the molecule (21). The observations of the current study which suggested that TFPI-2 binds heparin and dermatan sulfate but not other sulfated GAGs indicate that certain sulfation pattern in these GAGs may play a role in the binding of MSPI to these GAGs. Indeed, studies have documented a role for sulfation in the binding of basic fibroblast growth factor to heparin (22). It was shown that the binding of basic fibroblast growth factor to heparan sulfate proteoglycans of the endothelial cell ECM was inhibited when the sulfation in these GAGs was prevented by treatment of the cells with chlorate. At present, it is unknown whether sulfate residues are involved in the binding of TFPI-2 to heparin.

TFPI-2 inhibits the amidolytic activities of plasmin, trypsin, chymotrypsin, cathepsin G, and plasma kallikrein with  $K_i$  values 3, 2, 18, 200, and 25 nM, respectively (2). We previously showed that the ECM localized TFPI-2 triplet inhibits plasmin (10). In this study, we found that heparin accelerated the rate of inhibition of plasmin by TFPI-2, suggesting that TFPI-2 is more active in the heparin-bound form. Plasmin plays a multifunctional role in ECM degradation and remodeling, by degradation of ECM molecules (23, 24), by activation of promatrixmetalloproteases (MMPs) 1 and 3 (25, 26), and by release of certain growth factors and cytokines from the matrix (27, 28). Indeed, we found that TFPI-2 blocked the activation of proMMPs 1 and 3 by tumor cells, suggesting that the inhibitor negatively regulates ECM degradation by MMPs 1 and 3 (29). Thus, TFPI-2 and its two glycosylation variants, by being associated with ECM, could be readily accessible at the cell:ECM interface to function in ECM degradation and remodeling and thereby in the regulation of numerous processes, including tumor growth and metastasis, atherosclerosis, angiogenesis, and wound healing.

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