Effect of Heparin on the Inhibition of Factor Xa by Tissue Factor Pathway Inhibitor: A Segment, Gly^{212}−Phe^{243}, of the Third Kunitz Domain Is a Heparin-Binding Site†

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ABSTRACT: Tissue factor pathway inhibitor (TFPI) inhibits the tissue factor−factor VIIa complex and factor Xa with its first and second Kunitz domains (K1 and K2), respectively. The inhibitory activity is enhanced by heparin, and the C-terminal basic part has been shown to be a heparin-binding site (HBS-1). To characterize and localize a second heparin-binding site (HBS-2), we studied the effect of heparin on the inhibitory activity of two forms of recombinant human TFPI, the full-length TFPI (rTFPI), and TFPI lacking the C-terminal basic part (rTFPI-C), by assaying the inhibition of human factor Xa. rTFPI-C inhibited factor Xa with an initial K_i of 6.79 nM in the absence of Ca^{2+} and 22.3 nM in the presence of 5 mM $CaCl_2$. Heparin decreased the initial K_i to 1.79 nM in the absence of Ca^{2+} and 2.68 nM in the presence of 5 mM $CaCl_2$, indicating the presence of HBS-2 in rTFPI-C. The dissociation constant for the binding of HBS-2 with heparin was determined to be 830 nM using fluorescein-labeled heparin and rTFPI-C. Heparin enhanced the inhibitory activity of a fragment consisting of the K2 and K3 domains, but it did not stimulate the inhibitory activity of the K2 domain. A synthetic peptide mimicking from Gly^{212} to Phe^{243} in the K3 domain reduced the effect of heparin on the inhibition by rTFPI-C and rTFPI. These results defined the location of HBS-2 in the basic region of the K3 domain between Gly^{212} and Phe^{243}.

Tissue factor pathway inhibitor (TFPI)[1] is a protease inhibitor with three tandem Kunitz-type inhibitory domains (K1, K2, and K3), which inhibits coagulation factor Xa via the K2 domain and the factor VIIa−tissue factor complex via the K1 domain. Thus TFPI blocks the initial steps of the extrinsic coagulation pathway and regulates hemostasis [see reviews (Broze, 1992; Broze et al., 1991; Lindahl et al., 1992; Rapaport, 1991)]. TFPI is present in human plasma as three forms, *i.e.*, an LDL/VLDL-associated form, an HDL-associated form, and a free form (Novotny et al., 1989). Platelets release TFPI upon aggregation (Novotny et al., 1988), but the majority of *in vivo* TFPI is supposed to be associated with the vascular endothelial cells. Interaction of TFPI with heparin has been demonstrated in two ways: (1) the intravenous administration of heparin caused a marked increase of the plasma TFPI concentration within a few minutes. TFPI is possibly associated with proteoglycans on the surface of endothelial cells, and heparin releases surface-bound TFPI by forming a complex (Lindahl et al., 1990; Novotny et al., 1991; Sandset et al., 1988). (2) The tissue factor-mediated clotting time of human plasma was markedly prolonged by heparin (Lindahl et al., 1991; Nordfang et al., 1991; Wun, 1992). These findings indicate that TFPI is one of the plasma heparin-binding inhibitors like antithrombin III, heparin cofactor II, and protein C inhibitor (Pratt et al., 1992).

The heparin-binding site of TFPI (HBS-1) has been postulated to locate in its C-terminal portion, which contains a cluster of basic amino acid residues with a consensus sequence (Novotny et al., 1991). However, TFPI lacking the C-terminal portion (TFPI-C) still bound to a heparin column (Wesselschmidt et al., 1993), though with a weaker affinity than that of full-length TFPI, suggesting the presence of a second heparin-binding site. Wesselschmidt et al. (1993) suggested that, besides HBS-1, the K3 domain was involved in the interaction with heparin. However, direct evidence for the binding of heparin to the K3 domain has not been presented.

In the present study, we found that the inhibitory activity of rTFPI-C was markedly enhanced by heparin and confirmed the presence of HBS-2. HBS-2 was then localized in the basic region of the K3 domain as HBS-2 using each of the three Kunitz domains prepared by the limited cleavage of rTFPI-C and also synthetic peptides mimicking the amino acid sequence of a Kunitz domain. We also characterized the heparin-binding affinity of HBS-2. The functions of HBS-1 and HBS-2 for the inhibition of factor Xa by rTFPI will be discussed. A preliminary result of these studies has been reported elsewhere (Enjyoji et al., 1993).

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[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; CHO, Chinese hamster ovary; HPLC, high-performance liquid chromatography; K1, K2, and K3, the first, the second, and the third Kunitz domains; LMW heparin, low molecular weight heparin; MCA, methylcoumarin-7-amide; MTX, methotrexate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; Pyr, pyroglutamyl; rTFPI, full-length recombinant TFPI; rTFPI-C, carboxyl-terminal part deleted in rTFPI; TFPI, tissue factor pathway inhibitor; UF heparin, unfractionated heparin; Z, carbobenzoxy.

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MATERIALS AND METHODS

Materials were obtained from the following sources: factor Xa from Enzyme Research Laboratories, Inc. (South Bend, IN); human neutrophil elastase from Athens Research and Technology, Inc. (Athens, GA); UF heparin from Novo Nordisk (Copenhagen, Denmark); LMW heparin (M_r 4000−6000, average M_r 5000), phosphatidylserine, and phosphatidylcholine from Sigma Chemical Co. (St. Louis, MO); peptidyl-fluorogenic substrate (Z-Pyr-Gly-Arg-MCA) from Peptide Research Foundation (Osaka, Japan); heparin-5PW column from Tosoh Co. (Tokyo, Japan); Cosmosil $5C_{18}$ from Nacalai Tesque Co. (Kyoto, Japan); μBondasphere C_{18}-300A from Nihon Millipore Ltd. (Tokyo, Japan); Sephadex G-10 and Hitrap phenyl HP from Pharmacia Biotech Inc. (Tokyo, Japan); fluorescein isothiocyanate from Dojindo (Kumamoto, Japan). A monoclonal antibody against monkey recombinant TFPI was prepared as reported elsewhere (Kamei et al., 1994). PCR primers were synthesized using a 340A DNA synthesizer (Applied Biosystems, Foster City, CA).

Expression and Purification of Human Recombinant TFPI. The cDNA for the human TFPI coding region was obtained by PCR using the λgt11 human placental cDNA library (Clonetech Laboratory Inc., Palo Alto, CA) as a template and two primers with a restriction enzyme site for *Sal* I at the 5′ end (GGA TTC TGC AGT CGA CAT GAT TTA CAC AAT GAA GAA AGT A and CTC GAG AAT TCG TCG ACT TAG TAG AAT TAA TGT TAC ATT GC). The nucleotide sequence of TFPI cDNA was confirmed to be identical with that reported previously (Wun et al., 1988). The TFPI cDNA was connected with an expression vector, pCAG.dhfr, downstream of the chicken β actin promoter, which was followed by the SV40 polyadenylation site. This vector was constructed to coexpress dehydrofolate reductase as a selection marker, and it was introduced into DG44 cells, a dehydrofolate reductase-deficient strain of CHO cells, using Lipofectin (GIBCO BRL, New York) according to manufacturer's protocol (Yonemura et al., 1993) and a positive clone was selected in the presence of MTX. One transformant was seeded into Cell Factory (Nunc, Denmark) and cultured in ASF104 medium (Ajinomoto Co., Tokyo, Japan) containing 10% fetal calf serum and 1 mM butyric acid. The culture medium was concentrated to 1/10 of the original volume using a hollow-fiber membrane Amicon H1p30-20 (W. R. Grace & Co., Beverly, MA). From the concentrate, rTFPI and rTFPI-C that was derived from rTFPI by proteolysis during the culture, were isolated by an affinity column conjugated with an anti-monkey TFPI monoclonal antibody and by a Mono S column, as noted in our previous paper (Kamei et al., 1994) and another (Pedersen et al., 1990). Their N-terminal amino acid sequences were identical with that reported for human TFPI (Wun et al., 1988). rTFPI was recognized by an antibody raised against the C-terminal basic region (from Lys^{254} to the carboxyl terminus), whereas rTFPI-C did not react with this antibody. rTFPI and rTFPI-C showed a single band with 47.5 and 42.5 kDa, respectively, on SDS−PAGE in the presence of 2-mercaptoethanol. The amino acid sequences of rTFPI and rTFPI-C were confirmed by sequence analyses of the peptides produced by digestion with lysyl endopeptidase (Nakahara et al., 1994). The results indicated that rTFPI-C ends at Lys^{249}. The amino acid compositions obtained for rTFPI, rTFPI-C, K2−K3 (a fragment consisting of the K2 and K3 domains, obtained by the limited cleavage of rTFPI-C; see text), and K3 (obtained by limited proteolysis of rTFPI-C; see text) agreed with those expected for these molecules (Table 2). Concentration of rTFPI was determined by amino acid analysis. One absorbance unit of rTFPI and rTFPI-C at 280 nm corresponds to 41.6 and 46.4 μM, respectively.

Measurement of Inhibitory Activity of TFPI toward Factor Xa. The inhibition of factor Xa by TFPI was measured using Z-Pyr-Gly-Arg-MCA as a substrate. Three microliters of 10 mM substrate and 10 μL of 0.2 μM TFPI were mixed with 562 μL of the TBSA buffer (20 mM Tris-HCl, pH 8.0, 0.15 M NaCl, and 0.1 mg/mL bovine serum albumin) and preincubated at 37 °C. The reaction was initiated by the addition of 50 μL of 0.02 μM factor Xa. Release of 7-amino-4-methylcoumarin was monitored by measuring fluorescence intensity with excitation at 380 nm and emission at 440 nm using a Hitachi 650-40 fluorescence spectrophotometer. Residual factor Xa activity at 1 min was used to evaluate the inhibitory activity of TFPI. Various concentrations of heparin, synthetic peptides, or 5 mM $CaCl_2$ were also included in the reaction mixture before the addition of factor Xa.

Kinetic Analysis of Inhibition of Factor Xa by rTFPI. As described by Huang et al. (1993), the inhibition of factor Xa by TFPI can be described by a slow-tight binding mechanism:

$$E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{\text{slow}\ k_3}{\rightleftharpoons}} EI^* \qquad (1)$$

where E is enzyme; I, inhibitor; EI, initial encounter complex; and EI*, isomerized tight inhibitor−enzyme complex. The kinetic parameters [initial K_i ($=k_2/k_1$) and final K_i for the overall reaction] for the inhibition can be derived using four parameters (V_s, V_0, k_{obs}, F_0) obtained by curve fitting using eq 2 as described by Morrison and Walsh (1988):

$$F = V_s t + (V_0 - V_s)(1 - e^{-k_{obs}t})/k_{obs} + F_0 \qquad (2)$$

where V_s is velocity at steady state; V_0, initial velocity; F, fluorescence intensity at time t; F_0, fluorescence intensity at time 0; t, time; and k_{obs}, apparent rate constant for the steady-state complex formation.

Various concentrations of rTFPI (980 μL) were mixed with 10 μL of 20 mM fluorogenic peptide substrate for factor Xa, Z-Pyr-Gly-Arg-MCA, in a cuvette thermostated at 37 °C. The reaction was initiated by the addition of 10 μL of 5 nM factor Xa. Final concentrations are 0.5−8 nM for rTFPI, 50 pM for factor Xa, and 200 μM for synthetic fluorogenic substrate in 20 mM Tris-HCl, pH 8.0, containing 0.15 M NaCl and 0.1% BSA. The fluorescence change was monitored, and the data were collected at 60 points/min for 30 min on a Hitachi F-4500 fluorescence spectrophotometer. The curve fitting was performed using a nonlinear regression curve fitting program, Igor (Wave Metrics, Lake Oswego, OR). Consumption of substrate was below 0.5% of the initial substrate concentration in all cases. Heparin (1 unit/mL) and/or $CaCl_2$ (5 mM) was also included in the reaction mixture before the addition of factor Xa if needed.

Isolation of the Three Kunitz Domains of TFPI. TFPI was cleaved at Thr^{87}−Thr^{88} by treatment with human neutrophil elastase as reported by Higuchi et al. (1992) and Petersen et

al. (1992), while hydroxylamine treatment cleaved a peptide bond at Asn^{152}–Gly^{153}. rTFPI-C was treated with human neutrophil elastase according to the method by Higuchi et al. with a slight modification. The digest was diluted 5-fold with the starting buffer (20 mM Tris-HCl, pH 8.0) and then applied to a heparin-5PW column. Elution was performed with a linear gradient of NaCl from 0 to 1.0 M at a flow rate of 1 mL/min. A fragment containing the K1 domain (K1) and another fragment consisting of the K2 and K3 domains (K2–K3) were obtained in the breakthrough fraction and the eluate, respectively. K2–K3 in the eluate was lyophilized, dissolved in 0.5 mL of distilled water, and then treated with hydroxylamine according to the method of Balian et al. (1972). The digest was desalted and then loaded onto a heparin-5PW column. Elution was performed with a linear gradient of NaCl from 0 to 0.8 M at a flow rate of 0.5 mL/min. The fragment containing the K2 domain (K2) was recovered in the breakthrough fraction, whereas the fragment containing the K3 domain (K3) was coeluted with uncleaved K2–K3 in the eluate. Alternatively, rTFPI-C was treated with hydroxylamine, and the digest was separated on a heparin-5PW column. A fragment consisting of the K1 and K2 domains (K1–K2) was isolated in the breakthrough fraction, whereas K3 was recovered from the adsorbed fraction together with uncleaved rTFPI-C. The adsorbed fraction, a mixture of K3 and uncleaved rTFPI-C, was dialyzed against 20 mM Tris-HCl, pH 8.0, containing 50 mM NaCl and was applied to a Mono Q column equilibrated with the same buffer. Elution was performed with a linear gradient of NaCl from 0.05 M to 0.5 at a flow rate of 0.5 mL/min during a period of 180 min. K3 was eluted at 0.12 M NaCl, while rTFPI-C was eluted at 0.23 M NaCl. The amino acid compositions and the N-terminal amino acid sequences of each of the fragments thus isolated (K1, K2, K3, K1–K2 and K2–K3) are shown in Tables 2 and 3. These results indicate that these fragments were obtained by the specific cleavage at two peptide bonds, Thr^{87}–Thr^{88} by elastase and Asn^{152}–Gly^{153} by hydroxylamine.

The concentration of each fragment was determined by amino acid analysis.

Synthesis of Peptides Corresponding to the Third Kunitz Domain of TFPI. Peptides with the amino acid sequences of VPSLFEEHGPS (Val^{177}–Ser^{187}), LTPADRGL (Leu^{190}–Leu^{197}), RANENRFYYNSVIGK (Arg^{199}–Lys^{213}), GKCRPFKYSGAGGNENNFTSKQECLRAAKKGF (Gly^{212}–Phe^{243}), and KTKRKRKKQRVKIAYEEIFVKNM (Lys^{254}–Met^{276}) were synthesized using an Applied Biosystems 430A peptide synthesizer. The cysteine residues, Cys^{222} and Cys^{239} in the peptide Gly^{212}–Phe^{243}, were replaced by alanine. The other two cysteine residues (Cys^{214} and Cys^{235}) in Gly^{212}–Phe^{243} were oxidized to form a disulfide bridge as follows. The peptide was dissolved at 50 μg/mL in 0.05% trifluoroacetic acid, and the pH of the solution was adjusted to 7.5 with NH_4OH. Potassium ferricyanide was added incrementally to the solution until the yellow color remained, and the solution was incubated for 20 min at room temperature with stirring. Then oxidation was terminated by the addition of ascorbic acid, and the peptide was purified by HPLC. The peptide was eluted slightly faster than the untreated peptide on a Cosmosil $5C_{18}$ column. The peptide was found to be free of cysteinyl residues using Ellman's reagent. Its molecular weight was confirmed to be identical with the theoretical value as the oxidized monomer by plasma desorption mass spectrum analysis (BioIon 20, Applied Biosystems, Foster City, CA). In order to examine the effect of secondary structure, the peptide was further reduced and alkylated with methyl iodide according to the method of Rochat et al. (1970) and purified by HPLC using a Cosmosil $5C_{18}$ column. Methylation of the cysteinyl residue was confirmed by sequence analysis of the peptide. The amino acid sequence of the other synthetic peptides was confirmed by sequence analysis and the amino acid composition.

Preparation of Fluorescein-Labeled LMW Heparin. Four hundred fifty milligrams of LMW heparin was incubated with 67.5 mg of fluorescein isothiocyanate in 37 mL of 0.25 M sodium bicarbonate for 6 h at 35 °C. The reaction mixture was loaded to a column of Sephadex G-10 (4.5 cm × 12 cm) equilibrated with 0.1 M sodium phosphate buffer, pH 7.0, containing 1.0 M NaCl. LMW heparin was eluted in a void volume fraction, which was dialyzed against distilled water, lyophilized, and then dissolved in 50 mM sodium phosphate buffer, pH 6.2, containing 3.0 M ammonium sulfate. For separation of fluorescein-labeled LMW heparin from unlabeled LMW heparin, the sample was applied to a column of Hitrap phenyl HP equilibrated with the same buffer. The elution was performed with a linear gradient formed with the equilibration buffer and the same buffer without ammonium sulfate. Fluorescein-labeled LMW heparin was recovered in an adsorbed fraction, whereas unlabeled LMW heparin was found in a breakthrough fraction. The extent of fluorescein incorporated into LMW heparin was calculated to be 0.6 mol/mol of LMW heparin using a sodium fluorescein as a standard. Ten milligrams of fluorescein-labeled LMW heparin was obtained from 450 mg of LMW heparin. The average molecular weights of unlabeled and fluorescein-labeled LMW heparin were calculated to be 5230 and 5310, respectively, according to the method of Campbell (1974).

Analysis of Binding of Fluorescein-Labeled LMW Heparin with rTFPI-C. rTFPI-C was incubated with fluorescein-labeled LMW heparin in 200 μL of 0.15 M NaCl and 20 mM Tris-HCl, pH 8.0, containing 0.1 mg/mL BSA for 1 h at 25 °C. The change of fluorescence was measured in the quartz cuvette thermostated at 25 °C with excitation at 494 nm and emission at 505 nm using a Hitachi fluorescence spectrophotometer F4500.

Other Techniques. Amino acid composition and amino acid sequence analyses were performed as reported previously (Maeda et al., 1988). The nucleotide sequence was determined by the dideoxy chain termination method using a Sequenase version 2.0 sequencing kit (United States Biochemicals, Cleveland, OH). HPLC was carried out using a Waters LC 625 system. SDS–PAGE was performed using 8–25% polyacrylamide gel and a Phast System (Pharmacia LKB Biotechnology, Uppsala, Sweden) according to the manufacturer's protocol or using MINI-PROTEAN II ready gel containing 10–20% polyacrylamide (Bio-Rad Laboratories).

RESULTS

Factor Xa Inhibition by TFPI in the Presence of Heparin. Although UF heparin has been mainly used to enhance the inhibitory activity of TFPI, LMW heparin was used for the binding analysis of heparin with TFPI as described in the later section. Thus, we used both types of heparin to examine

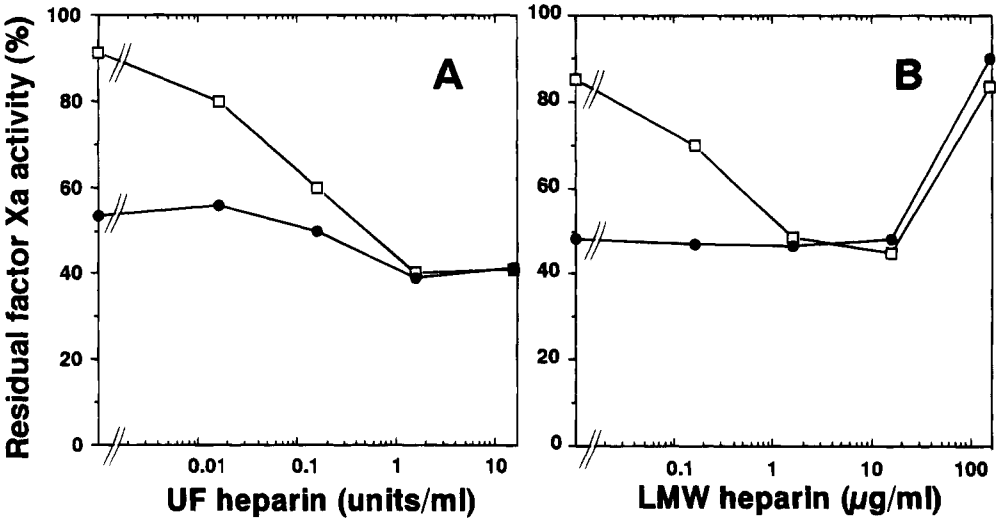

FIGURE 1: Inhibition of factor Xa by rTFPI or rTFPI-C in the presence of UF or LMW heparin. Three microliters of 10 mM substrate (Z-Pyr-Gly-Arg-MCA) and 10 μL of 0.2 μM TFPI were mixed with 562 μL of TBSA buffer (20 mM Tris-HCl, pH 8.0, containing 0.1 mg/mL bovine serum albumin and 0.15 M NaCl). The reaction was initiated by the addition of 50 μL of 0.02 μM factor Xa. Various concentrations of UF or LMW heparin were also included in the reaction mixture. The residual factor Xa activity was measured as described under Materials and Methods. Symbols: ●, rTFPI; □, rTFPI-C. Panels: (A) UF heparin; (B) LMW heparin.

Table 1: Comparison of the Kinetic Parameters for the Inhibition of Factor Xa by rTFPI and rTFPI-C

	rTFPI-C[a]				rTFPI[b]		
		Hep	Ca^{2+}	Ca^{2+}/Hep		Ca^{2+}	Ca^{2+}/Hep
K_i (nM)	6.79	1.79	22.3	2.68	1.24	42.7	3.13
K_i^* (pM)	110	162	48.1	39.6	26.4	85.2	30.9
k_3 (min^{-1})	0.970	0.936	2.00	2.86	0.741	2.50	3.077
k_4 (min^{-1})	0.0193	0.0767	0.0055	0.0483	0.0161	0.0050	0.0307

[a] Values indicate the mean values from two or three independent experiments. [b] Kinetic parameters for rTFPI were taken from Huang et al. (1993). 5 mM $CaCl_2$ and/or 1 unit/mL UF heparin in rTFPI-C or 10 units/mL UF heparin in rTFPI was included in the reaction mixture if needed.

the effect on the inhibitory activity of TFPI. Figure 1 shows the effect of UF heparin and LMW heparin on the inhibitory activity of rTFPI and rTFPI-C in the absence of Ca^{2+}. In these experiments, factor Xa was incubated with rTFPI or rTFPI-C in the presence of varying concentrations of heparin. After incubation of 1 min, the factor Xa substrate was added to measure the residual factor Xa activity as described in Materials and Methods, since the difference between rTFPI and rTFPI-C activity and the effect of heparin on the inhibition of factor Xa were not observed if rTFPI or rTFPI-C was incubated with factor Xa for long time (>60 min). This observation was supported by the kinetic analysis as described later. These results show that rTFPI-C has a weaker activity than rTFPI in the absence of heparin and are consistent with the previous report by Wesselschmidt et al. (1992). The inhibitory activity of rTFPI-C was significantly enhanced by UF heparin or LMW heparin over a range from 0.017 to 17 units/mL or 0.17 to 17 μg/mL with a broad optimal concentration from 1.7 to 17 units/mL or from 1.7 to 17 μg/mL, respectively. At the optimal heparin concentration, the inhibitory activity of rTFPI-C became comparable to that of rTFPI. On the other hand, the inhibition of factor Xa by rTFPI was not significantly enhanced by UF heparin or LMW heparin. Higher concentrations of LMW heparin decreased the inhibitory activity of both rTFPI and rTFPI-C.

In the presence of Ca^{2+}, the inhibitory activity of rTFPI was decreased, and 80% of the activity was lost at 2.6 mM $CaCl_2$ (physiological concentration) (data not shown). Wesselschmidt et al. (1993) also reported that the inhibitory activity of rTFPI was reduced almost to the same level as that of rTFPI-C in the presence of 5 mM $CaCl_2$, but it was restored with heparin in a similar manner as shown above.

Although the C-terminal basic region of TFPI is thought to be a heparin-binding site (HBS-1), the inhibitory activity of rTFPI-C was enhanced by heparin. As shown in Table 1, in the absence of heparin and Ca^{2+}, kinetic parameters for the inhibition of factor Xa by rTFPI-C were determined to be 6.79 ± 0.82 nM for an initial Ki and 110 ± 9 pM for a final K_i. In the presence of heparin, the initial K_i for the inhibition of factor Xa by rTFPI-C was decreased to 1.79 ± 0.08 nM, while the final K_i was slightly increased. Heparin also decreased an initial K_i for the inhibition of factor Xa from 22.3 ± 3.3 to 2.68 ± 0.86 nM without significant change in a final K_i in the presence of 5 mM $CaCl_2$. We compared the kinetic parameters of rTFPI-C with those of rTFPI which were reported by Huang et al. (1993). As shown in Table 1, in the absence of both calcium and heparin, rTFPI inhibited factor Xa more efficiently than rTFPI-C. However, in the presence of 5 mM $CaCl_2$, the inhibition of factor Xa by rTFPI and rTFPI-C showed the quite similar kinetic parameters in the absence and the presence of heparin, respectively. These results indicate that another heparin-binding site (HBS-2), which is also involved in the acceleration of inhibitory activity of TFPI by heparin, is present in TFPI and that heparin enhances the inhibition by rTFPI-C by stabilizing the formation of the initial encounter complex rather than the final steady-state enzyme−inhibitor complex.

Identification of the Third Kunitz Domain of TFPI as the Other Heparin-Binding Site (HBS-2). In order to localize

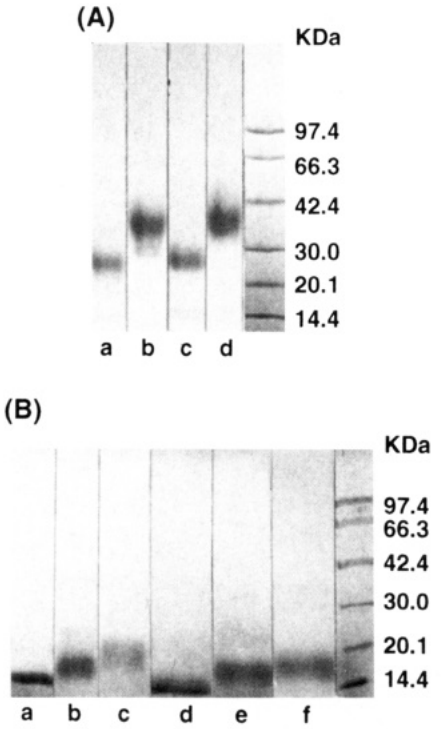

FIGURE 2: SDS−PAGE of fragments containing each Kunitz domain of rTFPI-C. Samples were subjected to SDS−PAGE using 8−25% (A) or 10−20% (B) polyacrylamide gel with a Laemmli buffer system followed by Coomassie brilliant blue staining as described under Materials and Methods. Panels: (A) a, K1−K2; b, K2−K3; c and d, reduced forms of lanes a and b, respectively; (B) a, K1; b, K2; c, K3; d−f, reduced forms of lanes a−c, respectively.

HBS-2 in rTFPI-C, we isolated each Kunitz domain of rTFPI-C as described in Materials and Methods and evaluated the factor Xa inhibitory activity and heparin-binding activity of each domain. As shown in Figure 2, K1−K2, K2−K3, and each isolated domain (K1, K2, and K3) showed a single band on SDS−PAGE. As summarized in Tables 2 and 3, these domains were identified by analysis of their amino acid compositions and N-terminal amino acid sequences. As shown in Figures 3C and 4 (open triangles), K1 did not bind to the heparin column equilibrated with 20 mM Tris-HCl, pH 8.0, and did not show significant inhibitory activity against factor Xa even at a concentration of 171-fold of rTFPI-C used in this assay. On the other hand, K2−K3 was retained on the heparin column and was eluted almost at the same NaCl concentration as TFPI-C (Figure 3B). K2−K3 also retained the inhibitory activity toward factor Xa, and the inhibition was enhanced by heparin (Figure 4, closed circles). These results indicate the presence of HBS-2 in K2−K3. As shown in Figures 3D and 4 (closed triangles), K2 was not retained on the heparin column and inhibited factor Xa but lost its heparin-dependent inhibitory activity for factor Xa. On the other hand, K3 was retained on the heparin column as shown in Figure 3E. As shown in Figure 3A, K1−K2 was not adsorbed on the heparin column. K1−K2 showed factor Xa inhibitory activity, but this inhibitory activity was not enhanced by heparin (open squares in Figure 4). These results clearly indicate that HBS-2 is present in the K3 domain but not in K1−K2 which also includes the amino-terminal region of TFPI.

In order to assign HBS-2 in the K3 domain, we synthesized four peptides, Val^{177}−Ser^{187}, Leu^{190}−Leu^{197}, Arg^{199}−Lys^{213}, and Gly^{212}−Phe^{243}, corresponding to various regions of K3 and examined the effect of these peptides on the LMW heparin-dependent enhancement of factor Xa inhibition by TFPI-C. As shown in Figure 5A, only Gly^{212}−Phe^{243} could abolish enhancement of the inhibitory activity of rTFPI-C by LMW heparin. Ninety-five percent of the enhancement caused by 0.33 μM LMW heparin was abolished in the presence of 26.7 μM the peptide. Since this peptide was also retained on the heparin column and was eluted with 0.5 M NaCl, this peptide probably competed with HBS-2 of rTFPI-C for the binding to LMW heparin. Reduced and methylated Gly^{212}−Phe^{243} did not abolish the LMW heparin-enhanced inhibitory activity of TFPI even at a maximum concentration (26.7 μM). These results indicate that a segment of Gly^{212}−Phe^{243} in the K3 domain is HBS-2 and that the binding requires a specific conformation formed by a disulfide bond and/or other secondary interactions. Since the inhibition of factor Xa by rTFPI was enhanced by heparin in the presence of Ca^{2+} (Huang et al., 1993), we examined the effect of this peptide on the inhibition by rTFPI in the presence of Ca^{2+} and heparin. Gly^{212}−Phe^{243} also reduced the heparin-enhanced inhibition of rTFPI in the presence of Ca^{2+} as shown in Figure 5B.

Binding of Fluorescein-Labeled LMW Heparin with rTFPI-C. Though one tryptophan residue (Trp^{188}) is present in rTFPI, it did not exhibit intrinsic fluorescence probably due to the presence of a cysteine residue next to the tryptophan. Therefore, the binding constant of rTFPI-C with LMW heparin was measured using fluorescein-labeled LMW heparin. As shown in Figure 6A, the peak of the emission spectrum of fluorescein-labeled LMW heparin was shifted to approximately 520 nm from 515 nm by the binding with rTFPI-C, and the shift reached a plateau at 16 μM TFPI. The change of the emission spectrum of fluorescein-labeled LMW heparin was measured quantitatively by monitoring the fluorescence intensity at 505 nm as shown in Figure 6B. This fluorescence quenching was canceled in a concentration-dependent manner by the addition of unlabeled LMW heparin as shown in Figure 7. The change of fluorescence quenching was proportional to the percentage of fluorescein-labeled LMW heparin to total LMW heparin; that is, the 50% substitution of fluorescein-labeled LMW heparin with unlabeled LMW heparin canceled 50% of fluorescence quenching. This result indicates that the affinity of LMW heparin to rTFPI-C was not altered by labeling of LMW heparin with fluorescein. Then we determined the kinetic parameters of the binding of LMW heparin to rTFPI-C using fluorescein-labeled LMW heparin. As shown in Figure 8, the fluorescence intensity was decreased by the addition of rTFPI-C, and the quenching reached a plateau at approximately 12 μM fluorescein-labeled LMW heparin. From the plot of the [bound fluorescein-labeled LMW heparin]/[free fluorescein-labeled LMW heparin] *versus* [bound fluorescein-labeled LMW heparin] (Figure 8, inset), rTFPI-C was determined to bind fluorescein-labeled LMW heparin at 1:1 stoichiometry ([*X* intercept]/[rTFPI-C]) with a single affinity of K_d = 830 nM (4.2 μg/mL, r = 0.93). This value is in good agreement with the range of LMW heparin concentration required for the acceleration of factor Xa inhibition by rTFPI-C as shown in Figure 1B. The value is comparable with those for the interaction of heparin with thrombin (8.0×10^{-7} M) (Jordan et al., 1980), factor IXa (2.6×10^{-7} M) (Jordan et al., 1980), factor Xa (8.7×10^{-6} M) (Jordan et

Table 2: Amino Acid Compositions of rTFPI and rTFPI-C, as Well as the Individual Domains and Their Fragments from rTFPI-C[a]

	residues/mol						
	K1	K2	K3	K1−K2	K2−K3	rTFPI-C	rTFPI
Asp	11.0 (11)	10.0 (10)	12.0 (12)	21.0 (21)	22.0 (22)	33.0 (33)	34.0 (34)
Thr	5.9 (6)	3.9 (4)	5.5 (5)	9.9 (10)	8.6 (9)	14.5 (15)	14.9 (16)
Ser	2.9 (3)		8.2 (8)	3.8 (3)	8.6 (7)	10.4 (11)	11.0 (11)
Glu	12.6 (13)	12.1 (12)	8.9 (9)	25.4 (25)	21.4 (21)	33.9 (34)	37.2 (37)
Pro	3.6 (3)	2.8 (3)	5.1 (5)	5.7 (6)	8.3 (8)	10.6 (11)	11.1 (11)
Gly	4.5 (4)	6.2 (6)	9.9 (9)	10.1 (10)	15.4 (15)	18.8 (19)	20.9 (21)
Ala	4.1 (4)		4.1 (4)	4.2 (4)	4.0 (4)	7.8 (8)	9.6 (9)
Val			3.6 (4)		3.6 (4)	3.7 (4)	5.9 (6)
Met	2.8 (3)	1.0 (1)		3.9 (4)	1.1 (1)	3.9 (4)	4.7 (5)
Ile	6.8 (7)	2.7 (3)	2.9 (3)	7.3 (10)	5.0 (5)	10.5 (13)	13.3 (16)
Leu	3.6 (4)	3.9 (4)	6.2 (6)	7.6 (8)	9.7 (10)	13.2 (14)	15.1 (15)
Tyr	1.2 (1)	3.7 (4)	3.9 (4)	4.7 (5)	7.3 (8)	8.1 (9)	9.4 (10)
Phe	8.2 (8)	4.9 (5)	7.3 (7)	11.3 (13)	11.6 (12)	18.8 (20)	19.8 (21)
Lys	6.9 (7)	3.8 (4)	6.8 (7)	9.6 (11)	10.9 (10)	16.0 (18)	23.7 (25)
His	1.9 (2)		1.0 (1)	2.1 (2)	1.1 (1)	2.8 (3)	2.8 (3)
Arg	5.5 (5)	3.2 (3)	6.5 (6)	8.6 (8)	9.4 (9)	14.0 (14)	18.2 (17)
Cys	(6)	(6)	(6)	(12)	(12)	(18)	(18)
Trp			(1)		(1)	(1)	(1)
total	(87)	(65)	(97)	(152)	(162)	(249)	(276)
position[b]	1−87	88−152	153−249	1−152	88−249	1−249	1−276

[a] Hydrolyzed in evacuated, sealed tubes at 110 °C for 20 h with 5.7 N HCl. Tryptophan and cysteine were not calculated. Numbers in parentheses represent the theoretical values calculated from the amino acid sequences (Wun et al., 1988). [b] Amino acid residue number was taken from Wun et al., (1988).

Table 3: Amino Acid Sequences of the Amino-Terminal Portion of Each Kunitz Domain and Their Fragments from rTFPI-C[a]

	K1		K2		K3		K1−K2		K2−K3	
cycle	PTH AA	yield	PTH AA	yield	PTH AA	yield	PTH AA	yield	PTH AA	yield
1	Asp	93.2	Thr	586.5	Gly	171.4	Asp	28.7	Thr	89.0
2	X		Leu	893.8	Phe	269.4	Ser	6.6	Leu	245.7
3	Glu	110.2	Gln	736.6	Gln	139.3	Glu	73.6	Gln	125.3
4	Glu	172.0	Gln	865.8	Val	277.5	Glu	76.7	Gln	134.9
5	Asp	76.1	Glu	597.4	Asp	76.6	Asp	28.6	Glu	101.9
6	Glu	124.0	Lys	530.7	Asn	100.6	Glu	52.0	Lys	116.3
7	Glu	150.7	Pro	540.4	Tyr	218.2	Glu	75.4	Pro	126.2
8	His	25.4	Asp	264.5	Gly	139.7	His	13.5	Asp	59.7
9	Thr	47.7	Phe	451.9	Thr	20.4	Thr	19.2	Phe	154.6
10	Ile	115.9	X		Gln	90.3	Ile	69.8	X	
11	Ile	177.9	Phe	483.3	Leu	191.8	Ile	69.8	Phe	156.5
12	Thr	48.0	Leu	319.5	Asn	75.7	Thr	19.5	Leu	143.5
13	Asp	50.0	Glu	235.9	Ala	154.9	Asp	10.6	Glu	91.8
14	Thr	39.6	Glu	429.6	Val	182.1	X		Glu	75.6
15	Glu	47.3	Asp	154.8	X		Glu	31.9	Asp	56.1
16	Leu	82.0	Pro	205.6	Asn	69.4	Leu	39.1	Pro	87.0
17	Pro	57.6	Gly	260.0	Ser	9.7	Pro	45.1	Gly	114.8
18	Pro	80.7	Ile	190.8	Leu	116.7	Pro	65.5	Ile	108.5
19	Leu	60.8	X		Thr	30.1	Leu	39.8	X	
20			Arg	77.1	Pro	73.0	Lys	30.8	Arg	43.2
21			Gly	230.8	Gln	35.8	Leu	49.1	Gly	110.6
22			Tyr	124.2	Ser	31.1	Met	17.9	Tyr	82.5
23			Ile	128.4	X		His	5.1	Ile	87.2
24			Thr	53.3	Lys	62.2	Ser	1.0	Thr	24.3

[a] X: not identified. Yield of PTH amino acid was expressed in picomoles.

al., 1980), or fibronectin ($1.18-2.17 \times 10^{-7}$ M) (Benecky et al., 1988).

DISCUSSION

The heparin-binding domain of heparin-binding proteins can be classified into two different types, one featuring a cluster of basic amino acid residues with the consensus heparin-binding sequence such as heparin cofactor II and protein C inhibitor (Cardin et al., 1989) and the other having a unique conformation that interacts with heparin as seen with antithrombin III (Evans et al., 1992). TFPI is a new heparin-binding plasma protease inhibitor, and its binding site was postulated to be its C-terminal basic portion (HBS-1), which has a cluster of basic amino acid residues as seen in the heparin-binding proteins of the first type. rTFPI-C that lacks the first-type binding site had a lower affinity to a heparin column than the full-length TFPI (rTFPI), and the presence of another heparin-binding domain (HBS-2) was demonstrated by the present study. We prepared fragments consisting of each Kunitz domain (K1, K2, and K3) by the specific cleavages of rTFPI-C and studied the relationships of factor Xa inhibitory activity and the affinity to heparin (Table 4). Only the K3 domain and a fragment containing K3 had as strong affinity to the heparin column as rTFPI-C. The factor Xa inhibitory activity of the K2−K3 fragment was potentiated by heparin, whereas the K2 domain did not

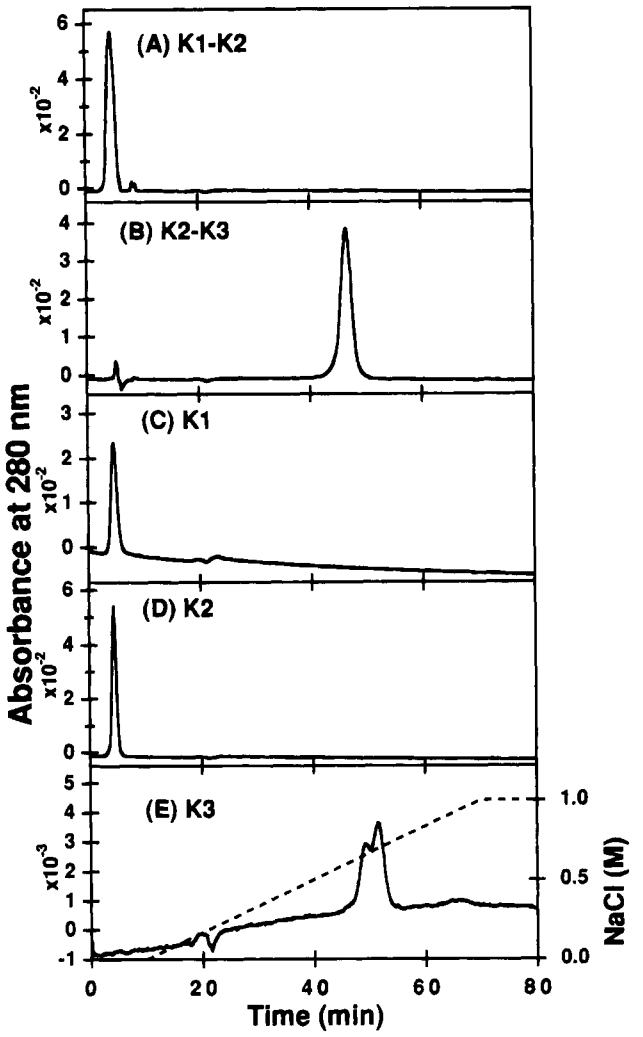

FIGURE 3: Binding of fragments containing each Kunitz domain from rTFPI-C to heparin-5PW. Samples were dialyzed against 20 mM Tris-HCl, pH 8.0, and then loaded to a heparin-5PW column equilibrated with the same buffer. After the column was washed with the same buffer, the elution was performed with a linear salt gradient of NaCl from 0 to 1.0 M during a period of 60 min at a flow rate of 0.5 mL/min using a Waters HPLC system LC625. Panels: (A) K1−K2; (B) K2−K3; (C) K1; (D) K2; (E) K3.

Table 4: Summary of Heparin-Binding Activity and Factor Xa Inhibitory Activity of rTFPI, rTFPI-C, and Fragments Derived from rTFPI-C

fragments	affinity to heparin[a] (M)	factor Xa inhibitory activity	enhancement of factor Xa inhibition by heparin
rTFPI	0.8	+	−[b]
rTFPI-C	0.45	+	+
K1−K2	na[c]	+	−
K2−K3	0.55	+	+
K1	na	−	−
K2	na	+	−
K3	0.55	nt[d]	nt
Gly^{212}−Phe^{243}	0.5	−	−

[a] The affinity to heparin was expressed as the NaCl concentration required for the elution on the column of heparin-5PW. [b] The inhibitory activity was enhanced by heparin in the presence of 5 mM Ca^{2+} but not in the absence of Ca^{2+}. [c] na, not adsorbed. [d] nt, not tested.

show affinity to heparin and its inhibitory activity was not enhanced by heparin. Therefore, HBS-2 was identified in the K3 domain. rTFPI-C required an almost 10-fold higher concentration of heparin for the optimal inhibition than K2−K3, as shown in Figure 4A. Wesselschmidt et al. reported that the N-terminal acidic region of TFPI forms a salt bridge with the C-terminal basic region using various recombinant TFPI mutants (Wesselschmidt et al., 1993). This mechanism also might be applied to rTFPI-C, in which the N-terminal acidic region may interact with the cluster of basic amino acids in K3 instead of the C-terminal basic region of TFPI; rTFPI-C required the higher concentration of heparin for the optimal inhibition to disrupt the salt bridge formed between the amino-terminal acidic region and the basic region in the K3 domain.

A synthetic peptide (Gly^{212}−Phe^{243}) that mimicked a part of the K3 domain had a strong affinity to the heparin column and completely abolished the enhancement of factor Xa inhibitory activity of rTFPI-C by heparin (Figure 5A). The synthetic peptide lost the function after disruption of a disulfide bond. These results indicate that HBS-2 is localized in a region (Gly^{212}−Phe^{243}) of the K3 domain, and a conformation assisted by a disulfide bond in this region is required. HBS-2 has a basic nature, since it has seven basic and two acidic amino acid residues, though these basic residues are not presented in the consecutive position. Superimposition of the basic amino acid residues of the K1, K2, and K3 domains on the tertiary structure of bovine pancreatic trypsin inhibitor (Wlodawer et al., 1987) suggests that heparin binds to these basic amino acid residues clustered by the specific conformation of K3 as illustrated in Figure 9 (indicated by a stippled circle), as has been indicated for the interaction of heparin with antithrombin III (Evans et al., 1992). Two acidic amino acid residues are present in this region as indicated in light blue. In contrast, the cluster of basic amino acid residues is not seen in the K1 and K2 while acidic amino acid residues in the K1 and K2 domains are relatively abundant compared with K3. The basic amino acid residues in the K3 were also highly conserved in rat (Enjyoji et al., 1992), rabbit (Belaaouaj et al., 1993;Warn-Cramer et al., 1992), Rhesus monkey (Kamei et al., 1994), and canine TFPI (Girard et al., 1994).

The fact that an excess of heparin reduced the inhibitory activities of rTFPI and rTFPI-C supports the speculation that heparin interacted with both TFPI and factor Xa, as occurs in the interaction of heparin with the antithrombin III−thrombin complex. However, enhancement of the inhibitory activity by heparin probably is not due to a conformational change of TFPI itself because the inhibitory activity of rTFPI or rTFPI-C against trypsin was not enhanced by heparin (Broze et al., 1990) and the CD spectrum for rTFPI or rTFPI-C also did not change in the presence of heparin (Enjyoji et al., unpublished data). From these results, we speculate that heparin simply increases the local concentration of factor Xa and rTFPI or rTFPI-C on the surface and stabilizes the initial complex formed on heparin. However, the possibility that rTFPI-C is in a less active conformation than rTFPI and heparin merely restores the inhibitory activity of rTFPI-C by changing the conformation cannot be completely excluded. Nonetheless, the mechanism of the heparin interaction with TFPI is different from that with antithrombin III. A conformational change of antithrombin III has been observed by the CD spectrum analysis following the addition of heparin (Evans et al., 1992).

As reported by Wesselschmidt et al. (1993), and also in this study, deletion of the C-terminal portion of TFPI reduced the inhibitory activity for factor Xa. The inhibitory activity

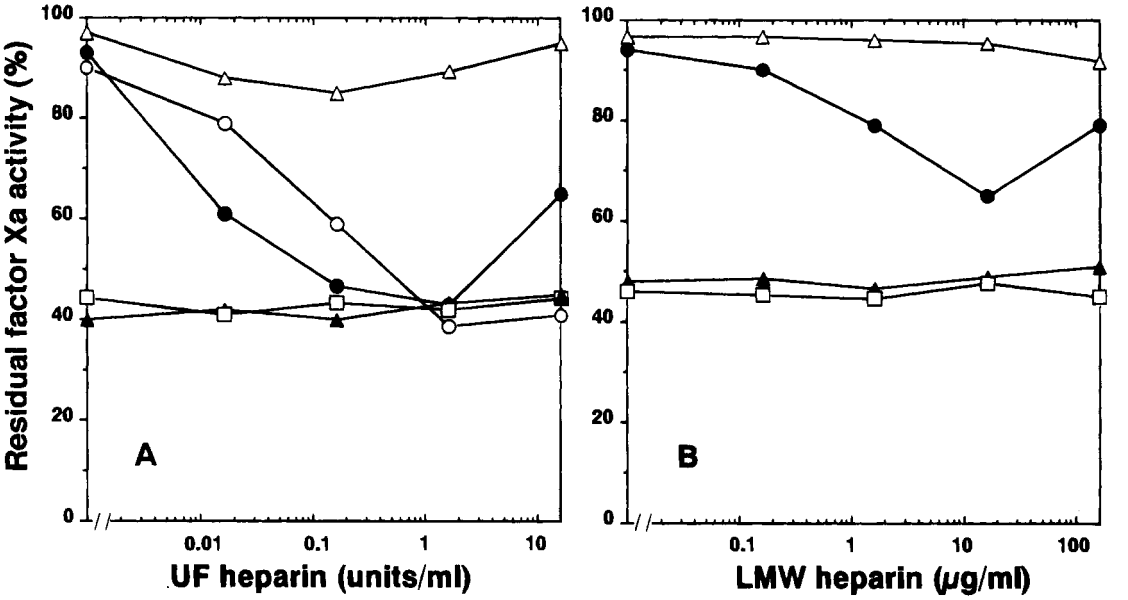

FIGURE 4: Inhibition of factor Xa by fragments containing each Kunitz domain derived from rTFPI-C in the presence of UF heparin or LMW heparin. Inhibition of factor Xa by various fragments of TFPI-C was examined as described under Materials and Methods. Symbols: ○, TFPI-C (3.2 nM); □, K1−K2 (35.5 nM); ●, K2−K3 (2.9 nM); △, K1 [550 nM (A) or 275 nM (B)]; ▲, K2 [111 nM (A) or 74.2 nM (B)]. Panels: (A) UF heparin; (B) LMW heparin.

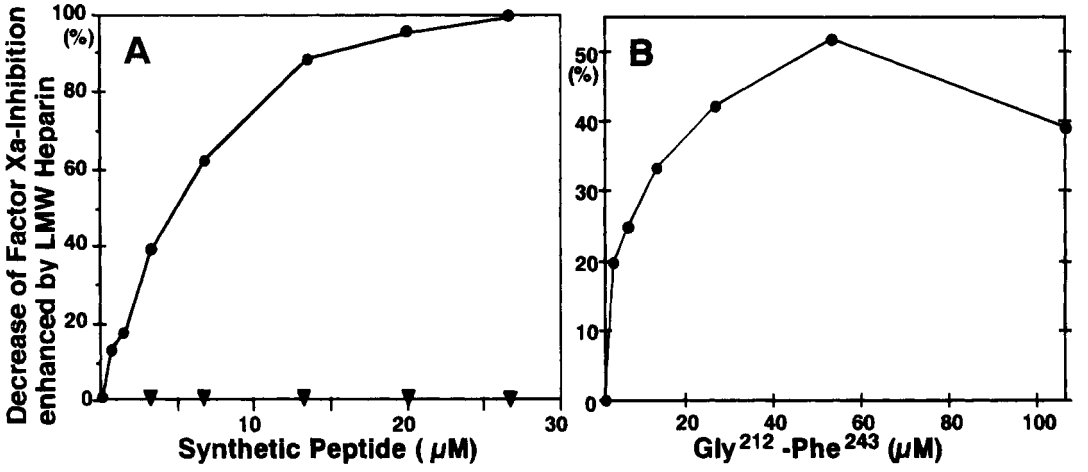

FIGURE 5: Effect of synthetic peptides corresponding to the K3 domain of TFPI on the heparin-enhanced inhibition of factor Xa by rTFPI-C or rTFPI. (A) Effect of synthetic peptides on the heparin-enhanced inhibition of factor Xa by rTFPI-C. Various concentrations of the synthetic peptide (10 μL) were added to a reaction mixture consisting of 10 μL of 20 μM LMW heparin, 10 μL of 0.2 μM rTFPI-C, 3 μL of 10 mM Z-Pyr-Gly-Arg-MCA, and 527 μL of TBSA buffer. The reaction was initiated by the addition of 50 μL of 0.02 μM factor Xa. (B) Effect of Gly^{212}−Phe^{243} on the heparin-enhanced inhibition of factor Xa by rTFPI in the presence of Ca^{2+}. A peptide with two cysteine residues, Gly^{212}−Phe^{243}, was synthesized and oxidized to make a disulfide bond as described under Materials and Methods. Various concentrations of Gly^{212}−Phe^{243} (10 μL) were added to a reaction mixture consisting of 10 μL of 2 μM LMW heparin, 10 μL of 0.2 μM rTFPI, 6 μL of 0.25 M $CaCl_2$, 3 μL of 10 mM Z-Pyr-Gly-Arg-MCA, and 541 μL of TBSA buffer. The reaction was initiated by the addition of 20 μL of 0.05 μM factor Xa. The difference in the inhibitory activity of rTFPI-C or rTFPI between the presence and absence of LMW heparin was defined as 100%. The ordinate shows the percent decrease of inhibition caused by the synthetic peptides in the presence of LMW heparin. Symbols: ●, Gly^{212}−Phe^{243} (oxidized); ▼, Val^{177}−Ser^{187}, Leu^{190}−Leu^{197}, Arg^{199}−Lys^{213}, or reduced and methylated Gly^{212}−Phe^{243}.

of rTFPI was also decreased when Gla-domain-less factor Xa was used, instead of intact factor Xa, as reported by Broze et al. (1988) and Wesselschmidt et al. (1993). The inhibition by rTFPI-C was enhanced by heparin whether Ca^{2+} is present or not, in which heparin stabilized the initial encounter complex as indicated by the initial K_i in Table 1. The inhibition of Gla-less factor Xa by rTFPI or rTFPI-C was enhanced by heparin in a similar manner (unpublished data). Thus, the enhancement of the inhibitory activity by heparin seems to be independent of the structure of the Gla domain of factor Xa. The binding of calcium to the Gla region is supposed to be important to prevent the binding of the C-terminal basic region of TFPI to the Gla region of factor Xa. These results also indicate that HBS-1 interacts directly with the Gla domain of factor Xa. In the presence of Ca^{2+}, the inhibitory activity of rTFPI was decreased but restored by heparin in a concentration dependency similar to that observed with rTFPI-C. This observation is consistent with the kinetic analysis by Huang et al. (1993) as shown in Table 1, and it suggests that, in the presence of calcium, heparin enhances the inhibitory activities of rTFPI-C and rTFPI by the same mechanism. The interaction of the Gla domain of factor Xa with HBS-1 is probably so strong that heparin cannot enhance the inhibitory activity when rTFPI and factor

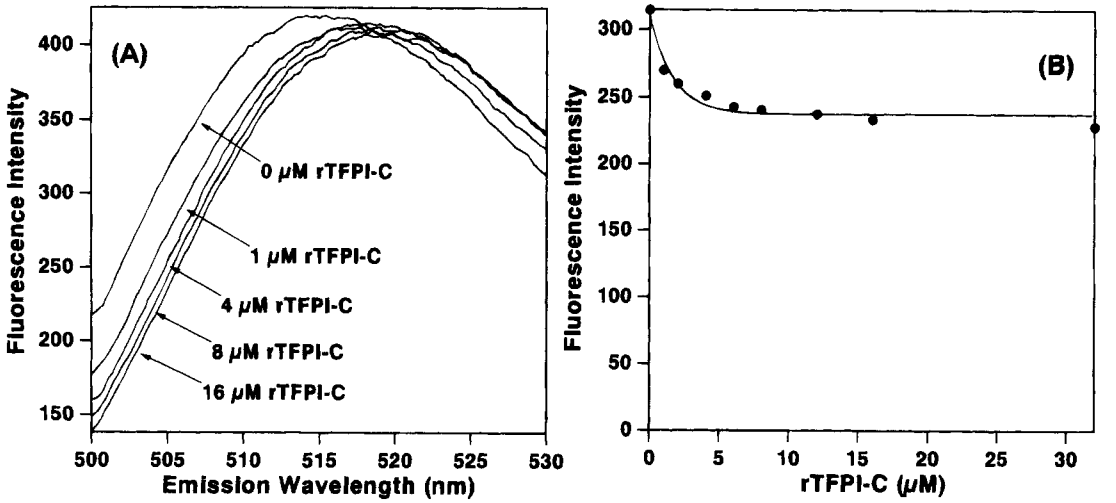

FIGURE 6: Fluorescence change of fluorescein-labeled LMW heparin by rTFPI-C. (A) Fluorescein-labeled LMW heparin (0.5 μM) was incubated with various concentrations of rTFPI-C in TBSA buffer at 25 °C for 1 h. The fluorescence intensity of fluorescein-labeled LMW heparin was monitored by scanning from 500 to 530 nm at 60 nm/min with an excitation at 494 nm. (B) The change of fluorescence intensity at 505 nm in (A) was replotted against the concentration of rTFPI-C added.

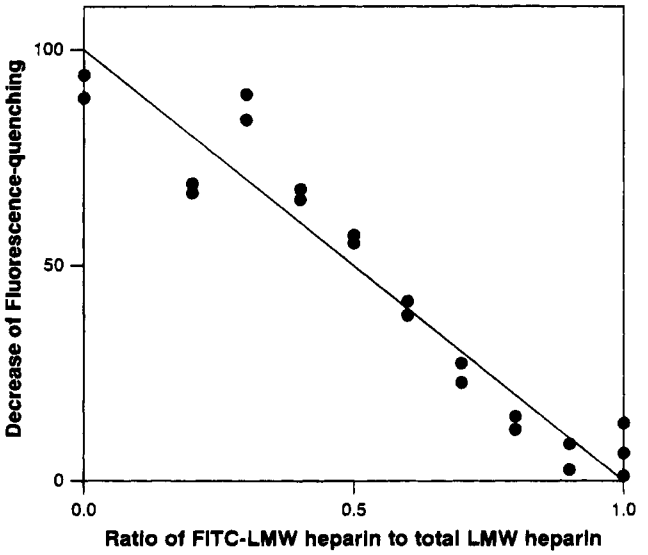

FIGURE 7: Competitive binding of fluorescein-labeled LMW heparin and unlabeled LMW heparin to rTFPI-C. One micromolar rTFPI-C was incubated with 10 μM LMW heparin as described in the legend to Figure 6. Fluorescein-labeled LMW heparin was substituted with unlabeled LMW heparin at the percentage indicated. The fluorescence intensity was measured as described under Materials and Methods. The degree of the cancellation of fluorescence quenching by the addition of unlabeled LMW heparin was calculated after the normalization, in which the raw fluorescence intensity was divided by the ratio of fluorescein-labeled LMW heparin to total LMW heparin so as to compare the fluorescence quenching derived from the same concentration of fluorescein-labeled LMW heparin.

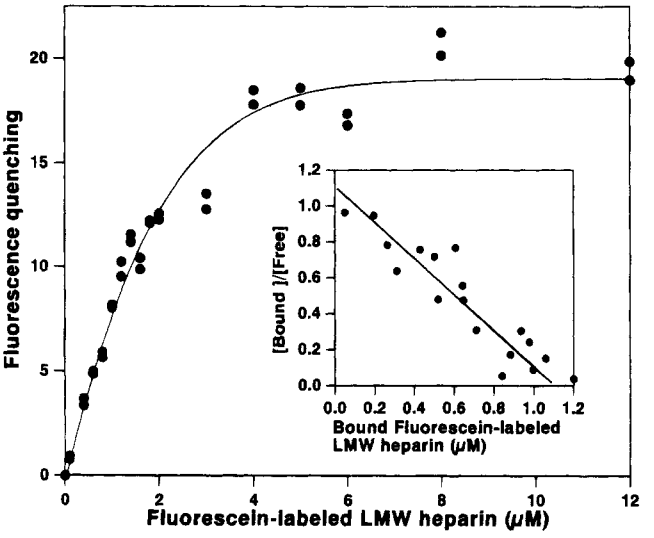

FIGURE 8: Direct binding of rTFPI-C with fluorescein-labeled LMW heparin. One micromolar rTFPI-C was incubated with various concentrations of fluorescein-labeled LMW heparin as described in the legend to Figure 6. The fluorescence intensity was measured as described under Materials and Methods. Inset: the amount of fluorescein-labeled LMW heparin bound with rTFPI-C was estimated from the fluorescence change of fluorescein-labeled LMW heparin on the basis of the maximum fluorescence change obtained with 16 μM fluorescein-labeled LMW heparin. The constant for the binding of TFPI-C to fluorescein-labeled LMW heparin was calculated by the method of Scatchard (1949).

Xa were used. However, when either of these portions is not present or when Ca^{2+} is included in the reaction, the interaction of the two molecules becomes weaker, and therefore heparin could accelerate the reaction. In this case, heparin may enhance the interaction between TFPI and factor Xa possibly through HBS-2 (a part of the K3 domain of TFPI) and some part of factor Xa. Huang et al. (1993) recently reported that Ca^{2+} prevented the formation of a stable initial encounter complex formed with factor Xa and rTFPI and that heparin improved the interaction of factor Xa with TFPI by concentrating TFPI and factor Xa onto the heparin, as shown in Table 1. These results indicate that, in the presence of Ca^{2+}, the interaction of HBS-1 of TFPI with the Gla domain of factor Xa diminishes and that heparin accelerates the interaction of TFPI with factor Xa through HBS-2. The binding of Ca^{2+} to the Gla region is important to prevent the binding of HBS-1 to the Gla region of factor Xa. If Ca^{2+} binds to the Gla region of factor Xa, the interaction of factor Xa with rTFPI would be achieved only by the interaction between the active site of factor Xa and the reactive site of rTFPI as in the case of TFPI-C. Thus, the binding affinity with heparin is similar for the rTFPI-C and rTFPI in the presence of 5 mM $CaCl_2$, as shown in Table 1. In the present study, we demonstrated that the effect of heparin on the inhibitory activity of rTFPI in the presence of Ca^{2+} was also decreased by the addition of Gly^{212}—Phe^{243},

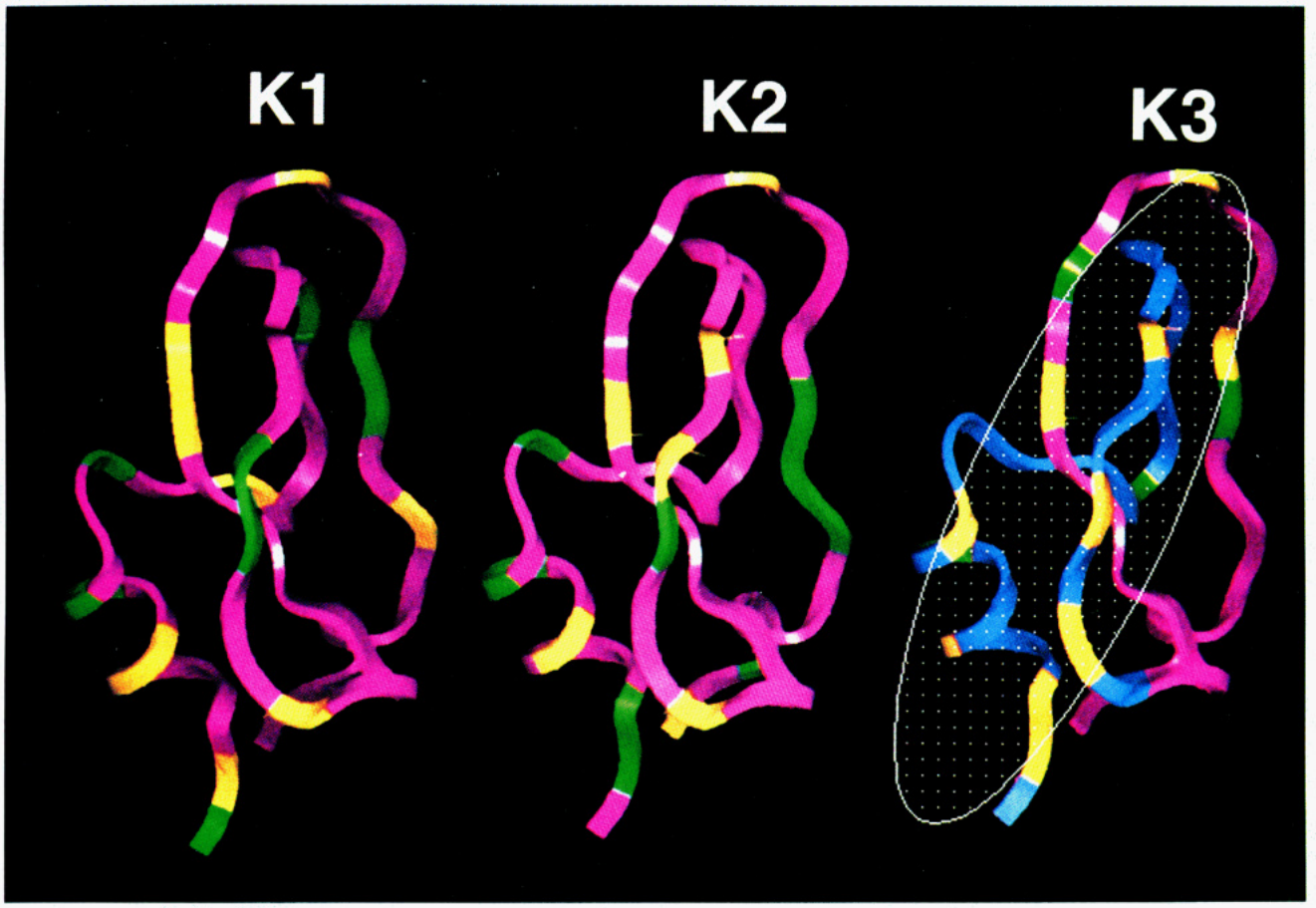

FIGURE 9: Distribution of basic and acidic amino acid residues in each Kunitz domain of TFPI. The basic amino acid residues (Arg and Lys) and the acidic amino acid residues (Asp and Glu) of each Kunitz domain of TFPI were superimposed on the tertiary structure of bovine pancreatic trypsin inhibitor (BPTI) using a software, InsightII (Biosym Technologies, San Diego, CA), and a computer, IRIS-4D (Silicon Graphics, Inc., Mountain View, CA). The backbone of each Kunitz domain was illustrated using a ribbon model, and side chains of amino acids were not included. Basic and acidic amino acid residues are indicated by yellow and green, respectively. The heparin binding region is indicated by a stippled circle. The region corresponding to Gly^{212}—Phe^{243} is indicated in light blue. The tertiary structure of BPTI is taken from Wlodawer et al. (1987).

as shown in Figure 5. All these results indicate that two heparin-binding sites, HBS-1 and HBS-2, in TFPI play an important role in the interaction with heparin.

It has been shown that TFPI circulates in the blood stream in association with lipoproteins such as VLDL/LDL and HDL as well as in a free form (Novotny et al., 1989). In addition, TFPI is known to be mainly synthesized and localized on endothelial cells (Bajaj et al., 1990; Werling et al., 1993). TFPI on the surface of endothelial cells is possibly present in association with proteoglycans on the surface, which is released by the infusion of heparin. Thus, TFPI on endothelial cells may have a very important role in the *in vivo* regulation of thrombosis. In fact, Grabowski et al. showed that the activation of factor X by tissue factor expressed on interleukin-1-stimulated endothelial cells was significantly reduced if the endothelial cells were not treated with anti-TFPI antibody (Grabowski et al., 1993). We did not examine whether TFPI-C could bind to proteoglycans on the cell surface or not. However, Callander et al. showed that TFPI isolated from the culture medium of transformed baby hamster kidney cells bound to OC-2008 cells even in the absence of factors Xa and VIIa and that 75% of the bound TFPI was released by heparin (Callander et al., 1992). Since TFPI isolated from the culture medium of transformed baby hamster kidney cells was shown to be essentially devoid of its C-terminal basic portion (Wun et al., 1992), these results suggest that TFPI-C can also bind to proteoglycans on the cell surface. Recently, the presence of a significant amount of TFPI-C in plasma has been indicated (Broze et al., 1994). Although TFPI-C is a truncated form lacking the C-terminal region of TFPI, our findings that, in the presence of calcium, TFPI-C shows the inhibitory activity equivalent to TFPI and the previous report on the binding ability onto the cell surface suggest the significant contribution of TFPI-C to the anticoagulability of plasma and the cell surface.

Our findings that both part of the K3 domain and the C-terminal part of TFPI are also heparin-binding domains may contribute to an increased understanding of the mechanisms involved in the interaction of TFPI with proteoglycans and the regulation of coagulation by TFPI on the endothelial cell surface.

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