Structure-Function Analysis of the Reactive Site in the First Kunitz-type Domain of Human Tissue Factor Pathway Inhibitor-2*

Received for publication, January 23, 2004, and in revised form, February 13, 2004
Published, JBC Papers in Press, February 16, 2004, DOI 10.1074/jbc.M40802200

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Human tissue factor pathway inhibitor-2 (TFPI-2) is a Kunitz-type protease inhibitor that regulates a variety of serine proteinases involved in coagulation and fibrinolysis through their non-productive interaction with a P1 residue (Arg-24) in its first Kunitz-type domain (KD1). Previous kinetic studies revealed that TFPI-2 was a more effective inhibitor of plasmin than several other serine proteinases, but the molecular basis for this specificity was unclear. In this study, we employed molecular modeling and mutagenesis strategies to produce several variants of human TFPI-2 KD1 in an effort to identify interactive site residues other than the P1 Arg that contribute significantly to its inhibitory activity and specificity. Molecular modeling of KD1 based on the crystal structure of bovine pancreatic trypsin inhibitor revealed that KD1 formed a more energetically favorable complex with plasmin versus trypsin and/or the factor VIIa-tissue factor complex primarily due to strong ionic interactions between Asp-19 (P6) and Arg residues in plasmin (Arg-644, Arg-719, and Arg-767), Arg-24 (P1) with Asp-735 in plasmin, and Arg-29 (P2) with Glu-606 in plasmin. In addition, Leu-26 through Leu-28 (P2–P4) in KD1 formed strong van der Waals contact with a hydrophobic cluster in plasmin (Phe-533, Met-585, and Phe-587). Mutagenesis of Asp-19, Tyr-20, Arg-24, Arg-29, and Leu-26 in KD1 resulted in substantial reductions in plasmin inhibitory activity relative to wild-type KD1, but the Asp-19 and Tyr-20 mutations revealed the importance of these residues in the specific inhibition of plasmin. In addition to the reactive site residues in the P1–P4′ region of KD1, mutation of a highly conserved Phe at the P1′n position revealed the importance of this residue in the inhibition of serine proteinases by KD1. Thus, together with the P1 residue, the nature of other residues flanking the P1′ residue, particularly at P2′ and P3′, strongly influences the inhibitory activity and specificity of human TFPI-2.

Protease inhibitors play a critical role in the regulation of several physiological processes such as blood coagulation, complement fixation, fibrinolysis, and fertilization (1). Most of these inhibitors are proteins having characteristic polypeptide scaffolds, and are grouped into a number of families, including the Kunitz (2), Kazal (2), Serpin (3), and mucus (4) families. The Kunitz-type family, serine proteinase inhibitors that include one or more Kunitz-type inhibitory domains, includes tissue factor pathway inhibitor (TFPI)1 and type-2 tissue factor pathway inhibitor (TFPI-2). These two inhibitors have been investigated extensively in the past decade and have been shown to play an important role in inhibiting serine proteinases involved in coagulation and fibrinolysis (5–8). Human TFPI-2, originally isolated from placenta and designated as placental protein 5, is a matrix-associated inhibitor consisting of three tandemly arranged Kunitz-type proteinase inhibitor domains flanked by a short acidic amino terminus and a highly basic C-terminal tail (7, 9). A wide variety of cells, including keratinocytes (10), dermal fibroblasts (10), smooth muscle cells (11), synovial fibroblasts (12), synovocytes (13), and endothelial cells (14), synthesize and secrete TFPI-2 primarily into their extracellular matrix. Three variants/isoforms of molecular mass 32, 30, and 27 kDa are synthesized by these cells and are thought to represent differentially glycosylated forms (15). TFPI-2 exhibits inhibitory activity toward a broad spectrum of proteinases, including trypsin, plasmin, chymotrypsin, cathepsin G, plasma kallikrein, and the factor VIIa-tissue factor complex. However, TFPI-2 exhibits little, if any, inhibitory activity toward urokinase-type plasminogen activator, tissue-type plasminogen activator, and a-thrombin (16). TFPI-2 presumably inhibits proteinases through a P1 arginine residue (Arg-24) in its first Kunitz-type domain, as an R24Q TFPI-2 mutant exhibited only 5–10% inhibitory activity toward trypsin, plasmin, and the factor VIIa-tissue factor complex (17). Recently, TFPI-2 expression by select tumors has been shown to play a significant role in inhibiting tumor growth and metastasis by a mechanism that involves its inhibitory activity (18, 19).

Several approaches have been employed to elucidate the structure-function relationship and broad specificity of Kunitz-type inhibitors using the well characterized bovine pancreatic trypsin inhibitor (BPTI) as a model. Detailed biophysical and biochemical studies have provided a greater insight into the

* This work was supported in part by National Institutes of Health Grants HL64119 (to W. K.) and HL70369 (to S. P. B.) and by the Orthopaedic Hospital Research Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported in part by Predoctoral Fellowship 0110191Z from the American Heart Association Heartland Affiliate.

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1 The abbreviations used are: TFPI, tissue factor pathway inhibitor; KD1, the first Kunitz-type domain of human TFPI-2; TFPI-2, tissue factor pathway inhibitor-2; VIIa, Factor VIIa; BPTI, bovine pancreatic trypsin inhibitor; APF, amyloid precursor protein inhibitor; APPH, amyloid precursor protein homolog; HAL, hepatocyte growth factor activator inhibitor; IsoTI, Inter-o-trypsin inhibitor; PLI, porcine leukocyte inhibitor; UPTI, uterine plasmin/trypsin inhibitor; SPI1, silk proteinase inhibitor-1; AsKCI, A. sulcata kalichudine1; TF, tissue factor; S-2251, H-O-Val-Leu-Lys-p-nitroanilide; S-2288, H-O-Ile-Pro-Arg-p-nitroanilide; BSA, bovine serum albumin; TBS, Tris-buffered saline; FPLC, fast-protein liquid chromatography.

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structural basis for the association of BPTI, or its homologues, to proteinases. Moreover, using semisynthetic (20, 21) or recombinant approaches (22, 23), it has been possible to change or enhance the inhibitory activity and spectrum of BPTI, as well as its homologues. Kunitz-type inhibitors possess a compact pear-shaped structure stabilized by three disulfide bonds containing a reactive site region featuring the principal determinant P₁ residue in a rigid conformation. These inhibitors competitively prevent access of the serine proteinase for its addition to the P₁ residue, other residues within the reactive site region of BPTI (P₁₋₋⁻⁻+') have been shown to interact with different serine proteinases, and it is generally recognized that the N-terminal side of the reactive site (P₁) is energetically more important than the P₁ C-terminal side (25). In all, about 10–12 amino acid residues in the inhibitor and 20–25 residues in the proteinase are in direct contact in the formation of a stable proteinase-inhibitor complex, and provide a buried area of 600–900 Å² (26). Although many proteins structurally similar to BPTI, such as TFPI KD2 (27), APPI (28), and bikunin (29), have been isolated and their three-dimensional structures determined, there are few studies that have assigned the relative contribution of residues flanking the reactive site residue in the formation of the proteinase-inhibitor complex and their effect on inhibitory activity and specificity (30–32).

In the case of TFPI-2, it is generally believed that its first Kunitz-type domain, in a BPTI-like manner, harbors most of its inhibitory activity, although no studies have definitively shown that this domain is sufficient to mediate this activity. In the present study, the complete first Kunitz domain of human TFPI-2 was expressed and purified, and its inhibitory activity toward selected proteinases was compared with that of full-length TFPI-2 and BPTI. In addition, molecular modeling was employed to obtain three-dimensional structural information on complexes of TFPI-2 KD1-plasmin, TFPI-2 KD1-trypsin, and TFPI-2 KD1-factor VIIa to identify residues in KD1 involved in its molecular recognition of each proteinase. From this analysis, residues primarily responsible for the interaction and proteinase specificity were then selected for mutagenesis. Select amino acid residues on both the N- and C-terminal side of the reactive site residue (P₁) were substituted individually, and the effects of these point-mutations on the proteinase specificity and inhibitory activity were investigated.

EXPERIMENTAL PROCEDURES

Materials—The chromogenic substrates H-o-Val-Leu-Lys-p-nitroanilide (S-2251) and H-o-Ile-Pro-Arg-p-nitroanilide (S-2288) were purchased from DiaPharma Group Inc. (West Chester, OH). Human plasmin was purchased from Hematologic Technologies, Inc. (Essex Junction, VT). Human recombinant factor VIIa, porcine trypsin and bovine aprotinin (BPTI) were generously provided by Novo Nordisk (Copenhagen, Denmark). Escherichia coli strain BL21(DE3)pLys and pET19b bearing a BPTI mutant ((35); pdb code 2ptc), and plasmin ((36); pdb code 1bm) served as templates in building these models. Bulk solvent was excluded from the proteinase-inhibitor complex, and, accordingly, it was anticipated that hydrogen bonds and ionic interactions that may play an important role in specificity could be accurately evaluated. The protocols for modeling these complexes have been described in detail earlier (8). Briefly, the relative positions of the inhibitor and proteinase domains were maintained and adjustments were only made to side chains. Hydrophobic/van der Waals, hydrogen bonds, and ionic interactions were observed between each proteinase-inhibitor complex. All of these interactions were taken into consideration in evaluating each proteinase-inhibitor complex, and it was assumed that all potential hydrogen bond donors and acceptors would participate in these interactions.

Expression and Purification of Wild-type and Mutant Proteins—The first Kunitz-type proteinase inhibitor domain of human TFPI-2 (KD1), and its mutants were overexpressed as N-terminal His-tagged fusion proteins in E. coli strain BL21(DE3)pLys using the T7 promoter system (37). The recombinant plasmid derived from pET19b bearing a decahistidine tag leader sequence followed by an enterokinase cleavage site and cDNA encoding the first Kunitz-domain of TFPI-2 was prepared according to standard procedures (38). Using this recombinant vector as a template, several other constructs containing the desired point mutations were generated using a QuikChange® site-directed mutagenesis kit according to the manufacturer's instructions. Each recombinant construct was examined for in-frame orientation, integrity, and desired mutation by nucleic acid sequencing. Wild-type and mutant His-tagged recombinant proteinases were expressed in E. coli grown in a rich media containing 100 mg/liter ampicillin, and induced at 37 °C with 1 mM isopropyl thiogalactopyranoside at mid log-phase (A₆₀₀ = 0.6–0.8). The over-expressed proteins were recovered from the cell lysates in the form of inclusion bodies following sonication in 50 mM Tris-HCl (pH 8.0) containing 0.5 M NaCl, 5 mM 2-mercaptoethanol, and 10 mM imidazole (buffer A). Inclusion bodies were recovered by high speed centrifugation (20,000 × g for 60 min) and thoroughly washed overnight at room temperature before solubilizing in buffer A containing 6 M guanidine hydrochloride. The solubilized inclusion bodies were recovered by high speed centrifugation and were filtered through 0.22-μm Nalgene® filters before application to His-Trap® column individually dedicated to each expressed protein. His-Trap affinity columns were used in a Amersham Biosciences FPLC® system and purification was carried out following the manufacturer's protocol. Peak fractions were identified by SDS-PAGE, pooled, and oxidatively refolded by initial dialysis against 50 mM Tris-HCl (pH 8.0) containing 3 mM 2-mercaptoethanol, followed by extensive dialysis against 50 mM Tris-HCl (pH 8.0).

The refolded proteins were purified to homogeneity by Mono Q FPLC® at room temperature. KD1 proteins were eluted from the column in a linear NaCl gradient consisting of 50 mM Tris-HCl (pH 8) and 50 mM Tris-HCl (pH 8) containing 1 M NaCl. Peak fractions were subjected to SDS-PAGE analysis, and pure fractions were pooled and concentrated on YM-3 ultrafiltration membranes.

General Methods—The concentration of each purified KD1 protein was determined by measuring its absorbance at 280 nm using calculated values for E₁₀₀ derived from its Tyr, Trp, and Cys content (39). The concentration of plasmin was provided by the supplier, whereas the concentrations of all other proteins used in this study were determined according to Bradford (40) using BSA as the reference protein. SDS-PAGE was performed according to Laemmli (41) using 4–20% polyacrylamide gradient gels.

Trypsin and Plasmin Inhibition Assays—Trypsin and plasmin inhibition assays were performed as described elsewhere (16). Briefly, trypsin and plasmin were incubated with various concentrations of inhibitor preparations for 15 min at 37 °C in a 96-well microtitration plate. The chromogenic substrate S-2251 was then added, and residual amidolytic activity was measured in a Molecular Devices UVmax kinetic microplate reader purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). Inhibition Assay for Factor VIIa-Tissue Factor Amidolytic Activity—Recombinant soluble human tissue factor (100 nM) and factor VIIa (50 nM) were incubated in a TBS (50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl)-BSA buffer/5 mM CaCl₂ for 15 min at 37 °C. Following this incubation, aliquots (100 μl) were dispensed into a 96-well microtiter plate and treated with serial dilutions of inhibitors dissolved in TBS buffer. After 15 min of incubation, 30 μl of S-2288 (final concentration, 1 μM) was added to each well and the absorbance at 405 nm was determined as described earlier.

Inhibition Kinetics—The apparent inhibition constant, Kᵢ, was determined using the non-linear regression data analysis program.
Inhibitor | NH$_2$--P$_6$ P$_5$ P$_4$ P$_3$ P$_2$ P$_1$' P$_2'$ P$_3'$ P$_4'$ P$_5'$--COOH
--- | ---
BPTI | --- Y T G P C K A R I I R --- --- F---
APP | --- E T G P C R A M I I R --- --- F---
APPH | --- M T G P C R A V M P R --- --- F---
TFPI Domain 1 | --- D D G P C K A I M K R --- --- F---
TFPI Domain 2 | --- D P G I C R G Y I T R --- --- F---
TFPI Domain 3 | --- D L G L C R A N E N R --- --- F---
TFPI-2 Domain 1 | --- D Y G P C R A L L L R --- --- F---
TFPI-2 Domain 2 | --- V D D Q C E G S T E K --- --- F---
TFPI-2 Domain 3 | --- D E G L C S A N V T R --- --- F---
HAL-1 Domain 1 | --- K V G R C R G S F P R --- --- F---
HAL-1 Domain 2 | --- D T G L C K E S I P R --- --- F---
HAL-2 Domain 1 | --- V V G R C R A S M P R --- --- F---
HAL-2 Domain 2 | --- V T G P C R A S F P R --- --- F---
IoTI | --- S A G P C M G M T S R --- --- F---
PLI | --- Y T G P C K A R M I K --- --- F---
UPTI | --- Y T G P C R A H F I R --- --- F---
SPI 1 | --- K T G P C K A A F Q R --- --- F---
AsKC-1 | --- D V G R C R A S H P R --- --- F---

Fig. 1. Amino acid sequences surrounding the P$_1$ reactive site residue in selected Kunitz-type inhibitors. BPTI sequence is from Creighton and Charles (43), APP is from Ponte et al. (44), APPH is from Sprecher et al. (45), TFPI is from Wun et al. (46), TFPI-2 is from Sprecher et al. (7), HAI-1 is from Shimomura et al. (47), HAI-2 is from Kawaguchi et al. (48), IoTI is from Kaumeyer et al. (49), PLI is from Drobnic-Kosorok et al. (50), UPTI is from Stallings-Mann et al. (51), SPI is from Nirmala et al. (52), and AsKC1 is from Schweitz et al. (53).

Ultrasoft v3.0 (Bio soft). Trypsin and plasmin inhibitory data were analyzed according to the following equation for a tight-binding inhibitor,

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

(Eq. 1)

where \( v_i \) and \( v_0 \) are the inhibited and uninhibited rates, respectively, and \([I]\) and \([E]\) are the total concentrations of inhibitor and enzyme, respectively. Factor VIIa-tissue factor inhibition data, where \( K_i > [E]_0 \), were analyzed according to Equation 2.

\[ v_i = v_0/(1 + [I]/K_i') \]

(Eq. 2)

The \( K_i \) values were obtained by correcting for the effect of substrate according to Bieth et al. (42), where \( K_i = K_i'/1 + [S]/K_m \).

RESULTS

Molecular Modeling and Selection of Mutations—Previous studies demonstrated that human TFPI-2 is a strong inhibitor of plasmin and trypsin, and a relatively weak inhibitor of the factor VIIa-tissue factor complex (16). The molecular basis of the specificity of TFPI-2 for plasmin and trypsin relative to the serine proteinase factor VIIa is unclear, but presumably involves residues other than the P$_1$ Arg in the first Kunitz-type domain of TFPI-2, as well as residues in the active site region of the proteinase. To address whether other residues in the reactive site region of TFPI-2 may play a role in its inhibitory potency and specificity, we employed a molecular modeling approach to guide subsequent mutagenesis studies designed to provide information on the functional importance of these residues. Because our preliminary data indicated that a recombination preparation of the first Kunitz-type domain of TFPI-2 (KD1) exhibited better inhibitory activity in comparison to the intact parent molecule (see below), we decided to model complexes of KD1 with plasmin, trypsin, and factor VIIa based on the crystal structure of BPTI and each proteinase, respectively. A preliminary inspection of the amino acid sequences surrounding the P$_1$ residue in a number of Kunitz-type inhibitors revealed highly conserved residues at the P$_6$, P$_1$, P$_5$, and P$_{18}$ positions (Fig. 1), and our molecular modeling studies thus initially focused on the contributions of these residues in the formation of an energetically stable complex between KD1 and the above proteinases. In the model structures of these complexes, no unfavorable contacts between atoms and no unnatural chiral centers were observed. In the Ramachandran plot of the main-chain \( \phi-\psi \) angles, all of the non-glycine residues are in the most favored or permissible regions. Moreover, there are no gross steric clashes that preclude the interaction of proteinases with KD1. For simplicity and consistency, the residue numbering system employed for KD1 and each proteinase is that of its linear sequence position. In addition, to relate each proteinase residue number to its corresponding position in the prototypical proteinase chymotrypsin, each proteinase residue number is followed by its position in chymotrypsin in braces and is preceded by the letter "c." Finally, the relationship between residues mutated in KD1 and their position in BPTI is indicated in Table 1.

In the KD1-proteinase complex, there is an interactive hydrophobic patch and an internal hydrophobic patch in KD1 (Fig. 2, A and B). The plasmin-interacting hydrophobic interface is formed by a number of residues in KD1, including Leu-26, Leu-27, Leu-28, Leu-43, and Tyr-55. The residues Leu-18, Tyr-20, Tyr-31, Phe-42, and Tyr-44 in KD1 are buried within and contribute to the formation of an internal hydrophobic pocket. Within the interactive patch, Leu-27 in KD1 interacts with Phe-583(c37), Met-585(c39), Phe-587(c41), and C$_B$ of Lys-607(c61) in plasmin (Fig. 2, A and B). In addition, Leu-28 interacts with Met-585(c39) in plasmin. Furthermore, the side-chain C$_D$ and C$_G$ of Lys-607(c61) could make hydrophobic interactions with C$_{D1}$ and C$_{G1}$ of Tyr-55 in KD1 (Fig. 2A). These flanking region interactions at the interface of plasmin and KD1 exhibit a marked variability in structural com-

Note: See Table 1 for details. The data include the average of three experiments, and the standard deviation was 10% or less. 
The effect of mutations at the P_{6}, P_{5}, P_{4}, P_{1}, P_{2}, P_{3}′, and P_{18}′ are also listed in Table I. An alanine substitution at the P_{8} position, Asp-19, showed a dramatic (~40-fold) loss of inhibitory activity toward plasmin but failed to show any significant loss of activity toward trypsin and factor VIIa. From the molecular graphics model (Fig. 1A), the most rational explanation for this effect is that Asp-19 interacts with a basic patch in plasmid consisting of Arg-644(c98), Arg-719(c173), and Arg-767(c221). Because there are no corresponding basic patches in trypsin or factor VIIa at these positions, the interaction of Asp-19 with the basic patch in plasmid appears to confer KD1 with enhanced reactivity, and inhibitory potency, toward plasmin. Mutagenesis of the neighboring P_{3} residue, Tyr-20, to Ala.
also had a significant negative effect on KD1 inhibition of plasmin, suggesting that this residue either contributes toward the formation of the KD1-plasmin complex, or is critical in maintaining the conformation of the KD1 reactive site toward plasmin. Mutagenesis of Tyr-20 to Ala, however, slightly enhanced its ability to inhibit trypsin and factor VIIa (Table I). The residue at the P4 position, Gly-21, also seems to play a supportive role in the interaction of KD1 with proteinases as shown earlier using BPTI mutants (32). In this study, we mutated Gly-21 to aspartic acid to increase the acidic patch on KD1 and enhance its interaction with the basic patch in plasmin, because Gly-21 is in close spatial proximity to Asp-19. However, mutation of Gly with Asp did not have the desired effect, and this mutant lost inhibitory activity toward all proteinases tested most probably due to perturbation in the main-chain conformation of the KD1 reactive site. In this regard, the phi angle (\( \phi \)) of Gly is +111\(^\circ\) and the psi angle (\( \psi \)) is −174\(^\circ\), which places Gly-21 in a region of the Ramachandran plot accessible only to Gly residues (43). Accordingly, any other residue would conceivably alter the backbone structure by changing the phi and psi angles resulting in an altered main-chain conformation in the vicinity of Gly-21.
The side chain of the P₁ residue primarily dictates the specificity of a proteinase inhibitor for its cognate proteinase. Systematic substitution at this position in a number of inhibitors revealed a large dynamic range of effects on its association with different proteinases (31, 32, 44–49). The glutamine substitution at the P₁ site resulted in a decreased inhibitory activity in KD1 (Table I), as was observed with the full-length R24Q TFPI-2 mutant (17). As observed in the model structure of KD1 with plasmin, trypsin, and factor VIIa, the Arg-24 in KD1 forms a salt bridge, in addition to two hydrogen bonds, with the carbonyl backbone of Asp{c₁₈₉} and Gly{c₂₁₉} to stabilize the complex. Mutation of Arg to Gln eliminates interaction with the S₁ site residue Asp{c₁₈₉} due to the shorter side chain and its lack of charge. Lysine substitution at this position restores the inhibitory activity of KD1, and the lower $K_i$ values obtained with R24K KD1 against plasmin and trypsin could be the result of an ionic interaction of the protonated amino group with the carboxylate group of Asp{c₁₈₉}, as well as water-mediated hydrogen bonding between the carbonyl group of Gly{c₂₁₉} and the hydroxyl group of Ser{c₁₉₀} with the P₁ Lys amino group. In view of this potential bonding pattern, it is curious as to why the inhibitory activity of R24K KD1 for factor VIIa was reduced 5-fold, inasmuch as factor VIIa also has a Ser-326{c₁₉₀}. The reason for its reduced inhibitory activity against factor VIIa is not known at this point but may be due to other residues in the substrate binding pocket of factor VIIa as opposed to that of plasmin and trypsin.

As mentioned above, KD1 contains a cluster of hydrophobic Leu residues at the P₂–P₄ region that interacts with a hydrophobic patch in plasmin, trypsin, and factor VIIa. To disrupt this cluster, Leu-26 was substituted with the highly hydrophilic residue, glutamine. This L26Q mutation resulted in at least a 10-fold reduction in inhibitory potency of KD1 toward each of the proteinases tested (Table I) and underscores the importance of this hydrophobic interaction in the inhibitory mechanism of KD1. Leu-26 is part of a hydrophobic patch and interacts with Leu-43 and Leu-28 of KD1. Leu-26 also has the potential to have hydrophobic interactions with Gln-738{c₁₉₂} in plasmin, with C₈ and C₉ of Gln-200{c₁₉₂} in trypsin, and with C₈ and C₉ of Lys-328{c₁₉₂} in factor VIIa. Thus, changing this residue to a non-hydrophobic residue such as Gln will disrupt these interactions and be disruptive for each proteinase.

Virtually all Kunitz-type domains studied have a highly
conserved Lys/Arg at the P₅' position (Fig. 1), and three point mutants were made at this position. In plasmin, Glu-606(c60) makes hydrogen bonds with Arg-29 in KD1, whereas Tyr-67(c59) and Asp-196(c60) in trypsin and factor VIIa, respectively, interact with this residue. Substitution of Arg-29 with alanine resulted in a marginal loss of inhibitory activity toward all three proteinases (Table I), whereas substitution with aspartic acid presumably caused charge repulsion, as well as disruption of hydrogen bonds, with a major effect on Kᵢ (Table I). Mutation of Arg-29 with lysine could possibly preserve the hydrogen bonding observed with Arg and resulted in minor changes in Kᵢ (Table I). Although the P₅' Arg/Lys residue is not a major determinant in KD1 specificity.

Finally, as expected, mutagenesis of the highly conserved Phe-42 at the P₆' position with alanine resulted in similar losses of inhibitory activity toward all three proteinases (Table I), presumably by disruption of the internal hydrophobic core in KD1 formed by Phe-42, Tyr-20, Leu-18, Tyr-31, Tyr-44, and the side chain of Arg-29.

**DISCUSSION**

In the present study, we have expressed and purified the human TFPI-2 Kunitz-type domain 1 (KD1) and compared its inhibitory activity toward plasmin, trypsin, and the factor VIIa-tissue factor (VIIa-TF) complex to that of full-length TFPI-2, BPTI, and nine human TFPI-2 KD1 constructs with mutations in the reactive site region (P₆–P₅'). The isolated TFPI-2 KD1 exhibited stronger inhibitory activity toward these proteinases in comparison to intact TFPI-2. Alainine substitution at the P₆ (D19A) and the P₅ (Y20A) positions had a marginal effect on its inhibitory activity toward trypsin and VIIa-TF but exhibited a marked decrease in activity toward plasmin. Substitution of aspartic acid for alanine was particularly deleterious to plasmin inhibition by KD1, and molecular modeling studies revealed that this was in all likelihood due to the modulation of an ionic interaction between an acidic patch in KD1, formed by Asp-19 and Glu-39, and a basic patch unique to plasmin composed of Arg-644(c95), Arg-719(c173), and Arg-767(c221). Thus, Asp-19 and Tyr-20 in KD1 appear to play a major role in the specificity of TFPI-2 for plasmin. In contrast, point mutations at the P₁(G21D), P₁(R24Q), P₂(L26Q), and P₅'(R29A) positions all exhibited substantial decreased inhibitory activity toward all of these proteinases. The importance for a highly conserved basic residue (Arg/Lys) at the P₅' position was evident from a substantial loss of inhibitory activity in the R29D KD1, presumably through the loss of either a stabilizing ionic interaction between Arg-29 and Glu-606/Asp-196(c60) in plasmin/VIIa, or by hydrogen bonding of Arg-29 to Tyr-67(c59) in trypsin. Finally, mutation of a highly conserved phenylalanine at the P₆' position (F42A) revealed the importance of this residue in the stabilization of the reactive site structure through internal hydrophobic interactions.

A lysine substitution at the P₁ position (R24K) in KD1 significantly increased its inhibitory activity toward both plasmin and trypsin, making it essentially as effective as BPTI toward these proteinases. In sharp contrast, R24K KD1 paradoxically exhibited a significantly increased its inhibitory activity toward both plasmin and/or trypsin IV (59), and the secretion of inhibitory TFPI-2 by these tumors markedly inhibits their growth and metastasis in animal models (18, 19). Moreover, in preliminary studies, we have shown that human KD1 exhibits dose-dependent inhibition of angiogenesis in a commercially available *in vitro* human endothelial cell angiogenesis assay. In addition, intravenous administration of human KD1 to ovalbumin-sensitized asthma mice resulted in a significant decrease in the number of airway macrophages and lymphocytes relative to vehicle-treated asthma mice, suggesting that KD1 inhibits the proteinase-mediated transepithelial migration of mononuclear cells from the bloodstream to the airways. Accordingly, administration of KD1, or a more potent KD1 mutant, may conceivably regulate these and other pathological processes dependent upon the activity of serine proteinases. In addition, the availability of human KD1 generated in these studies will facilitate x-ray crystallographic studies of either this inhibitor alone or in complex with serine proteinases, and these studies are currently ongoing in our laboratories.

In summary, these studies provide the initial, definitive evidence that the first Kunitz-type domain of human TFPI-2 contains all the structural elements for the inhibition of a variety of serine proteinases and underscores the importance of critical residues in its P₆–P₅' position in its inhibitory activity toward these proteinases. In addition, these studies reveal the importance of the Asp and Tyr residues at the P₆ and P₅ positions in the reactive site region of KD1 that appears to confer specificity for plasmin inhibition by TFPI-2.

**Acknowledgment**—The excellent technical assistance of Alex Boyd is gratefully acknowledged.

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Structure-Function Analysis of the Reactive Site in the First Kunitz-type Domain of Human Tissue Factor Pathway Inhibitor-2
Hitendra S. Chand, Amy E. Schmidt, S. Paul Bajaj and Walter Kisiel

J. Biol. Chem. 2004, 279:17500-17507.
doi: 10.1074/jbc.M400802200 originally published online February 16, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M400802200

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Additions and Corrections

Vol. 279 (2004) 6761–6768

DsbB elicits a red-shift of bound ubiquinone during
the catalysis of DsbA oxidation.

Kenji Inaba, Yoh-hei Takahashi, Nobutaka Fujieda, Kenji
Kano, Hideto Miyoshi, and Koreaki Ito

The names of Drs. Miyoshi, Kano, and Fujieda were inadvert-
ently omitted from the author list. All three of these authors
are from the Division of Applied Life Sciences, Graduate School
of Agriculture, Kyoto University, Kyoto 606-8502, Japan. The
corrected list is shown above.

Vol. 279 (2004) 17500–17507

Structure-function analysis of the reactive site in the
first Kunitz-type domain of human tissue factor
pathway inhibitor-2.

Hitendra S. Chand, Amy E. Schmidt, S. Paul Bajaj, and
Walter Kisiel

Page 17505, Fig. 4: The amino-terminal sequence of the first
Kunitz-type domain (KD1) of human TFPI-2 is incorrect at
positions 7 and 8 where a transposition occurred. The correct
sequence of the TFPI-2 KD1 is DAAQEPTG...KV. This cor-
rection does not have any impact on the conclusions drawn in
the study.

Vol. 279 (2004) 15402–15410

Double strand break repair by homologous recombination
is regulated by cell cycle-independent signaling via ATM
in human glioma cells.

Sarah E. Golding, Elizabeth Rosenberg, Ashraf Khalil,
Alison McEwen, Matthew Holmes, Steven Neill, Lawrence F.
Povirk, and Kristoffer Valerie

Page 15403: There are errors in the primer sequences. In the
right column, third line under “DNA Analysis” the primer
sequence should be: “top strand, 5’-CGT GCT GGT TAT TGT
GCT GTC T-3’; bottom strand, 5’-GAC TTG AAG AAG TCG
TGC TG-3’.”

Page 15404: Under “Chromatin Immunoprecipitation (ChIP)
Assay,” left column, lines 2 and 3 should read: “Primers used
were 5’-GAA GTT CAT CTG CAC CAC C-3’ (top strand) and
5’-TTG AAG TTC ACC TTG ATG CC-3’ (bottom strand). . . .”

Page 15406, Fig. 6A: The panels of the Western blot showing
p53 phosphorylation were flipped. The correct Fig. 6A is shown
below:

![Fig. 6A](image2)

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.