# Contribution of Regions Distal to Glycine-160 to the Anticoagulant Activity of Tissue Factor Pathway Inhibitor†

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ABSTRACT: The functions of the first two Kunitz domains of tissue factor pathway inhibitor (TFPI) are well defined as active site-directed inhibitors of factor VIIa and factor Xa. The anticoagulant properties of the third Kunitz domain and C-terminal region were probed using altered forms of TFPI. TFPI-160 contains the first two Kunitz domains. K1K2C contains the first two Kunitz domains and the basic C-terminus. Neither TFPI-160 nor K1K2C contains the third Kunitz domain. In amidolytic assays containing calcium, TFPI-160 is a less potent inhibitor of factor Xa than TFPI. However, addition of the C-terminus in K1K2C nearly restores inhibitory activity to that of TFPI, indicating that the third Kunitz domain is not required for direct inhibition of factor Xa. When compared in assays containing phospholipids and factor Va, K1K2C and TFPI-160 are poor inhibitors compared to TFPI, demonstrating that the third Kunitz domain is required for the full anticoagulant activity of TFPI. TFPI was further characterized in amidolytic assays performed with Gla-domainless factor Xa and in prothrombin activation assays using submicellar concentrations of short-chain phospholipids (C6PS). TFPI and K1K2C are worse inhibitors of Gla-domainless factor Xa, compared to wild-type factor Xa, while TFPI-160 inhibits both forms of factor Xa equally, suggesting a C-terminus/Gla domain interaction. TFPI is a potent inhibitor of thrombin generation by prothrombinase assembled with C6PS, while TFPI-160 and K1K2C are not. Conversely, TFPI does not inhibit prothrombin activation by prothrombinase assembled on a two-dimensional lipid bilayer. Together, the data indicate that the region between Gly-160 and the end of the third Kunitz domain contributes to TFPI function by orienting the second Kunitz domain so that it can bind the active site of phospholipid-associated factor Xa prior to prothrombinase assembly and/or by slowing formation of the prothrombinase complex.

Tissue factor pathway inhibitor (TFPI) is an ∼42 kDa glycoprotein containing an acidic N-terminal region, three tandem Kunitz-type serine proteinase inhibitor domains, and a basic C-terminal region (*1*). Through site-directed mutagenesis of the reactive site residues of the individual Kunitz domains, it was demonstrated that the second Kunitz domain directly inhibits factor Xa and, in a factor Xa-dependent manner, the first Kunitz domain inhibits factor VIIa, resulting in the formation of a quaternary factor Xa/TFPI/factor VIIa/tissue factor complex (*2*). The cooperative action between the first two Kunitz domains of TFPI makes it the most effective known inhibitor of the factor VIIa/tissue factor catalytic complex that initiates coagulation in vivo.

While the functional roles of the first two Kunitz domains as active site-directed serine proteinase inhibitors are well characterized, the physiologic importance of the other structural regions of TFPI is less well defined. Studies with forms of TFPI lacking the acidic N-terminal region have demonstrated that it is a fully functional inhibitor of factor Xa in both amidolytic and clot-based assays, suggesting that this region is not required for the anticoagulant activity of TFPI (*3*). The third Kunitz domain appears structurally to be a functional proteinase inhibitor, but it has never been shown to function as an inhibitor, either as a component of TFPI or as an independent Kunitz domain following expression in yeast (*4*). It weakly binds to heparin (*5*); however, the importance of interactions between heparin and the third Kunitz domain in the anticoagulant activity of TFPI is unclear. The basic C-terminal region is necessary for binding to heparin (*3*, *6*), thrombospondin-1 (*7*), and cell surfaces (*8*). It is also required for optimal inhibition of factor Xa by the second Kunitz domain in amidolytic assays and for the anticoagulant activity of TFPI in both factor Xa- and tissue factor-initiated coagulation assays (*3*, *6*). Interestingly, the C-terminal region is not required for effective inhibition of factor VIIa/tissue factor catalytic activity in two-stage amidolytic assays measuring the activation of factor X (*3*, *6*, *9*). Further, factor Xa inhibition is diminished following the limited proteolytic cleavage of TFPI by human leukocyte elastase, which separates the amino terminus and first Kunitz domain from the second Kunitz domain and the remainder of the molecule (*10*). Thus, the anticoagulant actions of TFPI observed in tissue factor-initiated coagulation assays are not

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mediated solely by the inhibition of factors VIIa and Xa by the first and second Kunitz domains; other regions of the protein must also have anticoagulant functions.

To address the potential anticoagulant function of the third Kunitz domain and surrounding sequence of TFPI, we have compared the inhibitory activity of full-length TFPI to that of TFPI-160, a form of TFPI truncated after the second Kunitz domain, and K1K2C, a form of TFPI that contains the first two Kunitz domains and the C-terminal region but lacks the third Kunitz domain. The data demonstrate that K1K2C and full-length TFPI are effective direct inhibitors of factor Xa in amidolytic assays where TFPI-160 is a relatively poor inhibitor. However, in assays containing the other components of prothrombinase, phospholipids and factor Va, the inhibitory activity of K1K2C is similar to that of TFPI-160 and much worse than full-length TFPI. The results indicate that the portion of TFPI between Gly-160 and the end of the third Kunitz domain is required for effective inhibition of phospholipid-associated factor Xa by TFPI and, consequently, for its anticoagulant activity.

## MATERIALS AND METHODS

*Materials*. The chromogenic substrates Spectrozyme Xa (methoxycarbonyl-D-cyclohexylglycylglycylarginine-*p*-nitroanilide acetate) for factor Xa and Spectrozyme TH (hexahydrotyrosyl-Ala-Arg-*p*-nitroanilide diacetate) for thrombin were purchased from American Diagnostica Inc. (Greenwich, CT). Rabbit brain cephalin, used as a source of phospholipids, was kindly provided by Marc Goldford (Sigma Chemical Co., St. Louis, MO). 1,2-Dihexanoyl-*sn*-glycero-3-phospho-L-serine (C6PS) was purchased from Avanti Polar Lipids (Alabaster, AL) and prepared according to the manufacturer's instructions. Freshly expired apheresis platelets were purchased from Lifeblood/Mid-South Regional Blood Center (Memphis, TN). Fresh frozen plasma was obtained from the Memphis Veterans Affairs Hospital Blood Bank.

*Proteins*. Recombinant full-length human Ala-TFPI produced in *Escherichia coli* was a gift of Chiron Corp. (Emeryville, CA) and Searle Corp. (Skokie, IL). TFPI-160, an altered form of TFPI truncated after Gly-160, was produced in *E. coli* and purified as described previously (*8*). K1K2C, an altered form of TFPI containing the first two Kunitz domains and the basic C-terminal region, was the gift of Dr. George Broze, Jr. (Washington University, St. Louis, MO). Specifically, the K1K2C form of TFPI contains a Met-Ala-Asp-Ser sequence connected to Glu-15 at the amino terminus. The protein is then truncated at Gly-150 following the second Kunitz domain, and the basic C-terminal region (amino acids from Phe-243 to Met-276) is attached. Concentrations of K1K2C and TFPI-160 were determined by titration with factor Xa and comparison to full-length TFPI of known concentration. These titration experiments also ensured that equal concentrations of the different forms of TFPI were used in the various assays. Factor Xa was purchased from Enzyme Research Laboratories Inc. (South Bend, IN). Prothrombin, thrombin, factor Va, and $\gamma$-carboxyglutamic acid (Gla) domainless factor Xa were purchased from Haematologic Technologies Inc. (Essex Junction, VT).

*Equipment*. A Spectra MAX Plus spectrophotometer from Molecular Devices Corp. (Sunnyvale, CA) was used to monitor amidolytic assays. Data were collected and analyzed by the SoftMax Pro 2.1 software package. An ST-4 clot detection system from American Bioproducts Co. (Parsippany, NJ) was used to monitor clot-based assays.

*Estimation of $K_i$(initial)*. The affinity of the initial encounter complex between factor Xa and TFPI, K1K2C, or TFPI-160 was estimated from a series of progress curves measuring factor Xa inhibition ($\Delta A_{405}$/min) during the first minute of the reaction. In a microtiter plate, varying concentrations of Spectrozyme Xa (500, 200, 100, and 50 $\mu$mol/L) are made in 50 mmol/L HEPES, pH 7.4, 100 mmol/L NaCl, 5 mmol/L $CaCl_2$, and 0.1% bovine serum albumin (HBSA). TFPI or K1K2C is added to 5 nmol/L final concentrations. TFPI-160 is added to 25 nmol/L final concentration. The reaction is initiated upon addition of factor Xa or Gla-domainless factor Xa (0.5 nmol/L), and the absorbance at 405 nm is continuously recorded. The $K_m$(apparent) for the inhibition of factor Xa by TFPI was derived from a plot (Lineweaver−Burk) of the reciprocal of the velocity ($\Delta A_{405}$/min) versus the reciprocal of substrate concentration. $K_i$ values were calculated using the equation $K_i(\text{initial}) = (K_m[I])/(K_m(\text{app}) - K_m)$, where [I] is the initial concentration of inhibitor present in the reaction mixture. $K_m$ was determined independently to be 124 $\mu$M for factor Xa and Spectrozyme Xa in the absence of calcium ions, 140 $\mu$M for factor Xa and Spectrozyme Xa in the presence of calcium ions, 164 $\mu$M for factor Xa and Spectrozyme Xa in the presence of calcium ions and phospholipids, and 168 $\mu$M for factor Xa and Spectrozyme Xa in the presence of calcium ions, phospholipids, and factor Va. The $K_m$ for Gla-domainless factor Xa and Spectrozyme Xa in the presence of calcium ions was determined independently to be 57 $\mu$M. EDTA (10 mmol/L) was added to HBSA to determine effects of calcium ions on the reaction. Rabbit brain cephalin (10%) alone, or with factor Va (18.7 nmol/L), was added in some experiments. For these experiments, the final concentration of factor Xa or Gla-domainless factor Xa used to initiate the reaction was 5 nmol/L, while TFPI and K1K2C were added to 25 nmol/L final concentration and TFPI-160 was added to 50 nmol/L.

*Determination of $K_i$(final)*. The kinetic constants for the final tightened complex were determined as described previously (*11*). In brief, varying concentrations of inhibitor (1, 2, 3, 4, and 5 nmol/L) are added to substrate (500 $\mu$mol/L Spectrozyme Xa). The reaction is initiated by the addition of 0.2 nmol/L factor Xa or Gla-domainless factor Xa and is monitored continuously until the $\Delta A_{405}$/min is linear (~1 h). The apparent $K_i$ is defined as the reciprocal of the slope of the line generated by a plot of $V_0/V_i - 1$ versus concentration of inhibitor, where $V_0$ is the velocity of the reaction in the absence of inhibitor and $V_i$ is the velocity of the reaction in the presence of inhibitor. These values are determined by calculating the slopes of progress curves at equilibrium. The $K_i$(final) is calculated by correcting for the concentration of the substrate, Spectrozyme Xa, using the equation $K_i(\text{final}) = K_i(\text{apparent})/(1 + ([S]/K_m))$, where [S] is the concentration of substrate used in the reaction. The $K_m$ for factor Xa and substrate in the absence of inhibitor was determined independently as described above. Rabbit brain cephalin (1%) alone, or with factor Va (18.7 nmol/L), was added in some experiments. In control experiments performed simultaneously using the same lots of reagents, the presence of

phospholipids and factor Va does not alter the rate of amidolytic substrate cleavage by factor Xa compared to that observed in the presence of calcium ions alone. However, variations in the rate of substrate cleavage by factor Xa were observed over time with different lots of substrate and factor Xa (compare noninhibited control reactions in Figures 2 and 5). These differences are likely accounted for by variations in the actual active substrate and/or enzyme concentration of the different lots and by experimental error. Importantly, calculation of the $K_i$(final) values does not require accurate estimation of the enzyme concentration as the ratio of $V_0/V_i - 1$, which is not dependent on enzyme or substrate concentration, is the kinetic parameter used to generate the plots from which $K_i$(final) is calculated. Student's *t*-test was used to determine the statistical significance between kinetic values calculated for each altered form of TFPI.

*Factor Xa-Induced Coagulation of Plasma*. In a cuvette, 50 $\mu$L of rabbit brain cephalin, prepared as described by the manufacturer (Sigma, St. Louis, MO) and diluted 10-fold into HBSA, 50 $\mu$L of $CaCl_2$ (5 mmol/L), and 50 $\mu$L of factor Xa (0.2 nmol/L) are incubated together at 37 °C. One hundred microliters of a 50/50 mixture of TFPI sample (of various concentrations) and normal human pooled plasma is incubated together for 15 min at room temperature. The clotting reaction is initiated upon the addition of the TFPI/plasma mixture to the cuvette. The degree of apparent factor Xa inhibition is determined by comparison of the clotting time to a standard curve generated by performing the assay at different concentrations of factor Xa without inhibitor.

*Direct Activation of Prothrombin by the Prothrombinase Complex*. In factor Xa initiated reactions, prothrombin (1.4 $\mu$mol/L), factor Va (3 nmol/L), rabbit brain cephalin (1:40 dilution of the stock preparation), $CaCl_2$ (2.5 mmol/L), and dilutions of TFPI, K1K2C, and TFPI-160 are mixed. The concentrations of prothrombin and calcium were chosen to approximate physiologic conditions. Factor Va at 3 nmol/L is saturating. After incubation for 2.5 min at 37 °C, the reaction is initiated by the addition of factor Xa (0.1 nmol/L). Every 15 s 10 $\mu$L samples are removed, diluted 100-fold into HBSA containing 5 mmol/L EDTA, and assayed for thrombin activity using the chromogenic substrate Spectrozyme TH (hexahydrotyrosyl-Ala-Arg-*p*-nitroanilide diacetate). No detectable thrombin is produced when factor Xa, phospholipids, or factor Va is omitted from the reaction. Reactions initiated with a mixture of TFPI, K1K2C, or TFPI-160 and prothrombin are performed identically except that factor Xa is incubated with the calcium, phospholipids, and factor Va for 2.5 min at 37 °C to allow assembly of the prothrombinase complex before the reaction is started. In some experiments, nonactivated platelets ($5 \times 10^8$/mL) isolated from apheresis units are used as a source of phospholipids and factor Va. For some assays, submicellar concentrations (3 mmol/L) (*12*, *13*) of C6PS were used as a source of phospholipids instead of rabbit brain cephalin. These reactions were incubated for 5 min to allow the assembly of the prothrombinase complex with C6PS prior to initiation with a mixture of prothrombin and inhibitor. Samples (10 $\mu$L) were then taken every 5 min for 30 min, diluted 100-fold into HBSA containing 5 mmol/L EDTA, and assayed for thrombin activity using the chromogenic substrate Spectrozyme TH.

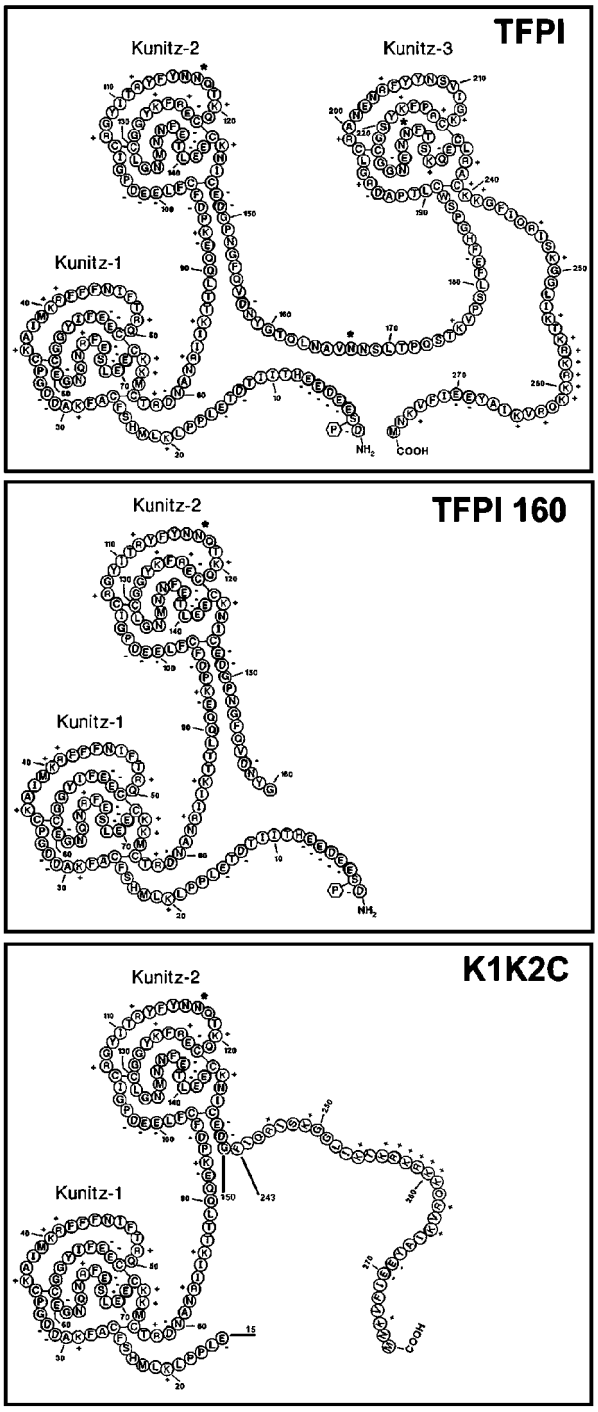


FIGURE 1: Primary structure of TFPI and altered forms of TFPI. The primary amino acid sequence and Kunitz domain structure of TFPI, TFPI-160, and K1K2C are diagramed to demonstrate the structural details that make these forms useful for studying the function of the third Kunitz domain and basic C-terminal region of TFPI.

## RESULTS

*Structures of TFPI and the Altered Forms of TFPI.* The functional significance of the third Kunitz domain and C-terminal region of TFPI was probed using three different forms of TFPI. Diagrams of the primary amino acid sequence and Kunitz domain structure of full-length TFPI, TFPI-160, and K1K2C are presented in Figure 1. These proteins have previously been characterized by SDS−PAGE and western analysis using an antibody directed against the C-terminal

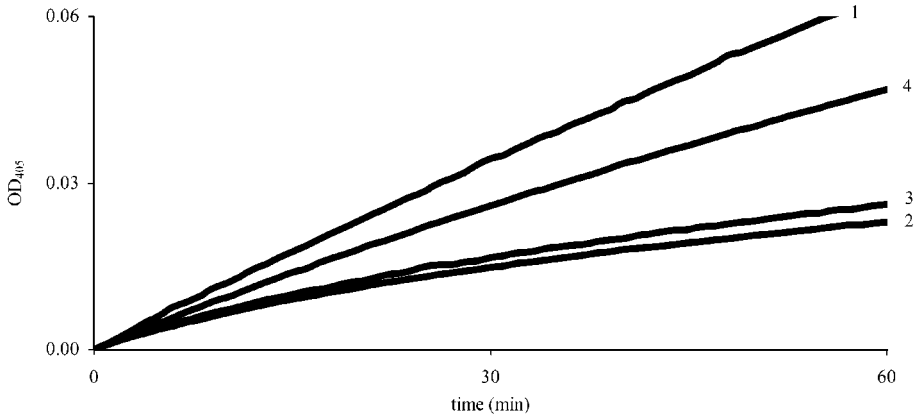


FIGURE 2: Inhibition of factor Xa by TFPI, K1K2C, and TFPI-160 in an amidolytic assay. Cleavage of 500 $\mu$mol/L Spectrozyme Xa is monitored continuously in reactions initiated with 0.2 nmol/L factor Xa, analyzing the effects of 5 nmol/L TFPI, K1K2C, and TFPI-160 in the presence of 5 mmol/L $CaCl_2$. Samples are as follows: (1) no inhibitor; (2) TFPI; (3) K1K2C; (4) TFPI-160. The progress curves shown are representative examples ($n = 3$) of curves used to generate the $K_i$(final) values presented in Table 1.

Table 1: Kinetic Constants for Inhibition of Factor Xa by TFPI, K1K2C, and TFPI-160

| | $K_i$(initial) (nM) | | | $K_i$(final) (pM) | | |
|---|---|---|---|---|---|---|
| | TFPI | K1K2C | TFPI-160 | TFPI | K1K2C | TFPI-160 |
| EDTA | 1.5 | 3.7 | 10.7 | 35 | 30 | 79 |
| $Ca^{2+}$ | 8.0 | 4.0 | 12.0 | 75 | 133 | 240 |
| $Ca^{2+}$, PL | 7.1 | 6.7 | 6.5 | 75 | 465[a] | 1382[a] |
| $Ca^{2+}$, Va, PL | 2.9 | 2.3 | 3.2 | 38 | 900[a] | 2367[a] |
| Gla-less Xa with $Ca^{2+}$ | 16.8 | 23.3 | 13.2 | 648[b] | 656[b] | 312 |

[a] Denotes statistical significance ($p < 0.01$) between altered forms and full-length TFPI. [b] Denotes statistical significance ($p < 0.01$) with TFPI or K1K2C using either wild-type Xa or Gla-less Xa.

region of TFPI to demonstrate purity and the presence of the C-terminal region in full-length TFPI and K1K2C but not TFPI-160 (*7*).

*TFPI and K1K2C Are More Effective Inhibitors of Factor Xa than TFPI-160 in Amidolytic Assays.* TFPI is similar to other Kunitz-type proteinase inhibitors in that it is a slow, tight-binding inhibitor of factor Xa that forms an immediate encounter complex, which slowly isomerizes to a final tight complex according to the mechanism (*14*):

$$E + I \underset{k_2}{\overset{k_1}{\rightleftarrows}} EI \underset{k_4}{\overset{k_3}{\rightleftarrows}} EI^*$$

where EI represents the immediate encounter complex with $K_i$(initial) $= k_2/k_1$ and EI* represents the isomerized, tightened complex with $K_i$(final) $= K_i$(initial) $[k_4/(k_3 + k_4)]$. To examine and quantify the factor Xa inhibitory activity of each of the three forms of TFPI, progress curves measuring hydrolysis of the chromogenic substrate, Spectrozyme Xa, were performed under conditions that allow determination of $K_i$(initial) and $K_i$(final) (see Materials and Methods). Previous studies by several groups have demonstrated that C-terminally truncated forms of TFPI, such as TFPI-160, have decreased inhibitory potency against factor Xa compared to TFPI (*4*, *6*, *15*, *16*), which we have confirmed here (Figure 2, Table 1). The reduced inhibitory activity is caused by an increase in both the $K_i$(initial) (in the absence of calcium ions) and the $K_i$(final) (in either the presence or absence of calcium ions) values, indicating that in the absence of the amino acids distal to Gly-160, including the third Kunitz domain and C-terminal tail, formation of the initial encounter complex is slowed and the final isomerized complex is not as tight as that formed between full-length TFPI and factor Xa. In contrast, the factor Xa inhibitory capacity of K1K2C is similar to that of full-length TFPI in both the absence and presence of calcium ions (Figure 2, Table 1), indicating that attachment of the C-terminal region to the end of the first two Kunitz domains of TFPI nearly restores its ability to inhibit factor Xa in solution phase amidolytic assays.

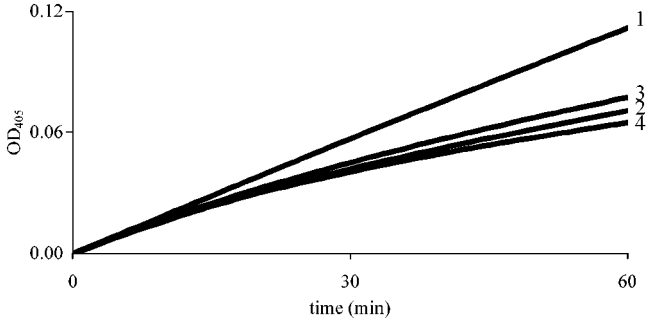


FIGURE 3: Inhibition of Gla-domainless factor Xa by TFPI, K1K2C, and TFPI-160 in an amidolytic assay. Cleavage of 500 $\mu$mol/L Spectrozyme Xa is monitored continuously in reactions initiated with 0.2 nmol/L Gla-domainless factor Xa, analyzing the effects of 5 nmol/L TFPI, K1K2C, and TFPI-160 in the presence of 5 mmol/L $CaCl_2$. Samples are as follows: (1) no inhibitor; (2) TFPI; (3) K1K2C; (4), TFPI-160. The progress curves shown are representative examples ($n = 3$) of curves used to generate the $K_i$-(final) values presented in Table 1.

*K1K2C and TFPI Are Poor Inhibitors of Gla-Domainless Factor Xa.* In amidolytic assays where Gla-domainless factor Xa is substituted for factor Xa, the factor Xa inhibitory capacity of both K1K2C and TFPI is greatly reduced, while that of TFPI-160 is not substantially altered in either the absence (data not shown) or presence of calcium ions (Figure 3, Table 1). Sequential addition of phospholipids and factor Va has only a minimal effect, consistent with the inability of Gla-domainless factor Xa to bind to phospholipid and assemble the prothrombinase complex (data not shown).

*Neither K1K2C nor TFPI-160 Is an Effective Inhibitor of Clot Formation in Factor Xa-Initiated Coagulation Assays.* When compared to TFPI, K1K2C and TFPI-160 are much less effective (~10- and 20-fold, respectively) inhibitors of clot formation in factor Xa-initiated coagulation assays

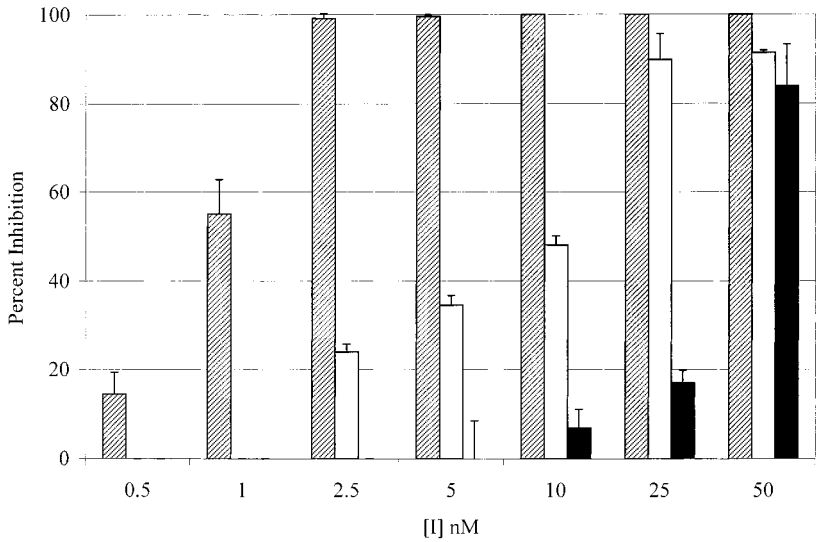


FIGURE 4: Inhibition of factor Xa-initiated plasma clotting assays by TFPI, K1K2C, and TFPI-160. The generation of a fibrin clot was measured in assays initiated by addition of 0.1 nmol/L factor Xa to mixtures of plasma, 5 mmol/L $CaCl_2$, 2% phospholipids, and inhibitor at the indicated concentrations. Clotting times were compared to a standard curve to determine apparent factor Xa concentration. Percent inhibition was determined by comparing the apparent factor Xa concentration in the presence of inhibitor to the factor Xa concentration in the absence of inhibitor. Hatched bars represent TFPI. Open bars represent K1K2C. Solid bars represent TFPI-160. The data presented are the average of quadruplicate experiments. Error bars represent one standard deviation.

(Figure 4). Thus, although the region between Gly-160 and the end of the third Kunitz domain is not required for rapid factor Xa inhibitory activity in solution phase amidolytic assays, it is critical for the anticoagulant activity of TFPI in factor Xa-initiated plasma coagulation assays.

*Sequential Addition of Phospholipids and Factor Va Has Opposite Effects on the Inhibitory Activity of TFPI and K1K2C.* The discrepancy between the factor Xa inhibitory activity of K1K2C in amidolytic and clot-based assays was further investigated by performing amidolytic assays in the presence of the other components of the prothrombinase complex, phospholipids and factor Va. It has previously been demonstrated that the interaction between TFPI and factor Xa is substantially weakened by the addition of physiologic concentrations of calcium ions (*17*). However, upon sequential addition of phospholipids and factor Va, TFPI increasingly inhibited factor Xa, nearly restoring the inhibitory activity to that observed in the absence of calcium ions (Figure 5a, Table 1) (*17*). In contrast to TFPI, the factor Xa inhibitory activity of both K1K2C and TFPI-160 progressively decreases with addition of phospholipids and factor Va, almost entirely through an effect on the $K_i$(final) (Figure 5b, Table 1). The addition of phospholipids to the reaction mixtures was responsible for most of the increase in $K_i$ (final) for both TFPI-160 and K1K2C (3−5-fold), with the subsequent addition of factor Va causing another 2-fold increase (Table 1). In reactions initiated with a mixture of Spectrozyme Xa and inhibitor, after the prothrombinase complex is allowed to form, the progress curves are identical to those initiated with factor Xa for all three forms of TFPI (data not shown).

*Comparison of TFPI, K1K2C, and TFPI-160 in Prothrombin Activation Assays Using Normal and Short-Chain Phospholipids.* To further investigate the interaction of the three forms of TFPI with phospholipids and the other components of the prothrombinase complex, prothrombin activation assays were performed. In these assays, the prothrombinase complex cleaves its physiologic substrate, prothrombin, rather than the chromogenic substrate used in the amidolytic assays. When TFPI, TFPI-160, and K1K2C are compared in factor Xa-initiated prothrombin activation assays, using either purified factor Va and phospholipids (Figure 6a) or platelets (Figure 6b) as a source of phospholipids and factor Va, TFPI-160 and K1K2C are much less effective inhibitors of thrombin generation than TFPI. These data are consistent with their decreased potency in the clot-based and amidolytic assays containing phospholipids and factor Va (Figures 4 and 5).

C6PS is a short-chain phospholipid that has been shown to assemble the prothrombinase complex in a calcium-dependent manner and enhance thrombin generation by ~60-fold (*12*). Prothrombinase assembled with C6PS at submicellar concentrations (<4.0 mM) allows study of thrombin generation by the prothrombinase complex in the absence of a two-dimensional phospholipid surface. Its mechanism of action is thought to be mediated largely through induction of the phospholipid-mediated change in the active site conformation of factor Xa, but it also binds prothrombin and alters its conformation (*12*). When submicellar concentrations of C6PS are substituted for the crude phospholipids in factor Xa-initiated reactions, all three forms of TFPI (10 nM final concentration) totally prevent thrombin generation (data not shown). Since prothrombinase assembly with C6PS is slow, it is likely that the inhibitory effect observed in these factor Xa initiated reactions is due to direct inhibition of factor Xa before it is incorporated into the prothrombinase complex. When the inhibitory activities of TFPI, TFPI-160, and K1K2C are compared in reactions initiated with a mixture of TFPI and prothrombin, after the C6PS-assembled prothrombinase complex is allowed to form, TFPI is a potent inhibitor of thrombin generation, while TFPI-160 and K1K2C have little inhibitory effect (Figure 7a). This is dramatically different from inhibition of a preformed prothrombinase complex assembled on a two-dimensional phospholipid surface where TFPI, as well as TFPI-160 and K1K2C, is a very weak inhibitor of thrombin generation (Figure 7b) (*18*).

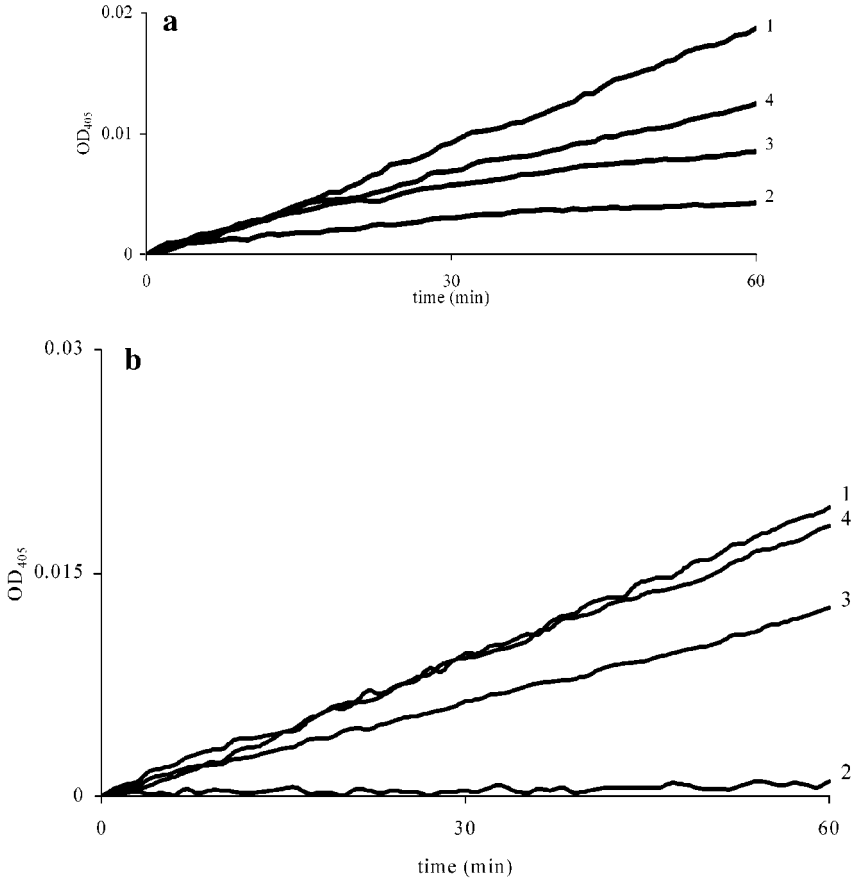


FIGURE 5: Inhibition of factor Xa by TFPI, K1K2C, and TFPI-160 in an amidolytic assay in the presence of phospholipids or phospholipids and factor Va. Cleavage of 500 $\mu$mol/L Spectrozyme Xa is monitored continuously in reactions initiated with 0.2 nmol/L factor Xa, analyzing the effects of 5 nmol/L TFPI, K1K2C, and TFPI-160 on the reaction. The reaction is performed in 5 mmol/L $CaCl_2$. In panel a, phospholipids (1%) were added to the reaction mixture. In panel b, phospholipids and factor Va (18.7 nmol/L) were added to the reaction mixture. Samples are as follows: (1) no inhibitor; (2) TFPI; (3) K1K2C; (4) TFPI-160. The progress curves shown are representative examples ($n = 3$) of curves used to generate the $K_i$(final) values presented in Table 1.

## DISCUSSION

Most TFPI is located on the endothelium where it regulates tissue factor-initiated blood coagulation. Its importance in maintaining anticoagulant surfaces within the vasculature is best demonstrated in mice lacking the first Kunitz domain of TFPI, which die *in utero* from disseminated intravascular coagulation (*19*). Localization of TFPI to the endothelial surface is thought to occur either through nonspecific association with glycosaminoglycans and phospholipids (heparin-releasable TFPI) (*8*, *20*, *21*) or through a glycosylphosphatidylinositol- (GPI-) anchored binding protein(s) (*22*−*25*). The C-terminal region of TFPI most likely binds to the cell surface, allowing the first and second Kunitz domains to interact with other membrane-associated and soluble proteins located on or near the cell surface. The third Kunitz domain is also thought to be accessible to interactions with soluble or membrane proteins (*26*, *27*), yet it has no identified function as a proteinase inhibitor.

We have used TFPI and two altered forms of TFPI, TFPI-160 and K1K2C, to probe potential anticoagulant functions of the third Kunitz domain and surrounding sequence. It has previously been shown that truncated forms of TFPI, including TFPI-160, which lack the C-terminal region, are relatively poor inhibitors of factor Xa (*4*, *15*, *16*). The data presented here demonstrate that when the C-terminal region is attached to the end of the second Kunitz domain to create K1K2C, the factor Xa inhibitory activity of TFPI is nearly restored in the absence of phospholipids. These data indicate that the C-terminal region, but not the third Kunitz domain, is required for rapid inhibition of factor Xa by TFPI in the solution phase. In amidolytic assays performed using Gla-domainless factor Xa, both TFPI and K1K2C have dramatically reduced inhibitory activity, primarily through an effect on the $K_i$(final), while the inhibitory activity of TFPI-160 is not substantially altered. These data indicate that interactions between either the factor Xa Gla domain or a region of factor Xa structurally dependent on an intact Gla domain and the C-terminal region of TFPI occur and are important for formation of the final tight complex between factor Xa and TFPI in the solution phase. The venom of *Deinagkistrodon acutus* (hundred pace snake) contains an anticoagulant protein that functions by binding to the Gla domain region of factor Xa, slowing its association with phospholipids and thereby reducing prothrombinase activity (*28*, *29*). The data presented here suggest that the C-terminal region of TFPI may inhibit formation of the prothrombinase complex in a similar manner.

It has been demonstrated that interactions between phospholipids and factor Xa alter the conformation of factor Xa such that it becomes a more efficient activator of prothrombin (*12*, *30*, *31*). The presence of factor Va has also been shown

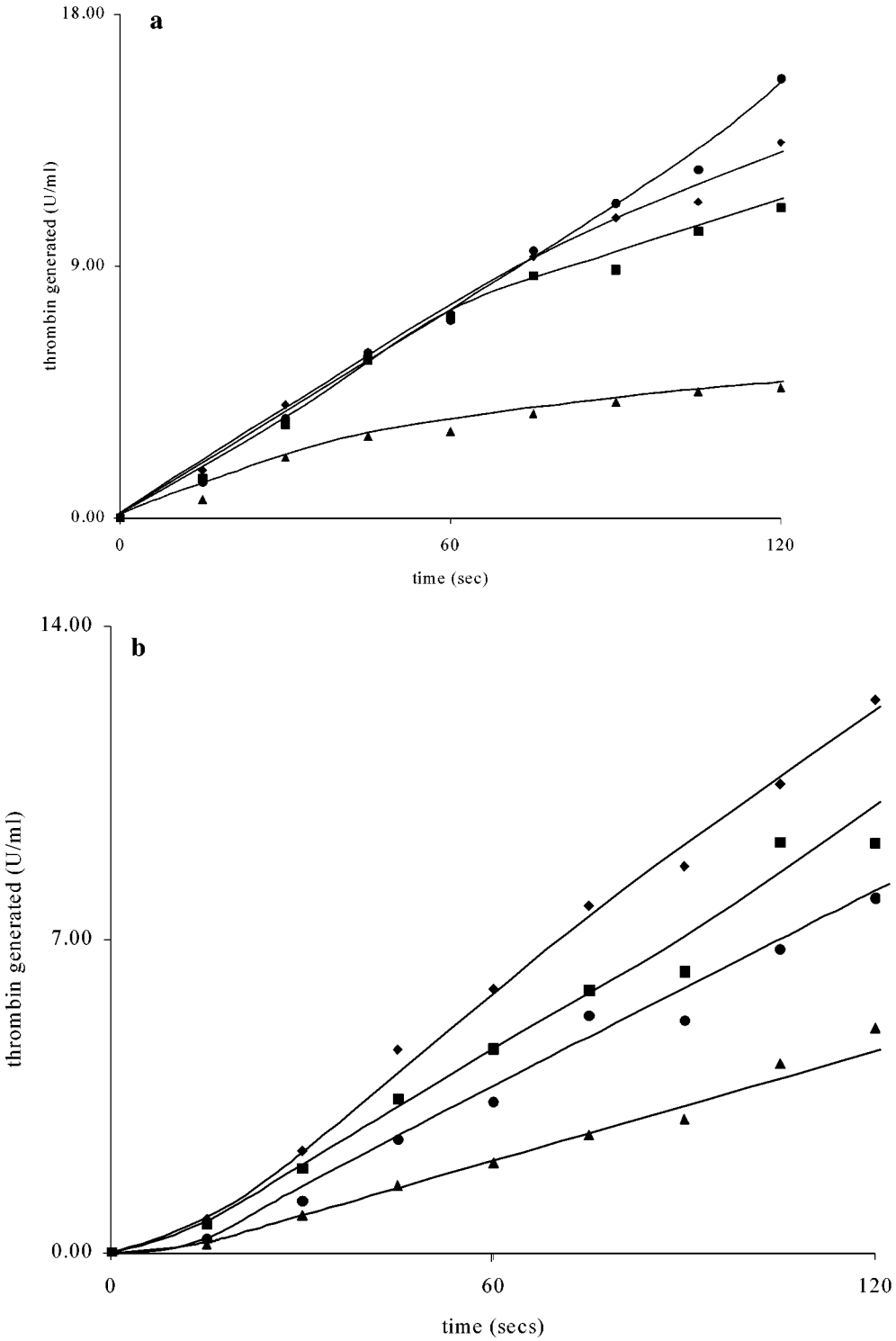


FIGURE 6: Inhibition of thrombin generation by TFPI, K1K2C, and TFPI-160 in factor Xa-initiated assays. In panel a, factor Xa (0.1 nmol/L) was added to reactions containing calcium (2.5 mmol/L), saturating phospholipids, factor Va (3 nmol/L), prothrombin (1.4 $\mu$mol/L), and inhibitor at 10 nmol/L. In panel b, platelets ($5 \times 10^8$/mL) were substituted for factor Va and saturating phospholipids. For each experiment, samples were removed at 15 s intervals, diluted 100-fold into 5 mmol/L EDTA, and assayed for thrombin activity. Samples are as follows: (◆) no inhibitor; (▲) TFPI; (●) K1K2C; (■) TFPI-160.

to alter the conformation of factor Xa (*32*−*34*). Although K1K2C inhibits factor Xa nearly as well as TFPI in the absence of phospholipids, it is a much worse inhibitor than TFPI in amidolytic, plasma coagulation, and prothrombin activation assays where phospholipids are present. The effect of phospholipids is demonstrated most clearly in amidolytic assays, where they produce opposite effects on TFPI, which becomes a more efficient inhibitor of factor Xa, and K1K2C, which becomes less efficient (Figure 5, Table 1). This effect is amplified by addition of factor Va to the reaction mixtures (Figure 5, Table 1). Thus, it appears that although the third Kunitz domain is not necessary for effective inhibition of factor Xa in the absence of phospholipids and factor Va, in the presence of a phospholipid surface and factor Va, the third Kunitz domain and/or the connecting region between the second and third Kunitz domains contribute(s) in a

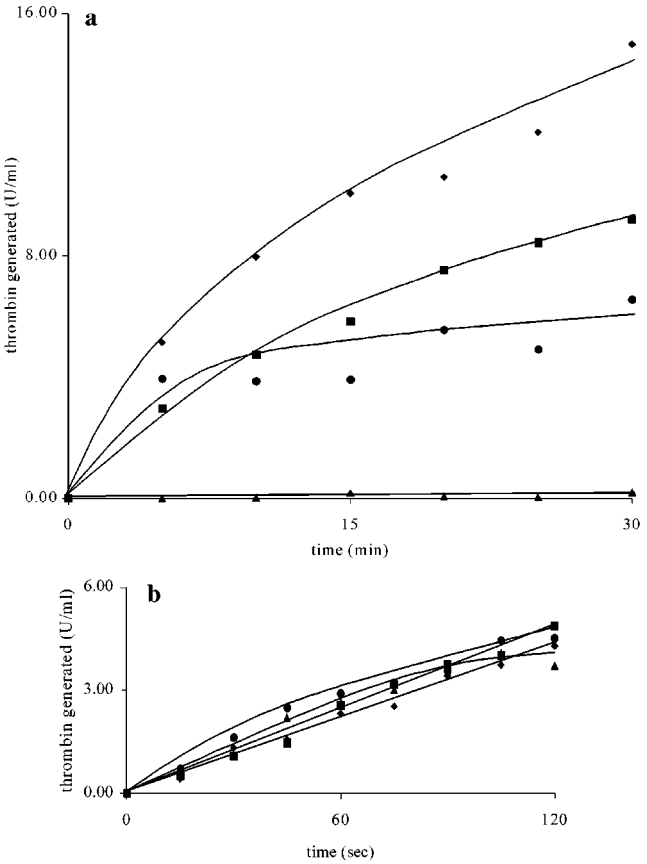


FIGURE 7: Inhibition of thrombin generation by a preformed prothrombinase complex by TFPI, K1K2C, and TFPI-160 in assays using normal phospholipids or C6PS mixtures. Prothrombin (1.4 $\mu$mol/L) and inhibitor (TFPI, K1K2C, or TFPI-160) at 10 nmol/L were added to mixtures of factor Xa (0.1 nmol/L), calcium (2.5 mmol/L), factor Va (3 nmol/L), and either submicellar concentrations (3 mmol/L) of C6PS (panel a) or saturating phospholipids (panel b). Samples in panel a were removed at 5 min intervals, diluted 100-fold into 5 mmol/L EDTA, and assayed for thrombin activity. Samples in panel b were removed at 15 s intervals, diluted into EDTA, and assayed for thrombin activity. Samples are as follows: (◆) no inhibitor; (▲) TFPI; (●) K1K2C; (■) TFPI-160.

physiologically significant manner to the anticoagulant activity of TFPI.

Prothrombin activation assays were performed in the presence of the three forms of TFPI to further characterize the anticoagulant function of the third Kunitz domain and surrounding sequence. We have previously shown that TFPI produces modest inhibition of thrombin generation in assays initiated using factor Xa (*18*). Both TFPI-160 and K1K2C are much less effective inhibitors of thrombin generation in these assays than TFPI, which is consistent with their reduced rate of factor Xa inhibition in the presence of phospholipids and factor Va. In reactions initiated with a mixture of inhibitor and prothrombin, after assembly of the prothrombinase complex on a two-dimensional phospholipid bilayer is allowed, TFPI, TFPI-160, and K1K2C are all poor inhibitors of thrombin generation at concentrations $\leq$10 nM. The inability of TFPI to prevent the prothrombinase complex from cleaving prothrombin in these assays is not caused by a conformational change at or near the reactive site of factor Xa that prevents binding of the second Kunitz domain because TFPI prevents prothrombinase from cleaving amidolytic substrates. Rather, it appears that interactions between prothrombinase and prothrombin on the lipid bilayer may block nonactive site interactions between TFPI and prothrombinase that are necessary for access of the second Kunitz domain to the active site of factor Xa. Similarly, the inhibition of thrombin generation observed in the factor Xa-initiated reactions is mediated either through inhibition of factor Xa before it assembles into the prothrombinase complex or by nonactive site interactions between TFPI and factors Va and/or Xa that slow the rate of prothrombinase assembly.

The function of the phospholipid bilayer in the interactions between TFPI, prothrombinase, and prothrombin was further investigated in prothrombin activation assays using submicellar concentrations of C6PS under conditions that allow assembly of the prothrombinase complex in the absence of a two-dimensional phospholipid surface. In these assays, TFPI effectively prevents activation of prothrombin, confirming that the interactions between prothrombin and the prothrombinase complex on a two-dimensional phospholipid surface prevent TFPI inhibitory activity in a manner that is independent of the interaction between the second Kunitz domain and the active site of factor Xa. It has previously been demonstrated that the lipid bilayer of the prothrombinase complex acts both as a surface for condensing proteins and as a functional element of the prothrombinase complex that directly alters the catalytic activity of factor Xa (*30*, *31*). The data presented here suggest that a third function of the bilayer is to allow efficient interactions between the prothrombinase complex and prothrombin that greatly reduce the susceptibility of the prothrombinase complex to inhibition by TFPI.

In contrast to TFPI, neither TFPI-160 nor K1K2C effectively inhibits thrombin generation by the prothrombinase complex assembled with submicellar concentrations of C6PS. These data indicate that the region between Gly-160 and the end of the third Kunitz domain maintains the second Kunitz domain of TFPI in the proper conformation for inhibition of phospholipid-associated factor Xa. This is independent from functioning as a spacer to properly distance the second Kunitz domain off the lipid bilayer for interaction with the active site of factor Xa. Instead, it is possible that nonactive site interactions occur between this region of TFPI and factor Xa that properly orient the second Kunitz domain such that it can bind to the active site of phospholipid-associated factor Xa.

The data presented here demonstrate that the third Kunitz domain and surrounding sequence participate in the anticoagulant activity of TFPI, most likely by maintaining the second Kunitz domain in the proper orientation such that it can efficiently bind the active site of phospholipid-bound factor Xa before the prothrombinase complex forms and/or by slowing formation of the prothrombinase complex. Since the activation of prothrombin to thrombin by phospholipid-associated factor Xa and the subsequent assembly of the prothrombinase complex are thought to be early events in the formation of a blood clot, these anticoagulant functions are consistent with TFPI's role as an inhibitor of the initial stages of blood coagulation.

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