Tissue Factor Residues Lys^{165} and Lys^{166} Are Essential for Rapid Formation of the Quaternary Complex of Tissue Factor·VIIa with Xa·Tissue Factor Pathway Inhibitor†

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ABSTRACT: The extrinsic coagulation pathway is initiated by the binding of plasma factor VII(a) (VIIa) to the cell surface receptor tissue factor (TF), which serves as the cofactor for the ligand protease VIIa in the activation of macromolecular substrate factors X and IX. The catalytic function of the TF·VIIa complex is regulated by a specific Kunitz-type inhibitor, tissue factor pathway inhibitor (TFPI), which forms a stoichiometric complex with the serine protease factor Xa (Xa), resulting in greatly accelerated inhibition of the extrinsic initiation complex as compared to free inhibitor. In the present study we identify specific residues in the TF·VIIa complex that are involved in the factor Xa-mediated acceleration of TFPI inhibitory function. VIIa residue Arg^{290}, which contributes to extended recognition of macromolecular substrate factor X, is not involved in the interaction with the TFPI·Xa complex. In contrast, TF residues Lys^{165} and Lys^{166}, which are important for the activation of factor X, are required for the accelerated inhibition of the TF·VIIa complex by TFPI mediated by factor Xa. These data indicate that similar interactions contribute to the assembly of substrate factor X as well as of product Xa after complex formation with TFPI, suggesting a central role for the carboxyl-terminal structural module of TF in regulating the proteolytic activity of TF·VIIa.

The *in vivo* initiation of the coagulation pathways is triggered by the exposure of circulating plasma factor VII(a) (VIIa)[1] to the cell surface receptor tissue factor (TF), which serves as the cofactor for VIIa (Rapaport & Rao, 1992; Ruf & Edgington, 1994). Complex formation of VIIa with TF results in a marked enhancement of the catalytic function of the bound protease. The enhanced catalytic activity of VIIa is due, in part, to its interaction with the cofactor, which apparently supports the reorientation and insertion of the amino terminus of the VIIa catalytic domain, leading to activation of the catalytic triad (Higashi et al., 1994). The activity of the catalytic triad can readily be monitored with small peptidyl substrates, which are hydrolyzed at accelerated rates in the presence of the cofactor (Ruf et al., 1991; Butenas et al., 1993). However, mutants of TF as well as of VIIa have been characterized that form TF·VIIa complexes exhibiting normal catalytic function toward small pseudosubstrates, but markedly reduced catalytic activity toward the natural macromolecular substrate factor X. Because the mutations did not reduce the affinity of the enzyme−cofactor interaction, this indicates that these residues, namely, Lys^{165} and Lys^{166} of TF (Roy et al., 1991; Ruf et al., 1992) and Arg^{290} of VIIa (Ruf, 1994), contribute either directly or indirectly to extended recognition of the macromolecular substrate factor X.

The principal physiological modulator of TF·VIIa activity is the tissue factor pathway inhibitor (TFPI) (Broze et al., 1990; Rapaport, 1991). TFPI is a multidomain protein (Wun et al., 1988) with an acidic aminoterminus, three typical Kunitz-type inhibitor domains, and a basic carboxyl terminus conferring affinity for glycosaminoglycans (Wesselschmidt et al., 1993). TFPI inhibits TF·VIIa activity in a two-step stoichiometric reaction (Broze et al., 1988; Warn-Cramer et al., 1988). In the first step, a reactive center arginine in the second Kunitz domain binds to the active site of factor Xa (Xa), resulting in the formation of a catalytically inactive TFPI·Xa complex (Girard et al., 1989). In the second step, a reactive center lysine in the first Kunitz domain of TFPI binds to the TF·VIIa complex, resulting in the formation of a quaternary TF·VIIa·TFPI·Xa complex with inhibited TF·VIIa catalytic activity (Girard et al., 1989). Although Xa enhances the inhibition of TF·VIIa function by TFPI, the inhibition is not absolutely dependent on the presence of Xa (Pedersen et al., 1990; Callander et al., 1992). The light chain of Xa has been identified as critical in mediating the interaction of TFPI·Xa with the TF·VIIa complex (Girard et al., 1990). Moreover, it has been shown that the γ-carboxyglutamic acid-rich domain (Gla domain) of Xa is important for TFPI·Xa-mediated inhibition of TF·VIIa activity (Warn-Cramer et al., 1988). Despite the considerable progress in understanding the biochemistry of the inhibition of TF·VIIa, no specific residues have been identified in either Xa, VIIa, or TF that are involved in the macromolecular assembly of the TF·VIIa·TFPI·Xa complex. In the present study, we test the hypothesis that recognition structures for substrate factor

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[1] Abbreviations: VIIa, coagulation factor VIIa; TF, tissue factor; Xa, coagulation factor Xa; TFPI, tissue factor pathway inhibitor.

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X present in the enzyme·cofactor complex contribute to the rate-enhancing effect of Xa during the quaternary complex formation. We identify Lys^{165} and Lys^{166} in TF as important residues for rapid inhibition of the TF·VIIa complex by TFPI in the presence of Xa.

EXPERIMENTAL PROCEDURES

Proteins. Wild-type TF was expressed in insect cells using baculovirus technology (Ruf & Edgington, 1991b). The cell line expressing the TF mutant Lys^{165}→Ala, Lys^{166}→Ala has been described (Ruf et al., 1992). Mutant TF was purified from cells after lipid extraction by acetone (Broze et al., 1985) and heptane/butanol (Morrissey et al., 1988), followed by solubilization of the protein in CHAPS and adsorption to an immunoaffinity matrix of monoclonal antibody TF8-5G9 (Morrissey et al., 1988). After extensive washes with 5 and 0.5 mM aqueous solutions of CHAPS, the protein was eluted with 50 mM acetate buffer at pH 2.7, and the volatile buffer was removed by lyophilization. Concentrations of TF were determined by immunoassay calibrated with purified wild-type TF of known concentration based on amino acid composition (Ruf et al., 1993). Purified wild-type and mutant TFs were reconstituted into mixed phosphatidylcholine/phosphatidylserine (70/30, w/w) vesicles as described previously (Ruf & Edgington, 1991a). The efficiency of relipidation was assessed by analyzing the catalysis of peptidyl substrates by TF·VIIa complexes at saturation with VIIa, and the concentrations given in this study are the effective concentrations determined by this assay. The expression, purification, and characterization of VIIa Arg^{290}→Ala have been described in detail (Ruf, 1994). Wild-type recombinant VIIa was a gift from Novo-Nordisk (Gentfote, Denmark). Recombinant TFPI was expressed in baby hamster kidney cells and purified as described previously in detail by Pedersen et al. (1990). These preparations of TFPI lack carboxyl-terminal basic residues (Nordfang et al., 1991), but nonetheless possess the same specific activity as full length TFPI in inhibiting TF·VIIa activity in the presence of Xa (Nordfang et al., 1991; Wesselschmidt et al., 1992). Xa was prepared by incubating purified factor X (Bajaj et al., 1981) with Russell's viper venom and then separating the Xa by benzamidine Sepharose chromatography (Rao et al., 1992).

Concentration Dependence of Inhibition of TF·VIIa by TFPI. Inhibition of TF·VIIa catalytic activity by free TFPI or TFPI·Xa was measured in an end stage amidolytic assay. In this assay, 20 nM VIIa was incubated with 20 nM TF in calcium (5 mM) containing buffer [50 mM Tris-HCl and 0.15 M NaCl (pH 7.5), supplemented with 5 mg/mL fatty acid free bovine serum albumin as carrier protein] in a microtiter plate to which varying concentrations of TFPI·Xa or TFPI were added. At the end of a 60 min incubation period at room temperature, Chromozym tPA (1.25 mg/mL) was added to the wells, and residual TF·VIIa activity was analyzed by monitoring the absorbance increase at 405 nm (in mOD/min) of a 150 μL reaction mixture on a microplate reader (Molecular Devices, Palo Alto, CA). For all experiments, the TFPI·Xa complex was preformed by incubating Xa with a slight molar excess (5%) of TFPI for 30 min at room temperature before the complex was added to TF·VIIa. Under these experimental conditions, the contribution of Xa toward the hydrolysis of synthetic substrate was negligible (less than 5%). All of the reagent concentrations listed in this and the following sections are the final concentrations before the addition of chromogenic substrate.

Kinetics of Inhibition of TF·VIIa by TFPI. The rate of inhibition of TF·VIIa complexes by TFPI·Xa or free TFPI was analyzed by monitoring the residual TF·VIIa amidolytic activity after incubation with TFPI·Xa or TFPI for varying times. To form TF·VIIa complexes, VIIa (20 nM) was incubated with TF (20 nM) in the presence of calcium ions (5 mM) in a microtiter well for 5 min at room temperature. Free TFPI (100 nM) or TFPI·Xa (20 nM) that was preformed as described earlier was added to the TF·VIIa complex and allowed to react for 15 s to 30 min. The chromogenic substrate Chromozym tPA (1.25 mg/mL) was added to the sample, and the rate of absorbance increase was immediately monitored in a kinetic microtiter plate reader for 2 min. The delay from the addition of the substrate to the beginning of the recording was about 20−30 s and care was taken to stay within those limits. On the basis of repeated short interval readings of chromogenic substrate hydrolysis, there was no evidence for significant ongoing inhibition of the TF·VIIa complex by TFPI after the addition of the chromogenic substrate.

Alternatively, the kinetics of inhibition was analyzed with limiting TF at 0.1 nM and a slight excess of VIIa (0.5 nM), which were reacted with preformed TFPI·Xa (0.2 nM) for a defined period. The reaction was quenched by dilution into an excess of factor X (2 μM). This concentration of factor X was sufficient to reduce by >95% the ongoing inhibition by TFPI·Xa. Factor X activation by residual TF·VIIa complex was determined after a 1 min reaction that was terminated with EDTA followed by chromogenic assay for Xa. The rate of inhibition was determined from linear regression analysis of semilogarithmic plots of residual TF·VIIa concentration versus time.

RESULTS

Effect of Amino Acid Exchanges in TF and VIIa on Inhibition by Free TFPI. Proteolytic activity of the TF·VIIa complex is dependent on key basic residues in TF and VIIa. Exchange of these residues by alanine resulted in a functional defect characterized by the diminished activation of factor X, yet unaltered complex formation and induced change in catalytic activity in the hydrolysis of small peptidyl pseudosubstrates (Ruf et al., 1992; Ruf, 1994). The unaltered amidolytic activity of the mutant TF·VIIa complexes was employed to monitor the inhibition by TFPI and to assess the contribution of Xa in the formation of the quaternary complex. The $TF_{A165A166}$ mutant in complex with wild-type VIIa was efficiently inhibited by free TFPI (Figure 1A) when analyzed in an end point assay that approached equilibrium of complex formation. Similarly, TFPI inhibition of mutant $VIIa_{A290}$ complexed with wild-type TF was indistinguishable from that of inhibition of wild-type VIIa complexed with wild-type TF (Figure 1A). These data indicate that the equilibrium interaction of the free inhibitor with the TF·VIIa complex is unaffected by the mutations in the cofactor or the enzyme.

We further evaluated the kinetics of assembly of free TFPI with TF·VIIa complexes. TFPI bound to the $TF_{A165A166}$·VIIa complex with a rate (50% inhibition in 1.5 ± 0.31 min, n = 4) comparable to that observed for the wild-type TF·VIIa complex (50% inhibition in 1.2 ± 0.32 min, n = 4) (Figure

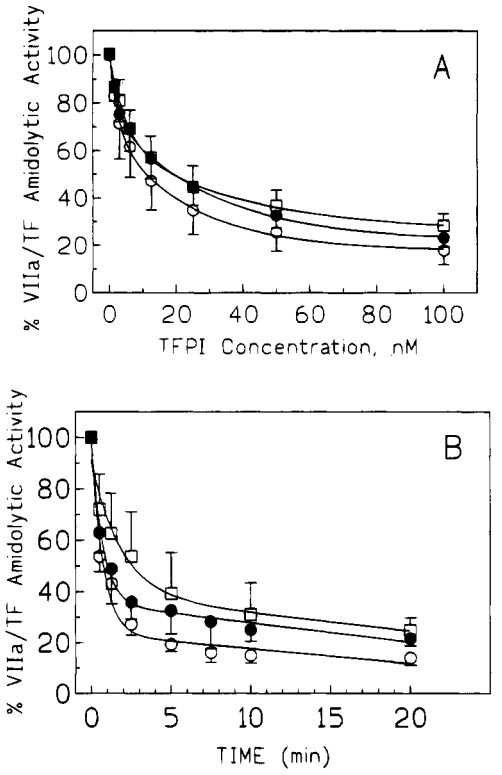

FIGURE 1: (A) Dose-dependent inhibition of TF·VIIa complexes by free TFPI. Inhibition of 20 nM wild-type TF (○) and mutant $TF_{A165A166}$ (●) in complex with 20 nM wild-type VIIa and inhibition of 20 nM mutant $VIIa_{A290}$ (□) in complex with wild-type TF (20 nM) by free TFPI at the indicated concentration are shown (n = 5). Residual TF·VIIa amidolytic activity was determined after 60 min of incubation at ambient temperature. (B) Time dependence of inhibition of TF·VIIa complexes by free TFPI. 20 nM wild-type TF (○) and mutant $TF_{A165A166}$ (●) were assembled with 20 nM wild-type VIIa and 20 nM mutant $VIIa_{A290}$ (□) was assembled with wild-type TF (20 nM) for 5 min, followed by incubation with 100 nM TFPI for the indicated time periods (n = 4). Means and standard deviations are shown.

1B). These data are consistent with the conclusions from previous analyses of the mutant, which indicated that the elimination of the two basic residue side chains in TF does not affect the active site environment of VIIa (Ruf et al., 1992), the presumed interactive site for the Kunitz domain 1 of TFPI. The inhibition of TF·$VIIa_{A290}$ complexes by free TFPI appeared to differ slightly from the inhibition of wild-type TF·VIIa complexes. The data in Figure 1B indicate a decreased rate (50% inhibition in 2.6 ± 0.35 min, n = 4) of complex formation as a consequence of the Arg^{290}→Ala mutation. A similar reduction in the rate of inhibition was apparent when the interactions of wild-type VIIa (50% inhibition in 6.0 ± 1.4 min, n = 3) or mutant VIIa (50% inhibition in 13.7 ± 0.8 min, n = 3) with TFPI were analyzed at a lower concentration of the inhibitor (Figure 2). These data indicate that the Arg side chain that is in close proximity to the catalytic triad of VIIa has subtle direct or local conformational effects on the interaction of the free inhibitor with the active site of VIIa.

VIIa Residue Arg^{290} Is Not Contributing to Interactions with TFPI·Xa. Residue Arg^{290} in VIIa had been shown to contribute to the efficient activation of factor X. To assess the role of this residue side chain in the interaction with the TFPI·Xa complex, the inhibition of TF·$VIIa_{A290}$ complexes was analyzed, both in kinetic analysis and after prolonged incubation with varying concentrations of TFPI·Xa. At all

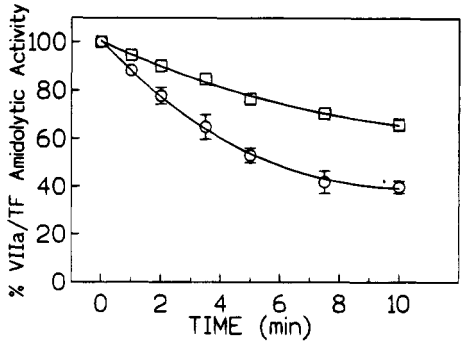

FIGURE 2: Time dependence of inhibition of TF·VIIa complexes by free TFPI. 20 nM wild-type TF was assembled with 20 nM wild-type VIIa (○) or 20 nM mutant $VIIa_{A290}$ (□) for 5 min, followed by incubation with 20 nM TFPI for the indicated time periods. Means and standard deviations are shown (n = 3).

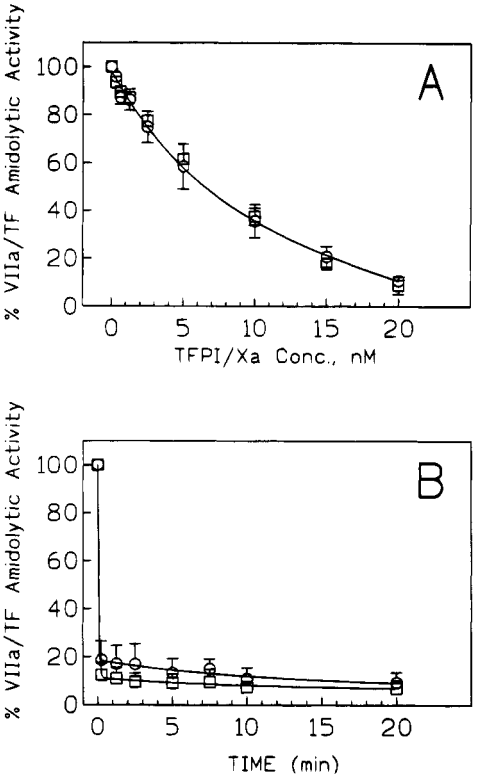

FIGURE 3: Effect of Xa on the inhibition of TF·$VIIa_{A290}$ by TFPI. The inhibition of TF complexed with mutant (□) or wild-type (○) VIIa was analyzed by monitoring the residual amidolytic function. (A) Concentration dependence of inhibition of 20 nM TF·VIIa complexes by varying concentrations of TFPI·Xa complexes after 60 min of incubation (n = 5). (B) Time course of inhibition of 20 nM TF·VIIa complexes by 20 nM TFPI·Xa complexes, which were preassembled for 30 min (n = 3). Means and standard deviations are shown.

concentrations, TFPI·Xa inhibited the mutant VIIa in complex with TF indistinguishable from complexes formed with wild-type enzyme (Figure 3A). This indicates that the Arg side chain is not required to form a stable quaternary complex. Despite the slightly reduced rate of inhibition by free TFPI (see Figures 1B and 2), TFPI complexed with Xa inhibited the mutant VIIa·TF complex efficiently. For both mutant and wild-type VIIa in complex with TF, the inhibition by TFPI·Xa was in excess of 80% within 15 s (Figure 3B). An apparent limitation of the data is the rapid reaction due to the high enzyme concentration in the reaction mixture, which was necessary to allow monitoring of VIIa/TF activity by

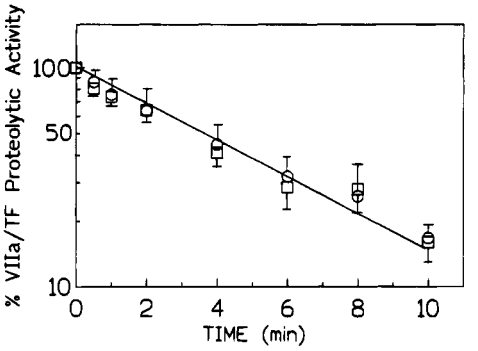

FIGURE 4: Kinetics of the inhibition of TF (0.1 nM) in complex with 0.5 nM wild-type VIIa (○) or mutant $VIIa_{A290}$ (□) by TFPI·Xa (0.2 nM). Residual TF·VIIa complexes were determined by monitoring the rate of factor X activation after the indicated reaction times. Means and standard deviations are shown ($n = 4$).

the chromogenic substrate. To exclude subtle differences in the rate of inhibition due to the mutation in VIIa, the kinetics of inhibition by much lower concentrations of TFPI·Xa (0.2 nM) was analyzed by using the activation of factor X as a measure for residual TF·VIIa complex function. Under these conditions, $TF{\cdot}VIIa_{A290}$ was also inhibited by TFPI·Xa at the same rate as the wild-type VIIa complex (Figure 4). These data emphasize that the Arg^{290} side chain does not play a substantial role in the rapid inhibition of the TF·VIIa complex by TFPI·Xa.

Cofactor Residues Lys^{165} and Lys^{166} Are Essential for Rapid Inhibition of TF·VIIa by TFPI·Xa. The $TF_{A165A166}$·VIIa complex was inhibited by free TFPI indistinguishable from wild-type complexes (see Figure 1). When tested after prolonged incubation, inhibition of the $TF_{A165A166}$·VIIa complex by low concentrations of TFPI·Xa was no different from the inhibition of wild-type TF·VIIa complexes (Figure 5A), although there appeared to be a slight decrease in the efficiency of inhibition with increasing concentrations of the inhibitor. Analysis of the kinetics of inhibition of the mutant TF·VIIa complex demonstrated that the rate of quaternary complex formation was severely reduced in comparison to wild-type TF·VIIa complexes (Figure 5B). Only half of the mutant TF·VIIa complexes were inhibited in approximately 3−4 min (3.4 ± 0.82 min, $n = 4$), as compared to >80% inhibition of wild-type complexes within 15 s. The inhibition of the mutant TF·VIIa complex was accelerated 2.5-fold by the presence of Xa (Figure 5), on the basis of comparison with the inhibition by free TFPI under identical conditions (50% inhibition in 8.8 ± 1.6 min, $n = 3$). This contrasts with the >25-fold increased rate in the reaction with the wild-type TF·VIIa complex. These data thus indicate the importance of the TF residues Lys^{165} and Lys^{166} for the rate-enhancing effect of Xa on the inhibition of TF·VIIa by TFPI.

DISCUSSION

The catalytic function of the TF·VIIa complex is regulated by the specific Kunitz-type inhibitor TFPI, and binding of TFPI to Xa greatly enhances the anticoagulant function of TFPI (Callander et al., 1992). This study addresses the hypothesis that Xa utilizes recognition structures in the enzyme·cofactor complex that mediate the assembly of macromolecular substrate factor X. Activation of factor X is dependent on basic residues in TF and VIIa that could potentially be involved in the formation of a larger charge

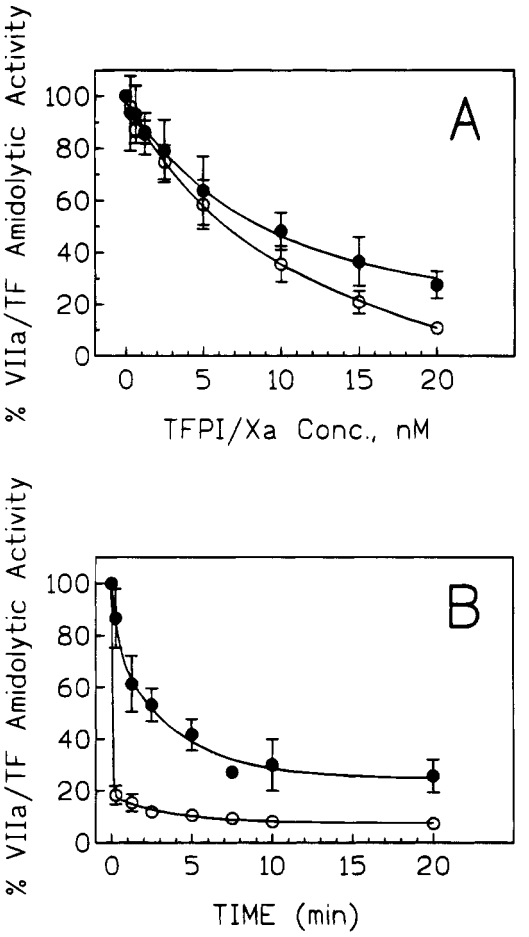

FIGURE 5: Effect of Xa on the inhibition of $TF_{A165A166}$·VIIa complexes by TFPI. Inhibition of wild-type TF (○) or $TF_{A165A166}$ (●) in complex with VIIa was analyzed by monitoring the residual amidolytic function. (A) Concentration dependence of inhibition of 20 nM catalytic complex by the indicated concentrations of TFPI·Xa complex ($n = 5$). (B) Time course of inhibition of 20 nM catalytic complex by 20 nM preformed TFPI·Xa complex ($n = 4$). Means and standard deviations are shown.

cluster, which supports the directed assembly of macromolecular substrate. Elimination of the charged side chain of Arg^{290} from enzyme VIIa did not affect the inhibition of TF·VIIa by free TFPI when analyzed in an end point assay. The rate of assembly of free TFPI with $TF{\cdot}VIIa_{A290}$ was reduced 2-fold, suggesting that this residue could contribute to the charge complementarity of the active site environment of VIIa, and the first Kunitz domain of TFPI may influence the assembly rate. However, the mutant complex was inhibited by TFPI·Xa at the same rate as the wild-type complex, indicating that Arg^{290} in the active site vicinity of VIIa is not contributing to assembly with the inhibitor in complex with Xa. The side chains of the VIIa protease domain that are involved in the extended recognition of inhibitor thus may contribute differently to the assembly of the free versus Xa-bound Kunitz-type inhibitor domains.

The exchange of TF residues Lys^{165} and Lys^{166}→Ala had little effect on the inhibition of TF·VIIa by free TFPI. However, elimination of the two basic side chains from TF essentially prevented the rate-enhancing effect observed in the presence of Xa. These data indicate that the basic residues are involved in interactions that depend on the presence of Xa. The residue side chains of Lys^{165} and Lys^{166} are highly solvent exposed and located in the carboxyl-

terminal module of TF. The residues are oriented away from the VIIa-binding site on TF, which is localized at the boundary between the two structural modules (Muller et al., 1994; Harlos et al., 1994; Ruf et al., 1994). VIIa-binding function of the $TF_{A165A166}$ mutant is normal in a variety of assays using different methodologies (Roy et al., 1991; Ruf et al., 1992, 1994), indicating a lack of energetic contributions of these residue side chains to VIIa interaction. Although indirect effects cannot be excluded, the spatial separation of the two Lys residues from the ligand-binding site (Muller et al. 1994; Harlos et al., 1994) may suggest that the residue side chains could be directly involved in the interaction with Xa during the assembly of the TFPI•Xa complex with TF•VIIa. An alternative possibility is that these TF residues could be involved in direct interactions with a specific site in TFPI that might have become exposed after complex formation with Xa.

Since no evidence was obtained that free Xa exhibits significant product inhibition in the activation of factor X by TF•VIIa (Krishnaswamy et al., 1992), any overlap in Xa and factor X recognition structures must be considered only partial. However, the structural requirements for activation of factor X by TF•VIIa have striking similarities with the demonstrated importance of the Xa light chain structure to enhance the inhibition of TFPI. The requirement for γ-carboxylation (Girard et al., 1990) or the presence of the Gla domain of Xa (Warn-Cramer et al., 1988) for efficient inhibition by TFPI is paralleled by the essential role of the Gla domain of factor X to allow efficient activation by TF•VIIa (Rezaie et al., 1993). Although the functional role of the Gla domain of factors X and Xa could in part relate to critical interactions with a charged phospholipid surface (Ruf et al., 1991; Krishnaswamy et al., 1992), the Gla domain of factor X is essential even in the absence of phospholipid (Rezaie et al., 1993). Protein−protein interactions critical for TFPI•Xa assembly with the TF•VIIa complex therefore may involve the Gla domain of Xa itself or the first epidermal growth factor domain, which has been suggested to be involved in the activation of factor IX by TF•VIIa (Zhong et al., 1994) and which appears to be conformationally dependent on the Gla domain (Valcarce et al., 1993). The present study provides the first evidence that specific residues in the cofactor TF are required for both substrate assembly and inhibitor interaction with the TF•VIIa complex. These findings indicate a central role for the carboxyl-terminal structural module of the TF extracellular domain in modulating the proteolytic activity of VIIa by directing the assembly of both substrate and inhibitor.

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