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Residues of the 39-Loop Restrict the Plasma Inhibitor Specificity of Factor IXa*

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Background: FXa and FIXa have homologous structures, but only FIXa is resistant to inhibition by plasma inhibitors.
Results: Replacing 39-loop residues with corresponding residues of FXa renders the FIXa chimera susceptible to inhibition by plasma inhibitors.
Conclusion: 39-Loop residues restrict the FIXa inhibitor specificity.
Significance: Insight into how plasma inhibitors regulate coagulation proteases is critical for understanding thrombosis and hemostasis.

The two plasma inhibitors, protein Z-dependent protease inhibitor (ZPI) and tissue factor pathway inhibitor (TFPI), effectively inhibit the activity of activated factor X (FXa); however, neither inhibitor exhibits any reactivity with the homologous protease activated factor IX (FIXa). In this study, we investigated the molecular basis for the lack of reactivity of FIXa with these plasma inhibitors and discovered that unique structural features within residues of the 39-loop are responsible for restricting the inhibitor specificity of FIXa. This loop in FXa is highly acidic and contains three Glu residues at positions 36, 37, and 39. On the other hand, the loop is shorter by one residue in FIXa (residue 37 is missing), and it contains a Lys and an Asp at positions 36 and 39, respectively. We discovered that replacing residues of the 39-loop (residues 31–41) of FIXa with corresponding residues of FXa renders the FIXa chimera susceptible to inactivation by both ZPI and TFPI. Thus, the inactivation rate of the FIXa chimera by ZPI in the presence of protein Z (PZ), negatively charged membrane vesicles, and calcium ions approached the same diffusion-limited rate ($>10^7\ M^{-1}\ s^{-1}$) that has been observed for the PZ-dependent inhibition of FXa by ZPI. Interestingly, sequence alignments indicated that, similar to FXa, residue 36 is a Glu in both mouse and bovine FIXa and that both proteases are also susceptible to inhibition by the PZ-ZPI complex. These results suggest that structural features within residues of the 39-loop contribute to the resistance of FIXa to inhibition by plasma inhibitors ZPI and TFPI.

The coordinated catalytic function of a series of trypsin-like serine proteases of the clotting cascade is responsible for thrombin generation in plasma during vascular injury (1, 2). This proteolytic cascade has been conventionally divided into two phases: initiation and amplification (3). The initiation phase is triggered when the subendothelial tissue factor (TF)[2] is exposed to circulating blood upon vascular injury. The high affinity binding of the first protease of the clotting cascade, factor VIIa (FVIIa), to exposed TF leads to the activation of homologous vitamin K-dependent zymogens factors IX and X to their activated forms (FIXa and FXa). Subsequent to activation of FX to FXa, the protease generates a small amount of thrombin through the activation of prothrombin, which is followed by thrombin amplifying its own generation by rapidly activating the homologous procoagulant cofactors V and VIII to their activated forms (FVa and FVIIIa). The complex formations of FVa with FXa and FVIIIa with FIXa on negatively charged membrane phospholipids in the presence of Ca^{2+} dramatically enhance the catalytic activity of these proteases toward their natural substrates, prothrombin and FX, respectively, thereby leading to generation of physiological levels of thrombin during the amplification phase of the clotting cascade (1–3).

Following zymogen activation and the formation of clots at injury sites, several plasma inhibitors must inhibit the proteolytic activities of coagulation proteases in both initiation and amplification phases of the cascade to prevent excessive clotting. The initiation phase of the cascade is regulated by the tissue factor pathway inhibitor (TFPI) (3, 4). TFPI is a multidomain Kunitz-type inhibitor that rapidly neutralizes the proteolytic activities of both FVIIa-TF and FXa by binding initially to the active site of FXa via its second Kunitz domain and thereafter by binding tightly to the active site of the FVIIa-TF complex via its first Kunitz domain, thereby trapping both proteases in inactive forms in a quaternary complex (4). The amplification phase of the clotting cascade is primarily regulated by antithrombin (AT). AT is a serine protease inhibitor (serpin) that

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[2] The abbreviations used are: TF, tissue factor; FIX, factor IX; FIXa, activated factor IX; FIXa-FX$^{39\text{-loop}}$, factor IXa mutant in which the residues of the 39-loop (residues 31–41) in the chymotrypsin numbering system (12) have been replaced with corresponding residues of FXa; FVIIIa, thrombin-activated factor VIII; FXa, activated factor X; FVIIa, activated factor VII; PZ, protein Z; ZPI, protein Z-dependent protease inhibitor; TFPI, tissue factor pathway inhibitor; AT, antithrombin; PC/PS, phosphatidylcholine/phosphatidylserine; SI, inhibition stoichiometry.

regulates the proteolytic activity of all coagulation proteases of the clotting cascade. It binds to the active site of its target protease through an exposed reactive center loop followed by undergoing a conformational change that leads to entrapment of the protease in the form of an inactive covalent serpin-protease complex (5–7). Another serpin that contributes to regulation of the clotting cascade is protein Z (PZ)-dependent protease inhibitor (ZPI) (8). However, in contrast to AT, ZPI has narrower protease specificity and thus is capable of only inhibiting FXa and factor XIa (FXIa) but not other coagulation proteases (8). The optimal inhibitory activity of ZPI toward FXa requires the cofactor function of PZ, which promotes the reactivity of the serpin with the protease by more than 3 orders of magnitude on the surface of negatively charged membrane phospholipids in the presence of calcium (9). Although structurally homologous to FXa, neither TFPI nor ZPI exhibit inhibitory activities toward FIXa. AT is the only known physiological inhibitor in plasma, which can inhibit FIXa, although with ~40-fold lower reactivity when compared with FXa (10).

The structural basis for the resistance of FIXa to inhibition by either ZPI or TFPI is not known. However, it is known that several surface loops surrounding the active site pockets have variant residues in FIXa and FXa that play critical roles in determining the substrate and inhibitor specificity of coagulation proteases. One of these surface loops, known as the variable region 1 or 39-loop (also referred to as the 37-loop), is known to participate in restricting the substrate and inhibitor specificity of all coagulation proteases (11, 12). In a recent study, we prepared a FIXa mutant in which the residues of this loop (residues 31–41) were replaced with the corresponding residues of FX (FIXa-FX$^{39\text{-loop}}$), and then we demonstrated that this loop plays a critical role in the reactivity of FIXa with AT and its catalytic activity in intrinsic tenase (10). In this study, we demonstrate that the 39-loop of FIXa also plays a critical role in restricting the ZPI and TFPI specificity of the protease. Thus, we discovered that the FIXa-FX$^{39\text{-loop}}$ chimera reacts with the ZPI-PZ complex with essentially the same rate as does FXa on negatively charged phospholipid vesicles in the presence of calcium. Interestingly, sequence alignments suggested that unlike human FIXa, residue 36 of both mouse and bovine FIXa is a Glu and that both of these proteases are also susceptible to inhibition by the ZPI-PZ complex. These results suggest that the residues of the 39-loop contribute to the lack of reactivity of human FIXa with the ZPI-PZ complex.

EXPERIMENTAL PROCEDURES

Construction, Mutagenesis, and Expression of Recombinant Proteins—The construction, expression, and purification of wild-type FIX using a novel expression/purification vector system in HEK-293 cells have been described (13). The expression and purification of the FIX mutant in which the residues of the 39-loop from residues Val^{31} to Phe^{41} (^{31}Val-Val-Leu-Asn-Gly-Lys-Val-Asp-Ala-Phe^{41}) in the chymotrypsin numbering (12) were replaced with the same 39-loop residues of FXa (^{31}Ala-Leu-Leu-Ilu-Asn-Glu-Glu-Asn-Glu-Gly-Phe^{41}) (FIX-FX$^{39\text{-loop}}$) using the same expression/purification vector system have been described (10). The activation of FIX derivatives by the FX-activating enzyme from Russell's viper venom and determination of their active site concentration by an amidolytic activity assay and active site titrations using known concentrations of AT in the presence of heparin have been described (13). The expression, purification, and characterization of ZPI, prepared from *Escherichia coli* using the SUMO fusion expression system, have been described (14, 15). The expression, purification, and characterization of protein Z in HEK-293 cells have been described (16).

Mouse and bovine plasma-derived FIXa and the FX-activating enzyme from Russell's viper venom were purchased from (Hematologic Technologies, Essex Junction, VT). Phospholipid vesicles containing 80% phosphatidylcholine and 20% phosphatidylserine (PC/PS) were prepared as described (17). TFPI was from Monsanto Chemical Co. (St. Louis, MO). The chromogenic substrates, Spectrozyme FXa was purchased from American Diagnostica (Greenwich, CT), and the FIXa-specific chromogenic substrate CBS 31.39 (LGR-pNA) was purchased from Midwest Bio-Tech, Inc. (Fishers, IN).

Inhibition Assays—A discontinuous assay method was used to monitor the time course of inhibition and to measure the second order association rate constants (k_2) for the ZPI inhibition of wild-type FIXa and the FIXa-FX$^{39\text{-loop}}$ chimera under pseudo-first order conditions both in the absence and presence of PZ as described (16). Briefly, FIXa (5–10 nM) was incubated with increasing concentration of ZPI (100–1000 nM) in 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5, and 5 mM Ca^{2+} (TBS/Ca^{2+}) containing 0.1 mg/ml BSA and 0.1% PEG 8000 at room temperature for 0.5–2 h. In the presence of PZ (5–160 nM), the inhibition of the FIXa derivatives (3–5 nM) by ZPI (200 nM) was monitored for 0.5–5 min on 50 μM PC/PS vesicles in the same TBS buffer system. All of the reactions were carried out in 50-μl volumes in 96-well plates and at different time points, 50 μl of the chromogenic substrate CBS 31.39 in TBS was added to each reaction, and the remaining enzyme activity was measured at 405 nm using a V_{max} kinetic microplate reader (Molecular Devices, Menlo Park, CA) as described (10, 16). The observed pseudo-first order rate constants (k_{obs}) were determined by fitting the time course data to a single exponential decay function with a non-zero end point, and the second order association rate constants (k_2) for uncatalyzed and PZ-catalyzed reactions were obtained from the slopes of the plots of k_{obs} *versus* concentrations of ZPI as described (10, 16). All of the values are presented as the average of at least three independent measurements ± S.D.

Determination of Inhibition Stoichiometry (SI)—The SI value for the ZPI-PZ inhibition of the FIXa-FX$^{39\text{-loop}}$ chimera was determined by titration of 200 nM protease with increasing concentrations of the serpin-cofactor complex corresponding to serpin/protease molar ratios of 0–5 and a PZ concentration equal to or 2-fold in molar excess of ZPI. The reaction was carried out in TBS/Ca^{2+} containing 50 μM PC/PS, and the residual amidolytic activity of the FIXa chimera was monitored for up to 30 min at room temperature from the hydrolysis of CBS 31.39 (1 mM) as described above. After completion of the inhibition reaction, the serpin/protease ratios were plotted *versus* the residual activity of the protease, and the SI value was determined from the x intercept of the linear regression fit of the inhibition data as described (15).

Analysis of the Stable Serpin-Protease Complex Formation—Complex formation of the FIXa-FX$^{39\text{-loop}}$ chimera with ZPI was monitored by SDS-PAGE as described (9). The reaction was carried out in 20 μl of volume using 2 μM protease and 4 μM ZPI in complex with 4 μM PZ in TBS/Ca^{2+} containing 50 μM PC/PS. Following 10 min of incubation at room temperature, 5 μl of 5× nonreducing SDS sample buffer was added, and the sample was loaded on a 10% SDS-PAGE and stained with Coomassie Blue R-250.

Interaction with TFPI—The ability of the FIXa-FX$^{39\text{-loop}}$ chimera to interact with TFPI was evaluated by incubating the protease (5 nM) with different concentrations of TFPI (12.5–200 nM) in TBS/Ca^{2+} in 50-ml volumes in a 96-well assay plate as described (18). Following 30 min of incubation at room temperature, 50 ml of CBS 31.39 was added to a final concentration of 1 mM, and the inhibition constant, K_i, was estimated by nonlinear regression analysis of data using an equation expressing the interaction of reversible inhibitors with proteases at equilibrium as described (18).

$$V_s = V_o/([I]/(K_i(1 + [S]/K_m)) + 1) \quad \text{(Eq. 1)}$$

In this equation, V_s and V_o are steady-state velocities of chromogenic substrate hydrolysis in the presence and absence of TFPI, respectively, [S] is the concentration of the chromogenic substrate CBS 31.39, K_m is the Michaelis-Menten constant for CBS 31.39 (~1 mM for both wild-type and FIXa chimera), and [I] is the TFPI concentration.

RESULTS

We have already characterized the amidolytic and proteolytic activities of the FIXa-FX$^{39\text{-loop}}$ chimera in a previous study and noted that the mutant exhibits a normal amidolytic activity toward the chromogenic substrate CBS 31.39, yielding K_m and k_{cat} values similar to those observed for the wild-type FIXa (10). However, although the FIXa mutant exhibited a normal affinity for FVIIIa, its catalytic activity (k_{cat}) toward the natural substrate FX in the presence of FVIIIa was decreased ~4-fold, suggesting that residues of the 39-loop contribute to the recognition specificity of the substrate in the intrinsic tenase complex (10). Further studies revealed that the reactivity of the FIXa mutant with AT has been improved ~5-fold specifically in the absence of pentasaccharide, suggesting that the 39-loop of FIXa contributes to the slower reactivity of the protease with the serpin in the absence of a heparin cofactor (10). In light of the latter observation that the 39-loop of FXa improves the recognition specificity of the FIXa chimera with the circulating native conformer of AT, we wondered whether the 39-loop of FIXa plays any role in rendering FIXa resistant to inhibition by other plasma inhibitors, which readily inhibit FXa in circulation. Thus, we evaluated the reactivity of the FIXa-FX$^{39\text{-loop}}$ chimera with the two specific inhibitors of FXa: ZPI and TFPI, which are known to participate in the physiological regulation of FXa during both the initiation and amplification phases of the clotting cascade.

Reaction with ZPI—In agreement with previous results, no decline in the amidolytic activity of the wild-type FIXa was observed upon its incubation with a high concentration of ZPI (0.5 μM) over a period of 1 h in TBS/Ca^{2+} in either the absence or presence of PZ and PC/PS vesicles (data not presented) (9). However, the FIXa chimera exhibited a moderate reactivity with ZPI in TBS/Ca^{2+}, thus yielding a second order association rate constant (k_2) of 1.2×10^2 M^{-1} s^{-1} for interaction with the serpin in the absence of any added cofactor. The rate of ZPI inhibition of the FIXa chimera was ~83-fold slower than the serpin inhibition of FXa under similar conditions (Table 1). Interestingly, however, the reactivity of ZPI with the FIXa chimera was dramatically enhanced if the reaction was monitored in TBS/Ca^{2+} in the presence of PZ on PC/PS vesicles. Thus, time course analysis of the ZPI (200 nM) inhibition of the FIXa chimera in the presence of PZ (20 nM) on PC/PS vesicles indicated that the reaction is rapid and reaches near completion in less than 40s (Fig. 1*A*). Nonlinear regression analysis of these data by a single exponential decay function with non-zero end point yielded a pseudo-first order rate constant (k_{obs}) of 0.129 ± 0.008 s^{-1} for the ZPI-PZ inhibition of the FIXa chimera (Fig. 1*A*). Noting the relatively slower rate in the absence of the cofactor, the serpin reactivity was monitored with a fixed concentration of ZPI (200 nM) as a function of increasing concentrations of PZ for 20s on PC/PS vesicles. The k_{obs} values for the ZPI-PZ inhibition of the FIXa chimera exhibited a saturable dependence on the concentration of the inhibitor-cofactor complex (Fig. 1*B*). Analysis of k_{obs} values according to a hyperbolic equation yielded $K_{d(app)}$ and k values of 10.2 nM and 0.189 s^{-1}, respectively, and a k_2 of 1.85×10^7 M^{-1} s^{-1} ($k_2 = k/K_{d(app)}$) for the inhibition reaction, suggesting that PZ promotes the reaction rate by approximately 5 orders of magnitude, exceeding the rate accelerating effect that has been observed for the PZ-mediated inhibition of FXa by ZPI on PC/PS vesicles (9, 15) (Table 1). These results clearly suggest that the residues of the 39-loop are primarily responsible for the lack of reactivity of FIXa with the PZ-ZPI complex on negatively charged phospholipid surfaces.

To directly monitor the extent of complex formation between the FIXa chimera and ZPI, SDS-PAGE analysis was carried out following incubation of the protease mutant (2 μM) with a 2 M excess of ZPI in the presence of PZ for 10 min at room temperature. As presented in Fig. 2 (*top panel*), the FIXa chimera formed a stable covalent complex with ZPI as evidenced by the appearance of a high molecular mass band migrating at ~95 kDa, which represents the combination of the molecular masses of ZPI plus the protease mutant (*lane 4*). The protease

TABLE 1

Inhibition constants for TFPI and second order association rate constants for the ZPI inhibition of FXa and FIXa-FX$^{39\text{-loop}}$ in the absence and presence of PZ and PC/PS vesicles

The K_i value for TFPI binding (derived from Fig. 3) and second order rate constants for the ZPI inhibition of FIXa-FX$^{39\text{-loop}}$ in the absence and presence of PZ and PC/PS vesicles (derived from Fig. 1) in TBS/Ca^{2+} were determined at room temperature as described under "Experimental Procedures."

	TFPI (K_i)	ZPI (k_2)	
		−PZ	+PZ
	nM	M^{-1} s^{-1}	
FXa	0.23 ± 0.03[a]	$1.0 \pm 0.1 \times 10^{4}$[b]	$2.3 \pm 0.5 \times 10^{7}$[b]
FIXa-FX$^{39\text{-loop}}$	48.3 ± 5.1	$1.2 \pm 0.2 \times 10^{2}$	$1.8 \pm 0.2 \times 10^{7}$

[a] The K_i value for the TFPI inhibition of FXa is derived from Ref. 20.
[b] The k_2 values for the ZPI inhibition of FXa in the absence and presence of PZ and PC/PS are derived from Ref. 9.

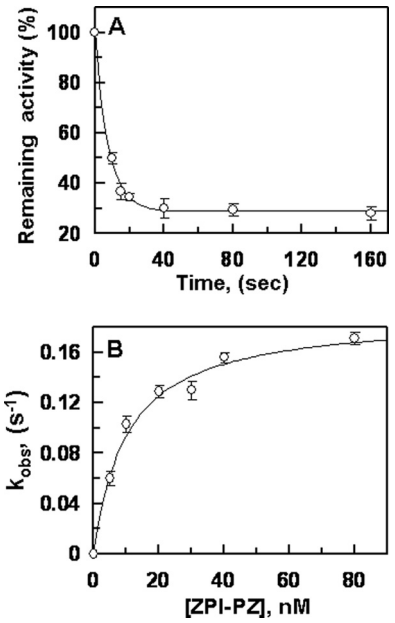

FIGURE 1. **Time course and concentration dependence of the PZ-mediated ZPI inhibition of FIXa-FX$^{39\text{-loop}}$ on PC/PS vesicles.** *A*, time course of the inhibition of the FIXa chimera (5 nM) by ZPI (200 nM) was monitored in the presence of PZ (20 nM) on PC/PS vesicles (50 μM) in TBS/Ca^{2+} as described under "Experimental Procedures." Nonlinear regression analysis by a single exponential decay function with a non-zero end point yielded a pseudo-first order rate constant (k_{obs}) of 0.129 $\pm$ 0.008 s^{-1} for the ZPI-PZ inhibition of the FIXa chimera. *B*, the same as *A* except that the inhibition of the FIXa chimera by ZPI (200 nM) was monitored as a function of increasing concentration of PZ (*x* axis). The k_{obs} values were calculated and plotted as a function of different concentrations of the PZ-ZPI complex. Nonlinear regression analysis of kinetic data using a hyperbolic equation yielded a rate constant (*k*) of 0.189 $\pm$ 0.009 s^{-1}, a $K_{d(app)}$ value of 10.2 $\pm$ 1.6 nM, and a second order association rate constant ($k/K_{d(app)}$) of 1.85 $\times$ 10^7 M^{-1} s^{-1} for the PZ-ZPI inhibition of the FIXa chimera. The data are derived from at least three independent measurements.

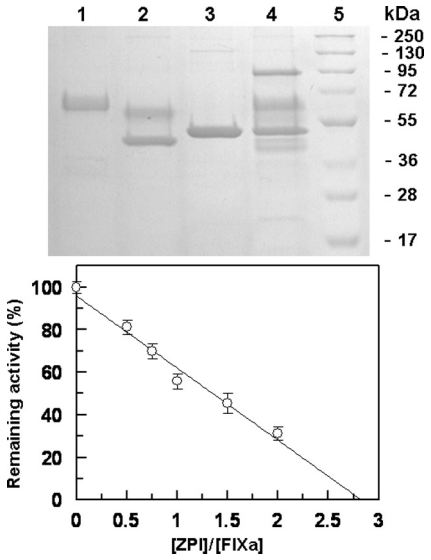

FIGURE 2. **SDS-PAGE analysis of the stable ZPI-FIXa-FX$^{39\text{-loop}}$ complex formation and stoichiometry of inhibition.** *Top panel*, the FIXa chimera (2 μM) was incubated with an equimolar concentration of PZ in complex with a two molar excess of ZPI for 10 min on PC/PS vesicles (50 μM) in TBS/Ca^{2+} in 20-μl reactions at room temperature. Five ml of nonreducing sample buffer was added to each sample, boiled for 5 min, and loaded on 10% SDS gel. *Lane 1*, PZ; *lane 2*, FIXa-FX$^{39\text{-loop}}$; *lane 3*, ZPI; *lane 4*, FIXa-FX$^{39\text{-loop}}$ + PZ + ZPI; *lane 5*, molecular mass standards (in kDa). *Bottom panel*, a fixed concentration of FIXa-FX$^{39\text{-loop}}$ (200 nM) was incubated with increasing concentrations of PZ-ZPI complex on PC/PS vesicles (50 μM) in TBS/Ca^{2+} for 10 min at room temperature. After completion of the reactions, the residual amidolytic activity of the protease was measured and plotted *versus* the [serpin]/[protease] ratios. The SI value was determined from the *x* intercept of the linear regression fit of the kinetic data. An SI value of 2.8 $\pm$ 0.2 was obtained for the PZ-ZPI inhibition of the FIXa chimera.

mutant migrated as a double band with apparent molecular masses of ~60 and ~45 kDa (*lane 2*), possibly representing activated single-chain and two-chain derivatives of the recombinant protease mutant, respectively. Analysis of the SDS-PAGE data (Fig. 2, *top panel*) suggests that both species of the FIXa chimera react with ZPI. This was evidenced by the observation that the higher molecular mass protease band (*lane 2*) also disappeared after its incubation with the ZPI-PZ complex (*lane 4*) (note that the band migrating at ~65 kDa is PZ). This was further confirmed by the SDS-PAGE of the FIXa chimera with AT, showing that the higher molecular mass band is fully reactive with AT (data not shown).

FXa has been demonstrated to react with ZPI in the substrate pathway of the reaction as well (9). To determine whether the FIXa chimera can also recognize the serpin as a substrate, the stoichiometry of the ZPI inhibition (SI) of the FIXa chimera was determined as described under "Experimental Procedures." As presented in Fig. 2 (*bottom panel*), ZPI exhibited an SI of ~2.8 for interaction with the FIXa chimera in the presence of PZ, which is essentially the same SI value that has been reported for the ZPI inhibition of FXa under the same experimental conditions (9). The SDS-PAGE analysis of an aliquot of the chimeric FIXa reaction with an equimolar concentration of ZPI in the absence of any added cofactor revealed that although the protease mutant can form a stable covalent complex with the serpin, it nevertheless does not recognize the inhibitor as a substrate because no cleavage reaction product could be detected on the gel (data not shown). By contrast, some cleavage product for the ZPI reaction with the FIXa chimera was observed in the presence of PZ as demonstrated in Fig. 2 (*top panel*). It is of interest to note that FXa has been demonstrated not to form an SDS-PAGE stable complex with ZPI unless the complex was denatured at the low pH of ~2 (9). However, the FIXa-FX$^{39\text{-loop}}$ chimera readily formed an SDS-PAGE stable complex with ZPI when denatured at neutral pH (Fig. 2, *top panel*), possibly suggesting that the covalent complex of the FIXa chimera with ZPI may dissociate at a slower rate than the corresponding complex of FXa with the serpin.

Interaction with TFPI—The reversible Kunitz-type inhibitor, TFPI, is a potent inhibitor of FXa exhibiting a K_i of less than 1 nM for interaction with the protease (19, 20). Unlike FXa, TFPI is not known to inhibit FIXa. To determine whether differences in the residues of the 39-loop also contribute to the differential specificity of TFPI recognition by the two proteases, the ability of TFPI to interact with FIXa-FX$^{39\text{-loop}}$ was examined by incubating the chimeric protease with different concentrations of TFPI and monitoring the amidolytic activity of the protease after 30 min (determined to be sufficient for establishing equilibrium). The results presented in Fig. 3 suggest that the chimeric FIXa is inhibited by TFPI with a K_i of ~48 nM that is ~210-fold weaker than the affinity of FXa for interaction with the inhibitor (Table 1). These results suggest that residues of the

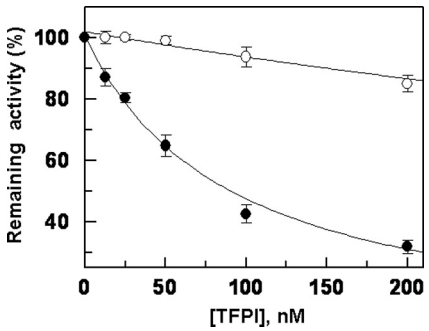

FIGURE 3. **TFPI inhibition of wild-type FIXa and FIXa-FX$^{39\text{-loop}}$ chimera.** Wild-type (○) and FIXa chimera (●) (5 nM each) were incubated with different concentrations of TFPI (*x* axis) in TBS/Ca^{2+}. After 30 min (sufficient to establish equilibrium), the remaining activity of enzymes were measured by an amidolytic activity assay using the chromogenic substrate CBS 31.39. Nonregression analysis of the kinetic data according to Equation 1 yielded a K_i of 48 ± 5 nM (±S.D., $n = 3$) for the TFPI inhibition of the FIXa chimera.

39-loop also contribute to restricting the specificity of FIXa interaction with this FXa-specific inhibitor.

Mouse and Bovine FIXa Are Susceptible to Inhibition by the PZ-ZPI Complex—Having established that residues of the 39-loop contribute to determining the specificity of human FIXa recognition of the FXa-specific plasma inhibitors, we decided to compare the sequence of residues of the 39-loop among several mammalian species whose FIXa protein sequences have been determined. The alignment of the residues of this loop for several species presented in Fig. 4 revealed that, similar to human FIXa, residue 36 is also a Lys in horse, pig, dog, and monkey; however, similar to human FXa, this residue is a Glu in mouse, rat, and bovine. Thus, we hypothesized that FIXa from these latter species may also react with ZPI. In agreement with our hypothesis, we noted that the ZPI-PZ complex can inhibit both proteases in the presence of calcium and negatively charged phospholipid vesicles (Fig. 4). Analysis of the data yielded similar second order association rate constants of 7.7×10^5 and 1.0×10^6 M^{-1} s^{-1} for the human ZPI-PZ complex inhibition of mouse and bovine FIXa, respectively (see the legend of Fig. 4 for the kinetic values). These results suggest that residue 36 is a determinant of ZPI specificity in FIXa. It should be noted that time course and concentration dependence of inhibition reactions indicated that the inhibitory activity of the ZPI-PZ complex toward both mouse and bovine FIXa was not complete but rather reached a maximum of 50% inhibition, and thereafter no further decline in the amidolytic activity of the proteases was observed. A similar incomplete inactivation for the FXa reaction with ZPI has been observed in similar amidolytic activity assays (8, 9). It has been found that this is due to a lesser stability of the complex of FXa with ZPI as compared with most other protease-serpin complexes (9). This appears to also be true, and even more pronounced, for complexes of both mouse and bovine FIXa with the human serpin. Nevertheless, further studies are required to establish this hypothesis because prolonged incubation (2 h) of either mouse or bovine FIXa with human ZPI-PZ did not result in significant recovery of the amidolytic activities. It should also be noted that human TFPI (up to 200 nM) did not effectively inhibit mouse and bovine proteases, possibly suggesting that residue 36 is a determinant of the specificity of ZPI but not TFPI in these species. Further studies in homologous systems are required to answer these questions.

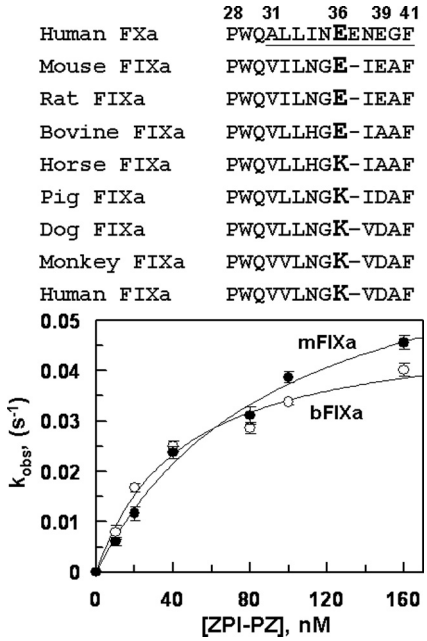

FIGURE 4. **Sequence alignment of the residues of the 39-loop among different mammalian species and the concentration dependence of the PZ-mediated ZPI inhibition of mouse and bovine FIXa.** *Top panel*, the sequence of amino acid residues of the 39-loop of human FXa from residues 28–41 (chymotrypsin numbering) are compared with the corresponding sequences of FIXa derived from several different species. *Bottom panel*, the inhibition of mouse FIXa (●) and bovine FIXa (○) (10 nM each) by ZPI (200 nM) was monitored in the presence of increasing concentration of PZ (*x* axis) on PC/PS vesicles (50 μM) in TBS/Ca^{2+} as described under "Experimental Procedures." The pseudo-first order rate constants (k_{obs}) were calculated and plotted as a function of different concentrations of the PZ-ZPI complex. Nonlinear regression analysis of kinetic data using a hyperbolic equation yielded *k*, $K_{d(app)}$, and k_2 values of 0.073 ± 0.007 s^{-1}, 94.6 ± 17.0 nM, and 7.7×10^5 M^{-1} s^{-1} and 0.049 ± 0.004 s^{-1}, 46.7 ± 8.7 nM, and 1.0×10^6 M^{-1} s^{-1} for the PZ-ZPI inhibition of the mouse FIXa and bovine FIXa, respectively. The data are derived from at least three independent measurements.

DISCUSSION

In this study we have demonstrated that unique structural features within residues of the 39-loop make a key contribution to determining the specificity of human FIXa interaction with the FXa-specific inhibitors in plasma. This is derived from the observation that the substitution of this loop with the corresponding loop of FXa resulted in a mutant (FIXa-FX$^{39\text{-loop}}$) that was rapidly inhibited by PZ-ZPI, and its affinity for TFPI was significantly improved. The inhibition rate of the FIXa chimera by ZPI in the presence of PZ and calcium on PC/PS vesicles approached the rate of FXa inhibition by the serpin-cofactor complex (Table 1), suggesting that the 39-loop of FIXa is primarily responsible for restricting the reactivity of the protease with this serpin. A significant improvement was also observed in the binding affinity of the FIXa chimera for the Kunitz inhibitor, TFPI. In a recent study, we demonstrated that the 39-loop of FIXa also plays a key role in determining the specificity of the protease interaction with the serpin, AT (10). Thus, the FIXa chimera exhibited ~5-fold improved reactivity with AT in the absence of a cofactor. However, unlike the improved reactivity with the plasma inhibitors, the FIXa mutant activated FX with ~4-fold impaired k_{cat} in the intrinsic tenase complex, suggest-

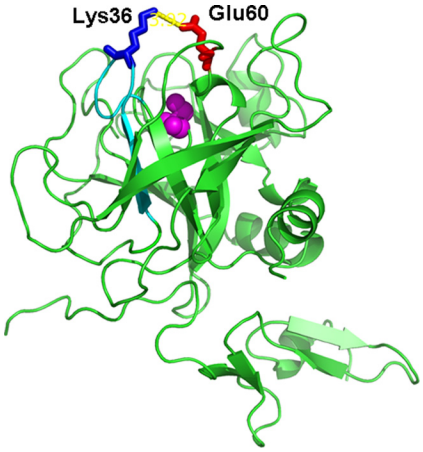

FIGURE 5. **Crystal structure of human Gla domain-less FIXa.** The relative three-dimensional locations of side chains of Lys^{39} (*blue*) and Glu^{60} (*red*) and a stabilizing salt bridge between them (*yellow*) are shown. The catalytic residue Ser^{195} is shown in *purple*. The coordinates (Protein Data Bank code 1RFN) was used to prepare the figure.

ing that the residues of the 39-loop contribute to a productive interaction of the protease with its physiological substrate (10). Taken together, these results indicate that the residues of the 39-loop have evolved to optimally catalyze the activation of the natural substrate FX but restrict the inhibitor specificity of the protease in plasma, thereby allowing the protease to circulate in plasma with a longer half-life.

A likely residue of the 39-loop that may be involved in restricting the inhibitor specificity of FIXa is the nonconserved residue Lys^{36}, which is a Glu in FXa. Further support for this hypothesis was provided by the observation that both mouse and bovine FIXa, which are similar to FXa, contain a Glu at this position were also susceptible to inhibition by the human ZPI-PZ complex on PC/PS vesicles in the presence of Ca^{2+}. This is the first study to demonstrate that mouse FIXa, unlike human FIXa, is susceptible to inhibition by the PZ-ZPI complex. Further studies will be required to determine whether ZPI contributes to the regulation of the intrinsic pathway in the mouse, assuming that mouse ZPI can also inhibit mouse FIXa in a homologous system.

Analysis of the structural and mutagenesis data can provide some insight into the mechanism through which residues of the 39-loop contribute to restricting the inhibitor specificity of FIXa. The x-ray crystal structures of human and porcine FIXa have indicated that both the 39- and 60-loops protrude out of the active site cleft of these proteases and that a salt bridge between Lys^{36} and Glu^{60} stabilizes these loops (21, 22) (Fig. 5). It appears that the salt bridge rigidifies the two loops as a wall on the S′ subsite, thus blocking the entrance of the substrate to the active site cleft and its subsequent interaction with the residues of the P′ side (23). Such a stabilizing salt bridge with the 60-loop is not expected to form in the FIXa-FX$^{39\text{-loop}}$ chimera because the mutant contains a Glu instead of a Lys at position 36 (24) (Fig. 5). Based on this model, it is likely that the lack of a salt bridge between the 39- and 60-loops renders the S′ site of the catalytic cleft open, thus allowing the inhibitor to interact with this binding pocket of the protease. The fact that the chromogenic substrates lack any P′ residue may explain the reason for the 39-loop not influencing the amidolytic activity of the protease and thus the FIXa chimera exhibiting a normal activity. FIXa is an allosterically regulated enzyme, and its catalytic activity toward the natural substrate FX is dramatically enhanced by FVIIIa without the cofactor affecting the activity of the protease with small synthetic substrates or the plasma inhibitors (25). Further study is required to determine whether a cofactor function for FVIIIa is to alleviate the inhibitory interaction of the substrate with the S′ subsite of the protease. Nevertheless, FVIIIa did not affect the reactivity of ZPI with either wild-type FIXa or the FIXa-FX$^{39\text{-loop}}$ chimera (data not shown); thus no evidence could be obtained in support of FVIIIa altering the conformation of the S′ binding pocket. This observation may not be surprising because recent structural and modeling data have indicated that only a concerted action by FVIIIa and the substrate FX can make the S′ site available for interaction with the substrate in the intrinsic tenase complex (26). It should be noted that although our interpretation of the data for the ZPI-PZ inhibition of FIXa-FX$^{39\text{-loop}}$ is consistent with x-ray structural data reported for FIXa in Refs. 23, 25, and 26, nevertheless the structure of the FIXa chimera must be resolved to validate the model.

Another surface loop that surrounds the catalytic cleft and is involved in influencing the substrate specificity of FIXa and FXa is the 99-loop (26, 27). Based on gain of function mutagenesis data with a highly catalytically active FIXa mutant containing FXa-like residues in the 99-loop, it has been hypothesized that unique structural features within this loop (in particular residues Tyr^{94}, Lys^{98}, and Tyr^{99}) trap FIXa in a low activity conformation (26). Thus, an important cofactor function for FVIIIa has been hypothesized to be the allosteric modulation of the conformation of the 99-loop of FIXa, which determines the S subsite specificity of the protease. Moreover, it has been hypothesized that the assembly of the zymogen FX to the intrinsic tenase complex is associated with a conformational change in the 60-loop of FIXa that results in Glu^{60} of the protease establishing an alternative electrostatic interaction with Tyr^{94} of the 99-loop, thereby improving the catalytic efficiency of the activation complex toward its natural substrate (26). In the context of this model, it can be hypothesized that such a substrate-assisted catalysis overcomes the inhibitory effect of the 39-loop by presumably breaking the salt bridge interaction between Glu^{60} and Lys^{36}, thereby allowing access of the S′ site of the protease by the substrate FX in the intrinsic tenase complex. Such a substrate-assisted catalysis cannot take place with the plasma serpins because, unlike FX, neither AT nor ZPI have recognition sites for the non-protease components of the intrinsic tenase complex.

In summary, we have demonstrated that replacing the 39-loop of FIXa with the corresponding loop of FXa renders the FIXa chimera susceptible to rapid inhibition by the PZ-ZPI complex with a rate constant that is essentially similar to that observed for the reaction of the serpin-cofactor complex with FXa, suggesting that differences in the residues of the 39-loop are primarily responsible for the differential reactivity of the two proteases with the inhibitory complex. The residues of this loop also contribute to restricting the specificity of human FIXa interaction with the FXa-specific Kunitz-type inhibitor, TFPI.

Thus, unique structural features within the 39-loop render FIXa resistant to inhibition by the plasma inhibitors, thereby increasing the half-life of the protease in circulation. This function of the 39-loop must be an evolutionary adaptation that is critical for the physiological function of the protease in the intrinsic tenase pathway during the amplification phase of the clotting cascade. Thus, upon injury-mediated exposure of vascular tissue factor and a simultaneous generation of some amount of both FXa and FIXa (3), the former protease proceeds to initiate the clotting cascade by activating a small amount of prothrombin before being rapidly neutralized by TFPI, whereas the latter protease freely circulates in plasma, ready to amplify the cascade upon demand that is regulated by thrombin itself as it activates the essential cofactors V and VIII.

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