Ixolaris: a Factor Xa heparin-binding exosite inhibitor

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Ixolaris is a two-Kunitz TFPI (tissue factor pathway inhibitor) from the tick salivary gland. In contrast with human TFPI, Ixolaris binds tightly to the zymogen FX (Factor X) and to dansyl-Glu-Gly-Arg-chloromethyl ketone-treated FXa (DEGR-FXa; active-site-blocked FXa), indicating that exosites are involved in the FX(a)–Ixolaris interaction. Here we provide evidence that Ixolaris binds specifically to the FXa HBE (heparin-binding exosite), since (i) it markedly decreases the inhibition of FXa by the anti-thrombin–heparin but not the antithrombin–pentasaccharide complex, (ii) it impairs FXa binding to Sepharose-immobilized heparin, and (iii) it allosterically modulates the catalytic activity of FXa for small chromogenic substrates (S-2765). By using a

series of recombinant FXa mutants in which the HBE is mutated, we have identified the importance of amino acids involved in the enzyme–inhibitor interaction as being in the following order: Arg-93 » Arg-165 ≥ Lys-169 > Lys-236 > Lys-96 > Arg-240 > Arg-125. Ixolaris at appropriate concentrations also inhibits thrombin formation *in vitro* by the assembled prothrombinase complex, a process that is critically dependent on the FXa HBE. Ixolaris is the first inhibitor characterized to date that binds specifically to the FXa HBE.

Key words: blood feeding, Factor Xa, heparin-binding exosite, prothrombin, tick saliva.

INTRODUCTION

FXa (Factor Xa) is a vitamin K-dependent coagulation serine proteinase that, upon binding to its cofactor FVa on membrane surfaces in the presence of Ca²⁺ ions, rapidly activates prothrombin to thrombin in the clotting cascade [1–4]. The activation complex, called prothrombinase, activates prothrombin with a catalytic efficiency that is more than 10⁵-fold greater than that of FXa alone [1–4]. FXa is composed of a light chain and a heavy chain that are held together by a disulphide bond. The light chain, consisting of an N-terminal Gla (γ-carboxyglutamic acid) residue and two epidermal growth factor-like domains, is involved in binding of FXa to the membrane and high-affinity binding of the proteinase to its protein cofactor [5]. The heavy chain of FXa contains the catalytic domain [6].

The catalytic domains of the serine proteinases of coagulation are highly similar to each other as well as to that of trypsin [7]. Despite these similarities, coagulation proteinases act on their protein substrates with narrow and distinctive specificities [8]; however, the molecular basis for the defined protein substrate specificity of coagulation enzyme complexes is not well understood. Although it is assumed that protein substrate specificity arises from specific binding interactions between the active site of the enzyme and distinctive amino acid residues surrounding the scissile bond, specific protein substrate recognition through extended macromolecular interaction sites (exosites) has been documented [9–11]. Exosite-dependent substrate recognition implies that extended surfaces on the enzyme complex, distinct from residues in the catalytic site, contribute to substrate affinity by favouring productive interactions between enzyme and substrate [9–11]. The existence of extended surfaces appears to be the rule rather than the exception for blood coagulation factors. In fact,

structural and functional studies have characterized exosites in AT (antithrombin) [12], thrombin [13], FXa [14], FIXa [15], protein C [16] and the FVIIa–TF (tissue factor) complex [17].

In the case of FXa, a series of mutagenesis and antibody inhibition studies has suggested a role for extended surface interactions between FXa and the FVIIa–TF complex [17,18]. It is also known that another extended surface mediates the interaction of FXa with heparin and FVa. This domain, called the FXa HBE (heparin-binding exosite) [14], is structurally similar to the well-studied heparin-binding site of thrombin [19], also known as TABE2 (thrombin anion binding exosite 2). TABE2 is located above the active-site pocket and contains 11 basic residues. Structural data indicate that seven basic residues of this region are conserved at the same or similar three-dimensional locations in FXa [20]. Site-directed mutagenesis studies have indicated that the basic residues Arg-93, Lys-96, Arg-125, Arg-165, Lys-169, Lys-236 and Arg-240 (chymotrypsin numbering system), which correspond to Arg-273, Lys-276, Arg-306, Arg-347, Lys-351, Lys-420 and Arg-424 respectively in FX numbering (mature peptide), constitute an exosite in the heavy chain of FXa, and that this region can bind heparin effectively [14]. Moreover, residues Arg-165 and Lys-169 seem to play a key role in FVa and/or prothrombin recognition by the enzyme in the prothrombinase complex [14]. Exosites also appear to participate in the interaction of FXa with inhibitors, including AT–heparin [21], and at least in part with TFPI (TF pathway inhibitor) [22].

In addition to AT and TFPI, specific blood coagulation inhibitors have been identified from exogenous sources, such as the salivary glands of blood-sucking insects [23]. Some of these inhibitors target coagulation factor exosites. Ixolaris, a 140-amino-acid protein from tick saliva that contains 10 cysteine residues and two Kunitz-like domains, inhibits the extrinsic tenase complex using

Abbreviations used: AT, antithrombin; [F]-Ixolaris, Ixolaris labelled at the N-terminus by fluorescein; DAPA, dansylarginine N-(3-ethyl-1,5-pentanediy) amide; FXa, Factor Xa; GDFXa, Gla-domainless FXa (i.e. FXa from which N-terminal residues 1–45 have been removed); Gla, γ-carboxyglutamic acid; HBE, heparin-binding exosite; PC, L-α-phosphatidylcholine; PEG, poly(ethylene glycol); PS, L-α-phosphatidylserine; TABE, thrombin-anion binding exosite; TF, tissue factor; TFPI, tissue factor pathway inhibitor.

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FX and FXa as scaffolds [24]. In contrast with TFPI [2], Ixolaris does not bind to the active-site cleft of FXa. Instead, complex formation is mediated through an unidentified exosite in the proteinase. Accordingly, we have studied further the mechanism of the interaction of Ixolaris with FXa. Our results using functional assays and FXa mutants identify Ixolaris as the first inhibitor described to date that specifically targets the FXa HBE.

EXPERIMENTAL

Proteins and chemicals

Recombinant Ixolaris was produced in High Five cells (Invitrogen, San Diego, CA, U.S.A.), purified and quantified as described previously [24]. Recombinant GDFXa (Gla-domainless FXa, i.e. FXa from which N-terminal residues 1–45 have been removed) forms (wild type and mutants R93A, K96A, R125A, R165A, K169A, K236A and R240A) were expressed and purified as described elsewhere [14]. AT was a gift from Dr Paulo A. Mourão (Federal University of Rio de Janeiro General Hospital-HUCFF/UFRJ). Human prothrombin and thrombin were purified from frozen human plasma following previously described procedures [25,26]. Protein concentration was estimated using the molar absorption coefficients ($A_{1\text{ cm}, 280}^{1\%}$) of human prothrombin (1.47) and human thrombin (1.83). S-2238 (*H*-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroaniline dihydrochloride) and S-2765 (*N*- α -benzyloxycarbonyl-D-Arg-Gly-Arg-*p*-nitroanilide) were from Chromogenix (Mölnådal, Sweden). Human FVa and human FXa were purchased from Haematologic Technologies (Essex Junction, VT, U.S.A.). Pentasaccharide (Fondaparinux sodium) was obtained from Organon Sanofi-Synthelabo LLC (West Orange, NJ, U.S.A.).

PC (L- α -phosphatidylcholine) and PS (L- α -phosphatidylserine) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Phospholipid vesicles composed of 75 % (w/w) PC and 25 % (w/w) PS were prepared by sonication. Briefly, phospholipids in chloroform were dried with a stream of N_2 , resuspended in 50 mM Tris/HCl, 150 mM NaCl, pH 7.5, and sonicated for 2 min. The solution was adjusted further to 500 μ M (final concentration).

Gel filtration

Gel filtration chromatography was performed using a Superdex G-75 HR column (Pharmacia, Uppsala, Sweden) attached to a Shimadzu HPLC system. The column was equilibrated with 50 mM Tris/HCl, 150 mM NaCl, pH 7.5, at a flow rate of 0.5 ml/min. [F]-Ixolaris (Ixolaris labelled at the N-terminus by fluorescein) was prepared by incubating the protein with fluorescein succinimidyl ester (PanVera, Madison, WI, U.S.A.), according to the manufacturer's instructions. Samples containing [F]-Ixolaris or [F]-Ixolaris-FXa were preincubated at room temperature for 20 min prior to 20 μ l injections. Elution was followed using two detectors: one for absorbance at 280 nm and another for fluorescein fluorescence (excitation at 490 nm, emission at 510 nm).

Amidolytic activity of FXa

Amidolytic activity was measured by hydrolysis of the synthetic substrate S-2765 in 50 mM Tris/HCl, 150 mM NaCl, 10 mM $CaCl_2$ and 0.1 % PEG [poly(ethylene glycol)] 6000, pH 7.5. FXa (1 nM final concentration) or GDFXa forms (0.3 nM final concentration) were incubated with various Ixolaris concentrations for 15 min at 37°C, and the reaction was initiated by the addition of S-2765 (200 μ M final concentration). Substrate hydrolysis was detected using a Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA, U.S.A.) equipped with a

microplate mixer and heating system as described [27]. Reactions were recorded continuously at 405 nm for 10 min at 37°C. The total volume of the reactions was 100 μ l. The apparent dissociation constants (K_d) were determined from the saturable Ixolaris-dependent increase in the amidolytic activities of FXa derivatives using the quadratic eqn (1) as described [28]:

$$V_s/V_o = (E + L + K_d) - [(E + L + K_d)^2 - 4E \cdot L]^{1/2} / 2E \quad (1)$$

where K_d is the apparent dissociation constant for the enzyme–ligand (Ixolaris) complex, V_s is the steady-state velocity in the presence of Ixolaris, V_o is the control velocity, L is the total Ixolaris concentration, and E is the total FXa concentration.

Binding to heparin–Sephacrose

FXa (7.5 μ g) was incubated for 2 min in the absence or presence of Ixolaris (3 μ g) in 20 mM Tris/HCl (pH 7.5) buffer containing 5 mM $CaCl_2$ and 0.1 % PEG 6000, and then applied on a 1 ml HiTrap heparin–Sephacrose (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) FPLC column pre-equilibrated with the same buffer. The column was washed with 5 ml of this buffer followed by elution with a 30 ml gradient of 0–1.0 M NaCl. Fractions (1 ml) were collected and their activity towards S-2765 was determined.

Inactivation by AT

The ability of Ixolaris to alter the inhibitory effect of the AT–heparin complex on FXa or α -thrombin, as well as that of the AT–pentasaccharide complex on FXa, was evaluated as follows. Various amounts of unfractionated heparin or pentasaccharide in 50 mM Tris/HCl, 150 mM NaCl, 10 mM $CaCl_2$ and 0.1 % PEG 6000, pH 7.5, were incubated for 10 min at 37°C with AT (20 nM) plus FXa (0.5 nM) or α -thrombin (0.5 nM) in the presence or absence of Ixolaris. Residual FXa or thrombin activities were determined by the addition of S-2765 or S-2238 (200 μ M) respectively, and substrate hydrolysis was detected using a Thermomax Microplate Reader. Reactions were recorded continuously at 405 nm for 10 min at 37°C. The total volume of the reactions was 100 μ l.

Prothrombin activation by FXa

Activation of prothrombin to thrombin by FXa was performed in 50 mM Tris/HCl, 150 mM NaCl, 10 mM $CaCl_2$ and 0.1 % PEG 6000, pH 7.5, using a discontinuous assay as described [29]. FXa (10 nM final concentration) was incubated with different Ixolaris concentrations for 15 min at 37°C. The reaction was started by the addition of human prothrombin (1 μ M final concentration), and aliquots of 25 μ l were removed every 10 min into microplate wells containing 25 μ l of 50 mM Tris/HCl, 150 mM NaCl, 50 mM EDTA and 0.1 % PEG 6000, pH 7.5. After addition of 50 μ l of 200 μ M S-2238, absorbance at 405 nm was recorded at 37°C for 10 min at 6 s intervals using a Thermomax Microplate Reader (Molecular Devices). Velocities (milli-absorbance units/min) obtained in the first few minutes of reaction were used to calculate the amount of thrombin formed using a standard curve.

Experiments in the presence of FVa were performed as follows. FXa (1 nM final concentration) was incubated in the presence of FVa (50 nM final concentration) and different Ixolaris concentrations for 15 min at 37°C. The reaction was started by addition of human prothrombin (1 μ M final concentration), and aliquots of 10 μ l were removed every 1 min into microplate wells

containing 40 μ l of 50 mM Tris/HCl, 150 mM NaCl, 50 mM EDTA and 0.1 % PEG 6000, pH 7.5. The amount of thrombin formed was determined as described above.

Experiments in the presence of phospholipids were conducted as follows. FXa (10 pM final concentration) was incubated with different Ixolaris concentrations in the presence of FVa (1 nM final concentration) and PC/PS vesicles (10 μ M) for 15 min at 37 °C. The reaction was started by the addition of human prothrombin (1 μ M final concentration). Then aliquots of 5 μ l were removed every 20 s into microplate wells containing 45 μ l of 50 mM Tris/HCl, 150 mM NaCl, 50 mM EDTA and 0.1 % PEG 6000, pH 7.5. The amount of thrombin formed was determined as described above.

Prothrombin activation as monitored by SDS/PAGE

Activation of purified human prothrombin by the prothrombinase complex was monitored by SDS/PAGE as follows. Assay medium (50 mM Tris/HCl, 100 mM NaCl, 10 mM CaCl_2 , 0.1 % PEG 6000, pH 7.5) containing FXa (1 nM final concentration), FVa (3 nM final concentration) and 30 μ M PC/PS vesicles was incubated in the absence (control) or presence of Ixolaris (40 nM final concentration) for 15 min at room temperature. The reaction was started by the addition of prothrombin (1.4 μ M final concentration), giving a total volume of 500 μ l. To reduce the formation of cleavage products by thrombin, reactions were carried out in the presence of 6 μ M DAPA [dansylarginine *N*-(3-ethyl-1,5-pentanediyl) amide]. Samples (50 μ l) were taken at various times, the reaction was stopped with 15 mM EDTA and 5 μ M D-Phe-Pro-Arg-chloromethyl ketone (20 μ l), and the samples were subjected to SDS/PAGE (12 % acrylamide) under non-reducing conditions and gels were stained with Coomassie Blue.

RESULTS

Complex formation between Ixolaris and FXa

Binding of Ixolaris to human FXa was examined first by Superdex G-75 gel filtration, using [F]-Ixolaris to monitor elution. In a typical experiment, the retention time of [F]-Ixolaris was $\sim$ 24.2 min (15.5 kDa) (Figure 1A, solid line), and that of FXa was $\sim$ 21.7 min (46 kDa) (results not shown). When Ixolaris and FXa were incubated in stoichiometric proportions and loaded into the column, a peak at $\sim$ 19.4 min was obtained (Figure 1A, broken line). These results were consistent with formation of a 1:1 complex of $\sim$ 60 kDa between [F]-Ixolaris (15.5 kDa) and FXa (46 kDa). Ixolaris behaves as a tight-and-fast-binding ligand of FXa without inhibiting the proteinase catalytic site [24]. On the other hand, Figure 1(B) shows that Ixolaris bound FXa tightly with an apparent K_d of 520 ± 18 pM that led to an increase in chromogenic substrate hydrolysis.

Ixolaris interacts with the HBE of FXa

Kinetic data strongly suggest that full-length heparin may effectively support the interaction between FXa and AT through a template mechanism [30]. This phenomenon occurs at physiological levels of calcium and is modulated by specific residues that form the FXa HBE [14]. Therefore we tested the ability of Ixolaris to interfere with inactivation of FXa by the AT-heparin complex. Figure 2(A) shows that increasing the concentration of heparin enhanced the inhibitory effect of AT on FXa, with complete inhibition observed above 1 μ g/ml heparin; however, in the presence of Ixolaris, inhibition of FXa by the AT-heparin complex was only 20 % at 1 μ g/ml and 40 % at 100 μ g/ml heparin. Accordingly, Ixolaris decreased the inactivation of FXa by the

Figure 1 Complex formation between Ixolaris and FXa

(A) Gel filtration chromatography on Superdex G-75 was performed at a flow rate of 0.5 ml/min using 50 mM Tris/HCl and 150 mM NaCl, pH 7.5, as buffer. Elution of [F]-Ixolaris (2 μ M) was monitored by measuring fluorescein fluorescence in the absence (solid line) or in the presence (broken line) of FXa (2 μ M). (B) Measurement of the effect of Ixolaris on the rate of hydrolysis of S-2765 (200 μ M) by FXa (1 nM) was performed as described in the Experimental section. Data are means $\pm$ S.D. of three independent determinations. Inset: progress curves for S-2765 hydrolysis by FXa in the absence (a) or in the presence of 0.6 nM (b) or 40 nM (c) Ixolaris. The solid line represents the fit of the data to eqn (1) [28] (see the Experimental section).

AT-heparin complex (1 μ g/ml) in a dose-dependent fashion, suggesting that Ixolaris and heparin compete for the same binding site on FXa (Figure 2B). Figure 2(C) shows that the effect of the inhibitor is specific for FXa, as thrombin inhibition by AT-heparin occurred to the same extent regardless of the presence of Ixolaris. Unlike AT-heparin, the mechanism of inhibition of FXa by the AT-pentasaccharide complex is independent of FXa HBE [31]. Thus, as expected, the presence of Ixolaris had only a minor effect on the pentasaccharide-accelerated reaction of AT with FXa (Figure 2D).

Finally, the ability of Ixolaris to compete with heparin for binding to FXa was documented further by affinity chromatography using a heparin-Sepharose column. FXa showed a high affinity for the resin and could be recovered by a linear gradient of NaCl, with a major peak eluted at $\sim$ 0.4 M NaCl (Figure 3, solid line). In contrast, FXa incubated with a 1.2-fold molar excess of Ixolaris was mostly eluted before the NaCl gradient started,

Figure 2 Effect of Ixolaris on the inactivation of FXa by AT

(A) Inactivation of FXa (0.5 nM) by AT (20 nM) as a function of increasing concentrations of heparin was performed in the absence (●) or in the presence (■) of Ixolaris (10 nM). (B) Inactivation of FXa (0.5 nM) by AT (20 nM) as a function of increasing concentrations of Ixolaris was performed in the presence of 1 µg/mL heparin. (C) Inactivation of α -thrombin (0.5 nM) by AT (20 nM) was measured as described in (A). (D) Inactivation of FXa (0.5 nM) by AT (20 nM) as a function of increasing concentrations of pentasaccharide was performed in the absence (●) or in the presence (■) of Ixolaris (10 nM). The remaining FXa activity towards S-2765 or α -thrombin activity towards S-2238 was measured as described in the Experimental section. Data are means $\pm$ S.D. of three independent determinations.

Figure 3 Impairment by Ixolaris of the interaction of FXa with immobilized heparin

Affinity chromatography on heparin–Sepharose was performed at a flow rate of 1 ml/min using 20 mM Tris/HCl, 5 mM CaCl₂ and 0.1% PEG 6000, pH 7.5, as buffer. Elution of FXa (7.5 µg) in the absence (solid line) or presence (broken line) of Ixolaris (3 µg) was monitored by measuring the hydrolysis of S-2765.

indicating that interaction of the enzyme with heparin was markedly impaired (Figure 3, broken line).

Ixolaris interacts with basic residues that comprise the FXa HBE

Studies using site-directed mutagenesis showed that seven basic residues in the heavy chain of FXa constitute the HBE of the enzyme, known to bind to heparin [14,30]. We used GDFXa mutants and chromogenic substrate hydrolysis to estimate the relative importance of the residues of the HBE for the Ixolaris–FXa inter-

Table 1 Complex formation between Ixolaris and wild-type and mutated forms of GDFXa

GDFXa forms (0.3 nM) were incubated with various amounts of Ixolaris (0–10 nM) for 15 min at 37 °C, and assayed for hydrolysis of S-2765 (200 µM). The relative affinity was calculated according to the tight-binding equation [28]. N.D., not detected (up to 10 nM).

Recombinant GDFXa	K_d (pM)	Relative affinity
Wt	78 ± 15	1.0
R93A	N.D.	N.D.
K96A	594 ± 93	0.13
R125A	365 ± 62	0.21
R165A	1087 ± 97	0.07
R169A	1015 ± 198	0.07
K236A	610 ± 63	0.12
R240A	472 ± 15	0.16

action. Analysis of these results indicated a similar, although apparently tighter, dissociation constant for wild-type GDFXa (K_d 78 ± 15 pM) compared with native FXa (K_d 520 ± 18 pM) (Figure 1B), suggesting that the Gla domain may somehow modulate the Ixolaris–FXa interaction. Table 1 shows the relative affinities obtained for the interaction between the various GDFXa mutants and Ixolaris, as compared with wild-type GDFXa. All mutations interfered with complex formation, with relative affinities ranging from 0.07 to 0.21. Most strikingly, however, was the observation that mutation of residue Arg-93 abolished the interaction of GDFXa with Ixolaris (Table 1).

Effect of Ixolaris on prothrombin activation

Mutagenesis experiments have identified several basic residues in the FXa HBE that are critical for substrate recognition by the prothrombinase complex [14]. Therefore we initially attempted to determine whether Ixolaris affects the ability of FXa to convert prothrombin into thrombin in the absence of phospholipids and cofactors [32]. Figure 4(A) shows that premixing of Ixolaris with FXa (10 nM) produced a concentration-dependent inhibition of prothrombin activation by the enzyme. The inhibitory effect of Ixolaris was then tested and confirmed when zymogen activation was promoted by FXa (1 nM) in the presence of FVa (50 nM) (Figure 4B). Figure 4(C) shows that Ixolaris was also effective in preventing prothrombin activation by fully assembled prothrombinase complex, i.e. FXa (10 pM) in the presence of FVa (1 nM), phospholipids (30 µM) and Ca²⁺ (see Discussion).

The effects of Ixolaris were documented further by monitoring prothrombin activation using SDS/PAGE, in the presence of PC/PS vesicles, cofactor and Ca²⁺. As shown in Figure 4(D) (upper panel), conversion of prothrombin into thrombin was almost complete after 20 min of zymogen incubation with the prothrombinase complex. On the other hand, incubation of FXa with Ixolaris prior to the addition of prothrombin significantly delayed the activation process, as shown by weaker bands corresponding to thrombin (Figure 4D, lower panel) as compared with the control (upper panel).

Figure 5 shows a working hypothesis for the mechanism of inhibition of the prothrombinase complex by Ixolaris (See Discussion and Figure legends).

DISCUSSION

The occurrence of functional exosites is a common feature in blood-clotting serine proteinases. A conserved HBE has been demonstrated in thrombin [19], FXa [14], FIXa [15] and protein C

Figure 4 Effect of Ixolaris on prothrombin activation by Fxa

(A) Activation of prothrombin (1 μ M) by Fxa (10 nM) was assayed with a discontinuous chromogenic assay using the thrombin substrate S-2238 (200 μ M) as described in the Experimental section. Zymogen activation reactions were carried out at the indicated concentrations of Ixolaris in 50 mM Tris/HCl, 150 mM NaCl, 10 mM CaCl₂ and 0.1% PEG 6000, pH 7.5. (B) Prothrombin (1 μ M) activation by Fxa (1 nM) in the presence of FVa (50 nM) was assayed as described above at the indicated concentrations of Ixolaris. (C) Prothrombin (1 μ M) activation by Fxa (10 pM) in the presence of FVa (1 nM) and 10 μ M PC/PS vesicles (prothrombinase complex) was assayed at the indicated concentrations of Ixolaris as described in the Experimental section. In (A)–(C), thrombin (Th) formation was calculated using a standard curve. (D) Time course of prothrombin (1.4 μ M) activation by Fxa (1 nM) in the presence of FVa (3 nM) and PC/PS vesicles (30 μ M). Reactions were performed in 50 mM Tris/HCl, 10 mM CaCl₂, 100 mM NaCl and 0.1% PEG 6000, pH 7.4, containing 6 μ M DAPA in the absence (control) or presence of 40 nM Ixolaris. Samples were further subjected to SDS/PAGE (12% acrylamide) under non-reducing conditions and gels were stained with Coomassie Blue.

[16]. These extended surfaces play critical roles in the formation of complexes between proteinases and their substrates, cofactors and inhibitors [9–11]. Exogenous sources such as salivary glands from blood-sucking insects and snake venoms are rich sources of exosite inhibitors, and the mechanisms of action of some of these molecules have been characterized in detail [23]. For instance, triabin from the bug *Triatoma pallidipennis* interacts with TAFE1 [33], haemadin from the leech *Haemadipsa sylvesteris* binds specifically to TAFE2 [34], and bothrojaracin from *Bothrops jararaca* snake venom recognizes both exosites of thrombin [35].

In the present paper, we have shown that Ixolaris from tick salivary gland is a specific ligand for the FXa HBE. Upon complex formation, Ixolaris induces changes in the catalytic groove of FXa, as shown by increased hydrolysis of synthetic substrates. Therefore Ixolaris binds to a site remote from the FXa catalytic site, i.e. the FXa HBE. This region consists of seven basic surface residues and has been shown to mediate the interaction of FXa with heparin [14,20,36,37]. Consistent with its interaction with the FXa HBE, Ixolaris markedly decreases heparin-catalysed inhibition by AT and also impairs the binding of FXa to immobilized heparin. The effects of Ixolaris appear to be specific to the

FXa HBE. In fact, Ixolaris did not affect the inhibition of thrombin by AT–heparin or the inactivation of FXa by the AT–penta-saccharide complex, a reaction that does not involve the FXa HBE [30]. The contention that Ixolaris interacts with the FXa HBE was confirmed using a series of exosite mutants for FXa (Table 1). The importance of the basic residues involved in inhibitor–enzyme recognition was in the order: Arg-93 > Arg-165 > Lys-169 > Lys-236 > Lys-96 > Arg-240 > Arg-125. These amino acids overlap in part with residues important for the FXa–heparin interaction [14]. Accordingly, direct heparin binding and kinetic data have suggested that two basic residues, Lys-236 and Arg-240, located on the C-terminal-helix make the greatest contribution to the binding of FXa to heparin, but are not the most critical for FXa–Ixolaris recognition. On the other hand, Arg-93, shown herein to be essential for the FXa–Ixolaris interaction, makes the least contribution in FXa heparin binding.

With regard to the roles of specific residues of the FXa HBE in the assembly of the prothrombinase complex, most mutants have inhibitory effects [14], further emphasizing the importance of this domain in the process [9,11,14,37]. However, the mutant R93A is the only one that activated prothrombin-2 at lower rates than

Figure 5 Working hypothesis for the mechanism of action of Ixolaris

(A) Non-inhibited prothrombinase: FXa binds to the membrane in a Ca^{2+} -dependent fashion and interacts with FVa, resulting in a productive prothrombin (PT) activation complex, leading to the formation of thrombin (Th) and prothrombin fragment 1-2 [1,4]. (B) Ixolaris at appropriate concentrations *in vitro* inhibits assembled prothrombinase (see Figure 4C): Ixolaris binding to the FXa HBE impairs the recognition of prothrombin, resulting in a non-productive prothrombinase complex. *In vivo*, inhibitory concentrations of Ixolaris may be reached only in the feeding cavity, a collection of blood produced after tick attachment and where salivary proteins are released and concentrated [29]. Otherwise, prothrombinase may be resistant to inhibition by Ixolaris in the presence of physiological concentrations of its substrate prothrombin, as described for TFPI (see Discussion and [38]).

wild-type FXa in the absence of FVa, PC/PS and Ca^{2+} [14]. Therefore Arg-93 appears to be critical for the direct interaction of prothrombin with FXa. Notably, interaction of Ixolaris with FXa in which Arg-93 is mutated was by far the most severely impaired among all mutants tested. This indicates that Arg-93 is critical in the recognition of the HBE by the inhibitor, and is consistent with the finding that Ixolaris effectively inhibits prothrombin activation by FXa in the absence of PC/PS and FVa (Figure 4A). Other amino acids may also participate in FXa–Ixolaris interactions. In fact, FXa mutants in which Arg-165 or Lys-169 was mutated also displayed lowered affinities for Ixolaris, and thus these residues are also important for the productive recognition of the assembled complex [14]. Figure 4(A) also suggests a secondary interaction of FXa with prothrombin, since FXa should be saturated with low concentrations of Ixolaris, and insignificant amounts of enzyme–substrate complex should be formed under these conditions. Finally, it has been reported that when the prothrombinase is assembled, FVa exposes a site on FXa that is recognized by prothrombin [36], and we cannot exclude at present the possibility that Ixolaris might also interact with these conformational sites. As far as the inhibitor is concerned, Ixolaris has a number of anionic amino acids (Asp-95, Glu-102, Glu-117, Glu-119, Glu-120, Glu-121 and Glu-124) in its second Kunitz domain that are atypical, with two disulphide bridges, and appear to have evolved to interact with sites other than the catalytic

domain [24]. We speculate that these acidic residues may contribute to Ixolaris–FXa HBE interactions.

Taken together, we suggest that inhibition of the HBE-mediated interaction of FXa with prothrombin by appropriate concentrations of Ixolaris destabilizes some of the forces involved in the formation of a productive prothrombinase complex *in vitro*. Of note, Figure 4 also shows that the effect of Ixolaris is less prominent when the prothrombinase is fully assembled. In fact, the ratio of the Ixolaris concentration producing 50% inhibition to the FXa concentration increases from ~3- to ~1000-fold when the prothrombinase is partially (Figure 4B) or fully (Figure 4C) assembled respectively. This suggests that the prothrombinase is protected from inactivation by low concentrations of Ixolaris. It is plausible that this is because of competition between prothrombin and Ixolaris for additional prothrombinase binding sites in addition to the HBE of FXa, e.g. sites involving FVa or phospholipids, or a distant site on the FXa molecule that is induced when FXa is bound within the prothrombinase complex [36,38]. This finding has also been demonstrated for other inhibitors, such as heparin–AT [21] and TFPI at physiological plasma concentrations [38]. As far as tick biology is concerned, one can speculate that Ixolaris may reach appropriate concentrations to inhibit the prothrombinase in the so-called feeding cavity, a collection of blood where the salivary secretions are released and concentrated during tick attachment, an event that can last for days [23]. Finally, it remains to be demonstrated whether Ixolaris also affects the direct interaction of FXa with FVa. It appears, however, that Ixolaris does not bind FVa, at least as a tight ligand. In fact, incubation of Ixolaris with FXa and PC/PS vesicles in the presence of a 10-fold excess of FVa does not affect the increase in the catalytic activity of FXa (towards S-2765) produced by Ixolaris (results not shown).

The observation that basic residues in different regions of FXa are involved in binding of Ixolaris is consistent with previous studies using synthetic peptides derived from selected regions of FXa and its effects on the prothrombinase complex [37]. In that study, peptide 267–283 [contains Arg-93 (chymotrypsin numbering system; equivalent to Arg-273 in FX; see Introduction)] was the most potent inhibitor, followed by peptide 417–431 (contains Lys-236) and peptide 344–362 (contains Arg-165 and Lys-169). Additional functional information on the FXa exosite comes from antibodies raised to this region that effectively block prothrombinase [10], and from NAPc2, a hookworm-derived TFPI that interacts with the C-terminus of FXa [39]. These various lines of evidence together support the hypothesis that regions remote from the catalytic site of FXa play a dominant role in the interaction of the enzyme with Ixolaris and in productive prothrombinase assembly. Ixolaris may be regarded as a useful tool with which to study FXa HBE.

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REFERENCES

- 1 Mann, K. G. (1999) Biochemistry and physiology of blood coagulation. *Thromb. Haemostasis* **82**, 165–174.
- 2 Broze, Jr, G. J. (1995) Tissue factor pathway inhibitor and the revised theory of coagulation. *Annu. Rev. Med.* **46**, 103–112.

- 3 Davie, E. W., Fujikawa, K. and Kisiel, W. (1991) The coagulation cascade: initiation, maintenance, and regulation. *Biochemistry* **30**, 10363–10370
- 4 Rosing, J., Tans, G., Govers-Riemsag, J. W., Zwaal, R. F. and Hemker, H. C. (1980) The role of phospholipids and factor Va in the prothrombinase complex. *J. Biol. Chem.* **255**, 274–283
- 5 Stenflo, J. (1999) Contributions of Gla and EGF-like domains to the function of vitamin K-dependent coagulation factors. *Crit. Rev. Eukaryotic Gene Expr.* **9**, 59–88
- 6 Persson, E., Valcarce, C. and Stenflo, J. (1991) The γ -carboxyglutamic acid and epidermal growth factor-like domains of factor X. Effect of isolated domains on prothrombin activation and endothelial cell binding of factor X. *J. Biol. Chem.* **266**, 2453–2458
- 7 Rose, T. and Di Cera, E. (2002) Substrate recognition drives the evolution of serine proteases. *J. Biol. Chem.* **277**, 19243–19246
- 8 Bode, W., Brandstetter, H., Mather, T. and Stubbs, M. T. (1997) Comparative analysis of haemostatic proteinases: structural aspects of thrombin, factor Xa, factor IXa and protein C. *Thromb. Haemostasis* **78**, 501–511
- 9 Krishnaswamy, S. and Betz, A. (1997) Exosites determine macromolecular substrate recognition by prothrombinase. *Biochemistry* **36**, 12080–12086
- 10 Wilkens, M. and Krishnaswamy, S. (2002) The contribution of factor Xa to exosite-dependent substrate recognition by prothrombinase. *J. Biol. Chem.* **277**, 9366–9374
- 11 Yegneswaran, S., Mesters, R. M. and Griffin, J. H. (2003) Identification of distinct sequences in human blood coagulation factor Xa and prothrombin essential for substrate and cofactor recognition in the prothrombinase complex. *J. Biol. Chem.* **278**, 33312–33318
- 12 Izaguirre, G., Zhang, W., Swanson, R., Bedsted, T. and Olson, S. T. (2003) Localization of an antithrombin exosite that promotes rapid inhibition of factors Xa and IXa dependent on heparin activation of the serpins. *J. Biol. Chem.* **278**, 51433–51440
- 13 Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R. and Hofsteenge, J. (1989) The refined 1.9 Å crystal structure of human α -thrombin: interaction with D-Phe-Pro-Arg chloromethylketone and significance of the Tyr-Pro-Pro-Trp insertion segment. *EMBO J.* **8**, 3467–3475
- 14 Rezaie, A. R. (2000) Identification of basic residues in the heparin-binding exosite of factor Xa critical for heparin and factor Va binding. *J. Biol. Chem.* **275**, 3320–3327
- 15 Yang, L., Manithody, C. and Rezaie, A. R. (2002) Localization of the heparin binding exosite of factor IXa. *J. Biol. Chem.* **277**, 50756–50760
- 16 Yang, L., Manithody, C. and Rezaie, A. R. (2002) Contribution of basic residues of the 70–80-loop to heparin binding and anticoagulant function of activated protein C. *Biochemistry* **41**, 6149–6157
- 17 Banner, D. W., D'Arcy, A., Chene, C., Winkler, F. K., Guha, A., Konigsberg, W. H., Nemerson, Y. and Kirchhofer, D. (1996) The crystal structure of the complex of blood coagulation factor VIIa with soluble tissue factor. *Nature (London)* **380**, 41–46
- 18 Konigsberg, W., Kirchhofer, D., Riederer, M. A. and Nemerson, Y. (2001) The TF:VIIa complex: clinical significance, structure-function relationships and its role in signaling and metastasis. *Thromb. Haemostasis* **86**, 757–771
- 19 Sheehan, J. P. and Sadler, J. E. (1994) Molecular mapping of the heparin-binding exosite of thrombin. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 5518–5522
- 20 Padmanabhan, K., Padmanabhan, K. P., Tulinsky, A., Park, C. H., Bode, W., Huber, R., Blankenship, D. T., Cardin, A. D. and Kisiel, W. (1993) Structure of human des(1–45) factor Xa at 2.2 Å resolution. *J. Mol. Biol.* **232**, 947–996
- 21 Rezaie, A. R. (2001) Prothrombin protects factor Xa in the prothrombinase complex from inhibition by the heparin-antithrombin complex. *Blood* **97**, 2308–2313
- 22 Krishnaswamy, S., Broze, G. J., Vlasuk, G. P. and Wilkens, M. (2001) Exosite-binding contributes significantly to the potent inhibition of factor Xa by tissue factor pathway inhibitor. *Blood* **98**, 528a
- 23 Ribeiro, J. M. C. and Francischetti, I. M. B. (2003) Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. *Annu. Rev. Entomol.* **48**, 73–88
- 24 Francischetti, I. M., Valenzuela, J. G., Andersen, J. F., Mather, T. N. and Ribeiro, J. M. (2002) Ixolaris, a novel recombinant tissue factor pathway inhibitor (TFPI) from the salivary gland of the tick, *Ixodes scapularis*: identification of factor X and factor Xa as scaffolds for the inhibition of factor VIIa/tissue factor complex. *Blood* **99**, 3602–3612
- 25 Ngai, P. K. and Chang, J. Y. (1991) A novel one-step purification of human α -thrombin after direct activation of crude prothrombin enriched from plasma. *Biochem. J.* **280**, 805–808
- 26 Bezeaud, A., Denninger, M. H. and Guillin, M. C. (1985) Interaction of human α -thrombin and γ -thrombin with antithrombin III, protein C and thrombomodulin. *Eur. J. Biochem.* **153**, 491–496
- 27 Francischetti, I. M., Valenzuela, J. G. and Ribeiro, J. M. (1999) Anophelin: kinetics and mechanism of thrombin inhibition. *Biochemistry* **38**, 16678–16685
- 28 Williams, J. W. and Morrison, J. F. (1979) The kinetics of reversible tight-binding inhibition. *Methods Enzymol.* **63**, 437–467
- 29 Monteiro, R. Q. and Zingali, R. B. (2002) Bothrojaracin, a proexosite I ligand, inhibits factor Va-accelerated prothrombin activation. *Thromb. Haemostasis* **87**, 288–293
- 30 Rezaie, A. R. (2000) Heparin-binding exosite of factor Xa. *Trends Cardiovasc. Med.* **10**, 333–338
- 31 Quinsey, N. S., Whisstock, J. C., Le Bonniec, B., Louvain, V., Bottomley, S. P. and Pike, R. N. (2002) Molecular determinants of the mechanism underlying acceleration of the interaction between antithrombin and factor Xa by heparin pentasaccharide. *J. Biol. Chem.* **277**, 15971–15978
- 32 Boskovic, D. S., Giles, A. R. and Nesheim, M. E. (1990) Studies of the role of factor Va in the factor Xa-catalyzed activation of prothrombin, fragment 1.2-prethrombin-2, and dansyl-L-glutamyl-glycyl-L-arginine-meizothrombin in the absence of phospholipid. *J. Biol. Chem.* **265**, 10497–10505
- 33 Fuentes-Prior, P., Noeske-Jungblut, C., Donner, P., Schleuning, W. D., Huber, R. and Bode, W. (1997) Structure of the thrombin complex with triabin, a lipocalin-like exosite-binding inhibitor derived from a triatomine bug. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 11845–11850
- 34 Richardson, J. L., Fuentes-Prior, P., Sadler, J. E., Huber, R. and Bode, W. (2002) Characterization of the residues involved in the human α -thrombin-haemadin complex: an exosite II-binding inhibitor. *Biochemistry* **41**, 2535–2542
- 35 Monteiro, R. Q., Raposo, J. G., Wisner, A., Guimarães, J. A., Bon, C. and Zingali, R. B. (1999) Allosteric changes of thrombin catalytic site induced by interaction of bothrojaracin with anion-binding exosites I and II. *Biochem. Biophys. Res. Commun.* **262**, 819–822
- 36 Yegneswaran, S., Fernandez, J. A., Griffin, J. H. and Dawson, P. E. (2002) Factor Va increases the affinity of factor Xa for prothrombin: a binding study using a novel photoactivable thiol-specific fluorescent probe. *Chem. Biol.* **9**, 485–494
- 37 Chattopadhyay, A., James, H. L. and Fair, D. S. (1992) Molecular recognition sites on factor Xa which participate in the prothrombinase complex. *J. Biol. Chem.* **267**, 12323–12329
- 38 Mast, A. E. and Broze, Jr, G. J. (1996) Physiological concentrations of tissue factor pathway inhibitor do not inhibit prothrombinase. *Blood* **87**, 1845–1850
- 39 Buddai, S. K., Touloukhouva, L., Bergum, P. W., Vlasuk, G. P. and Krishnaswamy, S. (2002) Nematode anticoagulant protein c2 reveals a site on factor Xa that is important for macromolecular substrate binding to human prothrombinase. *J. Biol. Chem.* **277**, 26689–26698

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