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Chapter 6

FXα–induced activation of FV is crucially important in initiating the coagulation system: lessons from a novel tick salivary protein

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Ticks are blood-sucking ectoparasites that transmit several diseases. A wide range of anti-haemostatic and anti-inflammatory salivary proteins enable ticks to feed for several days on mammalian hosts. Recently we identified the new coagulation inhibitor P23, produced by salivary glands of the tick *Ixodes scapularis*. *In silico* analysis showed that P23 is a member of an unknown protein family that is highly conserved in ticks. We here show that recombinant P23 inhibits thrombin generation initiated in plasma via the TF as well as the contact activation pathways. Further analysis showed that rP23 inhibits the common FXa pathway but does not inhibit the active site of FXa or thrombin. Thrombin generation initiated in the presence of preactivated factor V was not inhibited by rP23. Remarkably, factor V activation experiments demonstrated that rP23 does not inhibit FV activation by thrombin, but abrogated FV activation by FXa in the presence of phospholipids. Henceforth, P23 was renamed to the ‘Tick Inhibitor of factor Xa towards factor V’ (TIX-5) revealing the functional activity of TIX-5. *Ixodes scapularis* feeding was impaired on rabbits actively immunized with rTIX-5, displaying the *in vivo* importance of TIX-5 further delineating the molecular mechanisms by which ticks inhibit the host’s coagulation system. Furthermore, these data challenge the paradigm that thrombin is the only physiological activator of FV and demonstrate that FXa plays a crucial role in the activation of FV during initiation of coagulation using a novel tick protein.
**INTRODUCTION**

*Ixodes* ticks transmit various pathogens, including the causative agent of Lyme borreliosis, *Borrelia burgdorferi* [1] and feed for several days on a vertebrate host to obtain a blood meal. To counteract several host defense mechanisms ticks introduce tick saliva containing a range of salivary proteins with immunosuppressive and anticoagulant properties at the tick site. Ticks tear their way into the dermis and damage small blood vessels embedding their mouthparts in the host’s skin [2], which could initiate blood coagulation. Under normal circumstances, coagulation is triggered either by the contact activation (or intrinsic) pathway or by the tissue factor (or extrinsic) pathway [3,4]. When tissue factor (TF) is exposed to blood it forms a complex with plasma factor VIIa (FVIIa), which subsequently activates factors X (FX) and IX (FIX) [5,6]. Activated factor IX (IXa) together with FVIIIa will generate additional activated factor X (Xa). The contact activation pathway starts by reciprocal activation of factor XII (FXII) and prekallikrein on a foreign surface with high-molecular-weight kininogen (HMWK) as cofactor [7]. Activated FXII (FXIIa) converts factor XI (FXI) into an active enzyme (FXIa), which on its turn activates FIX leading to the formation of FXa. The contact and tissue factor pathways converge at the common pathway, which starts at the level of FXa. FXa forms the prothrombinase complex together with factor Va (FVa) on a phospholipid membrane surface, leading to thrombin generation [8]. Thrombin catalyzes many coagulation-related reactions and converts soluble fibrinogen to fibrin, which forms a solid blood clot together with erythrocytes and platelets [9]. Activation of the coagulation system can be divided into two phases; the “initiation phase” and “propagation phase”. The “initiation phase”, with major factor V (FV) and factor VIII (FVIII) activation and only limited FIX and FX activation [10], is characterized by low rates of thrombin generation while the “propagation phase” is characterized by rapid, quantitative activation of all prothrombin. During the initiation phase low concentrations of enzyme activate the first traces of FV necessary to generate the prothrombinase complex with FXa. Traces of thrombin are hypothesized to be responsible for generating the initial FVa under physiological conditions [11,12,13]. However, despite intensive research on the activating events of FV the question is still open as to whether FV has some intrinsic activity before activation, or whether FXa plays a significant role in FV activation. The latter is complicated by the high potency of thrombin to activate FV, forming a strong feed-forward loop to form prothrombinase and the lack of specific inhibitors of either thrombin dependent FV activation or FXa dependent FV activation.

Ticks have developed a broad array of anti-haemostatic proteins, secreted from the tick's salivary glands, which are essential for successful feeding and survival. These include platelet inhibitors, intrinsic and extrinsic pathway inhibitors, FXa inhibitors, thrombin...
inhibitors, and fibrinolytic agents [14], which maintain the tick’s blood meal in a fluid state. As such, anti-haemostatic tick proteins are potential pharmacological agents, which has been proven to be effective in the case of the tick anticoagulant peptide (TAP) from Ornithodoros mouhata [14,15] and potential vaccine candidates to thwart tick feeding and pathogen transmission.

Recently, we identified a novel anti-coagulant I. scapularis salivary protein, designated P23 according to its molecular weight, by probing an I. scapularis salivary gland yeast surface display library with tick immune rabbit sera [16]. We now demonstrate impaired feeding of adult ticks on rP23-immunized rabbits indicating a clear functional role of P23 during tick feeding. Moreover, we here characterize the anti-coagulant properties of P23, which dose-dependently prolongs activation of the coagulation system by specifically preventing activation of FV through FXa. Hence, the tick salivary protein P23 was renamed to ‘Tick Inhibitor of factor Xa towards factor V’ (TIX-5).

### RESULTS

**General properties of the anticoagulant Ixodes scapularis protein TIX-5.** Ixodes scapularis protein TIX-5 was recently identified as an antigenic salivary protein after performing a Yeast Surface Display screening with I. scapularis immune rabbit serum and showed anticoagulant activity (designated as P23, GenBank: AEE89467) [16]. Protein TIX-5 displays homology (54-58% similarity and 38-41% identity) with several annotated homologues in I. scapularis (GenBank: XP_002405271.1, GenBank: AAY66581.1 and GenBank: XP_002435217.1) indicating that TIX-5 is part of a novel family of I. scapularis proteins (Fig.S1A and Table S1). Interestingly, I. scapularis protein TIX-5 showed homology (46% similarity and 24% identity) with an annotated partial amino acid sequence from a putative secreted salivary protein from Ornithodoros coriaceus (GenBank: ACB70374.1), suggesting that this family of proteins is conserved in distinct tick families (Fig.S1A and Table S1). Since TIX-5 was found to be antigenic in I. scapularis immune rabbits it is likely that TIX-5 is a secreted protein [16]. Indeed, a signal peptide cleavage site was predicted between amino acid position 17 and 18 (Fig.S1A), using the web-based software SignalP (version 3.0). Analysis of the TIX-5 sequence using the web-based NetNGlyc server (version 1.0) revealed three predicted N-glycosylation sites (Fig.S1B). Removing N-glycans from the protein backbone of Drosophila-expressed recombinant protein TIX-5, using N-glycosidase (PNGase) F reduced the molecular weight (MW) with ~10 kDa and showed several partially deglycosylated forms in line with the three predicted N-glycosylation sites (Fig.S1C). Further in silico analysis of TIX-5 did not reveal homologies to other known proteins or functional domains.
Both contact activation and the tissue factor coagulation pathways are inhibited by recombinant TIX-5 in a dose-dependent manner. We have previously shown that rTIX-5 significantly prolonged the lag time of thrombin formation after initiating coagulation with 1 pM TF [16]. We now show that rTIX-5 significantly prolonged lag time and time to peak and inhibited the total amount of thrombin formed (Endogenous Thrombin Potential, ETP) and peak thrombin concentration after initiating coagulation by contact activation (Fig.1A, silica reagent APTT-based) and by 1 (Fig.1B) and 5 pM (Fig.1C) TF. When small amounts of thrombin are generated FXI becomes activated [21] which forms a positive feedback loop by activating FIX which leads to amplified FX formation. We assessed whether rTIX-5 had an effect on back-activation of FXI. This was not the case since rTIX-5 retained its anticoagulant properties after initiation of the coagulation pathway using 1 pM TF in FXI deficient plasma (Fig.1D). rTIX-5 also showed a prolonged lag time and decreased thrombin formation in platelet rich plasma when coagulation was initiated with 1 pM TF (Fig.1E). The effect of rTIX-5 was dose-dependent (Fig.1F), which was confirmed in the fibrinogen clotting assay as an alternative read-out assay of coagulation activation (Fig.S2). After rTIX-5 was degraded by proteinase K treatment, rTIX-5 lost its anticoagulant properties (Fig.S3). In recalcified human citrated whole blood, 3.25 μM rTIX-5 demonstrated a ~2-fold prolongation of clot time (5.5±0.3 minutes for PBS control versus 12.9±0.3 minutes for 3.25 μM rTIX-5, p<0.0001) and a ~4-fold prolongation of clot time (5.5±0.3 minutes for PBS control versus 19.5±1.2 minutes for 6.5 μM rTIX-5, p<0.0001) with 6.5 μM rTIX-5 (Fig.1G), further underscoring its biological importance.

Immunization with recombinant TIX-5 impairs adult tick feeding on rabbits. We immunized 3 rabbits with rTIX-5 which induced an antibody response that recognized native TIX-5 in salivary gland extract (SGE) of adult *Ixodes scapularis* nymphs (Fig.2A). Immune serum antibodies detected multiple bands in the range from 23 to 30 kDa representing the different glycosylated forms of TIX-5 (Fig.2A). Immune sera from 3 control rabbits immunized with OVA did not react with proteins in SGE while rP19 antisera, used as a tick salivary protein control, recognized native P19 but not native TIX-5 (Fig.2A). Upon challenge of the immunized rabbits with *Ixodes scapularis* adult ticks, comparable numbers of ticks fed to repletion and spontaneously detached on the control and experimental animals (40 and 32 adult ticks on the rP19 and the OVA control groups versus 34 on the rTIX-5 immunized group, respectively).
Figure 1. Both the contact activation and the tissue factor coagulation pathways are inhibited by recombinant rTIX-5 in a dose-dependent manner. Thrombin generation was initiated in normal human plasma by addition of 4 μM phospholipids and (A) 8 times diluted APTT-based reagent, (B, F) 1 pM TF, (C) 5pM TF or (D) in FXI deficient plasma with 1 pM TF. (E) Thrombin generation in platelet-rich plasma (PRP) was initiated with 1pM TF. Data are means ± SEM. (G) Recalcification of citrated whole blood in the presence of PBS (control), 3.25 μM rTIX-5 or 6.5 μM rTIX-5. The time to clot formation was measured. Unpaired t-tests were performed to determine significance (*p<0.01). Representatives of at least two experiments are shown.
Adult tick engorgement weights were significantly lower after feeding on rabbits immunized with rTIX-5 than adults that fed on the OVA control rabbits (Fig. 2B; 113.8 ± 17.7 mg versus 171.1 ± 17.6 mg, respectively, p=0.02) or on rP19 immune rabbits (Fig. 2B; 214.4 ± 17.2 mg, p=0.0001 compared to rTIX-5). Consistently, rTIX-5 inhibited coagulation in rabbit plasma as well, when initiated by both the TF (Fig. 2C) and the contact activation pathways (Fig. 2D).

**Figure 2. Impaired adult *Ixodes scapularis* feeding on rTIX-5 immunized rabbits.** (A) Adult salivary gland extract probed with antiserum from OVA, rP19 or rTIX-5 immunized rabbits. (B) *I. scapularis* post engorgement weights recovered from the OVA, rP19 and rTIX-5 immunized rabbits. Each group consisted of three rabbits. The horizontal bars represent the medians of the respective groups. Unpaired t-test was used to determine statistical significance. Thrombin generation was initiated in normal rabbit plasma by addition of 4 μM phospholipids and 8 times diluted APTT-based reagent (C) or 5 pM TF (D).

rTIX-5 inhibits coagulation in the presence of FXa and does not neutralize phospholipids. Since rTIX-5 inhibited the coagulation system initiated after both the contact activation and the TF pathways, we assessed whether rTIX-5 inhibited the common pathway of coagulation.
Figure 3. rTIX-5 has anticoagulant activity in the presence of FXa, but is not an active site inhibitor of FXa and is not dependent on phospholipids. Thrombin generation was initiated with 30 pM FXa in normal human plasma (A) or FX deficient plasma (B) in the absence or by addition of 0.25 μM (A), 1 μM (A) or 4 μM (A,B) phospholipids. (C) FXa mediated cleavage of substrate S2222 was measured in the presence (grey bars) or absence (black bars) of 6.5 μM rTIX-5. Unpaired t-test was used to determine statistical significance. (D) Coagulation was initiated with 1 pM TF in the absence or by addition of 4 μM or 20 μM phospholipids. Graphs show a representative of 2 separate experiments.

When thrombin generation was initiated in plasma by 30 pM FXa and varying concentrations of phospholipids, rTIX-5 inhibited thrombin generation (Fig.3A). To assess whether rTIX-5 inhibited FXa generation through feedback-activation we initiated coagulation with 30 pM FXa in FX deficient plasma and demonstrated that rTIX-5 retained its anticoagulant properties (Fig.3B). However, rTIX-5 did not show a direct effect on FXa in a chromogenic assay (Fig.3C), indicating that rTIX-5 is not an active site inhibitor of FXa. Since the effect of rTIX-5 was more evident in the presence of lower amounts of phospholipids (Fig.3A), we assessed whether rTIX-5 was able to neutralize phospholipids. In the presence of high concentrations of phospholipids (20 μM), rTIX-5 still inhibited thrombin generation (Fig.3D), indicating that rTIX-5 does not simply neutralize the phospholipids required for the prothrombinase complex.
Regulators of the initiation phase of coagulation support the anticoagulant effect of rTIX-5. We assessed whether the absence of physiological inhibitors of the human coagulation system influenced the anticoagulant properties of rTIX-5. Fibrinogen reduces thrombin generation by binding thrombin with high affinity, and fibrinogen is actually also referred to as antithrombin I [22]. Defibrination of normal human plasma resulted in a clear reduction of the lag time when coagulation was triggered with 1 pM or 5 pM of TF (Fig.4A), in line with a study performed by de Bosch et al. [23]. When coagulation was triggered with 1 pM TF, rTIX-5 prolonged the lag time by 1.5 min in the absence and 5.7 min in the presence of fibrinogen (Fig.4A). Activation of coagulation by 5 pM TF with rTIX-5 resulted in a prolongation of lag time by 2.0 and 3.2 min in the absence and presence of fibrinogen respectively (Fig.4A). These results demonstrate that the inhibitory effect of rTIX-5 is greatly reduced in fibrinogen depleted plasma. In line with this, the absence of other physiological inhibitors of the initiation phase, e.g. antithrombin, TF pathway inhibitor and protein S resulted in a reduced anticoagulant effect of rTIX-5 (Fig.S4). The absence of protein C did not influence the effect of rTIX-5 (Fig.S4).

Preactivation of prothrombin and FV impairs the anticoagulant effect of rTIX-5. Since the absence of fibrinogen severely hampered the anticoagulant activity of rTIX-5, we further investigated if rTIX-5 was still able to inhibit coagulation in the presence of preactivated prothrombin. In the presence of 3 nM thrombin, rTIX-5 was not able to inhibit further thrombin generation via the feedback loop through FXI activation (Fig.4B). Next, we studied the anticoagulant effect of rTIX-5 in the presence of preactivated FV. Interestingly, rTIX-5 significantly inhibited coagulation when 20 nM FV was added to FV deficient plasma, whereas this effect was for most part abrogated in the presence of 20 nM FVa (Fig.4C), which suggested that rTIX-5 postpones both the activation of prothrombin and FV. To further investigate the anticoagulant effect of rTIX-5 we used a purified system of coagulation factors to study thrombin generation. In this purified system, supplemented with TFPI (Fig.S5C) and FVIII (Fig.S5C and D) to make the system sensitive and potent, thrombin generation was inhibited by rTIX-5 after initiating activation of coagulation through the TF pathway using 1 pM TF (Fig.4D), as well as when initiated with 8 pM FIXa (Fig.S5A) or 32 pM FXa (Fig.S5B). These data clearly demonstrated that rTIX-5 inhibited coagulation activation by interfering with one or more factors present in the purified system. By using this purified system of coagulation factors, we confirmed that thrombin generation was postponed (Fig.4E) in the presence of rTIX-5. Moreover, FV activation was postponed in the presence of rTIX-5 (Fig.4F).
Figure 4. The effect of rTIX-5 is abrogated in the presence of preactivated FV and thrombin and in the absence of fibrinogen. (A) Coagulation was initiated with 1 pM (black lines) and 5 pM TF (grey lines) in human normal pool plasma (NHP) or in fibrinogen depleted pool plasma (Fib def) by addition of 4 μM phospholipids and lag time was measured. (B) Coagulation was initiated with 3 nM thrombin in normal human plasma in the presence of 4 μM phospholipids. FIX deficient and FXI deficient plasma were used as a negative control and both lie on the x-axis. (C) FV deficient human plasma was complemented with 20 nM purified human FV (grey lines) or FVa (black lines). Thrombin generation was initiated by addition of 4 μM phospholipids and 1 pM TF in the presence (dashed lines) or absence (solid lines) of 6.5 μM rTIX-5. Thrombin was generated in a purified system as described in Experimental Procedures and at specific time intervals aliquots were withdrawn for analysis of thrombin formation by the rate of conversion of the thrombin specific chromogenic substrate S2238 (D). Additionally, the aliquots were subjected to Western blot analysis (E) in non-denaturing (upper panel) and denaturing (lower panel) conditions were visualized with anti-thrombin antibody. Meizothrombin-des-fragment 1 and pre-thrombin 1 (50 kDa) and α-thrombin (39 kDa) were visualized in the upper panel. The thrombin B-chain (30 kDa) was visualized in the lower panel. Simultaneously, FV heavy chain (100 kDa) formation was visualized by Western blot analysis (F). Graphs show a representative of 2 separate experiments.

rTIX-5 specifically inhibits the activation of FV by FXa. To further elucidate the mechanism by which rTIX-5 prolonged FV activation we explored the role of rTIX-5 in the direct activation of
FV. Since thrombin is thought to be the most significant contributor in the formation of FVa [13], we determined if rTIX-5 inhibited activation of FV by thrombin. We show that rTIX-5 did not affect the activation of FV by thrombin (Fig.5A) as demonstrated by both Western blot analysis (left panel) and by a FVa clot assay (right panel). FXa has been shown to activate FV in the presence of phospholipids but clearly less efficiently compared to thrombin and it has been suggested that FXa does not contribute to the conversion of FV at physiologically relevant conditions [11,12,13]. Unexpectedly, rTIX-5 abrogated activation of FV by FXa (Fig.5B) demonstrated by both Western blotting of active fragments of FV (left panel) and a FVa clot assay (right panel). Activation of FV by FXa in the absence of phospholipids was negligible (data not shown). Presently it is assumed that small amounts of thrombin are generated by FXa on phospholipids which subsequently activate enough FV to generate prothrombinase. Direct activation of prothrombin to thrombin by FXa in the absence of FV was not impaired by rTIX-5 (Fig.5C), which corroborates that rTIX-5 is not an inhibitor of the active site of FXa, nor of an exosite of FXa involved in prothrombin activation. Collectively, these data show that rTIX-5 is a powerful inhibitor of FXa-mediated FV activation.

**DISCUSSION**

It is of crucial importance that enzymes of the coagulation system and their cofactors, including FV, circulate in an inactive form under normal conditions and are only activated when necessary. In the common pathway, FX and FV become activated to FXa and FVa respectively, which occurs early after activation via the contact and TF pathways of coagulation. FVa is a non-enzymatic cofactor and a crucial ingredient of the prothrombinase complex since it makes FXa 300,000 times more efficient in converting prothrombin to thrombin [24,25]. FVa forms a complex with FXa on negatively charged phospholipids provided by activated platelets in the presence of Ca²⁺ to form the prothrombinase complex. The initial activation of FV has been the subject of great investigative effort and of speculation and controversy. FXa and thrombin are both able to activate FV to FVa. Although FXa has been shown to activate FV in the presence of phospholipids [26], it did not appear to contribute to FVa formation at physiologically relevant conditions and it has been suggested that thrombin is the physiological activator of FV [11,12,13]. Previously, Foster et al. postulated factor Xa could be responsible for activation of FV needed for early prothrombinase activity without definite proof [27].

We now report a tick salivary protein - previously designated as P23 according to its molecular weight [16], but now renamed TIX-5 based on its function - as a tool to demonstrate that FXa is crucial in the activation of the first FV to form the initial prothrombinase complexes.
Figure 5. rTIX-5 specifically prevents activation of FV by FXa on a phospholipid membrane. Time course of FV activation by 1 nM thrombin (A) or 10 nM FXa (B) in the presence of phospholipids as described in Methods was evaluated by Western blotting (left panels) using an anti-FV heavy chain antibody. Reaction time points in minutes are indicated above the lanes. As an alternative approach, FV activation was determined by measuring FVa activity in FV deficient plasma (right panels) at the indicated time points as described in the Methods. (C) Thrombin concentration was measured by the rate of conversion of a thrombin specific chromogenic substrate at the indicated time points when 100 μg/ml FII was activated with 35 nM FXa in the presence of 0.7 μM phospholipids.
rTIX-5 inhibits both the contact activation and tissue factor (TF) pathways of coagulation by interfering with the conversion of FV by FXa on phospholipids. It has been proposed that the α-thrombin that is responsible for early FV activation is produced directly by FXa on phospholipids during the initial phase of coagulation [12,28]. Since rTIX-5 specifically hampered FV activation by FXa on phospholipids and did not affect direct activation of prothrombin to thrombin on phospholipids by FXa, we demonstrate the crucial role of early FV activation by FXa.

Because suboptimal concentrations of phospholipids increased the anticoagulant effect of rTIX-5 we postulated that rTIX-5 would lead to an enhanced anticoagulant phenotype in platelet rich plasma. However, the anticoagulant effects of rTIX-5 were reduced in platelet rich plasma. Roughly 80% of FV circulates in plasma, whereas 20% is found within the α-granules of platelets [29]. Activated platelets release several coagulation factors, including FVa [29,30,31] which is partially resistant to activated protein C [32] and a FV activating protease [33]. The data obtained with TF triggered thrombin generation in platelet rich plasma are thus consistent with the observations that in the presence of FVa the anticoagulant effect of rTIX-5 was almost completely ablated. Ticks produce various inhibitors of platelets and platelet activating factors in order to prevent platelet activation [14], which shows to be crucially important for the inhibiting effect of TIX-5 in the presence of platelets and TF.

Interestingly, the anticoagulant effect of rTIX-5 was almost entirely ablated in the absence of fibrinogen when coagulation was initiated with 1 pM TF in human plasma. Fibrinogen, also referred to as antithrombin I, strongly binds to thrombin [22,34]. In line with these data, others have shown that thrombin formation is increased in fibrinogen depleted plasma [23]. Also in this study we demonstrate that the absence of fibrinogen in human plasma decreased the lag time of coagulation. These data suggest that trace amounts of thrombin that are initially formed after initiation of coagulation are bound and captured by fibrinogen and are subsequently hampered to activate FV. Indeed, others have shown that thrombin binding to the γ chain of fibrinogen resulted in a reduction of FVIII activation [35]. We demonstrated that the anticoagulant effect of rTIX-5 was clearly reduced when fibrinogen depleted plasma was initiated with 1 pM TF compared to 5 pM TF. This is in line with the mechanism of rTIX-5, since generation of FXs occurs more rapid with higher concentrations of TF. In addition we found that the anticoagulant effect of rTIX-5 was absent when coagulation was initiated with 3 nM thrombin. Once significant amounts of thrombin are present, coagulation is activated through the positive feedback loops provided by thrombin which includes activation of the procofactors FV and FVIII, as well as FXI. TFPI and protein S are two other physiologic coagulation inhibitors that regulate the initiation phase of thrombin generation [36,37]. We
demonstrated that, in a fibrinogen-free but protein S and TFPI containing system, FV activation as well as thrombin generation were also inhibited by rTIX-5.

These experiments showed that rTIX-5 may inhibit thrombin generation profoundly when the initiating trigger is low and when thrombin formation and function are counterbalanced by the coagulation inhibitors of the host. Non-anticoagulated blood was kept in a fluid state for 19.5 minutes by rTIX-5 compared to 5.5 minutes in the absence of rTIX-5. Blood-feeding ticks alternate phases of sucking blood and secreting saliva at the bite site while each phase last as long as 5-20 minutes [38], enabling them to introduce new TIX-5, among others, at the bite site during each secretion phase. This process is crucial for hematophagous organisms, including *Ixodes* ticks, to suppress the host coagulation system in order to maintain blood in a fluid state so that it can absorb, store and digest its blood meal. This is necessary, since the coagulation system is triggered immediately after the mouthparts penetrate and damage the host's tissue. In order to do this efficiently ticks have shown to target several parts of the host coagulation system such as platelets, thrombin, FXa, fibrinogen etc. [14]. Considering ticks have a widely diverged set of anticoagulant proteins, the effect of TIX-5 on coagulation is most likely enhanced in the presence of these proteins. In line with this, in the absence of other physiological inhibitors of the initiation phase besides fibrinogen, c.q. AT, TFPI and protein S, the effect of rTIX-5 was reduced. Interestingly, by performing *in silico* analysis of TIX-5, a partial amino acid sequence coding for an annotated homologue in *Ornithodoros coriaceus* was found. Since *Ornithodoros* is part of a different family of ticks, c.q. Argasidae [39], this shows that TIX-5 is a member of larger protein family prevalent in ticks.

Since rTIX-5 was identified by probing a salivary gland expression library with tick immune rabbit sera [16] and an anti-tick vaccine is an interesting approach to thwart tick feeding and to block tick transmitted pathogens such as the Lyme borreliosis causing agent *Borrelia burgdorferi* [40], we immunized rabbits with rTIX-5. Anti-tick vaccines to prevent pathogen transmission are the topic of extensive investigation and one possible approach would be to target a group of tick proteins that inhibit the host coagulation system [40]. Activation of coagulation also results in induced inflammation and vice versa, since there is extensive crosstalk between these two systems [41]. After several tick infestations rabbits acquire an immune response resulting in tick rejection, also referred to as 'tick immunity' or 'tick resistance' [42]. One of the major parameters indicative of tick immunity is a reduction of tick weights after feeding. Western blot analysis of salivary gland extract from adult *I. scapularis* ticks probed with serum from rTIX-5 immunized rabbits indicated that rTIX-5 specific antibodies recognized native rTIX-5. Previously, we have shown that weights of *I. scapularis* nymphs were slightly but not significant reduced after feeding on rTIX-5 immunized rabbits [16]. Our findings here show that adult *I. scapularis* weights were dramatically reduced after feeding on rTIX-5 immunized
rabbits. This could be, at least partially, explained by the large amount of blood taken by adult ticks in a similar time frame of feeding on the host as compared to nymphal ticks, which makes suppressing the host coagulation system a more challenging task. Indeed, adult ixodid female ticks consume more than 100 times their unfed body [43]. Ticks have a wide range of strategies and several fall-back mechanisms to overcome activation of coagulation [14]. As we postulated before, to efficiently impair tick feeding a cocktail of several anticoagulant tick proteins could be targeted [40].

Collectively, the selected specific effect of rTIX-5 on FXa-mediated FV activation and the inhibitory effect of rTIX-5 on plasma thrombin/fibrin formation and on whole blood clotting provides, for the first time, evidence of the importance of direct activation of FV by FXa under in vitro, but physiologically relevant, conditions. Furthermore, FXa plays a crucial role in activating small amounts of FVa for the prothrombinase complex under physiological blood or plasma clotting conditions in order to facilitate rapid thrombin generation at a low initiating stimulus. This was accomplished by using a novel anticoagulant tick protein that inhibits the coagulation system in a unique way which has not been reported until now. This study has broad implications for a better understanding of the initiation phase of coagulation and since this protein targets FV activation specifically during the initiation phase it may provide novel therapeutic strategies and development of useful inhibitors for thrombotic diseases.

**MATERIALS AND METHODS**

**Ticks and rabbits.** *Ixodes scapularis* adult ticks were obtained from a tick colony at the Connecticut Agricultural Experiment Station in New Haven CT, USA. Ticks were maintained at 23°C and 85% relative humidity under a 14 hour light, 10 hour dark photoperiod. For the immunization studies, approximately 6 week old inbred New Zealand white rabbits (Charles River Laboratories, USA) were used. The work reported in this study is fully compliant with and approved by institutional policies pertinent to biosafety and animal care protocols. The protocol for the use of mice and rabbits was reviewed and approved by the Yale Animal Care and Use Committee (protocol number 2008-07941, approval date is 03/31/10 to 3/31/11).

**Purification of recombinant *Ixodes scapularis* salivary protein.** Cloning and expression of *TIX-5* and *p19* (as a control tick salivary protein) in the *Drosophila* Expression System (Invitrogen, USA) and purification of recombinant protein was performed as described before [16]. Briefly, for the purification of recombinant TIX-5 (rTIX-5) and recombinant P19 (rP19) the coding sequence of *TIX-5* and *p19* was cloned into the pMT/Bip/V5-HisA plasmid in frame with a His tag and a V5 epitope (Invitrogen, USA), and validated by sequencing. *Drosophila melanogaster* S2 cells were transfected with the plasmids containing *TIX-5* or *p19* and the blasticidin selection vector pCOBlast using the Calcium Phosphate Transfection Kit (Invitrogen, USA). Subsequently, cells expressing *TIX-5* or *p19* were selected with blasticidin (25 μg/ml), and were grown in large spinner flasks for 3 days. Thereafter, recombinant protein expression was induced in *Drosophila* cells with copper sulfate at a final concentration of 500 μM for 4 days and centrifuged at 1,000xg for 15 min. The supernatant was filtered using a 0.22 μm filter (Millipore, USA) and rTIX-5 or rP19 was purified from the supernatant by binding to a Ni-NTA Superflow column (Qiagen, USA) and elution with 250 mM imidazole. The eluted fractions were filtered through a 0.22 μm filter and concentrated with a 5-kDa concentrator (Sigma-Aldrich,
USA) through centrifugal concentration at 4°C, washed and dialyzed against PBS. The purity of the purified rTIX-5 and rP19 was checked by SDS-PAGE followed by Coomassie blue staining and the concentration was determined by BCA protein assay kit (Thermo Fisher Scientific inc., USA).

Deglycosylation of recombinant TIX-5. Deglycosylation of recombinant TIX-5 with N-Glycosidase (PNGase) F (Sigma, USA) (95.000 U/mg protein) was performed in 0.75% TRITON® X-100 in PBS for 24 hours at 37°C. As a control equal amounts of rTIX-5 was incubated in 0.75% TRITON® X-100 in PBS for 24 hours at 37°C. Equal amounts of purified recombinant salivary proteins (1 μg), were electrophoresed on 12% SDS-PAGE and protein was stained with Coomassie blue.

Human plasma and coagulation factors. Human FXa was purchased from Enzyme Research Laboratory (UK). Human FV and FVa were obtained from Haematologic Technologies Inc. (USA). FXI, FIX and Protein S deficient plasmas were purchased from Siemens Healthcare Diagnostics (Germany). Antithrombin deficient plasma was purchased from Affinity Biological (USA). TFPI deficient plasma was purchased from American Diagnostica (USA). Protein C deficient plasma was obtained from Kordia (NL). Defibrination of normal human pool plasma was carried out by mixing plasma with 0.4 BU (batoroxbina Units) / ml reptilase (Roche, USA) and was incubated for 10 min at 37°C, kept on room temperature for 10 min and the fibrin clot was removed. Platelet rich plasma (PRP) was prepared from citrated blood after centrifugation at 200 g for 10 min at 25 degrees. Recombinant full-length TFPI isolated from Escherichia coli was obtained from American Diagnostica Inc (USA). FVIII was obtained from Baxter (NL). Thrombin was kindly provided by Dr. W. Kisiel. FIXa was prepared by activation of FIX (Baxter) by FXIa and purified by an anti-FIX column.

Thrombin generation. Calibrated Automated Thrombogram (CAT) was used to assay the generation of thrombin in clotting plasma using a Fluoroskan Ascent microtiter plate reading fluorometer (Thermo Labsystems, Finland) and Thrombinoscope software (Thrombinoscope BV, the Netherlands) according to the manufacturer’s instructions and Hemker et al. [17]. Thrombin generation was initiated by recalcification of citrated pooled human plasma or citrated rabbit plasma (Harlan, UK) in the presence of recombinant human tissue factor (Innovin, Siemens Healthcare Diagnostics, Germany), 4 μM phospholipids (PC:PS:PE 60%:20%:20%), 2.5 mM fluorogenic substrate (Z-Gly-Gly-Arg-AMC from Bachem, Bubendorf, Switzerland) with or without rTIX-5. Thrombin formation was followed for 20-60 minutes and measurements were taken at 20 second intervals. Fluorescence intensity was detected at wavelengths of 355 nm (excitation filter) and 460 nm (emission filter). In some cases, coagulation was initiated with APTT based lyophilized silica reagent (Pathromtin SL 8 times diluted, Siemens Healthcare Diagnostics, Germany), FIXa or FXa. The following parameters were derived: ETP, the area under the curve represents the total amount of thrombin generated over time; lag time, the time to the beginning of the explosive burst of thrombin generation; peak, the maximal thrombin concentration; time to peak (TTP), the time until the thrombin peak is reached. Experiments were performed in triplicate and repeated three times. Thrombin generation in a purified system was determined in the presence of the prothrombin complex concentrate Cofact® (Sanquin), which is prepared from human plasma and contains the vitamin K-dependent proteins factors II, VII, IX, X, protein S and protein C. Cofact® was diluted so that the assay was performed under near physiological concentrations of these factors. Assays were performed in the presence of TFPI, FV, phospholipids (PC:PS:PE 60%:20%:20%), 3 mM CaCl₂ and FVIII. Thrombin activation was initiated by the addition of recombinant tissue factor, FIXa or FXa in the presence of rTIX-5 or rP19 as a control. At specific time intervals, aliquots were removed and diluted in 100 mM NaCl, 20 mM Tris-HCl, 10 mM EDTA (pH 7.5) to stop FII activation. Thrombin generation was quantified by adding a final concentration of 0.3 mM of thrombin chromogenic substrate S2238 (Chromogenix) and substrate hydrolysis was measured kinetically by determination of absorbances at 405 nm using a kinetic microplate reader. All experiments were carried out in triplicate. As an alternative approach FII
activation in the purified system was determined by Western blot assessment. At specific time intervals, aliquots were removed and added to SDS sample buffer with or without 2% 2-Mercaptoethanol. Samples were electrophoresed on a SDS 12% polyacrylamide gel and transferred to PVDF membranes. The membranes were blocked with PBS containing 5% milk powder and the immunoblots were probed with a sheep anti-human prothrombin antibody (Kordia, NL). Immunoreactive bands were visualized using horseradish peroxidase conjugated anti-mouse secondary antibodies (Sigma-Aldrich, MO) and the enhanced chemiluminescence Western Blotting Detection System (GE Healthcare, NJ).

**Fibrinogen and whole blood clotting assay.** Thrombin-generation time was measured spectrophotometrically by the fibrin polymerization method as previously described [18]. Thrombin generation was initiated by the addition of recombinant tissue factor and 12 mM CaCl₂, and results were expressed as T 1/2 (time to reach the midpoint of clear to maximal turbid density measured at 450 nm). Whole blood coagulation time was assessed by preincubating fresh citrated human blood with various concentrations of rTIX-5, or PBS as a control, at 37°C for 15 minutes and then recalified. Tubes were incubated at 37°C, tilted every 30 seconds and clotting times were recorded. Experiments were performed in triplicate.

**FXa chromogenic assay.** A single-stage chromogenic assay of FXa inhibition was used to assess the FXa inhibitory activity of rTIX-5. Human FXa was diluted to 2 nM in 10 mM HEPES (pH 7.5) containing 0.1% bovine serum albumin and 150 mM NaCl. Recombinant TIX-5 (with a final concentration 6.5 μM) was incubated with 100 μl of FXa for 15 min at 37°C. Fifty microlitres of 1 mM S-2222 (Chromogenix) was added subsequently and substrate hydrolysis was determined by measuring absorbance at 405 nm over a period of 5 min using a kinetic microplate reader. All experiments were carried out in triplicate.

**Immunization of rabbits with recombinant *I. scapularis* proteins.** Three rabbits were immunized subcutaneously with 3 doses containing 50 μg of rTIX-5 emulsified with Complete Freund’s Adjuvant (first dose) and two subsequent booster injections emulsified in Incomplete Freund’s Adjuvant at 3-week intervals. Control rabbits were immunized with adjuvant and 50 μg of ovalbumin (OVA) or rP19. To demonstrate that the sera from immunized rabbits recognize tick salivary proteins, 2 μg adult *I. scapularis* salivary gland extract (SGE) prepared as described earlier [19], was electrophoresed on a SDS 12% polyacrylamide gel and transferred to PVDF membranes. Immunoblotting was performed using the same methods as described above with the exception that the immunoblots were probed with a 1:250 dilution of rabbit serum and immunoreactive bands were visualized using horseradish peroxidase conjugated goat anti-rabbit secondary antibodies (Sigma-Aldrich, MO). Two weeks after the last immunization, rabbits were infested with 15 *I. scapularis* adult couples on the ear of each rabbit and were kept in place using small socks attached to each ear. Ticks were allowed to feed to repletion until they naturally detached from the host. From 90 hours post attachment the rabbits were examined twice a day for detached ticks and tick weights after repletion were recorded.

**Determination of rate of FV activation by thrombin and FXa in the presence of rTIX-5.** The effect of rTIX-5 on FV activation by FXa and FIIa was determined using a method previously described by Safa et al. [20]. Briefly, purified human FV (133 nM final concentration) was incubated at 37°C with purified human thrombin (1 nM final concentration) or with purified FXa (20 nM final concentration) and phospholipid vesicles (PC:PS:PE 60:20:20%) at a final concentration of 50 μg/ml in HBSA buffer containing 150 mM NaCl, 25 mM HEPES (pH 7.5), 3 mM CaCl₂, and 0.3 mg/mL bovine serum albumin. At specific time intervals, aliquots were removed and diluted in 100 nM NaCl and 20 mM Tris (pH 7.5) to evaluate FV activation immediately in a prothrombin time using FV-deficient plasma. As an alternative approach FV activation was determined by Western blot assessment. For this, a final concentration of 20 nM purified human FV was incubated at 37°C with purified human thrombin (1 nM final concentration) or with purified FXa (10 nM final concentration) in the presence or absence of phospholipid vesicles (50 μg/ml final
concentration) in HBSA buffer. At specific time intervals, aliquots were removed and added to SDS sample buffer.

Samples were electrophoresed on a SDS 5% polyacrylamide gel and transferred to PVDF membranes. Immunoblotting was performed using the same methods as described above with the exception that the immunoblots were probed with the mouse anti-human FV heavy chain monoclonal antibody AHV-5146 (Haematologic Technologies, USA).

Statistical analysis. The significance of the difference between the mean values of the groups was analyzed using the Student $t$ test with Prism 5.0 software (GraphPad Software, USA). $p \leq 0.05$ was considered statistically significant.
Figure S1. General properties of *Ixodes scapularis* protein TIX-5. (A) Multiple sequence alignment of *I. scapularis* TIX-5 aligned with three annotated homologues in *I. scapularis* and one annotated homologue in *Ornithodoros coriaceus*. Amino acids in white on a black background are identical; residues in white on a grey background are similar. Region inside the grey box shows the predicted signal sequence. (B) Predictive N-glycosylation sites using the web based NetNGlyc.
prediction software (http://www.cbs.dtu.dk/services/NetNGlyc). (C) Coomassie staining of rTIX-5 on SDS-PAGE before and after deglycosylation with PNGase F.

Table S1. Amino-acid and nucleotide identity and similarity of TIX-5 and its homologues in *Ixodes scapularis* and *Ornithodoros coriaceus*.

<table>
<thead>
<tr>
<th></th>
<th>L.scap TIX-5</th>
<th>L.scap H-1</th>
<th>L.scap H-2</th>
<th>L.scap H-3</th>
<th>O.coria H-1</th>
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Percent identity and similarity (italics) were calculated after alignment of the nucleotide sequences (down left corner) using the EMBOSS Pairwise Alignment Algorithms (https://www.ebi.ac.uk/Tools/emboss/align/) and amino acid sequences (upper right corner) using the web-based software from NCBI as described by Tatusova et al. (1999). L.scap TIX-5 (GenBank: AE89467), L.scap H-1 (GenBank: XP_002405271.1), L.scap H-2 (GenBank: AAY66581.1), L.scap H-3 (GenBank: XP_002435217.1) and O.coria H-1 (GenBank: ACB70374.1) were compared for homology.

Figure S2. rTIX-5 dose-dependently inhibits coagulation in a fibrinogen-clotting assay. Fibrinogen cloting assay was initiated with 0.25 μM (A), 1 μM (B) or 5 μM (C) TF in normal human plasma. Graphs show a representative of 2 separate experiments.
Figure S3. Activity rTIX-5 is lost after proteinase K treatment.
Purified rTIX-5 or BSA as a control was digested with Proteinase K from Tritirachium album (Sigma) treatment for 30 minutes at 37°C and inactivated by incubation at 70°C for 15 minutes.

Figure S4: Physiological inhibitors of the initiation phase of coagulation support the anticoagulant function of rTIX-5.
Thrombin generation was initiated with 1 pM TF by addition of 4 μM phospholipids in normal human plasma (NHS), protein C deficient (prot. C def), antithrombin deficient (AT def), TFPI deficient (TFPI def) or protein S deficient (prot. S def) human plasma in the presence (white bars) or absence (black bars) of 6.5 μM rTIX-5.

Figure S5: Thrombin generation measured in a purified system. Thrombin was generated in a purified system as described in Methods. At specific time intervals aliquots were withdrawn for analysis of thrombin formation by the rate of conversion of the thrombin specific chromogenic substrate S2238. Thrombin generation was initiated with 8 pM FIXa (A) or 32 pM FXa (B) in the presence of 6.5 μM rTIX-5 or 6.5 μM rP19 as a control.

(C) Varying concentrations of TFPI were tested in the purified system in order to make the system sensitive enough and thrombin generation was initiated with 1 pM TF. (D) Thrombin formation was initiated in the absence of FVIII together with 6.5 μM rTIX-5 or 6.5 μM rP19 as a control.
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<th>Reference</th>
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FXa-induced activation of FV: lessons from a tick salivary protein