Factor Xa Activation of Factor V is of Paramount Importance in Initiating the Coagulation System: Lessons from a Tick Salivary Protein

Running title: Schuit et al.; Factor Xa activation of factor V

Tim J. Schuijt, PhD1,2,3; Kamran Bakhtiar, PhD4; Sirlei Daffre, PhD2,5; Kathleen DePonte2; Simone J.H. Wielders6; J. Arnoud Marquart4; Joppe W. Hovius, MD, PhD1; Tom van der Poll, MD, PhD1; Erol Fikrig, MD2; Matthew W. Bunce, PhD7; Rodney M. Camire, PhD7; Gerry A. F. Nicolaes, PhD6; Joost C. M. Meijers, PhD4; Cornelis van ’t Veer, PhD1

1Center for Experimental and Molecular Medicine; 4Dept of Experimental Vascular Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; 2Dept of Internal Medicine, Yale University School of Medicine, New Haven, CT; 5Dept of Medical Microbiology, Leiden University Medical Center, Leiden, The Netherlands; 6Departamento de Parasitologia, Universidade de São Paulo, São Paulo, Brazil; 7Dept of Biochemistry, Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht, the Netherlands; 1Dept of Pediatrics, Division of Hematology, The Children's Hospital of Philadelphia, PA

Address for Correspondence:
Tim Schuijt, PhD
Center for Experimental and Molecular Medicine
University of Amsterdam
Meibergdreef 9 Room L01-145
1105 AZ, Amsterdam, The Netherlands
Tel: +31(0)20-5665910
Fax: +31(0)20-6977192
E-mail: T.J.Schuijt@outlook.com

Journal Subject Code: Anticoagulants:[159] Anticoagulant mechanisms
Abstract:

**Background**—Generation of active procoagulant cofactor FVα and its subsequent association with the enzyme FXa to form the prothrombinase complex is a pivotal initial event in blood coagulation and has been the subject of investigative effort, speculation and controversy. The current paradigm assumes that FV activation is initiated by limited proteolysis by traces of (meizo) thrombin.

**Methods and Results**—Recombinant tick salivary protein TIX-5 was produced and anticoagulant properties were studied using plasma, whole blood and purified systems. Here we report that TIX-5 specifically inhibits FXa-mediated FV activation involving the B-domain of FV and show that FXa activation of FV is pivotal for plasma and blood clotting. In line, tick feeding is impaired on TIX-5 immune rabbits displaying the in vivo importance of TIX-5.

**Conclusions**—Our data elucidate a unique molecular mechanism by which ticks inhibit the host’s coagulation system. Based on our data we propose a revised blood coagulation scheme wherein direct FXa-mediated FV activation occurs in the initiation phase during which thrombin-mediated FV activation is restrained by fibrinogen and inhibitors.

**Key words:** coagulation
Introduction

Ixodes ticks feed for several days on a vertebrate host to obtain a blood meal and potentially transmit various pathogens, including the causative agent of Lyme borreliosis Borrelia burgdorferi. Ticks tear their way into the dermis and damage small blood vessels after embedding their mouthparts in the host’s skin, which will initiate blood coagulation. To counteract host defense mechanisms ticks introduce saliva at the bite site, containing a variety of proteins with immunosuppressive and anticoagulant properties.

Coagulation can be activated via either the contact activation (or intrinsic) pathway or by the tissue factor (TF) (or extrinsic) pathway. The contact and TF pathway converge at the common pathway, which starts at the level of FX. Activated FX (FXa) forms the prothrombinase complex together with factor Va (FVa) on a phospholipid membrane surface, leading to thrombin generation. Thrombin catalyzes several coagulation-related reactions and converts soluble fibrinogen to fibrin, which forms a solid blood clot together with erythrocytes and platelets. Activation of the coagulation system can be divided into two phases; the “initiation phase” and “propagation phase”. The “initiation phase”, with concurrent FV and FVIII activation and only limited FIX and FX activation, is characterized by low rates of thrombin generation while the “propagation phase” is characterized by rapid, quantitative activation of all available prothrombin. During the initiation phase, low concentrations of enzyme activate the first traces of FV necessary to generate the prothrombinase complex. Traces of thrombin or the active prothrombin activation-intermediate meizothrombin are hypothesized to be responsible for initial FV activation under physiological conditions. However, despite several decades of intensive research on FV activation, the question is still open as to whether FV has some degree of intrinsic activity before activation, and/or whether FXa plays a significant role in FV activation.
The latter is complicated by the extremely rapid feed-forward activation of FV by thrombin, and the lack of specific inhibitors of either thrombin-dependent FV activation or FXa-dependent FV activation. Present knowledge of the behavior and function of the trace active reactants during the initiation phase of coagulation is partly based on extrapolation of clotting times\textsuperscript{12} and computational analysis using models constructed based on the kinetics of the isolated reactions in which FXa activation of FV was assumed to be insignificant\textsuperscript{13}.

Here we characterize the anticoagulant properties of a tick salivary protein previously designated as P23\textsuperscript{14}, which dose-dependently postpones 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 X\(a\) towards factor V’ (TIX-5). By means of TIX-5 we demonstrate that the activation of factor V (FV) by factor X\(a\) (FXa) is a crucial event in the initiation of thrombin generation.

Methods

Ticks and rabbits

\textit{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\(^\circ\)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 was 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 (previously designated as P23\textsuperscript{14}, GenBank: AEE89467) 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\textsuperscript{14}. 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 1,000g 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 were 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 (batroxobin 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 produced in *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. Stable R155A meizothrombin was prepared fresh and used immediately as previously described.\(^1\)

**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 Thrombinscope software (Thrombinscope BV, the Netherlands) according to the manufacturer’s instructions and Hemker *et al*.\(^1\) Thrombin generation was initiated by recalcification of citrated pooled human plasma or citrated rabbit plasma (Harlan,
UK) in the presence of recombinant human 1 or 5pM TF (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 a 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. TF or FIXa dependent 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 1 pM TF or 8 pM FIXa in the presence of rTIX-5 or rP19 as a control. At specific time intervals, aliquots were removed and diluted in 20 mM EDTA, 100 mM NaCl, 25 mM Tris (pH 7.5) to stop prothrombin activation. Prothrombinase experiments with purified components were performed as follows. The mediators were prepared at 2 times their final concentration in 25 mM Hepes 150 mM NaCl, 3 mM CaCl₂, 0.3% BSA and prewarmed to 37°C. Equal volumes of mixtures containing either FXa and phospholipids or FV
and Cofact®, as a thrombin free prothrombin source, were mixed at start of the incubation. rTIX-5 or control was added to the FXa phospholipid mixture immediately before mixing. Final concentrations were 50 pM FXa, 4 μM phospholipid, 5 ug/ml FV or FVa, and Cofact® at a concentration of 0.7 μM prothrombin. Samples were taken in time in 20 mM EDTA, 25 mM Tris 100 mM NaCl pH 7.4 to stop prothrombin 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. Prothrombin activation and FV activation were determined in the purified system by Western blotting. At specific time intervals, aliquots were removed and added to SDS sample buffer with or without 2% 2-Mercaptoethanol. Samples were electrophoresed on SDS polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked with PBS containing 5% milk powder and the immunoblots were probed with either a sheep anti-human prothrombin antibody (Kordia, NL) or mouse anti-human FV heavy chain monoclonal antibody AHV-5146 (Haematologic Technologies Inc, USA). Immunoreactive bands were visualized using horseradish peroxidase conjugated anti-sheep or anti-mouse secondary antibodies (Cell Signaling tech., MA) 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 described17. Thrombin generation was initiated in citrated plasma by addition of recombinant TF and 17 mM CaCl₂, and results were expressed as T 1/2 (time to reach the midpoint of clear to maximal turbid density of the polymerized fibrin 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 recalcified by mixing with IMDM medium containing 12 mM CaCl₂. 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 activity 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 0.3% 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 S2222 (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, 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 1:250 dilution of rabbit serum and immunoreactive bands were visualized using horseradish
peroxidase conjugated anti-rabbit secondary antibodies (Cell Signaling tech, MA). 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.

**Surface plasmon resonance**

Surface plasmon resonance experiments were performed using a BIACORE 2000 (GE Heathcare). rTIX-5 was immobilized on channel 1 of a research-grade CM5 sensor using amine-coupling chemistry according to the standard protocol of the manufacturer. An activated/deactivated surface served as a reference surface. To collect kinetic binding data, FV-variants (1 µM) were injected for 3 minutes in 10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM CaCl₂, 0.005% Tween-20 as flow buffer, followed by flow buffer alone to observe the dissociation. Phospholipids (PC:PS:PE 60%:20%:20%) were immobilized on a L1-chip according to the manufacturer. After stabilization, 100 nM TIX-5 was injected to flow over the surface for three minutes. Subsequently, rTIX-5 was replaced by the flow buffer (10 mM HEPES 7.4, 150 mM NaCl, 3 mM CaCl₂) and dissociation was followed. The curves were processed using the BiaEvaluation 4.1 software.

**Fluorescence binding assay of FV basic peptide**

The peptide encoding the basic region of the FV B-domain (residues 951-1008, “FVBR”) was expressed as a SUMO fusion using the SUMOpro bacterial expression system (LifeSensors, Malvern, PA). The SUMO-FVBR fusion was purified on HisTrapFF columns (Amersham), the SUMO tag was cleaved with SUMO protease (LifeSensors), and the FV peptide was purified by
ion exchange chromatography. For fluorescence anisotropy experiments, FVBR containing an N-terminal Cys was labeled with Oregon Green488 maleimide (“OG488-BR”) according to the manufacturer’s instructions (Invitrogen).

Steady state fluorescence anisotropy was measured at 25°C in a PTI QuantaMaster fluorescence spectrophotometer (Photon Technology International) using excitation and emission wavelengths of 480 nm and 520 nm, respectively with long pass filters (KV500, CVI Melles Griot) in the emission beam. Displacement experiments were performed by pre-incubating 30 nM OG488-BR with 20 nM FV-810 and 50 μM PCPS (75:25 PC:PS) in 20 mM Hepes, 150 mM NaCl, 2 mM CaCl2, and 0.1% (w/v) PEG-8000, pH 7.4 (“assay buffer”). Displacement of OG488-BR from FV-810 was monitored by the decrease in OG488-BR anisotropy throughout the titration of unlabeled FVBR peptide or rTIX-5. Fluorescence anisotropy measurements and data analysis were performed as described19, 20.

Recombinant FV variants

Cloning, expression and characterization of the recombinant FV variants that contain mutations at the three thrombin and FXa cleavage sites have been described previously21. The FV variants were named according to which amino acid replaced the Arg-residue present at each cleavage site22, i.e. WT FV (Arg709, Arg1018, and Arg1545), RIQ (Arg709, Ile1018, and Gln1545), QRQ (Gln709, Arg1018, and Gln1545), QIR (Gln709, Ile1018, and Arg1545) and QIQQQ (Gln709, Ile1018, Gln1545, Gln1761, and Gln1765). The FV variant lacking the B-domain region (FV des 827-1499) was named FV B-dom Δ. Transient expression and collection of the FV variants was performed as previously described22. Proteins were secreted and collected in serum-free medium (Optimem Glutamax, Gibco) and protein concentration was determined using a FV-ELISA as previously
described\textsuperscript{22}. FV-variants with partial deletions of the B-domain were expressed and purified as described\textsuperscript{23}.

**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, thrombin and meizothrombin was determined using a method previously described by Safa et al.\textsuperscript{24}. Briefly, purified human FV (133 nM final concentration) was incubated at 37°C with purified human thrombin (1 nM final concentration) with purified FXa (10 nM final concentration) or R155A meizothrombin (0.2 nM final concentration) and 4 μM phospholipid vesicles (PC:PS:PE 60%:20%:20%) in HBSA buffer containing 150 mM NaCl, 25 mM HEPES (pH 7.5), 3 mM CaCl\textsubscript{2}, and 0.3% bovine serum albumin. At specific time intervals, aliquots were removed and diluted in 100 mM 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 as described\textsuperscript{25}. 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 4 μM phospholipid vesicles (PC:PS:PE 60%:20%:20%) 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).

The effect of rTIX-5 on the activation of the various FV variants described above by FXa and thrombin was determined using a method previously described\textsuperscript{22}. In this assay, the FVa cofactor activity was determined via FXa-catalyzed thrombin generation. Briefly, 250 pM of various
recombinant FV variants were activated by 5 nM FXa or thrombin in the presence or absence of rTIX-5. At different time points FV concentrations were measured by prothrombin activation in a reaction mixture with 25 mM HEPES pH 7.5, 150 mM NaCl, 3 mM CaCl₂, 1 μM prothrombin, 0.33 nM FXa, 20 μM phospholipid vesicles (PC:PS 90%;10%) and 0.5 mg/ml ovalbumin. A final concentration of 1 μM Pefabloc TH was added to the mixture to prevent feedback activation of FV by thrombin. Activation of prothrombin was stopped by dilution in ice-cold EDTA buffer and generated thrombin was measured using the chromogenic substrate S2238 by measuring absorbance at 405 nm over a period of 5 min using a kinetic microplate reader.

Statistical analysis

Data are expressed as mean ± SEM. 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). For comparisons between multiple groups, results were analyzed by the Kruskal-Wallis test with Dunn’s posttest for multiple comparisons or 2-way ANOVA when appropriate. If overall significant by ANOVA, differences were analyzed by Bonferroni post tests. p ≤ 0.05 was considered statistically significant.

Results

Both contact- and TF-mediated activation of coagulation is inhibited by recombinant TIX-5 in a dose-dependent manner

Ixodes scapularis protein TIX-5 was recently identified as an antigenic salivary protein after a Yeast Surface Display screen with I. scapularis immune rabbit serum (designated as P23) and showed anticoagulant activity by significantly prolongation of the lag time of thrombin
formation in human plasma after initiation of coagulation with 1 pM TF\textsuperscript{14}. We now show that recombinant (r)TIX-5 dramatically prolonged lag time and time to peak of thrombin generation initiated in human plasma through the contact activation route (Fig.1a) as well as by 1 pM and 5 pM TF (Fig.1b,c), with more subtle effects on the total amount of thrombin formed (Endogenous Thrombin Potential, ETP). rTIX-5 retained its anticoagulant properties after initiation of the TF coagulation pathway in both FVIII (Fig.1d) and FXI (Fig.1e) deficient plasma, which indicates that TIX-5 does not exert its inhibitory action through interference with FVIII activity or generation of thrombin through feedback activation via FXI\textsuperscript{26}. 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.1f). The effect of rTIX-5 was dose-dependent (Fig.1g), and confirmed in a fibrinogen-dependent plasma clotting assay (Supplementary Fig.1). Upon degradation by proteinase K, rTIX-5 lost its anticoagulant properties (Supplementary Fig.2). Spontaneous clotting of human whole blood was also dose-dependently extended by rTIX-5 (Fig.1h), underscoring its efficacy in a more relevant system.

**Immunization with recombinant TIX-5 impairs adult tick feeding on rabbits**

To elucidate the biological importance of TIX-5 in tick feeding, rabbits were immunized with rTIX-5. Immunization induced an antibody response that recognized native TIX-5 in salivary gland extract (SGE) of *I. scapularis* adults (Fig.2a). Immune serum antibodies detected multiple bands in the range from 23 to 30 kDa representing differentially glycosylated forms of TIX-5 (Fig.2a and Supplementary Fig.3). Serum from OVA immunized rabbits showed no reaction with proteins in SGE. Antisera to a tick salivary control protein rP19 recognized native P19, but not native TIX-5 (Fig.2a). Adult tick engorgement weights after spontaneously detachment were significantly lower after feeding on rTIX-5 immune rabbits compared to rP19 immune control.
rabbits (Fig.2b). In line, rTIX-5 inhibited TF or contact activation pathway initiated thrombin generation in rabbit plasma as well (Fig.2c,d).

rTIX-5 inhibits FXa-driven thrombin generation independent of the active site and phospholipids

Since rTIX-5 inhibited the coagulation system initiated via either the contact activation or TF pathway, we assessed whether rTIX-5 inhibited the common pathway of coagulation. When thrombin formation 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). Tick saliva contains active site inhibitors of FXa, including Salp1427. 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 concentrations 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 TF-initiated thrombin generation (Fig.3d), indicating that rTIX-5 does not simply neutralize phospholipids.

Inhibitors of the initiation phase of coagulation reinforce the anticoagulant effect of rTIX-5

We assessed whether physiological inhibitors of the human coagulation system influenced the anticoagulant properties of rTIX-5. Fibrinogen, also referred to as antithrombin I, reduces thrombin generation by binding thrombin with high affinity28. 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 al29. 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). Thus, the inhibitory effect of rTIX-5 is greatly reduced in fibrinogen-depleted plasma. In line with this observation, the absence of other physiological inhibitors of the initiation phase, e.g. antithrombin, TF pathway inhibitor (TFPI) and protein S resulted in a reduced anticoagulant effect of rTIX-5 (Supplementary Fig.4). The absence of protein C, which is not an inhibitor of the initiation phase, did not influence the effect of rTIX-5 (Supplementary Fig.4).

Preactivation of prothrombin and FV bypasses the anticoagulant effect of rTIX-5

Since the absence of thrombin inhibitors or thrombin generation inhibitors dampened the anticoagulant activity of rTIX-5, we further investigated if rTIX-5 was 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 abrogated in the presence of 20 nM FVa (Fig.4c), which suggested that rTIX-5 postpones the activation of FV leading to reduced activation of prothrombin. Human plasma contains numerous factors or proteins that could be involved in the inhibitory effect of rTIX-5 on FV activation. We therefore investigated the anticoagulant effect of rTIX-5 on thrombin generation in a partially purified system containing the vitamin K dependent coagulation factors (Cofact®) supplemented with FV, FVIII, TFPI, phospholipids and CaCl₂. In this system thrombin generation was inhibited by rTIX-5 after initiation with 1 pM TF.
(Fig.4d), as well as when initiated with 8 pM FIXa (Supplementary Fig.5). Thus, by using this system of purified coagulation factors, we showed by Western blotting that thrombin generation (Fig.4e) as well as FV activation (Fig.4f) was postponed in the presence of rTIX-5. In line with these data, thrombin generation was inhibited by rTIX-5 in a prothrombinase experiment with procofactor FV, while rTIX-5 did not inhibit prothrombinase activity started with preformed FVa (Fig.4g). These data prompted us to study whether rTIX-5 inhibits coagulation activation by interference with FV activation.

**rTIX-5 specifically inhibits FXa-mediated FV activation**

To 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 and the active prothrombin activation-intermediate meizothrombin have been hypothesized to be the most significant contributors in formation of FVa (reviewed in 9,10), we determined whether FV activation by these was inhibited by rTIX-5. We here demonstrate that rTIX-5 did neither affect the activation of FV by 1 nM thrombin (Fig.5a) nor by 0.2 nM meizothrombin (Fig.5b) in the presence of phospholipids as evaluated by both Western blot analysis (upper panels) and FVa clot assay (lower panels). Strikingly, rTIX-5 abrogated activation of FV by 10 nM FXa-phospholipid (Fig.5c), demonstrated by both Western blotting of active fragments of FV and FVa clot assay. Activation of FV by FXa in the absence of phospholipids was negligible (data not shown). rTIX-5 dose-dependently inhibited FXa-mediated FV activation with a half maximal inhibitory concentration (IC50) for rTIX-5 of ~3.2 μM (Fig.5d). Presently it is assumed that small amounts of thrombin are generated by FXa when assembled on a membrane surface which subsequently activate sufficient FV to induce prothrombinase activity. Importantly, direct activation of prothrombin by FXa in the absence of FV was not impaired by rTIX-5 (Fig.5e), which corroborates that rTIX-5
is not a FXa active site inhibitor, nor binds to an exosite of FXa involved in prothrombin activation. Collectively, these data demonstrate that rTIX-5 is a highly specific inhibitor of FXa-mediated FV activation. In line with this, the inhibiting effect of TIX-5 on clot formation was essentially absent in factor V deficient human plasma initiated with 30 nM of FXa (Fig.5f). The nominal inhibiting effect of TIX-5 in factor V deficient plasma can be explained by traces of FV as shown by means of factor V neutralizing IgG and APC (Fig.5f).

Inhibitory action of rTIX-5 on FV activation is B-domain dependent

To further pinpoint the anticoagulant effect of TIX-5, we used FV mutants that either lack one, several or all Arg residues involved in the proteolytic activation of FV, or miss the central B-domain as described by Segers et al.22 Also using this method, rTIX-5 inhibited FXa-catalyzed (Fig.6b) but not thrombin-mediated (Fig.6a) FV activation. FXa activation of the FV mutants that lacked one or more activation sites was still inhibited by rTIX-5 (Fig.6c-f,h), while TIX-5 specifically failed to inhibit FXa activation of mutants lacking the central B-domain (Fig.6g,h). TIX-5 did not affect activation of the FV mutants by thrombin (data not shown). In line with this, rTIX-5 significantly inhibited coagulation when recombinant wild type FV was added to FV deficient plasma whereas this effect was abrogated in the presence of the FV mutant lacking the central B-domain (Fig.6i). These results indicate that TIX-5 is not simply inhibiting the proteolysis of one specific cleavage site but interferes via a B-domain dependent mechanism.

Both the acidic and basic region of the factor V B-domain support the inhibitory effect or TIX-5

Very recently it was shown that the B-domain, which contains basic and acidic regions (Fig.7a), hinders FXa binding to the high affinity FXa binding site normally exposed only after proteolysis of the B-domain.23 Since TIX-5 also harbors a basic region (Fig.7a) we next assessed whether
TIX-5 was able to compete for binding to the acidic region of FV-810, a FV B-domain derivative that contains the B-domain acidic region but not the basic region. Nevertheless, rTIX-5 did not compete with the FV basic region based on competitive binding experiments (Fig.7b). In line with these results, rTIX-5 inhibited thrombin formation when the various factor B-domain variants, with either the basic region (FV-B152) or acidic region (FV-810) or both basic and acidic regions (FV-1033) were added to FV deficient plasma (Fig.7c). Of note, thrombin formation was less inhibited by rTIX-5 in the presence of FV-B152 or FV-810 compared to plasma derived factor V (FV-WT) or FV-1033, indicating that both the acidic and basic regions of the FV B-domain support the inhibiting effect of rTIX-5 (Fig.7c). rTIX-5 abrogated activation of FV-810 (Fig.7d), FV-B152 (Fig.7e), FV-1033 (Fig.7f) by 10 nM FXa in the presence of phospholipids as demonstrated by Western blotting of active fragments of FV. To characterize specific interactions of rTIX-5 with FXa and/or FV, surface plasmon resonance experiments were performed which revealed that rTIX-5 bound to both FV-1033, FV-B152 and to a lesser extent to FV-810, but not to FXa under these conditions (Fig.7g). Binding analysis of TIX-5 to a phospholipid layer showed an additional interaction of TIX-5 with phospholipids (Fig.7h).

**Discussion**

We here report that TIX-5, a major antigen in tick saliva, inhibits blood coagulation by specific inhibition of FXa-dependent FV activation. The mechanism by which TIX-5 specifically inhibits FXa-mediated FV activation involves the central B-domain of FV, which contains the initial preferential cleavage site for FXa\(^1\). Most importantly, delineation of the molecular anticoagulant mechanism of TIX-5 provides for the first time evidence that FXa is a physiological relevant activator of FV, challenging the dogma that thrombin or the active prothrombin activation-
intermediate meizothrombin are the only physiologic activators of FV in the initiation phase of coagulation\textsuperscript{9-12,30}.

It is of crucial importance that enzymes of the coagulation system and their cofactors circulate in an inactive form under physiological conditions and are activated promptly, but only when necessary. Traces of activated FV are likely inactivated by activated protein C. The latter is a crucial antithrombotic mechanism as shown by the association of the FV-Leiden mutation with thrombosis, which renders APC-resistant FV\textsubscript{a}\textsuperscript{31}. In the common pathway of coagulation, FX and FV are activated to FX\textsubscript{a} and FV\textsubscript{a} respectively. FV\textsubscript{a} is the non-enzymatic cofactor of the prothrombinase complex, which accelerates the conversion of prothrombin to thrombin by FX\textsubscript{a} approximately 300,000 times\textsuperscript{32,33}. The high efficiency of FX\textsubscript{a} to generate thrombin in the presence of only minute traces of FV\textsubscript{a}, together with the relative poor activation of FV by FX\textsubscript{a} and extremely efficient FV activation by (meiz)thrombin, have hampered studies to the contribution of FV activation by FX\textsubscript{a} in relevant thrombin generating systems. Activation of FV involves cleavages in the procofactor form that release the central gatekeeper B-domain to generate active FV\textsubscript{a}, a non-covalent complex formed by the N-terminal derived heavy chain and the C-terminal derived light chain\textsuperscript{11} (Supplementary Fig.6). While thrombin directly cleaves FV at Arg709 and subsequently at Arg1545 to liberate the respective heavy chain and light chain, the sequence of events is different for FX\textsubscript{a}. FX\textsubscript{a} preferentially first cleaves the FV B-domain at Arg1018 and subsequently at position Arg709 and Arg1545\textsuperscript{21}. Using mutant FV molecules, we were able to show that TIX-5 only inhibited FV activation by FX\textsubscript{a} when the B-domain was present in the procofactor form. A FV mutant that lacks the B-domain was activated by FX\textsubscript{a} in a manner that is not inhibited by TIX-5, showing that TIX-5 inhibits FX\textsubscript{a}-catalyzed FV proteolytic activation in a B-domain dependent mechanism. Very recently it was shown that the B-domain,
which contains basic and acidic regions, hinders FXa binding to the high affinity FXa binding site normally exposed only after proteolysis of the B-domain. We hypothesize that TIX-5 cooperates with the inhibitory B-domain segments to impair FXa-mediated activation of FV. In line with this hypothesis, TIX-5 inhibited FXa-mediated activation of all four FV variants with mutated cleavage sites. Thus, TIX-5 does not block the cleavage sites themselves but prevents the accessibility of FXa to activate FV.

Although an obvious basic region is present in TIX-5, binding of TIX-5 to the acidic region of FV did not appear to dominate binding. The SPR experiments revealed that rather the basic region of the FV B-domain seems to support a larger part of the direct protein-protein interaction between TIX-5 and FV. Importantly, TIX-5 interacted with and inhibited FV variants that lack either the acidic or basic region in the B-domain, indicating that the action of TIX-5 is not caused by a simple one site binding event. Thus, the exact molecular mechanism by which TIX-5 inhibits FXa-mediated FV activation remains elusive. While FV variants that lack either the acidic or basic region in the B-domain behave as active cofactors, TIX-5 is still able to turn these derivatives into partially active or procofactor proteins. Since this is not the case for FVa or B-domainless FV, these findings emphasize a dependency of parts of the FV B-domain in the function of TIX-5 as coagulation inhibitor. In our current view, based on the notion that TIX-5 has some phospholipid binding properties, we construe that TIX-5 prevents functional low affinity activating Xa-FV interactions that drives the FXa-mediated FV activation on phospholipid membranes. Importantly, interaction of TIX-5 with the phospholipid surface does not interfere with other phospholipid-dependent reactions, including the activation of prothrombin by FXa and the phospholipid-dependent FV activation by meizothrombin. Additionally, in the presence of excessive amounts of phospholipids (20 μM), TIX-5 still...
inhibited TF-initiated thrombin generation. Altogether, these data exclude that TIX-5 is just a phospholipid scavenger and show that TIX-5 is a specific inhibitor of the phospholipid dependent activation of FV by FXa.

The ability of FXa to activate the FV QIQQQ variant, lacking all specific FXa activation sites is puzzling. However, Thorelli et al. demonstrated that FXa is more promiscuous in cleaving at other sites than the traditional 709, 1018 and 1545 thrombin cleavage sites21. Potential other exposed Arg are vulnerable to cleavage by FXa in the B-domain and/or Arg residues in the HC and LC that would generate truncated HC/LC fragments forming a partially active FVa, which could explain how FV QIQQQ gets activated by FXa.

It has been proposed that the α-thrombin responsible for early FV activation is produced directly by FXa on phospholipids during the initial phase of coagulation9,34. TIX-5 did not affect direct activation of prothrombin to thrombin on phospholipids by FXa, nor did it affect the enzymatic activity of FXa or thrombin towards small chromogenic substrates. Consistent with these observations and characteristic for its mode of action TIX-5 did not inhibit thrombin generation in plasma in the presence of FVa or started by traces of thrombin and positive feedback by FXI activation, whereas both the contact activation and TF pathway of coagulation were inhibited by TIX-5. Indeed, in a purified system, prothrombinase activity was exclusively inhibited by TIX-5 in the presence of procofactor FV, whereas inhibition of thrombin formation by TIX-5 was abrogated in prothrombinase with preactivated FVa.

Furthermore, by using TIX-5 as a tool we uncovered that the importance of FXa-dependent FV activation for thrombin generation in plasma is largely dependent on the presence of fibrinogen. This underscores the notion that fibrinogen, also referred to as antithrombin I, binds and inhibits thrombin28,35, thereby shifting the role of FXa to a crucial FV activator in
plasma. In a reconstituted system with purified coagulation factors we were able to confirm the inhibitory role of TIX-5 on thrombin generation in complete absence of fibrinogen which indicates that the action of TIX-5 is not dependent, but more efficient in the presence of fibrinogen. Similar observations were made regarding the other physiologic coagulation inhibitors that regulate the initiation phase of thrombin generation, among which antithrombin-III\textsuperscript{25}, TFPI and protein S\textsuperscript{36,37}, indicating that TIX-5 efficiently exploits the halt on thrombin generation provided by the host coagulation inhibitors. Thus, we observed that rTIX-5 inhibits thrombin generation profoundly when the initiating trigger is low and when thrombin formation and function are counterbalanced by coagulation inhibitors of the host.

The coagulation system is triggered immediately after the tick’s mouthparts penetrate and damage the host’s tissue. In order to secure their blood meal, ticks have been shown to target several parts of the host coagulation system, among which platelets, thrombin, FXa and fibrinogen\textsuperscript{38} by alternating phases of sucking blood and secreting saliva into the feeding pit with each phase lasting as long as 5-20 minutes\textsuperscript{39}. Interestingly, non-anticoagulated whole 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. Considering ticks have a wide repertoire of anticoagulant proteins, the anticoagulant effect of these additional inhibitors is most likely enhanced in the presence of TIX-5, since TIX-5 inhibits the generation of FVa, which stabilizes and protects FXa against inhibition\textsuperscript{40}. Interestingly, by performing a bioinformatics analysis of TIX-5, we identified beside several homologues in\textit{I. scapularis} also a partial amino acid sequence coding for an annotated homologue in\textit{Ornithodoros coriaceus} (Supplementary Fig.7 and Supplementary Table 1). Since\textit{Ornithodoros} is part of a distinct family of ticks, i.e. Argasidae\textsuperscript{41}, it is likely that TIX-5 is a member of a larger protein family in ticks. Anti-tick vaccines to prevent pathogen transmission
are the topic of extensive investigation and TIX-5 or its homologues could be employed, possibly in combination with other tick proteins, in efforts to develop vaccines that render ‘tick immunity’ or ‘tick resistance’\textsuperscript{42}. In line with this, we here show that adult \textit{I. scapularis} weights were dramatically reduced after feeding on rTIX-5 immunized rabbits, which is a major parameter indicative of tick immunity\textsuperscript{43}.

In conclusion, our findings show evidence for facilitation of rapid thrombin generation by direct FXa-mediated FV activation under physiological blood and plasma clotting conditions. This was accomplished by using a novel anticoagulant tick protein, TIX-5, that inhibits the coagulation system in a unique way. Thus, we propose a scheme for blood coagulation as shown in Fig.8 in which FXa-dependent FV activation is pivotal during the initiation phase while traces of initial thrombin are captured by physiological thrombin inhibitors. By formation of the initial prothrombinase complexes, thrombin generation enters the ‘transition phase’ with complete FV activation leading to the explosive propagation phase of thrombin generation. This study, using TIX-5 as a tool to inhibit FXa-mediated activation of FV, has broad implications for the comprehension of the initiation phase of coagulation and indicates that inhibition of FV activation by FXa may provide an alternative therapeutic strategy for thrombotic diseases.

\textbf{Acknowledgements:} Cofact® was generously provided by Ruud Zoethout (Sanquin). We thank Han Levels, Rolf Urbanus and Sukanya Narasimhan for technical support. We thank Nan van Geloven and Arjan Hoogendijk for statistical assistance.

\textbf{Funding Sources:} T.J.S. is supported by a grant from the Onze Lieve Vrouwe Gasthuis (OLVG) research fund. This study was supported by grants 41440, 49200, and 32947 from the NIH. J.W.H. is a recipient of a VENI stipend (91611065) from the Netherlands Organization for health research and development (ZonMw). E.F. is an Investigator of the Howard Hughes Medical
Institute. M.W.B is supported by grants T32 HL07439 from the NIH. R.M.C. is supported by grants R01-HL88010 and P01-HL74124, Project 2; NIH.

Conflict of Interest Disclosures: None.

References:


**Figure Legends:**

**Figure 1.** Both the contact activation and the TF coagulation pathway 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 silica-based APTT reagent, (b, f) 1 pM TF, (c) 5 pM TF or (d) in FVIII deficient plasma with 5 pM TF or (e) FXI deficient plasma with 1 pM TF. (f) Thrombin generation in platelet-rich plasma (PRP) was initiated with 1 pM TF. Data are means ± SEM. The asterisk indicates a statistically significant difference (P<0.01). (g) Thrombin generation in normal human plasma was initiated with 1 pM TF in the presence of various concentrations of rTIX-5 (h) 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. Representatives of at least two experiments are shown.

**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. Upon challenge of the immunized rabbits with *I. scapularis* adult ticks, similar numbers of ticks fed to repletion and spontaneously detached on the control and experimental animals. The horizontal bars represent the medians of the respective groups. Thrombin generation was initiated in normal rabbit plasma by addition of 4 μM phospholipids and 8 times diluted APTT reagent (c) or 5 pM TF (d). Lagtimes are shown in the box. The asterisk indicates a statistically significant difference (P<0.01).

**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 (a) normal human plasma or (b) FX deficient plasma in the absence (0 μM) or by addition of (a) 0.25 μM, (a) 1 μM or (a,b) 4 μM phospholipids. (c) FXa mediated cleavage of substrate S2222 was measured in the presence (white bars) or absence (black bars) of 6.5 μM rTIX-5. (d) Coagulation was initiated in normal human plasma 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. Lagtimes are shown in the box. The asterisk indicates a statistically significant difference (P<0.01).

**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 (solid lines) and 5 pM TF (dashed 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 or FVa. 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. (d) Thrombin was generated in a purified system as described in Methods 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. (e) Additionally, the aliquots were subjected to Western blot analysis under reduced conditions and the thrombin B-chain (FIla-BC;30 kDa) was visualized with anti-prothrombin antibody. (f) Simultaneously, FV heavy chain (FVa-HC;100 kDa) formation was visualized by Western blot analysis. (g) Thrombin was generated as described in Methods in a purified system with 50 pM FXa, 4 μM phospholipids, 5 μg/ml FV (circles, solid line) or FVa (squares, dashed line) with 6.5 μM rTIX-5 (red) or control rP19 (black) 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. Graphs show a representative of 2 separate experiments.

**Figure 5.** rTIX-5 specifically prevents activation of FV by FXa on a phospholipid membrane. Time course of FV activation by (a) 1 nM thrombin or (b) 0.2 nM meizothrombin or (c) 10 nM FXa in the presence of phospholipids as described in Methods was evaluated by Western blotting (upper 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 (lower panels) at the indicated time points as described in the Methods. (d) Left: FV was incubated with 10 nM FXa for 8 minutes in the presence of phospholipids and various concentrations of rTIX-5. FV activation was evaluated by
Western blotting using an anti-FV heavy chain antibody. Right: Band intensities were quantified by scanning densitometry using ImageJ software (National Institutes of Health). (e) Thrombin concentration was measured by the rate of conversion of a thrombin specific chromogenic substrate at the indicated time points when 1.4 μM prothrombin was activated with 35 nM FXa in the presence of 0.7 μM phospholipids. (f) Coagulation of human FV deficient plasma was initiated with 30 nM FXa in the fibrinogen clotting assay (right panel) in the presence of 6.5 μM rTIX-5, 10 μg/ml FV inhibiting antibody (aFV) and/or 30 nM APC (right panel). As a control normal human plasma was initiated with 0.25 pM or 1 pM TF in normal human plasma under the same conditions (left panel).

**Figure 6.** rTIX-5 inhibits FXa-mediated FV activation in a B-domain dependent mechanism. Activation of various FV variants was described by Segers et al\(^2\). Wild type FV was activated by either (a) 1 nM thrombin or (b) 10 nM FXa. In addition, several FV variants (c) QIR, (d) RIQ, (e) QRQ, (f) QIQQQ and the (g) FV B-domain deletion mutant was activated by 10 nM FXa. Rates of FVa generation per minute in the absence (dotted line) and presence (straight line) of 6.5 μM rTIX-5 were derived from the initial slopes of the curves. (h) Percentage of FVa generation was calculated by dividing the rate of FVa generation in the presence of TIX-5 by the rate of FVa generation in the absence of TIX-5 (x 100%). (i) Thrombin generation was initiated with 1 pM TF and 4 μM phospholipids in FV deficient human plasma supplemented with either wild-type factor V or the FV B-domain deletion mutant in the presence (white bars) or absence (black bars) of 6.5 μM rTIX5. Normal human plasma (NHP) was used as a control.
Figure 7. Both the acidic and basic regions of the FV B-domain support inhibition of FXa-mediated FV activation by rTIX-5. (a) Schematic representation of TIX-5 and several recombinant FV B domain variants as described previously. Top: the factor V B-domain (yellow) is located at position 709-1545 and contains a basic region (position 963-1008) as well as an acidic region (position 1493-1537). Middle: TIX-5 harbors a basic region (position 148-172). Bottom: Recombinant FV variants with variable B-domains. FV-1033 is a procofactor-like variant that contains both the basic and acidic regions, whereas FV-810 contains only the acidic region and FV-B152 contains only the basic region. Residues that are deleted from the B-domain are indicated on the right. (b) Labeled FV basic peptide was pre-incubated with FV-810. Either unlabeled FV basic peptide (as a positive control) or rTIX-5 was titrated and the change in fluorescence anisotropy of the labeled FV peptide at each point was measured as previously described\textsuperscript{23}. (c) Thrombin generation was initiated with 1 pM TF and 4 μM phospholipids in FV deficient human plasma supplemented with either 2 nM FVa, FV-810, FV-B152, FV-1033, FV-WT in the presence (white bars) or absence (black bars) of 6.5 μM rTIX5. Normal human plasma (NHP) was used as a control. Time course of (d) FV-810, (e) FV-B152, (g) FV-1033 activation by 10 nM FXa in the presence of phospholipids as described in Methods was evaluated by Western blotting using an anti-FV heavy chain antibody. Reaction time points in minutes are indicated above the lanes. (h) Binding of FV-810, FV-B152, FV-1033 or FXa to rTIX-5 was investigated with surface plasmon resonance. rTIX-5 was immobilized to a CM5 sensor chip and FV-810, FV-B152, FV-1033 or FXa was applied to the chip by injection for 180s. (i) Phospholipids (PC:PS:PE 60%:20%:20%) were immobilized on a L1 chip and rTIX-5 was applied to the chip.
**Figure 8.** Molecular anticoagulant mechanism of TIX-5 and an adapted model for the subsequent phases of thrombin generation depicting the role of FXa in FV activation. Ticks introduce tick salivary proteins at the feeding pit in order to suppress host inflammation and coagulation responses including the anticoagulant protein TIX-5. The specific anticoagulant action of TIX-5 through interference with FXa-catalyzed FV activation on a phospholipid surface shows that this pathway is of paramount importance in the initiation phase of coagulation activation (left panel). In this model (right panel), the FXa dependent activation of FV becomes of crucial importance because traces of thrombin formed in the ‘initiation phase’ are captured by physiological thrombin inhibitors such as fibrinogen and antithrombin (AT). Once FXa activates sufficient FV to form the prothrombinase complex, prothrombin conversion is amplified resulting in rapid thrombin-catalyzed FV activation in the ‘transition phase’ and the formation of a blood clot. Next, thrombin generation accelerates once more and all prothrombin is converted during the propagation phase.
Figure 1

**Figure 1**

(a) Control and 6.5 μM rTIX-5 FLB (nM) over time (min).

(b) Control and 6.5 μM rTIX-5 FLB (nM) over time (min).

(c) Control and 6.5 μM rTIX-5 FLB (nM) over time (min).

<table>
<thead>
<tr>
<th>APTT reagent</th>
<th>Control</th>
<th>rTIX-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag time (min)</td>
<td>4.3 ± 0.3</td>
<td>12.7 ± 0.1*</td>
</tr>
<tr>
<td>ETP (nM/min)</td>
<td>1455 ± 23</td>
<td>1241 ± 19*</td>
</tr>
<tr>
<td>tTPeak (min)</td>
<td>6.2 ± 0.2</td>
<td>15.7 ± 0.2*</td>
</tr>
<tr>
<td>Peak (nM)</td>
<td>353 ± 33</td>
<td>178 ± 25*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TF 1pM</th>
<th>Control</th>
<th>rTIX-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag time (min)</td>
<td>4.7 ± 0.8</td>
<td>13.8 ± 0.8**</td>
</tr>
<tr>
<td>ETP (nM/min)</td>
<td>1685 ± 55</td>
<td>1445 ± 23*</td>
</tr>
<tr>
<td>tTPeak (min)</td>
<td>9.4 ± 0.5</td>
<td>17.0 ± 0.3*</td>
</tr>
<tr>
<td>Peak (nM)</td>
<td>197 ± 20</td>
<td>167 ± 18*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TF 5pM</th>
<th>Control</th>
<th>rTIX-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag time (min)</td>
<td>2.3 ± 0.2</td>
<td>6.3 ± 0.5*</td>
</tr>
<tr>
<td>ETP (nM/min)</td>
<td>1786 ± 15</td>
<td>1505 ± 16*</td>
</tr>
<tr>
<td>tTPeak (min)</td>
<td>5.1 ± 0.3</td>
<td>10.1 ± 0.3*</td>
</tr>
<tr>
<td>Peak (nM)</td>
<td>331 ± 35</td>
<td>209 ± 21*</td>
</tr>
</tbody>
</table>

(d) FVIIIdef control and FVIIIdef + 6.5μM rTIX-5 FLB (nM) over time (min).

(e) FXIdef control and FXIdef + 6.5μM rTIX-5 FLB (nM) over time (min).

<table>
<thead>
<tr>
<th>TF 5pM</th>
<th>FVIII def</th>
<th>Control</th>
<th>rTIX-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag time (min)</td>
<td>2.8 ± 0.1</td>
<td>7.0 ± 0.2*</td>
<td></td>
</tr>
<tr>
<td>ETP (nM/min)</td>
<td>1073 ± 23</td>
<td>936 ± 20</td>
<td></td>
</tr>
<tr>
<td>tTPeak (min)</td>
<td>8.2 ± 0.1</td>
<td>15.2 ± 0.1*</td>
<td></td>
</tr>
<tr>
<td>Peak (nM)</td>
<td>59 ± 1.4</td>
<td>48 ± 1.6*</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TF 1pM</th>
<th>FXI def</th>
<th>Control</th>
<th>rTIX-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag time (min)</td>
<td>4.3 ± 0.3</td>
<td>12.7 ± 0.1*</td>
<td></td>
</tr>
<tr>
<td>ETP (nM/min)</td>
<td>1455 ± 23</td>
<td>1241 ± 19*</td>
<td></td>
</tr>
<tr>
<td>tTPeak (min)</td>
<td>10.2 ± 0.2</td>
<td>17.7 ± 0.2*</td>
<td></td>
</tr>
<tr>
<td>Peak (nM)</td>
<td>122 ± 13</td>
<td>89 ± 15*</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PRP-TF 1pM</th>
<th>Control</th>
<th>rTIX-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag time (min)</td>
<td>7.8 ± 0.6</td>
<td>12.7 ± 0.4*</td>
</tr>
<tr>
<td>ETP (nM/min)</td>
<td>2243 ± 51</td>
<td>2074 ± 44</td>
</tr>
<tr>
<td>tTPeak (min)</td>
<td>17.3 ± 0.4</td>
<td>33.2 ± 0.6*</td>
</tr>
<tr>
<td>Peak (nM)</td>
<td>167 ± 13</td>
<td>147 ± 13*</td>
</tr>
</tbody>
</table>

**Table 1**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control</th>
<th>rTIX-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag time (sec)</td>
<td>(*** p&lt;0.0001)</td>
<td></td>
</tr>
<tr>
<td>Clot time (minutes)</td>
<td>(*** p&lt;0.0001)</td>
<td></td>
</tr>
</tbody>
</table>

**Graphs a, b, and c** show FLB (nM) over time (min) for Control and 6.5 μM rTIX-5.

**Graphs d, e, and f** show FLB (nM) over time (min) for FVIIIdef and FXIdef controls and their respective treatments.

**Graph g** depicts the relationship between concentration protein (μM) and lag time (sec), with a significant difference indicated by \(*** p<0.0001\).

**Graph h** illustrates the clot time (minutes) for Control and two concentrations of rTIX-5, showing a significant difference indicated by \(*** p<0.0001\).
Figure 2
Figure 3
Figure 4

(a) Plotted data showing time (min) versus Flia (nM) for different concentrations of rTIX-5 with or without TF.

(b) Modified plot showing time (min) versus Flia (nM) for different conditions including Control, rTIX-5, FXI def, and FXII def.

(c) Graph displaying time (min) versus Flia (nM) for different concentrations of FV and FVa.

(d) Graph showing thrombin formation (μM) over time (min) with Control and 6.5 μM rTIX-5.

(e) Trend of Flia formation estimated from a log-linear model.

(f) Graph illustrating thrombin formation over time (min) with Control, rTIX-5, 6.5 μM TIX-5, FV, and FVa.

(g) Time (seconds) versus thrombin formation (μM) for Control - FV, Control - FVa, and 6.5 μM TIX-5 - FVa.

Legend:
- Pool
- Pool + rTIX-5
- Fl def
- Fl def + rTIX-5
- Pool + Fl def + rTIX-5

Bar graphs displaying lag times (minutes) for different conditions.
Figure 5

(a) Gel electrophoresis showing protein bands for Control and 6.5 μM rTIX-5 conditions.

(b) Time-course of clotting for FIIa and Meizo-FIIa in Control and 6.5 μM rTIX-5 conditions.

(c) Similar time-course as (b) but for FXa.

(d) Western blot showing protein bands at different time points for Control and 6.5 μM rTIX-5.

(e) Graph showing thrombin formation over time for Control and rTIX-5 with no significant difference (n.s.).

(f) Bar graph showing clot times for different conditions including Normal human plasma and FV-deficient plasma.
Figure 6

(a) FIIa - FV WT

(b) FXa - FV WT

(c) FXa - FV Mutant QIR

(d) FXa - FV Mutant RIQ

(e) FXa - FV Mutant QRQ

(f) FXa - FV Mutant QIQQQ

(g) FXa - FV B-dom Δ

(h) FV generation in the presence of rTIX-5 (%)

(i) Lagtime (min)

WT FIIa, WT, QIR, RIQ, QRQ, QIQQQ, B-dom Δ

Control, rTIX-5

Activation by FXa

FV deficient plasma
Figure 8
Factor Xa Activation of Factor V is of Paramount Importance in Initiating the Coagulation System: Lessons from a Tick Salivary Protein

Tim J. Schuitj, Kamran Bakhtiari, Sirlei Daffre, Kathleen DePonte, Simone J. H. Wielders, J. Arnoud Marquart, Joppe W. Hovius, Tom van der Poll, Erol Fikrig, Matthew W. Bunce, Rodney M. Camire, Gerry A. F. Nicolaes, Joost C. M. Meijers and Cornelis van’t Veer

_Circulation_. published online July 1, 2013;
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circ.ahajournals.org/content/early/2013/06/25/CIRCULATIONAHA.113.003191

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2013/06/25/CIRCULATIONAHA.113.003191.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/
SUPPLEMENTAL MATERIAL.

Supplemental Tables.

Supplementary Table 1. 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>I.scap TIX-5</th>
<th>I.scap H-1</th>
<th>I.scap H-2</th>
<th>I.scap H-3</th>
<th>O.coria H-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.scap TIX-5</td>
<td></td>
<td>41%</td>
<td>40%</td>
<td>38%</td>
<td>24%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>58%</td>
<td>56%</td>
<td>54%</td>
</tr>
<tr>
<td>I.scap H-1</td>
<td>51.7%</td>
<td></td>
<td>36%</td>
<td>37%</td>
<td>21%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>60%</td>
<td>45%</td>
</tr>
<tr>
<td>I.scap H-2</td>
<td>52.3%</td>
<td>49.2%</td>
<td>81%</td>
<td>48%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>45%</td>
</tr>
<tr>
<td>I.scap H-3</td>
<td>38.5%</td>
<td>34.9%</td>
<td>59.3%</td>
<td>88%</td>
<td>24%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O.coria H-1</td>
<td>35.7%</td>
<td>38.7%</td>
<td>35.8%</td>
<td>32.3%</td>
<td></td>
</tr>
</tbody>
</table>

Percent identity and similarity (italics) were calculated after alignment of the nucleotide sequences (down left corner) using the EMBOSs Pairwise Alignment Algorithms (http://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). I.scap TIX-5 (GenBank: AEE89467), I.scap H-1 (GenBank: XP_002405271.1), I.scap H-2 (GenBank: AAY66581.1), I.scap H-3 (GenBank XP_002435217.1) and O.coria H-1 (GenBank: ACB70374.1) were compared for homology.

Supplemental Figures and Figure Legends.

Supplementary Fig.1 rTIX-5 dose-dependently inhibits coagulation in a fibrinogen-clotting assay. Fibrinogen cloting assay was initiated with (a) 0.25 pM, (b) 1 pM or (c) 5 pM TF in normal human plasma. Graphs show a representative of 2 separate experiments.
Supplementary Fig. 2 Proteinase K treatment abolished the anticoagulant effect of rTIX-5. Purified rTIX-5 or BSA (Control) was digested with Proteinase K (Sigma) treatment for 30 minutes at 37°C followed by incubation at 70°C for 15 minutes to inactivate Proteinase K. Thrombin generation was initiated in normal human plasma by addition of 4 µM phospholipids and 1 pM TF.

Supplementary Fig. 3 N-linked glycosylation of *Ixodes scapularis* protein TIX-5. (a) Predictive N-glycosylation sites using the web based NetNGlyc prediction software (http://www.cbs.dtu.dk/services/NetNGlyc). (b) Coomassie staining of rTIX-5 on SDS-PAGE before and after deglycosylation with PNGase F.
Supplementary Fig.4 Physiological inhibitors of the initiation phase of coagulation support the anticoagulant function of rTIX-5. Thrombin generation was initiated with 1 pM TF and 4 µM phospholipids in normal human plasma (NHP), 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.

Supplementary Fig.5 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 in the presence of 6.5 µM rTIX-5 or 6.5 µM rP19 as a control.
Supplementary Fig.6 Schematic representation of FV activation by thrombin and FXa.

Thrombin-mediated activation of FV proceeds via sequential cleavage at Arg\textsuperscript{709}, Arg\textsuperscript{1018} and Arg\textsuperscript{1545}, while FXa-catalyzed FV activation proceeds via initial proteolysis at Arg\textsuperscript{1018}, followed by limited proteolysis at Arg\textsuperscript{709}, Arg\textsuperscript{1545} and Arg\textsuperscript{1761}/Arg\textsuperscript{1765}. Scissors symbolize cleavage sites at Arg\textsuperscript{709}, Arg\textsuperscript{1018} and Arg\textsuperscript{1545} and Arg\textsuperscript{1761}/Arg\textsuperscript{1765}.
Supplementary Fig. 7  TIX-5 shows homology with several *I. scapularis* proteins and with a protein in *Ornithodoros coriaceus*  Multiple sequence alignment of *I. scapularis* TIX-5 aligned with three annotated homologues in *I. scapularis* (GenBank: XP_002405271.1, GenBank: AAY66581.1 and GenBank: XP_002435217.1) and one annotated homologue in *Ornithodoros coriaceus* (GenBank: ACB70374.1). 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.