Selective factor VIII activation by the tissue factor–factor VIIa–factor Xa complex

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Key Points

- A coagulation initiating pathway is revealed in which the TF-FVIIa-nascent FXa complex activates FVIII apart from thrombin feedback.
- Direct activation of the intrinsic pathway by TF may preserve hemostasis under anticoagulant therapy targeting thrombin amplification.

Safe and effective antithrombotic therapy requires understanding of mechanisms that contribute to pathological thrombosis but have a lesser impact on hemostasis. We found that the extrinsic tissue factor (TF) coagulation initiation complex can selectively activate the antihemophilic cofactor, FVIII, triggering the hemostatic intrinsic coagulation pathway independently of thrombin feedback loops. In a mouse model with a relatively mild thrombogenic lesion, TF-dependent FVIII activation sets the threshold for thrombus formation through contact phase-generated FXa. In vitro, FXa stably associated with TF-FVIIa activates FVIII, but not FV. Moreover, nascent FXa product of TF-FVIIa can transiently escape the slow kinetics of Kunitz-type inhibition by TF pathway inhibitor and preferentially activates FVIII over FV. Thus, TF synergistically primes FXa-dependent thrombin generation independently of cofactor activation by thrombin. Accordingly, FVIIa mutants deficient in direct TF-dependent thrombin generation, but preserving FVIIIa generation by nascent FXa, can support intrinsic pathway coagulation. In ex vivo flowing blood, a TF-FVIIa mutant complex with impaired free FXa generation but activating both FVIII and FX supports efficient FVIII-dependent thrombus formation. Thus, a previously unrecognized TF-initiated pathway directly yielding FVIIIa-FXa intrinsic tenase complex may be prohemostatic before further coagulation amplification by thrombin-dependent feedback loops enhances the risk of thrombosis. (Blood. 2017;130(14):1661-1670)

Introduction

Blood clotting in response to tissue injury is key for hemostasis1 and innate immunity2 but can cause vascular thrombosis, leading to serious diseases.1,3 In the current coagulation scheme (Figure 1A), the extrinsic pathway initiation complex of tissue factor (TF) with active factor VIIa (FVIIa) promotes a cascade of proteolytic reactions yielding FXa; this combines with FVa in the prothrombinase complex, converting prothrombin to thrombin. This initially generated thrombin activates the FVIII and FV cofactors in feedback reactions that amplify coagulation,4 but active cofactor generation prior to significant thrombin production is also possible, and indeed, FXa is now viewed as the relevant FV activator during coagulation initiation.4 Whether FXa5-7 or TF-FVIIa8,9 contribute to coagulation initiation through direct FVIII activation remains unclear.

Extrinsic coagulation initiation is controlled by the TF pathway inhibitor (TFPI), which by inactivating FVIIa and FXa within a quaternary complex with TF10,11 attenuates thrombosis.12 Moreover, a positively charged TFPIα carboxyl terminal region interacts with a specific acidic sequence in partially processed FV interfering with FXa formation of active prothrombinase13,14. These mechanisms reducing direct thrombin generation are compensated for by TF-FVIIa activating the intrinsic pathway FIX15 in a kinetically favored reaction in the presence of physiologic plasma inhibitors.16 Alternatively, FXa is generated by FXa activated in a thrombin feedback loop,17 also promoting vascular inflammation,18 or by contact phase FXIIa.19 In mouse models,20 FXIIa contributes to amplified thrombin generation in experimental thrombosis but, consistent with human data,21 has no role in hemostasis.

The current coagulation paradigm, with the expanded function of TFPI tightly controlling both TF-dependent initiation and prothrombinase generation, cannot readily explain how initially produced thrombin can be the origin of FVIIa cofactor for FXa produced by the contact phase pathway or by TF-FVIIa itself. Here, we outline a novel function of the extrinsic coagulation initiation complex whereby nascent FXa associated with TF-FVIIa directly activates
FVIII, resisting inhibitor control by TFPI. Such a mechanism may be relevant for the function of TF in hemostasis and provides new perspectives for interpreting the distinct roles of coagulation reactions in physiologic and pathologic thrombus formation.

**Materials and methods**

**Blood perfusion experiments**

TF-coated glass coverslips were perfused with venous blood at a wall shear rate of 300 s⁻¹ (see the supplemental Materials and methods, available on the Blood Web site). A Zeiss Axiovert 135M/LSM 410 microscope with a Plan-Apochromat ×40/1.40-NA oil immersion objective (Carl Zeiss AG, Oberkochen, Germany) was used to visualize platelets/leukocytes and fibrin stained with mepacrine and a specific antibody, respectively. Image analysis to calculate volumes was performed as has been described.²²

**Thrombin generation (TG) analysis in human native or reconstituted platelet-rich plasma**

TG in platelet-rich plasma (XPR) or reconstituted PRP was evaluated as has been described.²¹ Platelets in PRP were adjusted to 180 • 10³ per microliter with homologous platelet-poor plasma (PPP), and corn trypsin inhibitor (CTI) was added at 30-50 µg/mL. Reconstituted PRP was prepared with washed platelets resuspended at 180 • 10³ per microliter into PPP. TG was initiated by adding recombinant TF (rTF), FIXa, or both at defined concentrations with 18 mM CaCl₂ into microtiter-plate wells containing 360 µM benzoylarginyl-glycyl-glycyl-l-arginine coupled to fluorogenic 7-amido-4-methylcoumarin (Bachem, D-CHA-Ala-Arg-AMC (Pefalfluor TH, Pentapharm, Basel, Switzerland) at 50 µM.

**Analysis of FVIII and FV activation by immunoblotting**

Coagulation products were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions; only reactions containing anti-FVIII monoclonal antibody (MoAb) were processed under nonreducing conditions to avoid confounding effects from immunoglobulin G (IgG) heavy chains with a molecular mass comparable to FVIII A1 domain. Proteins transferred to polyvinylidene fluoride membranes were probed with anti-FVIII MoAb C5 (0.5 µg/mL) or anti-FV AHV-5146 (1 µg/mL). FVIIa and FVa were quantified by infrared detection with the Odyssey infrared imager (LI-COR, Lincoln, NE), calibrated with known FVIIa and FVa quantities.

**Coagulation activation in reactions with purified components**

Reactions in 50 mM Tris-buffered saline, pH 7.4, with 0.1% bovine serum albumin included 0.7 nM FVIII, 3 nM FV, 135 nM FX, and 1 µM prothrombin without or with 4 µM dansylarginine -N-(3-ethyl-1,5-pentanediyl)amide (DAPA), TFPIα and other inhibitors were added as indicated. Reactions were initiated by rTF (50 or 400 pM) with FVIIa (200 or 500 pM), FIXa (2 or 10 nM), or both added with 2.5 mM CaCl₂ and incubated at 37°C for the indicated times. After quenching the reaction with 10 mM EDTA, generated FXa was measured with S-2765 (180 µM). FVIIa procoagulant activity was measured as FXa-dependent FXa generation; FVIIa procoagulant activity generated by the nascent TF-FVIIa-FXa complex was calculated by subtracting the amount of FXa produced in reactions initiated by FIXa and FIXa individually from that produced in reactions initiated by FVIIa/FIXa combined. When indicated, 200 nM lepirudin was used to inactivate possible thrombin contamination.

**Ferric chloride-induced thrombosis in mice**

Animal procedures complied with the Guide for Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of The Scripps Research Institute. Vascular injury was induced in C57BL/6J mice by 10.8-µL drop of 7% (0.26 M) or 8% (0.30 M) FeCl₃, or 6H₂O applied on the carotid artery for 3 min; or a 0.4-µL 4% (0.15 M) drop for 1 min on the femoral vein, followed by rinsing. Antibodies were administered by bolus injection into the catheterized jugular vein. FVIII and FVIIa were administered by a bolus injection (1.4 pmol), followed by maintenance with continuous infusion at the rate of 0.47 pmol/min for 15 min. Time to first occlusion after injury and flow index were quantified as has been described.²⁵ Additional details for experimental procedures are described in the supplemental Methods.

**Study approval**

Studies involving human subjects were approved by appointed institutional review boards. Human volunteers gave informed consent to participate in the studies before blood collection, and experiments were performed in accordance with the Declaration of Helsinki.

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**Figure 1. Schematic representation of coagulation initiation and amplification.** (A) In the current paradigm, activation of the FVIII and FV cofactors is considered to be primarily a thrombin-mediated feedback reaction, although a key role of FXa in relation to thrombin has been demonstrated for FV activation. (B) The coagulation scheme supported by the present studies highlights in addition the newly identified selective activation of FVIII by nascent FXa within the extrinsic TF-FVIIa-FXa initiation complex prior to inhibition by TFPI. By concurrently activating FIX, the extrinsic TF-FVIIa complex can initiate directly the FVIIa-FIXa antihemophilic pathway. The 2 depicted mechanisms of coagulation activation may be variably integrated in response to different stimuli.
Figure 2. TF pathway and FVIII activation in vivo. (A) Carotid artery occlusion after injury by 7% (0.26 M) or 8% (0.3 M) FeCl₃ in C57BL/6J mice treated with anti-TF 21E10, anti-FXI 14E11 MoAbs, or both, as indicated (n = 5-20 in different groups); control mice (panel C) were injected with buffer or isotype-matched nonimmune mouse IgG. (B) Femoral vein occlusion after injury by 4% (0.15 M) FeCl₃ in mice (n = 3-7) receiving FVIII or FVIIIa (1.4 pmol bolus followed by 0.47 pmol/min for 15 min) before injury and MoAb treatment. Results in panel A (top) and panel B (dot plots; median and interquartile range) were analyzed with ANOVA/Tukey tests; results in panel A (bottom) (dot plot; mean and 95% confidence interval) were analyzed with Kruskal-Wallis/Dunn tests; results in panel A (bottom) (dot plot; mean and 95% confidence interval) were analyzed with ANOVA/Tukey tests. (C) Fibrin formation in the femoral vein. Control mice were injected with phosphate-buffered saline (top left). FVIII injection does not prevent the antithrombotic effect of 9 μg/g anti-TF/65 ng/g anti-FXI MoAbs combined (top right). FVIIIa injection bypasses inhibition by this antibody combination (bottom left). FVIIIa cannot bypass inhibition by a full dose (125 ng/g) of anti-FXI MoAb alone (bottom right). (D) Representative TG (n = 3) induced by 0.15 μM rTF, 20 μM FIXa, or both in citrated human PRP (180 × 10⁶ platelets/μL) recalcified with 18 mM CaCl₂ at 37°C and containing 30 μg/mL CTI to block FIXa and 40 μg/mL rabbit anti-TFPI IgG (left) or nonimmune IgG (right). (E) Representative TG (n = 2) induced by rTF, FIXa, or both as above in recalcified FIX-deficient PPP containing 50 μg/mL CTI and 180 × 10⁶ normal washed platelets per microliter. *P < .05. **P < .01. ***P < .001.

Statistics

Group variances were evaluated with Levene’s median and Bartlett’s tests; differences were evaluated with 1-way analysis of variance (ANOVA) or the Kruskal-Wallis test, followed by Tukey’s and Dunn’s tests, respectively, for multiple comparisons. Data were transformed as y = log₁₀ y when necessary to obtain homoscedasticity. Software packages used were GraphPad Prism (version 7; GraphPad Software, La Jolla, CA) and XLSTAT (Addinsoft, Paris, France).

Results

The TF pathway activates FVIII in vivo

As was previously shown, contact phase FXII²⁰,²⁵,²⁶ and TF²²,²⁷ contribute to experimental thrombosis in the ferric chloride-induced carotid artery occlusion model, but how this happens remains unclear. We found that MoAbs blocking the TF function²² or FXI activation by FXIIa²² independently reduced occlusion after a vessel wall lesion caused by 7% (0.26 M) FeCl₃ (Figure 2A). After a lesion by 8% (0.3 M) FeCl₃ the same MoAb concentrations were ineffective, but a higher dose of the anti-FXI MoAb, not of the anti-TF, still prevented thrombosis (Figure 2A). Thus, even after the more severe lesion, thrombogenesis required FIXa generation by FXIIa-FXIa.²⁰,²⁵ Remarkably, under the latter conditions, combining individually inactive low doses of anti-TF and anti-FXI MoAbs reduced arterial occlusion significantly (Figure 2A), confirming a role for the TF pathway in this intrinsic coagulation-dependent model of thrombus formation. To explain such findings, we hypothesized that TF might contribute to activate FVIII, the essential cofactor for the intrinsic tenase protease, FIXa. As visualized in the femoral vein, FVIIIa, but not FVIII, prevented thrombogenesis by FXIIa-FXIa,²²,²⁶ and TF-FVIIa,²⁷,²⁸ FIXa generation by TF-FVIIa or alternative pathways used exogenously provided FVIIIa to trigger thrombosis, supporting the idea that TF-FVIIa contributes to FVIII activation in vivo.

To challenge this concept, we measured TG in PRP. In reactions containing an inhibitory polyclonal antibody blocking TFPI and CTI preventing FXI activation by FXIIa,²⁸ we defined the concentrations of FIXa or relipidated rTF yielding comparable TG (Figure 2D, left). At the same concentration but without TFPI blockade (Figure 2D, right), rTF produced little thrombin and late in the reaction, but enhanced TG with FIXa added concurrently. This amplification of FIXa-triggered TG required FVIII at < 10% plasma concentration (supplemental Figure 1) but not FIX (Figure 2E), excluding additional TF-dependent FIXa generation.¹⁶,¹⁷ Because FVIII concentrations sufficient to prevent severe spontaneous bleeding in FVIII-deficient patients²⁹ could support synergistic TG amplification, the conditions of this assay appear to be relevant for assessing hemostatic competence in PRP.
The TF-FVIIa-FXa complex activates FVIII

We explored the mechanism of TF-induced priming of intrinsic coagulation using a sensitive 2-stage TG assay. In FVII-deficient reconstituted PRP, 0.15 pM rTF with FVIIa wild-type (WT) produced little thrombin (<20 pM in 11 min), similar to that generated by the active site mutant FVIIa S195A (iFVIIa) or FIXa alone (Figure 3A). However, combining FIXa with rTF and FVIIa WT, but not inactive iFVIIa, amplified TG ~5-20 times in 5-11 min (Figure 3A). Thus, the additive quantities of thrombin produced in this assay by TF-FVIIa and FIXa separately were far less than those yielded by the 2 combined, demonstrating a synergistic interaction linking TF-initiated and intrinsic coagulation upstream of thrombin generation.

To identify the reaction that, apart from thrombin, could yield FVIIIa for FIXa-dependent coagulation, we considered TF-FVIIa, FVa, and FXa as potential FVIII activators. First, we determined that TF-FVIIa without FX (or TF-FX without FVIIa) generated no or minimal FVIIIa activity but that TF-FVIIa with FX (generating FXa) produced substantial amounts of FVIIIa with all reagents at physiologically relevant concentrations (Figure 3B). To distinguish between functions of FXa released from or still associated with TF-FVIIa, we formed a stable TF-FVIIa-FXa complex with the nematode anticoagulant protein NAPc2.31,32 Use of iFVIIa excluded FVIIa catalytic activity, whereas FXa is known to retain catalytic function in the formed TF-FVIIa-FXa complex with the nematode anticoagulant protein NAPc2.31,32 This complex failed to induce TG in FVII-deficient plasma (Figure 3C). As was expected, a similar complex formed with TFPI, which inhibits FXa, was inactive alone and did not support FIXa-dependent TG in
activator akin to its function in generating the prothrombinase cofactor, with TF-FVIIa can exert the same function (Figure 3E).

Importantly, although thrombin could generate more FVIIIa, as was indicating that FXa bound to FVa cannot efficiently activate FVIII (Figure 3H) by the same FXa concentration was contrast, generation of FVIIIa activity (Figure 3G) or proteolytic marks the transition to direct TF-induced coagulation.

Thus, nascent FXa associated with TF-FVIIa preferentially activates thrombin conversion by a low concentration of FXa (Figure 3F). In above, increasing concentrations of FVa markedly stimulated pro-

The TF-FVIIa-FXa complex activates FVIII independently of thrombin

To assess the relative roles of the nascent TF-FVIIa-FXa complex and thrombin in generating FVIIIa, we studied procofactor activation on rTF-bearing phospholipid vesicles mixed with purified FX, prothrombin, FVIII, and FV. Activation of FVIII after FVIIa addition was partially inhibited by blocking thrombin with DAPA or by replacing normal prothrombin with the inactive S195A mutant, but ~15% FVIIIa was still detectable under both conditions (Figure 4A). Importantly, although thrombin could generate more FVIIIa, as was expected from efficient FVIII cleavage in the solution phase, the amount of VIIIa activated by the TF-initiated reaction in the absence of active thrombin was sufficient for full function of membrane assembled FVIIa-FXa intrinsic tenase complex (Figure 4B; supplemental Figure 2A). In agreement with the results in PRP containing endogenous coagulation inhibitors (see Figure 2D), TFPIα that markedly suppressed direct FXa generation by TF-FVIIa (supplemental Figure 2B) had limited effect on the formation of functional FVIIa-FXa complex in TF-initiated reactions (Figure 4C). The latter was also resistant to inhibition by TFPIα with the cofactor protein S34-35; partial inhibition by protein S alone likely resulted from competition for limited procoagulant surfaces (supplemental Figure 2C). Consistent with the observed thrombin-independent FVIII activation by nascent FXa in PRP, FVIII activation by TF-FVIIa-FXa was not influenced by von Willebrand factor (VWF) binding FVIII (supplemental Figure 2D).

To demonstrate directly that rTF supports FVIII activation in a physiological plasma milieu independently of thrombin feedback reactions, we used hirugen (63-O-sulfo-tyr-hirudin[55-65]) to block thrombin exosite I required for cofactor activation.36,37 Hirugen dose-dependently inhibited FVIII activation by thrombin (supplemental Figure 3A) but not FXa generated by TF-FVIIa-FXa (supplemental Figure 3B). In PRP with CTI and anti-FXI MoAb to block feedback TG activity calculated from FXa generation in reactions as in panel A but with 10 nM FIXa added and incubated for 180 s at 37°C (n = 4-12). FXa generation dependent on FVIIIa-FXa activity was calculated by subtracting FXa generated by FVIIIa and FIXa added individually from that by FVIIIa/FIXa added together. (C) FVIIIa activity generated and calculated in reaction as in panels A and B but with the addition of 10 nM TFPIα. Results in panel A (bottom) and panels B and C (shown as 29th-75th percentile bars, min-to-max whiskers, line at the median; or min-to-max floating bars with line at the mean when n ≤ 3) were analyzed by ANOVA/Tukey tests. (D) Effect of different hirugen concentrations on TG initiated by 10 pM FIXa in normal PRP (180 s at 37°C) containing 30 μg/mL CTI and 20 μg/mL anti-FXa blocking MoAb O1A6 (n = 3). (E) Representative thrombograms initiated in normal PRP, containing CTI and anti-FXa MoAb as in D, by 0.15 μM rTF, 10 μM FIXa, or both without (left) or with (right) 2 μM hirugen (n = 3). ***p < .001; NS, not significant.
that cleave FX but display very low substrate turnover because of the pathway TG, we studied 2 FVIIa mutants, T99Y and E154A, that contributes to intrinsic pathway activation as opposed to direct extrinsic pathway function.

To elucidate further how FXa generation by TF-FVIIa distinctly occurs also on a natural procoagulant surface with human or mouse TF-FVIIa (supplemental Figure 3C).

**Intrinsic coagulation pathway activation by the nascent TF-FVIIa-FXa complex contributes to thrombin generation independently of direct extrinsic pathway function**

To elucidate further how FXa generation by TF-FVIIa distinctly contributes to intrinsic pathway activation as opposed to direct extrinsic pathway TG, we studied 2 FVIIa mutants, T99Y and E154A, that cleave FX but display very low substrate turnover because of impaired FXa release. In a phospholipid-free assay or with phospholipid-reconstituted rTF, the FVIIa mutants produced an initial burst but, in contrast to FVIIa WT, could not sustain FXa generation occurred also on a natural procoagulant surface with human or mouse TF-FVIIa (supplemental Figure 3C).

Figure 5. FVIIa mutants with impaired FXa product turnover support FVIII activation by nascent FXa when thrombin feedback is blocked. (A) Time course (mean ± SEM) of 1 μM FX activation by 2 μM phospholipid-free soluble rTF with 10 nM FVIIa WT (n = 4-7), T99Y (n = 2-3), or E154A mutants (n = 3-4); incubation at 37°C (left), FXa generation in reactions with 50 μM phospholipid-reconstituted rTF, 200 pM FVIIa WT or mutants, 135 nM FX, and 700 pM FVIII incubated for 2 (n = 2-3), 4 (n = 4-5), or 6 (n = 6-9) min (right). (B) Representative immunoblots (n = 2) of FVIIIa activity as measured by FXa produced by 10 nM FIXa incubated with or without 10 nM TFPI incubated for 180 s. (C) Representative immunoblots (n = 2) generated as in panel B but without TFPI, measured as FXa produced by 10 nM FIXa, 135 nM FX, 700 pM FVIII, 3 nM FV, and 200 nM lepirudin, with or without 10 nM TFPI incubated for 180 s. (D) Representative immunoblots (n = 2) of FVIIIa activity generated as in panel B but without TFPI, measured as FXa produced by 10 nM FIXa incubated for 180 s. (E) Representative TG (n = 3) initiated by 2.5 μM rTF and 400 pM FVIIa WT or mutants in FVII-deficient reconstituted PRP containing 30 μg/mL CTI, 8 μg/mL anti-FVIII MoAb 8D4-blocking FVIIIa cofactor activity. (F) Representative TG (n = 3) initiated by 2.5 μM rTF and 400 pM FVIIa WT or mutants in FVII-deficient reconstituted PRP containing 30 μg/mL CTI, 8 μg/mL anti-FVIII MoAb 8D4-blocking FVIIIa cofactor activity. (G) Representative TG (n = 3) initiated by 2.5 μM rTF and 400 pM FVIIa WT or mutants in FVII-deficient reconstituted PRP containing 30 μg/mL CTI, 8 μg/mL anti-FVIII MoAb 8D4-blocking FVIIIa cofactor activity. (H) Representative TG as in panel E but without anti-FVIII MoAb and without (left) or with (right) 20 μg/mL anti-FXI MoAb C1A6-blocking FXI activity in F IX activation. (I) Representative TG as in panel H but with added 3 nM FVa with (left; n = 4) or without (right; n = 5) anti-FVIII MoAb. **P < .01, ***P < .001.
Both FVIIa mutants with impaired FXa turnover formed a functional FVIIa-FXa intrinsic tenase complex when FIXa was available, but only the exosite mutant E154A produced FVIIa-FXa activity when zymogen FIX was present instead (Figure 5D), in agreement with the fact that FVIIa T99Y, unlike E154A, cannot activate FIX (supplemental Figure 4B). Thus, complementing the ability to generate FIXa, direct activation of the antihemophilic FVIII cofactor by nascent FXa product of TF-FVIIa enables intrinsic pathway coagulation before TFPI inhibitory control. We tested these conclusions in FVII-deficient reconstituted PRP in which, besides endogenous plasma coagulation inhibitors, platelets are an additional source of TFPIs. Under these conditions, FVIIa WT, but not E154A or T99Y mutants, induced TG in the presence of a neutralizing anti-FVIII MoAb (Figure 5E), confirming that the mutants could not directly generate thrombin in a plasma milieu. Without TFPI inhibition, TG by FVIIa E154A was only slightly slower than that by WT, whereas TG by FVIIa T99Y was clearly decreased. FXIa inhibition reduced markedly TG by FVIIa T99Y but less by WT or E154A (Figure 5F; supplemental Figure 4C), in agreement with the latter generating FIXa as well as FVIIIa.

To prove directly that thrombin-independent FVIII activation occurred in these reactions, we first verified that the thrombin exosite blocker, hirugen, abolished FIXa-initiated TG in FVII-deficient plasma (Figure 5G). In the presence of the same hirugen concentration, TG by mutant FVIIa E154A, not by FVIIa WT, was entirely FVIII dependent, and without FXIII inhibition, TG induced by FVIIa WT and E154A was of similar magnitude, but TG by the latter was clearly delayed (Figure 5H). To explain the delay, we reasoned that impaired direct FXa generation by the mutant FVIIa could reduce FVa cofactor generation for initial prothrombinase assembly. Indeed, adding FVa normalized the delay in FVIIIa-dependent TG by FVIIa E154A (Figure 5I). Accordingly, adding FVa to normal PRP accelerated TF-initiated but not more than did blocking TFPI function (supplemental Figure 4D). These data indicate that prothrombinase activity is regulated by TFPI control of FVa generation that contributes to FV activation and reinforce the concept that FVIII activation during TF-initiated coagulation generates FVIIIa-FIXa intrinsic tenase activity independently of thrombin feedback reactions and escaping TFPI control.

We then screened a library of MoAbs to FVIIa to identify a proof of principle inhibitor that could recapitulate the shift in functional properties (loss of efficient FXa and FIXa generation but not of FVIII activation) seen with the FVIIa mutant T99Y. In contrast to the fully inhibitory MoAb 3G12, antibody 12C7 had no effect on FVIII activation (Figure 6A). Moreover, it had no significant effect on the generation of intrinsic tenase activity when FIXa was present, but it markedly inhibited when FIX was supplied instead (Figure 6B). MoAb 12C7 attenuated TG in PRP but, as seen with FVIIa T99Y, rendered TG FXa dependent (Figure 6C). Thus, results with mutant FVIIa molecules and inhibitory antibodies concordantly show that the TF-FVIIa complex can initiate intrinsic and extrinsic coagulation pathways in distinct reactions.

**Intrinsic pathway activation by TF leads to fibrin formation in flowing blood**

To evaluate whether TF-FVIIa initiates thrombus formation in flowing blood ex vivo when direct initial thrombin generation is limited, we surface-immobilized rTF at a low concentration sufficient for FVIII-dependent fibrin formation at a wall shear rate of 300 s⁻¹. Under these conditions established with WT FVIIa, FVIIa T99Y were less thrombogenic in spite of supporting more platelet adhesion than were inactive FVIIaS195A (Figure 7A-B). Addition of 10 pM FIXa to blood containing FVIIa T99Y, but not containing inactive FVIIa S195A, restored FVIII-dependent fibrin formation (Figure 7A-B). In contrast, mutant FVIIa E154A, which is as defective as T99Y in direct TG (Figure 5E), supported FVIIIa-dependent thrombus formation similar to FVIIa WT when added without FIXa to FXa to FVIII-deficient reconstituted blood (Figure 7A,C). This confirmed that the nascent FXa product of TF-FVIIa can directly generate FVIIIa functioning in the intrinsic tenase complex with the potential to enhance hemostasis in low-TF environments with limited direct TF-dependent thrombin generation activating feedback loops.

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**Figure 6. Anti-FVIIa MoAb 12C7 mimics FVIIa T99Y functional properties.**

(A) Representative immunoblot showing the effect of anti-FVIIa MoAbs 3G12 and 12C7 (20 μg/mL) on TF-dependent FVIIIa generation in reactions including 50 pM rTF, 200 pM FVIIa, 135 nM FX, 3.5 nM FVIII, 3 nM FV, and 200 nM lepirudin incubated for 120 s at 37°C (left). Quantification of the data on the left (n = 3; min-to-max floating bars, line at the mean) (right); differences were evaluated by Welch-corrected 2-tailed t test. (B) Anti-FVIIa MoAb 12C7, but not 3G12, preserves FVIIIa-dependent FXa generation by 10 nM FIXa (left; n = 3), but not 90 nM FIXa (right; n = 3-5) in reactions containing 50 pM rTF, 200 pM FVIIa, 700 pM FVIII, 3 nM FV, 135 nM FX, 10 nM TFPIa, 200 nM lepirudin, and 2.5 mM CaCl₂ incubated for 180 or 360 s, respectively, at 37°C. Anti-FVIIa MoAbs or control mouse IgG were added at 20 μg/mL. Results (min-to-max floating bars, line at the mean) were analyzed by ANOVA/Tukey tests. (C) Representative thrombograms (n = 2) showing the effect of anti-FVIIa MoAbs 3G12 and 12C7 (20 μg/mL) on 1.2 pM rTF-induced TG in normal PRP with CTI (30 μg/mL) and without (left) or with (right) addition of anti-FXa MoAb 01A6 (20 μg/mL) blocking FIX activation by FXa. **P < .01. ***P < .001.
Discussion

Our findings delineate a novel function of the extrinsic TF-FVIIa complex, namely, providing selective feed-forward activation of the FVIII antihemophilic cofactor independently of thrombin feedback loops (Figure 1B). This specific reaction of nascent FXa escapes control by physiologic coagulation inhibitors in PRP or TFPIα in purified systems. Together with generation of the FIXa antihemophilic protease by TF-FVIIa, direct FVIII activation by TF-FVIIa-nascent FXa completes a pathway to FVIIIa-FIXa intrinsic tenase activity fully integrated within TF-initiated coagulation preceding direct activation of the common coagulation pathway.

Nascent TF-FVIIa-FXa generates FVIIIa, facilitating the formation of intrinsic tenase but without providing FXa for prothrombinase activity. Generating the latter requires FXa undocking from TF-FVIIa, thus exposing free FXa to inhibitory control. Therefore, the newly identified TF-FVIIa-FXa function allows for accumulation of active prohemostatic antihemophilic FVIIa cofactor without increasing prothrombotic FXa. This may be of relevance for targeted FXa anticoagulants that, with comparable antithrombotic potency, have a lesser impact on hemostasis than do vitamin K antagonists.42–44 Of note, such a mechanism is independent of thrombin feedback reactions and FXI activity and may thus support hemostasis during treatment with thrombin inhibitors or recently validated strategies targeting FXI.45

Selectivity for cofactor activation indicates distinct functional properties of FXa in complex with or released from TF-FVIIa. Although coagulation cofactor-enzyme complexes are typically geared toward efficient substrate turnover for rapid thrombin generation, throughout evolution the TF initiation complex appears to have preserved mechanisms favoring its stability. FX interacts with TF-FVIIa through an extended interface that is minimally affected toward efficient PAR activation, FVIIIa generation, and innate immunity.32 Moreover, as seen for FXIa generation, resistance to functional inhibition by TFPIα is also an important feature of PAR signaling induced by TF-FVIIa-FXa in endothelial cells.49

![Diagram of TF-FVIIa-FXa function](https://example.com/diagram.png)

Figure 7. FVIIIa-dependent thrombus formation induced by the TF-FVIIa initiation complex in flowing blood. (A) Blood reconstituted with washed type 0 blood cells added to the original count into FVIII-deficient citrated PPP with 200 pM WT or mutant FVIIa and without or with the inhibitory anti-FVIII MoAb C5 (25 μg/mL) was recalcified to 1.29 mM CaCl2 and perfused for 3.5 min at 300 s⁻¹ wall shear rate. Where indicated, FIXa (20 pM) was added to blood. Representative confocal images are shown with superimposed green (platelet aggregates and leukocytes) and red (fibrin) fluorescence channels. Image side = 312 μm. (B) Quantification of the volume of platelet aggregates and deposited fibrin after adding FVIIa WT, T99Y, or S195A without or with anti-FVIII MoAb (n = 4-6 for the different conditions). (C) As in panel B, but after adding FVIIa WT or E154A (n = 3-8 for the different conditions). Results in panels B and C (shown as 25th-75th percentile bars, min-to-max whiskers, line at the median; or min-to-max floating bars, line at the mean when n ≤ 3) were evaluated by the ANOVA/Tukey tests. *P < .05; **P < .01; ***P < .001.
signaling complex is additionally stabilized by recruitment of the FXa-binding partner, endothelial protein C receptor (EPCR), in mouse and man.50,51 A key innate immune signaling role for the TF-FVIIa-FXa-EPCR complex recently emerged in dendritic cells,52 in which it is essential for toll-like receptor 4 induction of interferon-regulated genes. Negative regulation of this pathway by the alternative EPCR ligand, activated protein C, utilizes the canonical anticoagulant cofactor functions of FV and protein S.53 Thus, these and other nontraditional functions of the coagulation system54 likely use the same mechanistic features that simultaneously serve diverse roles in immunity, hemostasis, and injury repair.

Our findings provide the biochemical bases for defining distinct roles of TF in supporting hemostasis or contributing to thrombosis. One can envision application of mutants of FVIIa or the described roles of TF in supporting hemostasis or contributing to thrombosis. The novel concepts on coagulation presented here may also have implications for the development and evaluation of hemostatic agents, providing protection from severe bleeding complications while avoiding adverse thrombotic complications in patients with underlying vascular disorders.

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References


Selective factor VIII activation by the tissue factor–factor VIIa–factor Xa complex

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