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Supplemental Information

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

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Detailed description of materials and methods

Materials. Mouse and rabbit IgG, quinacrine-HCl and apyrase were from Sigma-Aldrich (St. Louis, MO). Human prothrombin (FII), thrombin (FIIa), FV, FVa, FIX, FIXa, FX, FXa, CTI, dansylarginine N-(3-ethyl-1,5-pentanediyl)amide (DAPA) and anti-human FV MoAb AHV-5146 (binding to A1-A2 domain residues 306-506) were from Haematologic Technologies (Essex Junction, VT). Recombinant tissue factor (rTF; Dade Innovin®) was from Siemens Healthcare Diagnostics (Deerfield, IL). Each lot used in these studies was calibrated against a reference lot (# 53691; 13.9 nM rTF) measuring FXa generation in reactions including varying rTF concentration, 100 pM FVIIa and 135 nM FX. The reference lot contained 101.4 μM procoagulant phospholipids by prothrombinase activity assay calibrated with 80% phosphatidylcholine (PC)/20% phosphatidylserine (PS) mol/mol phospholipid vesicles (Avanti Polar Lipids, Alabaster, AL) sonicated in 10 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), 0.15 M NaCl, pH 7.4. Bovine serum albumin was from Calbiochem (San Diego, CA); human protein S from Enzyme Research Laboratories (South Bend, IN); nematode anticoagulant protein (NAP) c2 and tick anticoagulant peptide (TAP) from Corvas International (San Diego, CA). Of the anti-human FVIII MoAbs used, ESH-8 was from Sekisui Diagnostics (Stamford, CT); 8D4 was a gift of Dr. Marc Jacquemin (Leuven, Belgium); and C5, directed against the A1 domain, was prepared in our laboratories.[1] FVIII was a gift from Bayer Healthcare (Berkeley, CA). Thrombin-generated FVIIIa was prepared incubating 190 nM FVIII, 19 nM FIIa and 5 mM $CaCl_2$ for 30 s at 37 °C, followed by 36 nM lepirudin (recombinant [Leu1-Thr2]-63-desulfohirudin; Refludan, Bayer Corp, Pittsburgh, PA) to neutralize thrombin. Recombinant TFPI; soluble TF (residues 1-218); human FVIIa WT and mutants S195A (chymotrypsin numbering; iFVIIa), T99Y and E154A; and prothrombin S195A were produced and characterized as described.[2-8] Hirugen,[9] MoAbs anti-mouse TF 21E10,[10] anti-human FVIIa 12C7,[11] anti-mouse FXI 14E11[12] and anti-human FXI O1A6[13] were previously characterized. Inhibitory anti-TFPI polyclonal antibody,[14] anti-human FVIIa 3G12 and anti-human TF 5G9 MoAbs were produced by us.

Blood perfusion experiments. Glass coverslips treated with 0.2 mg/ml poly-L-lysine for 6 h at 37 °C were coated with rTF for 18-20 hours at 37 °C, rinsed with buffered-saline and assembled in a rectangular flow chamber with a 125 μm-high silicon gasket. Venous blood was collected into final 10.9 mM trisodium citrate using a plastic syringe. $CaCl_2$ was added to obtain 1.29 mM Ca^{2+} before perfusion at a wall shear rate of 300 s^{-1} maintained with a syringe pump (Harvard Appa-

ratus, Holliston, MA). For experiments with WT and mutant FVIIa, cells in citrated group 0 blood, supplemented with 50 μg/ml CTI and apyrase (10 U/ml ADPase activity), were washed free of plasma by sequential cycles of centrifugation at 1500 g for 7 min followed by resuspension. In the first cycle, plasma was replaced with an equal volume of calcium-free Tyrode buffer, pH 6.5, containing 5 U/ml apyrase; then, with buffer and 1.25 U/ml apyrase; and, finally, with human FVII deficient PPP (<1% of normal plasma; George King Bio-Medical, Overland Park, KS) up to the original blood volume. Hematocrit and platelet counts of reconstituted and native blood were within ±10%. Platelets/leukocytes were visualized by incorporation of quinacrine hydrochloride (mepacrine; Sigma) added at a concentration of 10 μg/ml and incubated for 15 min at room temperature before perfusion. Fibrin was visualized by binding of mouse monoclonal IgG (HB-8545; American Type Culture Collection, Manassas, VA) labeled with Alexa Fluor 546 (Invitrogen) and used at 50 μg/ml concentration in blood; the antibody interacts with the human/mouse fibrin β but not fibrinogen Bβ chain.

TG analysis in human native or reconstituted PRP. PRP was prepared from blood containing 10.9 mM trisodium citrate centrifuged at 250 *g* for 10 min at 25 °C. Platelets were adjusted to $180 \cdot 10^3$/μl by dilution with homologous PPP obtained from PRP (or citrated blood) centrifuged at 1,500 *g* for 10 min at 25 °C. Reconstituted PRP was prepared with washed platelets isolated from normal PRP mixed with $1/5^{th}$ volume of acid-citrate-dextrose (71 mM citric acid, 85 mM trisodium citrate, and 111 mM dextrose, pH 4.5) and 5 U/ml apyrase. After centrifugation at 1,500 *g* for 10 min, the platelet pellet was resuspended into PPP either normal or lacking a specific coagulation factor and in which 30-50 μg/ml CTI had been added in advance; the final platelet count was $180 \cdot 10^3$/μl. PPP from patients with severe congenital deficiency of FVIII or FIX (<1% of normal plasma) was from George King Bio-Medical; FIX-deficient PPP prepared by immunoaffinity depletion of normal plasma was from Haematologic Technologies. Fluorescence intensity in reactions generated by thrombin cleavage of the substrate Gly-Gly-Arg-AMC was measured continuously at 37 °C for up to 40 min in a spectrofluorometer. The thrombin burst slope (nM/min) was calculated by dividing the peak height of thrombin concentration by the time from induction to its occurrence minus the lag time, defined as the time from induction to 3 nM thrombin generated. The endogenous thrombin potential (ETP, i.e., total generated thrombin activity) was determined from the area under the TG curve. The discontinuous 2-stage TG assay was initiated by adding 0.15 pM rTF and 18 mM $CaCl_2$ into FVII-deficient PRP with

30 µg/ml CTI and 400 pM WT FVIIa or iFVIIa without/with 20 pM FIXa. In other experiments, TG was induced by TF-iFVIIa-FXa-TFPI or TF-iFVIIa-FXa-NAPc2. Stable complexes were pre-formed by incubating 5 nM FXa, 10 nM TF, 10 nM iFVIIa and 40 nM TFPI or NAPc2 in the presence of 2.5 mM $CaCl_2$ for 120 s at 37 °C. Complexes were added into FVII-deficient PRP, followed by incubation for 8 min at 37 °C.

Coagulation activation in reactions with purified components. FVIII activation by stable FXa complexes with TF-FVIIa was tested in reactions containing FVIII, FV and lepirudin with or without TFPIα, with incubation for 30-120 s at 37 °C. Stable complexes with FXa as the only active protease were prepared in reactions incubated for 120 s at 37 °C including: 1) 200 pM FXa, 400 pM rTF, 500 pM iFVIIa, 40 nM NAPc2 and 2.5 mM $CaCl_2$; 2) 100 pM FXa, 50 pM rTF, 100 pM iFVIIa, 5 nM NAPc2 and 2.5 mM $CaCl_2$. The effect of added FVa on FVIIIa generation by free FXa was tested in reactions with 10 pM FXa incubated with 50 pM rTF and substrates for 180 s at 37°C. The inhibitory effect of hirugen on FVIIIa generation by FIIa was tested in reactions with 0.5 nM FIIa incubated with 50 pM rTF and FVIII for 180 s at 37 °C.

Substrate turnover by TF-FVIIa was evaluated from FXa generation in reactions without phospholipids containing 10 nM FVIIa, 1 µM FX and 2 µM soluble rTF1-218. FVa was titrated in a prothrombin activation assay in which 10 pM FXa was incubated with 1 µM prothrombin 50 pM rTF and 700 pM FVIII for 180 s.

Activation of FIX by TF-FVIIa was tested in reactions containing 150 nM FIX with 50 pM rTF, 200 pM FVIIa and 2.5 mM $CaCl_2$ incubated for 30 min at 37 °C. After terminating reactions with 10 mM EDTA, FIXa activity was determined in the presence of ethylene glycol (37%, volume/volume) by measuring kinetically amidolytic activity with the chromogenic substrate CH_3SO_2-(D)-CHG-Gly-Arg- para-nitroanilide·AcOH (Pefachrome FIXa, Pentapharm, Basel, Switzerland; 1 mM). The calibration curve of FIXa was constructed with known concentrations of FIXa.

Measurement of FVIIIa clotting activity. Aliquots of reactions in which FVIIIa was generated were added into FVIII-deficient plasma and then mixed with 10 nM FIXa, 20 µM PL and 8 mM $CaCl_2$. FVIIIa clotting activity was quantified using calibration curve constructed with known concentrations of thrombin-activated FVIIIa.

Ferric chloride-induced thrombosis in mice. Time to first occlusion after injury is that required for a decrease of blood flow to <10% of that measured in the uninjured artery. Flow index is the

ratio between blood volume flowing through the injured artery in 30 min (integration of flow measured in ml/min and sampled every second) and that expected in the uninjured artery (calculated from the flow measured in 1 minute before injury multiplied by 30).

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Supplemental Figures and Figure Legends

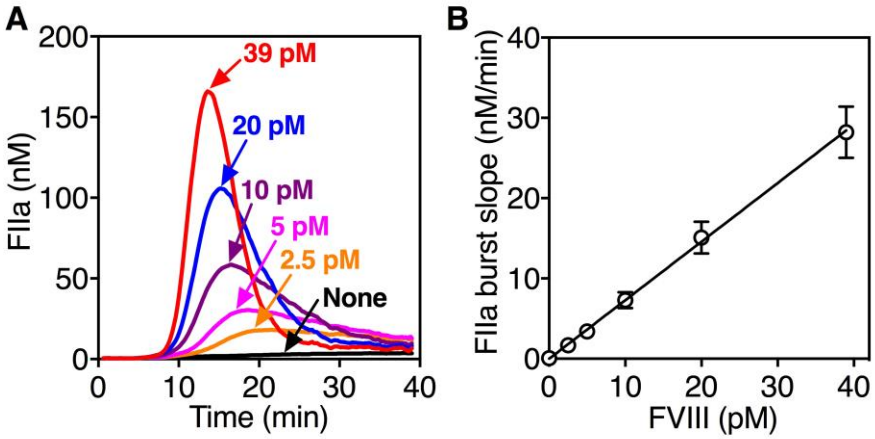

Figure S1. FVIII dose-dependent synergistic enhancement of thrombin generation triggered by TF/FIXa in FVIII-deficient PPP. (A) Representative thrombograms (n =3) of thrombin generation (TG) induced by 0.15 pM relipidated recombinant TF (rTF) and 200 pM FIXa in FVIII-deficient PPP containing 30 µg/ml CTI and reconstituted with increasing concentrations of FVIII at 37°C. (**B)** Thrombin (FIIa) burst slope (nM/min; mean ±95 CI; n =3) in the reactions shown in panel A.

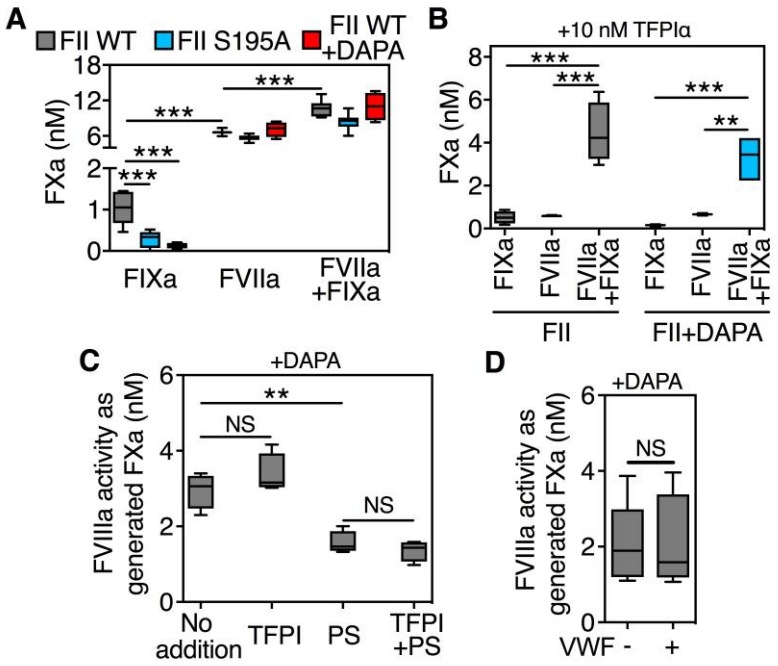

Figure S2. FVIIIa generated by nascent TF-FVIIa-FXa supports intrinsic tenase activity. (A) Synergistic FXa generation by combined addition of TF-FVIIa and FIXa. FXa generation was induced by 10 nM FIXa or 200 pM FVIIa or the two combined added into 50 pM rTF, 135 nM FX, 700 pM FVIII, 3 nM FV, 1 μM prothrombin and 2.5 mM $CaCl_2$ without (n =7) or with (n =12) 4 μM DAPA; incubation for 180 s at 37°C. In 4 experiments, inactive S195A mutant substituted for WT prothrombin without DAPA. (**B**) Limited effect of 10 nM TFPIα on FXa generation by combined TF-FVIIa and FIXa - as opposed to inhibition of direct FXa generation by TF-FVIIa - in reactions as in (A) containing FII WT with (n =3) or without (n =4) 4 μM DAPA. (**C)** Effect of 2.5 nM TFPIα without/with 150 nM protein S (PS) on thrombin-independent FVIIIa generation measured as (A) with FII WT and 4 μM DAPA added (n=4). **(D)** Thrombin-independent FVIIIa generation without or with (n= 4-5) added VWF (18 nM) measured as in (A) but in reactions induced by 400 pM rTF-500 pM FVIIa containing 40 nM TFPIα, FII WT and 4 μM DAPA. Results in (A-D) shown as 25^{th}-75^{th} percentile bars with min-to-max whiskers and line at the median; statistical analysis by ANOVA/Tukey tests. **P<0.01, ***P<0.001.

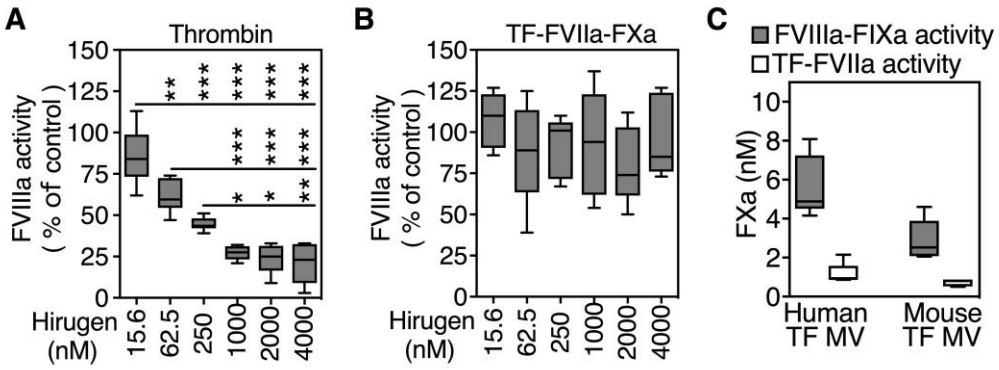

Figure S3. FVIII activation by TF-FVIIa-FXa initiation complex. (A) Effect of increasing hirugen concentrations on FVIIIa generation in reactions containing 0.5 nM thrombin F(IIa), 50 pM rTF, 700 pM FVIII and 2 nM FIXa incubated for 180 s at 37 °C (n =5-6). FVIIIa activity was calculated by determining its cofactor activity in FIXa-dependent FXa generation. **(B)** Hirugen has no effect on FVIIIa generation by TF-FVIIa-FXa complex induced by adding 200 pM FVIIa and/or 2 nM FIXa into reactions containing 50 pM rTF, 135 nM FX and 700 pM FVIII incubated for 180 s at 37°C (n =5). FVIIIa activity generated by TF-FVIIa-FXa was calculated by subtracting the sum of FXa generated in reactions containing FVIIa and FIXa alone from that generated when FVIIa and FIXa were added together. (**C**) FVIIIa generation on procoagulant microvesicles (MV) from mouse macrophages[15] expressing knocked-in human TF or endogenous mouse TF. MV suspensions were added into reactions containing 10 nM species-matched FVIIa, 700 pM FVIII, 3 nM FV, 135 nM FX and 200 nM lepirudin, without/with 2 nM FIXa, and incubated for 180 s at 37 °C (n =2-3). FXa generation by FVIIIa-IXa was calculated as in (B). Results in (A-C) are shown as 25^{th}-75^{th} percentile bars with min-to-max whiskers and line at the median; statistical analysis by ANOVA/Tukey tests. *P<0.05, **P<0.01, ***P<0.001.

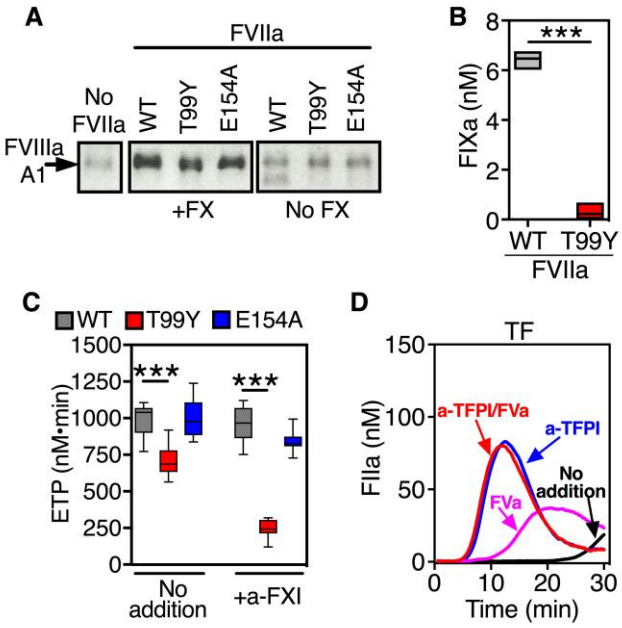

Figure S4. Activation of the intrinsic pathway induced by TF- FVIIa mutants with impaired FXa product turnover. (A) Representative immunoblotting (n =2) of FVIII activation in reactions containing 50 pM rTF, 200 pM FVIIa WT or indicated mutants (T99Y and E154A), 700 pM FVIII, 3 nM FV and 200 nM lepirudin without/with 135 nM FX, incubated 120 s at 37 °C. The smaller fragment seen in reactions with FVIIa WT and no FX is inactive FVIII $A1^{337-372}$-$A2^{16}$, indicating that activating cleavages are preferentially by nascent FXa. (**B**) FIXa generation in reactions (n =3) containing 150 nM FIX with 50 pM rTF, 200 pM FVIIa WT or mutant T99Y and 2.5 mM $CaCl_2$, incubated for 30 min at 37°C. After terminating the reaction with 10 mM EDTA, FIXa activity was determined by measuring amidolytic activity towards the chromogenic substrate Pefachrome FIXa (1 mM). The results are shown as min to max floating bars with a line at the mean and were analyzed by a two-tailed t-test. ***P<0.001. (**C**) Quantification of endogenous thrombin potential (ETP) calculated from the area under the TG curve. TG was induced in recalcified FVII-deficient PRP reconstituted with 400 pM FVIIa WT, or mutants T99Y or E154A, by adding 2.5 pM TF (n =3). Results are shown as 25th-75th percentile bars with min to max whiskers and line at the median. Differences were evaluated with ANOVA/Tukey tests. ***P<0.001. (**D**) TFPI function controlling FVa generation. Representative thrombograms (n =2) obtained by adding 0.15 pM rTF into recalcified normal PRP ($180 \cdot 10^3$ platelets/µl) containing 30 µg/ml CTI without/with 40 µg/ml anti-TFPI IgG to neutralize TFPI activity and 3 nM FVa, as shown.