SUPPLEMENTARY APPENDIX

A mutated factor X activatable by thrombin corrects bleedings *in vivo* in a rabbit model of antibody-induced hemophilia A

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Supplemental Information

Methods

List of material

Human plasmatic factor X (pdFX), plasma-derived activated FX (pdFXa), human α-thrombin (IIa), r-Hirudin, FX-deficient plasma, phospholipids, Russell viper venom-fraction X (RVV-X), pNAPEP-1065 substrate (S2765), Glu-Gly-Agr chloromethylketone (GGACK), mouse activated FXa (mFXa), the polyclonal sheep anti-human FIX antibody and the polyclonal sheep anti-human FX antibody were from Haematologic Technologies (Cryopep, Montpellier, France). Hemophilia A plasmas with 292 BU and without inhibitors were from Cryopep. Recombinant FVIII (200 U/mL) was from Shire (Vienna, Austria). Dade Innovin standard plasma and FVIII-deficient plasma were from Siemens (Saint Denis, France). FluCa Kit, Owren-Koller Buffer, Thrombin Calibrator, PRP reagent, MP reagent, rabbit anti-human FX Peroxidase Conjugated, FIX- and FXI-deficient plasma were from Stago (Asnières, France). The polyclonal sheep anti-human FVIII antibody was from Tebu-Bio (Le Perray-en-Yvelines, France). Monoclonal antibody against human Gla domain was from Sekisui (Dusseldorf, Germany). Rabbit plasma was from Clinisciences (Nanterre, France). Peroxidase AffiniPure Goat Anti-Mouse IgG (H+L) and the peroxidase AffiniPure F(ab')2 Fragment Rabbit Anti-Sheep IgG (H+L) were from Jackson ImmunoResearch (Montluçon, France). Super signal West Pico Substrate Chemiluminescent, 293Fectin reagent, PageBlue Protein Staining Solution, Magic Mark and Gel NuPAGE Novex Bis-Tris 4-12% were from ThermoFisher (Villebon sur Yvette, France). Precision Plus Protein All Blue Standards and Trans-Blot Turbo Mini PVDF Transfer Packs were from Biorad (Marne la Coquette, France). The couple of anti-FVIII monoclonal antibody inhibitors was produced and characterized at LFB Biotechnology. They totally inhibit thrombin generation in human and rabbit plasma at 0.65 µg/mL each.

Plasmid construct generation

Actiten construct was generated by gene synthesis using FX protein sequence (UNIPROT P00742), in which a 10 amino acid coding sequence for a thrombin cleavage site was inserted after the activation peptide sequence (Figure 1). Codon optimization was performed to enhance protein expression (GeneArt for Actiten and GeneScript for FX-WT). Actiten cDNA was cloned into homemade vector at unique restriction site whereas FX-WT cDNA was cloned into pCEP4. The coding sequences were

checked by DNA sequencing and endotoxin-free plasmid preparations were produced for cell transfection.

Cell culturing and product production

FX-WT was transiently produced in Expi293F cells using ExpiFectamine (Thermo Fisher Scientific) for co-transfection with a plasmid encoding the human VKOR enzyme, pCEP4-VKOR. Stable clones of HEK293F cells expressing the Actiten molecule were generated. Briefly, cells were transfected with the expression vector containing the Actiten construct using 293Fectin reagent. From day 2, Neomycin (1 g/L) was added and maintained during the selection steps. Cells were maintained in F17 medium supplemented with 8 mM L-glutamine. The production of Actiten and recFX-WT were performed in batch mode. Vitamin K1 (5 µg/mL) was added 24h before the production and every 2 or 3 days until the cells were harvested at day 7. For Actiten, the first day of production, cells were transfected with pCEP4-hVKOR. At day 7, the supernatant was clarified by centrifugation. PMSF (0.5 mM) and benzamidine (5 mM) were added followed by a filtration at 0.2 µm before storage at -80°C.

Material for RP-HPLC

RVV Factor X activator (RVV-X) was from Pentapharm (Basel, Switzerland). Dithiothreitol (DTT), iodoacetamide (IAM), urea, ammonium bicarbonate, ethylenediaminetetraacetic acid (EDTA), phosphate buffered saline (PBS) and calcium chloride ($CaCl_2$) were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Peptide-N-glycosidase F (PNGase F) was from Prozyme (San Leandro, USA). HPLC reagent grade acetonitrile (MeCN) and trifluoroacetic acid (TFA) were purchased from JT Baker (Philipsburg, NJ, USA). All the aqueous solutions were prepared using ultra-pure water (18.2 MΩ-cm resistivity at 25°C, total organic carbon (TOC) < 5 ppb).

RP-HPLC Protocol

For intact mass analysis by LC-MS, lyophilized product (150 µg) was dissolved in 50 µL of a PBS solution pH 7.4 and N-deglycosylated by incubation with 7.5 mU of PNGase F overnight at 37°C. Then the sample was activated using 7.5 mUI of RVV-X in presence of 25 mM $CaCl_2$, for 8 minutes at 37°C. After vacuum-drying, Actiten was recovered in 50 µL of denaturing buffer (8 M urea, 0.4 M ammonium bicarbonate pH 8.0 and 10 mM EDTA). Reduction was done by adding 15 µL of a 100 mM DTT solution in water and incubating the resulting mixture for 15 min at 55°C. After cooling at room temperature, 15 µL of a 200 mM IAM solution in water were added and the solution was incubated at room temperature for 15 min in the dark. RP-HPLC was performed using an ACQUITY UPLC system (Waters, Milford, MA, USA) coupled to an UV detector and an electrospray mass spectrometer (Synapt G2S, Waters, Milford, MA, USA). Around 20 µg of sample was injected on a Pursuit 200Å Diphenyl reverse phase column (150x2.0 mm, 3 µm) equilibrated at 40°C and operated at a flow rate of 250 µL/min

(Agilent, Santa Clara, CA, USA). An aqueous solution containing 0.1% TFA and MeCN containing 0.1 % TFA were respectively used as buffer A and buffer B; proteins were eluted by using an increasing gradient of buffer B. The mass spectrometer was operated in the positive mode, at a 20,000 FWHM resolution, using a capillary voltage of 3 kV, a source block temperature of 80°C and a desolvation temperature of 150°C. Calibration was carried out according to manufacturer's procedure using NaI cluster ions. Data were recorded from m/z 500 to m/z 4000. Protein mass spectra were deconvoluted using Transform and Maxent 1 tools available in the MassLynx software.

Binding to phospholipids

The following buffers were used during the assay: Coating buffer: Absolute Ethanol (Carlo Erba), Saturating buffer (SB): 50 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$, pH 7.5 + 1% BSA (w/v), Washing and diluting buffer: 50 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$, pH 7.5 + 0.1% BSA (w/v). Phospholipids were diluted to 12.5 µM, loaded in 96-wells plate flat bottom, coated overnight at room temperature (RT). Then, the plate was saturated for 2h at RT with SB and washed before loading the diluted sample (12.5 ng/mL to 200 ng/mL) for 2h at RT. Washes were then performed before adding the HRP-coupled revelation anti-FX antibody for 1h at RT. Washes were finally performed before adding TMB and stopping the reaction with 0.45 M H_2SO_4. The optical density was read at 450 nm and is proportional to the amount of bound FX.

Pharmacokinetic study in rabbit

At day 1 (D1), one single dose of purified pdFX or Actiten was administered by IV (marginal ear vein) on conscious animals. The rabbits included in the experiment were weighed at the day of treatment to adjust the dose injected per rabbit. Injection volumes were limited to 2 mL/kg max. Blood samples of approximately 2.5 mL of whole blood on citrated tube for plasma preparation was performed at the central artery of the ear at the following times: D_{-3} (T_0), prior to the product administration: the sample was compensated by an injection of 0.9% NaCl with an equivalent volume of 2.5 mL to avoid hypovolemic stress. Samples were performed at the following times: T_{5min}, T_{30min}, T_{60min}, T_{180min}, $T_{300min,}$ $T_{1440min}$ for pdFX and, T_{14min}, T_{40min}, T_{70min}, T_{120min}, T_{150min} and $T_{1450min}$.

Plasmas obtained were divided into at least 2 cryotubes. It was stored at -80 °C until the product concentration was monitored in plasma sample by a commercial ELISA specifically detecting human FX purchased from Abnova (ref. KA073). The administrated product (pdFX or Actiten) was utilized to establish the standard curve for dosing.

Activation by thrombin

Activation of Actiten (100 nM) by thrombin (10 nM) was studied at 37°C in reaction buffer (25 mM Hepes, 175 mM NaCl, 5 mg/mL BSA, 5 mM $CaCl_{2,}$ pH 7.4). After different times of incubation, the

reaction was quenched by adding a STOP buffer (50 mM Tris-HCl, pH 8.8, 475 mM NaCl, 9 mM EDTA). The FXa formation was monitored by measuring the hydrolysis rate of pNAPEP 1065 (250 µM) at 405 nm. For all the activations, as a standard, pdFXa dilutions (from 20 nM to 1.25 nM) were introduced into stop buffer and revealed as the samples. Determination of the k_{obs} and the ratio k_{cat}/Km was performed as described based on a one-phase exponential association (Louvain-Quintard et al. J. Biol. Chem 2005, 280, 41352-41359)

Activation of FX by FVIIa/TF

Activation of FX (100 nM) by TF-FVIIa (50 pM) was studied at 37°C in reaction buffer. After 10 min incubation, the reaction was quenched by adding the stop buffer. The FXa formation was monitored at different points by measuring the hydrolysis rate of pNAPEP 1065 (250 µM) at 405 nm.

Activation of FX by FIXa-FVIIIa

The reaction was performed in the reaction buffer with 10 µM phospholipids. The complex of activated factors (FIXa/FVIIIa) was generated by mixing 44 nM of FIXa to 1.85 nM of FVIIIa. To activate FVIII, it was incubated at 1.85 nM for 2 min at 37°C then 3.7 nM of thrombin was added and the mixture was incubated 1 min more at 37°C. The reaction was neutralized by 1.2 U/mL of hirudin and immediately added to FIXa. The purified FX was diluted to 200 nM and further serially diluted by half in a plate to obtain 100, 50 and 25 nM. The FIXa/FVIIIa complex was rapidly added (50 µL) to the dilutions of FX (50 µL). After 5 min incubation at 37°C, the reaction was quenched by adding stop buffer. The FXa formation was monitored by measuring the hydrolysis rate of pNAPEP 1065 (250 µM) at 405 nm in kinetic mode for 10 min.

In vivo evaluation of Actiten

KBL/NZW male rabbits weighting between 2.191 kg and 2.762 kg were used. The animals received either a single intravenous dose of 2 monoclonal anti-FVIII antibodies (99 µg/kg each; N=4) or saline solution (NaCl 0.9%; N=8) 45 min before cuticle bleeding induction. Rabbits were then pre-anesthetized with diazepam (0.4 mg/kg) in the marginal ear vein using a catheter and anaesthesia was induced by administration of pentobarbital sodium (24.62 mg/kg, IV route). From that time, animals were kept under anaesthesia with a continuous infusion of pentobarbital sodium (2.46 mg/kg). One forepaw was placed 5 min after anaesthesia induction in a beaker containing a saline solution at 37°C. After another 10 min, cuticle bleeding was induced by cutting 2 mm of the nail of the third digit. The paw was replaced in the beaker with saline at 37°C, followed by a 60 min observation period, after which the animals were euthanized by pentobarbital. Before euthanasia, blood samples were collected from the central artery of the ear. A sample was collected from the saline solution, in which the bleeding occurred to measure the concentration of haemoglobin.

Actiten or recFX-WT efficacy at 1.7 mg/kg was assessed with the same study design by administering the treatment 35 min before the bleeding induction. In contrast, recombinant FVIIa (500 µg/kg) was infused 5 min before.

Dosage of thrombosis markers

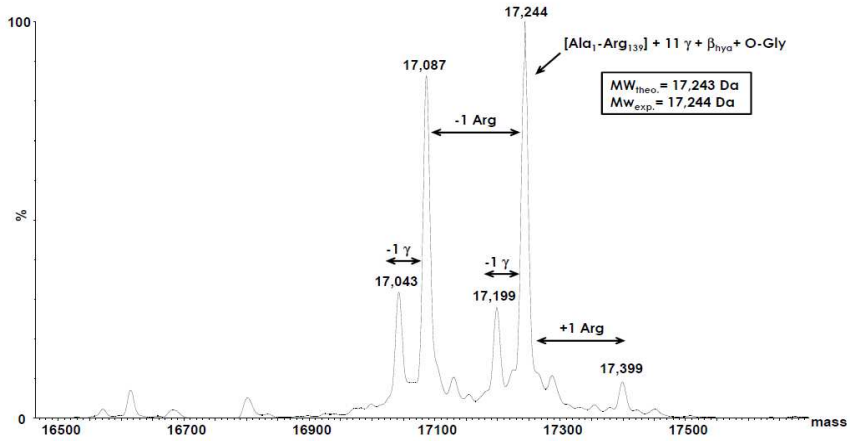
Rabbit plasmas from the *in vivo* evaluation experiment of Actiten were collected and analyzed for the presence of Thrombin-AntiThrombin complexes (TAT), fragment 1 and 2 from prothrombin (F1+2) and D-dimers from fibrinogen (D-dimers). All products were dosed using ELISA dedicated to analyze rabbit products according to manufacturers instructions. Two assays from two different providers were performed to analyze each marker. TAT were dosed using the Rabbit Thrombin-Antithrombin ELISA Kits from Cryopep (Montpellier, France) and MyBiosource.com (San Diego, Ca, USA). Prothrombin 1+2 fragments were dosed using Rabbit Prothrombin Fragment 1+2 ELISA Kits from Cryopep and from Elabscience (Houston, Tx, USA). D-dimers were dosed using the Rabbit D-Dimer ELISA Kits from Cryopep and Elabscience.

Supplemental Table 1: Rabbit thrombosis markers following infusion of saline or Actiten

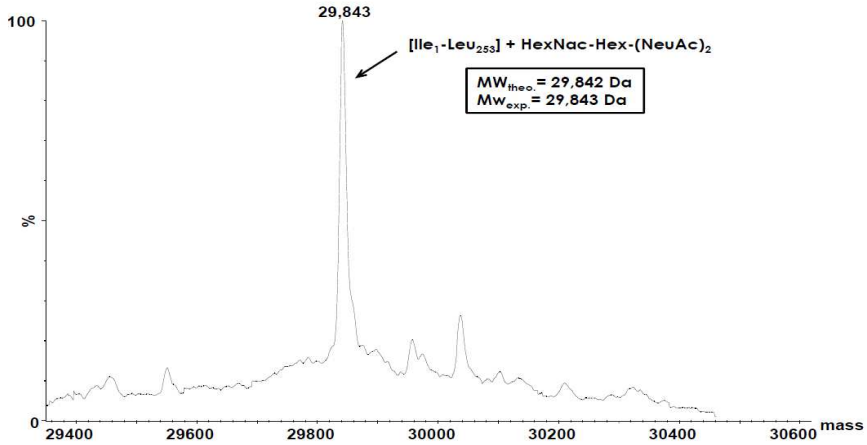
	Rabbit TAT Cryopep (pg/mL)	**Rabbit TAT My Biosources** (pg/mL)	**Rabbit F1+2 Cryopep** (pg/mL)	**Rabbit F1+2 Elabscience** (pg/mL)	**Rabbit D-dimers Cryopep** (ng/mL)	**Rabbit D-dimers Elabscience** (ng/mL)
NaCl 9/1000	<75	215.4 +/- 9.7	<6	1.48+/-0.21	<150	<6.25
Actiten (1.7 mg/kg)	<75	218.6+/-12.8	<6	2.00+/-0.79	<150	<6.25

Plasmas from NZW rabbits infused with the couple of anti-FVIII antibodies and with, either saline or Actiten (1.7 mg/kg), were dosed for thrombin-antithrombin complexes (TAT), prothrombin fragment 1+2 (F1+2) and fibrinogen D-dimers by two different commercial assays. In each assays, samples were dosed in triplicate.

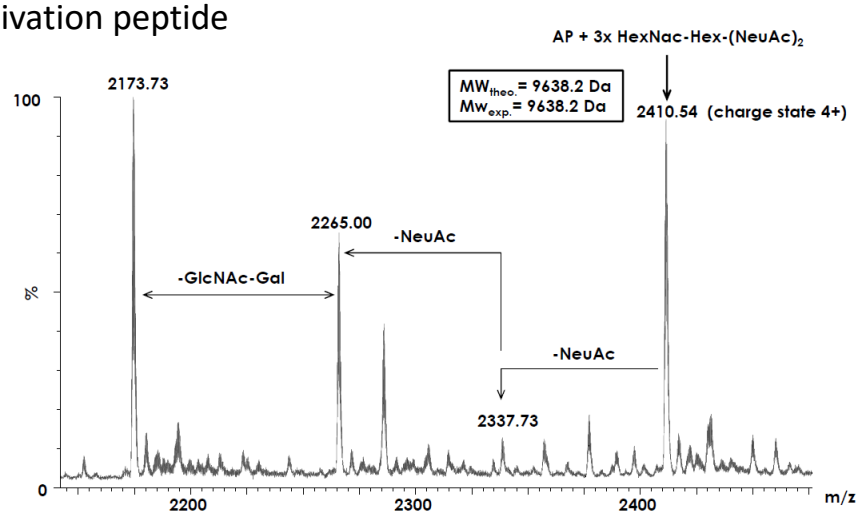
A-Actiten light chain



B-Actiten heavy chain

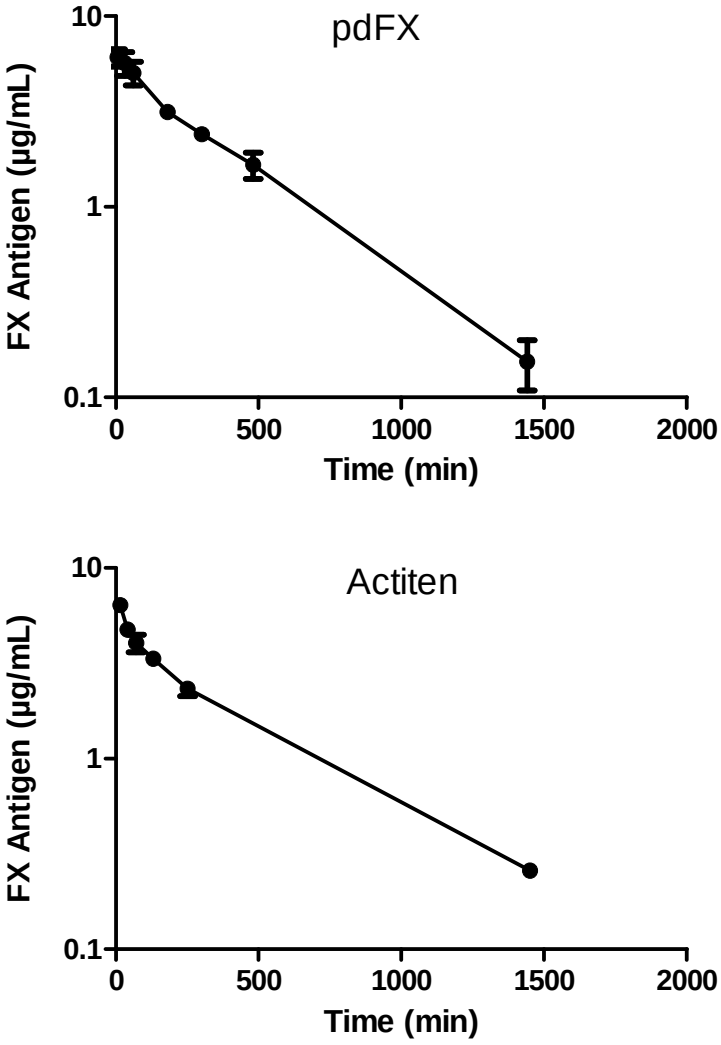


C- Actiten activation peptide



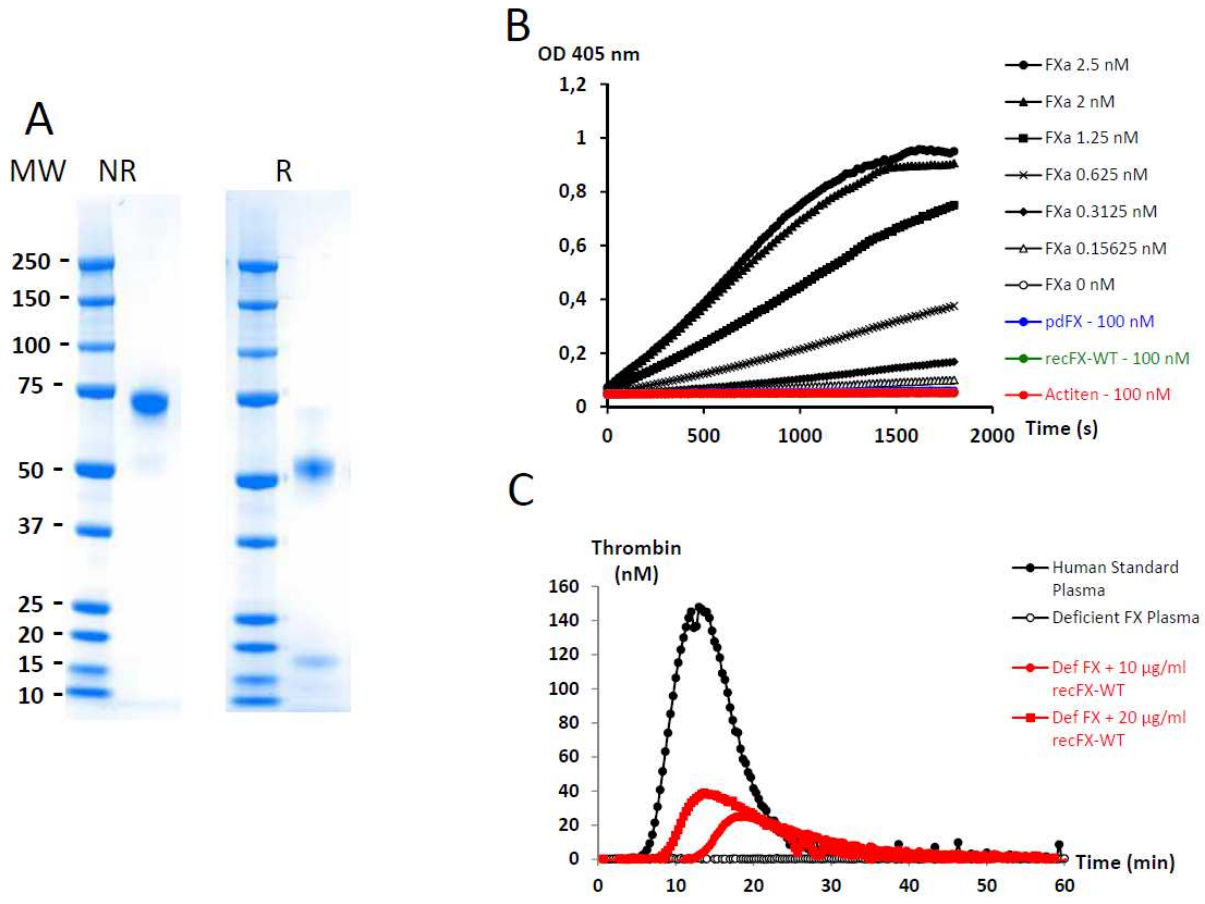
Supplemental Figure 1: Mass spectrometry analysis of separated domains of Actiten

Anti-Gla purified Actiten was treated by PNGase F and RVV-X before being separated by RP-HPLC and mass spectrometry. Representations of the mass corresponding to the light chain (A), the heavy chain (B) and the activation peptide (C) of Actiten are presented.



Supplemental figure 2: PK of pdFX (0.3 mg/kg) and Actiten (0.33 mg/kg) in wild-type rabbit

Plasma-derived FX (0.3 mg/kg) and Actiten (0.33 mg/kg) were infused in New Zealand White rabbits. At different times following infusion, plasma samples were collected and FX was dosed by ELISA recognizing specifically human FX. The ELISA was controlled to recognize identically pdFX and Actiten. N=3-4 rabbits.



Supplemental figure 3: Production of a recombinant wild-type Factor X

Panel A, RecFX-WT was produced in HEK293F cells and purified as Actiten. Following anti-gla aptamer purification, the molecule was purified to homogeneity with a migration following SDS-PAGE and PAGE-blue coloration as a single band in non-reducing (NR) condition When the molecule was DTT-reduced (R), it migrates under the form of 2 bands, corresponding respectively to the heavy and light chains of FX. **Panel B,** To insure the absence of contaminating FXa in the different preparations, pdFX, Actiten and recFX-WT were prepared at 100 nM and incubated in presence of pNAPEP 1065, a FXa substrate. A standard curve was established with different concentrations of FXa (from 0 to 2.5 nM). Down to 0.16 nM of FXa (0.16% of 100 nM) can be detected in this setting. No FXa can be detected in the three preparations evaluated. **Panel C,** The activity of recFX-WT was then evaluated in TGA. In contrast to Actiten, recFX-WT was able to correct a FX deficiency with no delay. TGA and other enzymatic assays (RVV-X, FVIIa/TF and FVIIIa/FIXa activations) revealed an activity for recFX-WT that vary from 20 to 40 % of pdFX being comparable to the activity detected for Actiten (Panel C and data not shown).