Supporting Information

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SI Materials and Methods

Materials. Human factor Xa (FXa), prothrombin, and thrombin were from Enzyme Research Laboratories. Corn trypsin inhibitor (CTI) and the thrombin inhibitor dansylarginine N-(3-ethyl-1,5-pentanediy)lamide (DAPA) were from Haematologic Technologies. BSA, heparin, polyphosphate, fucoidan, and the protease inhibitors E-64 and 3,4-dichloroisocoumarin were from Sigma-Aldrich. Recombinant tissue factor pathway inhibitor (TFPI)α, K1K2, and K1K2C were as described (1). Monoclonal antibodies against the active site of K2 (anti-K2) and the two residues immediately following K3 (anti-K3C) domains of TFPI were provided by Novo Nordisk. The monoclonal antibody against the active site of the K1 domain (anti-K1) was a gift from George Broze, Jr (Washington University, St. Louis).

Human factor V (FV) was isolated from plasma (2, 3). The altered forms of FV (FV810, FV810R709Q, FV810R1545Q, FV810ΔQ, and FV-B152) were as described (4). Monoclonal antibodies against the FV light chain (anti-FV_{LC}) (5) and residues 1019–1545 (anti-FV_{HH}), and a rabbit polyclonal antibody against human FVII, were from the Antibody Core Facility (University of Vermont, Burlington, VT). Activated FV (FVa) was produced by incubation of FV (50 nM) with FXa (1 nM) or thrombin (2 nM) in 20 mM Hepes, 150 mM NaCl, 5 mM CaCl\(_2\), 0.1% PEG-8000 pH 7.4 (HBS/Ca\(_2^+\)/PEG), for 10 min at 37 °C, generating FV_{ΔAR} or FV_{ΔTAR}, respectively. Phospholipid vesicles containing 40% (vol/vol) phosphatidylcholine, 20% (vol/vol) phosphatidylserine, and 40% (vol/vol) phosphatidylethanolamine (PCPSPE) (20 μM) were included when activating FV with FXa. For some reactions, FV810 (4.6 μM in HBS/Ca\(_2^+\)/PEG) was treated with thrombin (20 nM) for 20 min at 37 °C, followed by addition of Phe-Pro-Arg-chloromethylketone and purification of FV_{TAR} (4). Replicated tissue factor (TF) (Hemoliance RecombiPlasTin) was from Instrumentation Laboratory and was reconstituted in 5 mL of 0.9% NaCl.

FV and TFPI Basic Peptides. Peptides encoding the FV B-domain basic region (residues 951–1008; FV-BR) and the TFPIα basic region (residues 240–265; TFPI-BR) were expressed as SUMO fusions using the SUMOpro bacterial expression system (Life-Sensors). To facilitate its purification, TFPI-BR included an N-terminal fusion of FV residues 951–962, which include a Trp to allow for absorbance detection at 280 nm. The SUMO fusions were purified on HisTrapFF columns (Amersham), the SUMO tag was cleaved with SUMO protease (LifeSensors), and the peptides were purified by ion exchange chromatography. For fluorescence anisotropy experiments, FV-BR and TFPI-BR, containing N-terminal Cys residues, were labeled with Oregon Green 488 (OG488) maleimide, according to the manufacturer’s instructions (Invitrogen).

Cells. Chinese hamster ovary (CHO) cells transfected with human TFPI{\(\alpha\)} (CHO-TFPI{\(\alpha\)}) were previously generated and characterized (6). Cells were harvested with EDTA (5 mM), washed with PBS, and resuspended in HBS containing 5 mM CaCl\(_2\) and 0.1% BSA (HBS/Ca\(_2^+\)/BSA). Total cellular protein was determined by bicinchoninic acid assay (ThermoScientific). Cell surface TFPI{\(\alpha\)} activity was measured by injection of cleavage of a chromogenic substrate (Spectrozyme Xa; Sekisui Diagnostics) by FXa and found to be 5.9 nmol/g total cellular protein.

Human Platelets and Platelet-Rich Plasma. All experiments using human subjects were approved by the institutional review board at the Blood Center of Wisconsin (Milwaukee). Blood was drawn from consenting adults into 3.2% (wt/vol) sodium citrate and 50 μL/mL CTI. The first collection tube was discarded to minimize contaminating TF. Platelet-rich plasma was prepared by centrifugation (150 × g, 15 min). Washed platelets (3 × 10\(^8\) per mL in HBS/Ca\(_2^+\)/BSA) were prepared as previously described (7) and activated by incubation with collagen (0.1 mg/mL, 10 min, 37 °C) or collagen followed by thrombin (0.1 nM, 10 min, room temperature).

Phospholipid Vesicles. Phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine were from Avanti Polar Lipids. PCPSPE was prepared as described (8). Those containing 75% (vol/vol) phosphatidylcholine and 25% (vol/vol) phosphatidylserine (PCPS) were prepared as described (4).
**Fig. S1.** Inhibition of FVII has no effect on the K2 and K3C-dependent anticoagulant activity of TFPIα in PRP. (A) Tissue factor (1/2,000), factor VIIa (20 pM), and PCPSPE (20 μM) were incubated with rabbit anti-FVII (0–100 nM), reactions initiated by addition of factor X (20 nM), and quenched after 15 min by addition of EDTA (30 mM). FXa generation was measured using a chromogenic substrate (0.5 mM). (B) Calibrated automated thrombography (CAT) assays were performed as in Fig. 1 in the absence (solid lines) or presence (dashed lines) of rabbit anti-FVII (100 nM) and in the absence (black) or presence of 50 nM anti-K1 (red), anti-K2 (blue), or anti-K3C (orange).

**Fig. S2.** Platelets contain multiple forms of FV/Va, some containing the acidic region of the B domain attached to the light chain. Washed platelets were solubilized with Triton X-100, subjected to SDS/PAGE, and analyzed by immunoblotting first with a monoclonal antibody against the FV light chain (Left). The blot was then stripped and reprobed with a monoclonal antibody against residues 1,019–1,545 of the FV B domain (Right). Indicated are FV fragments which contain the light chain and an extended stretch of the B domain (*), as well as fragments that contain the light chain and a small part of the B domain (◄). All of these represent potential acidic region-containing, functional forms of FVa.

**Fig. S3.** The inhibition of FXa amidolytic activity by TFPIα is independent of the type of FVa present. TFPIα (0 nM, black; 1 nM, red; 5 nM, green; and 10 nM, blue) was incubated with 0.1 nM FVaAR (solid lines) or FVaIIa (dashed lines), 20 μM PCPSPE, and 0.5 mM chromogenic substrate. Reactions were initiated with FXa (0.5 nM) and cleavage of the chromogenic substrate was monitored. Shown are the average chromatograms for two independent experiments.
Fig. S4. Antibodies against the K2 and K3 domains of TFPIα block its inhibition of FVα AR prothrombinase. Prothrombin activation assays were performed using FVα AR, as described in Fig. 5, in the presence of 1 nM TFPIα and varying concentrations of anti-K1 (■), -K2 (△), or -K3C (shaded circle). The dashed line indicates thrombin generation in the absence of TFPIα, and the solid line indicates thrombin generation in the presence of TFPIα and the absence of antibody. Shown are the mean ± range values from two independent experiments.