Supplemental Data

Supplemental Methods

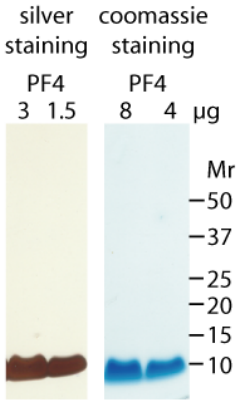
Purification of protein C – Plasma-derived protein C was purified from factor concentrate Prothromplex-T TIM4 (Baxter, Vienna, Austria) by affinity chromatography on anti-protein C (HPC4)-coupled Sepharose and anion exchange chromatography. Briefly, 4800 IU Prothromplex-T dissolved in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.4) containing 2 mM $CaCl_2$ was applied to HPC4-Sepharose equilibrated in the same buffer. After extensive washing with TBS containing 0.5 M NaCl and 2 mM $CaCl_2$, protein C was eluted in TBS with 5 mM EDTA. Pooled fractions containing protein C were diluted 1:1 with 20 mM Tris pH 7.4 and applied to fast flow Q-Sepharose (GE Healthcare, Piscataway, NJ). Purified protein C was recovered from a 0-600 mM NaCl gradient in 20 mM Tris pH 7.4. APC was generated from purified protein C by incubation with thrombin (1/50, w/w) in the presence of 2 mM EDTA for 2.5 - 3 h at 37 °C, followed by the addition of hirudin to inactivate the thrombin and fast flow Q-Sepharose chromatography to remove thrombin (1).

Generation of stable K239 cell lines expressing TM and TM-His – TM was cloned from EA.hy926 endothelial cells following RNA isolation (RNAqueous-4PCR; Ambion, Austin, TX), RT-PCR with oligo dT primers (Retroscript, Ambion) and PCR with TM specific primes (forward: 5'-GCACGGCCCTGTCGCAGTGC-3' and reverse: 5'-AGGCTCCTGGACGGAGCCA-3' and nested primers forward: 5'-GCACGCGGCAAGCTTGGGTAACA-3' and reverse: 5'-GCTCCTGGATCCAGGCCGCTCAG-3') using SuperTaq (Ambion). The obtained PCR product for TM was introduced into pCR 2.1-TOPO (Invitrogen, Carlsbad, CA) and transferred to pcDNA 3.1 (+) neo (Invitrogen) using the HindIII and XhoI restriction sites. The sequence of the resulting wt-TM was verified and transfected into HEK-K293 cells using polyfect (Qiagen, Valencia, CA). Full length TM with a C-terminal His-tag (TM-His) was generated by introducing a BspEI restriction site in place of the stop codon using Quickchange mutagenesis (Stratagene, La Jolla, CA) and inserting a synthetic double stranded oligomer encoding a short flexible spacer, 6xHis tag, stop codon and the appropriate BspEI and NotI (3' MCS) overhang (forward: 5'-CCGGGCCCGCGGTTCGAAGGTCATCATCATCATCATTGA-3' and reverse: 5'-GGCCTCAATGATGATGATGATGACCTTCGAACCGCGGGC-3'). The sequence of the resulting TM-His was verified and transfected into HEK-293 cells for generation of stable expressing cells (TM-His-K293).

Clot lysis assay – Clot lysis was studied in a plasma system of thrombin-induced clot formation and tPA-mediated fibrinolysis as described (2). The change in turbidity (405 nm) at 37° C was measured

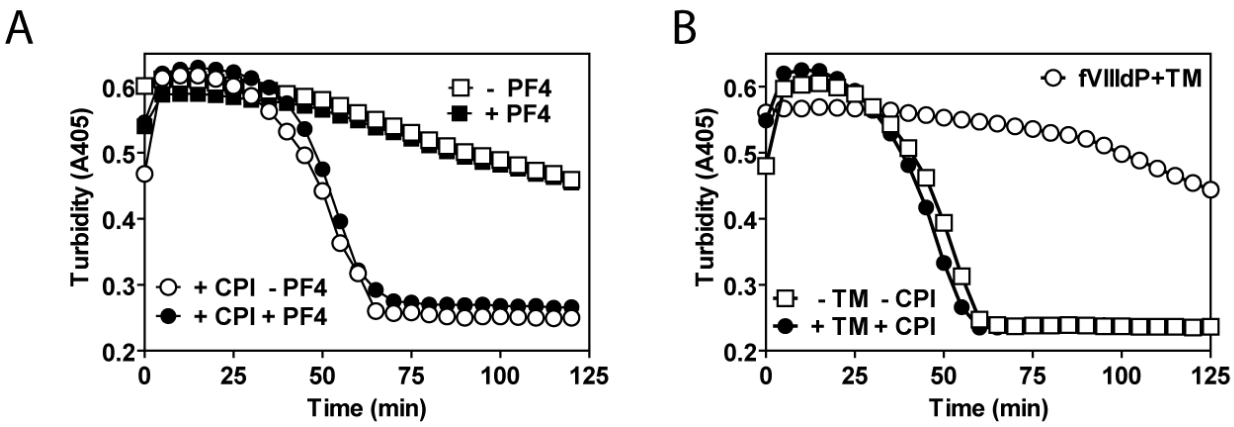
(Thermomax; Molecular Devices) in 50% normal pooled plasma (v/v) in the presence of rl-TM, PF4, 10 nM thrombin, 17 mM $CaCl_2$ and tPA. The clot lysis time was defined as the time to reach a half-maximal decrease in turbidity. CPI was used at 20 µg/ml to inhibit TAFIa. A blocking anti-TM antibody (clone #RTM96; Cell Sciences) was used at 10 µg/ml and was preincubated with the TM-K293 cells for 15 min.

Supplemental Figures



<u>Supplemental Fig.1.</u> SDS-PAGE analysis of PF4 purified from plasma.
Purified PF4 was analyzed for homogeneity on 4-12% Bis-Tris gels (Invitrogen) with MES running buffer (Invitrogen). Protein bands were detected by silverstain (left) or Coomassie Blue (right). These results indicate that the purified PF4 from plasma migrated at the anticipate apparent molecular weight of 8,000 Da for the PF4 monomer and was >95% homogeneous.

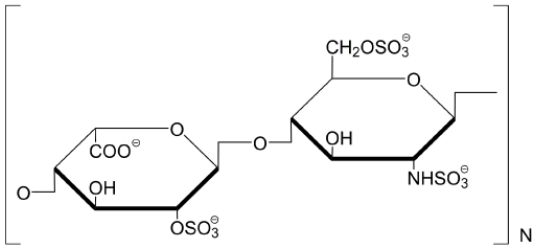
Supplemental Figure 2



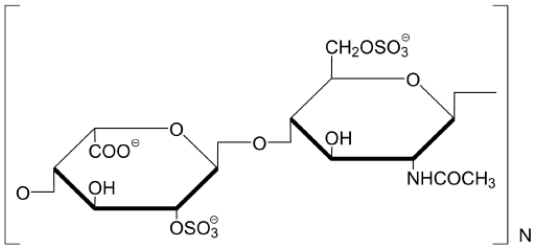
<u>Supplemental Fig.2.</u> Clot-lysis in normal and factor VIII deficient plasma

(**A**) Clot formation was initiated in normal plasma in the absence of rl-TM by thrombin (10 nM) and fibrinolysis was initiated by tPA (30 U/ml) in the absence (open symbols) and presence (closed symbols) of PF4 (50 μg/ml). No apparent differences are observed in the absence (□,■) of CPI (20 μg/ml), indicating that under the conditions employed PF4 did not notably affected secondary thrombin formation via thrombin-mediated factor XI activation and subsequent amplification by the intrinsic pathway that is required for thrombin-mediated TAFI activation in the absence of TM. No apparent differences are observed in the presence (○,●) of CPI (20 μg/ml), indicating that under the conditions employed PF4 did not notably affected tPA-mediated activation of the fibrinolytic system. (**B**) Characterization of clot-lysis in the factor VIII deficient plasma (fVIIIdP) used. Clot lysis was initiated by thrombin (10 nM) and tPA (30 U/ml) (□) supplemented with either 1 nM rl-TM (○) or 20 μg/ml CPI (●). No apparent differences are observed in the presence (●) or absence (□) of CPI (20 μg/ml), indicating that under the conditions employed insufficient secondary thrombin formation occurred to sustain thrombin-mediated TAFI activation in the absence of TM. In the presence of rl-TM (○), TAFI activation occurred readily by the available thrombin concentrations used to initiate clot formation, thereby eliminating the requirement for secondary thrombin formation to activate TAFI. Thus, TAFI activation and TAFIa-mediated inhibition of fibrinolysis in fVIIIdP is entirely dependent on the availability of thrombin-TM complexes.

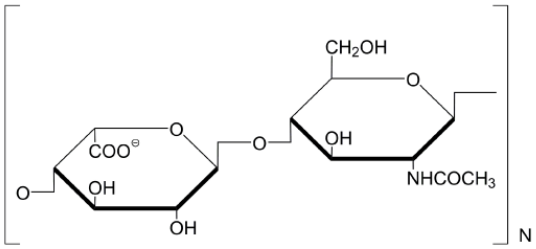
Supplemental Figure 3



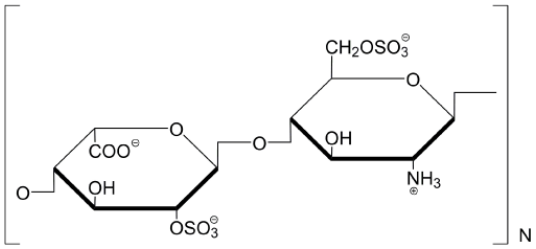
Heparin (Hep)



N-Acetylheparin (NAc-Hep)



N-Acetyl-de-O-sulphated Heparin
(NAc-deO-Hep)



De-N-sulphated Heparin
(deN-Hep)

Supplemental Fig.3. Heparin and heparin-derivatives.

Schematic representation of heparin (Hep) and the heparin-derivatives N-Acetylheparin (NAc-Hep), N-Acetyl-de-O-sulphated heparin (NAc-deO-Hep) and De-N-sulphated heparin (deN-Hep).

Heparins	PF4 binding to Hep-biotin IC_{50} (95% CI) (µg/ml)	PF4 binding to TM IC_{50} (95% CI) (µg/ml)
Hep	0.29 (0.19-0.43)	0.079 (0.048-0.13)
NAc-Hep	0.22 (0.17-0.29)	0.084 (0.064-0.11)
NAc-deO-Hep	-	433 (225-834)
deN-Hep	91.6 (19.1-440.0)	0.47 (0.31-0.71)

Supplemental Table 1. Affinity of heparin-derivatives for PF4.

Concentrations of heparin and heparin-derivatives required for 50% inhibition (IC_{50}) of PF4 binding to biotinylated-heparin (Hep-biotin) immobilized on avidin coated plates (left) or immobilized human recombinant TM-his (right). The IC_{50} values are derived from data presented in figures 4A and 4D. In separate experiments, the apparent affinities of PF4 for Hep-biotin ($K_{d,\,app}$ = 1.0 ± 0.02 nM), rabbit lung TM ($K_{d,\,app}$ = 12.7 ± 5.0 nM) and human recombinant TM-his ($K_{d,\,app}$ = 11.4 ± 1.2 nM) were determined and are in agreement with previously reported values (3,4).

Supplemental References

1. Yan, S. C., Razzano, P., Chao, Y. B., Walls, J. D., Berg, D. T., McClure, D. B., and Grinnell, B. W. (1990) *Biotechnology* **8,** 655-661

2. Mosnier, L. O., Von dem Borne, P. A. K., Meijers, J. C. M., and Bouma, B. N. (1998) *Thromb. Haemost.* **80,** 829-835

3. Dudek, A. Z., Pennell, C. A., Decker, T. D., Young, T. A., Key, N. S., and Slungaard, A. (1997) *J. Biol. Chem.* **272,** 31785-31792

4. Slungaard, A. and Key, N. S. (1994) *J. Biol. Chem.* **269,** 25549-25556