SUPPLEMENTAL MATERIALS

Cardiac myosin promotes thrombin generation and coagulation in vitro and in vivo

Jevgenia Zilberman-Rudenko[1,2]*, Hiroshi Deguchi,[1]* Meenal Shukla,[1] Yoshimasa Oyama,[3] Jennifer N. Orje[1], Zihan Guo[1], Tine Wyseure[1], Laurent O. Mosnier[1], Owen J.T. McCarty[2,4], Zaverio M. Ruggeri[1], Tobias Eckle,[3] John H. Griffin[1,5]

[1]Department of Molecular Medicine, The Scripps Research Institute, La Jolla, CA; [2]Department of Biomedical Engineering, School of Medicine, Oregon Health and Science University, Portland, OR; [3] Department of Anesthesiology, University of Colorado School of Medicine, Aurora, CO; [4]Department of Hematology-Oncology, School of Medicine, Oregon Health and Science University, Portland, OR; [5]Division of Hematology, Department of Medicine, University of California, San Diego, CA.

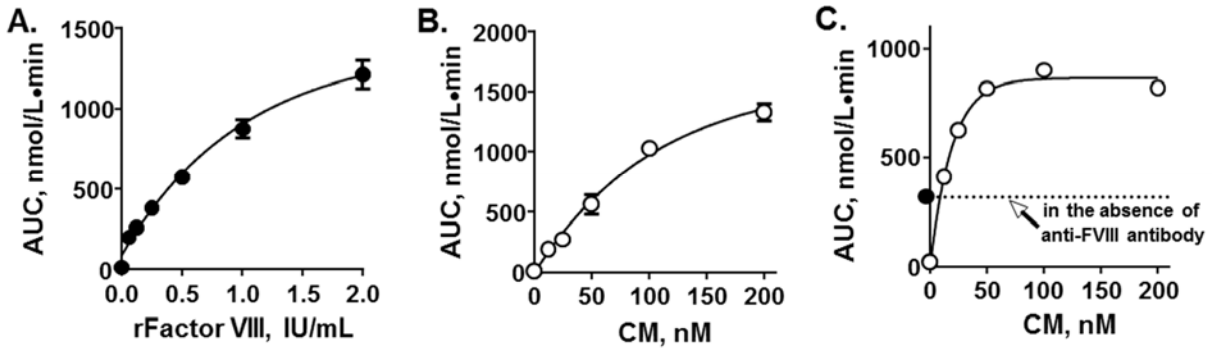
*Equal-contributors

Methods

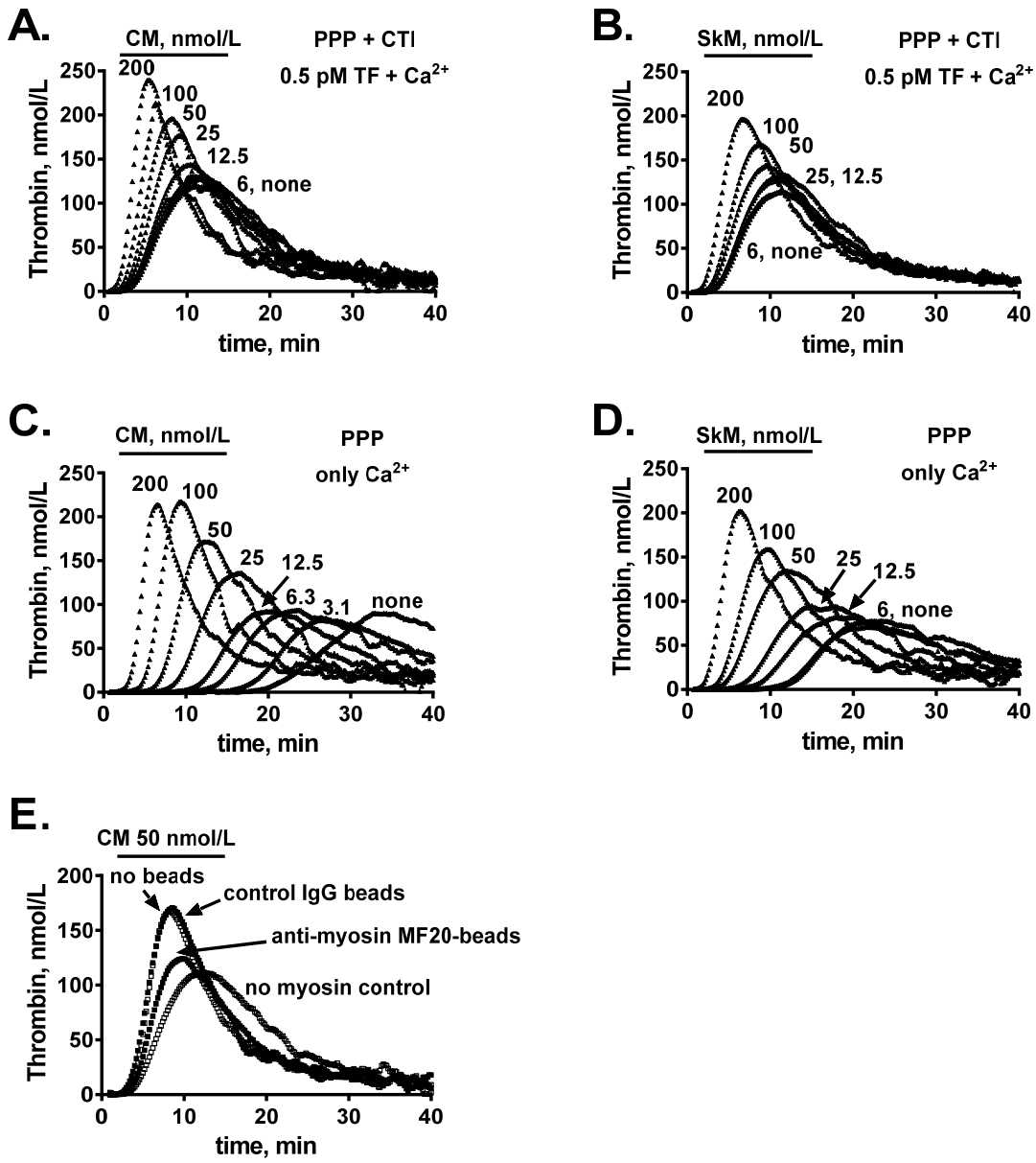
TAFI activation

TAFI activation as presented in Supplemental Figure S7 was determined as described previously.[1, 2] Purified human plasma TAFI was mixed with myosin or dialysis buffer in HBS buffer (20 mmol/L HEPES, 137 mmol/L NaCl, pH 7.4 containing 3 mmol/L KCl) and activated with thrombin (20 nmol/L) or thrombin (20 nmol/L) and thrombomodulin (5 nmol/L) in the presence of calcium chloride (5 mmol/L) in a volume of 60 µl for 10 min at room temperature. Thrombin was inactivated by the addition of PPACK (20 µl, 150 µm). In parallel studies, TAFI was incubated with plasmin (0.5 µmol/L) or HBS buffer containing 3 mmol/L KCl in the presence of calcium and either myosin or dialysis buffer. After 15 or 30 min, plasmin was inactivated with aprotinin (20 µl, 32.5 µg/mL). As controls, TAFI free reactions with either myosin or dialysis buffer were set up and quenched with PPACK after 5 and 10 min of incubation. The substrate for activated TAFI, hippuryl-Arg (20 µl, 20 mmol/L), was added was added to 80 µL of the test reaction mixture and, after thorough mixing, substrate conversion was allowed for either 5 or 10 min. The substrate reaction was stopped by adding 1 N HCl (20 µl) and neutralized with the same volume of 1 N NaOH. After the addition of 25 µl of 1 M disodium phosphate (pH 7.4), 60 µl of 4% (w/v) cyanuric chloride dissolved in 1,4-dioxan was added and color was allowed to develop, during which the samples were extensively vortexed. The mixture was centrifuged (2 min, 14 000 rpm) and the absorbance at 405 nm of the supernatant was measured.

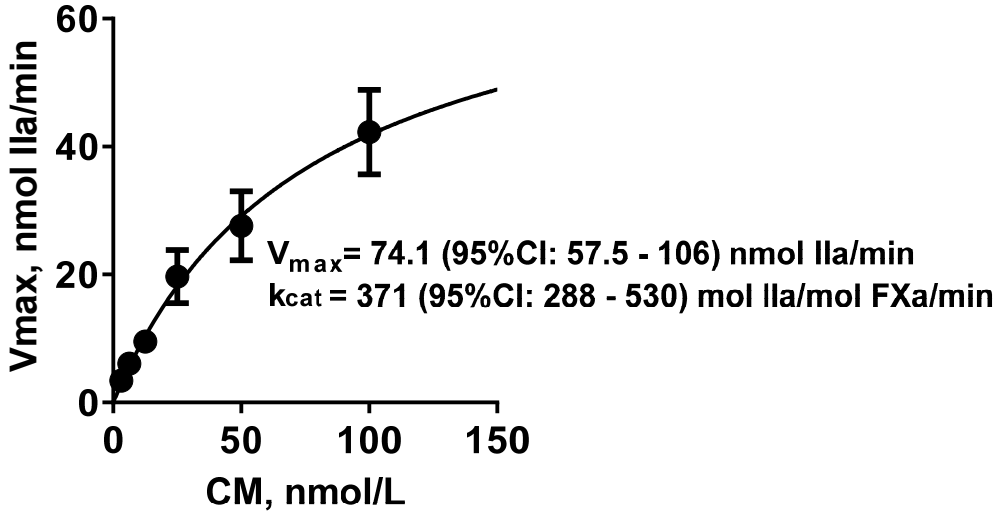
SUPPLEMENTAL FIGURES



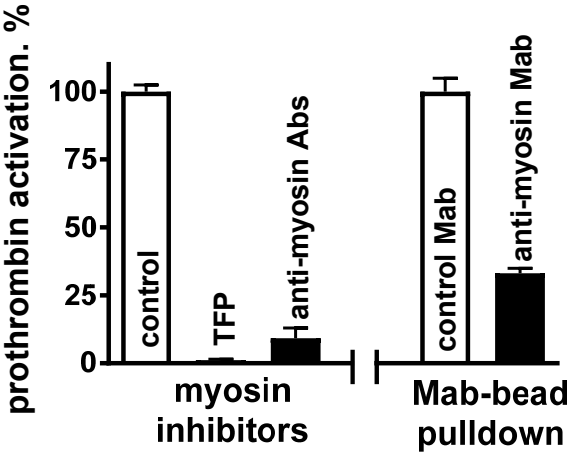
Supplemental Figure I. CM enhances TF-induced thrombin generation in human hemophilia plasma and mouse hemophilia plasma. (A) The effects of recombinant (r) factor VIII (Kogenate) or **(B)** of CM on thrombin generation induced by TF (0.4 pM, final) was evaluated in human hemophilia A plasma (50% final concentration) that contained 4 μM PC/PS (80%:20%) vesicles (final concentration). Thrombin generation was monitored as area under the curve of thrombin generation (AUC). **(C)** The effects of CM (open circle) on thrombin generation induced by TF (0.4 pM, final) was evaluated in C57BL/6J mouse plasma that contained 4 μM PC:PS (80%:20%) in the presence of 5 μg/mL anti-factor VIII antibody (GMA-8015; 5 μg/mL final) (acquired murine hemophilia A model). This anti-factor VIII antibody totally inhibited thrombin generation (>98%) in the absence of added CM. The dotted line and the filled circle point indicate thrombin generation in the absence of anti-FVIII antibody or of added CM, respectively. Thrombin generation was followed continuously for 40 min. The first derivative of fluorescence versus time was used to produce thrombin generation curves with the correction for substrate consumption and inner filter effect. Each value represents the mean [SD] of at least triplicate determinations.



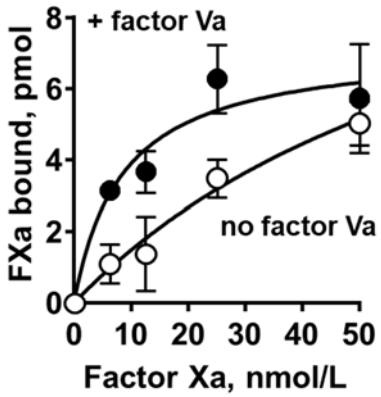
Supplemental Figure II. CM promotes thrombin generation in pooled human plasma. (**A-D**) Pooled normal human platelet poor plasma (PPP) (George King Bio Medical Inc., Overland Park, KS) was incubated with various indicated concentrations of CM (**A**) or skeletal muscle myosin (SkM) (**B**) for 10 min at 37ºC. Thrombin generation was initiated by recalcification and addition of TF (0.5 pM final), phospholipid vesicles (4 μM final), and CTI (50 μg/mL final). In separate experiments, thrombin generation was initiated by recalcification with phospholipid vesicles (4 μM final), in the presence of either (**C**) CM or (**D**) SkM. (**E**) The specificity for the CM molecule for the CM preparation's enhancement of prothrombinase activity was tested by depleting CM from reaction mixtures using anti-myosin MF20 antibody-conjugated beads. Assays using absorption with nonimmune IgG-conjugated control beads and using no beads provided controls.



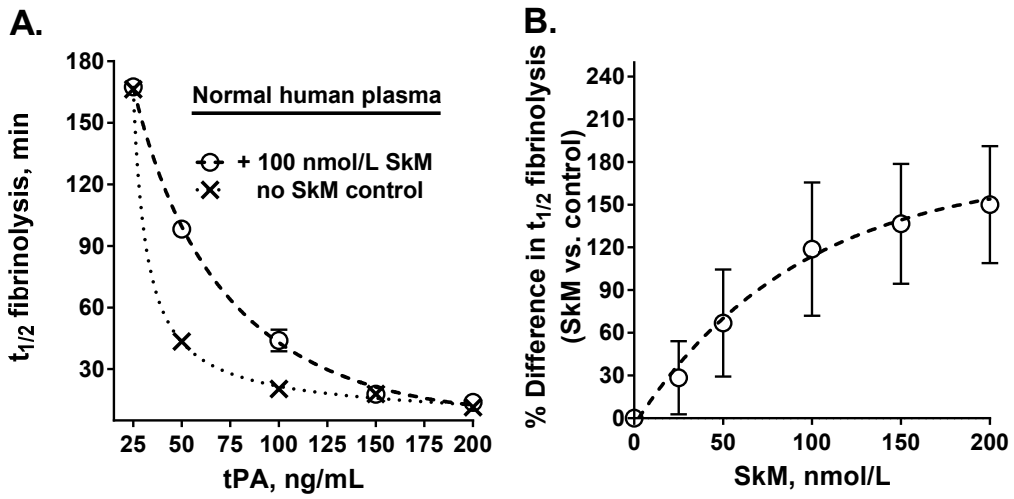
Supplemental Figure III. Vmax and kcat for CM-enhanced prothrombinase activity. Data in the Supplemental Table 1 were plotted and the curve fitting obtained by Prizm software gave the maximal value for V_{max} for CM-enhanced prothrombinase as 74.1 nmol thrombin (IIa) per min. Value for k_{cat} of 371 min^{-1} was derived based on the use of 0.2 nM factor Xa in the protocol. Each value represents the mean [SD] of quadruplicate determination.



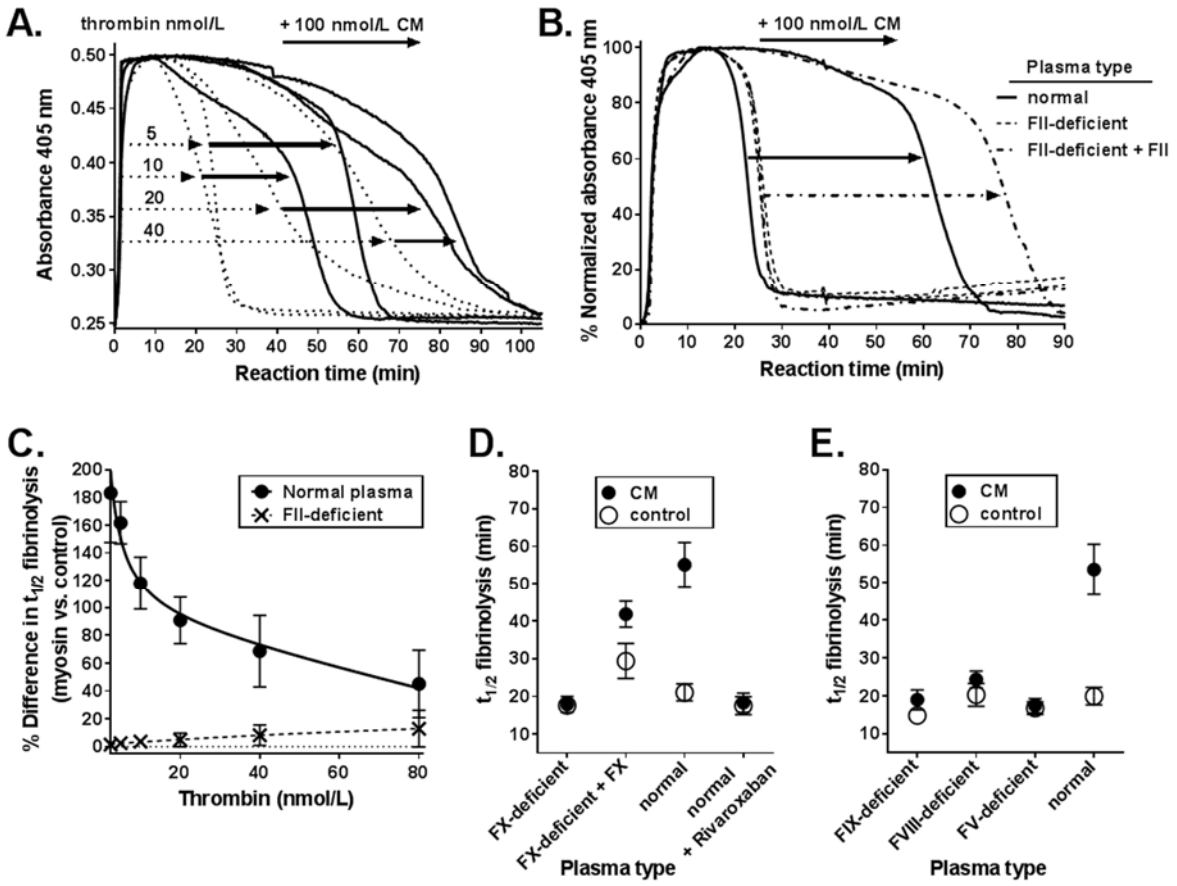
Supplemental Figure IV. CM specifically promotes thrombin generation in purified prothrombinase reaction mixtures. The specificity of the effect of CM's enhancement of purified prothrombinase activity was tested in reaction mixtures containing purified factors Xa, Va, prothrombin, Ca^{2+} and CM plus either an allosteric myosin inhibitor (100 μM trifluoperazine (TFP)) or soluble anti-myosin antibodies (Abs: 0.9 mg/mL total protein). Specificity was further tested by depletion of CM from reaction mixtures using anti-myosin monoclonal antibody (MAb)(MF20)-conjugated beads in the absence of Ca^{2+} prior to recalcification. Nonimmune control Mab-conjugated beads were used as control. Each value represents the mean [SD] of at least triplicate determinations.



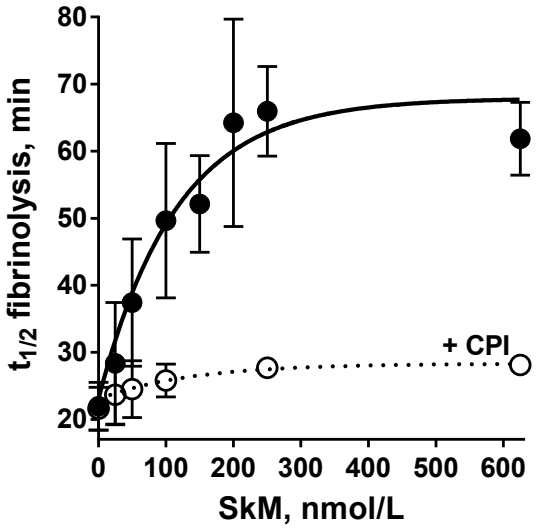
Supplemental Figure V. Factor Xa binds to immobilized SkM. Binding studies of factor Xa to immobilized SkM in the absence or presence of factor Va was measured using factor Xa chromogenic assays to quantify bound factor Xa. Each value represents the mean [SD] of at least triplicate determinations.



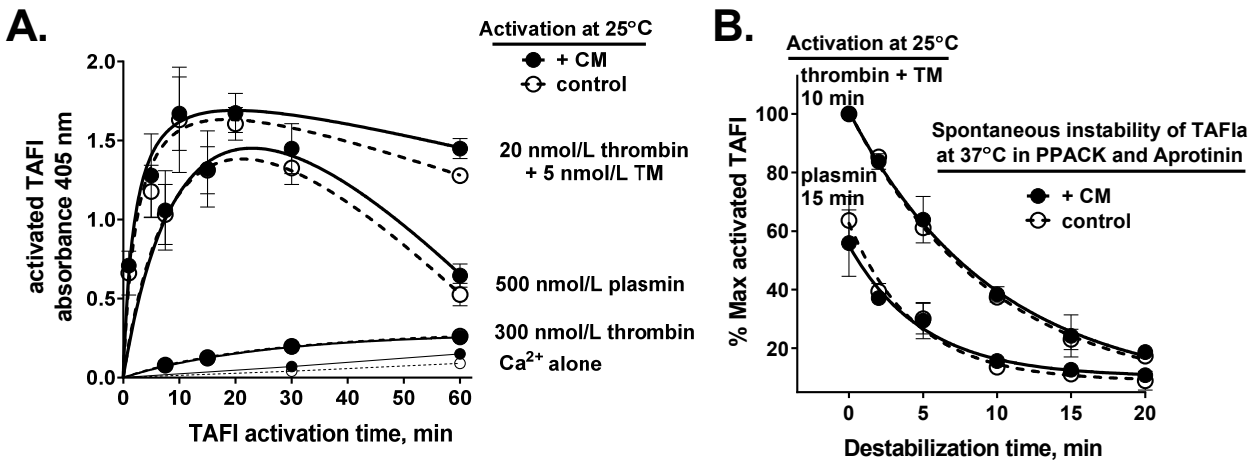
Supplemental Figure VI. SkM attenutes tPA-induced plasma clot lysis. (**A**) The plasma clot lysis time ($t_{1/2}$, min for 50 % clot lysis) was quantified in the presence of SkM (O) at different tPA levels or control buffer (**X**). (**B**) The effect of varying concentration of SkM on the difference in plasma clot lysis time ($t_{1/2}$) for tPA at SkM concentration minus that for the control lacking myosin. Each value represents the mean [SD] of at least triplicate determinations.



Supplemental Figure VII. Prothrombin is required for CM's inhibition of tPA-induced plasma clot lysis. (**A**) Normal human pooled plasma was used for tPA-induced clot lysis studies, as above, when clot formation was initiated with different levels of thrombin, using 100 ng/mL tPA to promote clot lysis. Plasma was pre-incubated with either CM (100 nmol/L) (solid lines) or control buffer (dotted lines). At each thrombin level, myosin prolonged clot lysis time. (**B**) Normal plasma (solid line), prothrombin (FII)-deficient plasma (dashed lines), or FII-deficient plasma reconstituted with purified prothrombin (100% final concentration) (dashed-dotted lines) was used for clot lysis studies using 5 nmol/L thrombin, 100 ng/mL tPA, and either 100 nmol/L CM or control buffer, following procedures as described above. (**C**) The effects of different thrombin concentrations on the difference in plasma clot lysis ($t_{1/2}$) as compared to no myosin controls for normal plasma (solid line) and for prothrombin (FII)-deficient plasma (dashed line with X). (**D**) The effect of CM (●) or control buffer(○) on plasma clot lysis time ($t_{1/2}$) was studied for FX-deficient plasma without or with supplementation by purified factor X (FX), and for normal plasma pre-incubated without or with a factor Xa inhibitor (Rivaroxaban). (**E**) The effect of CM (●) or control buffer (○) on plasma clot lysis time ($t_{1/2}$) was studied as in (**B**) for factor (F) IX-, FVIII-, and FV-deficient plasmas with normal plasma as control. Data for the absence and presence of myosin (open and closed circles, respectively) were overlapping if FX- and FV-deficient plasmas as well as in normal human plasma were pretreated with Rivaroxaban (data not shown). Each value represents the mean [SD] of at least triplicate determinations.



Supplemental Figure VIII. Inhibition of tPA-induced plasma clot lysis by SkM requires thrombin activated fibrinoysis inhibitor (TAFI) activity. Normal human pooled plasma was pre-incubated with either carboxypeptidase inhibitor (CPI) (10 µg/mL final) (O) that inhibits TAFI or control buffer (●), and plasma clot lysis times ($t_{1/2}$) were quantified in the presence of different levels of SkM, 5 nmol/L thrombin and 100 ng/mL tPA as described in Methods. Each value represents the mean [SD] of at least triplicate determinations.



Supplemental Figure IX. CM does not directly affect TAFI activation or the spontaneous instability of activated TAFIa. (**A**) TAFI purified from pooled normal human plasma was incubated at 25°C with thrombin, plasmin or a combination of thrombin and thrombomodulin (TM) in the presence of Ca^{2+} (5 mmol/L final), or control buffer, for varying activation times. Identical reaction mixtures were analyzed in parallel for the presence (●, solid line) or absence (O, dashed line) of CM (100 nmol/L final) as indicated. TAFI activation was quenched with PPACK and aprotinin, and then TAFIa activity was measured as described in Methods. (**B**) To measure the effect of CM on spontaneous instability of active TAFIa, TAFI was activated either by the combination of thrombin and thrombomodulin for 10 min or by plasmin for 15 min at 25°C (as in **A**) and the activation reactions were quenched with PPACK and Aprotinin, and then active TAFIa was incubated at 37°C in the presence of CM (100 nmol/L final)(●, solid line) or control buffer (O, dashed line). The spontaneous loss of TAFIa activity was monitored as described in Methods where the maximum initial TAFIa level was defined as 100%. Each value represents the mean [SD] of at least triplicate determinations.

SUPPLEMENTAL TABLES

Supplemental Table I. Kinetic analysis of cardiac myosin's (CM's) enhancement of prothrombin activation in purified reaction mixtures. The K_m for prothrombin based on data presented in Figure 5A were calculated by Prizm software curve-fitting. Vmax data for varying myosin concentrations were analyzed further in Supplemental Figure 3 to enable calculation of k_{cat} shown in Supplemental Table 2. CI denotes confidence intervals.

CM (nmol/L)	K_m (95% CI) for prothrombin (μmol/L)	Observed V_{max} (95% CI) (nmol IIa/min)
3.2	0.42 (0.30 - 0.53)	3.4 (3.1 - 3.8)
6.3	0.43 (0.35 - 0.52)	6.1 (5.6 - 6.6)
12.5	0.42 (0.33 - 0.51)	9.5 (8.6 - 10.4)
25	0.47 (0.22 - 0.71)	19.7 (15.1 - 24.3)
50	0.41 (0.21 - 0.62)	27.6 (21.8 - 33.4)
100	0.45 (0.28 - 0.63)	42.3 (35.1 - 49.4)

Supplemental Table II. Human Prothrombinase k_{cat} Comparison

Source	Prothrombinase complex surface	PL Conc.	FXa Conc.	FVa Conc.	$\frac{k_{cat}}{min^{-1}}$
This study	**CM**	**-**	0.2 nM	5.0 nM	371
Moyer et al [3]	PC/PS (75/25)	5.0 μM	5.0 nM	5.0 nM	480
Deguchi et al[4]	SkM	-	0.2 nM	5.0 nM	490
Deguchi et al[4]	PC/PS (80/20)	20 μM	0.2 nM	5.0 nM	396
Krishnaswamy et al[5]	PC/PS (75/25)	30 μM	1 nM	4.1 nM	1344
Tracy et al[6]	Platelet ($1x10^8$/mL)		5.0 nM	5.0 nM	1140
Bunce et al[7]	Platelet ($1x10^8$/mL)		0.1 nM	10 nM	560

Vmax for maximal optimum CM concentration was obtained from Supplemental Figure 3 to give value of k_{cat} (V_{max}/[FXa]) for CM. For comparisons in this Table, note that experimental conditions included modest variations, inter alia, in assay buffer contents (Tris vs. HEPES, 2 mM vs. 5 mM, $CaCl_2$, etc.), different temperatures and different PC/PS ratios. See references[3-7] for details about differences in assay contents and conditions. This Table is modified from a previous paper.[4] PL, phospholipid; FXa, factor Xa; FVa, factor Va; PC/PS, phosphatidylcholine/phosphatidylserine.

Suplemental References

1. Mosnier LO, von dem Borne PA, Meijers JC and Bouma BN. Plasma TAFI levels influence the clot lysis time in healthy individuals in the presence of an intact intrinsic pathway of coagulation. *Thromb. Haemost*. 1998;80:829-835.
2. Mosnier LO. Platelet factor 4 inhibits thrombomodulin-dependent activation of thrombin-activatable fibrinolysis inhibitor (TAFI) by thrombin. *J. Biol. Chem*. 2011;286:502-10.

3. Moyer MP, Tracy RP, Tracy PB, van't Veer C, Sparks CE and Mann KG. Plasma lipoproteins support prothrombinase and other procoagulant enzymatic complexes. *Arterioscler. Thromb. Vasc. Biol*. 1998;18:458-465.
4. Deguchi H, Sinha RK, Marchese P, Ruggeri ZM, Zilberman-Rudenko J, McCarty OJ, Cohen MJ and Griffin JH. Prothrombotic skeletal muscle myosin directly enhances prothrombin activation by binding factors Xa and Va. *Blood*. 2016;128:1870-1878.
5. Krishnaswamy S, Church WR, Nesheim ME and Mann KG. Activation of human prothrombin by human prothrombinase. Influence of factor Va on the reaction mechanism. *J. Biol. Chem.* 1987;262:3291-3299.
6. Tracy PB, Eide LL and Mann KG. Human prothrombinase complex assembly and function on isolated peripheral blood cell populations. *J. Biol. Chem.* 1985;260:2119-2124.

7. Bunce MW, Toso R and Camire RM. Zymogen-like factor Xa variants restore thrombin generation and effectively bypass the intrinsic pathway in vitro. *Blood*. 2011;117:290-298.

Major Resources Table

In order to allow validation and replication of experiments, all essential research materials listed in the Methods should be included in the Major Resources Table below. Authors are encouraged to use public repositories for protocols, data, code, and other materials and provide persistent identifiers and/or links to repositories when available. Authors may add or delete rows as needed.

Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex	Persistent ID / URL
mouse	Rodent Breeding Colony at the Scripps Research Institute	C57BL/6J	M/F	
mouse	The Jackson Laboratory	C57BL/6J	M	

Genetically Modified Animals

Not applicable

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot # (preferred but not required)	Persistent ID / URL
muscle myosin	DEVELOPMENTAL STUDIES HYBRIDOMA BANK	MF-20 (hybridoma)	Immobilized on beads		
Myosin heavy chain	Fisher	PA1-28037	0.9 mg/mL total protein		
Factor VIII	Green Mountain Antibodies	GMA-8015	0.25 mg/kg		

DNA/cDNA Clones

Not applicable

Cultured Cells

Not applicable

Data & Code Availability

Not applicable

Other

Not applicable

DOI [to be added]