Supplementary Material

Re: "The roles of selected arginine and lysine residues of TAFI (proCPU) in its activation to TAFIa by the thrombin-thrombomodulin complex" (M8:04745)

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Experimental Procedures

Analysis of Kinetics of Activation of TAFI Variants by the IIa-TM Complex using a single pathway model – The rates of activation of TAFI variants were subjected to analysis by a single pathway model (Eq. S1) rather than the enzyme central, parallel assembly model described previously (14, 23, 32 in the main manuscript)

$$T + TM \xleftrightarrow{K_d} T-TM + TAFI \xleftrightarrow{K_m} T-TM-TAFI \xrightarrow{k_{cat}} TAFIa \quad \text{(Eq. S1)}$$

In this model, the enzyme thrombin (T) interacts with thrombomodulin (TM) to form the T-TM complex with the dissociation constant K_d. The T-TM complex can then interact with TAFI to form the T-TM-TAFI complex with the dissociation constant K_m. The ternary T-TM-TAFI complex then turns over to TAFIa with the first order rate constant, k_{cat}.

The rate of the reaction is given by Equation S2.

$$v = k_{cat} \cdot [T-TM-TAFI] = k_{cat} \cdot \frac{[T] \cdot [TM] \cdot [TAFI]}{K_m \cdot K_d} \quad \text{(Eq. S2)}$$

The total concentration of thrombin, $[T]_0$, is given by Equation S3, which can be rearranged to give Equations S4 and S5.

$$[T]_0 = [T] + [T-TM] + [T-TM-TAFI] \quad \text{(Eq. S3)}$$

$$[T]_0 = [T] + \frac{[T]\cdot[TM]}{K_d} + \frac{[T]\cdot[TM]\cdot[TAFI]}{K_m \cdot K_d} \qquad \text{(Eq. S4)}$$

$$[T]_0 = [T]\left(1 + \frac{[TM]}{K_d} + \frac{[TM]\cdot[TAFI]}{K_m \cdot K_d}\right) \qquad \text{(Eq. S5)}$$

The TAFI activation rate divided by the total concentration of thrombin is obtained by dividing Equation S2 by Equation S5. The result, after algebraic rearrangement, is given by Equation S6.

$$\frac{v}{[T]_0} = \frac{k_{cat}\cdot[TM]\cdot[TAFI]}{K_m\cdot(K_d + [TM]) + [TM]\cdot[TAFI]} \qquad \text{(Eq. S6)}$$

All of the rate data were fit simultaneously by non-linear regression to Equation S6 with the NONLIN module of SYSTAT (SPSS Inc., Chicago, IL). The best fit parameters were k_{cat} and K_m for wild-type and each variant. K_d (the dissociation constant for the IIa-TM interaction) was a best fit parameter also, but its value was assumed to be the same for wild-type TAFI and the variants, because all reactions have this interaction in common. The regression analysis returned best fit values along with their asymptotic standard errors (A.S.E.). The analyses were applied to no less than four and no more than five independent experiments with wild-type TAFI and each of the variants. The mean and standard deviations for k_{cat}, K_m and k_{cat}/K_m values were calculated. Statistical analysis on the variance of catalytic efficiencies (k_{cat}/K_m) of activation of the TAFI derivatives with respect to the wild-type was performed using independent samples T test in SPSS (SPSS Inc., Chicago, IL).

RESULTS

Kinetics of Activation of TAFI and Variants Analysed by the One Pathway Model — The one pathway model was fit to the initial rates of TAFIa formation measured as the concentrations of TAFI and TM were systematically varied. Using the best fit k_{cat} and K_m values for each variant of TAFI ,and the K_d value

for the wild-type TAFI, Equation S6 was used to generate regression lines (Fig. S1). The regression analysis yielded a K_d value of 29.7 ± 7.3 nM. The best fit rate constants for each variant are listed in Table S1. The rate constants are very similar to those determined using the enzyme central, parallel assembly model. The one pathway model, however, resulted in a poorer regression line fit to the rates observed (Fig. S1).

The TAFI-K42/43/44A variant shows a 7.9-fold decrease in its catalytic efficiency (k_{cat}/K_m) in the activation by IIa-TM complex when compared to the wild-type (Table S1). When the residues K133, K211, K212, and R220 of one surface patch of TAFI were substituted by alanine individually, the catalytic efficiencies of TAFI activation by the IIa-TM complex decreased from 2.1-fold to 2.9-fold. When residues K240 and R275 on another surface patch were individually substituted by alanine, the catalytic efficiencies of TAFI-K240A and TAFI-R275A activation decreased 1.8-fold and 2.4-fold, respectively. Statistical analysis shows that the catalytic efficiency of wild-type TAFI is significantly higher than those of all of the mutants, with p-values ranging from less than 0.001 to 0.002 (Table S1).

The binding energies for the interactions between the various forms of TAFI and the IIa-TM complex were calculated using the K_m values as described in the manuscript. The losses of binding energy range from a low of 2.1% (K240A) to a high of 21% (K42/43/44A) (Table S1). The sum of losses of all of the mutants is 59.9%. Thus, no single mutation eliminated the binding of TAFI to the IIa-TM complex, but the nine residues together account for 59.9% of the binding energy. This sum is close to the value of 54.8% as determined by the enzyme central, parallel assembly model.

TABLE S1

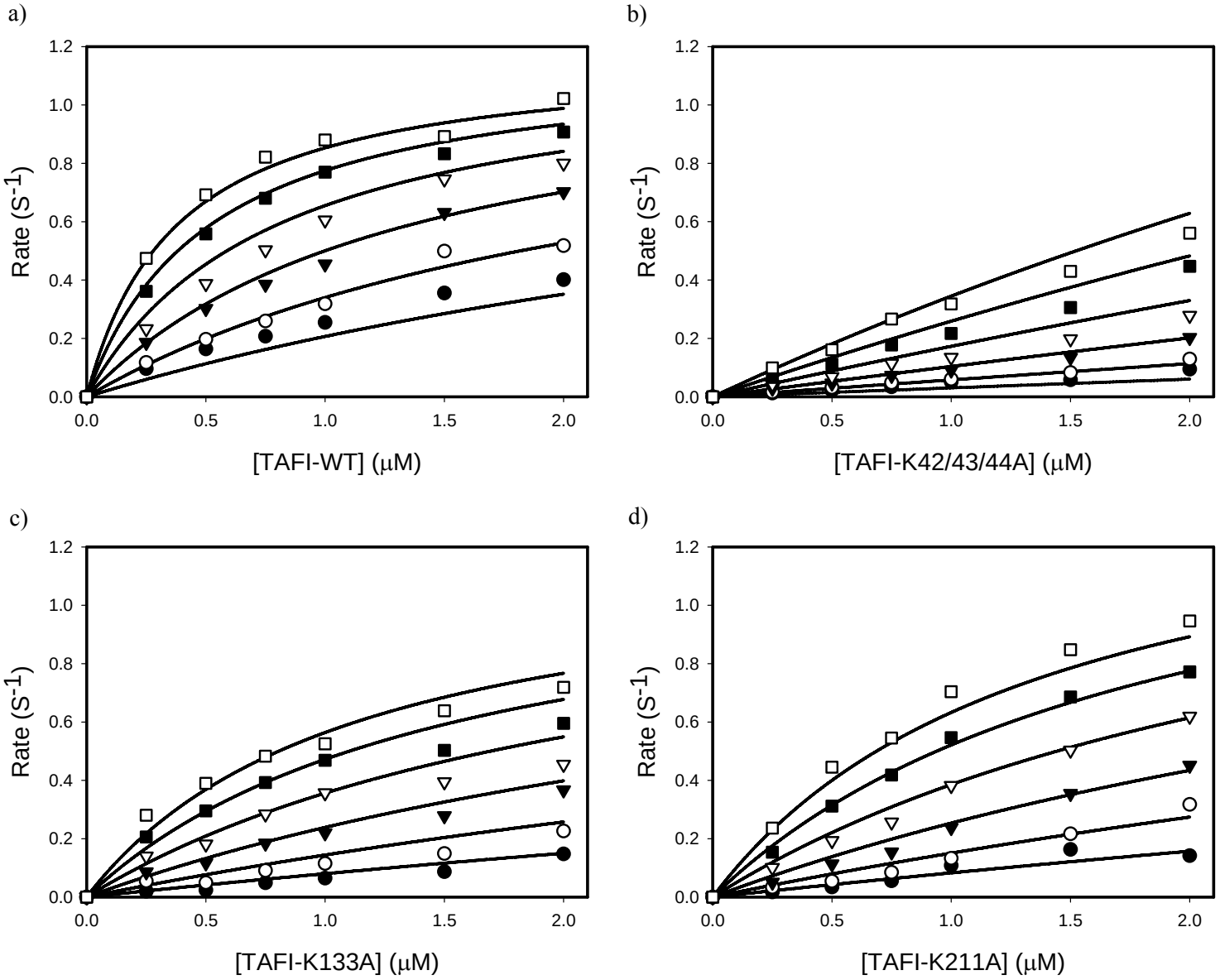
Comparison of kinetic parameters for TAFI wild-type and mutants. The k_{cat}, K_m and K_d values were estimated by non-linear regression of the data presented in Figure S1 to the rate Equation S6. The rate constant values listed here are given as the mean ± S.D. of the estimates of at least four and at most five independent experiments. P values were calculated using independent samples T test for the catalytic efficiencies (k_{cat}/K_m) of each derivative in comparison to the wild-type.

	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	Relative Binding Energy	P Value
WT	1.18 ± 0.24	0.24 ± 0.02	4.90 ± 0.79	1.000	---
K42/43/44A	3.54 ± 1.02	5.87 ± 2.00	0.62 ± 0.12	0.790	<0.001
K133A	1.20 ± 0.17	0.71 ± 0.15	1.72± 0.28	0.929	<0.001
K211A	1.51 ± 0.15	0.88 ± 0.12	1.72 ± 0.07	0.915	<0.001
K212A	0.85 ± 0.51	0.60 ± 0.68	2.11 ± 0.88	0.940	<0.001
R220A	1.86 ± 0.44	0.83 ± 0.23	2.29 ± 0.47	0.919	<0.001
K240A	0.85 ± 0.28	0.33 ± 0.15	2.74 ± 0.43	0.979	0.002
R275A	1.45 ± 0.60	0.71 ± 0.26	2.08 ± 0.65	0.929	<0.001

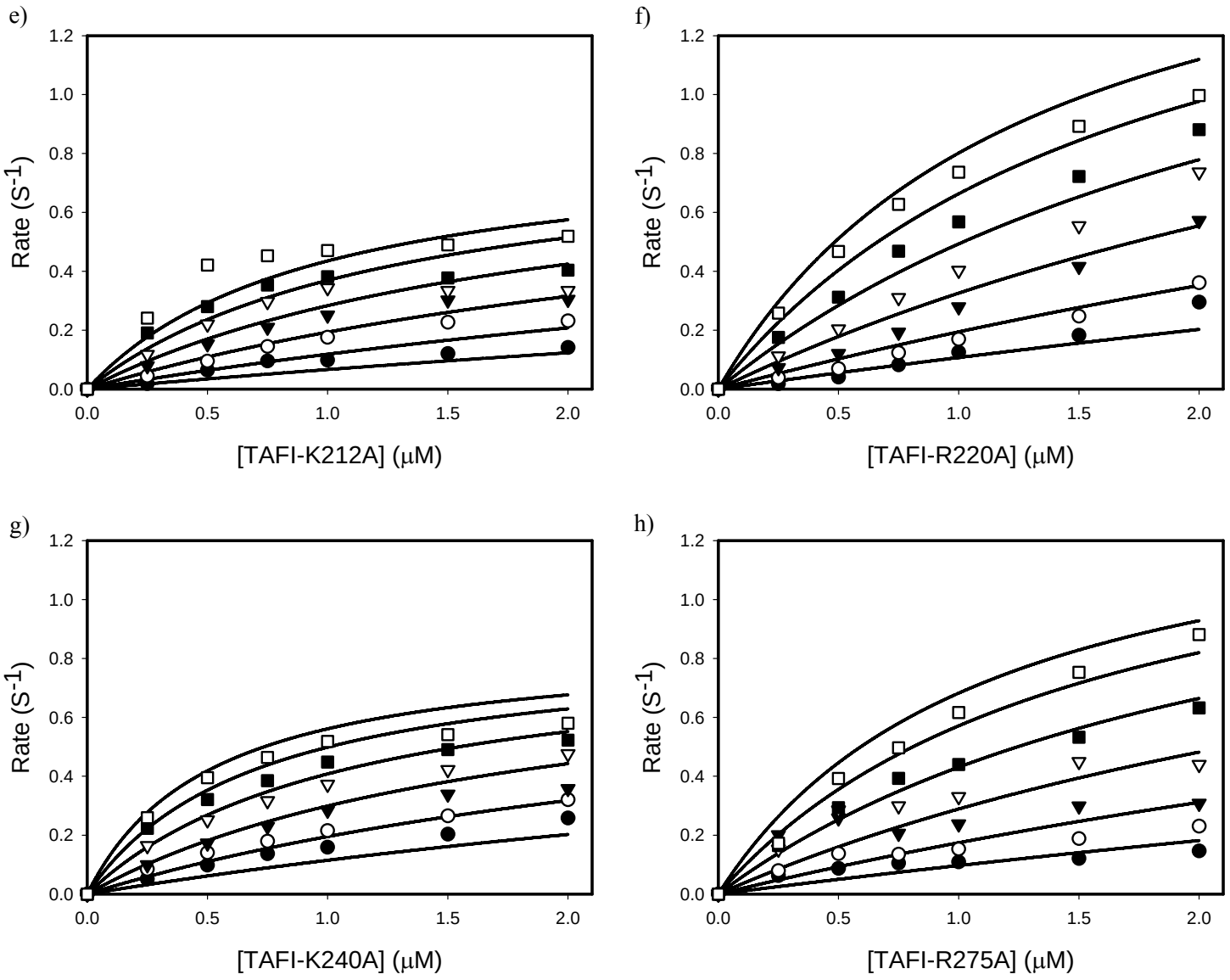
TABLE S2

Primers of TAFI mutants. A pair of primers was designed to construct each of the seven mutants as described in "Experimental Procedures". The triple mutant TAFI-K42/43/44A was constructed by three steps, in which single sequential mutations were made, using the new construct as the template for the next mutation.

Mutation	Primer Sequence
1) TAFI-K133A	Sense: 5'-CACATTGGATCCTCATTTGAG**GCG**TACCCACTCTATG-3'
	Anti-sense: 5'-CATAGAGTGGGTACGCCTCAAATGAGGATCCAATGTG-3'
2) TAFI-K211A	Sense: 5'-GACGGTTATGACTACTCATGG**GCA**AAGAATCGAATGTGG-3'
	Anti-sense: 5'-CCACATTCGATTCTTTGCCCATGAGTAGTCATAACCGTC-3'
3) TAFI-K212A	Sense: 5'-CTACTCATGGAAA**GCG**AATCGAATGTGGAGAAAGAACC-3'
	Anti-sense: 5'-GGTTCTTTCTCCACATTCGATTCGCTTTCCATGAGTAG-3'
4) TAFI-R220A	Sense: 5'-CGAATGTGGAGAAAGAAC**GCT**TCTTTCTATGCGAACAATC-3'
	Anti-sense: 5'-GATTGTTCGCATAGAAAGAAGCGTTCTTTCTCCACATTCG-3'
5) TAFI-K240A	Sense: 5'-GGAACTTTGCTTCC**GCA**CACTGGTGTGAGGAAGG-3'
	Anti-sense: 5'-CCTTCCTCACACCAGTGTGCGGAAGCAAAGTTCC-3'
6) TAFI-R275A	Sense: 5'-GGCAGTGGCTAGTTTCTTG**GCA**AGAAATATCAACCAG-3'
	Anti-sense: 5'-CTGGTTGATATTTCTTGCCAAGAAACTAGCCACTGCC-3'
7) TAFI-K42A	Sense: 5'-CGGTAACAGCTGACCTTATTGTG**GCG**AAAAAACAAGTCC-3'
	Anti-sense: 5'-GGACTTGTTTTTTCGCCACAATAAGGTCAGCTGTTACCG-3'
TAFI-K42/43A	Sense: 5'-GTAACAGCTGACCTTATTGTG**GCGGCA**AAACAAGTCC-3'
	Anti-sense: 5'-GGACTTGTTTTGCCGCCACAATAAGGTCAGCTGTTAC-3'
TAFI-K42/43/44A	Sense: 5'-CTGACCTTATTGTG**GCGGCAGCA**CAAGTCCATTTTTTTG-3'
	Anti-sense: 5'-CAAAAAAATGGACTTGTGCTGCCGCCACAATAAGGTCAG-3'



<u>Fig S1.</u> Kinetics of activation of TAFI variants by the IIa-TM complex. The zymogens TAFI-WT (a), TAFI-K42/43/44A (b), TAFI-K133A (c), TAFI-K211A (d), TAFI-K212A (e), TAFI-R220A (f), TAFI-K240A (g), and TAFI-R275A (h), each at various concentrations, were incubated with IIa (0.5 nM) and TM at concentrations of 1.56 nM (●), 3.13 nM (○), 6.25 nM (▼), 12.5 nM (▽), 25 nM (■), and 50 nM (□). TAFIa formation rates (mol of TAFIa formed / mol of thrombin / second) were calculated and the complete data set was fit globally to Equation S6. The lines are best fit regression lines.



<u>Fig S1.</u> Kinetics of activation of TAFI variants by the IIa-TM complex. The zymogens TAFI-WT (a), TAFI-K42/43/44A (b), TAFI-K133A (c), TAFI-K211A (d), TAFI-K212A (e), TAFI-R220A (f), TAFI-K240A (g), and TAFI-R275A (h), each at various concentrations, were incubated with IIa (0.5 nM) and TM at concentrations of 1.56 nM (●), 3.13 nM (○), 6.25 nM (▼), 12.5 nM (▽), 25 nM (■), and 50 nM (□). TAFIa formation rates (mol of TAFIa formed / mol of thrombin / second) were calculated and the complete data set was fit globally to Equation S6. The lines are best fit regression lines.