Activated thrombin activable fibrinolysis inhibitor (TAFIa), generated upon activation of TAFI, exerts an antifibrinolytic effect. TAFIa is a thermolabile enzyme, inactivated through a conformational change. The objective of the current study was to generate a stable variant of human TAFIa. Using a site-directed as well as a random mutagenesis approach to generate a library of TAFIa mutants, we identified two mutations that increase TAFIa stability, i.e. a Ser305 to Cys and a Thr329 to Ile mutation, respectively. Combining these mutations in TAFI-Ala147-Ile325, the most stable isoform of TAFIa (half-life of 9.4 ± 0.4 min), revealed a TAFIa half-life of 70 ± 3.1 min (i.e. a 11-fold increase versus 6.3 ± 0.3 min for TAFIa-Ala147-Thr325, the most frequently occurring isoform of TAFI in humans) at 37 °C. Moreover, clot lysis (induced by tissue plasminogen activator) experiments in which TAFI-Ala147-Cys305, Ile325-Ile329 was added to TAFI-depleted plasma revealed a 50% clot lysis time of 313 min (i.e. a 3.0-fold increase versus 117 ± 10 min for TAFIa-Ala147-Thr325). The availability of a more stable TAFIa variant will facilitate the search for inhibitors and allow further structural analysis to elucidate the mechanisms of the instability of TAFIa.

The zymogen thrombin activable fibrinolysis inhibitor (TAFI), also known as procarboxypeptidase U (proCPU), can be activated by the thrombin/thrombomodulin complex (T/TM) or by plasmin into its active form (i.e. TAFIa or CPU). TAFIa exerts an antifibrinolytic effect by removing C-terminal lysines from partially degraded fibrin, thereby removing the fibrin cofactor function for tissue plasminogen activator-mediated plasminogen activation (1–4). Even though TAFIa is a powerful antifibrinolytic enzyme, there are no known physiologic inhibitors of TAFIa (5). Instead, TAFIa is regulated by its intrinsic temperature-dependent instability (i.e. half-life between 5 and 15 min at 37 °C) (6–9). Subsequent to this conformational change, TAFIa can be cleaved at Arg302 by thrombin and plasmin and at Lys327 or Arg330 by plasmin (10).

The antifibrinolytic effect of TAFIa is half-maximal at 1 nM, whereas the plasma concentration of total TAFI ranges between 75 and 250 nM (11–13). Therefore, only a small fraction of TAFI in plasma needs to be activated to exert the maximal antifibrinolytic effect (observed at 20 nM TAFIa). Thus, increasing the concentration (above 20 nM TAFIa) does not result in an increased antifibrinolytic effect. Consequently, the maximal antifibrinolytic effect can only be further increased by increasing the TAFIa stability (6).

TAFIa and pancreatic carboxypeptidase B (pCPB) are highly homologous members of the carboxypeptidase A family. In contrast to TAFIa, pCPB is a stable enzyme. Although TAFIa and pCPB share 48% sequence identity, the sequence identity in the 300–330 amino acid region is only 13%.

Two polymorphisms in the TAFI coding region, resulting in two amino acid variations (i.e. Ala147T and Thr325I), have been reported (14, 15). The allele frequencies of the corresponding four single nucleotide polymorphisms are 0.72, 0.28, 0.64, and 0.36 for the Ala147-, Thr147-, Thr325-, and Ile325-encoding alleles, respectively (15). However, Ile at position 325 extends the half-life of TAFIa from 8 to 15 min regardless of the residue at position 147 (6). The increased stability of the Ile325 variants is associated with a 1.6-fold higher antifibrinolytic effect, indicating that the thermal (in)stability is an important determinant for the antifibrinolytic potential of TAFIa. To date, this T325I polymorphism is the only known amino acid variation that increases the TAFIa half-life. Attempts have been undertaken to increase the stability of TAFIa by mutagenesis (7) or by construction of chimeric variants (16). Although some variants exhibit an increased TAFIa stability (16), this effect was never associated with an increased antifibrinolytic effect. The objective of the current study was to create a more stable TAFIa variant that retains its antifibrinolytic potential.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant TAFI isoforms (Ala147-Thr325 (TAFI-AT), Ala147-Ile325 (TAFI-AI)) and monoclonal antibodies raised against TAFI purified from plasma were generated as described before (17). Oligonucleotides for mutagenesis and sequencing were purchased from Sigma-Genosys. dNTP mix was obtained from Roche Applied Science. Mutagenesis was performed using the GeneMorph™ II EZClone domain mutagenesis kit or the QuikChange™ XL site-directed mutagenesis kit (both from Stratagene). Pfu Turbo™ polymerase was obtained from Stratagene. PCR reactions were carried out with the Mastercycler® Gradient from Eppendorf. Plasmid DNA purification was performed with the Nucleobond™ AX500 kit (Machery-Nagel) or the QIAquick™ spin column (Qiagen). DNA was sequenced using the Automated Laser Fluorescent ALF™ apparatus according to the protocol for the ALFExpress™ Autoread Sequencing kit (both from Amersham Biosciences).

Human thrombin, rabbit thrombomodulin, and H-D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) were obtained from Sigma, American Diagnostica, and Biomol Research Labs, respectively. Aprotinin was purchased from Fluka. Hippuryl-L-arginine (Hip-Arg) and potato tuber carboxypeptidase inhibitor (PTCI) were obtained from Sigma.

**RESULTS**

The pCPB TAFIa variant was generated in order to study the stability of this variant without the influence of thrombin. The pCPB-TAFIa variant revealed a half-life of 158 min versus 8 min for the parental TAFIa (Figure 1). The pCPB-TAFIa variant was stable at 37 °C when incubated with thrombin, whereas the parental TAFIa was inactivated (Figure 1). The pCPB-TAFIa variant was also active in clot lysis (Figure 1). The zymogen thrombin activable fibrinolysis inhibitor (TAFI), also known as procarboxypeptidase U (proCPU), can be activated by the thrombin/thrombomodulin complex (T/TM) or by plasmin into its active form (i.e. TAFIa or CPU). TAFIa exerts an antifibrinolytic effect by removing C-terminal lysines from partially degraded fibrin, thereby removing the fibrin cofactor function for tissue plasminogen activator-mediated plasminogen activation (1–4). Even though TAFIa is a powerful antifibrinolytic enzyme, there are no known physiologic inhibitors of TAFIa (5). Instead, TAFIa is regulated by its intrinsic temperature-dependent instability (i.e. half-life between 5 and 15 min at 37 °C) (6–9). Subsequent to this conformational change, TAFIa can be cleaved at Arg302 by thrombin and plasmin and at Lys327 or Arg330 by plasmin (10).

The antifibrinolytic effect of TAFIa is half-maximal at 1 nM, whereas the plasma concentration of total TAFI ranges between 75 and 250 nM (11–13). Therefore, only a small fraction of TAFI in plasma needs to be activated to exert the maximal antifibrinolytic effect (observed at 20 nM TAFIa). Thus, increasing the concentration (above 20 nM TAFIa) does not result in an increased antifibrinolytic effect. Consequently, the maximal antifibrinolytic effect can only be further increased by increasing the TAFIa stability (6).

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from Bachem and Calbiochem, respectively. Lipofectamine™ 2000 and Opti-MEM 1 medium containing glutamax were purchased from Invitrogen. Horseradish peroxidase-conjugated goat anti-mouse antibody was purchased from Bio-Rad.

Citrated plasma of 23 healthy individuals was pooled for clot lysis experiments. TAFI-depleted plasma was obtained by adsorption on monoclonal antibodies T4E3, raised against human plasma-derived TAFI, and covalently coupled to CNBr-activated Sepharose 4B as described (17).

Construction, Expression, and Purification of TAFI Variants—The pcDNA3.1(+) vector (Invitrogen) with the cDNA coding for TAFI (pcDNA-TAFI) was used as the template for mutagenesis (17). The Genemorph® II EZClone domain mutagenesis kit (Stratagene) was used to create a library of random TAFI mutants harboring Thr147 and Ile325 (pcDNA-TAFI-TI) using the primers 5'-CGTACTATAGAACGATTCACTCAC-3' and 5'-CATTCCTAATGACATGCCAAGCTAT-3' (mutagenesis was restricted to the enzyme moiety, amino acid 96–401). Site-directed mutagenesis of TAFI, harboring Ala147 and Thr325 (pcDNA-TAFI-AT), to introduce Cys point mutations was performed using the QuikChange™ XL site-directed mutagenesis kit (Stratagene). Mutants were transiently transfected in HEK293T cells using the Lipofectamine™ 2000 protocol (Invitrogen). Media were harvested 96 h after transfection. Purification of TAFI mutants was performed by immunoaffinity chromatography using monoclonal antibodies T4E3 as described previously (17).

Determination of TAFIa Stability—The TAFIa activity was measured as described earlier (13). TAFI (90 nM) was activated with thrombin (20 nM), thrombobodulin (5 nM), and CaCl2 (5 mM) in 60 μl of Hapesbovine serum albumin (BSA) buffer (25 mM Hapes, 137 mM NaCl, 3.5 mM KCl, 3 mM CaCl2, pH 7.4, containing 0.1% BSA) for 2 min at 37 °C. Thrombin-induced activation was stopped at the indicated time points by the addition of 20 μl of PPACK (30 μM final concentration). Samples were incubated at 37 °C for different time intervals. Twenty μl of the substrate Hip-Arg (5 mM final concentration) was added, and substrate conversion was allowed to proceed for 10 min at 22 °C. Reactions were stopped by the addition of 20 μl of 1 N HCl followed by 20 μl of 1 N NaOH and 25 μl of 1 M sodium phosphate buffer (pH 7.4). Subsequently, 30 μl of 6% cyanuric chloride dissolved in 1,4-dioxane was added, and the mixtures were vortexed and centrifuged. One hundred microliters of supernatant was transferred into a 96-well microtiter plate, and the absorbance at 405 nm was measured using an EL808 Ultra Microplate Reader (Bio-Tek Instruments Inc). The calculated activity was expressed as a fraction of the initial activity (i.e. of the sample that was not incubated at 37 °C after the addition of PPACK). Data were analyzed according to a one-phase exponential decay function. Decay constants (K) were used to calculate the half-lives according to the equation

\[ K = 0.693/\tau. \]

Inhibition of TAFIa Activity by PTCI—TAFI (90 nM) was activated with T/TM for 2 min as described above. Reactions were stopped with PPACK (30 μM) followed by 10 min of incubation at 22 °C with PTCI (0.05–5 μM) before the addition of the chromogenic substrate (Hip-Arg) for determination of TAFIa activity as described above.

TAFI Activation by Thrombin/Thrombomodulin—TAFI (0.06–1 μM) was activated for different time intervals (ranging between 0 and 4 min) in the presence of CaCl2 (5 mM), thrombin (4 nM), and thrombobodulin (1 nM) in Hapes-bovine serum albumin buffer. Under these conditions less than 20% of total TAFI was converted to TAFIa, and rates of activation were linear over the time of measurements. Activation was stopped by the addition of PPACK (30 μM). Samples were incubated for 10 min at 25 °C with Hip-Arg. The amount of TAFIa generated after each activation time was obtained using standard calibration curves (absorbance/min/μM TAFIa). The kcat and Km values for activation of each TAFI variant were determined by nonlinear regression of the data to the Michaelis-Menten equation using Graph Pad Prism 4.01.

TAFIa Hydrolysis of Hippuryl-l-arginine—Characterization of TAFIa hydrolysis of Hip-Arg was determined as described previously with some modifications (5). Briefly, TAFI (1 μM) was incubated for 15 min at 22 °C in the presence of CaCl2 (5 mM), thrombin (20 nM), and thrombobodulin (80 nM) in Hepes buffer. Under these conditions, TAFI is quantitatively converted to TAFIa without conversion to TAFIai (confirmed with SDS-PAGE analysis in parallel with measurement of TAFIa activity). Thrombin activity was stopped by the addition of PPACK (30 μM). Hydrolysis of Hip-Arg (0.1–2 mM) was monitored at 254 nm in a Powerwave X spectrophotometer thermostatted at 25 °C (Bio-Tek Instruments, Inc). Reactions were performed in half area UV 96-well microtiter plates (Elscolab). For each substrate concentration, the rate of hydrolysis was linear over the time of measurement, and initial rates were determined under conditions where less than 10% of the substrate was hydrolyzed. The formation of hippuric acid was calculated from the change in absorbance at 254 nm using the change in extinction coefficient that occurs upon hydrolysis of Hip-Arg (Δε Hip-Arg → hippuric acid = 0.524 μM−1 cm−1) as published previously (5). Kinetic constants for the hydrolysis of Hip-Arg by the different TAFI variants were obtained by fitting the rates of substrate hydrolysis to the Michaelis-Menten equation using Graph Pad Prism 4.01.

Evaluation of Generated TAFI Fragments upon Activation with Thrombin/Thrombomodulin—TAFI (410 nM) was incubated with thrombin (20 nM), thrombobodulin (5 nM), and CaCl2 (5 mM) in 60 μl of Tris buffer (20 mM Tris, 0.1 mM NaCl, pH 7.4) at 37 °C for 0.5, 10, 20, 30, 60, 90, 120, 180, 210, 240, or 280 min. The reactions were stopped at the indicated time points by the addition of SDS (1% final concentration). The samples were heated for 30 s at 100 °C and subjected to SDS-PAGE followed by silver staining.

Clot Lysis Assay—A series of identical clots was formed: TAFI (0.7–90 nM in Hepes buffer, Hepes 20 mM, pH 7.4), tissue plasminogen activator (40 ng/ml in Hepes buffer containing 0.1% Tween 20), thrombobodulin (5 nM in Hepes buffer), thrombin (20 nM in Hepes buffer), and CaCl2 (12.5 mM in Hepes buffer) were added to TAFI-depleted plasma (½ final dilution). The change in turbidity was followed every 5 min at 405 nm (during a 7-h time course) with an EL808 Ultra Microplate Reader (Bio-Tek instruments Inc) to determine the 50% clot lysis time (i.e. the time from the maximum turbidity to the midpoint between maximum and return to base line). Additional experiments were performed in which clot formation and dissolution were monitored in the presence of 4 μM PTCI.

Statistical Analysis—Quantitative data were summarized by the mean and S.D. Statistical analyses were performed with Graph Pad Prism 4.01 using the unpaired t test (Graph Pad Software, Inc., San Diego, USA). p values less than 0.05 were considered statistically significant.

RESULTS

Generation of TAFI Mutants; Targeted Strategy—The rationale for the site-directed mutagenesis was to hamper the conformational change of TAFIa, which consequently leads to the inactivation of TAFIa followed by an increased susceptibility to cleavage at position Arg302. Therefore, connecting the 25- and 11-kDa moieties with disulfide bridges (formed by introducing two new Cys residues in the TAFIa molecule) seems to be a reasonable approach. Thus, 25 single and double Cys point
mutants were constructed in the Glu99-Tyr101, Ala167-Leu189, Ile294-Ile329, and Ala315, and Glu176, Ile396 regions. These mutants were generated in pcDNA-TAFI-AT and transiently transfected in eukaryotic cells. Conditioned media of the different TAFI variants were screened for TAFI protein expression, TAFI activity, and TAFIa stability (data not shown). Only one TAFI variant, carrying a Ser305 to Cys mutation, had an increased half-life at 37 °C (i.e. 5.8-fold increased versus TAFIa-AT). The introduction of all other mutations in the TAFI molecule revealed no significant effect on TAFIa stability except for the Trp271 to Cys mutan, which exhibited a 2.4-fold increased TAFIa half-life versus TAFIa-AT.

**Generation of TAFI Mutants; Random Strategy**—A library of random TAFIa-TI mutants was generated and transiently transfected in eukaryotic cells. Initially, conditioned media of 250 clones were screened for TAFI protein expression, TAFIa activity, and TAFIa stability (data not shown). This resulted in the identification of a Thr329 to Ile mutation, revealing a 1.7-fold increased TAFIa half-life at 37 °C. Subsequent to these findings the Cys305 mutation (cfr targeted strategy) and the Ile329 mutation (cfr random strategy) were introduced in TAFIa either individually or combined, resulting in TAFI-Al-Cys305, TAFI-Al-Ile329, TAFI-Al-Cys305-Ile329, TAFI-AT, TAFI-AI, TAFI-Al-Cys305, TAFI-Al-Ile329, and TAFI-Al-Cys305-Ile329 were purified and characterized.

**Effect of Mutations on TAFIa Stability**—The stability of the different TAFIa variants was evaluated at 37 °C. The half-lives of TAFIa-AT and TAFIa-Al were 6.3 ± 0.3 and 9.4 ± 0.4 min, respectively. TAFI-Al-Ile329, TAFI-Al-Cys305, and TAFI-Al-Cys305-Ile329 revealed half-lives of 15 ± 1.6, 41 ± 3.1, and 70 ± 3.1 min, respectively, corresponding to a 2.4-fold (p < 0.05), 6.4-fold (p < 0.005), and 11-fold (p < 0.005) increase versus TAFIa-AT, respectively (Table 1; Fig. 1).

**Inhibition of TAFIa Activity by PTCI—**TAFIa-AT, TAFIa-AI, TAFIa-Al-Ile329, TAFIa-Al-Cys305, and TAFIa-Al-Cys305-Ile329 were equally well inhibited by PTCI, a specific inhibitor of TAFIa (Table 1). Fifty percent inhibition was observed upon the addition of 78–97 nM PTCI.

**TAFI Activation by Thrombin/Thrombomodulin**—The ability of the T/TM complex to activate the different TAFIa variants (0.06–1 μM) was evaluated. The initial rates of TAFIa formation were assessed by measurement of Hip-Arg hydrolysis. Double-reciprocal plots of activation rate versus substrate (TAFIa) concentration were linear (r² = 0.98), confirming Michaelis-Menten kinetics. The kinetic parameters were determined by non-linear regression of the data to the Michaelis-Menten equation. The combination of a more than 2-fold increased affinity (Km) (Table 2) and an almost 2-fold increased catalytic rate (kcat) lead to 3–6-fold increased catalytic efficiency (kcat/Km) of TAFI activation by T/TM for the TAFI variants bearing Cys305 (compared with TAFIa-AT). TAFIa-Al and TAFIa-Al-Ile329 revealed a 2-fold higher catalytic efficiency for activation by T/TM compared with TAFIa-AT.

**TAFIa Hydrolysis of Hippuryl-L-arginine**—To compare the enzymatic properties of the different TAFIa variants, the ability of the respective enzymes to hydrolyze Hip-Arg (0.1–2 mM) was evaluated. Double reciprocal plots of hydrolysis rate versus substrate (Hip-Arg) concentration were linear (r² = 0.95), confirming Michaelis-Menten kinetics. The kinetic parameters were determined by non-linear regression of the data to the Michaelis-Menten equation. The catalytic efficiency (Kcat/Km) (Table 3) for converting the substrate Hip-Arg by TAFIa-Al-Ile329, TAFIa-Al-Cys305, and TAFIa-Al-Cys305-Ile329 was significantly lower than those for TAFIa-AT and TAFIa-Al (p < 0.05) due to a strongly decreased catalytic rate (kcat).

**Fragmentation Pattern of TAFI Variants upon Activation with Thrombin/Thrombomodulin**—Upon activation of TAFIa-AT, a 36-kDa band corresponding to TAFIa is generated after 5 min at 37 °C (Fig. 2A). Subsequently, after 20 min, the typical degradation products of 25 and 11 kDa are formed. The activation peptide of TAFI has a theoretical increase of the 50% clot lysis time (Δt50) of 1.7-fold increased TAFIa half-life at 37 °C. Subsequent to these findings the Cys305 mutation (cfr targeted strategy) and the Ile329 mutation (cfr random strategy) were introduced in TAFIa either individually or combined, resulting in TAFI-Al-Cys305, TAFI-Al-Ile329, TAFI-Al-Cys305-Ile329, TAFI-AT, TAFI-AI, TAFI-Al-Cys305, TAFI-Al-Ile329, and TAFI-Al-Cys305-Ile329 were purified and characterized.

**Effect of Mutations on TAFIa Stability**—The stability of the different TAFIa variants was evaluated at 37 °C. The half-lives of TAFIa-AT and TAFIa-Al were 6.3 ± 0.3 and 9.4 ± 0.4 min, respectively. TAFI-Al-Ile329, TAFI-Al-Cys305, and TAFI-Al-Cys305-Ile329 revealed half-lives of 15 ± 1.6, 41 ± 3.1, and 70 ± 3.1 min, respectively, corresponding to a 2.4-fold (p < 0.05), 6.4-fold (p < 0.005), and 11-fold (p < 0.005) increase versus TAFIa-AT, respectively (Table 1; Fig. 1).

**Inhibition of TAFIa Activity by PTCI—**TAFIa-AT, TAFIa-AI, TAFIa-Al-Ile329, TAFIa-Al-Cys305, and TAFIa-Al-Cys305-Ile329 were equally well inhibited by PTCI, a specific inhibitor of TAFIa (Table 1). Fifty percent inhibition was observed upon the addition of 78–97 nM PTCI.

**TAFI Activation by Thrombin/Thrombomodulin**—The ability of the T/TM complex to activate the different TAFIa variants (0.06–1 μM) was evaluated. The initial rates of TAFIa formation were assessed by measurement of Hip-Arg hydrolysis. Double-reciprocal plots of activation rate versus substrate (TAFIa) concentration were linear (r² = 0.98), confirming Michaelis-Menten kinetics. The kinetic parameters were determined by non-linear regression of the data to the Michaelis-Menten equation. The combination of a more than 2-fold increased affinity (Km) (Table 2) and an almost 2-fold increased catalytic rate (kcat) lead to 3–6-fold increased catalytic efficiency (kcat/Km) of TAFI activation by T/TM for the TAFI variants bearing Cys305 (compared with TAFIa-AT). TAFIa-Al and TAFIa-Al-Ile329 revealed a 2-fold higher catalytic efficiency for activation by T/TM compared with TAFIa-AT.

**TAFIa Hydrolysis of Hippuryl-L-arginine**—To compare the enzymatic properties of the different TAFIa variants, the ability of the respective enzymes to hydrolyze Hip-Arg (0.1–2 mM) was evaluated. Double reciprocal plots of hydrolysis rate versus substrate (Hip-Arg) concentration were linear (r² = 0.95), confirming Michaelis-Menten kinetics. The kinetic parameters were determined by non-linear regression of the data to the Michaelis-Menten equation. The catalytic efficiency (Kcat/Km) (Table 3) for converting the substrate Hip-Arg by TAFIa-Al-Ile329, TAFIa-Al-Cys305, and TAFIa-Al-Cys305-Ile329 was significantly lower than those for TAFIa-AT and TAFIa-Al (p < 0.05) due to a strongly decreased catalytic rate (kcat).

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TABLE 3
Kinetic parameters for hippuryl-L-arginine hydrolysis by TAFIa variants (mean ± S.D., n = 3)

| TAFI variant | K_m (μM) | k_cat (s⁻¹) | k_cat/K_m (s⁻¹ μM⁻¹) |
|--------------|----------|-------------|-----------------------|
| TAFI-AT      | 2.4 ± 0.3| 23 ± 3      | 10 ± 2                |
| TAFI-Al      | 1.5 ± 0.4| 16 ± 3      | 11 ± 1                |
| TAFI-Al-Ile329| 1.4 ± 1.3| 4.2 ± 2.6   | 3.7 ± 1.1             |
| TAFI-Al-Cys305| 0.7 ± 0.3| 2.4 ± 0.7   | 3.8 ± 1.1             |
| TAFI-Al-Cys305-Ile329| 1.0 ± 0.2| 4.4 ± 1.2  | 4.2 ± 0.5            |

FIGURE 2. Fragmentation pattern of different TAFI variants. SDS-PAGE followed by silver staining of the reaction products formed upon activation of TAFI-AT (A) and TAFI-Al-Ile329-Cys305 (B) with T/TF between 0 and 280 min. TAFI (56 kDa, arrow) is activated to TAFIa (36 kDa, dashed arrow). TAFI is inactivated through a conformational change to TAFI (36 kDa, dashed arrow) followed by proteolytic cleavage resulting in fragments of 25 kDa (dotted arrow) and 11 kDa (arrowhead).

FIGURE 3. Antifibrinolytic potential of different TAFI variants. A, clots were formed by adding 0.7–90 nM TAFI-AT ( ), TAFI-Al-Ile329 ( ), TAFI-Al-Cys305 ( ), TAFI-Al-Ile329-Cys305 ( ), and TAFI-Al-Cys305-Ile329 ( ) to TAFI-deficient plasma in the presence of CaCl₂ (12.5 mM), tissue plasminogen activator (40 μM), thrombin (20 nM), and thrombomodulin (5 μM). The 50% clot lysis time of the different TAFI variants is plotted in function of the TAFI concentration. (mean ± S.D., n = 3). B, the 50% clot lysis time of the different TAFI variants (TAFI-AT ( ), TAFI-Al-Ile329 ( ), TAFI-Al-Cys305 ( ), TAFI-Al-Cys305-Ile329 ( ), and TAFI-Al-Cys305-Ile329 ( ) used at 90 nM final concentration is plotted in the function of the corresponding half-life at 37 °C. (*, at the intercept on the y-axis) represent clot lysis time of TAFI-deficient plasma (mean ± S.D., n = 3).

min, p < 0.005) (Table 1, Fig. 3A). The addition of TAFI-Al (90 nM) to TAFI-depleted plasma results in a 50% clot lysis time of 164 ± 16 min, corresponding to a 1.5-fold increased antifibrinolytic effect versus TAFI-AT (p < 0.005). The addition of TAFI-Al-Ile329, TAFI-Al-Cys305, and TAFI-Al-Cys305-Ile329 mutants (90 nM) to TAFI-depleted plasma results in 50% clot lysis times of 185 ± 50, 276 ± 37, and 313 ± 77 min, respectively, corresponding to a 1.7-fold (p < 0.05), 2.7-fold (p < 0.05), and 3.0-fold (p < 0.05) increased antifibrinolytic effect, respectively, versus TAFI-AT. PTCI used at a concentration of 4 μM fully reverses the effect of all TAFI variants in the clot lysis assay (Table 1).

DISCUSSION

TAFIa (36 kDa) is formed upon activation of TAFI (56 kDa) and exerts an antifibrinolytic effect. Regulated by an intrinsic temperature-dependent instability (i.e. half-life between 5 and 15 min at 37 °C), TAFIa converts into an inactive conformation that is prone to further proteolytic cleavage resulting in the formation of 25- and 11-kDa fragments. The exact nature of the TAFIa instability is still unknown. However, it is shown that TAFIa variants bearing Ile at position 325 have a 2-fold increased half-life at 37 °C that results in a 1.6-fold higher antifibrinolytic effect (6). Boffa et al. (7) demonstrated that replacement of Arg302, Arg320, and Arg330 by Gln decreases TAFIa stability. We hypothesized that this region, which bears only 13% sequence identity with pCPB, a stable protease with an overall sequence identity of 48% with TAFIa, determines TAFIa stability. Marx et al. (16) created a TAFIa-pCPB chimera in which the 293–401-amino acid region of TAFIa was substituted with the corresponding amino acids of pCPB. Although this chimera revealed an increased TAFIa stability, it lost most of its antifibrinolytic potential. Based on the three-dimensional structure of pCPB, Barbosa-Pereira et al. (21) proposed a TAFI model and suggested that Ile182 and Ile183 contribute to the instability of TAFIa. However, recent data of Marx et al. (9) revealed that replacing Ile182 by Arg and Ile183 by Glu (as in pCPB) does not result in an increased TAFIa stability. In contrast, mutating these residues resulted in a lower rate of TAFI activation and, in accordance, in a 6-fold reduced antifibrinolytic potential.

The goal of the current study was to create a TAFIa mutant with an increased half-life at 37 °C and a concomitant increased antifibrinolytic effect. Therefore, we combined two different approaches. First, site-directed mutagenesis in well defined regions yielded a TAFIa mutant (TAFI-AT-Cys305) with a 5.8-fold increased TAFIa half-life upon activation. Second, random mutagenesis identified a 1.7-fold stabilizing effect of Ile at position 329. These two stabilizing mutations, either individually or combined, were introduced in the TAFI isoform, which yields the most stable TAFIa (i.e. TAFI-Al). Combining Cys305, Ile325, and Ile329 revealed a synergistic effect on
both TAFIa half-life (i.e. 11-fold increased at 37 °C) and 50% clot lysis time (3.0-fold increased when 90 nM TAFI is added). From these data we can deduce that the introduction of Cys305, Ile325, and Ile329 slows down the conformational change responsible for the inactivation of TAFIa (7).

The delayed formation of 25- and 11-kDa products of the TAFI-AI-Cys305 and TAFI-Al-Cys305-Ile329 variants support these data (Fig. 2).

The stronger antifibrinolytic effect of all TAFIa variants is in agreement with their increased functional half-life and can be explained at least in part by the threshold phenomenon (22, 23). According to the threshold phenomenon, clot lysis is prevented from proceeding into the propagation phase as long as TAFIa activity remains above a threshold value. As demonstrated before by Schneider et al. (6), the increase in clot lysis time cannot be overcome by adding increasing amounts of TAFI (Fig. 3A). As expected, clot lysis time could only be further increased by increasing the stability of TAFIa (Fig. 3A). Under the current conditions, clot lysis times reached a maximum when TAFI concentrations between 45 and 90 nM were used (for all TAFI variants). This maximum could only be increased by increasing the TAFIa half-life to 40 min. Using these TAFIa variants, a maximum is reached between 270 and 320 min (Table 1; Fig. 3, A and B). Although we cannot exclude that this maximal effect is due to different activation rates by T/TM and/or to different enzyme activities, this maximal effect was also previously observed by Schneider and Nesheim (24) and Walker et al. (18), who reported maximal clot lysis times of 350 and 250 min, respectively.

In contrast to previous published results (6), we found that the catalytic efficiency for activation of TAFI-Al by T/TM is 2-fold higher than for TAFI-AT. Introduction of Cys305 increases the catalytic efficiency for activation of TAFI by T/TM again 2-fold (Table 2, k_{cat}/K_{m}). However, introducing either Cys305 or Ile329 leads to a more than 2-fold lower catalytic efficiency for converting the substrate Hip-Arg compared with the two naturally occurring variants, TAFIa-AT and TAFIa-Al (Table 3, k_{cat}/K_{m}). This is probably due to the influence of the introduced mutations, Cys305 and Ile329, on the active site conformation.

The 3 stabilizing mutations are located in the 300–330-amino acid region (Fig. 4), forming mainly an α-helix (residues 308–325) and two loops (residues 297–307 and 326–331, respectively) connecting the helix to the main part of the molecule. It is worth mentioning that the residues at positions 305 and 329 are in very close proximity to the α-helix (4.96 and 4.21 Å, respectively). Most likely this α-helix, the two loops, and their mutual interactions govern in part the conformational change accompanied with the inactivation process. These data are also in line with previous studies indicating that mutations in this region result in a destabilization (7, 16).

In conclusion, we were able to identify, in addition to the naturally occurring Ile325, two stabilizing mutations i.e. Cys305 and Ile329, that increase synergistically the TAFIa half-life. Subsequently, our study demonstrates that the half-life of TAFIa has an important impact on its antifibrinolytic potential. We observed that by increasing the TAFIa stability, the clot lysis time can be maximally increased to 300 min. Because the development of a TAFI(a) inhibitor is limited by the half-life of TAFIa, the availability of a more stable TAFIa variant will raise new opportunities in the search for TAFIa inhibitors. Moreover, the availability of a stabilized variant will also facilitate further structural analysis to obtain more insights in the underlying mechanisms of the instability of TAFIa.

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