Two Naturally Occurring Variants of TAFI (Thr-325 and Ile-325) Differ Substantially with Respect to Thermal Stability and Antifibrinolytic Activity of the Enzyme*

Received for publication, May 16, 2001, and in revised form, October 10, 2001
Published, JBC Papers in Press, October 29, 2001, DOI 10.1074/jbc.M104444200

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Thrombin-activable fibrinolysis inhibitor (TAFI) is a carboxypeptidase B-like zymogen that is activated to TAFIa by plasmin, thrombin, or the thrombin-thrombomodulin complex. The enzyme TAFIa attenuates clot lysis by removing lysine residues from a fibrin clot. Screening of nine human cDNA libraries indicated a common variation in TAFI at position 325 (Ile-325 or Thr-325). This is in addition to the variation at amino acid position 147 (Ala-147 or Thr-147) characterized previously. Thus, four variants of TAFI having either Ala or Thr at position 147 and either Thr or Ile at position 325 were stably expressed in baby hamster kidney cells and purified to homogeneity. The kinetics of activation of TAFI by thrombin/thrombomodulin were identical for all four variants; however, Ile at position 325 extended the half-life of TAFIa from 8 to 15 min at 37 °C, regardless of the residue at position 147. In clot lysis assays with thrombomodulin and the TAFI variants, or with pre-activated TAFI variants, the Ile-325 variants exhibited an antifibrinolytic effect that was 60% greater than the Thr-325 variants. Similarly, in the absence of thrombomodulin, the Ile-325 variants exhibited an antifibrinolytic effect that was 30–50% greater than the Thr-325 variants. In contrast, the variation at position 147 had little if any effect on the antifibrinolytic potential of TAFIa. The increased antifibrinolytic potential of the Ile-325-containing TAFI variants reflects the fact that these variants have an increased ability to mediate the release of lysine from partially degraded fibrin and suppress plasminogen activation. These findings imply that individuals homozygous for the Ile-325 variant of TAFI would likely have a longer lived and more potent TAFIa enzyme than those homozygous for the Thr-325 variant.

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1 The abbreviations used are: TAFI, thrombin-activable fibrinolysis inhibitor; TAFIa, activated TAFI; AAFK, anisylazoformyllysine; f-Pl-Pro-Arg chromomethyl ketone; tPA, tissue-type plasminogen activator; TAFI-TT, TAFI (Thr-147/Thr-325); TAFI-AT, TAFI (Ala-147/Thr-325); TAFI-TI, TAFI (Thr-147/Ile-325); TAFI-AI (Ala-147/Ile-325); f-plasminogen, fluorescein-labeled plasminogen.

2 Published, JBC Papers in Press, October 29, 2001, DOI 10.1074/jbc.M104444200

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briefly, a cDNA fragment encoding the Ala variant at position 147 (11) was amplified from a human liver first strand cDNA library using primers 3 (5′-CTTGGTCCAGACCTGGAAG-3′) and 4 (5′GTCTGG-GGATAGTATAAGTACGTT-3′) (7). A cDNA fragment encoding the Ile variant at position 325 was amplified from a human liver first strand cDNA library using primer above). The PCR products were individually cloned into pBluescript II SK+ (Stratagene, La Jolla, CA) and subjected to DNA sequence analysis. The clones were identical in sequence to the TAFI cDNA reported by Eaton et al. (21) with the exception of the respective single nucleotide substitutions at the codons corresponding to amino acid positions 147 and 325. The variant fragments were inserted into the multiple cloning site of the TAFI-SK plasmid (7) using the BglII and SpeI and the SpHl and XhoI restriction sites, respectively. The resultant three variant full-length TAFI cDNA sequences were then excised by digestion with XbaI and XhoI. The ends were made blunt using a Klenow fragment of Escherichia coli DNA polymerase I and were inserted into pNUT that had been digested with Smal. The nomenclature for the four TAFI variants is as follows: TAFI-TT, TAFI (Thr-147/Thr-325) (corresponds to the original cDNA isolated by Eaton et al. (21)); TAFI-AT, TAFI (Ile-147/Thr-325); TAFI-TI, TAFI (Thr-147/Ile-325); TAFI-AI, TAFI (Ile-147/Ile-325). Baby hamster kidney cells were transfected with the respective expression plasmids, using calcium-phosphate precipitation (22). Stably expressing lines were selected by culturing cells in the presence of the recombinant TAFI. For routine activation of TAFI to TAFIa, TAFI (1 μg) was incubated at 24 °C for 4 h in the presence of 0.2M glycine, pH 3, into 1-ml aliquots of 1 M Tris-HCl, pH 8. Protein-containing fractions were pooled and concentrated 10-fold in the Ultrafree-4 centrifugal filter device at 4 °C. Concentrated TAFI was diluted 10-fold with HBs/Tween 0.01% and re-concentrated as before. This process was repeated 5 times before the TAFI was removed and passed through a 0.22-μm filter. Purified TAFI was quantified by measuring protein-containing fractions using a Spectramax Plus plate reader (Molecular Devices, Sunnyvale, CA). Wavelengths longer than 350 nm were required for higher concentrations (0.2 μM). Absorbance at 280 nm (ε280–284 = 56.1, M, 10 μM) and stored at −20 °C.

Activation of TAFI and Characterization of TAFIa Hydrolysis of AAFK—For routine activation of TAFI to TAFIa, TAFI (1 μg) was incubated with thrombin (25 μg), Solulin (100 μg), and CalC12 (5 μg) in HBs/Tween 0.01% at 24 °C for 10 min. Where appropriate, the thrombin was quenched with PPAck (1 μg) before the mixture was placed on ice.

AAFK was dissolved in HBs/Tween 0.01%, protected from light, and stored at 4 °C until use. The concentration of AAFK was quantified by absorbance at 349 nm (ε = 18,400 M−1 cm−1) (16). To determine the kinetics of TAFIa hydrolysis of AAFK, TAFIa was formed as described, diluted, and placed on ice. TAFIa (25 ng) was then incubated with AAFK at various concentrations (0–4000 μM) at 24 °C in a microtiter plate that had been precooled in HBs/Tween 0.1% and thoroughly rinsed with deionized distilled water. The absorbance of the mixtures at 350, 375, and 400 nm were monitored over time using a Spectramax Plus plate reader (Molecular Devices, Sunnyvale, CA). Wavelengths longer than 350 nm were required for higher concentrations of AAFK to compensate for absorbances that exceed the measurable absorbance range of the instrument. Initial rates were determined using the first 20% of the total potential change in absorbance that would have occurred with complete substrate hydrolysis and were converted to moles of AAFK hydrolysed/mol of TAFIa (ε−1). The kcat and Km values for each variant were determined by nonlinear regression of the data to the Michaelis-Menten equation using SigmaPlot 4 (SPSS Inc., Chicago, IL).
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200 μl. The reactions were monitored at 350 nm, and the initial rate of hydrolysis was determined from the initial slope of the absorbance versus time relationship. To quantify the TAFIa formed, standard curves with TAFIa and TAFI for each variant were measured with AAFK (120 μM) and PPAck (1 μM). The TAFI zymogen shows modest activity toward the AAFK substrate compared using TAFIa). Thus, the following analysis was used: \( K_r \) is the slope determined from the rate of hydrolysis of AAFK versus the concentration of TAFI, and \( K_f \) is the slope determined from the rate of hydrolysis of AAFK versus the concentration of TAFIa. The initial rate of hydrolysis \( (r = \frac{d[A]}{dt}) \) is shown in Equation 1,

\[ r = K_r \cdot [TAFI] + K_f \cdot [TAFIa] \]  

(Eq. 1)

Because the total concentration of TAFI, [TAFI] = [TAFI] + [TAFIa], this relation can be re-arranged as shown in Equation 2,

\[ [TAFIa] = (r - K_r \cdot [TAFI])/(K_f - K_r) \]  

(Eq. 2)

Calculated [TAFIa] was corrected for dilution and converted to \( r/\text{TAFI formed per s per thrombin (s}^{-1}} \). The complete data set was fit globally to the model (Equation 2) described previously (5), where \( T \) is the concentration of Solulin, and \( K_r \) represents the interaction between thrombin and thrombomodulin as shown in Equation 3,

\[ v = (k_m \cdot [TAFIa] \cdot [TM])/(K_m \cdot [TAFIa] + K_m + K_f \cdot [TM]) \]  

(Eq. 3)

The calculated \( k_m, K_m, \) and \( k_f \) values are reported with the S.E. of the regression calculated by the regression algorithm (Nonlin module of SYSTAT 9, SPSS Inc., Chicago, IL).

TAFI-deficient Plasma—To make TAFI-deficient plasma, 150 ml of fresh-frozen, citrated human plasma was thawed and passed over a 1-ml monoclonal antibody 16-Sepharose 4B column at room temperature. The plasma was passed over the column 3 times, and between each pass the column was washed copiously with HBS/Tween 0.01%; bound TAFI was eluted with 0.2 M glycine, pH 3, and the column was re-equilibrated with HBS/Tween 0.01%. To be certain the plasma was TAFI-deficient, a clot lysis assay (see below) was performed in the absence of TAFI and either in the absence or presence of Solulin (10 nM). There was no difference between the clot lysis times, and the plasma bound TAFI was eluted with 0.2M glycine, pH 3, and the column was stored as a single band at 4°C. The supernatants were neutralized with potassium hydroxide, and the insoluble potassium perchlorate was removed by placing the samples on ice for 10 min followed by centrifugation. The concentration of free lysine was determined enzymatically using methods described by Wang et al. (6). Briefly, 100 μl of deproteinated clot supernatant was incubated with 80 μl of 25 μM NADH (Roche Molecular Biochemicals) and 2.5 mM α-ketoglutaric acid (Sigma) in 0.05 M HEPES, 150 mM NaCl, pH 7.0. The reaction was initiated by the addition of 0.1 units of saccharopine dehydrogenase (Sigma). Oxidation of NADH was monitored using a Spectramax Gemini XS fluorescence plate reader with excitation and emission wavelengths set to 340 and 450 nm, respectively, with a 435 nm cut-off filter in the emission beam. The concentration of free lysine in clot supernatants was determined by comparing decreases in NADH fluorescence after 4 h with those obtained in experiments using known concentrations of l-lysine (Sigma).

RESULTS

Identification of a Novel Amino Acid Sequence Variant of TAFI—DNA sequence analysis of reverse transcriptase-PCR products obtained from a number of different human liver cDNA libraries revealed a single nucleotide difference from the cDNA sequence published by Eaton et al. (21). This was a C to T mutation at position 1057 of the TAFI cDNA (numbering is per Boffa et al. (24)) and would result in the conversion of a Thr codon (ACU) to an Ile codon (AUU) at amino acid position 325. Because the mutation also resulted in the destruction of a SpeI restriction site (ACTAGT to ATTAGT), we amplified a fragment of the TAFI cDNA from liver cDNA libraries corresponding to nine individuals and subjected the PCR products to SpeI digestion followed by agarose gel electrophoresis to determine the genotype of these individuals with respect to the mutation. In five cases, the 425-bp PCR product was completely digested with SpeI resulting in 298- and 127-bp fragments. In four cases, however, ~50% of the PCR product was digested under identical conditions; a representative agarose gel containing digestion products for seven individuals is shown in Fig. 1. These findings indicate that five of the individuals are homozygous for the wild-type (Thr-325) allele, whereas four individuals were heterozygotes. In this small sample, no individuals were identified that are homozygous for the mutant (Ile-325) allele. However, Brouwers et al. (15) have recently determined the frequency of this allele in a much larger population of 152 blood donors and found that 17 individuals were homozygous for the Ile-325 allele, whereas 76 individuals were heterozygous.

Isolation of the Recombinant TAFI Variants—If we consider the Ile/Thr polymorphism at position 325 and the previously described Thr/Ala polymorphism at position 147, there are four possible variants of TAFI. Therefore, these variants were constructed, stably expressed in baby hamster kidney cells with fresh-frozen, citrated human plasma was thawed and passed over a Spectramax Gemini XS fluorescence plate reader with excitation and emission wavelengths set to 490 and 535 nm, respectively, with a 530 nm cut-off filter in the emission path. Fluorescein-plasminogen with fluorescein-labeled cysteine in place of the active site was used to test the ability of these variants to suppress plasminogen activation. Plasminogen activation was initiated and monitored as described above, in the presence of 0.5 nM activated TAFI-TT or TAFI-TI, and the samples were thermostatted to 25 °C. At intervals, clots were solubilized, and reactions were quenched with acetic acid (0.1 M final). Solutions were deproteinated with 0.2 M perchloric acid (final concentration) followed by centrifugation at 16,000 × g for 5 min. The supernatants were neutralized with potassium hydroxide, and the insoluble potassium perchlorate was removed by placing the samples on ice for 10 min followed by centrifugation. The concentration of free lysine was determined enzymatically using methods described by Wang et al. (6).

where \([Pg]_1\) and \([Pg]_2\) are the concentrations of plasminogen at time 1 \((t_1)\) and time 2 \((t_2)\), respectively.

\[ k = \ln([Pg]_1/[Pg]_2)(t_2 - t_1) \]  

(Eq. 4)

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Table I. Kinetic parameters for the hydrolysis of AAFK by TAFIa variants

| TAFI variant | k_{cat} (s^{-1}) | K_{m} (µM) | k_{cat}/K_{m} (s^{-1} µM^{-1}) |
|--------------|-----------------|-------------|-------------------------------|
| TAFI-TT      | 29.5 ± 1.1      | 979 ± 91    | 0.030 ± 0.003                 |
| TAFI-AT      | 26.8 ± 1.0      | 1260 ± 97   | 0.021 ± 0.002                 |
| TAFI-TI      | 27.4 ± 1.5      | 1111 ± 143  | 0.025 ± 0.003                 |
| TAFI-AI      | 27.0 ± 1.1      | 1098 ± 112  | 0.025 ± 0.003                 |

Fig. 1. Demonstration of the existence of the wild-type Thr-325 allele, whereas four were heterozygotes. An additional two individuals (not shown on this gel) were also found to be homozygous for the wild-type Thr-325 allele.

15 min compared with 8 min for TAFIa-TT and TAFIa-AT. The difference in thermal stability conferred by this variation in TAFI may also resolve some discrepancy in the half-lives for plasma-derived TAFI which have been reported as both 8–9 (7, 8) and 15 min (9) at 37 °C.

Activation of the TAFI Variants by Thrombin and Solulin—To determine whether the variation at position 325 could affect the kinetics of activation, each variant was activated by thrombin and Solulin. The k_{cat}, K_{m}, and K_{d} parameters were determined by fitting the data to the model for TAFI activation described previously (5).

To determine whether the difference in the stability of TAFI variants to attenuate clot lysis. Because we have identified a natural variant that has an increased thermal stability, it seemed likely that there would be a corresponding increase in the ability of the more stable variants to attenuate clot lysis.

We also found that AAFK can be hydrolyzed by the TAFI variants to attenuate clot lysis. The k_{cat}/K_{m} values ranged between 0.9 and 1.3 µM^{-1} s^{-1}.

Attenuation of Clot Lysis by the TAFI Variants—Our previous work (7, 8) indicated that differences in thermal stability correspond to differences in the ability of TAFI to attenuate clot lysis. Because we have identified a natural variant that has an increased thermal stability, it seemed likely that there would be a corresponding increase in the ability of the more stable variants to attenuate clot lysis.

To determine whether the difference in the stability of TAFI variants corresponds to a change in clot lysis time, we measured the attenuation of clot lysis by each variant in vitro. Fig. 4 shows the prolongation of clot lysis time by each variant at various concentrations in the presence of 10 nM Solulin. Under these conditions, the attenuation of clot lysis is half-maximal at a concentration of about 2 nM TAFI aI zymogen. TAFI-TI and TAFI-AI can increase the maximum attenuation of clot lysis by about 60% over that obtained with TAFI-TT and TAFI-AT. The difference in lysis times persist even at high concentrations of the variants; thus, the lower antifibrinolytic activity of the Thr-325 variants compared with the Ile-325 variants cannot be overcome by using high concentrations of the Thr-325 variants. As predicted by the half-life data, the variation at position 147 has little effect on the ability of the TAFI variants to attenuate clot lysis.

Fig. 5 presents a similar experiment using TAFIa instead of the zymogen for each variant. TAFIa has a similar effect on the attenuation of clot lysis in that a half-maximal effect is observed at about 2 nM TAFIa. At higher concentrations, TAFI-TI and TAFI-AI can prolong clot lysis 60% more than TAFI-TT and TAFI-AT. Each variant reaches a maximum attenuation of clot lysis with respect to TAFIa variant concentration, and this maximum depends on the residue at position 325. Whereas the tendency to reach a maximum time to clot lysis with TAFIa is similar to the tendency with TAFI activated in situ by thrombin.
bin/thrombomodulin (Fig. 4), the lysis times in the presence of the TAFI variant enzymes are marginally smaller. This probably reflects the lag time to complete TAFI activation in the results of Fig. 4, which is not a factor in this case. Nevertheless, the similarity between the behavior of pre-activated TAFI as compared with TAFI zymogen added in the presence of thrombin/thrombomodulin.

Fig. 6 shows clot lysis experiments performed in the absence of thrombomodulin, using the variant TAFI zymogens and PCPS (20 μM) to enhance endogenous prothrombin activation and thereby partially activate TAFI (albeit to a lesser degree than in the presence of thrombomodulin). TAFI-TI and TAFI-AI had a 30–50% greater antifibrinolytic effect than either TAFI-TT or TAFI-AT. The concentration of TAFI required for half-maximal effect is increased 15-fold (30 nM) for each variant as compared with lysis assays done in the presence of activated TAFI, which is in agreement with similar studies conducted previously (8) using TAFI-TT.

Finally, Fig. 7 shows a series of clot lysis assays performed in the presence of mixtures of TAFI-TT and TAFI-TI. With increasing amounts of the more stable TAFI-TI variant, there was a corresponding increase in the antifibrinolytic potential of

**Table II**

| Half-lives of TAFI activity at indicated temperatures | 37 °C | 33 °C | 30 °C | 25 °C |
|------------------------------------------------------|------|------|------|------|
| TAFI-TT                                              | 8.0 ± 0.2 | 14.5 ± 0.3 | 28.6 ± 0.4 | 77.2 ± 4.7 |
| TAFI-AT                                              | 7.8 ± 0.2 | 15.4 ± 0.3 | 28.3 ± 0.7 | 73.8 ± 1.7 |
| TAFI-TI                                              | 15.0 ± 0.6 | 27.4 ± 1.1 | 54 ± 2 | 147 ± 5 |
| TAFI-AI                                              | 14.7 ± 1.0 | 27.2 ± 0.6 | 54 ± 1.2 | 162 ± 13 |

**Fig. 2.** Thermal stability of the TAFI-TT and TAFI-TI variants. Purified recombinant TAFI-TT (A) and TAFI-TI (B) were activated in the presence of thrombin and Solulin; the thrombin was then quenched with PPAck (1 μM). The TAFI variants were placed in thermostated waterbaths at 37 (open triangles), 33 (closed triangles), 30 (open circles), and 25 °C (closed circles). Timed aliquots were removed, and the remaining TAFI activity was determined using AAFK. The rates are reported as a fraction of the initial activity and were fit by nonlinear regression of the data to the equation for first order decay to determine the half-lives. Half-lives were estimated for each TAFI variant at least twice at 37 and 30 °C, and the variation in the estimated values between trials was always less than 10%.
the mixtures. In this experiment we see that mixtures of TAFI variants with differing stabilities do not show positive or negative synergy, rather, the antifibrinolytic potential increases linearly in proportion to the amount of each TAFI variant present. This property is highlighted by the inset to Fig. 7 that plots the times to 50% clot lysis at 90 nM TAFI with respect to the proportion of each variant present. Therefore, in plasma with a mixture of TAFI mutants, the fraction of each mutant present may be more important in determining the antifibrinolytic potential than the total concentration of TAFI.

Effect of TAFIa Variants on the Kinetics of Plasminogen Activation—Plasmin potentiates plasminogen activation in the presence of fibrin by partially degrading fibrin, thereby exposing new plasminogen- or t-PA-binding sites on fibrin (25). Recently, we demonstrated that high concentrations of TAFIa eliminate plasmin-mediated up-regulation in plasminogen activation (6). To determine whether variation at position 325 of TAFI affects its ability to suppress plasminogen activation, various concentrations of activated TAFI-TT or TAFI-TI were added to reactions containing fixed concentrations of f-plasminogen, t-PA, fibrinogen, and thrombin. Concentrations of f-plasminogen were calculated from decreases in fluorescence, and rate constants for plasminogen activation were determined from these data. To determine the effect of native plasmin on plasminogen activation, experiments were performed in the absence or presence of Glu-plasminogen. Under the conditions outlined under “Experimental Procedures,” plasmin increases the rate constant for plasminogen activation 3.5-fold (from 4 × 10⁻⁵ to 1.4 × 10⁻⁴ s⁻¹), with a half-maximal effect 21 min after the

![Fig. 3. Activation kinetics of TAFI-TT and TAFI-TI by thrombin and Solulin. TAFI-TT (A) or TAFI-TI (B) at various concentrations were incubated with thrombin (0.5 nM), CaCl₂ (5 mM), and Solulin at 1.56 (closed circles), 3.13 (open circles), 6.25 (closed triangles), 12.5 (open triangles), 25 (closed squares), and 50 nM (open squares). Each reaction was incubated at 22 °C for 10 min and then quenched with PPack (1 μM) and AAFK (120 μM). The initial rates of AAFK hydrolysis were measured, and the amount of TAFIa formed per unit time was calculated as described under “Experimental Procedures.” Each activation matrix was repeated at least twice, and the lines represent the result of the nonlinear regression of the data to the rate equation (Equation 2).

| TAFI Variant | k_cat (s⁻¹) | K_d (nM) | K_m (μM) | k_cat/K_m (μM⁻¹ s⁻¹) |
|--------------|-------------|----------|----------|-----------------------|
| TAFI-TT      | 1.06 ± 0.07 | 12.5 ± 1.0 | 0.84 ± 0.09 | 1.3 ± 0.2 |
| TAFI-AT      | 0.74 ± 0.02 | 10.7 ± 0.4 | 0.82 ± 0.04 | 0.9 ± 0.1 |
| TAFI-TI      | 0.85 ± 0.04 | 8.4 ± 0.5  | 0.83 ± 0.07 | 1.0 ± 0.1 |
| TAFI-AI      | 0.74 ± 0.03 | 7.6 ± 0.5  | 0.67 ± 0.06 | 1.1 ± 0.1 |
initiation of plasminogen activation. This increase is consistent with that determined by Wang et al. (6). For comparison purposes, rate constants for plasminogen activation in the presence of increasing concentrations of activated TAFI-TT or TAFI-TI were determined at 21 min after the initiation of plasminogen activation. As can be seen in Fig. 8, each TAFIa variant decreases the rate constant in a concentration-dependent fashion and, at a high concentration, eliminates the increase in rate constant caused by plasmin. However, the variants differ with respect to their potencies of plasminogen activation suppression. Whereas 5.0 nM TAFIa-TT abolishes the increase in rate constant, only 0.5 nM TAFIa-TI is needed for the same effect.

**DISCUSSION**

While screening human cDNA libraries, we found a single nucleotide polymorphism in TAFI cDNA clones that corresponds to a substitution of Ile for Thr at amino acid position 325 of the protein. This is the second variation to be found in human TAFI, with the first being a variation at position 147 (14) that was found to have little effect on its thermal stability or its antifibrinolytic potential. Previously we found that mutations in residues at positions 302, 320, and 330 can have a marked effect on the stability of TAFI, albeit a negative effect. Because the present variation lies in the middle of this region, we were particularly interested in characterizing the effect of the Ile-325 variation with respect to thermal stability and antifibrinolytic potential.

As expected from previous work (14), the variation at position 147 did not affect the behavior of TAFI; however, the
presence of an Ile at position 325 nearly doubles the thermal stability of the enzyme. Again, we have found that altering a residue in the 302–330 region of TAFI can affect thermal stability, and the variation at position 325 could be particularly relevant since it occurs naturally. Most notably, the presence of the Ile at position 325 results in a 30–60% greater antifibrinolytic effect for TAFIa than the Thr at this position.

The increased antifibrinolytic potential of Ile-325-containing TAFIa variants reflects the fact that these variants have a decreased ability to suppress the up-regulation in plasminogen activation that normally occurs during fibrinolysis. In turn, the main difference between the two was that in the absence of Solulin, the half-maximal effect occurred at much higher concentrations of the initial TAFI concentration. Each point represents the mean of three independent experiments and all measured values varied by less than 15% between trials.

Why this portion of TAFIa is so important for stability is not immediately clear; however, this stretch of amino acids differs substantially from that of the homologous carboxypeptidase B of the pancreas (21), which is stable. Previously, we found (8) that the TAFIa inactivation process is highly unfavorable enthalpically but highly favorable entropically. Possibly, the Arg to Gln mutations in TAFI eliminate charge interactions, which decrease the enthalpic component of the active/inactive transition and thereby destabilize TAFIa. In contrast, in the case of the Ile-325 variant of TAFI, changing the hydrophilic Thr residue to a hydrophobic Ile residue may diminish the effect of the favorable entropic change that accompanies the inactivation process, thereby stabilizing TAFIa.

Most likely, when tissue is damaged, physiologically relevant amounts of TAFIa can be generated from the plasma zymogen, either by small amounts (several nanomolar) of thrombin complexed with thrombomodulin or by large amounts (several hundred nanomolar) of thrombin generated after clotting via the intrinsic pathway. We were able to see a change in antifibrinolytic potential under conditions that simulate both proposed activation pathways. The main difference between the two was that in the absence of Solulin, the half-maximal effect occurred at much higher concentrations of the initial zymogen. Nevertheless, there was a significant prolongation in clot lysis time.

Fig. 6. Antifibrinolytic effect of the TAFIa variants in the absence of Solulin with PCPS. Clots formed from TAFI-deficient human plasma diluted 3-fold in HBS/Tween in the presence of thrombin (5 nM), tPA (0.3 nM), PCPS (20 μM), and CaCl₂ (10 mM) were incubated with TAFI-TT (closed circles), TAFI-AT (open circles), TAFI-TI (closed triangles), and TAFI-AI (open triangles) at various concentrations. The turbidity of each clot was monitored at 37 °C, and the times to 50% clot lysis are plotted as a function of the initial TAFI concentration. Each point represents the mean of three independent experiments and all measured values varied by less than 15% between trials.

Fig. 7. Antifibrinolytic effect of TAFIa-TT and TAFIa-TI mixtures in the presence of Solulin. Clots formed from TAFI-deficient human plasma diluted 3-fold in HBS/Tween 0.01% in the presence of thrombin (5 nM), tPA (0.3 nM), PCPS (20 μM), and CaCl₂ (10 mM) were incubated with the following mixtures of TAFI-TT and TAFI-TI: 100% TAFI-TT (closed circles), 75% TAFI-TT and 25% TAFI-TI (open circles), 50% TAFI-TT and 50% TAFI-TI (closed triangles), 25% TAFI-TT and 75% TAFI-TI (open triangles), and 100% TAFI-TI (closed squares). The turbidity of each clot was monitored at 37 °C, and the times to 50% clot lysis are plotted as a function of the initial TAFI concentration. The inset graph shows the times to 50% clot lysis at 90 nM TAFI plotted as a function of fraction of the TAFI mixture consisting of TAFI-TI. Each point represents the mean of two independent experiments, and all measured values varied by less than 10% between trials.
mediated by the more stable variants (TAFI-TI or TAFI-AI) as compared with the less stable variants (TAFI-TT or TAFI-AT). Thus, the more stable variants would be more antifibrinolytic regardless of whether thrombin or the thrombin-thrombomodulin complex is the activator. Under both types of conditions, a difference in the antifibrinolytic potential between the Thr/Ile-325 variants persists throughout a range of TAFI concentrations used.

All of the clot lysis experiments presented here demonstrate that increased stability of TAFI results in the increased ability to maintain a fibrin clot. This increase in clot lysis time cannot be overcome by adding increasing amounts of TAFI. Both zymogen and pre-activated TAFI tend toward saturation, and the lysis time when saturation is reached depends on the thermal stability of the TAFIa variant used. This phenomenon is not restricted to this study. Previously, we found (8) that less stability corresponded to a smaller maximum in the time of clot lysis, while similar to this study, increased stability conferred an increase in the maximum time to clot lysis. Because the kinetics of AAFK hydrolysis were similar for all of the variants, active site differences between variants are probably not significant in mediating the variant-dependent change in the saturation of time to clot lysis. To resolve why all TAFI variants do not reach the same maximum time to clot lysis even at higher concentrations, a thorough understanding of the dynamics of lysine residue exposure during clot lysis by plasmin, and subsequent removal by TAFIa, is required.

Finally, here we have used AAFK as a small substrate to quantify TAFIa activity. A limitation of the furylacryloyl substrate analogues is their meager loss of absorbance upon hydrolysis, typically about 15%. The large loss of absorbance by AAFK (about 95%) can improve the signal to noise ratio when quantifying TAFIa. In addition, the substrate has an absorbance maximum in the visible light range, and even wavelengths as high as 400 nm or greater are adequate for consistent measurement of carboxypeptidase B activity. An unexpected property of this analogue is the activity of the TAFI zymogen, which we found to have one-fiftieth the activity of TAFIa. Among the pancreatic carboxypeptidases that are evolutionarily related to TAFI, activity of the zymogen against small substrates has been observed for procarboxypeptidase A1 and A2 but not for procarboxypeptidase B (26, 27). Based on x-ray crystallographic analysis of the respective zymogen structures, the interactions between the activation peptides and the latent active sites of procarboxypeptidase A1 and A2 result in conformations of the active site residues that are compatible with the execution of this activity. This conclusion is supported by our observation that no TAFIa activity is evident when TAFIa was expressed in a cell line that lacks both procarboxypeptidase A1 and A2 (1029).

FIG. 8. Effect of TAFIa variants on the kinetics of plasminogen activation. Clots were formed with fibrinogen (3 μM) and thrombin (10 nM) in the presence of t-PA (0.3 nM) and f-plasminogen (0.2 μM) and the absence or presence of native Glu-plasminogen (10 nM) (open bars). Plasminogen activation was monitored as the diminution of the extrinsic fluorescence of f-plasminogen, and rate constants for plasminogen activation were determined at 21 min, the time required to reach the half-maximal rate constant in the presence of plasmin. To compare the effect of Thr or Ile at position 325 of TAFIa, experiments were repeated in the presence of various concentrations of TAFIa-TT (closed bars) or TAFIa-TI (gray bars), respectively. Each point represents the mean of two independent experiments, and all measured values varied by less than 10% between trials.

FIG. 9. Free lysine released by TAFIa variants during fibrinolysis. Fibrinogen (3 μM) was mixed with t-PA (0.3 nM), Glu-plasminogen (10 nM), and TAFIa-TT (open circles) or TAFIa-TI (closed circles) (0.5 nM each). Clotting and plasminogen activation were initiated by the addition of thrombin (10 nM). At various times, all reactions were quenched with acetic acid (0.1 M) and deproteinized with perchloric acid (0.2 M). Concentrations of free lysine in supernatants were determined using saccharopine dehydrogenase-mediated oxidation of NADH. Each point represents the mean of two independent experiments, and all measured lysine concentrations varied by not more than 0.3 μM from the mean.
with carboxypeptidase activity against substrates small enough to enter the partially occluded active site cleft (26, 27). The activation peptide of procarboxypeptidase B, by contrast, completely blocks access to the critical S1 and S1’ subsites of the latent active site (27). The activation peptide of TAFI may therefore primarily serve to occlude macromolecular substrates from the latent active site while still allowing a latent active site of reduced catalytic efficiency that is accessible to some small substrates.

In this study we have found that human TAFI can have either a Thr or an Ile residue at position 325, and this variation can significantly alter the behavior of the active enzyme. Ile confers stability to TAFIa, and this increased stability has an effect on the antifibrinolytic potential. Because this variability affects antifibrinolytic potential in vitro, the Thr/Ile variation at position 325 may account for a proportion of the variable risk for the thrombogenic disorders observed in human populations.

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Two Naturally Occurring Variants of TAFI (Thr-325 and Ile-325) Differ Substantially with Respect to Thermal Stability and Antifibrinolytic Activity of the Enzyme

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J. Biol. Chem. 2002, 277:1021-1030.
doi: 10.1074/jbc.M104444200 originally published online October 29, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M104444200

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