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ORIGINAL ARTICLE

Role of isoleucine residues 182 and 183 in thrombin-activatable fibrinolysis inhibitor

P. F. MARX,* S. R. HAVIK,* B. N. BOUMA†‡ and J. C. M. MEIJERS*

**Department of Vascular Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; †Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA, USA; and ‡Thrombosis and Haemostasis Laboratory, Department of Haematology, University Medical Center, Utrecht, The Netherlands*

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Summary. Thrombin-activatable fibrinolysis inhibitor (TAFI) is a procarboxypeptidase that, once activated, can attenuate fibrinolysis. The active form, TAFIa, is a labile enzyme, with a half-life of a few minutes at 37 °C. Understanding the molecular mechanisms of TAFIa inactivation will allow the development of compounds that modulate TAFIa activity. Based on their three-dimensional model of TAFI, Barbosa Pereira *et al.* [*J Mol Biol* (2002), vol. 321, pp. 537–547] suggested that Ile182 and Ile183 were involved in the instability of TAFIa. However, these carboxypeptidases are, unlike TAFIa, stable proteases. Therefore, we constructed, expressed and characterized a TAFI mutant in which Ile182 and Ile183 were changed into the residues found in pancreas carboxypeptidase B at corresponding positions, Arg and Glu. The active form of the mutant, TAFIa-I182R-I183E, had a similar half-life as wild-type TAFIa, showing that Ile182 and Ile183 were not involved in the regulation of TAFIa stability. Remarkably, however, TAFI-I182R-I183E was activated at a lower rate by thrombin–thrombomodulin (mutant: 45 ± 2 U L^{-1} s^{-1} and wild type: 103 ± 3 U L^{-1} s^{-1}), thrombin (mutant: 1 ± 0.1 U L^{-1} s^{-1} and wild type 3 ± 0.2 U L^{-1} s^{-1}) and plasmin (mutant: 0.8 ± 0.04 U L^{-1} s^{-1} and wild type: 5.0 ± 0.2 U L^{-1} s^{-1}) compared with wild-type TAFI. Accordingly, it had a sixfold reduced antifibrinolytic potential. In conclusion, analysis of TAFI-I182R-I183E showed that I182 and I183 are not involved in TAFIa inactivation by conformational instability but that these residues may be involved in the activation of TAFI and stabilization of the fibrin clot.

Keywords: carboxypeptidase, coagulation, fibrinolysis, mutagenesis, TAFI.

Correspondence: Pauline F. Marx, Department of Vascular Medicine, G1-114, Academic Medical Center, P.O. Box 22660, 1100 DD Amsterdam, The Netherlands
Tel.: -31-20-5665842; fax: -31-20-6916972; e-mail: p.f.marx@amc.uva.nl

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Introduction

Thrombin-activatable fibrinolysis inhibitor (TAFI, procarboxypeptidase U, procarboxypeptidase R, or procarboxypeptidase B, EC3.4.17.20) is a pro-enzyme of a carboxypeptidase B (CPB), the active form of TAFI (TAFIa), that is specific for C-terminal lysine and arginine residues. TAFIa is assumed to attenuate the fibrinolytic system by removing the C-terminal lysine residues of partially degraded fibrin. These lysine residues act as cofactors for the stimulation of tissue-type plasminogen activator-mediated plasmin formation. Furthermore, they are involved in the protection of plasmin from inactivation by antiplasmin [1]. Hence, the removal of the C-terminal lysine slows down breakdown of the fibrin clot [2–4].

Thrombin-activatable fibrinolysis inhibitor consists of two structural domains: the activation peptide and the catalytic domain. The activation peptide shields the catalytic site and needs to be cleaved at Arg92 by enzymes such as thrombin and plasmin resulting in the release of the activation peptide from the catalytic domain [5], giving substrate access to the catalytic center. TAFIa is a labile enzyme that has a half-life of only a few minutes at 37 °C [5–7], but at lower temperatures the enzyme is more stable [7]. The current hypothesis for TAFIa inactivation is that it inactivates as a result of instability of its conformation [8,9]. The changes occurring within the molecule that result in conversion of the active form TAFIa into the inactivated active form TAFIai are, however, unclear. Once in its inactive conformation, it becomes more susceptible to proteolytic degradation by thrombin [8,9].

Thrombin-activatable fibrinolysis inhibitor is classified as a member of the metallocarboxypeptidase subfamily. Barbosa Pereira [10] proposed a three-dimensional model of TAFI based on the known crystal structures of other pro-CPAs and pro-CPBs, which share approximately 40% identity with TAFI. Although residues of carboxypeptidase A and B that were implicated in catalysis (Glu363, Arg217; residue numbering of TAFI), substrate binding (Arg235, Tyr341, Asn234) and zinc binding (His159, Glu162, His288) are conserved in the 309-amino acid catalytic domain of TAFIa [5], CPBs are stable proteases whereas TAFIa is spontaneously inactivated.

Analysis of the TAFI model showed the presence of two hydrophobic residues, Ile182 and Ile183, that are unique for TAFI and that show high positive values of pseudo-potential energy [10]. Ile182 and Ile183 are located in a surface-located loop that precedes an exposed α-helix that has little intrinsic tendency to form such a helix [10]. Based on their analyses, Barbosa Pereira *et al.* [10] hypothesized that destabilization of the region containing and surrounding these two residues could lead to TAFIa inactivation.

Understanding the molecular mechanisms of TAFIa inactivation will allow the development of compounds that modulate TAFIa activity. These compounds may show if inhibition of TAFIa is a useful therapeutic approach to treat cardiovascular diseases. In this study, we constructed and characterized a TAFI mutant in which Ile182 and Ile183 were mutated into the corresponding residues of pancreas pro-CPB, an Arg and a Glu residue, respectively.

Methods

Materials

Rabbit-lung thrombomodulin was purchased from American Diagnostica (Greenwich, CT, USA), hippuryl-arginine and H-D-Phe-Pro-Arg-chloromethylketone (PPACK) from Bachem (Bubendorf, Switzerland), and potato carboxypeptidase inhibitor from Calbiochem (La Jolla, CA, USA). Thrombin was a generous gift from Dr W. Kisiel (University of New Mexico, Albuquerque, NM) and plasmin from Dr A. Reijerkerk (University Medical Center, Utrecht, the Netherlands). Tissue-type plasminogen activator (t-PA) was obtained from Chromogenix (Mölndal, Sweden). Plasminogen and human fibrinogen free of von Willebrand factor and fibronectin were purchased from Enzyme Research Laboratories (South Bend, UK).

Cloning, expression and purification of recombinant TAFI and TAFI-I182R-I183E

The cloning of TAFI was described previously [8]. The I182R and I183E mutations were generated according to the QuikChange™ site-directed mutagenesis protocol (Stratagene, La Jolla, CA, USA), using the forward primer 5′-GGC CAT CTA ACT CAA TTC TAT GGG AGA GAA GGG CAA TAT ACC AAT CTC C-3′ and the reverse primer 5′-GGA GAT TGG TAT ATT GCC CTT CTC TCC CAT AAA GTT GAG TTA TAT GGC C-3′ (mutated bases underlined). The construct was confirmed by sequencing, and found to contain the Thr147 and the Thr325 isoform of TAFI as our wild-type TAFI. The constructs were used to make stably transfected baby hamster kidney cells as described [11]. Stably expressing clones were cultured to confluency in cell factories, the medium was changed to UltraCHO (BioWhittaker, Walkersville, MD, USA), and medium was harvested twice a week. The mutant was named TAFI-I182R-I183E. TAFI was purified from the media as described [8]. Concentrations were determined using a BCA kit (Pierce, Rockford, IL, USA) using bovine serum albumin (BSA) as the standard.

TAFI activation and TAFIa activity assay

The assay was basically performed as described [12]. To follow the activation of TAFI and inactivation of TAFIa in time, TAFI and TAFI-I182R-I183E (12.5 nM, all concentrations are final concentrations) were added to a premix of thrombin (16 nM) and thrombomodulin (32 nM), or plasmin (50, 100 or 200 nM), both in the presence of $CaCl_2$ (5 mM), in 20 mM HEPES, pH 8.0/0.1% BSA. The mixtures were incubated at 37 °C. To measure the rate of activation, 12.5 nM TAFI or TAFI-I182R-I183E, 4 nM thrombin, 8 nM thrombomodulin, and 5 mM $CaCl_2$ were used and the mixtures were incubated at 4 °C. Moreover, the rate of activation by thrombin (500 nM) or plasmin (200 nM) was determined at 37 °C. At several time points, 20 μL of samples were withdrawn and added to 50 μL of a mixture of PPACK (30 μM) to stop thrombin activity or aprotinin to stop plasmin activity, and hippuryl-arginine (21.4 mM) as the substrate. To determine the rate of TAFIa inactivation, TAFI or TAFI-I182R-I183E were incubated with thrombin/thrombomodulin for 1 min or with plasmin for 5 min (with plasmin more TAFI-I182R-I183E (87.5 nM) than TAFI (12.5 nM) was used to reach more or less similar levels of activity after 5 min) after which thrombin or plasmin activity was stopped by addition of PPACK or aprotinin, respectively, and the mixture remained at 37 °C to allow spontaneous TAFIa inactivation. At different time points, samples were withdrawn and added to substrate as described above. After 30 min of incubation with the substrate at 37 °C, substrate conversion was stopped by adding 50 μL of 1 M HCl. Then, *o*-methyl hippuric acid (22.5 μM) was added as an internal standard, and both *o*-methyl hippuric acid and hippuric acid were extracted using a solid-phase extraction unit according to the manufacturer's recommendation (Waters Oasis, Wexford, Ireland) and samples were analyzed by high-performance liquid chromatography (HPLC) as described [12]. The TAFIa activity is expressed in units per liter (U L^{-1}). One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 μmol of substrate per minute at 37 °C under the conditions described [12].

Fibrinogen and plasminogen binding

Fibrinogen or plasminogen (3 μg mL^{-1}) in carbonate buffer (50 mM Na_2CO_3/$NaHCO_3$, pH 9.6) was immobilized overnight at 4 °C to a 96-well plate (Maxisorp; Nunc International, Roskilde, Denmark). The wells were blocked by incubation with blocking buffer, Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.4) containing 1.5% BSA, at 37 °C for 2 h. Samples of TAFI and TAFI-I182R-I183E (0–1.7 μM for fibrinogen binding and 0–333 nM for plasminogen binding, in duplicate) were allowed to bind at room temperature for 1.5 h. Binding was detected using a mouse anti-TAFI monoclonal

antibody (Nik-9H10, 3 μg mL^{-1}) [13] and a swine antimouse peroxidase-conjugated secondary antibody (diluted 1:1000 in blocking buffer).

All binding data were analyzed by fitting them to the one site-binding equation $Y = B_{max} \cdot X/(K_D + X)$, where B_{max} is the maximal binding and K_D the concentration of ligand required to reach the half-maximal binding.

Affinities of anti-TAFI antibodies for TAFI and TAFI-I182R-I183E

Thrombin-activatable fibrinolysis inhibitor and TAFI-I182R-I183E (3 μg mL^{-1}, 50 μL per well) were immobilized overnight at 4 °C to a 96-well plate (Maxisorp, Nunc International). The wells were blocked by incubation with blocking buffer, TBS containing 1.5% BSA (100 μL per well), at 37 °C for 2 h. Samples of a polyclonal sheep anti-TAFI antibody or a mouse anti-TAFI monoclonal antibody (Nik-9H10) (0–67 nM, 50 μL per well) were allowed to bind to the proteins at room temperature for 1.5 h. After washing with TBST (TBS containing 0.1% Tween 20) the plates were incubated with a peroxidase-conjugated rabbit antisheep antibody or a swine antimouse peroxidase-conjugated antibody, diluted 1:1000 in blocking buffer (50 μL per well) at room temperature for 1 h. After washing, the peroxidase activity was detected with *o*-phenylenediamine (0.4 mg mL^{-1}) in phosphate citrate buffer (100 mM Na_2HPO_4, 50 mM citric acid, pH 5.0) with 0.035% H_2O_2 (50 μL per well). Color was allowed to develop, after which the reaction was stopped by the addition of 1 M sulphuric acid to each well (25 μL per well) and absorbance was measured at 490 nm.

Clot-lysis assay

The clot-lysis assay was performed essentially as described previously [13]. Briefly, 70 μL of citrated human TAFI-depleted plasma [14] was mixed with various concentrations of TAFI or TAFI-I182R-I183E. The volumes were adjusted to 100 μL with HBS (25 mM HEPES, 137 mM NaCl, 3.5 mM KCl, pH 7.4) containing 0.1% BSA. A mixture (50 μL) of thrombin (final concentration: 20 nM), $CaCl_2$ (final concentration: 10 mM) and tissue-type plasminogen activator (final concentration: 30 U mL^{-1}), was added to the plasma. The volume was adjusted to 150 μL with HBS/0.1% BSA. The experiments were performed in the presence or absence of 5 μL carboxypeptidase inhibitor (final concentration: 7.8 μM). A sample of 100 μL was then transferred to a microtiter plate and turbidity was measured in time at 37 °C at 405 nm in a Thermomax microplate reader (Molecular Devices Corporation, Menlo Park, CA, USA). The clot-lysis time was defined as the time at which half-maximal lysis occurred.

Results

In order to identify the amino acids involved in TAFIa inactivation, we synthesized a mutant form of TAFI, TAFI-I182R-I183E, in which the residues Ile182 and Ile183 were mutated into an Arg and a Glu residue, respectively. Wild-type TAFI and TAFI-I182R-I183E were stably expressed in baby hamster kidney cells and the recombinant proteins were purified from culture media. TAFI-I182R-I183E was characterized and compared with recombinant human TAFI.

SDS-PAGE and Western blotting of TAFI and TAFI-I182R-I183E

To characterize TAFI-I182R-I183E, the migration pattern of the mutant on an sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was compared with that of wild-type recombinant TAFI. Purified TAFI-I182R-I183E migrated, similar to TAFI, just above the molecular weight marker of 55 kDa on an SDS-PAGE gel (Fig. 1A, lane 1 and 2). Both wild-type TAFI and the mutant were recognized by a monoclonal antibody raised against human plasma TAFI, Nik-9H10 (Fig. 1B) and a polyclonal sheep anti-TAFI antibody (Fig. 1C). We also determined the affinities of these two anti-TAFI antibodies. The K_Ds of the monoclonal antibody Nik-9H10 were similar for TAFI and TAFI-I182R-I183E (1.5 and 0.6 nM, respectively) (Fig. 2A) and so were the K_Ds of the sheep anti-TAFI antibody (1 and 0.9 nM, respectively) (Fig. 2B).

Cleavage pattern of TAFI by thrombin–thrombomodulin

During incubation with the thrombin–thrombomodulin complex TAFI (approximately 55 kDa) is cleaved at Arg92, releasing the activation peptide (approximately 19 kDa, not visible on Coomassie-stained SDS-PAGE gels [15]) from the catalytic domain (36 kDa). The catalytic domain is degraded further by cleavage at Arg302, resulting in fragments of 25 and 11 kDa. SDS-PAGE analysis of samples of the activation mixtures after 30 min incubation revealed that the cleavage pattern of TAFI-I182R-I183E was identical to the cleavage pattern of TAFI (Fig. 1A, lanes 3 and 4).

Binding of TAFI and TAFI-I182R-I183E to fibrinogen and plasminogen

To further characterize the TAFI mutant, the binding to fibrinogen and plasminogen was studied. Fibrinogen or plasminogen was immobilized on a 96-well plate, several dilutions of TAFI or TAFI-I182R-I183E were applied to the plates and bound TAFI was detected using a TAFI specific antibody. TAFI and TAFI-I182R-I183E bound to the same extent to plasminogen (K_D of 19 and 11 nM, respectively) (Fig. 3A) and fibrinogen (K_D of 445 and 665 nM) as wild type TAFI (Fig. 3B).

Activity profile of TAFI during incubation with thrombin–thrombomodulin

Thrombin-activatable fibrinolysis inhibitor and TAFI-I182R-I183E were incubated with the thrombin–thrombomodulin

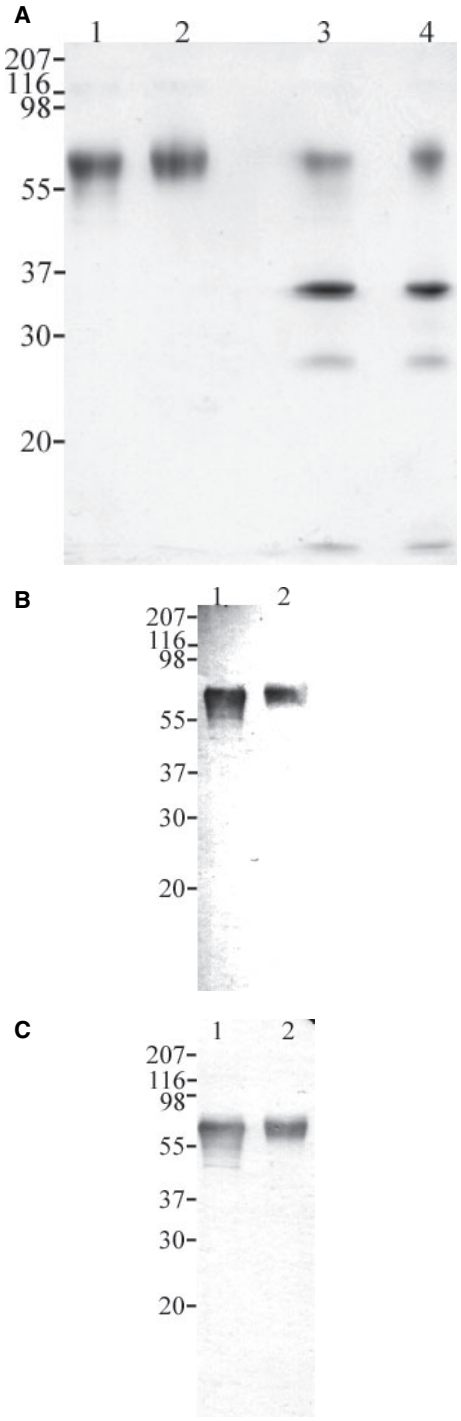

Fig. 1. SDS-PAGE and Western blot analysis of purified recombinant TAFI and TAFI-I182R-I183E. (A) Purified TAFI (lane 1) and TAFI-I182R-I183E (lane 2) were subjected to SDS-PAGE (12.5% gel) under reducing conditions. TAFI (lane 3) and TAFI-I182R-I183E (lane 4) were also incubated with thrombin–thrombomodulin at 37 °C. Aliquots of the activation mixtures were removed after 30 min and subjected to SDS-PAGE. Protein bands were visualized by staining with Coomassie Blue and the positions of molecular mass standards (kDa) are indicated to the left of the gel. (B/C) Proteins (TAFI, lane 1; TAFI-I182R-I182E, lane 2) were blotted on a PDVF membrane and detected with a B, mouse anti-human TAFI monoclonal antibody (9H10) or (C) a sheep anti-TAFI polyclonal antibody.

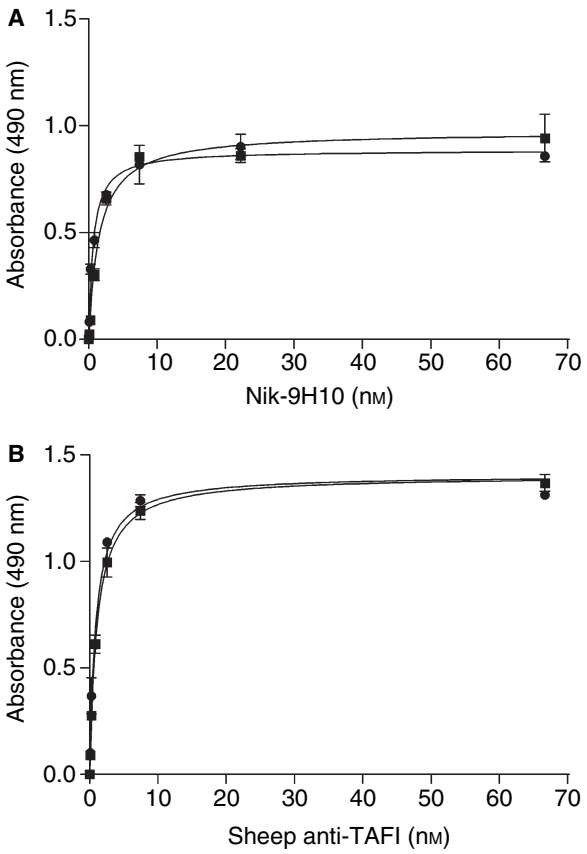

Fig. 2. Affinity of two antibodies for TAFI and TAFI-I182R-I183E. The binding affinity of (A) Nik-9H10 and (B) the sheep anti-TAFI antibody was determined by coating TAFI (squares) or TAFI-I182R-I183E (circles) to a well. The antibodies were allowed to bind and the complexes were detected using a rabbit antimouse HRP-conjugated antibody or a rabbit antisheep HRP-conjugated antibody, respectively. All the data are represented as mean ± SEM of duplicates. The lines represent the results of nonlinear regression of the data to the binding equation described in the Methods section.

complex at 37 °C. At various time points samples were removed, the reactions were stopped by addition of PPACK, and TAFIa activity was measured using hippuryl-arginine as the substrate. Under these conditions, the maximal TAFIa activity of TAFI and TAFI-I182R-I183E was reached after approximately 1 min, after which it decreased (Fig. 4A).

Stability of activated TAFI and TAFI-I182R-I183E

To investigate the stability of TAFIa and TAFIa-I182R-I183E, the zymogens were activated for 1 min by the thrombin–thrombomodulin complex. Then, thrombin activity was inhibited by addition of PPACK to prevent proteolytic inactivation of TAFIa by thrombin, whereas spontaneous inactivation could still proceed. In the meanwhile, the samples were kept at 37 °C to allow spontaneous conformational inactivation. The rate of inactivation of TAFIa, and TAFIa-I182R-I183E in the presence of PPACK did not differ from the rate of inactivation in the absence of PPACK (Fig. 4A), suggesting that inactiva-

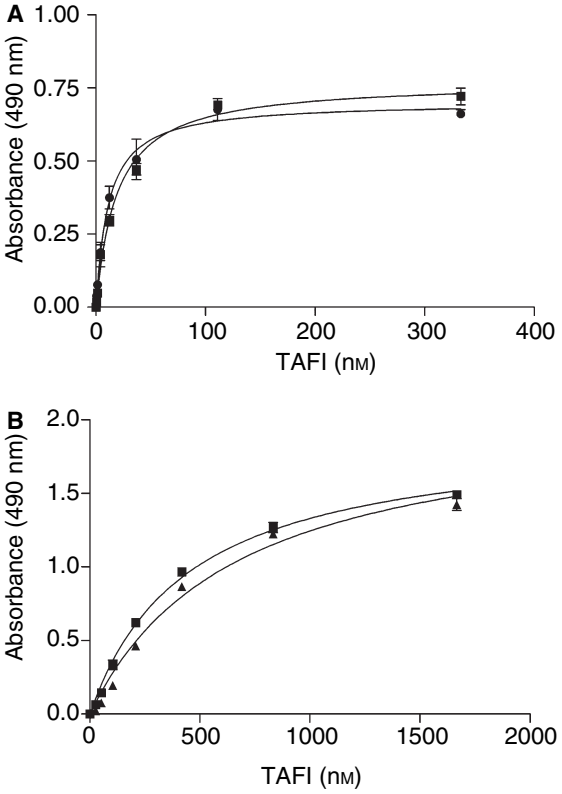

Fig. 3. Comparison of the binding of TAFI and TAFI-I182R-I183E to plasminogen and fibrinogen. (A) The capacity of TAFI (squares) and TAFI-I182R-I183E (circles) to bind to (A), plasminogen and (B), fibrinogen was assessed in an enzyme-linked immunosorbent assay (ELISA)-type of assay in which plasminogen or fibrinogen were coated to a well, TAFI was allowed to bind, and the complex was detected using an anti-TAFI antibody (Nik-9H10). All the data are represented as mean ± SEM of duplicates. The lines represent the results of non-linear regression of the data to the binding equation described in the Methods section.

tion results from the intrinsic instability of the enzyme and not proteolysis. The half-life of TAFI-I182R-I183E (4.0 ± 0.5 min) was similar to that of wild-type TAFIa (4.9 ± 1.1 min).

Activation of TAFI by thrombin–thrombomodulin or thrombin

To study the rate of activation of TAFI and TAFI-I182R-I183E in more detail, the system had to be modified to slow down the reaction. To this end, the concentrations of thrombin and thrombomodulin were reduced and the reaction mixtures were incubated at 4 °C. The rate of activation of TAFI-I182R-I183E (45 ± 2 U L^{-1} s^{-1}) was approximately twofold lower than the rate of activation of TAFI (103 ± 3 U L^{-1} s^{-1}) (Fig. 4B).

In addition, the rate of activation of TAFI and TAFI-I182R-I183E by thrombin alone was assessed. In the absence of thrombomodulin much higher concentrations of thrombin were required for TAFI activation. When using 500 nM thrombin (at 37 °C), TAFI-I182R-I183E (1 ± 0.1 U L^{-1} s^{-1})

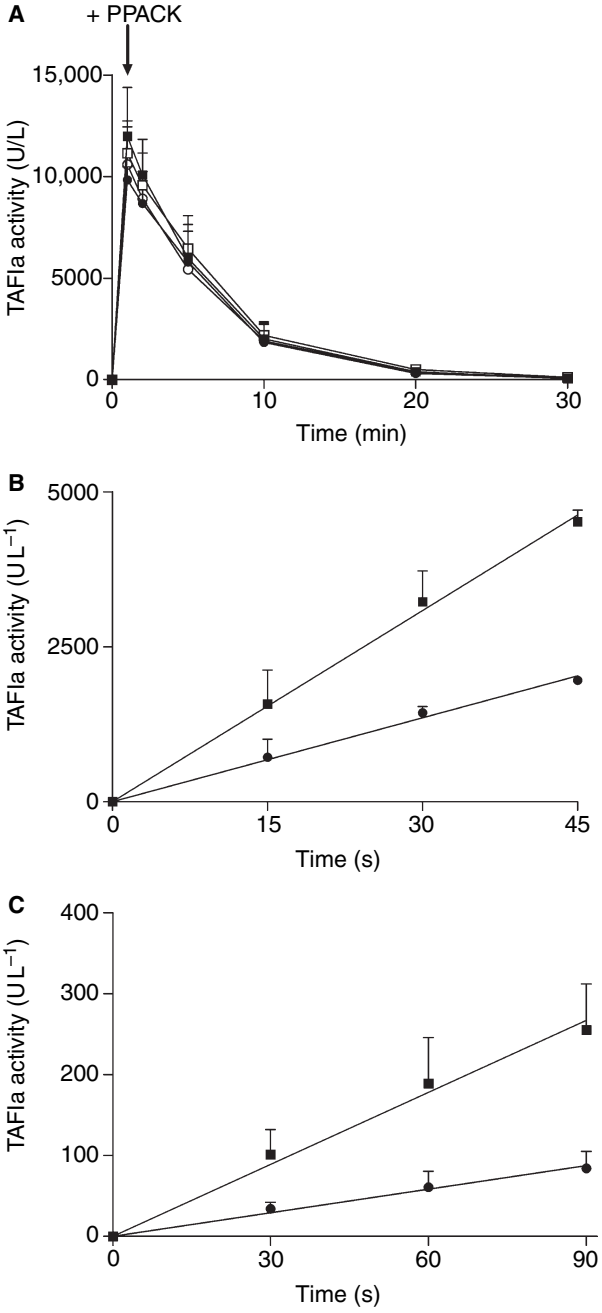

Fig. 4. Activation and inactivation of TAFI and TAFI-I182R-I183E during incubation with thrombin–thrombomodulin or thrombin. (A) TAFI (squares) and TAFI-I182R-I183E (circles) were activated for 1 min by incubation with thrombin–thrombomodulin at 37 °C. Then, thrombin activity was (open symbols) or was not (closed symbols) stopped by addition of PPACK while the activated species remained at 37 °C. At various times, aliquots were removed and the TAFIa activity was measured (data are expressed as mean ± SD, $n = 3$). (B/C) TAFI (squares) and TAFI-I182R-I183E (circles) were incubated with thrombin–thrombomodulin at 4 °C (B) or thrombin alone (C). At various time points, aliquots were taken from the activation mixtures and thrombin activity was inhibited by addition of PPACK. The TAFIa activity toward the substrate hippuryl-arginine was measured. The lines represent the results of linear regression of the data (data are expressed as mean ± SD, $n = 3$). Note the difference in time scale in Fig. 4A compared with 4B and 4C.

was activated at a rate of approximately three fold lower than TAFI (3 ± 0.2 U L^{-1} s^{-1}) (Fig. 4C).

Activation of TAFI and inactivation of TAFIa by plasmin

In the past, plasmin was shown to be able to activate TAFI and to inactivate TAFIa by proteolysis [16]. Therefore, we investigated the activation of TAFI and the inactivation of TAFIa by incubating TAFI and TAFI-I182R-I183E with various concentrations of plasmin and measuring the TAFIa activity over time. TAFI and TAFI-I182R-I183E reached maximal TAFIa activity at the same time, after which the activity decreased again (Fig. 5A). Activation of TAFI-I182R-I183E (0.8 ± 0.04 U L^{-1} s^{-1}) was seven times less efficient than activation of TAFI (5.0 ± 0.2 U L^{-1} s^{-1}) (Fig. 5B). When plasmin activity was inhibited by aprotinin after 5 min, TAFIa and TAFIa-I182-I183 activity decreased at a faster rate than when plasmin activity was not inhibited. The half-life of TAFIa-I182R-I183E (4.8 ± 0.4 min) was similar to that of wild-type TAFIa (4.3 ± 0.6 min) (Fig. 5C) and no differences in the cleavage pattern were observed by SDS-PAGE analysis (Fig. 5D).

Functionality of TAFI and TAFI-I182R-I183E in a plasma system

The ability of TAFI and TAFI-I182R-I183E to protect a fibrin clot from lysis was studied in a clot-lysis assay in which tissue-type plasminogen activator-mediated clot lysis of a thrombin-induced clot was followed spectrophotometrically in time. TAFI-depleted human plasma was reconstituted with different concentrations of TAFI or TAFI-I182R-I183E, either in the absence or presence of a TAFIa-specific inhibitor, potato carboxypeptidase inhibitor (CPI). TAFI-I182R-I183E could prolong the lysis time in a concentration-dependent manner, although it was sixfold less efficient than wild-type TAFI (Fig. 6). Addition of CPI completely inhibited the TAFI or TAFI-I182R-I183E-dependent prolongation of the lysis time.

Discussion

So far, the process that leads to TAFIa inactivation is not well understood on a molecular level. Previous research revealed several amino acids of TAFI that seem to influence TAFIa stability as changes in these amino acids resulted in TAFIa

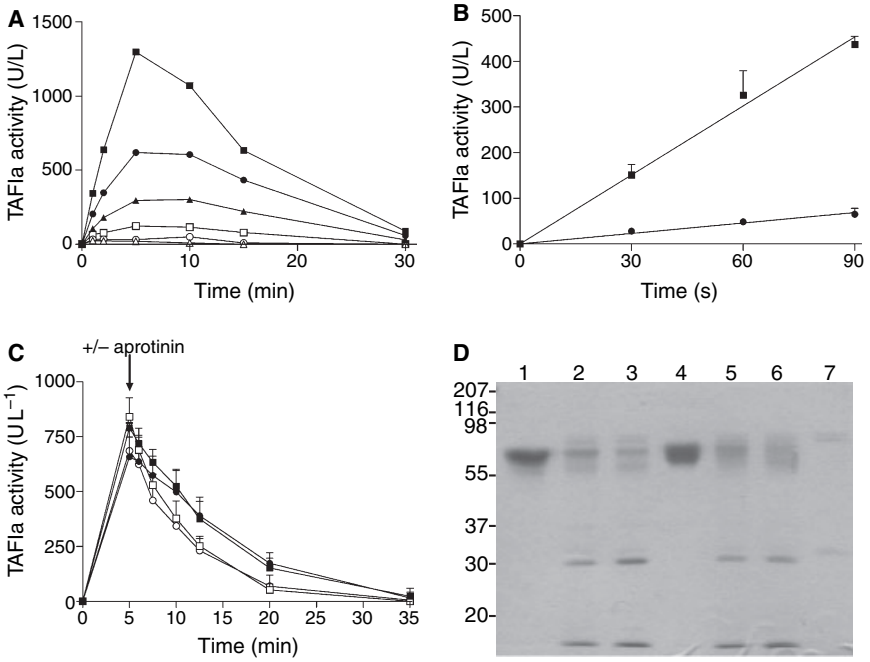

Fig. 5. Activation and inactivation of TAFI and TAFI-I182R-I183E by plasmin. (A) TAFI (solid symbols) and TAFI-I182R-I183E (open symbols) were incubated with plasmin (50 nM triangles, 100 nM circles, and 200 nM squares) at 37 °C. At various time points, aliquots were removed from the reaction mixtures and the TAFIa activity towards the substrate hippuryl-arginine was measured. (B) Rate of activation of TAFI and TAFI-I182R-I183E by plasmin (200 nM). The lines represent the results of linear regression of the data (data are expressed as mean ± SD, $n = 3$). (C) TAFI (squares) and TAFI-I182R-I183E (circles) were activated for 5 min by incubation with plasmin at 37 °C. As TAFI-I182R-I183E was activated less efficient than wild-type TAFI, much higher concentrations of TAFI-I182R-I183E (87.5 nM) than TAFI (12.5 nM) were used. Then, plasmin activity was (open symbols) or was not (closed symbols) stopped by addition of aprotinin while the activated species remained at 37 °C. At various times, aliquots were removed and the TAFIa activity was measured (data are expressed as mean ± SD, $n = 3$). Note the difference in time scale in Fig. 5A/C compared with 5B. (D) purified TAFI (lane 1) and TAFI-I182R-I183E (lane 4) were subjected to SDS-PAGE (12.5% gel) under reducing conditions. TAFI (lane 2, 30 min; lane 3, 60 min) and TAFI-I182R-I183E (lane 5, 30 min; lane 6, 60 min) were also incubated with plasmin (lane 7) at 37 °C and samples were subjected to SDS-PAGE. Protein bands were visualized by staining with Coomassie Blue and the positions of molecular mass standards (kDa) are indicated to the left of the gel.

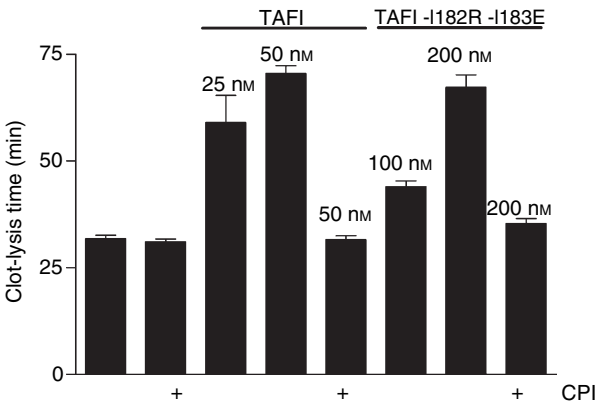

Fig. 6. Anti-fibrinolytic potential of TAFIa and TAFI-I182R-I183E. Clot-lysis times were determined in TAFI depleted plasma by measuring the turbidity of a thrombin-induced fibrin clot and tissue-type plasminogen activator-mediated fibrinolysis. The TAFI depleted plasma was supplemented with various concentrations of TAFI or TAFI-I182R-I183E, as indicated in the figure. The experiments were done in the absence or presence of CPI (+) to visualize the TAFIa-dependent prolongation of the clot-lysis time. Each bar represents the mean of at least three independent experiments ± SEM.

forms with altered stabilities. Among these amino acids are Thr/Ile325 [17], Arg302 and Arg330 [9]. Our study of two TAFI-CPB chimeras showed that replacing the C-terminal stretch of amino acids from His333 to Val401 by the corresponding amino acids of pancreas CPB results in a TAFIa form that is far more stable than wild type TAFIa, with a half-life of approximately 1.6 h compared with approximately 3 min of wild-type TAFIa [18]. Hence, these data suggested that certain elements within the C-terminal region are important for the stability of TAFIa. However, based on a three-dimensional model of TAFI, it was suggested that two other amino acids, i.e. Ile182 and Ile183, are important for TAFIa's stability [10]. To investigate whether this was indeed the case, we mutated those two residues. We choose to mutate Ile182 and Ile183 into the residues found in pancreas CPB at corresponding positions, an Arg and a Glu, as pancreas CPB and TAFI share a high degree of identity but differ tremendously in their stability. In addition, a similar approach of making TAFI-CPB chimeras had been successful in the past [18]. Nevertheless, the Arg and Glu differ considerably from Ile residues, and more subtle changes might render a different outcome.

Similar to wild-type TAFI, the mutant, TAFIa-I182-I183, was expressed in baby hamster kidney cells, it was recognized by anti-TAFI antibodies, and it bound to plasminogen and fibrinogen. The mutant had a half-life (4.0 ± 0.5 min) that was similar to that of wild-type TAFIa (4.9 ± 1.1 min), hence showing that Ile182 and/or Ile183 are not involved in TAFIa stability. The half-lives determined after activation with plasmin (TAFIa-I182R-I183E: 4.8 ± 0.4 min and wild-type TAFIa: 4.3 ± 0.6 min) were not statistically different from those found when thrombin–thrombomodulin was used as activator. Surprisingly, however, TAFI-I182-I183 (45 ± 2 U L^{-1} s^{-1}) was activated at a rate twice as slow as wild-type TAFI (103 ± 3 U L^{-1} s^{-1}) when activated by thrombin–thrombomodulin, threefold slower when activated with thrombin alone (1 ± 0.1 and 3 ± 0.2 U L^{-1} s^{-1}, respectively), and sixfold slower when plasmin was used (0.8 ± 0.04 and 5.0 ± 0.2 U L^{-1} s^{-1}, respectively). This showed that Ile182 and/or Ile183 are somehow involved in the TAFI-activating process. The mutations might have resulted in a slight change in the positioning of the activation peptide relative to the catalytic domain. Alternatively, one or both the amino acids might be involved in optimal positioning of the activator for TAFI activation. It also showed that the decreased rate of activation was determined by characteristics of the TAFI molecule as it was observed when thrombin–thrombomodulin was used, but also when thrombin alone or plasmin was used. Although thrombomodulin accelerated activation of both TAFI and the mutant by thrombin, it did not rigorously influence the ratio between the rates of activation of TAFI and TAFI-I182R-I183E. Moreover, TAFI-Ile182R-Ile183E was six times less efficient in prolonging the clot-lysis time of TAFI-depleted plasma, nicely reflecting its reduced rate of activation. Possibly, however, the radical changes (Ile to Arg and Glu substitutions) may have had far reaching effects on the structure of the protein and hence protein movement at a distant site may affect the way the protein is activated.

So far, it remains unclear how TAFIa is inactivated. Previous research showed that TAFI is not inactivated by proteolysis [8,9], although this may play a role when plasmin is present [16]. Moreover, TAFIa inactivation was not the result of release of the zinc ion that is coordinated to the active center of TAFIa [19]. In this study, we showed that Ile182 and Ile183 are not involved in this process. Analysis of TAFI-CPB chimeras demonstrated the importance of the C-terminal part of TAFI [18] and it is therefore likely that further research into this region will give valuable information on the process of TAFIa inactivation.

In conclusion, analysis of TAFI-I182R-I183E showed that I182 and I183 are not involved in TAFIa inactivation by conformational instability but that they may play a role in the activation of TAFI and the stabilization of the fibrin clot.

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References

1 Schneider M, Nesheim M. A study of the protection of plasmin from antiplasmin inhibition within an intact fibrin clot during the course of clot lysis. *J Biol Chem* 2004; **279**: 13333–9.

2 Bajzar L, Manuel R, Nesheim ME. Purification and characterization of TAFI, a thrombin-activable fibrinolysis inhibitor. *J Biol Chem* 1995; **270**: 14477–84.
3 Redlitz A, Nicolini FA, Malycky JL, Topol EF, Plow EF. Inducible carboxypeptidase activity – a role in clot lysis in vivo. *Circulation* 1996; **93**: 1328–30.
4 Wang W, Boffa M, Bajzar L, Walker JB, Nesheim ME. A study of the mechanism of inhibition of fibrinolysis by activated thrombin-activable fibrinolysis inhibitor. *J Biol Chem* 1998; **273**: 27176–81.
5 Eaton DL, Malloy BE, Tsai SP, Henzel W, Drayna D. Isolation, molecular cloning, and partial characterization of a novel carboxypeptidase B from human plasma. *J Biol Chem* 1991; **266**: 21833–8.
6 Wang W, Hendriks DF, Scharpé SS. Carboxypeptidase U, a plasma carboxypeptidase with high affinity for plasminogen. *J Biol Chem* 1994; **269**: 15937–44.
7 Boffa M, Wang W, Bajzar L, Nesheim M. Plasma and recombinant thrombin-activatable fibrinolysis inhibitor (TAFI) and activated TAFI compared with respect to glycosylation, thrombin/thrombomodulin-dependent activation, thermal stability, and enzymatic properties. *J Biol Chem* 1998; **273**: 2127–35.
8 Marx PF, Hackeng TM, Dawson PE, Griffin JH, Meijers JCM, Bouma BN. Inactivation of activated thrombin-activable fibrinolysis inhibitor takes place by a process that involves conformational instability rather than proteolytic cleavage. *J Biol Chem* 2000; **275**: 12410–5.
9 Boffa M, Bell R, Stevens WK, Nesheim ME. Roles of thermal instability and proteolytic cleavage in regulation of activated thrombin-activable fibrinolysis inhibitor. *J Biol Chem* 2000; **275**: 12868–78.
10 Barbosa Pereira PJ, Segura-Marin S, Oliva B, Ferrer-Orta C, Aviles FX, Coll M, Gomis-Ruth FX, Vendrell J. Human procarboxypeptidase B: three-dimensional structure and implications for thrombin-activatable fibrinolysis inhibitor (TAFI). *J Mol Biol 2* 2002; **321**: 537–47.
11 Meijers JC, Davie EW, Chung DW. Expression of human blood coagulation factor XI: characterization of the defect in factor XI type III deficiency. *Blood* 1992; **79**: 1435–40.
12 Schatteman KA, Goossens FJ, Scharpé SS, Neels HM, Hendriks DF. Assay of procarboxypeptidase U, a novel determinant of the fibrinolytic cascade, in human plasma. *Clin Chem* 1999; **45**: 807–13.
13 Mosnier LO, Von dem Borne PA, Meijers JC, Bouma BN. Plasma TAFI levels determine the clot lysis time in healthy individuals in the presence of an intact intrinsic pathway of coagulation. *Thromb Haemost* 1998; **80**: 829–35.
14 Marx PF, Wagenaar GTM, Reijerkerk A, Tiekstra MJ, Van Rossum AG, Gebbink MF, Meijers JC. Characterization of mouse thrombin-activatable fibrinolysis inhibitor. *Thromb Haemost* 2000; **83**: 297–303.
15 Guimarães AHC, Barrett-Bergshoeff MM, Gils A, Declerck PJ, Rijken DC. Migration of the activation peptide of thrombin-activatable fibrinolysis inhibitor (TAFI) during SDS-polyacrylamide gel electrophoresis. *J Throm Haem* 2004; **2**: 780–4.
16 Marx PF, Dawson PE, Bouma BN, Meijers JC. Plasmin-mediated activation and inactivation of thrombin-activatable fibrinolysis inhibitor. *Biochemistry* 2002; **41**: 6688–96.
17 Schneider M, Boffa M, Stewart RJ, Rahman NL, Koschinsky ML, Nesheim M. Two naturally occurring variants of TAFI (Thr-325 and Ile-325) differ substantially with respect to thermal stability and antifibrinolytic activity of the enzyme. *J Biol Chem* 2002; **277**: 1021–30.
18 Marx PF, Havik SR, Marquart JA, Bouma BN, Meijers JCM. Generation and characterization of a highly stable form of activated thrombin-activable fibrinolysis inhibitor. *J Biol Chem* 2004; **279**: 6620–8.
19 Marx PF, Bouma BN, Meijers JCM. Role of zinc ions in activation and inactivation of thrombin-activatable fibrinolysis inhibitor. *Biochemistry* 2002; **41**: 1211–6.