Plasmin-Mediated Activation and Inactivation of Thrombin-Activatable Fibrinolysis Inhibitor†

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ABSTRACT: Activated thrombin-activatable fibrinolysis inhibitor (TAFIa) attenuates the fibrin cofactor function of tissue-type plasminogen activator-mediated plasmin formation and subsequently fibrin degradation. In the present study, we focused on the role of plasmin in the regulation of TAFIa activity. Upon incubation with plasmin, TAFIa activity was generated, which was unstable at 37 °C. Analysis of the cleavage pattern showed that TAFI was cleaved at Arg^{92}, releasing the activation peptide from the 35.8-kDa catalytic domain. The presence of the 35.8-kDa fragment paralleled the time course of generation and loss of TAFIa activity. This suggested that, in the presence of plasmin, TAFIa is probably inactivated by proteolysis rather than by conformational instability. TAFI was also cleaved at Arg^{302}, Lys^{327}, and Arg^{330}, resulting in a ∼44.3-kDa fragment and several smaller fragments. The 44.3-kDa fragment is no longer activatable since it lacks part of the catalytic center. We concluded that plasmin can cleave at several sites in TAFI and that this contributes to the regulation of TAFI and TAFIa.

Coagulation and fibrinolysis refer to cascades of enzymatic reactions involved in the formation and degradation of fibrin. Thrombin-activatable fibrinolysis inhibitor (TAFI)[1] (*1*), also known as procarboxypeptidase B (*2*), U (*3*, *4*), or R (*5*) is a zymogen, which provides a link for these counteracting processes (*6*, *7*). Activated TAFI (TAFIa) exerts carboxypeptidase B activity, i.e., it can remove C-terminal arginine and lysine residues. C-terminal lysines are generated when plasmin partially degrades fibrin, and these lysine residues are ligands for the lysine-binding sites of plasminogen and tissue-type plasminogen activator (*8*). In this way, they play an important role in the upregulation of fibrinolysis. Removal of those lysines by TAFIa attenuates the fibrin cofactor function of tissue-type plasminogen activator-mediated plasmin formation and consequently fibrin degradation (*9*). When plasmin degrades cross-linked fibrin, one of the major products is a complex of D-dimer (DD) and fragment E, (DD)E. Recently, Stewart et al. (*10*) showed that TAFIa reduces the affinity of the fibrin degradation product (DD)E for t-PA. This results in loss of the stimulatory effect of (DD)E on plasmin formation. Furthermore, TAFIa inhibits the degradation of (DD)E to DD and E, which prevents impairment of fibrin polymerization by DD (*10*). C-terminal lysines also play a role in the conversion of Glu-plasminogen to Lys-plasminogen, which is more readily activated to plasmin (*6*, *11*). Furthermore, TAFIa was found to inhibit fibrin degradation by plasmin (*11*), suggesting that TAFIa attenuates fibrinolysis by inhibiting plasmin directly.

Activation of TAFI results from cleavage of the peptide bond between Arg^{92} and Ala^{93} by trypsin-like enzymes such as thrombin and plasmin, releasing the activation peptide from the catalytic domain (*2*). Thrombomodulin accelerates TAFI activation by thrombin ∼1250-fold (*12*), whereas glycosaminoglycans act as cofactors for plasmin-mediated TAFI activation (*13*). Previous research using thrombin/thrombomodulin as TAFI activator showed that the 35.8-kDa catalytic domain is degraded by cleavage at Arg^{302} into 24.5- and 11.1-kDa fragments (*14*). However, proteolysis of the 35.8-kDa fragment did not correlate with TAFIa activity, which was lost much faster than the 35.8-kDa fragment was cleaved (*12*, *14*, *15*). Inhibition of thrombin did not prevent TAFIa from inactivation, although no further proteolysis was observed (*14*). A mutant form of TAFI, in which Arg^{302} was changed into a glutamine residue, was still inactivated (*14*, *16*). Furthermore, a decrease in the intrinsic fluorescence of TAFIa was observed during incubation at 37 °C (*15*). These observations indicated that TAFIa is inactivated by a

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[1] Abbreviations: ϵ-ACA, ϵ-amino caproic acid; MALDI-MS, matrix-assisted laser desorption ionisation (time-of-flight) mass spectrometry; TAFI, thrombin-activatable fibrinolysis inhibitor; TAFIa, activated TAFI; TAFIai, inactivated TAFIa; CPI, carboxypeptidase inhibitor, HPLC, high performance liquid chromatography, PPACK, E-D-Phe-Pro-Arg-chloromethyl ketone, PAGE, polyacrylamide gel electrophoresis.

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conformational change within the enzyme rather than by proteolysis. Addition of the lysine analogue ϵ-ACA stabilized TAFIa and slowed proteolysis (*14*, *17*, *18*), suggesting that the conformational change results in exposure of the cleavage site. Proteolysis may make the process of inactivation irreversible. The presence of a substrate, however, may keep TAFIa in its active conformation, in analogy with what was observed with the competitive inhibitor ϵ-ACA. No physiological inhibitors of TAFIa have been identified to date. Since TAFIa exerts antifibrinolytic activity, profibrinolytic enzymes such as plasmin may play a role in the regulation of TAFIa. In the present study, we focused on the role of plasmin in the regulation of TAFIa activity.

EXPERIMENTAL PROCEDURES

Materials. Fresh frozen plasma was obtained from the local blood bank. Cyanuric chloride and casein were purchased from Merck (Amsterdam, The Netherlands), hippuryl-arginine, and H-D-Phe-Pro-Arg-cloromethyl ketone (PPACK) were from Bachem (Bubendorf, Switzerland). Human thrombin was a generous gift from Dr. W. Kisiel (University of New Mexico, Albuquerque, NM) and plasmin from Dr. A. Reijerkerk (University Medical Centre Utrecht, The Netherlands). Thrombomodulin was purchased from American Diagnostica (Greenwich, CT). Carboxypeptidase inhibitor (CPI) was purchased from Calbiochem (La Jolla, CA).

Purification of Plasma Derived TAFI. Human TAFI was purified essentially as described previously (*14*). Briefly, 3 L of citrated plasma supplemented with 0.1 M ϵ-ACA were applied to a PD-10 Sephadex G-25M column (Amersham Pharmacia Biotech) coupled to a CNBr-activated Sepharose column to which an antibody against TAFI (MoAb Nik-9H10, 4.5 mg/mL, 10 mL) was coupled. The columns were equilibrated in TBS (50 mM Tris, 150 mM NaCl, pH 7.4). Unbound and nonspecifically bound proteins were washed away with 0.5 M NaCl in TBS, and bound protein was eluted with 0.1 M glycine, pH 2.7. The eluted fractions were collected in 1/10 (v/v) 1 M Tris, pH 9, pooled, and diluted four times in H_2O and applied to a CNBr-activated Sepharose column to which an antibody against human serum albumin was coupled. The flow through was applied to a protein G-Sepharose column. Both columns were equilibrated with 20 mM Tris, pH 7.4. The flow through was applied to a MonoQ column, equilibrated with the same buffer. Bound protein was eluted with a linear gradient (0−250 mM NaCl in 20 mM Tris, pH 7.4). Fractions containing TAFI were pooled and stored at −70 °C. TAFI concentrations were determined with a BCA kit (Pierce) using bovine serum albumin as a standard.

TAFIa Activity Assay. TAFIa activity was measured as described (*14*). Purified TAFI (5.5 μM) was incubated with a mixture of thrombin (20 nM) and thrombomodulin (5 nM), or plasmin (500, 200, or 50 nM), both in the presence of $CaCl_2$ (5 mM), at 37 °C. In some experiments, ϵ-ACA (5 mM) was added. At several time points, aliquots of the activation mixtures were added to a mixture of PPACK (30 μM), when thrombin was used as activator or trasylol (100 u/mL) when plasmin was used, with the substrate hippuryl-arginine (4 mM) in TBS containing 0.1% bovine serum albumin. Substrate conversion was allowed to proceed for 10 min when thrombin was used and 30 min when plasmin was used as activator. Reactions were stopped by addition of 1 M HCl (20 μL). Subsequently, 20 μL of 1 M NaOH was added. Twenty-five microliters of 1 M sodium phosphate and 30 μL of 6% cyanuric chloride dissolved in 1,4-dioxane were added, and color was allowed to develop under extensive vortexing. Denatured proteins and excess of cyanuric chloride were removed by centrifugation and 100 μL of the supernatant was transferred to a 96-well microtiter plate. Absorbance was measured at 405 nm in a multiscan photometer (V_{max} reader, Molecular Devices, Menlo Park, CA). The TAFIa concentrations were calculated from the kinetic parameters for the hydrolysis of hippuryl-arginine by TAFIa ($K_M = 144$ μM, $k_{cat} = 21$ s^{-1}) (*12*).

Plasmin Activity Assay. Plasmin (500 nM) was incubated in the presence or absence of TAFI (5.5 μM) and/or ϵ-ACA (5 mM) at 37 °C. Volumes were adjusted to 15 μL with HBS containing 1% BSA. Then TAFIa activity was inhibited by addition of 15 μL CPI (40 μM) and 30 μL of 1% casein in 0.2 M glycine-NaOH, pH 9.0 was added. The mixtures were incubated at 37 °C for 30 min. The reactions were stopped by addition of 90 μL of stopping solution containing 0.11 M trichloroacetic acid, 0.22 M sodium acetate, and 0.33 M acetic acid. The reactions were placed on ice for 1 h. Precipitates were removed by centrifugation, and the absorbance at 280 nm of the supernatants was measured relative to a blank in which stopping solution had been added immediately after addition of proteinase.

Gel Electrophoresis. Purified TAFI (5.5 μM) was incubated with a mixture of thrombin (20 nM) and thrombomodulin (5 nM), or plasmin (500 nM), both in the presence of $CaCl_2$ (5 mM), at 37 °C. In some experiments, ϵ-ACA (5 mM) or CPI (35 μM) was added. After different time points, the reactions were stopped by the addition of reducing sample buffer, and samples were boiled for 3 min and subjected to SDS−PAGE. SDS−PAGE gels (12.5%) were used for Coomassie Brilliant Blue staining. For N-terminal sequencing, 10−20% ready gels (Bio-Rad Laboratories, The Netherlands) were used, and proteins were transferred to poly(vinylidene difluoride) membranes. Sequencing was performed according to the Edman degradation method on a Perkin-Elmer/Applied Biosystems type 476A.

Separation of Proteolytic Fragments by Reverse Phase HPLC. Purified TAFI (5.5 μM) was incubated with plasmin (500 nM) and $CaCl_2$ (5 mM), at 37 °C. Experiments were done in the presence and absence of CPI (35 μM). The reaction mixtures were applied to a C4 column (5 μm, 0.46 × 15 cm) connected to a Hewlett-Packard HPLC 1050 system. The column was equilibrated in 0.1% trifluoroacetic acid in H_2O. Proteins were eluted by a linear gradient from 0 to 60% acetonitrile in 0.1% trifluoroacetic acid in H_2O (1 mL/min and 2%/min). Peaks were collected separately and lyophilized.

Identification of Proteolytic Fragments by Mass Spectrometry. Lyophilized fragments were dissolved in 5 μL of 50% acetonitrile in H_2O, 0.1% trifluoroacetic acid. One microliter was mixed with one microliter of a saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid in 50% acetonitrile in H_2O, 0.1% trifluoroacetic acid and applied to MALDI-MS (Voyager Applied Biosystems, CA). Theoretical masses of proteins and protein fragments were calculated using Gene Runner 3.00 (Hastings Software Inc.).

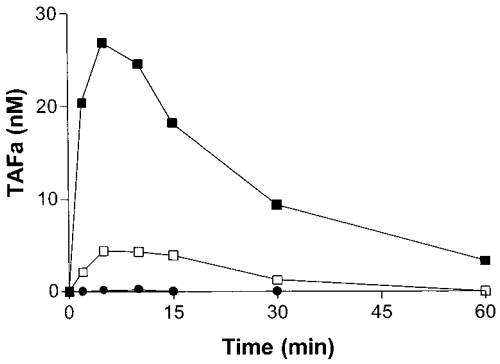

FIGURE 1: Activation of TAFI and inactivation of TAFIa by plasmin. TAFI was incubated with plasmin (500 nM, ■; 200 nM, □; 50 nM, ●), and $CaCl_2$ at 37 °C. At various time points, samples were taken, plasmin was inhibited by trasylol, and TAFIa activity was measured.

RESULTS

Activation of TAFI and Inactivation of TAFIa. TAFI was incubated with plasmin in the presence of $CaCl_2$ at 37 °C. At various time points, samples were removed, the reactions were stopped by the addition of trasylol, and TAFIa activity was measured using hippuryl-arginine as a substrate (Figure 1). Depending on the plasmin concentration, TAFIa activity was generated, which was unstable and became undetectable after ~60 min.

Identification of Proteolytic Fragments by HPLC and MALDI-MS. TAFI (~55-kDa) is proteolytically activated by thrombin/thrombomodulin by cleavage at Arg^{92}, which releases the activation peptide (19.4-kDa, not visible on Coomassie stained SDS−PAGE gels) from the catalytic domain (35.8-kDa). The 35.8-kDa fragment is further cleaved at Arg^{302}, resulting in fragments of 24.5- and 11.1-kDa. The cleavage pattern observed after incubation with plasmin was different from that after incubation with thrombin/thrombomodulin. To identify the proteolytic fragments of TAFI generated by plasmin, samples of the activation mixtures were analyzed by HPLC (Figure 2A) and MALDI-MS (Figure 2B). At 0 min, one major peak was seen on the HPLC spectrum, with a molecular mass of 55 kDa. The broadness of the peak reflected the heterogeneity of TAFI due to glycosylation.

After 60 min activation, the HPLC spectrum showed additional peaks compared to the starting material. Peak 2 contained a heterogeneous fragment of 19.4 kDa. TAFI contains four potential N-linked glycosylation sites (Asp^{22}, Asp^{51}, Asp^{63}, and Asp^{86}), which are all located in the activation peptide, suggesting that the 19.4-kDa fragment is the activation peptide (amino acid 1−92). Peak 3 contained two small fragments of 8.1- and 8.4-kDa. These two fragments were also found in peak 4 and 5 due to poor separation by HPLC of these three peaks. Peak 4 contained, beside the precursor, a fragment of 44.3-kDa, which was also heterogeneous. This suggested that this fragment was TAFI lacking a C-terminal fragment. Peak 5 contained fragments of 24.5 and 24.7 kDa and several smaller fragments indicating further degradation.

Identification of Cleavage Sites. To identify the proteolytic fragments within the TAFI sequence, the experimentally obtained masses were matched to theoretical masses. However, no theoretical masses could be calculated for the fragments containing the glycosylated activation peptide (55, 44.3, and 19.4 kDa). To confirm their identity, the N-termini of the proteins and peptides were determined by N-terminal sequencing. The results are summarized in Table 1. The mass of 55 kDa for TAFI was in agreement with the mass of TAFI reported previously and started with TAFI's N-terminal sequence Phe^{1}-Gln-Ser-Gly-Gln. The 19.4-kDa fragment is most likely the activation peptide (Phe^{1}-Arg^{92}), based on the presence of the glycosylated residues. The N-termini of the ~8-kDa fragments revealed two sequences, one starting at Asn^{328}, the other at Tyr^{331}. The 8.1-kDa matched to the theoretical mass of a fragment spanning from Tyr^{331} to Val^{401} (8053 Da), whereas the 8.4-kDa fragment matched to a peptide starting at Asn^{328} to Val^{401} (8424 Da). This indicated that plasmin could cleave at both Lys^{327} and Arg^{330}, resulting in the formation of the ~44.3-kDa fragment. The N-termini of the 24.5- and 24.7-kDa fragments were analyzed together and revealed one sequence, Ala^{93}-Ser-Ala-Ser-Tyr-Tyr-Glu. The 24.7-kDa fragment matched best to a fragment spanning from Ala^{93} to Arg^{302}, whereas the 24.5-kDa fragment matched better to a fragment spanning from Ala^{93} to Thr^{301}. This suggested that plasmin had cleaved at Arg^{302}, after which TAFIa removed the C-terminal Arg from this fragment. The fragment Tyr^{303}-Lys^{327} was not detected. After 60 min, hardly any TAFIa activity was measured and no 35.8-kDa fragment was observed by mass spectrometry.

Removal of C-Terminal Residues of TAFI Fragments by TAFIa. To investigate whether TAFIa is responsible for the removal of the C-terminal Arg^{302} from the 24.7-kDa fragment, TAFI was incubated with plasmin, $CaCl_2$, and CPI for 30 min at 37 °C. CPI had been reported to exert antiplasmin activity (*19*). The batch of CPI used in this study only marginally inhibited plasmin activity toward a chromogenic substrate (approximately 15%, results not shown) and not toward casein (results not shown), and was therefore unlikely to influence the outcome of our experiments. The incubation mixture of TAFI and plasmin with or without CPI was applied to HPLC and the peak containing the ~25-kDa fragment was analyzed by MALDI-MS (Figure 3A). In the presence of CPI (lower panel), only a polypeptide of 24.7-kDa was observed, whereas in the absence of CPI (upper panel) also a 24.5-kDa fragment was detected. The arrow indicates the theoretical position of the 24.5 kDa fragment (lower panel). This suggested that TAFIa indeed exerted carboxypeptidase B activity toward the C-terminal Arg of the 24.7-kDa fragment. Samples analyzed on SDS−PAGE showed that addition of CPI resulted in accumulation of the 35.8-kDa fragment by plasmin (Figure 3B), indicating that CPI prevents C-terminal proteolysis of TAFI and TAFIa.

Influence of TAFIa on Plasmin. Remarkably, a considerable amount of the 55-kDa protein (Figure 3B) is still present after 60 min incubation with plasmin, whereas no TAFIa activity is detected anymore. To investigate if this is due to inactivation of plasmin during the experiment, plasmin was incubated at 37 °C in the presence and absence of TAFI (Figure 4A). In both cases, only ~25% of the initial plasmin activity remained after 60 min. To exclude that some change in TAFI had occurred that could not be appreciated on SDS−PAGE, such as C-terminal cleavage of a few residues or a conformational change which prevented further TAFI activation, we incubated TAFI with or without plasmin at 37 °C for 2 h, after which we added thrombin/thrombomodulin

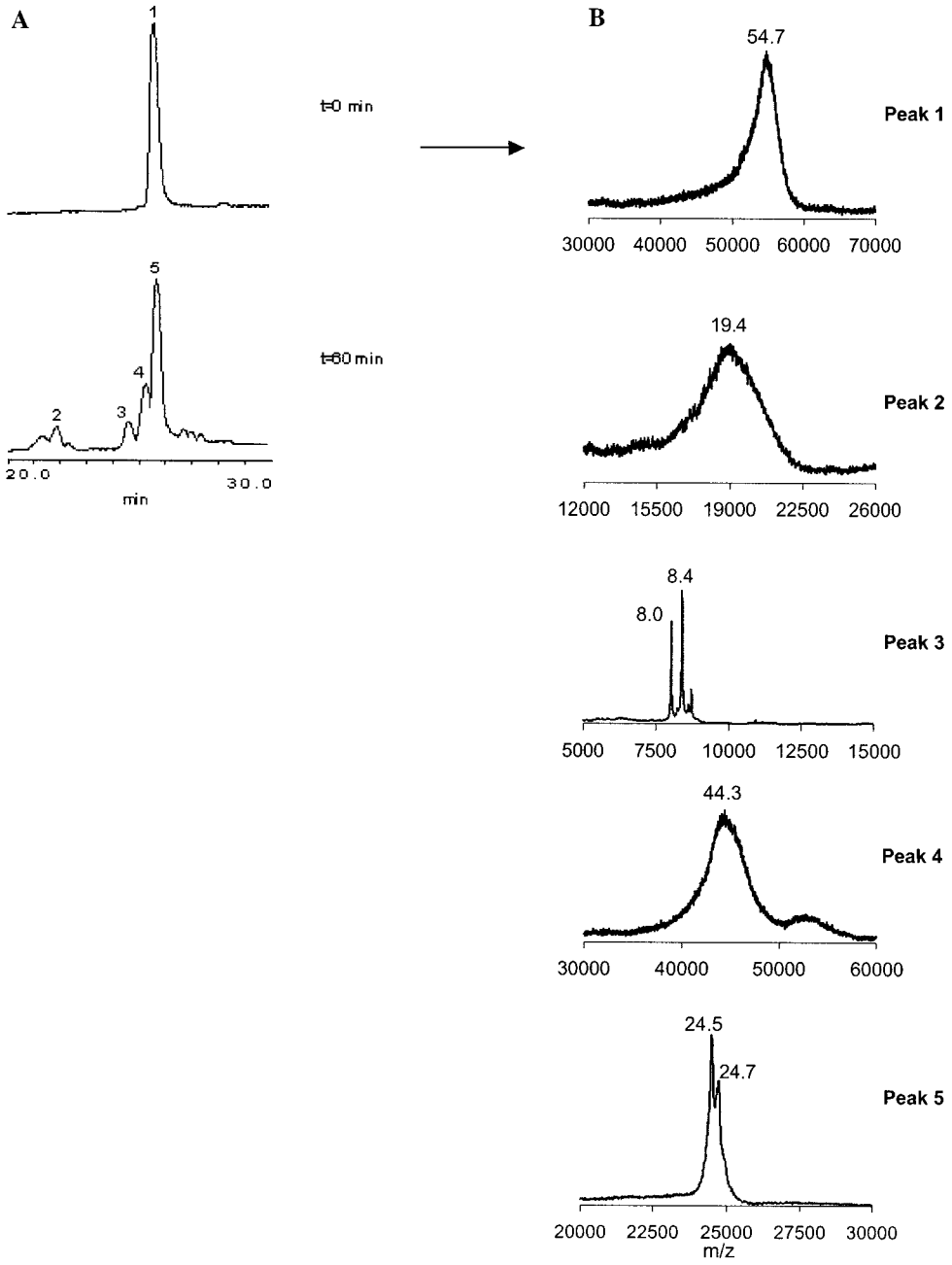

FIGURE 2: HPLC profile and MALDI spectra of TAFI incubated with plasmin. TAFI was incubated with plasmin and $CaCl_2$ for 60 min, after which generated fragments were purified by HPLC (A) and analyzed by MALDI-MS (B). Masses are expressed as the mean of four experiments (kDa). No ~36-kDa fragment was detected. Note broad MALDI-MS peaks due to glycosylation (peaks 1, 2, and 4). See text for further analyses of the peaks.

(Figure 4B). TAFIa activity was generated in both samples, showing that TAFI that had not been converted to TAFIa by plasmin within the 2 h incubation period, was still activatable. This indicated that no more TAFIa was detected because plasmin was partly inactivated rather than that TAFI had been compromised.

Importance of Proteolysis in Regulation of TAFIa Activity. Upon incubation of TAFI with thrombin/thrombomodulin at 37 °C, there is a discrepancy between the persistent presence of the 35.8-kDa catalytic domain and the time course for loss of TAFIa activity. This led to the conclusion that cleavage of the 35.8-kDa fragment at Arg^{302} was not the cause of inactivation of TAFIa, but was more likely to occur after a conformational change that converted TAFIa into its inactive state TAFIai (*14*, *16*). Subsequent proteolysis may make the process of inactivation by a conformational change irreversible. However, when plasmin was used, the presence of the 35.8-kDa fragment paralleled the time course of generation and loss of TAFIa activity (Figure 5A,B,C). Furthermore, generation of the 44.3-kDa fragment indicated that no conformational change is required to make Lys^{327} and Arg^{330} accessible for proteolysis by plasmin. These observations suggested that in the presence of plasmin, TAFIa is proteolyzed prior to conversion into TAFIai, and as a consequence, inactivation may result from proteolysis rather than from conformational instability. Removal of a C-terminal fragment from TAFI presumably prevented formation of a fragment exerting carboxypeptidase B activity,

Table 1: Estimated Masses and N-Terminal Sequences of TAFI Fragments Generated upon Incubation of TAFI with Plasmin[a]

exp mass (kDa)	N-terminal sequence	fragment	theoretical mass (kDa)
54.7	Phe-Gln-Ser-Gly-Gln	Phe^{1}-Val^{401}	45.999 + glycosylation
44.3	Phe-Gln-Ser-Gly-Gln	Phe^{1}-Arg^{302}/ Lys^{327}/Arg^{330}	≥37.575 + glycosylation
24.7	Ala-Ser-Ala-Ser-Tyr-Tyr-Glu	Ala^{93}-Arg^{302}	24.701
24.5	Ala-Ser-Ala-Ser-Tyr-Tyr-Glu	Ala^{93}-Thr^{301}	24.526
19.4	n.d.	Phe^{1}-Arg^{92}	10.200 + glycosylation
8.4	Asn-Thr-Arg-Tyr-Thr-His	Asn^{328}-Val^{401}	8.424
8.1	Tyr-Thr-His-Gly-His-Gly	Tyr^{331}-Val^{401}	8.053

[a] Mass analysis and fragment identification of TAFI cleavage fragments. Theoretical masses of protein fragments were calculated and matched to experimental masses. For proteins containing the activation peptide (amino acid 1−92), masses were calculated without carbohydrates (n.d. = not determined).

since residues considered to be involved in substrate binding and specificity (Tyr^{341} and Asp^{349}), and more importantly, Glu^{363}, which is one of the three catalytic amino acids (*2*), are located C-terminally from the cleavage site Arg^{330}. The significance of this proteolytic pathway is substantiated by the observations that only limited amounts of the 35.8-kDa fragment are formed during plasmin incubation and low activity is measured.

Stability of TAFIa. Addition of the lysine analogue ϵ-ACA during incubation of TAFI with plasmin resulted in a steep increase in TAFIa activity (Figure 6A), accumulation of the 35.8-kDa fragment, and no generation of the 44.3-kDa fragment (Figure 6B,C). This indicated that ϵ-ACA prevented cleavage of TAFI at all sites but Arg^{92}, slowed proteolysis of the 35.8-kDa fragment, and kept TAFIa in its active conformation. In the presence of ϵ-ACA, spontaneous inactivation of plasmin was prevented and co-incubation with TAFI let to an enhanced plasmin inactivation (Figure 6D).

DISCUSSION

To investigate the role of plasmin in the regulation of TAFI activation and TAFIa activity, we incubated TAFI with plasmin and correlated the formation of proteolytic fragments with the activation profile of TAFI. During incubation of TAFI with plasmin, TAFIa activity was generated, which was unstable at 37 °C. Similar to thrombin, plasmin was found to activate TAFI (55-kDa) by cleavage at Arg^{92}, releasing the 19.4-kDa activation peptide from the catalytic domain (35.8-kDa). The presence of a 24.7-kDa fragment, starting at Ala^{93} indicated that cleavage of the 35.8-kDa fragment had occurred at Arg^{302}, similar to the cleavage pattern of TAFIa by thrombin/thrombomodulin (*14*, *16*). The C-terminal Arg^{302} of the 24.7-kDa fragment is probably removed by the carboxypeptidase B activity of TAFIa, since addition of the TAFIa inhibitor CPI prevented the generation of a 24.5-kDa fragment found in the absence of CPI. A glycosylated fragment of 44.3-kDa starting with Phe^{1} suggested that plasmin not only cleaved TAFI at Arg^{92}, but also at one or more C-terminal sites. Two smaller fragments were observed, one of 8.4 kDa and one of 8.0 kDa. The mass of the 8.4-kDa fragment matched to the theoretical mass of a fragment spanning from Asn^{328} to Val^{401}, whereas the 8.0-kDa fragment matched to a fragment spanning from Tyr^{331} to Val^{401}. This indicated that plasmin cleaved TAFI both at Arg^{92}, but also at Lys^{327} and Arg^{330}, which resulted in a ∼44.3-kDa fragment. All cleavage sites were confirmed by N-terminal sequencing. However, the fragment spanning Arg^{302} to Lys^{327} was not observed. Schematic overviews of TAFI activation and inactivation by thrombin/thrombomodulin or plasmin are given in Figure 7.

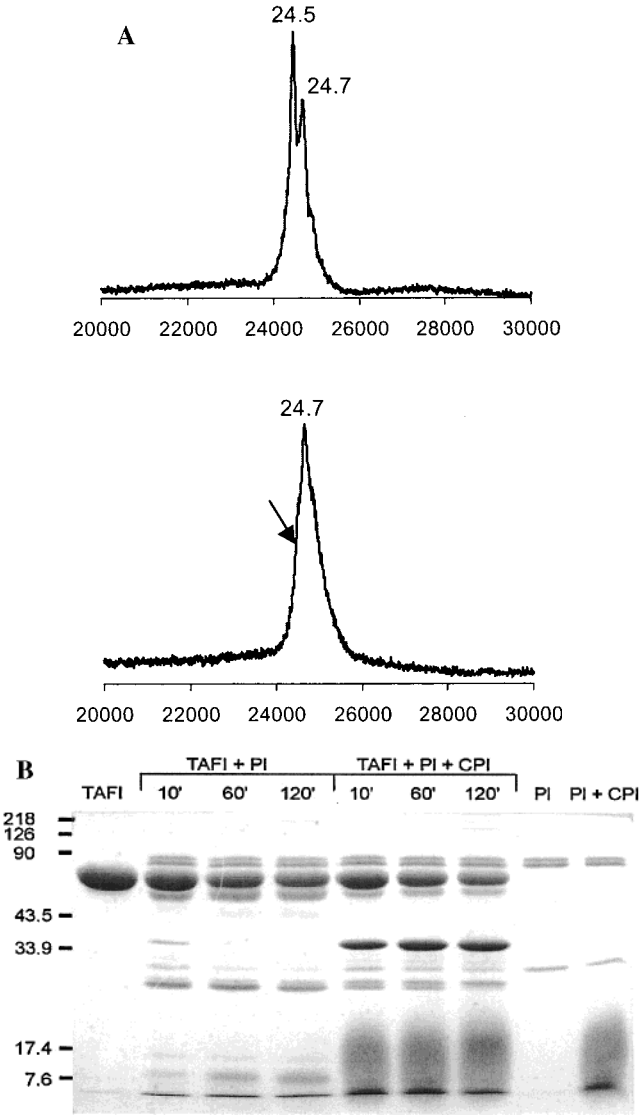

FIGURE 3: Influence of CPI on TAFI fragment generation. (A) TAFI was incubated with plasmin and $CaCl_2$ in the absence (upper panel) or presence (lower panel) of CPI for 30 min at 37 °C, and the reaction mixtures were purified by HPLC. The 25-kDa TAFI fragments were analyzed by MALDI-MS. CPI prevented removal of the C-terminal Arg^{302}. The arrow indicates the theoretical position of the 24.5 kDa fragment. (B) Analysis of the reaction mixtures by SDS−PAGE at indicated times (minutes) showed that CPI inhibited proteolysis of the 35.8-kDa fragment (positions of molecular mass standards (kDa) are indicated on the left; abbreviation used: Pl, plasmin).

Upon incubation of TAFI with plasmin, only a small amount of TAFIa activity was generated, and the precursor

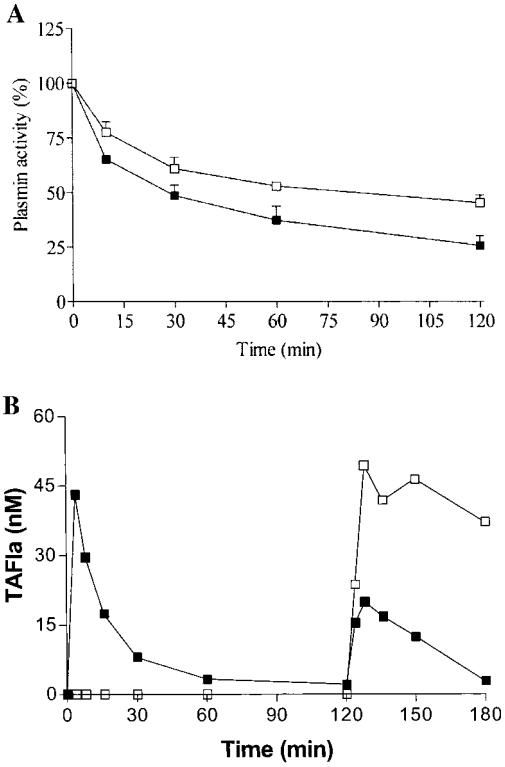

FIGURE 4: Activatability of TAFI. (A) Plasmin was incubated with (■) or without (□) TAFI in the presence of $CaCl_2$ at 37 °C. At indicated time points, the TAFIa activity was quenched by addition of CPI, and the plasmin activity was measured (data are expressed ± SD, $n = 3$). (B) TAFI was incubated with (■) or without (□) plasmin in the presence of $CaCl_2$ at 37 °C. At various times samples were taken, plasmin activity was inhibited by trasylol. After 2 h, thrombin/thrombomodulin was added and incubation was continued. At indicated time points samples were withdrawn and thrombin activity was inhibited by PPACK. The TAFIa activity was measured in all samples.

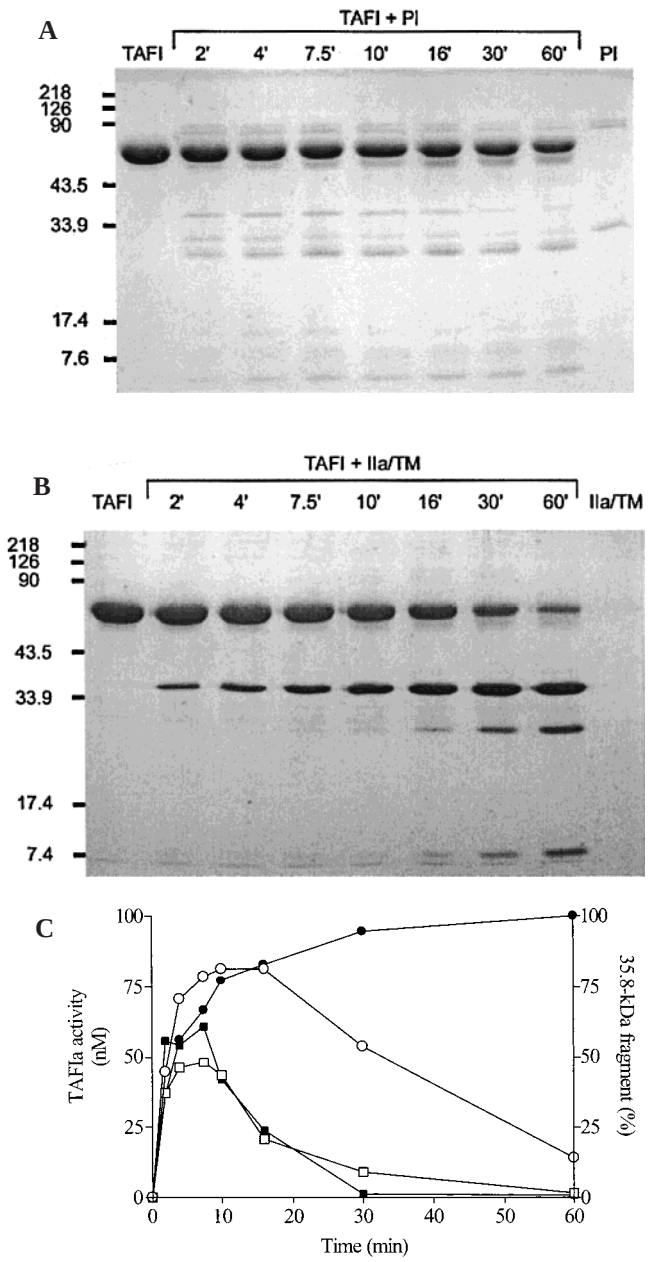

FIGURE 5: Relation between TAFIa activity and presence of the 35.8-kDa fragment. TAFI was incubated with thrombin/thrombomodulin and $CaCl_2$ or plasmin and $CaCl_2$ at 37 °C. (A/B) Samples of the reaction mixtures were analyzed by SDS−PAGE at indicated times (minutes). Masses of molecular standards are indicated on the left (kDa). (C) Samples were taken, PPACK or trasylol was added to inhibit thrombin activity or plasmin activity, respectively, and TAFIa activity was measured. The 35.8-kDa fragment was quantified; data were expressed as percentage of maximal density of the 35.8-kDa fragment generated by thrombin-thrombomodulin after 60 min (closed symbols) and plotted in one graph with the TAFIa activity (open symbols). The 35.8-kDa fragment (●) was persistently present upon incubation with thrombin/thrombomodulin, whereas TAFIa activity (○) was lost rapidly. Upon incubation of TAFI with plasmin, the time course of loss of TAFIa activity (□) coincided with the presence of the 35.8-kDa fragment (■) (abbreviations used: Pl, plasmin; IIa/TM, thrombin/thrombomodulin).

was only partly processed. Both TAFI activation and TAFIa inactivation occur simultaneously during incubation of TAFI with plasmin. Starting with high concentrations of TAFI, some TAFIa is detected in the first ~60 min. Upon prolonged incubation however TAFIa formation slows down due to a decreasing TAFI concentration, whereas inactivation by conformational instability and proteolysis continues at the same rate, and consequently TAFIa activity becomes undetectable in time (Figure 1). In addition, plasmin activity decreased to ~25% during the incubation period, whereas the residual TAFI could still be converted to TAFIa upon addition of thrombin/thrombomodulin (Figure 4). This indicated that the reason that the residual TAFI was not converted to TAFIa was that plasmin activity attenuated. In agreement with the low activity measured, no 35.8-kDa fragment is detected after 60 min (Figure 3B and Figure 5). Mao and co-workers (*13*) already showed previously that TAFIa catalyzed the release of arginines from plasmin and compromised the ability of plasmin to process TAFI, which further slows down the TAFIa generation. Moreover, TAFIa was also reported to inhibit fibrin degradation by plasmin, suggesting that TAFIa attenuates fibrinolysis by inhibiting plasmin directly (*11*, *13*).

The generation of the 44.3-kDa fragment provides an elegant mechanism by which plasmin prevents TAFIa formation. Tyr^{341} and Asp^{349}, which are considered to be involved in substrate binding and determination of substrate specificity, are located C-terminally of Arg^{330} and will therefore be absent in the 44.3-kDa fragment. More importantly, also Glu^{363}, which is one of the amino acids of the catalytic center of TAFIa, will be removed.

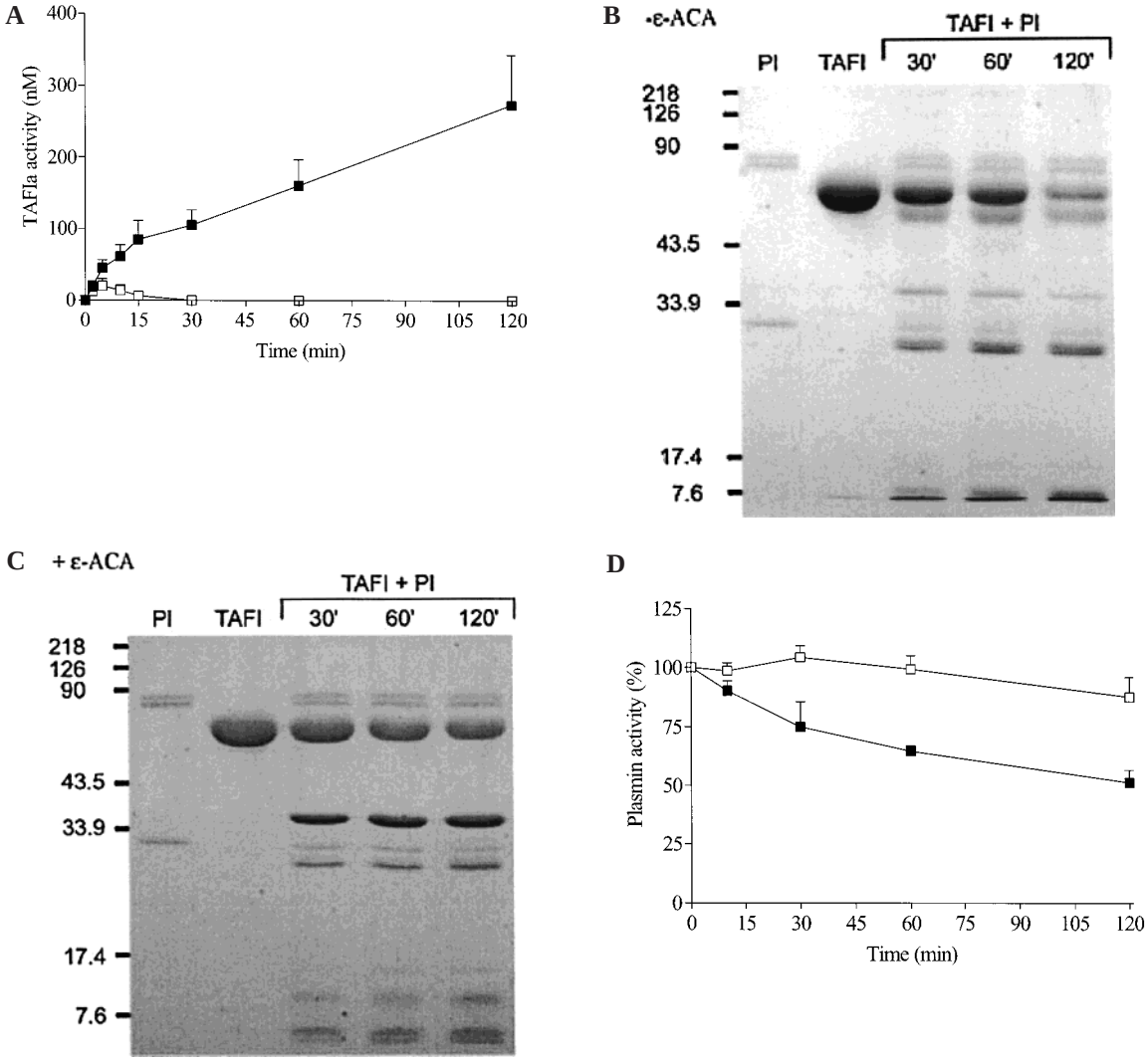

FIGURE 6: Influence of ϵ-ACA on TAFI activation and fragment generation by plasmin. TAFI was incubated with plasmin and $CaCl_2$ at 37° C. Experiments were done in the absence (□) or presence (■) of ϵ-ACA. (A) At various time points samples were taken, trasylol was added to inhibit plasmin activity, and TAFIa activity was measured (data are expressed as mean ±SD, $n = 3$). (B/C) Activation mixtures were also analyzed on SDS−PAGE to visualize fragment generation. Masses of molecular standards are indicted on the left (kDa). (D). Plasmin was incubated with ϵ-ACA and $CaCl_2$ at 37° C in the absence (□) and presence (■) of TAFI and the plasmin activity was measured at several time points (data are expressed ± SD, $n = 3$).

The physiological importance of plasmin as a regulator of TAFIa is not clear to date. Normally, thrombin generation will precede plasmin formation and the role of plasmin mediated TAFI activation, or prevention thereof, during clot lysis might be relatively limited. However, TAFI fragments resembling those generated by plasmin, like the 44.3-kDa fragment, were observed during tissue-type plasminogen activator (t-PA) mediated lysis of thrombin induced clots, and the 44.3-kDa fragment was observed in plasmas of patients treated with recombinant t-PA after myocardial infarction and not in plasmas of untreated patients (*20*). Decreased levels of functional TAFI might enhance the effect of increased fibrinolysis induced by t-PA administration. Decreased levels of activatable TAFI (40%) were also found in plasmas of patients suffering from acute promyelocytic leukaemia, whereas antigen levels were normal (*21*). In vitro experiments revealed that plasmin slightly reduced antigen levels but severely reduced the amount of TAFIa that could be generated by thrombin/thrombomodulin (*21*). This is likely the result of the formation of the 44.3-kDa fragment, since antigen levels were measured using a sandwich ELISA with an antibody directed against the activation peptide. This deficiency of functional TAFI may contribute to the severity of the hemorrhagic diathesis because the protection of the fibrin clot from fibrinolysis is compromised (*21*). These examples illustrate that plasmin is likely to play a role in the regulation of TAFIa activity in vivo.

In the absence of thrombomodulin, the activation of TAFI by thrombin is an inefficient process (K_m 0.5−2.1 μM, k_{cat} 0.0021 s^{-1}) (*12*, *15*, *22*), and consequently large amounts of thrombin are required. Thrombomodulin accelerates the activation of TAFI ~1250-fold (*12*, *15*, *22*). The expression of thrombomodulin varies substantially throughout the vasculature, and therefore also its relative contribution to the activation of TAFI. Compared to thrombin, the K_m for plasmin-mediated TAFI activation is much lower (55 nM), whereas the k_{cat} is 5-fold lower (0.0004 s^{-1}) (*13*). But glycosaminoglycans, which are present in the extracellular matrix and on endothelial cells, increase the k_{cat} and K_m for

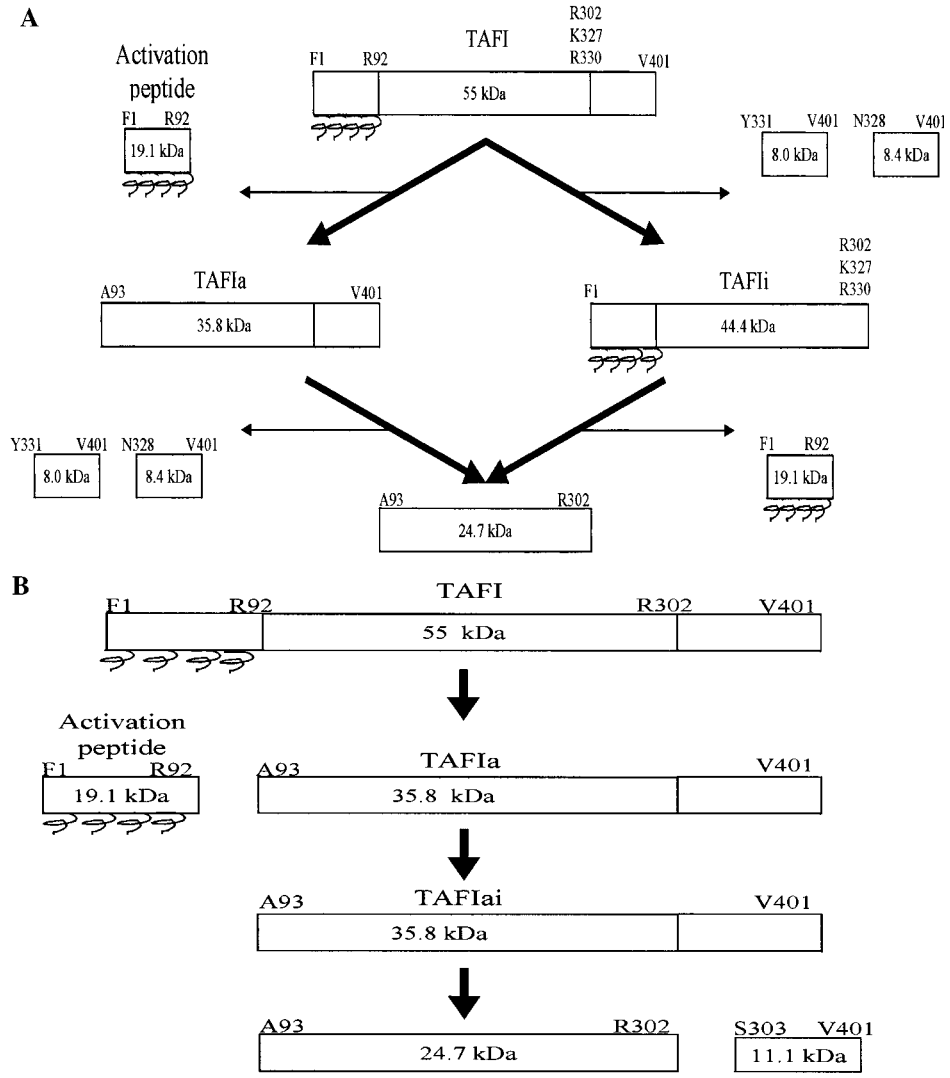

FIGURE 7: Overview of generated TAFI fragments upon incubation with plasmin or thrombin/thrombomodulin. (A) Plasmin proteolyses TAFI at several sites. Plasmin is able to cleave off the glycosylated N-terminal activation peptide of 19.4 kDa from TAFI (55 kDa), which results in the formation of activated TAFI (TAFIa, 35.8 kDa). TAFIa can subsequently be cleaved resulting in 24.7-, 24.5-, 8.0-, and 8.4-kDa polypeptides, and probably some small, nondetected fragments. However, little TAFIa activity is measured and low amounts of the 35.8-kDa fragment are transiently detected on SDS−PAGE. The second possibility is that plasmin removes the C-terminal 8.0-kDa and/or 8.4-kDa fragments by cleavage at Arg^{330} and Arg^{327} respectively, prior to removal of the activation peptide, which results in a 44.3-kDa form of TAFI (TAFIi). The 44.3-kDa fragment is probably no longer activatable, since it lacks a substrate binding site and residues involved in substrate specificity and substrate hydrolysis. The activation peptide may still be removed, which will result in convergence of both pathways. (B) Activation of TAFI by thrombin/thrombomodulin results from cleavage at Arg^{92}, releasing the activation peptide from the catalytic domain. TAFIa is unstable at 37 °C and is inactivated (TAFIai) by a temperature-dependent conformational change. TAFIai is degraded by proteolysis at Arg^{302} resulting in 24.7- and 11.1-kDa fragments. The conformational change may make the protein more susceptible to proteolysis.

plasmin-mediated activation of TAFI, resulting in a catalytic efficiency of only 1/10 that of thrombin/thrombomodulin (*13*), indicating that plasmin may contribute substantially to the regulation of TAFIa activity in vivo.

The observation that ϵ-ACA not only stabilized TAFIa activity, but also increased TAFIa generation while preventing C-terminal cleavage, emphasizes the importance of C-terminal proteolysis of TAFI by plasmin in the prevention of TAFIa formation. Addition of both CPI and ϵ-ACA resulted in accumulation of the 35.8-kDa fragment. CPI is a TAFIa inhibitor, and since more of the 35.8-kDa fragment is formed, the inhibition of TAFIa by CPI is not due to reduced activation of the protein. Apparently, CPI protected TAFI and TAFIa against C-terminal cleavage because more of the 35.8-kDa fragment and less of the 44.3 and 25-kDa fragments were observed. An explanation for the accumulation of the 35.8-kDa fragment during the inhibition of TAFIa by CPI is that TAFIa is inactivated by autoproteolysis and that CPI prevents this. This implies that first TAFIa has to be internally cleaved to generate a C-terminal lysine or arginine residue. However, previous research established that TAFIa generated by thrombin/thrombomodulin inactivates by conformational instability, not proteolysis (*14*, *16*). Mass determination of TAFIa generated by thrombin/thrombomodulin even in the absence of CPI by electrospray analysis and N-terminal sequencing showed clearly that TAFIa stretches from Ala^{93} to Val^{401} (*14*), indicating that TAFIa is not C-terminally proteolyzed. CPI is a 39 amino acid long polypeptide present in potato (*23*). The mechanism by which CPI inhibits carboxypeptidases is probably mediated by the

flexible C-terminal tail of CPI, which docks in the catalytic center of the carboxypeptidase (*23*). X-ray crystallographic analysis of CPI in complex with carboxypeptidase A showed that CPI's C-terminal Gly^{39} is cleaved off but remains associated with the complex, which is stabilized by Tyr^{37} of CPI (*24*, *25*). The exact mechanism by which CPI inhibits TAFIa is unknown. CPI might induce accumulation of the 35.8-kDa fragment by hindering access of proteolytic enzymes to cleavage sites in TAFIa, while preventing access of substrates to the catalytic center or binding sites. Alternatively, CPI may induce a conformational change in TAFIa, which results in an inactive 35.8-kDa fragment. Addition of ϵ-ACA also resulted in accumulation of the 35.8-kDa fragment and, although ϵ-ACA is considered a TAFIa inhibitor, at the concentrations used it did not inhibit conversion of the substrate hippuryl-arginine. ϵ-ACA completely abolished formation of the 44.3-kDa fragment and reduced breakdown of the 35.8-kDa fragment. This suggested that ϵ-ACA protected against proteolysis at Lys^{327} and Arg^{330} and slowed proteolysis at Arg^{302}. Although both ϵ-ACA and CPI are reversible inhibitors of TAFIa, ϵ-ACA is a small molecule, which may, in contrast to CPI, not completely prevent access of small substrates to TAFIa's catalytic center, while keeping TAFIa in its active conformation.

We conclude that plasmin can cleave at several sites in TAFI and that this contributes to the regulation of TAFI and TAFIa. Plasmin is able to proteolyze TAFI at Arg^{92}, which results in TAFIa formation. The generated TAFIa is inactivated by C-terminal proteolysis at Arg^{302}/Lys^{327}/Arg^{330}. Cleavage at the C-terminal cleavage sites in TAFI prior to cleavage at Arg^{92} results in a fragment, which can no longer be converted into an active enzyme.

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