Inactivation of Active Thrombin-activable Fibrinolysis Inhibitor Takes Place by a Process That Involves Conformational Instability Rather Than Proteolytic Cleavage*

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Thrombin-activable fibrinolysis inhibitor (TAFI) is present in the circulation as an inactive zymogen. Thrombin converts TAFI to a carboxypeptidase B-like enzyme (TAFIa) by cleaving at Arg92 in a process accelerated by the cofactor, thrombomodulin. TAFIa attenuates fibrinolysis. TAFIa can be inactivated by both proteolysis by thrombin and spontaneous, temperature-dependent loss of activity. The identity of the thrombin cleavage site responsible for loss of TAFIa activity was suggested to be Arg330, but site-directed mutagenesis of the cleavage site responsible for loss of TAFIa activity was accelerated by thrombin cleavage site and, furthermore, suggested that inactivation of TAFIa is based on its conformational instability rather than proteolytic cleavage at Arg302.

When 125I-TAFI was incubated with thrombin, the generation of a 25-kDa fragment became apparent. During the inactivation, this fragment was further proteolyzed into 25- and 14-kDa fragments (1). The N-terminal residue of the 25-kDa band was identified as Ala30. A TAFI mutant was generated in which Arg330 was changed to Ala. This TAFI mutant was inactivated by thrombin/thrombomodulin, the R330Q-TAFI mutant was activated and inactivated at similar rates as the two wild type forms.

Besides by proteolysis by thrombin, TAFIa is also inactivated by spontaneous, temperature-dependent inactivation (3, 8, 17, 18). The intrinsic fluorescence of TAFIa was quenched during incubation at 37 °C, suggesting that the decrease in activity is due to conformational changes of TAFIa (17).

We studied the mechanism of proteolytic activation of TAFI and inactivation of TAFIa by thrombin/thrombomodulin using HPLC in combination with matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). Furthermore, we analyzed a mutant form of TAFI in which the putative thrombin cleavage site at Arg302 was altered.

EXPERIMENTAL PROCEDURES

Materials—Fresh frozen plasma was obtained from the local blood bank. Bovine serum albumin, hippuryl-Arg, and cyanuric chloride were purchased from Sigma, CNBr-activated Sepharose, Protein G-Sepharose, Q-Sepharose, and PD-10 Sephadex G-25M columns were purchased from Amersham Pharmacia Biotech. Rabbit lung thrombomodulin was purchased from American Diagnostica (Greenwich, CT), HPLC grade acetonitrile from Fisher (Pittsburgh, PA), trifluoroacetic acid from Halocarbon (River Edge, NJ), 1,4-dioxane and 3,5-dimethoxy-4-
hydroxycinnamic acid from Aldrich Chemicals Co. (Milwaukee, WI), and H-γ-Phe-Pro-Arg-chloromethylketone (PPACK) from Bachem (Bubendorf, Switzerland). PefablocSC, restriction enzymes and DNA polymerase were obtained from Roche Molecular Biochemicals (Mannheim, Germany). Thrombin was a generous gift from Dr. W. Kielsi (University of New Mexico, Albuquerque, NM). Dulbecco’s modified medium/F-12 nutrients mixture, penicillin, streptomycin, and T4 DNA ligase were purchased from Life Technologies, Inc. (Paisley, United Kingdom). Other chemicals used were the best grade available.

**Cloning and Expression of Recombinant TAFI (rTAFI) and R302Q-TAFI**—The TAFI cDNA IM.A.G.E. Consortium Clone ID 194171 (19) was amplified by polymerase chain reaction using the forward primer TTCTCCAGGGGATGGAGGAGGACCAGGCTCACTGCC (sequence underlined, XhoI and XbaI sites in italics) and the reverse primer CGGGGTACCTTTAAAACTTCTATGGAC (KpnI site in italic). Polymerase chain reactions (100 μl) contained approximately 50 ng of each primer, 200 μM dNTPs, 2 μg of template DNA, and 5 units of Pwo polymerase. The polymerase chain reaction cycling conditions were as follows: 1 min 95 °C, 1 min 55 °C and 2 min 72 °C for 25 cycles. The polymerase and 200 μM dATPs at 72 °C for 30 min to generate 3′ overhangs for cloning it into the PCR-RII-TOPO vector according to the manufacturer’s recommendations. TAFI was excised from this vector using XhoI and KpnI and ligated into the eukaryotic expression vector pcDNA3.1 (Invitrogen, San Diego, CA). The Arg 302 to Gln mutation was introduced using the PCR system with primers GCCACCATGAAGCTTTGCAGCCTTG (Kozak sequence underlined) and AGACC and the reverse primer GGTCTTTGCTTTTACTTTGTGTATA- CGAATATGGAAACAC (mutated base underlined). Both constructs were confirmed by sequencing, and were found to be in the Thr147 isoform of TAFI (20). Baby hamster kidney cells were grown in Dulbecco’s modified Eagle’s medium/F-12 nutrients mixture supplemented with 5% newborn calf serum and penicillin/streptomycin, at 37 °C in 5% CO2, 5% CO2. Transfections were performed as described previously (21). One day after transfection, G418 (1 mg/ml) was added as selecting agent. Ten days later, surviving clones were picked and TAFI expression was detected by an enzyme-linked immunosorbent assay for TAFI (22). Stably expressing clones were cultured in 300 ml of 175-cm2 flasks. Once cells had grown to confluency, the medium was changed to UltraCHO (30 ml/flask, BioWhittaker, MD). The medium was refreshed twice a week for 3 weeks. The media was in ovo, PefablocSC was added to 0.2 μM and stored at −20 °C until use.

**Purification of TAFI**—Fresh frozen citrated human plasma was obtained from the local blood bank. One unit of plasma (∼300 ml) was supplemented with 5 mM benzamidine-HCl, 3 mM EDTA, 0.05 mg/ml Polybrene, and 0.1 mM e-aminoacproic acid (e-ACA) and applied to a PD-10 Sephadex G-25M column linked to a CNBr-activated Sepharose column to which a monoclonal antibody directed against TAFI (MoAb Nik-9H10 (22), 1 mg/ml, 5 ml) was coupled. Both columns were equilibrated in H2O, 0.1% trifluoroacetic acid. A linear gradient from 0 to 60% acetonitrile in H2O, 0.1% trifluoroacetic acid (1 ml/min, 2%/min) was used to elute bound protein. Eluting peaks were collected separately and lyophilized. Lyophilized HPLC samples were dissolved in 5% acetonitrile in H2O, 0.1% trifluoroacetic acid, extensively vortexed, and subjected to mass spectral analysis.

**Mass Spectrometry**—MALDI-MS was performed on a Dynamo DY-100 mass spectrometer (Thermo BioAnalysis) using 3,5-dimethoxy-4-hydroxycinnamic acid as a matrix. Electrospay ionization mass spectrometry (ESI-MS) was performed on an API-III triple quadrupole electrospray mass spectrometer (PE-Sciex). From the experimental mass to charge (m/z) ratios from all the observed protonation states of the protein, the mass was calculated using MacSpec software (Sciex). The mass found for TAFIa (35,808 Da fragment) was used to calibrate MALDI-MS spectra. Theoretical masses of proteins and protein fragments were calculated using MacProMass software (Beckman Research Institute, Duarte, CA) and Protein Analysis Worksheet Software, PAWS 6.0b2.

**RESULTS**

**Activation of TAFI and Inactivation of TAFIa**—To investigate the regulation of TAFIa activity, TAFI was incubated with thrombin, thrombomodulin, and CaCl2 at 37 °C. At various time points aliquots were taken from the reaction mixture, thrombin activity was inhibited by the addition of PPACK and TAFIa activity toward the substrate hippuryl-Arg was measured. Under these conditions, maximal TAFIa activity was reached within 5 min, after which the activity decreased (Fig. 1).

**Analysis by HPLC and Mass Spectrometry**—To identify the proteolytic fragments generated upon activation and inactivation by thrombin/thrombomodulin, we analyzed samples of the activation mixtures after 0, 10, 30, 60, and 120 min by HPLC (Fig. 2A) and MALDI-MS (Fig. 2B).

At 0 min, one major peak on the HPLC spectrum was observed, with a molecular mass of 55.6 kDa on the MALDI spectrum and corresponding with the molecular mass of TAFI of 55.6 kDa as measured on SDS-PAGE (2). The 55.6-kDa peak was broad, reflecting the heterogeneity of TAFI glycosylation. After 10 min activation, the HPLC spectrum revealed two additional peaks (peak 2 and 3) compared with the starting material. Peak 3 contained a fragment of 19.4 kDa. This fragment, like the 55.6-kDa protein, appeared as a broad peak on fragment.
the MALDI spectrum, reflecting a heterogeneous glycosylated protein. TAFI has four potential N-linked glycosylation sites located in its activation peptide, Asp22, Asp51, Asp63, and Asp86 (2). Therefore, the 19.4-kDa fragment is most likely the N-terminal activation peptide (amino acid 1–92). Peak 2 yielded a mass of 35.8 kDa and a small amount of a 24.5-kDa fragment on MALDI-MS. The peaks on the MALDI spectrum were sharp, indicating that these fragments were homogenous and not glycosylated.

After 30 min, TAFIa activity was no longer detectable (Fig. 1). The HPLC spectrum showed the same peaks as after 10 min with one additional peak, peak 4, which appeared as a shoulder of peak 2. Peaks 2 and 4, analyzed together by MALDI-MS, contained 2 fragments of 35.8 and 24.5 kDa. The amount of the 24.5-kDa fragment had increased considerably compared with 10-min TAFI activation, whereas the 55.6-kDa TAFI peak had decreased. After 60 min peaks 2 and 4 containing the 24.5- and 35.8-kDa forms were the major peaks on HPLC and MALDI.

After a 120-min incubation, the major fragment found was the 24.5-kDa fragment whereas minor amounts of the starting material and the 35.8-kDa form were still detected. Peak 5 was also analyzed, it contained a 11.1-kDa polypeptide.

**Mass Matching**—To identify the proteolytic fragments within the TAFI sequence, the experimentally obtained masses were matched to theoretical masses. In addition, the N-terminal sequences of the fragments were determined. The masses of the 55.6- and 19.4-kDa proteins could not be calculated due to the presence of glycosylated residues. The observed 55.6-kDa protein corresponded to the reported mass of TAFI (2) and started with TAFI’s N-terminal sequence Phe-Gln-Ser-Gly-Gln, thus confirming its identity. The 19.4-kDa fragment most likely represented the activation peptide (amino acid 1–92). The observed 35,808 Da fragment was analyzed by ESI-MS and revealed a molecular mass of 35,808 ± 9 Da (Fig. 3), which corresponds to the mass of TAFIa. This mass was used to calibrate the masses found by MALDI-MS, whenever the 35.8-kDa fragment was present in the sample.

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Fig. 1. Activation of TAFI and inactivation of TAFIa by thrombin/thrombomodulin. TAFI (2.8 μM) was incubated with thrombin (20 nM), thrombomodulin (5 nM), and CaCl₂ (5 mM) at 37 °C. At various time points aliquots were taken from the activation mixture, diluted to 0.2 μM TAFI, and thrombin activity was inhibited by the addition of PPACK. Then TAFIa activity toward the substrate hippuryl-Arg was measured. Data are expressed as mean ± S.D. (n = 3).

Fig. 2. HPLC profile (A) and MALDI spectrum (B) during TAFI activation and TAFIa inactivation. TAFI (5.4 μM) was incubated with thrombin (20 nM), thrombomodulin (5 nM), and CaCl₂ (5 mM) for 10, 30, 60, or 120 min as indicated, after which generated fragments were purified by HPLC (0–60% acetonitrile, 2%/min) (A) and analyzed by MALDI-MS (B). Note broad MALDI-MS peaks (peaks 1 and 3) due to glycosylation heterogeneity. Peak 2 contains TAFIa. See text for further analysis of the fragments.

Fig. 3. ESI-MS analysis of the 35,808-Da TAFIa fragment. TAFI (5.4 μM) was incubated with thrombin (20 nM), thrombomodulin (5 nM), and CaCl₂ (5 mM) at 37 °C. After 10 min thrombin activity was stopped by addition of PPACK. The generated fragments were separated by HPLC and the peak containing the 35.8-kDa fragment (peak 4 in Fig. 2A) was analyzed by ESI-MS. Mass reconstruction using the 15th-40th charged state revealed a molecular mass of TAFIa of 35,808 Da.

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tidase B activity of TAFIa itself. The results are summarized in Table I.

Stability of TAFIa—Comparison of the appearance and disappearance of the activity with the generation of fragments indicated that the presence of TAFIa did not correlate very well with the presence of the 35.8-kDa form. The 35.8-kDa fragment was present for at least 60 min, whereas the activity was no longer detectable after 30 min. Analysis of the reaction mixture under reducing conditions on SDS-PAGE yielded similar molecular weight fragments as obtained under nonreducing conditions with MALDI-MS. This suggests that the 35.8-kDa form of TAFIa was inactivated spontaneously, without detectable proteolytic cleavage. To study the discrepancy between the presence of the 35.8-kDa fragment and TAFIa activity, TAFI was activated for 10 min by thrombin/thrombomodulin whereafter thrombin activity was inhibited by PPACK. In this way, proteolytic inactivation of TAFIa by thrombin was prevented, whereas spontaneous inactivation was allowed. TAFIa activity and fragment formation were analyzed in time by the activity assay (Fig. 4A) and SDS-PAGE (Fig. 4B), respectively. In the absence of PPACK, proteolysis continued whereas in the presence of PPACK, no further proteolysis was observed (Fig. 4B). Despite the absence of proteolytic fragmentation, the rate of TAFIa inactivation in the presence of PPACK was identical to the rate of TAFIa inactivation in the absence of PPACK (Fig. 4A). Addition of e-ACA, which was shown before to prevent spontaneous inactivation of TAFIa (11, 17, 18), resulted in reduced rates of inactivation both in the presence and absence of PPACK (Fig. 4A). Presence of e-ACA seemed to slow down proteolysis in the absence of PPACK (Fig. 4B). These experiments suggest, in agreement with the results obtained with the MALDI-MS, that inactivation of TAFIa takes place in a process involving spontaneous inactivation rather than proteolysis.

Analysis of R302Q-TAFI—The role of proteolytic inactivation of TAFIa by thrombin was investigated by analysis of a mutant form of TAFIa in which the putative cleavage site Arg302 was changed to Gln (R302Q-TAFI). R302Q-TAFI exhibited activity upon incubation with thrombin/thromobomodulin toward the substrate hippuryl-Arg (Fig. 5A), although its specific activity was approximately half that of either TAFI or plasma-derived TAFI. SDS-PAGE analysis of the activation mixtures of R302Q-TAFI and rTAFI at the indicated time points (Fig. 5B), showed that the 35.8-kDa fragment of R302Q-TAFI was not proteolyzed by thrombin, even when 10 times higher concentrations of thrombin/thromobomodulin were used. This suggested that Arg302 is indeed the major cleavage site involved in proteolytic degradation of TAFIa by thrombin/thrombomodulin. Furthermore, this suggested that cleavage at Arg302 is not involved in TAFIa inactivation. As was observed for plasma-derived TAFIa, the presence of e-ACA stabilized R302Q-TAFIa and rTAFIa and resulted in the generation of more TAFIa activity.

DISCUSSION

TAFI is activated by thrombin in a process that is stimulated by thrombomodulin. Inactivation of TAFIa was first reported to take place by proteolytic cleavage at Arg330 by thrombin, plasmin, and trypsin. However, a mutant form of TAFIa in which Arg330 was replaced by Gln was still inactivated by thrombin/thrombomodulin, indicating that cleavage at Arg330 was not

### Table I

**Mass analysis and fragment identification of TAFI cleavage fragments**

Theoretical masses of protein fragments based on average isotope composition were calculated and matched to experimental masses. For proteins containing the activation peptide (residues 1–92) masses are calculated without the carbohydrate content. The identity of the fragments was confirmed by N-terminal sequencing.

| Fragment          | Theoretical mass | Experimental mass | N-terminal sequence |
|-------------------|------------------|------------------|--------------------|
| Phe<sup>1</sup>-Val<sup>101</sup> | 45,999 | 55.6 kDa | Phe-Gln-Ser-Gly-Gln |
| Phe<sup>1</sup>-Arg<sup>28</sup> | 10,200 | 19.4 kDa | ND<sup>b</sup> |
| Ala<sup>33</sup>-Val<sup>101</sup> | 35,813 | 35,808 Da | Ala-Ser-Ala-Ser-Tyr-Tyr-Glu |
| Ala<sup>33</sup>-Thr<sup>201</sup> | 24,526 | 24,494 Da | Ala-Ser-Ala-Ser-Tyr-Tyr-Glu |
| Ser<sup>202</sup>-Val<sup>401</sup> | 11,149 | 11,120 Da | Ser-Lys-Ser-Lys-Asp-His |

<sup>a</sup> ND, not determined.

<sup>b</sup> Average mass of 4 MALDI-MS measurements.
Our results thus suggest that the major thrombin/thrombomodulin cleavage site in TAFIa, is Arg302. This was supported by a recombinant TAFI mutant in which Arg302 was replaced by Gln. R302Q-TAFI was not proteolysed by thrombin/thrombomodulin into the 24.5- and 11.1-kDa fragments indicating that Arg302 is indeed the major cleavage site of thrombin/thrombomodulin in TAFIa. This is in contrast to previous reports that identified Arg330 as the major cleavage site (2). However, trypsin and plasmin were used in a part of those studies and it is possible that those enzymes cleaved TAFIa at different sites then Arg302. In addition, small differences in molecular weight might have been undetectable on SDS-PAGE, when plasmin, trypsin, and thrombin/thrombomodulin cleavage patterns were compared. It should be noted that thrombin/thrombomodulin contains both free thrombin and thrombin/thrombomodulin complex.

There was a discrepancy between the time course for loss of TAFIa activity and the persistent presence of the 35.8-kDa polypeptide that is considered to be the activated form of TAFI. Notably, the fragment was still present at times that TAFIa activity was no longer detectable. This was previously reported (8) and explained by intrinsic instability of TAFIa, suggesting that inactivation of TAFIa can take place by a process that does not involve proteolysis. In agreement with this, we found that R302Q-TAFI, which cannot be cleaved at Arg302, was still inactivated. This strongly supports that inactivation of TAFIa does not require any proteolytic cleavage but can be entirely caused by conformational instability of TAFIa. This hypothesis was further supported by experiments in which thrombin activity was inhibited by PPACK after activation of TAFI. Inactivation of TAFIa continued at the same rate as in the absence of PPACK, despite the fact that proteolysis by thrombin was prevented, suggesting that cleavage at Arg302 was not essential for inactivation. Furthermore, ε-ACA was shown to stabilize TAFIa, whereas proteolytic cleavage still continued at a slower rate.

Our results indicate that inactivation of TAFIa is the result of the conformational instability of TAFIa and not a direct result of proteolysis of TAFIa. It is possible that the conformational changes that take place as a result of the conformational instability make the inactivated 35.8-kDa form more susceptible to proteolysis. The mechanism by which ε-ACA stabilizes TAFIa is unknown, but is likely to involve lysine-binding sites. C-terminal lysine residues of partially degraded fibrin may have a similar stabilizing effect on TAFIa. If so, TAFIa would be most effective in situations where partially degraded fibrin is present because fibrin-bound TAFIa would presumably be resistant to spontaneous loss of TAFIa activity.

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**FIG. 5. Analysis of R302Q-TAFI.** A, rTAFI (2.8 μM, closed symbols) and R302Q-TAFI (2.8 μM, open symbols) were incubated with thrombin (20 nM), thrombomodulin (5 nM), and CaCl₂ (5 mM) at 37 °C in the presence (squares) or absence (circles) of ε-ACA (5 mM). At indicated times aliquots were taken from the activation mixtures, diluted to 0.2 μM rTAFI and 0.4 μM R302Q-TAFI and TAFIa activity toward hippuryl-L-Arg was measured. B, rTAFI (2.8 μM) and R302Q-TAFI (2.8 μM) were incubated with thrombin (200 nM), thrombomodulin (50 nM), and CaCl₂ (5 mM) at 37 °C. Aliquots of the activation mixtures were removed and analyzed on SDS-PAGE (15%) under reducing conditions. Molecular weights of the fragments are indicated on the left.
Regulation of Active TAFI

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Inactivation of Active Thrombin-activable Fibrinolysis Inhibitor Takes Place by a Process That Involves Conformational Instability Rather Than Proteolytic Cleavage

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