A Study of the Mechanism of Inhibition of Fibrinolysis by Activated Thrombin-activable Fibrinolysis Inhibitor*

(Received for publication, November 19, 1997, and in revised form, July 28, 1998)

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TAFI (thrombin-activable fibrinolysis inhibitor) is a recently discovered 60-kDa single-chain plasma protein that can be activated by thrombin-catalyzed proteolysis to a carboxypeptidase B-like enzyme that inhibits fibrinolysis (1, 2). It is present in plasma at a concentration of about 75 nM (3). TAFI was recently discovered plasma carboxypeptidase B by Eaton et al. (6) and subsequently plasma carboxypeptidase B (pro-plasma carboxypeptidase B) by Tan and Eaton (7). In addition, on the basis of its instability in serum, it probably can be identified as carboxypeptidase R described by Campbell and Okada (8).

Recent studies show that the thrombin-thrombomodulin complex, rather than free thrombin, is probably the physiologic activator of TAFI (2). In addition, activated TAFI (TAFIa) down-regulates tissue plasminogen activator (t-PA)-induced fibrinolysis half-maximally at a concentration of 1.0 nM (2). Because this is only about 1.3% of the level of the zymogen in plasma, ample TAFIa could be generated to modulate fibrinolysis very significantly in vivo (2). Bajzar et al. (3) showed that the apparent pro-fibrinolytic effect of activated protein C is absent in TAFI-deficient plasma or when plasma is supplemented with an anti-TAFI monoclonal antibody. They also showed in plasma systems supplemented with soluble thrombomodulin or in systems utilizing cultured endothelial cells as a source of thrombomodulin, that fibrinolysis is inhibited when and only when TAFI is activated (9). In addition, Redlits et al. (10) showed that activated pro-plasma carboxypeptidase B and plasma carboxypeptidase N diminish the binding of plasminogen to U937 cells and that fibrinolysis occurs more rapidly in pro-plasma carboxypeptidase B (TAFI)-deficient compared with normal plasma. These observations suggest that the coagulation and fibrinolytic cascades are linked through TAFI and that TAFI might participate fundamentally in the regulation of the fibrinolytic response. As a consequence, the following studies were carried out to elucidate the mechanism(s) by which TAFIa suppresses fibrinolysis.

EXPERIMENTAL PROCEDURES

Materials—The synthetic carboxypeptidase substrate hippuryl-l-arginine, and l-arginine, l-lysine, octopine dehydrogenase, and saccharo-pine dehydrogenase were obtained from Sigma. The chromogenic substrate S-2251 was purchased from Helena Laboratories (Beaumont, TX). The carboxypeptidase B inhibitor 2-guanidinoethylmercaptosuccinic acid and the plasmin inhibitor VKF-CMK were purchased from Calbiochem. NADH and carboxypeptidase B were purchased from Boehringer Mannheim. Na125I (100 mCi/ml) was purchased from ICN Biomedicals (Montreal, PQ), and IODO-BEADs were purchased from Pierce Chemical Company. DAPA, a specific thrombin inhibitor, was synthesized and isolated according to the method of Nesheim et al. (11). The human proteins fibrinogen, plasminogen, prothrombin, factor V, and antithrombin III were isolated from plasma; the enzymes thrombin, factor Xa, and plasmin and phospholipid vesicles containing 75% phosphatidylycholine and 25% phosphatidylserine were prepared as described previously (1). t-PA (Activase) was a generous gift of Dr. Gordon Vehar of Genentech (South San Francisco, CA). Recombinant soluble thrombomodulin (Solulin) was obtained as a generous gift from Dr. John Morser and colleagues at Berlex Biosciences (Richmond, CA). Recombinant human α1-antiplasmin was isolated from culture supernatants of baby hamster kidney cells transfected with the human cDNA and grown in serum-free medium, as described before (1). TAFI was purified from human plasma according to the method of Bajzar et al. (1).
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Measurement of Inhibition of t-PA-Mediated Fibrinolysis by TAFIa—
TAFIa was activated to the active species, TAFIa, by the thrombin-
thrombomodulin complex according to the method of Bajzar et al. (2).
The inhibition of t-PA-mediated fibrinolysis by TAFIa was then meas-
ured by lysis assays performed in a system of purified fibrinolytic con-
stituents preincubated fibrin clots.

Determination of the Release of Free Arginine and Lysine by TAFIa during Clot Lysis—Free arginine and lysine released from fibrin in the presence of TAFIa were measured during t-PA-mediated fibrinolysis. Clots were produced in the presence of various concentrations of TAFIa (0–63 nM), and lysis times were monitored. Once the clot lysed completely, the samples were deproteinized by perchloric acid (0.2 M, final), and subsequently neutralized by KOH. The insoluble potassium perchlorate was removed by centrifugation at 4 °C. The supernatants were used for determination of arginine and lysine. In separate experiments, the time courses of the release of arginine and lysine by TAFIa were measured by solubilizing fibrin clots with acetic acid (0.1 M, final), thereby quenching all reactions, at regular intervals over 3 h. The concentrations of arginine and lysine were determined according to methods similar to those described by Gaede et al. (12) and Nakatani et al. (13). 100 µl of supernatant or a standard solution containing known concentrations of arginine or lysine was added to the wells of a microtiter plate reader attached to a Perkin-Elmer model LS50B spectrophotofluorometer. The excitation and emission wavelengths were 340 and 450 nm, respectively, and a 430 nm cutoff filter was employed in the emission beam. The concentrations of arginine and lysine were determined according to standard curves that were constructed by plotting 2ΔEm,295 of the standard samples versus the concentration of arginine or lysine.

Effect of TAFIa on Plasmin-induced Clot Lysis—To study the effect of TAFIa on plasmin-induced clot lysis, clots were produced by the addition of 100 µl of a solution of fibrinogen (3.0 µM, final) to wells containing 2 µl of plasmin (2 nm, final), 2 µl of thrombin (6 nm, final) and CaCl2 (10 mm, final), and 7 µl of TAFIa (0–120 nm, final). Turbidity at 405 nm was then recorded at 37 °C over 10 h. One series of identical clots was formed in the absence of TAFIa and the other two in the presence of 25 and 50 nM TAFIa, respectively. As described previously (1), clots were solubilized at various times and reactions quenched by adding acetic acid (0.1 M, final). Equal volumes of a solution of S-2251 (500 µM) in 437 mM HEPES, containing 10 mM EDTA, 2 mM enantiomeric capric acid, and 40 µM DAPA, pH 8.0, were added to the wells, and the time courses of the increase of absorbance at 405 nm were then monitored. The initial rates of S-2251 hydrolysis, relative to that at zero time, were determined to infer plasmin levels. Similar experiments were performed in the absence of fibrinogen.

RESULTS

Release of Arginine and Lysine from Fibrin by TAFIa during Fibrinolysis—Previous studies showed that the inhibitory effect of TAFIa on fibrinolysis correlates with carboxypeptidase activity measured with hippuryl-L-arginine (1), suggesting that the antifibrinolytic effect of TAFIa can most likely be attributed to the removal of COOH-terminal arginine and lysine residues from partially degraded fibrin in the clot. We therefore measured free arginine and lysine in completely lysed clots. Released arginine and lysine were found in all samples that contained TAFIa. The lysis times were 33 min in the absence of TAFIa and 92 min in the presence of 63 nM TAFIa. No free arginine or lysine was detected in the absence of TAFIa. We then determined the time course of the release of free arginine and lysine from fibrin by TAFIa during clot lysis (Fig. 1). The arginine level rose to about 9.8 µM immediately after clotting, most likely because of the release of fibrinopeptides A and B during the clotting of the 3.0 mM input fibrinogen (two fibrinopeptides A and B were released from fibrin molecules). The arginine level then increased gradually to about 25 µM by 180 min. The lysine level rose smoothly to about 6 µM over the same interval. In separate experiments, the effects of free arginine and lysine on fibrinolysis, at the levels found with TAFIa, were tested by adding them to the lysis assay system, and no prolongation of lysis time was observed. Thus, the...
inhibition of lysis by TAFIa correlates with the release of, but is not caused by, free arginine and lysine in the clot.

Effects of TAFIa on Fibrin-dependent Plasminogen Activation—Plasminogen activation by t-PA is stimulated several hundredfold by fibrin, and this cofactor activity is augmented by plasmin-catalyzed feedback modification of fibrin, whereby carboxyl-terminal lysine and arginine residues are generated (14, 15, 17, 18). Because TAFIa is a carboxypeptidase B-like enzyme, it might possibly prevent or attenuate the effect of the plasmin-catalyzed feedback cleavages in fibrin. To explore this possibility the effects of TAFIa on the kinetics of cleavage of Glu-I and Lys29(S741C-fluorescein) plasminogen were investigated. These plasminogen derivatives do not generate plasmin; thus, plasmin feedback does not occur unless native plasminogen is included also. Results obtained when the time course of cleavage of the fluorescent plasminogen derivative, catalyzed by one-chain or two-chain t-PA within a clot, are shown in Fig. 3. The control results (no plasma plasminogen, no TAFIa) are shown by the solid circles. The results obtained when 5.0 nM plasminogen was included are shown by the solid squares. These data show that initially, rates of fluorescent plasminogen consumption are the same. Later in the course of the reaction, however, the fluorescent plasminogen is consumed more rapidly when native plasminogen is present to generate plasmin, thereby modifying fibrin and accelerating the reaction. This effect is attenuated progressively when TAFIa is included at increasing concentrations. When the TAFIa concentration is 5.0 nM, the effect of included native plasminogen is eliminated completely. This occurs with both one- and two-chain t-PA. These effects are quantified in Fig. 3, where the rate constants for plasminogen activation are shown as a function of time over the first 1,600 s of the reaction. With both one- and two-chain t-PA the rate constant evaluates to $1 \times 10^{-4}/s$ in the absence of added plasma plasminogen or TAFIa (solid circles). The near constancy of this value over time in the controls indicates that the reaction is approximately first order, which is consistent with the fairly high $K_m$ (0.5 μM) for this reaction under these conditions (15). They also show that one- and two-chain t-PA are identically active under these conditions. When a trace of plasma plasminogen is included, the value of the rate constant increases up to about $2.5 \times 10^{-4}/s$ (solid squares), presumably as a result of plasmin-catalyzed modification of fibrin by plasmin, with a concomitant enhancement of cofactor activity. This effect is attenuated progressively by TAFIa, with the half-maximal effect at approximately 1.0 nM TAFIa (solid triangles). At 5.0 nM TAFIa (open squares) no increase in the rate constant is observed. The effects with one- and two-chain t-PA were very similar. Although the data are not presented, the magnitude of the increase in rate constants was not increased by higher levels of plasminogen. These data thus confirm that plasmin formation in the early stages of fibrinolysis can promote the kinetics of t-PA-induced plasminogen activation as shown by others (17, 18) and that TAFIa eliminates this effect, presumably by removing newly formed carboxyl-terminal lysine and possibly arginine residues within fibrin. The inclusion of plasma plasminogen had no effect on the kinetics of cleavage of Lys29-Plg(S741C-fluorescein) (not shown).

Effect of TAFIa on the Activation of Plasminogen and Conversion of Glu-plasminogen to Lys-plasminogen during Fibrinolysis—Previous work, in which the flow of plasminogen to plasmin during the fibrinolytic process within a clot was analyzed, showed that about 50% of the plasmin generated by the time the clot lysed was obtained through Lys-plasminogen (19). The following experiments were performed to determine whether TAFIa influences this process. With radiolabeled Glu-plasminogen, we monitored by urea/acetic acid-polyacrylamide
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absence of TAFI both forms were evident. We conclude that TAFIa not only prevents the feedback enhancement of plasminogen activation but also interferes with the fibrin-dependent conversion of Glu-plasminogen to Lys-plasminogen and attenuates the contribution of this reaction to the accelerated phase of plasminogen activation. These data are consistent with the conclusions of others that partially degraded fibrin, but not intact fibrin, is the cofactor for the conversion of Glu-plasminogen to Lys-plasminogen (17, 18).

Effect of TAFIa on Plasmin-induced Fibrinolysis and Plasmin Activity—To determine whether TAFIa inhibits fibrinolysis by modes other than inhibition of plasminogen activation and the conversion of Glu-plasminogen to Lys-plasminogen, the lysis of fibrin directly catalyzed by plasmin was studied in the presence and absence of TAFIa. Fig. 5 shows the time courses of plasmin-induced lysis of clots. In the absence of TAFIa, the clot lysed at about 60 min with 2 nM plasmin. In the presence of TAFIa, the lysis times were prolonged in a TAFIa concentration-dependent manner. The concentrations of TAFIa required to prolong lysis initiated by plasmin are considerably greater than those needed to prolong lysis initiated by t-PA plus plasminogen (the half-maximal effect is observed at 1.0 nM TAFIa (2)). Nonetheless, because the concentration of TAFI in human plasma is about 75 nM (9) and the TAFI is exquisitely sensitive to activation by thrombin-thrombomodulin (2), sufficient TAFIa possibly could be generated in vivo to attenuate plasmin-catalyzed fibrin degradation directly. The observation that TAFIa markedly inhibits lysis of fibrin by plasmin suggests that TAFIa can inhibit plasmin activity directly. Therefore, plasmin activity during fibrinolysis was measured (Fig. 6).

In the absence of TAFIa, plasmin activity in the clot was stable. In the presence of TAFIa, however, plasmin activity was progressively lost. Thus, the inhibition of plasmin activity in the presence of TAFIa is consistent with prolongation of lysis initiated by plasmin. Although the data are not shown here, very similar results were obtained when fibrinogen was deleted from the experiment, thus indicating that fibrin or fibrin degradation products are not in some way responsible for the apparent inhibition. Binding of TAFIa to plasmin does not explain the inhibition because the kinetics of inhibition are too slow to be consistent with simple binding (7). To determine whether TAFIa can catalyze removal of the carboxyl-terminal

**gel electrophoresis the activation of plasminogen and the conversion of Glu-plasminogen to Lys-plasminogen during fibrinolysis in the absence and presence of TAFIa.** Fig. 4A shows the time course of Glu-plasminogen consumption and concomitant formation of plasmin-antiplasmin complexes for clots formed in the presence and absence of TAFIa. The lysis times were 53 min in the absence of TAFIa and 104 min in the presence of TAFIa (arrows).

**Effect of TAFIa on the activation of plasminogen and the conversion of Glu-plasminogen to Lys-plasminogen during fibrinolysis.** Shown in panel A are the time courses of the concentrations of Glu-plasminogen (○, □) and plasmin-antiplasmin complexes (▲, ■) when fibrinolysis was initiated by t-PA in a clot. The triangles represent results obtained without TAFIa and the squares, results with it. The concentrations of Lys-plasminogen are shown in panel B. Lysis times were 53 min in the absence of TAFIa and 104 min in the presence of TAFIa (arrows).

![Fig. 5. Inhibition of plasmin-induced clot lysis by TAFIa.](image-url)

**FIG. 5. Inhibition of plasmin-induced clot lysis by TAFIa.** TAFIa solutions (7 μl) were pipetted into the wells of a microtiter plate containing small, separated aliquots of plasmin (2 nM, final), thrombin (6 nM, final), and CaCl₂ (10 mM, final). Clots were produced by adding 100 μl of a fibrinogen solution (3 μg, final) to the wells. Turbidity at 405 nm was then recorded at 37 °C over 10 h. The final TAFIa concentrations for the profiles from left to right were 0, 30, 60, 80, 100, and 120 nM.
argine of the heavy chain, active-site blocked plasmin (4.0 μM) was incubated with TAFIa (100 nM) for 30 min. Over this interval arginine (3.6 μM) was released. Thus, TAFIa is able to remove the single carboxyl-terminal arginine that appears when plasminogen is converted to plasmin. This, however, is not sufficient to account for loss of plasmin activity because porcine pancreatic carboxypeptidase B also catalyzes release of arginine from active-site-blocked plasmin but does not inactivate native plasmin (data not shown). Perhaps TAFIa has other exo peptidase activities not revealed by our current studies.

**DISCUSSIONS**

Numerous previous studies have shown that a carboxypeptidase with specificity for COOH-terminal arginine and lysine residues can attenuate several reactions and interactions associated with fibrinolysis. For example, de Vries et al. (20) showed that immobilized, plasmin-treated fibrin, after exposure to porcine carboxypeptidase B, exhibits reduced capacity for the binding of t-PA. Miles et al. (21) showed that treatment of U937 cells with the same carboxypeptidase reduces their capacity to bind plasminogen, presumably by removing the COOH-terminal lysine residue of the enolase moiety that appears to serve as a plasminogen receptor. Carboxypeptidase B-catalyzed removal of the COOH-terminal lysine residue of the heavy chain of urokinase decreases its catalytic efficiency about 2-fold in the activation of plasminogen (22). Fleury and Angles-Cano (23) demonstrated increased plasminogen binding capacity of immobilized fibrin that had been pretreated with plasmin, and the excess capacity could be eliminated with pancreatic carboxypeptidase B (23). In addition, carboxypeptidase B treatment of partially degraded fibrin reduces urokinase-mediated plasminogen activation (24) and reduces the quantity of plasminogen associated with fibrin during the lytic process (25).

The studies cited above clearly indicate the importance of carboxyl-terminal lysine and possibly arginine residues of partially degraded fibrin or cellular receptors in modulating binding of fibrinolytic components and fibrinolysis. These studies, however, did not allow the inference to be drawn that physiologically significant regulation could be associated with removal of these residues because the results were obtained with the pancreatic carboxypeptidase B. Eaton et al. (6), however, showed that plasma contains a precursor of carboxypeptidase B-like enzyme. Because it binds plasminogen they suggested that it might influence fibrinolysis when activated. Further work reported by Redlitz et al. (10) showed that this protein reduces the rate of whole blood clot lysis induced by t-PA. Other work led to the isolation of the protein (1, 4, 26) and showed that it was activated by the thrombin-thrombomodulin complex to the expected carboxypeptidase B-like enzyme (2). The activated enzyme was shown to suppress fibrinolysis potently in a system of defined components as well as in plasma (1, 2, 26). Thus, because the precursor is found in plasma and is activated by a physiologic activator, physiologic modulation of fibrinolysis by this carboxypeptidase B-like enzyme is plausible.

The present studies show that when fibrin is exposed to plasmin, stoichiometric levels of COOH-terminal arginine and lysine residues are generated which can be removed by TAFIa. To determine whether the removal of these residues influences plasminogen activation, the kinetics of cleavage of Glu-Plg(S741C)-fluorescein were measured in the presence and absence of TAFIa under conditions where limited plasmin degradation was allowed by including a trace of plasma plasminogen. The results showed that the rate constant for cleavage increases 2.5-fold during the reaction when plasma plasminogen is included, an effect that is eliminated completely with TAFIa present at 5.0 nM. A half-maximal effect is achieved at a TAFIa concentration of 1.0 nM, which is the same concentration needed to prolong fibrinolysis half-maximally (2). We conclude therefore that one of the means by which TAFIa suppresses fibrinolysis is by down-regulating the cofactor activity of partially degraded fibrin.

We also showed that TAFIa, at relatively elevated concentrations, interferes directly with plasmin-catalyzed fibrin degradation. The interplay between plasmin and TAFIa is complex. TAFIa can inhibit plasmin and vice versa. In addition, TAFIa activity decays spontaneously, especially at 37 °C (27). When TAFIa and plasmin are together, the long term result is that plasmin activity declines to either none or a finite, stable amount, whereas TAFIa decays completely. The decline of plasmin activity to a stable, finite level that depends on the initial concentration of TAFIa is evident in Fig. 6.

A previous study from this laboratory indicated that when plasminogen, α2-antiplasmin, and t-PA are present within fibrin, approximately 50% of the plasmin formed during the lytic process is produced through the Lys-plasminogen intermediate (19). Because Lys-plasminogen is a much better substrate than Glu-plasminogen, the conversion of Glu-plasminogen to Lys-plasminogen potentially creates positive feedback in plasminogen activation and thereby promotes fibrinolysis. The formation of Lys-plasminogen is promoted by fibrin (18, 28) and consequently might be susceptible to modulation by TAFIa. The present work indicates that the accumulation of Lys-plasminogen during plasminogen activation and subsequent fibrinolysis is eliminated by TAFIa, and the time to achieve lysis is increased. These observations suggest that one of the means by which TAFIa suppresses fibrinolysis is by down-regulating the conversion of Glu-plasminogen to Lys-plasminogen. They also suggest that the reaction is dependent on, and promoted by, COOH-terminal lysine and/or arginine residues in fibrin. Notably, the data of Bajzar et al. (1) indicate that TAFIa has little influence on the activation of Lys-plasminogen. Thus, the conversion of Glu-plasminogen to Lys-plasminogen provides a means through which the fibrinolytic system can attenuate the inhibition elicited by TAFIa.

**FIG. 6. Inhibition of plasmin activity by TAFIa during fibrinolysis.** Three series of 12 identical clots containing 4 nM plasmin were formed at 37 °C, one in the absence (●) of TAFIa and another two in the presence of 25 nM (▲) and 50 nM (▼) TAFIa, respectively. At various times clots were solubilized and quenched by adding acetic acid (0.1 M, final). Equal volumes of a solution of S-2251 (500 μM) in 437 mM HEPES, 10 mM EDTA, 2 mM e-amino caproic acid, and 40 μM DAPA, pH 8.0, were added to the wells, and the time courses of increase of absorbance at 405 nm were then monitored. The initial rates of S-2251 hydrolysis, relative to that at zero time, were determined and are plotted versus time.
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Hortin et al. (29) demonstrated that the COOH-terminal lysine residue of α2-antiplasmin is removed in the presence of porcine pancreatic carboxypeptidase B with a consequent substantial loss of functional activity. Whether TAFIa modulates the properties of α2-antiplasmin was not investigated in this work. TAFIa activity expressed \textit{in vivo}, however, could account for the observation that plasma has two forms of α2-antiplasmin, one of which both binds to plasminogen and has a COOH-terminal lysine and the other of which has neither of these properties (30, 31).

A recent study by Sakharov et al. (32) showed that the time to lyse clotted fibrin could be prolonged up to 4-fold by adding thrombomodulin, and this effect correlated with the activation of procarboxypeptidase B (TAFI). The effect was sustained over a wide range of t-PA concentrations, including those that would be expected during thrombolytic therapy. In addition, they showed that fluorescently labeled plasminogen accumulates on fibrin in the absence of added thrombomodulin but not in its presence. This latter effect could be eliminated with an inhibitor of TAFIa. From these observations, the conclusion was reached that TAFIa attenuates fibrinolysis by eliminating, by virtue of its carboxypeptidase B activity, plasminogen binding sites in partially degraded fibrin. The work by Sakharov and the present work are thus highly complementary. Sakharov et al. demonstrated TAFIa-dependent diminishing of plasminogen binding to fibrin but did not study the impact of this on plasminogen activation. In the present work, the binding of plasminogen to fibrin was not studied, but loss of the up-regulation of fibrin cofactor activity was demonstrated. The two studies suggest that loss of plasminogen binding and attenuation of cofactor activity are related, and both are caused by the TAFIa-catalyzed removal of carboxyl-terminal lysine (and possibly arginine) residues.

In summary, the current work shows that TAFIa suppresses fibrinolysis by down-regulating plasminogen activation and the conversion of the Glu-plasminogen to Lys-plasminogen, effects most likely caused by the removal of COOH-terminal lysine and/or arginine residues from partially degraded fibrin. These effects occur at very low relative concentrations of TAFIa. In addition, at higher concentrations, TAFIa also inhibits plasmin directly. Whether TAFIa also influences α2-antiplasmin or other interactions associated with fibrinolysis is not known, but further work relating to modulation by TAFIa of other fluid phase or cellular reactions or interactions will contribute new insights into the role or roles of this carboxypeptidase B-like molecule in regulating fibrinolysis and coupling the coagulation and fibrinolytic cascades. A recent report by Redlitz et al. (33) is particularly intriguing. They showed, in a canine model of coronary thrombosis and thrombolysis, that a carboxypeptidase B-like activity, which is induced in serum and can be suppressed by a carboxypeptidase inhibitor from potatoes, correlates with both the time needed for reperfusion \textit{in vivo} and for clots to lyse \textit{in vitro}. The latter observations suggest that carboxypeptidase B-like activity modulates fibrinolysis not only \textit{in vitro} but also \textit{in vivo}.

Acknowledgment—We thank Tom Abbott for helping with the preparation of the manuscript and figures.

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