Thrombin-activatable Fibrinolysis Inhibitor Attenuates
(DD)E-mediated Stimulation of Plasminogen Activation
By Reducing the Affinity of (DD)E for Tissue Plasminogen Activator

A potential mechanism for enhancing the fibrin specificity of tissue plasminogen activator (t-PA) (1, 2). Plasmin then degrades fibrin, yielding soluble fibrin degradation products. Through a positive feedback mechanism, fibrin enhances its own degradation by stimulating t-PA-mediated Pg activation. To potentiate this reaction, fibrin acts as a template onto which both t-PA and Pg bind (3). The activator and its substrate bind to independent sites on intact fibrin because the t-PA interaction is primarily mediated by its fibronectin finger-like domain, whereas Pg binding is kringle-dependent (3–5). As a functional consequence of t-PA and Pg interaction with fibrin, the catalytic efficiency of t-PA-mediated Pg activation is 2–3 orders of magnitude greater in the presence of fibrin than in its absence (1, 3, 6). In contrast to the potent stimulatory effect of fibrin, fibrinogen (Fg) produces only a 25-fold enhancement in the catalytic efficiency of Pg activation by t-PA (1, 7). Because t-PA preferentially activates Pg in the presence of fibrin rather than Fg, it is designated a fibrin-specific Pg activator.

When cross-linked fibrin is solubilized by plasmin, a major degradation product is (DD)E, a complex of d-dimer (8, 9). Recently, we demonstrated that (DD)E compromises the fibrin specificity of t-PA, because this soluble fragment is as potent as fibrin at stimulating Pg activation by t-PA (10, 11). Like fibrin, (DD)E binds t-PA and Pg with high affinity (5, 10, 12). In contrast to its predominantly finger-dependent interaction with fibrin, t-PA binds to (DD)E via its second kringle domain (4, 5, 11). Although Pg also binds to (DD)E in a kringle-dependent fashion, the activator and substrate do not compete for (DD)E binding, indicating that they have distinct binding sites (5).

A complex of d-dimer noncovalently associated with fragment E ((DD)E), a degradation product of cross-linked fibrin that binds tissue plasminogen activator (t-PA) and plasminogen (Pg) with affinities similar to those of fibrin, compromises the fibrin specificity of t-PA by stimulating systemic Pg activation. In this study, we examined the effect of thrombin-activatable fibrinolysis inhibitor (TAFI), a latent carboxypeptidase B (CPB)-like enzyme, on the stimulatory activity of (DD)E. Incubation of (DD)E with activated TAFI (TAFIa) or CPB (a) produces a 96% reduction in the capacity of (DD)E to stimulate t-PA-mediated activation of Glu- or Lys-Pg by reducing $K_{ca}$ and increasing $K_{m}$ for the reaction; (b) induces the release of 8 mol of lysine/mol of (DD)E, although most of the stimulatory activity is lost after release of only 4 mol of lysine/mol of (DD)E; and (c) reduces the affinity of (DD)E for Glu-Pg, Lys-Pg, and t-PA by 2-, 4-, and 160-fold, respectively. Because TAFI or CPB-exposed (DD)E produces little stimulation of Glu-Pg activation by t-PA, (DD)E is not degraded into fragment E and d-dimer, the latter of which has been reported to impair fibrin polymerization. These data suggest a novel role for TAFIa. By attenuating systemic Pg activation by (DD)E, TAFIa renders t-PA more fibrin-specific.

Intravascular fibrinolysis is initiated when plasminogen (Pg) is converted to plasmin by tissue-type plasminogen activator (t-PA) (1, 2). Plasmin then degrades fibrin, yielding soluble fibrin degradation products. Through a positive feedback mechanism, fibrin enhances its own degradation by stimulating t-PA-mediated Pg activation. To potentiate this reaction, fibrin acts as a template onto which both t-PA and Pg bind (3). The activator and its substrate bind to independent sites on intact fibrin because the t-PA interaction is primarily mediated by its fibronectin finger-like domain, whereas Pg binding is kringle-dependent (3–5). As a functional consequence of t-PA and Pg interaction with fibrin, the catalytic efficiency of t-PA-mediated Pg activation is 2–3 orders of magnitude greater in the presence of fibrin than in its absence (1, 3, 6). In contrast to the potent stimulatory effect of fibrin, fibrinogen (Fg) produces only a 25-fold enhancement in the catalytic efficiency of Pg activation by t-PA (1, 7). Because t-PA preferentially activates Pg in the presence of fibrin rather than Fg, it is designated a fibrin-specific Pg activator.

When cross-linked fibrin is solubilized by plasmin, a major degradation product is (DD)E, a complex of d-dimer (DD)E noncovalently associated with fragment E (8, 9). Recently, we demonstrated that (DD)E compromises the fibrin specificity of t-PA, because this soluble fragment is as potent as fibrin at stimulating Pg activation by t-PA (10, 11). Like fibrin, (DD)E binds t-PA and Pg with high affinity (5, 10, 12). In contrast to its predominantly finger-dependent interaction with fibrin, t-PA binds to (DD)E via its second kringle domain (4, 5, 11). Although Pg also binds to (DD)E in a kringle-dependent fashion, the activator and substrate do not compete for (DD)E binding, indicating that they have distinct binding sites (5).

A complex of d-dimer noncovalently associated with fragment E (DD)E), a degradation product of cross-linked fibrin that binds tissue plasminogen activator (t-PA) and plasminogen (Pg) with affinities similar to those of fibrin, compromises the fibrin specificity of t-PA by stimulating systemic Pg activation. In this study, we examined the effect of thrombin-activatable fibrinolysis inhibitor (TAFI), a latent carboxypeptidase B (CPB)-like enzyme, on the stimulatory activity of (DD)E. Incubation of (DD)E with activated TAFI (TAFIa) or CPB (a) produces a 96% reduction in the capacity of (DD)E to stimulate t-PA-mediated activation of Glu- or Lys-Pg by reducing $K_{ca}$ and increasing $K_{m}$ for the reaction; (b) induces the release of 8 mol of lysine/mol of (DD)E, although most of the stimulatory activity is lost after release of only 4 mol of lysine/mol of (DD)E; and (c) reduces the affinity of (DD)E for Glu-Pg, Lys-Pg, and t-PA by 2-, 4-, and 160-fold, respectively. Because TAFI or CPB-exposed (DD)E produces little stimulation of Glu-Pg activation by t-PA, (DD)E is not degraded into fragment E and d-dimer, the latter of which has been reported to impair fibrin polymerization. These data suggest a novel role for TAFIa. By attenuating systemic Pg activation by (DD)E, TAFIa renders t-PA more fibrin-specific.
TAFIa Attenuates (DD)E Stimulation of Pg Activation

Associated with partial degradation of fibrin by plasmin.

Given its mechanism of action, we speculated that TAFIa or CPB would compromise the cofactor activity of (DD)E by abrogating Pg and/or t-PA binding. To explore this possibility, the ability of (DD)E to potentiate Pg activation by t-PA was examined before and after exposure of (DD)E to TAFIa or CPB. Herein we demonstrate that the stimulatory activity of (DD)E is nearly abolished upon exposure to TAFIa or CPB. Because plasmin generation in the presence of TAFIa- or CPB-exposed (DD)E is less than that with native (DD)E, TAFIa or CPB attenuates degradation of (DD)E into its constituent fragments, E and DD, the latter of which can impair fibrin polymerization. To explore the mechanism responsible for this phenomenon, light scattering spectroscopy was used to compare the affinities of TAFIa- or CPB-exposed (DD)E for Glu-Pg, Lys-Pg, or t-PA with those of native (DD)E. TAFIa or CPB exposure reduces the affinity of (DD)E for Glu-Pg and Lys-Pg 2- and 4-fold, respectively. In contrast, the affinity of (DD)E for t-PA is reduced 160-fold. These data raise the possibility that by attenuating the systemic plasmin generation induced by (DD)E, TAFIa renders t-PA more fibrin-specific.

EXPERIMENTAL PROCEDURES

Materials

Plasminogen Activator—Wild-type recombinant t-PA, kindly provided by Dr. B. Keyt (Genentech Inc., S. San Francisco, CA) was 93% single chain when analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 4–15% gradient gels (Ready-Gel; Bio-Rad) as determined using an ImageMaster Video Documentation System (Amersham Pharmacia Biotech). Active site blocked t-PA was prepared by incubating the activator with a 5-fold molar excess of N-phenyl-prolyl-arginine chloromethyl ketone (VFKCK; Calbiochem) and 1 mM Glu-Pg, and 4.3 nM VFKCK and 10 mM PPACK to inhibit plasmin and t-PA, respectively, as described previously (5, 20). The molecular weight and extinction coefficient used for t-PA were 65,000 and ε280 = 20,000, respectively (4).

Plasminogen—Native Glu-Pg was isolated from fresh frozen plasma by lysine-Sepharose affinity chromatography as described previously (5, 21). Isolated Glu-Pg was free of Lys-Pg based on urea/acetic acid PAGE analysis (22) and contained no plasmin as assessed using the plasmin-directed substrate β-Val-β-leucyl-lysine p-nitroanilide dihydrochloride (S-2251; Chromogenix, Mississauga, Canada). Glu-Pg concentrations were calculated by measuring the absorbance of 500 nm to distinguish tryptophan and phenylalanine absorbance from light scattering, respectively (23), using a molecular weight of 90,000 and ε280 = 16.1 (21). Lys-Pg was purchased from Enzyme Research Laboratories Inc. (South Bend, IN). Lys-Pg was free of Glu-Pg and contained no plasmin, determined as described above.

Fibrinogen—Human Fg-depleted Pg was purchased from Enzyme Research Laboratories and rendered fibrinogen-free by batch adsorption with gelatin-agarose (Sigma) for 30 min at room temperature followed by centrifugation at 3000 X g for 10 min (11). Fg concentration was calculated by measuring absorbances at 280 and 320 nm and using a molecular weight of 340,000 and ε280 = 16.0 (24).

(DD)E—The fibrin degradation product, (DD)E, was prepared by plasmin-mediated lysis of cross-linked fibrin clots as described previously (11). Briefly, 100 mg of Fg was clotted with 64 nM human plasmin (Enzyme Research Laboratories) and rendered fibronectin-free by batch adsorption with gelatin-agarose (Sigma) for 30 min at room temperature followed by centrifugation at 3000 X g for 10 min (11). Fg concentration was calculated by measuring absorbances at 280 and 320 nm and using a molecular weight of 340,000 and ε280 = 16.0 (24).

Effect of TAFIa or CPB on the Rate of (DD)E-stimulated Glu- and Lys-Pg Activation by t-PA—To examine the effect of TAFIa or CPB on the ability of (DD)E to stimulate Pg activation by t-PA, 1.0 ml of a 5 μM (DD)E solution was incubated with 20 mM t-PA or CPB for 40 min at 22 °C. At intervals, 20-μl aliquots were removed, and 2 μl of 40 μM CPI was added to inhibit the TAFIa or CPB. Complete TAFIa or CPB inhibition was achieved because no residual activity was detected using the CPB-directed synthetic substrate hippuryl-l-arginine. The ability of TAFIa- or CPB-treated (DD)E to stimulate Pg activation was then compared with that of the starting material. Because TAFIa activity is unstable at room temperature (27, 28), TAFIa was used immediately or kept on ice until used. Like CPB, the activity of 20 mM TAFIa was completely inhibited by 1 μM CPI. CPB was used for the majority of experiments because its activity is more stable than that of TAFIa and it does not require preactivation. To demonstrate that TAFIa has effects similar to CPB, however, confirmatory experiments were done using TAFIa.

Methods

Effect of TAFIa or CPB on the Rate of (DD)E-stimulated Glu- and Lys-Pg Activation by t-PA—To examine the effect of TAFIa or CPB on the ability of (DD)E to stimulate Pg activation by t-PA, 1.0 ml of a 5 μM (DD)E solution was incubated with 20 mM t-PA or CPB for 40 min at 22 °C. At intervals, 20-μl aliquots were removed, and 2 μl of 40 μM CPI was added to inhibit the TAFIa or CPB. Complete TAFIa or CPB inhibition was achieved because no residual activity was detected using the CPB-directed synthetic substrate hippuryl-l-arginine. The ability of TAFIa- or CPB-treated (DD)E to stimulate Pg activation was then compared with that of the starting material. Because TAFIa activity is unstable at room temperature (27, 28), TAFIa was used immediately or kept on ice until used. Like CPB, the activity of 20 mM TAFIa was completely inhibited by 1 μM CPI. CPB was used for the majority of experiments because its activity is more stable than that of TAFIa and it does not require preactivation. To demonstrate that TAFIa has effects similar to CPB, however, confirmatory experiments were done using TAFIa.

Molecular Weight Determination of (DD)E and Fragment E—(DD)E and fragment E, respectively (8, 25). (DD)E concentrations were calculated by measuring absorbances at 280 and 320 nm and using a molecular weight of 250,000 and ε280 = 16.0 (8).

Carboxypeptidase B and TAFIa—CPB, potato tuber-derived CPB inhibitor (CPI), and the CPB-directed synthetic substrate hippuryl-l-arginine were purchased from Sigma. CPB activity was assessed by incubating 20 mM CPB with 0.12 μM hippuryl-l-arginine for 30 min at 22 °C in 0.02 M Tris-HCl, 0.15 M NaCl, 0.1% Tween 20, pH 7.4 (TBS) in a quartz cuvette. Increases in absorbance at 254 nm were monitored for 20 min at 22 °C using a DU 7400 Spectrophotometer from Beckman (Mississauga, Canada). Under these conditions, CPB has a specific activity of 41 units/mg, where one unit hydrolyzes 1 μmol of hippuryl-l-arginine/min. The lowest concentration used to inhibit CPB prior to Pg activation assays, no increase in A at 254 nm was observed, indicating complete CPB inhibition. TAFIa was isolated from fresh frozen human plasma by Pg-Sepharose affinity chromatography and activated with thrombin and soluble thrombomodulin as described elsewhere (15, 26). The specific activity of the resultant TAFIa against hippuryl-l-arginine was similar to that of CPB. Because TAFIa activity is unstable at room temperature (27, 28), TAFIa was used immediately or kept on ice until used. Like CPB, the activity of 20 mM TAFIa was completely inhibited by 1 μM CPI. CPB was used for the majority of experiments because its activity is more stable than that of TAFIa and it does not require preactivation. To demonstrate that TAFIa has effects similar to CPB, however, confirmatory experiments were done using TAFIa.
deprotonated by bringing the solution to 0.2 m with perchloric acid followed by centrifuging at 12,000 × g for 5 min. After the supernatants were neutralized with potassium hydroxide, the samples were placed on ice, and insoluble potassium perchlorate was removed by centrifugation at 12,000 × g for 5 min. The concentration of lysine and arginine in the supernatants was determined by method described by Nakatani et al. (29) and Gaede et al. (30), respectively. For lysine determination, 50 µl of supernatant was added to 40 µl of 0.5 mM NADH (Roche Diagnostics, Laval, Canada) and 2.5 mM o-ketoglutaric acid (Roche Diagnostics) in 50 mM HEPES, 150 mM NaCl, pH 7.0 (HBS). Dehydration of NADH was initiated by the addition of 0.33 units of saccharopine dehydrogenase (Sigma) that was diluted in 10 µl of HBS. Decreases in fluorescence intensity were monitored over 25 min in a Spectra Max Gemini XS fluorescent plate reader ( Molecular Devices, Sunnyvale, CA), with excitation and emission wavelengths set to 340 and 450 nm, respectively, and fitted with a 435-nm emission cut-off filter. The concentration of lysine was calculated based on a standard curve generated by plotting changes in fluorescence intensity produced by known concentrations of l-lysine (Sigma). Determination of free arginine was accomplished in the same manner using pyruvate (Roche Diagnostics) in place of o-ketoglutaric acid and 0.5 units of octopine dehydrogenase (Sigma) in place of saccharopine dehydrogenase. Standard curves for free arginine determination were generated by plotting changes in fluorescence intensity produced by known concentrations of l-arginine (Sigma).

Effect of (DD)E Exposure to TAFIa or CPB on the Kinetics of (DD)E-stimulated Glu- and Lys-Pg Activation by t-PA—To determine the effects of exposure of (DD)E to TAFIa or CPB on the kinetics of (DD)E-stimulated Pg activation, kinetic parameters for Glu- and Lys-Pg activation by t-PA, measured in the presence of TAFIa or CPB-exposed (DD)E, were compared with those obtained with native (DD)E or in the absence of a cofactor. After exposure of 8 µM (DD)E to 20 µM TAFIa or CPB for 40 min at 22 °C, the reaction was terminated with CPI. Different concentrations of CPI were used to maintain a concentration of CPI in the Pg activation assay between 0.2 and 0.8 µM. This range of CPI concentrations was maintained to ensure CPI levels were well above the Kd for inhibiting CPI (31) but low enough so as not to inhibit plasmin (5 µM CPI causes a 7% reduction in the activity of 0.01 µM plasmin). 0.8 µM CPI has no measurable effect on the rate of plasmin formation (data not shown). Glu- or Lys-Pg, in concentrations ranging from 0 to 16 µM, was incubated with 0.1 µM t-PA in the absence or presence of native (DD)E or TAFIa- or CPB-exposed (DD)E at concentrations up to 6 µM. Plasmin formation was then monitored using the plasmin-directed substrate S-2251 and rates of Pg activation were determined above, were divided by the concentration of activator (t-PA) and subjected to nonlinear regression analysis (Table Curve; Jandel Scientific, San Rafael, CA) according to the Michaelis-Menten equation, 

\[
\frac{I}{I_0} = 1 + \frac{[S]}{K_m + [S]} = 1 + \frac{L}{K_d + L} - \frac{L}{K_d + L} = 1 + \frac{L}{K_d + L} - \frac{L}{K_d + L}
\]

where \( I_0 \) is the total concentration of ligand added, \( P \) is the concentration of target protein, \( K_d \) is the dissociation constant, \( n \) is the stoichiometry, and \( \alpha \) is the maximum change in emission intensity. To confirm the binding parameters obtained in this fashion, reverse titrations also were performed wherein 0.1 µM active site-blocked t-PA, Glu-Pg, or Lys-Pg was titrated with native or TAFIa- or CPB-exposed (DD)E.

**RESULTS**

Effect of CPB or TAFIa on the Ability of (DD)E to Stimulate t-PA-mediated Pg Activation—To determine whether CPB or TAFIa treatment of (DD)E modulates its ability to stimulate Pg activation by t-PA, (DD)E was incubated with CPB or TAFIa for intervals up to 40 min. After inhibiting CPB or TAFIa with CPI, the stimulatory activity of CPB- or TAFIa-treated (DD)E was then compared with that of the starting material. When t-PA-mediated activation of Glu-Pg is monitored, plasmin formation is decreased with increasing exposure of (DD)E to CPB (Fig. 1, inset) or TAFIa (data not shown). From these data, rates of plasmin formation were calculated (Fig. 1). After a 40-min exposure of (DD)E to CPB or TAFIa, the rate of Glu-Pg activation in the presence of CPB-treated (DD)E is similar to that in the absence of a cofactor. Analogous results are obtained when Lys-Pg is substituted for Glu-Pg. Although rates of plasmin formation are higher with Lys-Pg than with Glu-Pg, 40-min exposure of (DD)E to CPB or TAFIa almost completely abolishes its stimulatory activity (Fig. 2).

Plasmin can degrade (DD)E into DD and fragment E (8, 9). Because DD and fragment E are less potent stimulators of Pg activation than intact (DD)E (12), this phenomenon could influence the kinetics of (DD)E-stimulated Pg activation. However, when analyzed by PAGE, no detectable (DD)E degradation is observed during the first 10 min of Pg activation (data not shown), the time over which the initial rates of plasmin formation are calculated. These observations suggest that degradation of (DD)E does not influence the kinetics of Pg activation in the presence of (DD)E.

Effect of CPB or TAFIa on the Degree to Which (DD)E Is Degraded during t-PA-mediated Pg Activation—Fig. 3, which illustrates the formation of (DD)E from fibrin and its subsequent degradation by plasmin, helps to describe the gels presented in Fig. 4. (DD)E, which is formed when plasmin degrades two-stranded fibrin prototibrils between adjacent D and E domains, consists of two cross-linked D domains noncovalently associated with an E domain (Fig. 3). The E domain remains bound to DD provided that the amino-terminal portion of at least one α and β chain within the E domain (α5 and β5, respectively) remains intact (25, 33). Plasmin first cleaves β5 at Lys533 and then α5 at Arg539. The E moiety is characterized by changes in molecular weight that occur upon β5 cleavage (33, 37). Fragment E5, which has a molecular mass of ~60 kDa, has the Lys533-Arg539 peptide bond of both β5 chains intact whereas fragment E5, with a molecular mass of 55 kDa, has one β5 chain cleaved at Lys533. Both fragments E5 and E5 remain associated with DD. In contrast, fragment E5, which has a molecular mass of ~50 kDa, no longer binds DD, because both β5 chains are cleaved at Lys533.
When (DD)E is exposed to plasmin, it is degraded to DD and fragment E (8, 9). Since TAFIa attenuates fibrin degradation by limiting plasmin formation, we explored the possibility that CPB or TAFIa modulates (DD)E degradation (15, 16). (DD)E migrates as single species in (DD)E migrates as two bands corresponding to DD and fragment E, respectively (Fig. 4B, lane 1). Fragment E from (DD)E is a single species corresponding to fragment Eγ, characterized by β chains with amino-terminal Lys residues at position 54 (33). Following a 1-h incubation in the presence of CPB and Pg, DD mobility is unchanged, but fragment Eγ is degraded to Eγ3, DD and fragment E, respectively (Fig. 4C, lanes 1 and 9). When native (DD)E stimulates Pg activation for 1 h, the βγ chain is degraded, and, based on amino-terminal sequence analysis, three amino acids are removed from the amino-terminal portion of the αγ chain, although there is no noticeable shift in mobility of the αγ chain (Fig. 4C, lane 2). All other chains remain intact. These data are consistent with previously reported plasmin cleavage sites (33). When CPB-exposed (DD)E is used to stimulate Pg activation, the βγ chain is not degraded (Fig. 4C, lanes 3–8). The αγ chain also remains intact, as determined by its amino-terminal sequence.

The amount of βγ chain in each lane in Fig. 4C was quantified by measuring its optical density and comparing this value to the total optical density of all chains in the same lane. As illustrated in Fig. 5, the extent of βγ chain degradation is inversely related to the rate of plasmin formation measured prior to PAGE analysis (r = -0.986).

To determine whether TAFIa-mediated attenuation of plasmin formation also limits the degradation of (DD)E, (DD)E was incubated with 20 nM TAFIa for 40 min. At the end of the incubation period, CPI was added to stop the reaction. Like the results with CPB, when TAFIa-exposed (DD)E is used to stimulate Pg activation, the βγ and αγ chains are not degraded, and
the structural integrity of (DD)E is preserved (data not shown).

**CPB- or TAFIa-mediated Release of Lysine and Arginine from (DD)E**—To explore the mechanism by which CPB or TAFIa attenuates the stimulatory activity of (DD)E, we determined whether CPB or TAFIa induces the release of free lysine and arginine residues from (DD)E. Under the conditions outlined under “Methods,” CPB or TAFIa causes the release of approximately 4 mol of lysine/mol of (DD)E within 10 and 2 min, respectively (Fig. 6). By 60 min, an additional 4 mol of lysine/mol of (DD)E are released by both enzymes. The biphasic lysine release from (DD)E is not the result of reduction in the activity of CPB, because CPB activity against hippuryl-L-arginine remains constant over the 60-min incubation period. Although the activity of TAFIa against hippuryl-L-arginine is 25% lower at 60 min than it is at start, there is no detectable change in TAFIa activity at 10 min, a point well beyond the transition from slow to rapid lysine release. Thus, the carboxyl-terminal lysine residues on (DD)E exhibit different susceptibilities to CPB or TAFIa. Neither CPB nor TAFIa causes the release of arginine from (DD)E (Fig. 6).

**Effect of (DD)E Exposure to TAFIa or CPB on the Kinetics of (DD)E-Stimulated Glu- and Lys-Pg Activation by t-PA**—The kinetic parameters for (DD)E-stimulated Glu- or Lys-Pg activation by t-PA were measured in the absence or presence of native (DD)E, fibrin or Fg. These results were then compared with those obtained with TAFIa- or CPB-exposed (DD)E. Glu-or Lys-Pg was activated with a constant amount of t-PA in the presence of varying concentrations of Pg and cofactor. Data obtained with each cofactor concentration were fit to the Michaelis-Menten equation by nonlinear regression to determine the values of $k_{cat}$ and $K_m$ (Fig. 7). Cofactor concentrations were considered saturating when the catalytic efficiency ($k_{cat}/K_m$) reached a maximum. Maximum catalytic efficiencies were achieved with 0.4 $\mu$M native (DD)E and 5 $\mu$M (DD)E previously exposed to TAFIa or CPB.

As summarized in Table I, native (DD)E is as potent as fibrin at potentiating both Glu- and Lys-Pg activation by t-PA, stim-
TAFIa Attenuates (DD)E Stimulation of Pg Activation

The relative amounts of released arginine residues from (DD)E incubation with either CPB or TAFIa. Neither CPB (○) nor TAFIa (□) causes the release of 8 mol of lysine/mol of (DD)E. Under the conditions employed here, ~4 mol of lysine are released within the first 10 and 2 min of CPB and TAFIa incubation, respectively. The remaining 4 mol of lysine are released within 60 min of incubation with either CPB or TAFIa. Neither CPB (○) nor TAFIa (□) releases arginine residues from (DD)E.

Effect of TAFIa or CPB on the Binding of t-PA, Glu-Pg, and Lys-Pg to (DD)E—To determine whether the reduced stimulatory activity of TAFIa- or CPB-treated (DD)E reflects changes in its affinity for t-PA and/or Pg, light scattering spectroscopy was used to compare the affinities of t-PA or Pg for native (DD)E with those for TAFIa- or CPB-exposed (DD)E. The relative scatter plots for the interactions of t-PA or Glu-Pg with (DD)E before and after CPB treatment are illustrated in Fig. 8, A and B, respectively. Under the conditions outlined under "Methods," the scattering intensity of 0.1 μM (DD)E is 15 (I₀). At saturating levels of t-PA, the maximum relative scattering intensity (II₅₀) in the presence of untreated (DD)E is 1.5 (Fig. 8A), a value in good agreement with a calculated maximum relative scattering intensity of 1.6 if the stoichiometry is 1:1 (5, 35). The maximum II₅₀ when (DD)E was titrated with Glu-Pg was 2.1 (Fig. 8B), a value similar to a predicted II₅₀ of 1.9 for a 1:1 substrate interaction with (DD)E (5, 35). Data were fit to Equation 1 by nonlinear regression analysis to determine dissociation constants, and these results are summarized in Table II.

FIG. 6. CPB- or TAFIa-mediated lysine and arginine release from (DD)E. 8 μM (DD)E was incubated with 20 nm CPB or TAFIa for various times at 22 °C. After the addition of CPI, the amount of free lysine or arginine release was determined by monitoring dehydrogenation of NADH using saccharopine or octopine dehydrogenase, respectively. The total concentration of lysine or arginine was divided by the concentration of (DD)E to calculate mol of free amino acid released/mol of (DD)E. CPB (○) or TAFIa (□) causes the release of 8 mol of lysine/mol of (DD)E. Under the conditions employed here, ~4 mol of lysine are released within the first 10 and 2 min of CPB and TAFIa incubation, respectively. The remaining 4 mol of lysine are released within 60 min of incubation with either CPB or TAFIa. Neither CPB (○) nor TAFIa (□) releases arginine residues from (DD)E.

FIG. 5. Correlation between βₐ chain degradation and the ability of (DD)E to stimulate Pg activation. The relative amounts of βₐ in each lane of Fig. 4C (■), determined by densitometry, are plotted against the time that (DD)E was exposed to CPB prior to Pg activation. The rate of Pg activation by CPB-exposed (DD)E (■), measured prior to PAGE analysis, is plotted against time of CPB exposure on the secondary y axis. Both of these values are compared with those obtained with native (DD)E. These data demonstrate that the integrity of the βₐ chain is inversely correlated (r = −0.986) with the extent to which (DD)E stimulates Pg activation.

FIG. 7. Effect of CPB on the ability of (DD)E to potentiate t-PA-mediated activation of Glu-Pg. 0.1 nM t-PA was incubated with various concentrations of Glu-Pg in the presence of 0.4 μM native (DD)E (●), or 5 μM CPB-treated (DD)E (■) or in the absence of (DD)E (○). Plasmin formation was monitored using the plasmin-directed substrate S-2251, and rates of plasmin formation were calculated. CPB markedly reduces the rate of (DD)E-stimulated Pg activation at all Pg concentrations. These data were fit to the Michaelis-Menten equation by nonlinear regression (solid lines) to determine k cat and K m (Table I).
the possibility that TAFIa modulates the stimulatory activity of (DD)E via a similar mechanism.

(DD)E is as potent as fibrin at stimulating t-PA-mediated Pg activation. When (DD)E is incubated with TAFIa or CPB, its ability to stimulate the activation of either Glu- or Lys-Pg by t-PA is reduced by 96% and becomes comparable with that

| Cofactor                        | $k_{cat}$ | $K_m$   | $k_{cat}/K_m$ | Stimulation |
|---------------------------------|-----------|---------|---------------|-------------|
| Glu-Pg                          |           |         |               |             |
| No cofactor                     | 0.25 ± 0.10 | 0.78 ± 0.21 | 0.32 ± 0.04  | 360         |
| Native (DD)E                    | 0.080 ± 0.021 | 7.5 ± 2.3  | 0.011 ± 0.003 | 12          |
| CPB- (or TAFIa-) exposed (DD)E | 0.27 ± 0.13  | 0.87 ± 0.36 | 0.31 ± 0.12  | 350         |
| Fibrin                          | 0.21 ± 0.07  | 9.5 ± 1.4   | 0.022 ± 0.007| 25          |
| Fg                              |           |         |               |             |
| Lys-Pg                          |           |         |               |             |
| No cofactor                     |           |         |               |             |
| Native (DD)E                    | 0.10 ± 0.02 | 0.022 ± 0.004 | 4.5 ± 0.5  | 450         |
| CPB- (or TAFIa-) exposed (DD)E | 0.049 ± 0.010 | 0.27 ± 0.06 | 0.18 ± 0.05 | 18          |
| Fibrin                          | (0.081)    | (0.31)   | (0.26)       |             |
| Fg                              | 0.060 ± 0.010 | 0.033 ± 0.005 | 4.8 ± 1.3  | 480         |

$^a$-Fold stimulation is calculated by dividing the catalytic efficiency ($k_{cat}/K_m$) obtained in the presence of the indicated cofactor by that obtained in the absence of a cofactor.

The TAFIa Attenuates (DD)E Stimulation of Pg Activation

**Table I**

Kinetic parameters for Glu- and Lys-Pg activation in the absence of a cofactor, in the presence of (DD)E before and after its exposure to CPB or TAFIa, or in the presence of fibrin or Fg

All kinetic parameters are presented as the mean ± S.E. of at least three experiments, except those for TAFIa-exposed (DD)E, which are in parentheses and reported as the mean of two experiments.

**Fig. 8.** Effect of CPB on the affinity of (DD)E for t-PA or Glu-Pg. 0.1 μM native (DD)E (○) or CPB-exposed (DD)E (□) was titrated with active site blocked t-PA (A) or Glu-Pg (B), and scattering intensities obtained in the presence of titrant (I) were compared with those obtained in its absence (Io). Solid lines represent nonlinear regression analysis of the data to determine $K_d$ values, which are summarized in Table II. Whereas exposure of (DD)E to CPB causes a modest reduction in its affinity for Glu-Pg, CPB markedly reduces the affinity of (DD)E for t-PA.
Effect of CPB or TAFIa on the affinity of (DD)E for t-PA, Glu-Pg, or Lys-Pg

| Ligand | $K_a$ of native (DD)E | $K_a$ of CPB- or TAFIa-exposed (DD)E | Decrease in affinity $^a$ |
|--------|----------------------|-------------------------------|---------------------------|
| t-PA   | 0.04 ± 0.01          | 6.5 ± 1.5 (5.5)               | 160                       |
| Glu-Pg | 5.5 ± 1.8            | 10.8 ± 5.1 (9.8)              | 2                         |
| Lys-Pg | 0.09 ± 0.01          | 0.35 ± 0.10 (0.46)            | 4                         |

$^a$ Values for native and CPB-exposed (DD)E are presented as the means ± S.E. of at least three titrations.

$^b$ Fold decrease in affinity is calculated by dividing the affinity of native (DD)E for the indicated ligand by the affinity of CPB-exposed (DD)E for the same ligand.

Values in parentheses are for TAFIa-exposed (DD)E and are presented as the mean of two titrations.

**TABLE II**

TAFIa Attenuates (DD)E Stimulation of Pg Activation

Consequently, (DD)E is a poor stimulator of Pg activation by b-PA (5, 40). These data support the concept that only Pg activators that bind to (DD)E are significantly potentiating by this fragment.

Although it does not bind to (DD)E, b-PA binds fibrin with high affinity via its finger domain and is stimulated by fibrin to the same extent as t-PA (5, 41). Because (DD)E and fibrin stimulate t-PA to a similar extent, whereas b-PA is potentiated only by fibrin, t-PA is less fibrin-specific than b-PA. Thus, (DD)E compromises the fibrin specificity of t-PA. Our current findings raise the possibility that by decreasing the capacity of (DD)E to bind t-PA, TAFIa enhances the fibrin specificity of t-PA.

Upon exposure to TAFIa or CPB, the affinity of (DD)E for Glu- or Lys-Pg is only modestly reduced, suggesting that Pg binds to internal lysines as well as carboxyl-terminal lysine residues. In contrast, our data suggest that t-PA predominantly binds to carboxyl-terminal lysine residues on (DD)E because the affinity of t-PA for (DD)E is markedly reduced when (DD)E is exposed to TAFIa or CPB. These findings are consistent with the previously reported noncompetitive binding of t-PA and Pg to (DD)E (5).

Like their effect on (DD)E, TAFIa and CPB also release lysine residues from fibrin partially degraded by plasmin (19, 42). This results in a reduction in the affinity of plasmin-exposed fibrin for Glu- or Lys-Pg and blocks the accumulation of fluorescently labeled Pg on fibrin (16, 43). TAFIa also prevents the conversion of Glu-Pg to Lys-Pg, an early event in the course of t-PA-mediated clot lysis that serves as a positive feedback mechanism because Lys-Pg, which has higher affinity for fibrin than Glu-Pg, is more readily activated by t-PA (18, 19).

Although the effects of TAFIa on the affinity of the activator for fibrin have yet to be investigated, CPB blocks the 2–3-fold increase in t-PA binding that occurs when fibrin is exposed to plasmin, suggesting that this increase reflects kringel-dependent binding of t-PA to newly exposed carboxyl-terminal lysine residues (42). The inhibitory effect of TAFIa or CPB on these positive feedback events results in a 3–4-fold prolongation of the rate of t-PA-mediated fibrinolysis (15, 26).

Our results with (DD)E have similarities to those with fibrin. TAFIa or CPB causes the release of free lysine residues from (DD)E, reduces the affinity of the substrate and activator for the cofactor, and attenuates cofactor degradation. However, other features distinguish the effects of TAFIa or CPB on (DD)E from those on fibrin. Although fibrin degradation is attenuated by TAFIa or CPB, it is not inhibited, suggesting that even in the presence of these enzymes, fibrin remains a competent stimulator of Pg activation by t-PA (26). Furthermore, the inhibitory effects of TAFIa on fibrinolysis can be overcome by substituting Lys-Pg for Glu-Pg (18, 19). In contrast, exposure of (DD)E to TAFIa or CPB produces a 96% reduction in its ability to stimulate the activation of either Glu- or Lys-Pg by t-PA. These differences between fibrin and (DD)E are explained by the observation that TAFIa or CPB reduces the affinity of t-PA for (DD)E more than its affinity for fibrin. Whereas t-PA maintains high affinity binding to intact or partially degraded fibrin in the presence of CPB (42, 44), TAFIa or CPB almost abolishes t-PA binding to (DD)E.

(DD)E compromises the fibrin specificity of t-PA because it binds t-PA with high affinity and stimulates Pg activation to the same extent as fibrin. By reducing the affinity of (DD)E for t-PA, TAFIa converts (DD)E from a fibrin-like stimulator to one that has minimal stimulatory activity, much like Pg. Thus, our data suggest a novel role for TAFIa. By reducing the stimulatory activity of (DD)E and attenuating systemic Pg activation, TAFIa may enhance the fibrin specificity of t-PA. Furthermore,
since DD can impair fibrin polymerization, limiting (DD)E degradation may augment the anti-fibrinolytic properties of TAFIa.

REFERENCES

1. Hoylaerts, M., Rijken, D. C., Lijnen, H. R., and Collen, D. (1982) J. Biol. Chem. 257, 2912–2919
2. Collen, D., and Lijnen, H. R. (1986) Haemostasis 16, Suppl. 3, 25–32
3. Horrevoets, A. J., Pannekoek, H., and Nesheim, M. E. (1997) J. Biol. Chem. 272, 2183–2189
4. Nesheim, M., Fredenburgh, J. C., and Larsen, G. R. (1990) J. Biol. Chem. 265, 21541–21548
5. Stewart, R. J., Fredenburgh, J. C., and Weitz, J. I. (1998) J. Biol. Chem. 273, 18292–18299
6. Mosesson, M. W., Siebenlist, K. R., Voskuilen, M., and Nieuwenhuizen, W. (1999) Thromb. Haemostasis 82, 796–801
7. Nieuwenhuizen, W., Voskuilen, M., Vermond, A., Hoegee-de Nobel, B., and Traas, D. W. (1988) J. Biochem. (Tokyo) 174, 163–169
8. Olexa, S. A., and Budzynski, A. Z. (1979) Biochemistry 18, 991–995
9. Gaffney, P. J., and Joe, F. (1979) Thromb. Res. 15, 673–687
10. Stewart, R. J., Fredenburgh, J. C., Lee, A. Y., Rischke, J. A., and Weitz, J. I. (1998) Blood Coagul. Fibrinolysis 9, 691–692
11. Stewart, R. J., Fredenburgh, J. C., Leslie, B. A., Keyt, B. A., Rischke, J. A., and Weitz, J. I. (2000) J. Biol. Chem. 275, 10112–10129
12. Weitz, J. I., Leslie, B., and Ginsberg, J. (1991) J. Clin. Invest. 87, 1082–1090
13. Eaton, D. L., Malloy, B. E., Tsai, S. P., Henzel, W., and Drayna, D. (1991) J. Biol. Chem. 266, 21335–21338
14. Wang, W., Hendriks, D. F., and Scharpé, S. S. (1994) J. Biol. Chem. 269, 15937–15944
15. Bajzar, L., Manuel, R., and Nesheim, M. E. (1995) J. Biol. Chem. 270, 14477–14484
16. Sakharov, D. V., Plow, E. F., and Rijken, D. C. (1997) J. Biol. Chem. 272, 14477–14482
17. Nesheim, M. E. (1999) Fibrinolysis and Proteolysis 13, 72–77
18. Nesheim, M., Wang, W., Boffa, M., Nagashima, M., Morser, J., and Bajzar, L. (1997) Thromb. Haemostasis 78, 386–391
19. Wang, W., Boffa, M. B., Bajzar, L., Walker, J. B., and Nesheim, M. E. (1998) J. Biol. Chem. 273, 27176–27181
20. Higgins, D. L., and Lamb, M. C. (1986) Arch. Biochem. Biophys. 249, 418–426
21. Castellino, F. J., and Powell, J. R. (1981) Methods Enzymol. 80, 365–379
22. Fredenburgh, J. C., and Nesheim, M. E. (1992) J. Biol. Chem. 267, 26150–26156
23. Bloom, J. W., Nesheim, M. E., and Mann, K. G. (1979) Biochemistry 18, 4419–4425
24. Dellenback, R. J., and Chien, S. (1979) Proc. Soc. Exp. Biol. Med. 162, 353–355
25. Moskowitz, K. A., and Budzynski, A. Z. (1994) Biochemistry 33, 12837–12844
26. Bajzar, L., Morser, J., and Nesheim, M. (1996) J. Biol. Chem. 271, 16603–16608
27. Boffa, M. B., Bell, R., Stevens, W. K., and Nesheim, M. E. (2000) J. Biol. Chem. 275, 12868–12878
28. Marx, P. F., Hackeng, T. M., Dawson, P. E., Griffin, J. H., Meijers, J. C. M., and Bouma, B. N. (2000) J. Biol. Chem. 275, 12441–12445
29. Nakatani, Y., Fujikawa, M., and Higashino, K. (1972) Anal. Biochem. 49, 225–231
30. Gaede, G., and Grieshaber, M. (1974) Anal. Biochem. 66, 393–399
31. Ryan, C. A., Hass, G. M., and Kuhn, R. W. (1974) J. Biol. Chem. 249, 5495–5499
32. Boskovic, D. S., Giles, A. R., and Nesheim, M. E. (1990) J. Biol. Chem. 265, 10497–10505
33. Olexa, S. A., Budzynski, A. Z., Doolittle, R. F., Cottrell, B. A., and Greene, T. C. (1981) Biochemistry 20, 6139–6145
34. Olexa, S. A., and Budzynski, A. Z. (1979) J. Biol. Chem. 254, 4925–4932
35. Blanchon, L., Didry, D., Carlier, M. F., and Pantaloni, D. (1990) J. Biol. Chem. 271, 12380–12386
36. Walker, J. B., and Nesheim, M. E. (1999) J. Biol. Chem. 274, 5201–5212
37. Husain, S. S., Weisel, J. W., and Budzynski, A. Z. (1989) J. Biol. Chem. 264, 11414–11420
38. Olexa, S. A., and Budzynski, A. Z. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 1374–1378
39. Budzynski, A. Z., Olexa, S. A., and Brizuela, B. S. (1979) Biochim. Biophys. Acta 534, 284–287
40. Weitz, J. I., Stewart, R. J., and Fredenburgh, J. C. (1999) Thromb. Haemostasis 82, 974–982
41. Bringmann, P., Gruber, D., Liese, A., Toschi, L., Kratzschmar, J., Schleuning, W. D., and Donner, P. (1995) J. Biol. Chem. 270, 25596–25603
42. de Vries, C., Veerman, H., and Pannekoek, H. (1989) J. Biol. Chem. 264, 12604–12610
43. Fleury, V., and Angles-Cano, E. (1991) Biochemistry 30, 7630–7638
44. Higgins, D. L., and Vehar, G. A. (1987) Biochemistry 26, 7786–7791