Plasmin-mediated fibrinolysis by variant recombinant tissue plasminogen activators

(activity of variant tissue plasminogen activators/domains of tissue plasminogen activator/effectors of fibrinolysis)

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ABSTRACT   A rapid and quantitative fibrinolytic assay has been used to measure the overall activity of a recombinant tissue plasminogen activator (rTPA) preparation for dissolution of a fibrin clot by its ability to activate [Glu¹]plasminogen (containing glutamic acid at position 1) to plasmin. A standard curve constructed for wild-type two-chain rTPA that contains, from the amino terminus, the finger (F)–growth factor (E)–kringle 1 (K_1)–kringle 2 (K_2)–serine protease (P) domains was used to assess the overall fibrin-dissolving abilities of variant recombinant molecules. Two-chain deletion mutants lacking the E domain, the F–E domains, the F–E–K_1 domains, and the K_1–K_2 domains yielded activities ranging from 22% to 35% of the overall activity of wild-type two-chain rTPA, suggesting that both the K_2 and F domains are individually responsible for a portion of the function of the molecule. Comparison of variant molecules containing F–K_1–K_2–P and F–K_2–K_2–P domains showed that the latter variant possessed a 4-fold higher activity (1.4-fold greater than that of wild-type two-chain rTPA), indicating that, for the activity measured, the presence of K_2 leads to a greater effectiveness than that of K_1. A plasmin cleavage-resistant mutant (Arg-275 → Ser) has been used to assess possible differences in one- and two-chain rTPA in this overall activity, the former displaying 86% of the activity of the latter, suggesting that such differences are indeed small. Finally, the proper covalent attachment of the light and heavy chains of two-chain rTPA are very important to its overall fibrinolytic activity, since replacement of Cys-264 with glycine and concomitant disruption of one of the covalent attachment sites of the two chains provides a variant of rTPA with <2% of the activity of the wild-type two-chain molecule. The effector molecule, ε-amino hexanoic acid (εAhx; ε-aminocaproic acid), inhibits the overall fibrinolytic effect of rTPA in this system, with an effective K_i of ≈1.5 mM. Its efficacy, as measured by the K_i, is independent of the presence of the εAhx binding regions of plasminogen and rTPA and is similar to the efficacy obtained when urokinase was the activator in place of wild-type two-chain rTPA or when activation of plasminogen was bypassed as a result of provision of preformed plasmin to the assay. The results suggest that in the overall clot lysis system, an important εAhx binding site may exist on fibrin that inhibits its dissolution by plasmin.

Tissue plasminogen activator (TPA) is an important enzyme in the provision of the fibrinolytic response as a result of its ability to activate plasminogen to plasmin, this latter enzyme possessing the capability to degrade fibrin and its precursor molecule, fibrinogen. The effectiveness of TPA as a direct plasminogen activator is greatly modulated by certain plasma components, as revealed by the inhibition of this activation by physiological levels of Cl⁻ (1, 2) and its stimulation by

fibrinogen (2), fibrin (3), and the important effector ε-aminohexanoic acid (εAhx; ε-aminocaproic acid) (2).

TPA is synthesized and secreted in vascular endothelial cells as a one-chain protein containing 527 amino acid residues and ≈10% carbohydrate (4). In the presence of low levels of plasmin (4) and enzymes with a similar specificity (5), this protein is converted to a two-chain, disulfide-linked form by hydrolysis of the peptide bond between Arg-275 and Ile-276. The overall structure of TPA is predicted from amino acid sequence homology analysis to include at least five domains. The sequence from Ser-1 through His-43 is homologous to the fibronectin finger (F) domains (6); the sequence from Ser-50 through Asp-87 shows significant similarity to the sequence of human epidermal growth factor (E) (6); two consecutive kringle (K) domains (4) are included in the sequence from Cys-92 through Cys-172 (K_1) and from Cys-180 through Cys-261 (K_2); and the serine protease region (P) from Ile-276 through Phe-527 contains the catalytic triad of His-322, Asp-371, and Ser-478 (4). Additional evidence for the functional domain structure of TPA is obtained from the observation that at least one intron separates each of these regions and that four exist within the protease domain, similar to the gene structures of other serine proteases (7).

In comparisons of the activities of one- and two-chain TPA, it has been reported that the two-chain form is more active than the one-chain form toward small substrates (8, 9) and toward [Glu¹]plasminogen (8) and is more reactive than the one-chain form with diisopropyl fluorophosphate and α_2-antiplasmin (10). The amidolytic activity of one-chain TPA is stimulated by fibrin(ogen), while that of two-chain TPA shows no such effect (11). In the presence of fibrin, it appears as though both forms of TPA are very similar (12), but not identical (8), in their respective abilities to activate [Glu¹]plasminogen.

Because of the importance attached to TPA as a commercial thrombolytic agent, many variant molecules have been generated through recombinant DNA technology in attempts to improve its specificity for fibrin (13–15), its activity toward plasminogen activation (13–15), and its circulatory half-life (16). With any such mutant recombinant TPA (rTPA), it is of paramount importance to evaluate its activity in a fibrin clot system containing [Glu¹]plasminogen and physiological levels of Cl⁻. In this investigation, we have used a greatly improved fibrin lysis system, which can provide quantitative data on the abilities of variant rTPA derivatives to generate the plasmin required for fibrin clot lysis.

MATERIALS AND METHODS

Proteins. Human [Glu¹]plasminogen (containing glutamic acid at position one) was prepared from fresh human plasma by our modification (17) of the Deutsch and Mertz (18)

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affinity-chromatography technique. Affinity chromatography form 2 of human [Glu^1]plasminogen was used in the studies described herein. No significant differences in the interpretation of the data resulted when affinity chromatography form 1 was used.

Human fibrinogen was purchased from Helena Laboratories and percolated over Sepharose-lysine to remove contaminating plasminogen. Human thrombin was purchased from Sigma.

Wild-type rTPA and all rTPA variant proteins were provided by Monsanto or Eli Lilly (F-E-P domains). The human melanoma cDNA for rTPA was expressed in mouse mammary C127 cells. Mutant rTPA derivatives were prepared by site-directed mutagenesis of the wild-type gene and expressed in this same system. The strategy was to delete regions between introns. In cases where the F region was deleted, the amino-terminal five amino acids were retained, since this aided expression of the recombinant proteins. Specifically, the proteins used (and amino acids contained in the sequences) were designated by the domains they contain: F-K$_1$-K$_2$-P (1-50, 87-527), K$_1$-K$_2$-P (1-5, 87-527), K$_2$-P (1-5, 176-527), F-K$_2$-K$_2$-P (1-50, 176-262, 176-527), and F-E-P (1-86, 262-527).

Each mutant cDNA was subjected to nucleotide sequence analysis to verify the accuracy of the mutation. The recombinant proteins yielded the expected mobilities on reduced and nonreduced NaDodSO$_4$/polyacrylamide gels before and after treatment with plasmin. All proteins (except, of course, the plasmin cleavage site-resistant mutant) were converted to their two-chain forms as a consequence of treatment with Sepharose-plasmin (19). The concentration of active protein was determined by titration with 4-methylumbelliferyl p-guanidinobenzoate (19).

All proteins were equilibrated with 10 mM Hepes brought to pH 7.4 with NaOH.

Clot Lysis Assay. A total of 20 μl of the desired two-chain rTPA (0.25 nM; final concentration of titrated protein, 8 nM), containing εAhx when desired, was placed in individual wells of a 96-well microtiter plate. To these same wells were added 20 μl of a solution containing thrombin (final concentration, 5 international units/ml) and [Glu^1]plasminogen (final concentration, 0.1 μM). After this, 160 μl of a solution of fibrinogen (final concentration, 8 μM) and NaCl (final concentration, 0.1 M) was added, and the turbidity of the wells was measured (as the optical density at 405 nm) at various times by using an automatic Artek model 210 (Dynatech) microtiter plate reader. The entire plate was scanned within 30 sec.

The OD/time data were plotted and the $t_{1/2}$ for clot lysis was obtained from the graphs as a function of the concentration of rTPA. A standard curve for the wild-type protein was constructed to which all variant rTPA preparations were compared for determination of their activities relative to the wild-type protein. In the case of evaluation of the efficacy of εAhx toward inhibition of clot lysis with each rTPA examined, a titration with εAhx at the above concentrations of all components (4 nM rTPA) was performed. Plots of $(1/t_{1/2})^{-1}$ against εAhx concentration were constructed to obtain the apparent inhibition constants of this negative effector as the negative values of the respective abscissa intercepts.

RESULTS

A turbidimetric assay for fibrin clot dissolution was performed in microtiter assay plates, wherein the decrease in light scatter at 405 nm of fibrin clots generated under various conditions in individual wells was recorded automatically at different times and used as a quantitative measure of clot dissolution. Use of an automated microtiter plate reader facilitated this assay considerably and allowed many assays to be performed rapidly,
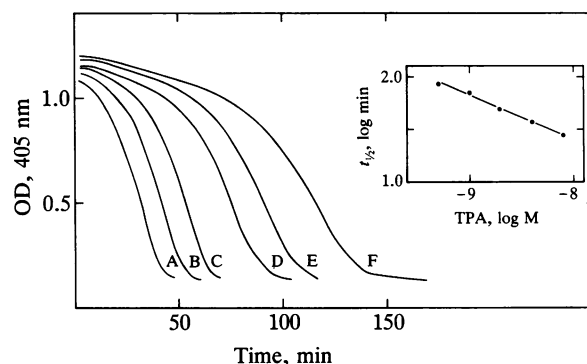

Fig. 1. Fibrin lysis by wild-type rTPA. The decrease in light scatter of a fibrin clot, measured as the optical density at 405 nm, was determined as a function of time with various concentrations of wild-type rTPA. (*Inset*) Double logarithmic plot of $t_{1/2}$ for clot lysis versus the concentration of two-chain rTPA, which has been used as the standard curve for comparison of the fibrin lysis activities of variant rTPA preparations. The wild-type rTPA concentrations were: 8 nM (curve A), 4 nM (curve B), 2 nM (curve C), 1 nM (curve D), 0.5 nM (curve E), and 0.25 nM (curve F).

with a large amount of replication. In all assays, we averaged at least three replicate wells for each time point, a procedure that allowed considerable precision to exist in the data. A similar assay has been used by Carlson *et al.* (20) with a microcentrifugal analyzer system.

The rate of dissolution of a [Glu^1]plasminogen-containing fibrin clot at various concentrations of wild-type two-chain rTPA, as monitored by the temporal decrease in light scatter of the clot, is illustrated in Fig. 1. Here, the rate of formation of the clot is rapid because of the levels of thrombin used and is not a factor in these experiments. Since the initial and final light-scattering values are similar under all conditions used, it is likely that the clots are uniform in their physical properties and dissolve in the same manner under the conditions of importance to this manuscript. From the data of Fig. 1, we constructed a clot-dissolution standard curve (Fig. 1 *Inset*) for various concentrations of wild-type two-chain rTPA to which all variants have been compared for analysis of their relative overall activities in this assay. From each $t_{1/2}$ obtained for the variant proteins, a wild-type two-chain rTPA concentration was calculated from the standard curve of Fig. 1 and was compared to the actual concentration of the rTPA sample used in each assay in Fig. 2 that provided the particular $t_{1/2}$ value. This process allowed calculation of the activity of the sample tested as a percentage of that of wild-type two-chain rTPA
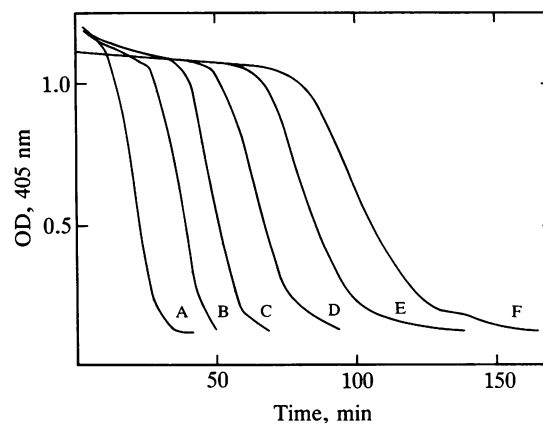

Fig. 2. The fibrin lysis times, determined as in Fig. 1, for wild-type two-chain rTPA (4 nM) as a function of the concentration of εAhx. The εAhx concentrations were: 0 (curve A), 1 mM (curve B), 2 mM (curve C), 3 mM (curve D), 4 mM (curve E), and 5 mM (curve F).

Table 1. Relative fibrinolytic activities of mutant rTPA preparations

Domain structures	Relative activity*	$K_{i_{\varepsilon Ahx}}$,[†] mM
F–E–K_1–K_2–P[‡]	1.00	1.5
F······K_1–K_2–P	0.35	ND
·········K_1–K_2–P	0.31	ND
···············K_2–P	0.31	2.4
F···············K_2–K_2–P	1.38	1.2
F–E···············P	0.22	1.2
F–E–K_1–K_2–P (Cys–264 → Gly)	0.02	ND
F–E–K_1–K_2–P (Arg–275 → Ser)	0.86	1.3

*The activity of wild-type rTPA was assigned an activity of 1.00. All other activities were obtained by comparison of the average $t_{1/2}$ for a range of variant rTPA concentrations (0.25–8.0 nM) with the standard curve (Fig. 1 Inset) for wild-type rTPA.

[†]The K_i for clot lysis of εAhx, obtained as -(abscissa intercept) of plots similar to those of Fig. 3. ND, not determined.

[‡]Wild-type rTPA, consisting of finger (F), growth factor (E), kringle 1 (K1), kringle 2 (K2), and serine protease (P) domains.

activity, which was averaged for all concentrations examined. The final value is listed as the relative activity in Table 1 for all rTPA samples examined.

The ability of εAhx to inhibit overall clot dissolution was examined by this same general assay procedure. Plots of the rate of fibrin dissolution mediated by wild-type rTPA as a function of the εAhx concentration (at constant concentrations of wild-type two-chain rTPA and [Glu^1]plasminogen) are presented in Fig. 2. From plots of the $t_{1/2}$ values against εAhx concentration, an operational constant ($K_{i_{\varepsilon Ahx}}$) identifying the concentration of εAhx that gives 50% of the maximal clot lysis rate was calculated from the negative values of the abscissa intercept. Examples of such plots for wild-type two-chain rTPA, F–K_2–K_2–P, and F–E–P are shown in Fig. 3, and the constants obtained are listed in Table 1.

DISCUSSION

We have examined the clot lytic activity of domain-variant rTPA derivatives by use of an assay system that reflects differences in the overall potency of these agents. In this study, two-chain rTPA was used, since data analysis with the
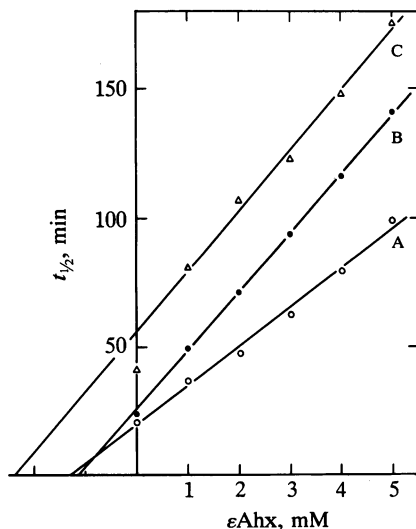

FIG. 3. Plots of the $t_{1/2}$ for fibrin lysis as a function of the εAhx concentration for wild-type two-chain rTPA (○), two-chain rTPA containing the F–K_2–K_2–P domains (●), and two-chain rTPA containing the F–E–P domains. The K_i for εAhx is calculated as the negative value of the abscissa intercept.

one-chain derivative would have been complicated because of feed-back conversion by plasmin of one-chain rTPA to two-chain rTPA. However, we were able to analyze possible differences between one- and two-chain rTPAs by comparing the wild-type two-chain rTPA with a plasmin-cleavage site-resistant mutant, (Arg-275 → Ser)rTPA. As seen from the data of Table 1, the [Glu^1]plasminogen activation activities of both proteins in the presence of the fibrin clot are approximately the same. The small difference that exists may well be due to the nature of the amino acid substitution, and studies with several additional variants would be required prior to establishing a foundation for this difference. Investigations with a different cleavage site-resistant variant, (Arg-275 → Glu)rTPA, also showed similar plasminogen activation activity as that of wild-type two-chain rTPA in the presence of fibrinogen (15), strengthening the conclusion that one- and two-chain rTPAs possess similar activities toward [Glu^1]-plasminogen in the presence of fibrin(ogen).

The plasmin-mediated clot lysis activity of the two-chain rTPA variant lacking the E domain (F–K_1–K_2–P) was ≈35% of that of the wild-type enzyme (Table 1). Since fibrin specificity has not been directly attributed to this domain in previous investigations, it would appear that this effect of the E domain may be due to conformational alterations induced in rTPA consequent to the removal of this region. Indeed, we have found that this rTPA variant is more thermally unstable than the wild-type protein (21), confirming this conclusion. The conclusions are in basic agreement with those of Larsen et al. (22), who demonstrated that rTPA derivatives lacking the F and E domains have lower fibrinolytic activity than the wild-type control. Additional deletions of the F (providing K_1–K_2–P) and K_1 (providing K_2–P) domains do not result in further decreases in the overall activity of the F–K_1–K_2–P variant, suggesting that this residual activity is due to the presence of the K_2 domain. The importance of K_2 in this overall clot lysis activity is emphasized upon comparison of the activities of the variants F–K_1–K_2–P and F–K_2–K_2–P; the latter variant had 4-fold greater activity than the former. Removal of both kringle domains from rTPA results in the derivative F–E–P (Table 1) possessing 22% of the activity of wild-type two-chain rTPA, either implicating directly the importance of F domain in the activity of the molecule and/or the importance of the E region in the overall stability of the molecule. Previous investigations with variant rTPA derivatives have shown that its fibrin-binding properties are mediated through both the F and K_2 domains (14). That covalent linkage of components of the heavy chain of two-chain rTPA is crucial to its overall plasmin-mediated clot lytic activity is amply illustrated by noting that the activity of the mutant (Cys-264 → Gly)rTPA is only 2% of the wild-type material, an amount barely above background levels. In this derivative, covalent attachment of the heavy chain of two-chain rTPA to its light chain has been disrupted.

It has been known for some time that εAhx possesses antifibrinolytic activity. This property is not based upon its direct inhibition of plasminogen activation, since, in fact, εAhx stimulates greatly the activation rate of [Glu^1]plasminogen by urokinase (23, 24) and rTPA (2). This stimulatory effect is based in the reversal (providing the low-S form of [Glu^1]plasminogen) by εAhx of a conformation alteration (high-S form of [Glu^1]plasminogen) and concomitant inhibition of activation induced in [Glu^1]plasminogen by physiological concentrations of Cl$^-$ (25). In the case of plasminogen activation by TPA, the antifibrinolytic effect of εAhx has been assumed to result from dissociation from fibrin of TPA or plasmin or both. It is clear that TPA interacts with fibrin through both the F (14) and K_2 (13) domains and that plasminogen binds to fibrin through its kringle regions, with the K_1 region possessing a tight fibrin site (26, 27) and the K_4 domain displaying weaker fibrin-binding properties (27). In

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addition, it has been shown that εAhx inhibits the binding of the K_2 domain of rTPA to fibrin (13). The results provided in Table 1 suggest that the antifibrinolytic activity of εAhx is not completely accounted for by these mechanisms, and in the assay procedure described here, a more important mechanism exists. As seen in Table 1, the overall K_i for εAhx toward clot lysis is similar for a variety of two-chain rTPA molecules, ranging from wild-type to deletion mutants, the latter lacking the F, E, and K_1 domains (providing K_2-P) and the K_1 and K_2 domains (F-E-P). Additionally, the K_i for εAhx is not substantially affected by insertion of a K_2 domain for a K_1 domain (providing F-K_2-K_2-P) in two-chain rTPA. This inhibitory effect of εAhx does not correlate with the macroscopic binding of rTPA variants to fibrin (13, 14).

The overall antifibrinolytic effect of εAhx also does not correspond to the ability of plasminogen to interact, on a macroscopic level, with fibrin. When a proteolytic fragment of plasminogen, plasminogen-(443–970) with Val-443 at the amino-terminal end, which does not contain the K_1-K_4 domain regions, is substituted for [Glu^1]plasminogen in the assay described herein, the overall K_i for εAhx remains essentially unchanged regardless of the form of two-chain rTPA used or regardless of substitution of the one-chain rTPA variant (Table 1) for two-chain rTPA. Similarly, when two-chain urokinase, which does not supposedly possess fibrin-binding properties and is not stimulated by fibrin to the same extent as TPA to activate [Glu^1]plasminogen (28), is substituted for rTPA in this assay, a similar effect of εAhx is noted, with an apparent K_i of 1.6 mM. This shows that direct fibrin binding of the activator of [Glu^1]plasminogen is not necessary for the inhibition of plasmin-mediated clot lysis by εAhx. In fact, when plasminogen activation is bypassed in this system by addition of a preformed plasmin containing all plasminogen kringles, which we call "(Lys^{78})-plasmin,"† or a preformed plasmin containing only kringle 5, which we call "(Val^{443})-plasmin,"‡ the K_i values for εAhx are within range of those in Table 1. Further, εAhx at the levels used for the clot lysis assays has no effect on the activity of the two preformed plasmin derivatives for casein or for a synthetic small peptide substrate, a result that suggests that the effect of εAhx is fibrin-specific under our assay conditions. Given the results presented herein regarding the antifibrinolytic effect of εAhx, at least two important mechanistic conclusions are possible: (i) that an important εAhx-mediated association of plasmin(ogen) and fibrin occurs that is independent of the K_1-K_4 domains, the location of this site existing within the K_5-P region; and/or (ii) that an important εAhx-fibrin(ogen) interaction is present that results in inhibition of plasminolysis of fibrin. It is important to note that a previous work has shown that an εAhx dissociable interaction of fibrin with (Val^{443})-plasmin does exist (27). However, data in that same study (27) also showed that fibrin(ogen) fragment E contained the major macroscopic binding site(s) for plasminogen and its fragments, and that fibrin(ogen) fragment D did not interact with plasminogen to the same extent. Since ensuing work clearly demonstrated that fragment E was not nearly as effective as fibrinogen fragment D in stimulation of plasminogen activation (29), it would appear that macroscopic binding may not be fully reflective of events that are responsible for stimulation of [Glu^1]plasminogen activation by fibrin(ogen). Different, but more kinetically

productive binding phenomena than those revealed by simple interactive systems obviously have importance in this system. On the other hand, mechanism ii above is particularly appealing and is supported by previous work, suggesting that εAhx interacts with the fibrin clot (30).

In conclusion, use of domain-variant and point-mutated rTPA and plasminogen derivatives in an overall clot lysis system has revealed important aspects of the mechanism of activation of native [Glu^1]plasminogen by rTPA.

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†[Glu^1]Plasminogen-(78–561), a derivative with cleavage between Arg-561 and Val-562 and between Lys-77 and Lys-78, containing Lys-78 as the N terminus of the nonprotease chain.
‡[Glu^1]Plasminogen-(443–561), a derivative with cleavage between Arg-561 and Val-562 and between position 442 and Val-443, containing Val-443 as the N terminus of the nonprotease chain.