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Variants of human tissue-type plasminogen activator that lack specific structural domains of the heavy chain

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The heavy chain of tissue plasminogen activator (t-PA) consists of four domains [finger, epidermal-growth-factor (EGF)-like, kringle 1 and kringle 2] that are homologous to similar domains present in other proteins. To assess the contribution of each of the domains to the biological properties of the enzyme, site-directed mutagenesis was used to generate a set of mutants lacking sequences corresponding to the exons encoding the individual structural domains. The mutant proteins were assayed for their ability to hydrolyze artificial and natural substrates in the presence and absence of fibrin, to bind to lysine-Sepharose and to be inhibited by plasminogen activator inhibitor-1. All the deletion mutants exhibit levels of basal enzymatic activity very similar to that of wild-type t-PA assayed in the absence of fibrin. A mutant protein lacking the finger domain has a 2-fold higher affinity for plasminogen than wild-type t-PA, while the mutant that lacks both finger and EGF-like domains is less active at low concentrations of plasminogen. Mutants lacking both kringles neither bind to lysine-Sepharose nor are stimulated by fibrin. However, mutants containing only one kringle (either kringle 1 or kringle 2) behave indistinguishably from one another and from the wild-type protein. We conclude that kringle 1 and kringle 2 are equivalent in their ability to mediate stimulation of catalytic activity by fibrin.

Key words: epidermal growth factor/exon-deleted variants/finger/fibrin activation/kringle/tissue-type plasminogen activator/serine protease

Introduction

Tissue plasminogen activator (t-PA), an enzyme widely used as a thrombolytic agent in the treatment of acute myocardial infarction, does not dissolve blood clots directly. Instead, t-PA specifically cleaves a single peptide bond in plasminogen, thereby converting the inactive proenzyme into the powerful but non-specific protease, plasmin, which efficiently solubilizes the fibrin mesh that forms the core of the clot (Collen, 1980). The rate of activation of plasminogen by t-PA is accelerated by 2-3 orders of magnitude in the presence of polymerized fibrin, to which both plasminogen and t-PA bind (Hoylaerts *et al.*, 1982; Ranby, 1982; Rijken *et al.*, 1982). This combination of properties leads to a preferential production of plasmin on the surface of the clot, where its activity will be most beneficial.

The amino acid sequence of the human t-PA precursor, deduced from the nucleotide sequence of cloned cDNAs (Pennica *et al.*, 1983; Harris *et al.*, 1986) is 563 residues in length, including a signal sequence of 20-23 hydrophobic amino acids and a hydrophilic 'pro' sequence of 12-15 amino acids. Oligosaccharide groups are added at three potential glycosylation sites on the molecule and the signal and pro sequences are cleaved from the newly synthesized enzyme as it is transported along the secretory pathway (Pohl *et al.*, 1984, 1987; Vehar *et al.*, 1984; Sambrook *et al.*, 1986). t-PA is secreted from endothelial cells as a single polypeptide chain that is subsequently cleaved (between Arg-275 and Ile-276) into two chains held together by a single disulfide bond (Rijken and Collen, 1981). Both the single- and two-chain forms of the enzyme can bind to fibrin (Rijken *et al.*, 1982; Higgins and Vehar 1987), although the two-chain form is catalytically more active (Wallen *et al.*, 1982; Ranby, 1982; Tate *et al.*, 1987; Boose *et al.*, 1988; Petersen *et al.*, 1988). In addition, both forms of the enzyme (Jorgensen *et al.*, 1987; Boose *et al.*, 1988) are inactivated by a fast-acting plasminogen activator inhibitor (PAI-1), a member of the serpin family that is secreted from endothelial cells and forms a covalent bond with Ser-478 of t-PA (Levin, 1983; for review, see Sprengers and Kluft, 1987).

Mature t-PA (527 amino acids) is organized by 16 disulfide bridges into a series of five discrete structural domains that are strikingly homologous to similar domains found in other secreted and cell surface proteins, including several serine proteases of plasma (Ny *et al.*, 1984; Patthy, 1985). Residues 4-50 of the mature protein form a 'finger' domain (F), closely related to the fibrin-binding finger structures of fibronectin (Banyai *et al.*, 1983). Residues 51-87 share homology with the precursor to epidermal growth factor (EGF) (Doolittle *et al.*, 1984) and with similar domains in a variety of other proteins, including urokinase (Verde *et al.*, 1984), protein C (Foster and Davie, 1984), coagulation factors IX and X (Anson *et al.*, 1984; Leytus *et al.*, 1984), the receptor for low-density lipoprotein (Russell *et al.*, 1984) and differentiation-specific proteins encoded by *Drosophila melanogaster* (Wharton *et al.*, 1985) and *Caenorrhabditis elegans* (Greenwald, 1985). This EGF-like domain is referred to as E. Residues 88-175 and 176-263 form two sequential 'kringle' domains (K_1 and K_2), each built around three characteristic intradomain disulfide bonds (Olsson *et al.*, 1982; Park and Tulinsky, 1986; Holland *et al.*, 1987). Domains of similar structure are found in plasminogen, prothrombin, urokinase and lipoprotein(a) (Patthy *et al.*, 1984; Patthy, 1985; McLean *et al.*, 1987). The remainder of the molecule (residues 277-527) constitutes the catalytic domain, which shares homology with other members of the

serine protease family and contains a typical catalytic triad of amino acids (His-322, Asp-371 and Ser-478).

t-PA, like many other plasma proteins, has evolved by exon shuffling (Doolittle, 1985; Blake *et al.*, 1987). The 29-kb gene for human t-PA contains 14 exons, whose boundaries show a striking correspondence to the boundaries between the putative structural domains of the enzyme (Ny *et al.*, 1984; Freizner-Degen *et al.*, 1986). From this and other evidence, Patthy (1985) argued persuasively that the gene for t-PA evolved into its present mosaic form by duplication and exchange of individual exons and groups of exons. This led to the idea that the structural domains of the protein might contribute autonomous functions to the enzyme. A number of groups have tested aspects of this hypothesis by analyzing the properties of mutant proteins that lack various segments of the wild-type enzyme (Kagitani *et al.*, 1985; van Zonneveld *et al.*, 1986a,b,c; Verheijen *et al.*, 1986; Larsen *et al.*, 1988; Browne *et al.*, 1988; Kalyan *et al.*, 1988; for review, see Harris, 1987). Particular effort has been made to identify the domain(s) of the heavy chain responsible for binding of the enzyme to fibrin and the consequent stimulation of its catalytic activity. The results of previous experiments indicate that, in the absence of plasminogen and at low concentrations of fibrin, binding of t-PA to fibrin is due mainly to the finger domain. However, at physiological concentrations of plasminogen, both binding of the enzyme to fibrin and stimulation of its activity have been ascribed to kringle 2 (van Zonneveld *et al.*, 1986a,b,c; Verheijen *et al.*, 1986). Kringle 1 has been thought to play no part in these processes. These conclusions were based on analyses of mutants that were constructed by deletion of sequences lying between naturally occurring restriction sites within t-PA cDNA. While many of the sites map near the borders of putative structural domains of the protein, others lie some distance away. This raises the possibility that the loss of function exhibited by certain of the mutants is due to malfolding rather than the absence of a particular structural domain from the protein. We have therefore used site-directed mutagenesis to delete precisely the individual domains of the heavy chain, both singly and in combination. We have expressed the mutants in simian cells and have used a variety of *in vitro* assays to characterize the proteins secreted into the conditioned medium.

Results

Construction and expression of t-PA mutants lacking structural domains

To investigate the function of the structural domains of the heavy chain of t-PA, oligonucleotide-directed mutagenesis was used to construct cDNAs encoding mutant enzymes that lack either a single domain (F^-, E^-, K_1^- or K_2^-), two adjacent domains (F^-E^- or $K_1^-K_2^-$) or all four structural domains (HC^-) of the heavy chain, but retain the signal and pro sequences and the complete catalytic light chain. In each case the mutant enzymes were designed to contain no unpaired cysteine residues that might form improper intra- or interdomain disulfide bonds and generate abnormally folded proteins. Figure 1 shows a schematic comparison of the structure of wild-type t-PA and t-PAs encoded by the deletion mutants.

The mutant t-PA sequences were inserted into plasmids containing a copy of the bacteriophage SP6 promoter (Melton *et al.*, 1984). RNAs synthesized *in vitro* were translated in reticulocyte lysates and the resulting proteins were immunoprecipitated with anti-t-PA serum and analyzed by SDS−PAGE. The deletion mutants encode proteins that migrate with mobilities approximately corresponding to their expected mol. wts relative to wild-type t-PA (Figure 2). However, some degree of anomalous migration was observed. For example, although the F^-E^-, K_1^- and K_2^- proteins should differ in size by only one to four amino acids, their apparent mol. wts differ by 1−2 kd (Figure 2). The possibility that the deletion mutants might carry additional alterations was ruled out both by a detailed analysis of restriction digests and by DNA sequencing of the mutagenized regions. In addition, the entire cDNA coding for the K_1^- mutant (which displayed the most anomalous migration) was sequenced. The nucleotide sequence exactly matched that of wild-type t-PA apart from the expected deletion that removed the two exons encoding kringle 1. Finally, the enzymatic properties of the K_1^- and K_2^- mutants synthesized in mammalian cells are indistinguishable from those of wild-type t-PA (see below), a result that would not be expected if these mutants contained multiple alterations. We therefore believe that any anomalous migration of the mutants through SDS−polyacrylamide gels is a consequence of changes in the net charge of the proteins or in their ability to bind detergent.

Two different systems were used to express the wild-type and mutant t-PA cDNAs in simian cells. SV40-based viruses encoding the mutant proteins were generated as described previously (Doyle *et al.*, 1985) after substituting the mutant cDNAs for the wild-type t-PA sequences in the SV/t-PA3 recombinant genome (Sambrook *et al.*, 1986). The recombinant viruses were then used to infect CV-1 cells. Alternatively, the wild-type and mutant t-PA cDNAs were inserted into the pSVT7 transient expression vector (Bird *et al.*, 1987) and introduced into COS-1 cells. No significant differences were observed in the characteristics of the t-PA enzymes synthesized using the two expression systems. When metabolically radiolabeled forms of t-PA secreted from simian cells expressing the wild-type or mutant cDNAs were analyzed by SDS−PAGE, all of the deletion mutants encoded glycosylated proteins with approximately the expected electrophoretic mobilities (results not shown, but see for example Figure 4).

Wild-type t-PA and mutants lacking structural domains exhibit identical activities in a direct amidolytic assay

A direct amidolytic assay was used to measure the enzymatic activities of antigenically equal amounts of wild-type and mutant t-PAs secreted from simian cells. This assay (see Materials and methods) employs the chromogenic substrate, Spectrozyme tPA, cleavage of which results in release of free *p*-nitroaniline that is detected spectrophotometrically. The specific amidolytic activities of all of the mutant t-PAs (which varied between 22 000 and 25 000 U/mg) were very similar to that of the wild-type enzyme (24 000 U/mg). We conclude that removal of the structural domains of the heavy chain, either individually or *en masse*, has little or no effect on the ability of the catalytic, light chain of t-PA to hydrolyze a low-molecular weight substrate.

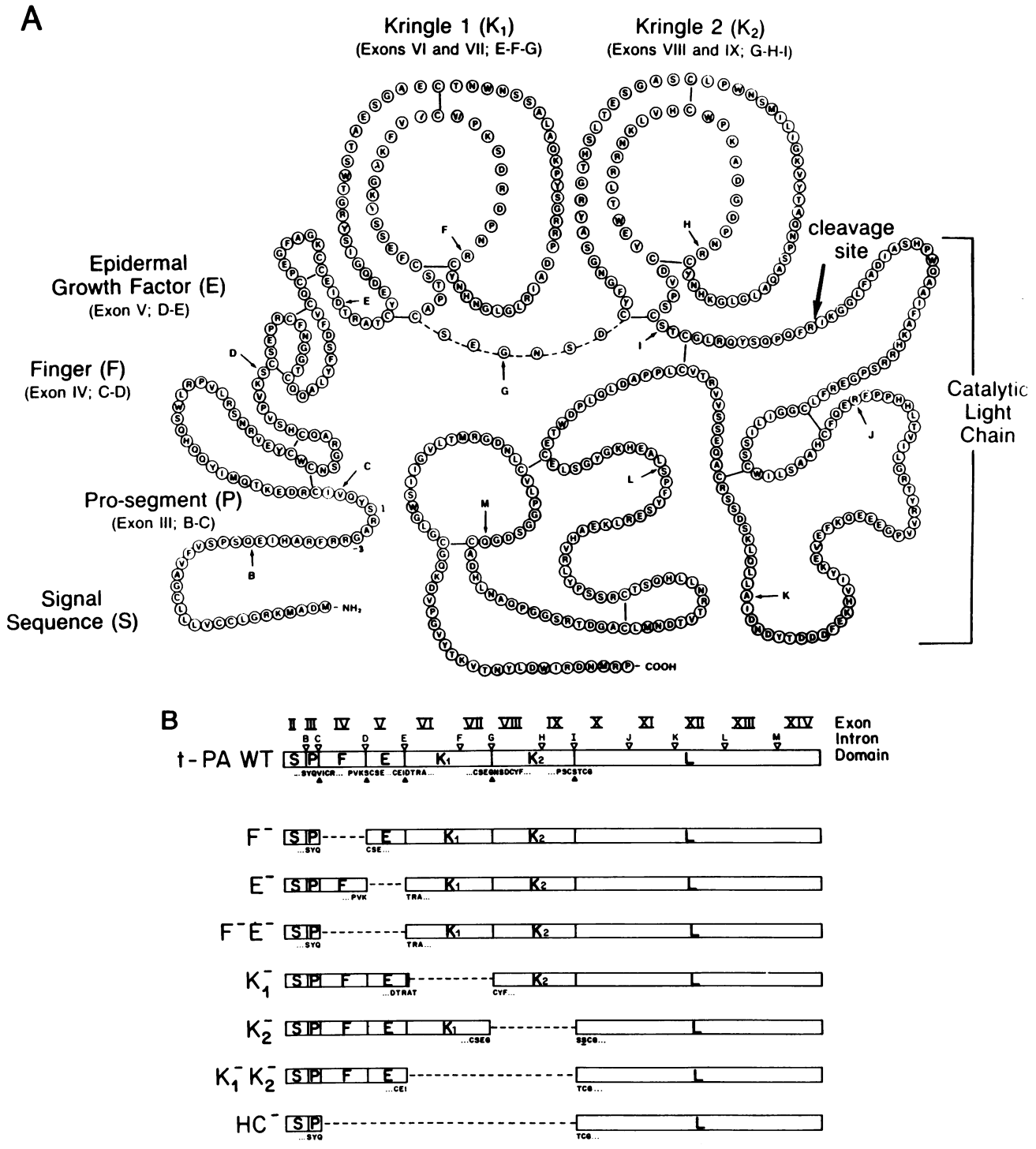

Fig. 1. Schematic representation of the structures of wild-type and mutant t-PA pròteins. **Panel A** illustrates the amino acid sequence (Pennica *et al.*, 1983) and the predicted secondary structure (Ny *et al.*, 1984) of the precursor of wild-type t-PA, including the signal sequence (S) and the pro-segment (P). The solid bars indicate the potential disulfide bridges, and the site of proteolytic cleavage into heavy (H) and catalytic light (L) chains is shown by a bold arrow. The arrows marked with letters B–M indicate the map positions of individual exons in the protein (Ny *et al.*, 1984). The locations of the finger (F), EGF-like (E) and kringle 1 (K_1) and kringle 2 (K_2) domains of the heavy chain are shown, together with notation (roman numerals) of the exon(s) that encode each domain. This diagram is modified from Ny *et al.* (1984). **Panel B** illustrates the domain structure of the wild-type and mutant t-PA proteins in a linear format, with the deleted sequences shown as dotted lines. The amino acid sequences flanking the point of deletion are shown beneath each diagram.

Removal of structural domains can affect the ability of t-PA to activate plasminogen in the presence of soluble fibrin

Wild-type and mutant forms of t-PA secreted from simian cells were tested in an indirect assay for their ability to activate plasminogen in the presence of soluble fibrin. In this assay, t-PA converts plasminogen to plasmin which, in turn, hydrolyzes a synthetic chromogenic substrate to release free *p*-nitroaniline (see Materials and methods). The addition of fibrin to the assay mixture stimulates the activity of the

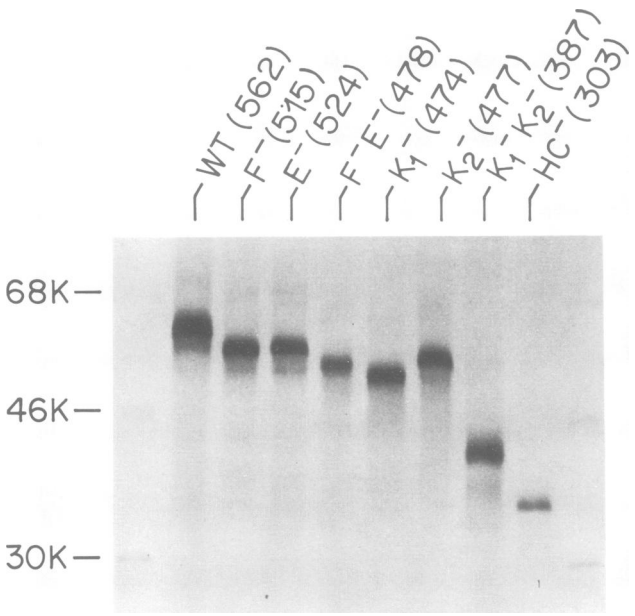

Fig. 2. Comparison of the apparent mol. wts of wild-type and domain-deleted t-PA proteins. The cDNAs encoding the wild-type and mutant t-PA proteins were inserted into plasmids containing a copy of the bacteriophage SP6 promoter. RNAs synthesized *in vitro* were translated in reticulocyte lysates and the resulting polypeptides were immunoprecipitated with anti-t-PA serum and analyzed by SDS–PAGE and autoradiography (see Materials and methods). The number of amino acids in each polypeptide is shown in parentheses.

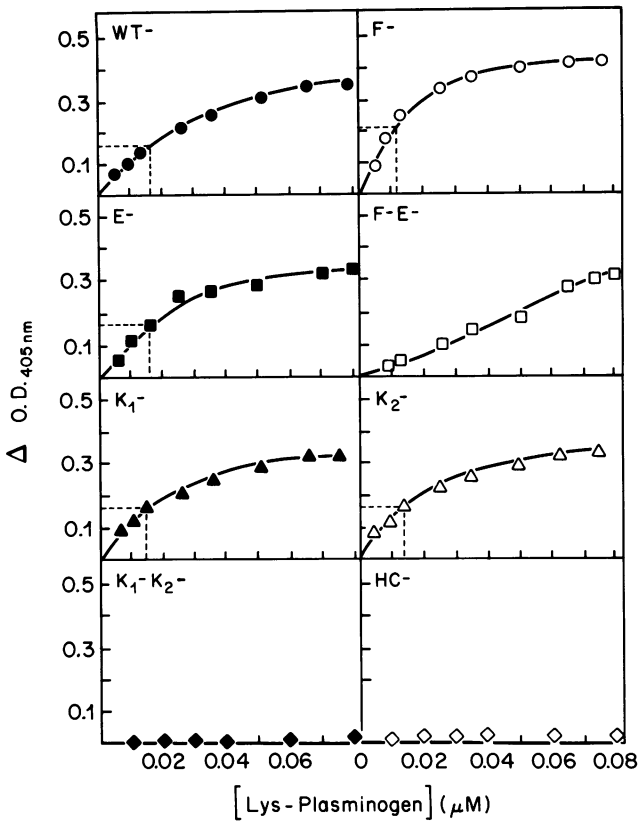

Fig. 3. Effect of plasminogen concentration on the activities of wild-type and mutant t-PA enzymes. The indirect chromogenic assay (see Materials and methods) was used to determine the activity of wild-type and mutant t-PAs over a range of lys–plasminogen concentrations in the presence of a saturating concentration of fibrin (DESAFIB, 25 μg/ml).

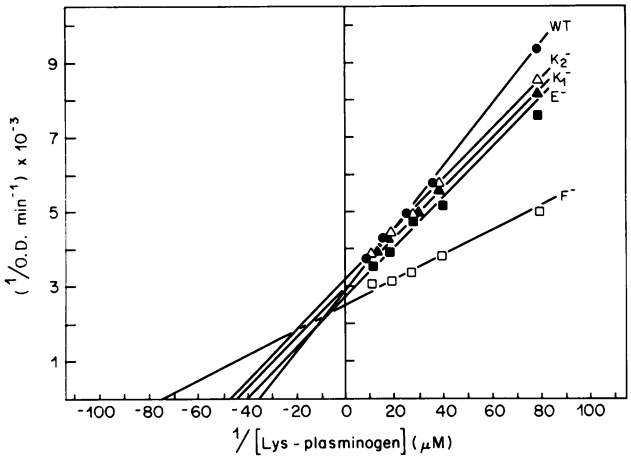

Fig. 4. Effect of plasminogen concentration on the activity of wild-type and mutant t-PA proteins. The data presented in Figure 3 are replotted in the Lineweaver–Burke format.

wild-type enzyme (Hoylaerts *et al.*, 1982; Ranby, 1982; Rijken *et al.*, 1982; see also below). The results obtained when wild-type and mutant t-PA enzymes were tested over a range of plasminogen concentrations in the presence of saturating amounts of fibrin are shown in Figures 3 and 4. Wild-type t-PA and the deletion mutants E^-, K_1^- and K_2^- exhibited apparent Michaelis–Menten kinetics in the assay, displaying very similar values for K_m and V_{max}. The F^- mutant also exhibited apparent Michaelis–Menten kinetics, but its affinity for plasminogen (K_m = 0.013 μM) was ~2-fold higher than that of wild-type t-PA (K_m = 0.028 μM). By contrast, the reaction catalyzed by the mutant enzyme that lacks both finger and EGF-like domains (F^-E^-) deviated significantly from Michaelis–Menten kinetics, displaying a sigmoidal response to increasing concentrations of plasminogen. To eliminate the possibility that the altered reaction kinetics displayed by the F^- and F^-E^- mutants were the result of accelerated or delayed conversion of the single-chain forms of the enzymes to the more active two-chain forms during the assay (Boose *et al.*, 1988), we used SDS–PAGE to analyze the molecular forms of radiolabeled wild-type and mutant t-PAs that were present following incubation for various times under the reaction conditions used in the assays shown in Figure 3. The results obtained for wild-type t-PA and the F^- and F^-E^- mutants are shown in Figure 5. Densitometric analysis of shorter exposures of the autoradiographs shown in the figure indicated that deletion of the finger or EGF-like domains did not affect the rate of cleavage. For every mutant, conversion to the two chain form was complete after 25 min of incubation (Figure 5). Thus the data depicted in Figures 3 and 4, which were derived from a series of measurements initiated 30 min after the start of the reaction, correspond entirely to the two-chain forms of the wild-type and mutant proteins, ruling out the explanation that altered reaction kinetics of these mutants are the result of altered rates of proteolytic conversion.

Mutants lacking both kringles ($K_1^-K_2^-$) or the entire heavy chain of t-PA (HC^-) showed a dramatic reduction in their ability to activate plasminogen (Figure 3). In the

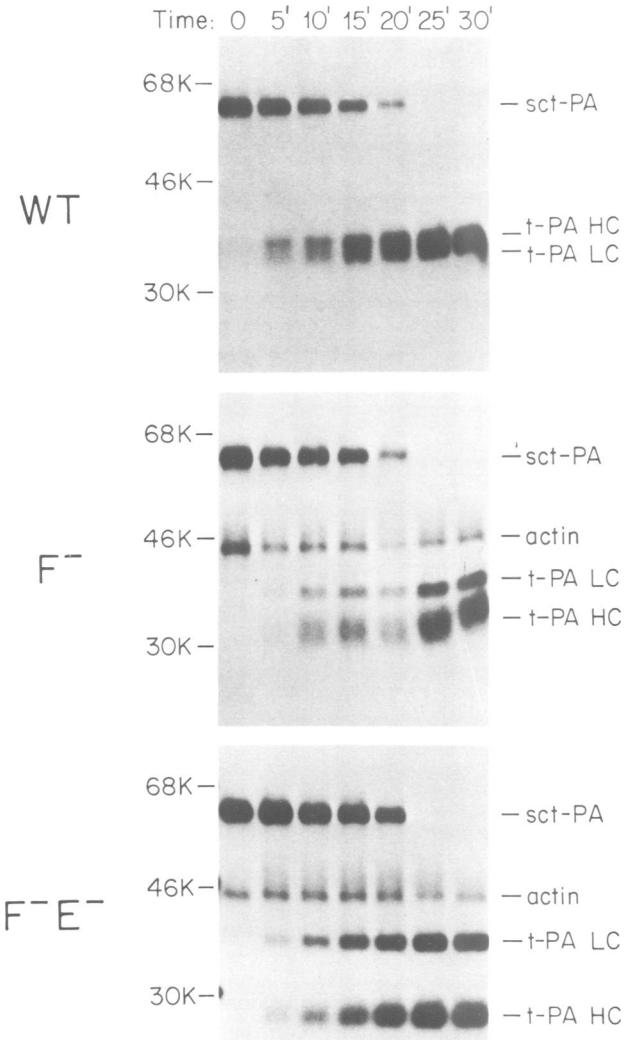

Fig. 5. Time course of conversion of wild-type and mutant t-PAs from the single-chain to the two-chain form during the indirect chromogenic assay. Wild-type (WT) t-PA and mutants lacking the finger domain (F^-) or both the finger and the EGF-like domains (F^-E^-) were labeled with [^{35}S]cysteine as described in Materials and methods and then incubated for various periods under assay conditions identical to those used in the experiment shown in Figure 3. The t-PA species were then immunoprecipitated, separated by SDS–PAGE and visualized by autoradiography. Bands corresponding to single-chain t-PA (sct-PA), the t-PA heavy chain (t-PA HC) and the light chain (t-PA LC) are indicated on the figure. The size of the HC band is reduced in the mutant proteins, due to the deletion of one or more domains from the heavy chain. In some experiments, contamination by cellular actin was not avoided during the immunoprecipitation procedure.

presence of plasminogen concentrations that were saturating for wild-type t-PA, the mutants lacking both kringles were 10- to 20-fold less active than the wild-type enzyme. As discussed below, this is a consequence of the failure of these mutants to respond to fibrin.

Removal of structural domains can affect the ability of fibrin to activate t-PA

The wild-type and mutant forms of t-PA were also tested in the indirect assay for their ability to activate plasminogen in the absence or presence of increasing concentrations of

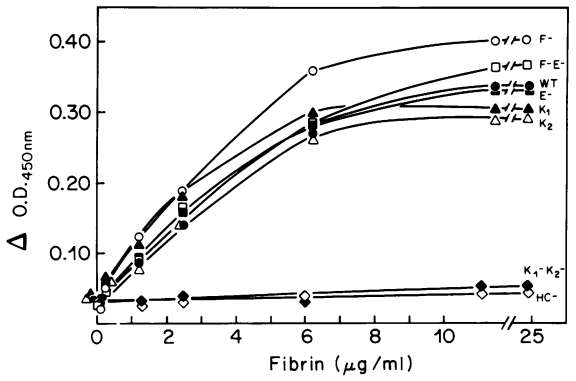

Fig. 6. Effect of fibrin concentration on the activity of wild-type and mutant t-PA proteins. Wild-type and mutant t-PAs were assayed as described in Materials and methods using lys–plasminogen as substrate in the presence of varying concentrations of fibrin (DESAFIB).

soluble fibrin (Figure 6). In the absence of fibrin the abilities of the wild-type and mutant proteins to activate plasminogen were not significantly different, indicating that the basal activity of the catalytic light chain on its natural substrate plasminogen was not affected by the removal of any or all of the structural domains from the heavy chain. As reported previously (Hoylaerts *et al.*, 1982), the addition of increasing concentrations of fibrin caused stimulation of the activity of wild-type t-PA. Maximum stimulation (20- to 30-fold) was achieved at concentrations of soluble fibrin >25 μg/ml. This degree of stimulation of t-PA activation of lys–plasminogen is similar to that reported by others using the soluble fibrin DESAFIB (e.g. Karlan *et al.*, 1987).

Only the mutants that lacked both kringle domains (K_1^- K_2^-) or the entire heavy chain (HC^-) failed to display any significant stimulation by soluble fibrin of the basal t-PA activity. It seems unlikely that this lack of response to fibrin is a consequence of gross malfolding of the mutant t-PA proteins, since the $K_1^-K_2^-$ and HC^- proteins displayed approximately wild-type levels of enzymatic activity on the synthetic substrate Spectrozyme tPA and on plasminogen in the absence of fibrin (see above). Furthermore, both mutants could be cleaved efficiently by plasmin into the two-chain forms without further degradation (results not shown). By contrast, both the mutants that contain a single kringle (K_1^- and K_2^-) were stimulated by fibrin to the same extent as the wild-type enzyme. These results demonstrate that (i) the presence of one kringle is both necessary and sufficient for stimulation of plasminogen activation by soluble fibrin, and (ii) either kringle, K_1 or K_2, can facilitate the stimulation of t-PA by fibrin.

The remaining mutants (F^-, E^- and F^-E^-) displayed levels of stimulation by soluble fibrin that were similar to or slightly higher than that of the wild-type enzyme. The increased level of activity of the F^- mutant at the higher fibrin concentrations was a consistent observation, although the amount of additional stimulation over that displayed by the wild-type enzyme showed some variation from experiment to experiment.

t-PA mutants that lack both kringles fail to bind to lysine–Sepharose

It has been proposed previously that the interaction of t-PA with fibrin, like that of plasminogen (Collen, 1980), is mediated by lysine-binding sites (van Zonneveld *et al.*,

1986b). To determine if the results discussed above reflected the inability of mutants lacking both kringle domains to bind to COOH-terminal lysine residues on fibrin, we measured the binding of the wild-type and mutant proteins to lysine–Sepharose. Samples of wild-type and mutant t-PAs (2.5 ng) were incubated overnight at 4°C with a slurry of lysine–Sepharose. The beads were then collected by centrifugation and the t-PA antigen remaining in the supernatant was determined by radioimmunoassay. The results demonstrated that the $K_1^-K_2^-$ and HC^- mutants failed to bind detectably to lysine–Sepharose. By contrast, mutants lacking only one (K_1^-, 89% binding) or the other (K_2^-, 87% binding) of the kringle mutants were indistinguishable from the wild-type t-PA (89% binding) in their ability to associate with lysine–Sepharose. Mutants lacking the finger domain (F^-, 80% binding) or both the finger and EGF-like domains (F^-E^-, 75% binding) also bound efficiently to the resin. These results indicate that each of the kringles in t-PA must contain a functional lysine-binding site.

Deletion of structural domains from the t-PA heavy chain does not prevent inhibition by the serpin, PAI-1

Human endothelial cells secrete a fast-acting inhibitor (the serpin PAI-1, M_r = 50 000) that forms stable 1:1 complexes with wild-type t-PA (Levin, 1983; van Mourik *et al.*, 1984; for review, see Sprengers and Kluft, 1987). We used the indirect chromogenic assay to determine whether PAI-1 was able to inhibit the activity of the t-PA deletion mutants. Equal amounts of wild-type t-PA and those deletion mutants that contain at least one kringle domain (F^-, E^-, F^-E^-, K_1^- and K_2^-) were preincubated with various amounts of PAI-1 isolated from cultured human endothelial cells (Levin *et al.*, 1984) and then assayed for enzymatic activity. The degree of inhibition by PAI-1 of the $K_1^-K_2^-$ and HC^- mutants was compared to that of the wild-type t-PA diluted to yield an approximately equal level of activity. The results (Figure 7) show that all of the mutant t-PAs were inhibited by PAI-1 to the same extent as the wild-type enzyme. We conclude that none of the structural domains of the heavy chain of t-PA are required for inhibition of catalytic activity by PAI-1. A similar conclusion has been published by van Zonneveld *et al.* (1986c).

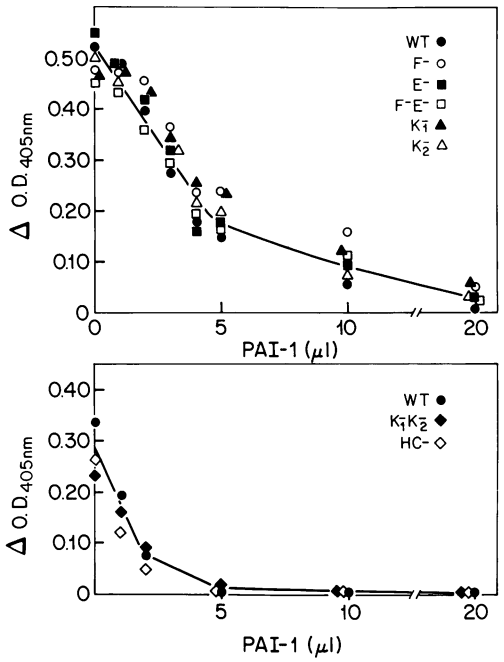

Fig. 7. Inhibition of wild-type and mutant t-PAs by PAI-1. **Upper panel.** Antigenically equal amounts of wild-type (WT) t-PA and those deletion mutants that contain at least one kringle domain (F^-, E^-, F^-E^-, K_1^- and K_2^-) were incubated with increasing amounts of PAI-1 for 1 h at room temperature prior to analysis by the indirect chromogenic assay using a 2-h reaction time (see Materials and methods). **Lower panel.** The degree of inhibition by PAI-1 of the $K_1^-K_2^-$ and HC^- mutants was compared to that of the wild-type t-PA diluted to yield an approximately equal level of activity. Following incubation with PAI-1 for 1 h at room temperature, indirect assays were carried out using a 12-h reaction time.

Discussion

t-PA contains several motifs that resonate through a number of mammalian proteins, sometimes appearing once and sometimes as several copies in tandem (Patthy, 1985). Because each of these motifs is encoded by a single exon or group of exons (Ny *et al.*, 1984), it seems likely that they behave as autonomous structural domains. To test this hypothesis and to assign functions to the individual domains, we have constructed and analyzed a series of t-PA deletion mutants that lack sequences corresponding to the exons encoding each of the four principal domains of the heavy chain of the enzyme. The results obtained by assaying these mutants in a standard set of *in vitro* reactions can be summarized as follows.

(i) Removal of the finger domain increases ~2-fold the affinity of t-PA for its natural substrate plasminogen. The mutant enzyme retains wild-type capacity for stimulation by fibrin under *in vitro* assay conditions.

(ii) The absence of the EGF-like domain has no detectable effect on the behavior of t-PA in the *in vitro* assays used in this study.

(iii) Deletion of both finger and EGF-like domains yields a form of the enzyme that responds in a sigmoidal fashion to increasing concentrations of plasminogen. This enzyme therefore requires ~3-fold more plasminogen than wild-type t-PA to attain half-maximal activity. However, at saturating concentrations of the substrate, the mutant enzyme is stimulated normally by fibrin.

(iv) t-PA must carry at least one kringle domain in order to be stimulated by fibrin. Both kringles 1 and 2 are equally effective in these processes. However, t-PAs carrying both kringles are no more effective than t-PAs that carry either kringle alone.

(v) Removal of both kringles (or all four structural domains) from the heavy chain generates mutant enzymes with basal levels of activity that are not significantly stimulated by soluble fibrin. However, the catalytic activities of these kringle-minus mutants on a low-molecular weight substrate, or on plasminogen in the absence of fibrin, are very similar to that of wild-type t-PA.

(vi) All the deletion mutants are inhibited by the serpin PAI-1 to an extent comparable to that of wild-type t-PA. Following removal of the signal and pro sequences, the HC^- mutant encodes, in addition to the light chain, only 17–20 amino acids of the heavy chain. Thus the primary target for the inhibitor almost certainly lies in the light chain of t-PA.

This is the first report of a complete set of deletion mutants, constructed by oligonucleotide-directed mutagenesis, that lack each of the individual structural domains of the t-PA heavy chain. Other workers have utilized the same technique to construct mutants corresponding to a subset of those described in this paper. In studies utilizing mammalian expression systems, Larsen *et al.* (1988) have analyzed the activity and fibrin-binding capacity of t-PA mutants lacking the finger and/or the EGF-like domains, while Kalyan *et al.* (1988) have characterized another mutant that lacks both of these domains. Browne *et al.* (1988) have tested the *in vivo* clearance of a growth-factor-minus t-PA, but have not reported any functional assays on the mutant enzyme. Since the intention in all these studies was to delete individual exons as precisely as possible, the various mutants comparable to ours differ only slightly in the choice of the amino acid positions at which the domain deletions begin and end. A quite different approach has been taken by two other groups who have utilized restriction sites within the t-PA cDNA sequence to construct mutants that lack multiple domains of the heavy chain (Verheijen *et al.*, 1986; van Zonneveld *et al.*, 1986a,b,c). These studies were aimed at analyzing which functions are retained by molecules that contain only the light chain and chosen domains of the heavy chain. The positions of available restriction sites dictated the end points of the deletions, although in most cases these lie reasonably close to the exon junctions.

Where different groups have reported data for mutants of similar structure [e.g. the F^-, E^- and F^-E^- mutants described by ourselves and by Larsen *et al.* (1988)], the results have been consonant whenever comparable conditions were employed. However, the F^-E^- mutant described by Kalyan *et al.* (1988) is insensitive to fibrin stimulation, in contrast to the mutants described by ourselves and by Larsen *et al.* (1988). It is possible that this difference is due to conformational changes in the other domains of the heavy chain caused by the more extensive deletion (residues 2–89) created by Kalyan *et al.* compared with those created by ourselves (residues 4–87) or Larsen *et al.* (residues 6–86). As discussed below, it is likely that a similar explanation accounts for the loss of fibrin responsiveness in several mutants constructed using a *Sca*I restriction site located within the sequences encoding kringle 2 (van Zonneveld *et al.*, 1986a,b,c; Verheijen *et al.*, 1986). These results underscore the need to interpret loss-of-function phenotypes with caution, since they may be caused by conformational changes in the remaining portions of the molecule rather than by loss of an individual functional domain. On the other hand, retention of function by a mutant provides incontrovertible evidence that the deleted domain is not required for that function. Since we have constructed a complete set of mutants each lacking only one of the domains of the heavy chain, we have been able not only to correlate loss of function with a deletion of a domain, but also to verify that this function is expressed by all the mutant proteins that retain the domain.

It is generally accepted that the finger domain is involved in the high affinity interaction between t-PA and fibrin (van Zonneveld *et al.*, 1986b; Verheijen *et al.*, 1986; Larsen *et al.*, 1988), a role consistent with this domain's homology with the finger structures required for the fibrin affinity of fibronectin (Sekiguchi *et al.*, 1981; Petersen *et al.*, 1983). We and others (van Zonneveld *et al.*, 1986b) have observed that the interaction of finger [and/or the EGF-like domain (Larsen *et al.*, 1988)] with fibrin plays little or no direct role in the stimulation of t-PA activity by fibrin. Van Zonneveld *et al.* (1986b) have proposed a two-phased mechanism of fibrinolysis in which t-PA is initially bound to intact fibrin via the high-affinity sites on the finger domain. Although the enzyme is not stimulated by this interaction with fibrin, sufficient plasmin is generated to partially degrade the fibrin matrix, exposing carboxy-terminal lysine residues which interact with both kringles (see below) causing enhancement of the t-PA activity in the second phase of the reaction. Impairment of the fibrinolytic activity of an F^- mutant at low enzyme concentration (Larsen *et al.*, 1988), or of our F^-E^- mutant at low substrate concentration, might be explained in terms of insufficient generation of plasmin in the first phase to initiate the second phase of enhanced activity. Alternatively, conformational alterations in the deletion mutants might provide the basis for the observed phenotypic changes. We have observed that the affinity of t-PA for plasminogen was increased in the F^- mutant, suggesting that deletion of the finger domain of the heavy chain in some way alters the conformation of the light chain, increasing its affinity for the substrate. It is also possible that binding of plasminogen to the F^-E^- mutant may alter the conformation of the enzyme, resulting in increased affinity of the heavy chain for fibrin and consequent stimulation of enzyme activity.

Perhaps the most important finding of this study is the observation that kringle 1 and kringle 2 are equivalent in their ability to mediate stimulation of catalytic activity by fibrin. However, our conclusions stand in sharp contrast to reports that only one of the two kringles (kringle 2) of t-PA is active in fibrin-binding and stimulation (van Zonneveld *et al.*, 1986a,b,c; Verheijen *et al.*, 1986). These conclusions, which were surprising in view of the high degree of homology between the two kringles (Pennica *et al.*, 1983), were based on analyses of the properties of deletion mutants of t-PA created by excision of restriction fragments from t-PA cDNA. One of the restriction sites used in these studies (the*Sca*I site at nucleotide 950) cleaves within the sequences coding for kringle 2, eight amino acids upstream of the exon/intron border that marks the end of this kringle (Ny *et al.*, 1984). These eight amino acids contain two cysteine residues that normally form disulfide bonds with two of the other cysteine residues of the kringle (Park and Tulinsky, 1986). None of the mutants created with this *Sca*I site either bind efficiently to fibrin or are stimulated by it. In the light of the data presented in this paper, it seems that the defects exhibited by these mutants may result from malfolding of the protein, perhaps mediated by formation of improper disulfide bonds, rather than as a direct consequence of deletion of kringle 2.

In general, our results support the idea that individual exons or groups of exons encode structural domains of t-PA that fold autonomously and carry autonomous functions. However, there are indications that deletion of some domains may result in conformational alterations in the remainder of the molecule, suggesting that the individual domains may interact to some extent with each other. Further *in vitro* and *in vivo* analyses of our deletion mutants should reveal more information about the role of the individual domains in the protein's structure, its function and its rate of turnover. Once functions have been ascribed to domains, site-specific

mutagenesis can be carried out to assess the role of individual amino acids. Since both kringle 1 and kringle 2 confer full activity on t-PA, it now seems likely that fibrin binding and stimulation are mediated by sequences that they hold in common. To test this hypothesis, we are currently carrying out a detailed mutagenic analysis of both kringles.

Materials and methods

Reagents

The chromogenic substrates Spectrozyme tPA (methylsulfonyl-D-cyclohexyltyrosyl-glycyl-arginine-*p*-nitroaniline acetate) and Spectrozyme PL (H-D-norleucyl-hexahydrotyrosyl-lysine-*p*-nitroanilide diacetate salt), soluble fibrin (DESAFIB) and a goat anti-human melanoma t-PA polyclonal antiserum were purchased from American Diagnostica Inc., Greenwich, CT. Lys-plasminogen was from Calbiochem, San Diego, CA. Plasmin, aprotinin and soybean trypsin inhibitor were obtained from the Sigma Chemical Co. (St Louis, MO).

Recombinant DNA techniques

Buffers and reaction conditions for restriction enzymes, T4 DNA ligase, and the Klenow fragment of DNA polymerase I were those listed by the commercial source, New England Biolabs (Beverly, MA). Isolation of DNA fragments, preparation of plasmid DNAs, and other standard recombinant DNA techniques were carried out as described (Sambrook *et al.*, 1988, and other references cited therein). Transformation of *Escherichia coli* DH1 or TG1 cells was carried out by the method of Hanahan (1983).

Construction of t-PA cDNAs lacking sequences encoding structural domains

The cloning of a full-length human t-PA cDNA, beginning with polyadenylated mRNA purified from the Bowes melanoma cell line, has been described elsewhere (Sambrook *et al.*, 1986). Plasmid pSV/t-PA3, which contains a full-length cDNA copy of the t-PA gene (Sambrook *et al.*, 1986), was digested with *Xba*I restriction endonuclease, and a 1970-bp DNA fragment encoding the full-length t-PA polypeptide was purified by gel electrophoresis. This fragment was inserted at the *Xba*I site within the polylinker of the double-stranded (ds) replicative form of M13mp19 bacteriophage DNA, and *E.coli* TG1 cells were transfected with the ligated DNA molecule. A recombinant bacteriophage was selected in which the single-stranded (ss) DNA purified from M13 virions contained the coding sequences of t-PA cDNA. Table I lists the oligonucleotides that were designed to generate deletion mutants by the technique of loop-out mutagenesis, or to facilitate removal of the sequences following the introduction of new restriction sites by oligonucleotide-directed mutagenesis. The procedures used to carry out mutagenesis have been described in detail in Zoller and Smith (1985) and in Doyle *et al.* (1986). Where possible, the yield of bacteriophage carrying t-PA sequences with the desired deletions was enriched by treating the ds DNAs, generated by the extension-ligation reaction (Zoller and Smith, 1985), with a restriction endonuclease that cleaves only within those sequences that should be deleted from the mutant cDNA. Shorter, radiolabeled oligonucleotides designed to span the deletion sites (Table I) were used as probes for DNA filter hybridization to identify the desired mutants. DNA sequence analysis (Sanger *et al.*, 1977) was carried out to confirm that the correct deletions hads been made and then, for each desired deletion, ds replicative forms of the mutant bacteriophage DNAs were prepared and purified by centrifugation on CsCl gradients. For unknown reasons, we were unsuccessful in using loop-out mutagenesis to generate a kringle 2 deletion mutant. Construction of this mutant utilized a version of the t-PA cDNA (E.L.Madison *et al.*, in preparation) whose nucleotide sequence has been altered to contain an *Sph*I site at nucleotide 746 (resulting in no amino acid change) and an *Xho*I site at nucleotide 1018 (resulting in the substitution of serine for threonine at residue 298 but no change in the properties of the wild-type enzyme). To delete the sequences encoding kringle 2, the cDNA was cleaved with *Sph*I and *Xho*I and the restriction fragment encoding the kringle was replaced with an adaptor consisting of two partially complementary oligonucleotides (Table I). DNA sequence analysis was carried out to confirm that the desired deletion had been introduced. The *Xba*I fragments encoding the mutant t-PAs were isolated and used to replace the equivalent wild-type sequences in the various expression vectors used in this study (see below).

Expression of t-PA cDNAs by in vitro transcripton/translation

The *Xba*I DNA fragments encoding wild-type t-PA and the various deletion mutants were subcloned into the *Xba*I site of the pSP64 vector (Melton *et al.*, 1984). Unlabeled RNAs were synthesized from *Bam*HI-linearized plasmid DNA using SP6 polymerase as described previously (Melton *et al.*, 1984). The RNAs were then translated using nuclease-treated rabbit reticulocyte lysates according to the instructions of the supplier, Promega Biotech (Madison, WI). The [^{35}S]cysteine-labeled translation products were diluted directly into NET-gel buffer (Gething *et al.*, 1986) and immunoprecipitated with goat anti-human t-PA antibodies as described previously (Doyle *et al.*, 1985; Sambrook *et al.*, 1986).

Generation of SV40/t-PA recombinant virus stocks and infection of CV-1 cells

The mutant t-PA cDNAs were substituted for the wild-type t-PA sequence in the SV40/t-PA3 vector described previously (Sambrook *et al.*, 1986). Vectors containing the t-PA sequences in the correct orientation were identified by restriction endonuclease digestion. Following removal of the plasmid sequences (Sambrook *et al.*, 1986), the recombinant genomes containing the wild-type and mutant forms of the t-PA gene were transfected into CV-1 cells using DEAE-dextran and chloroquine as previously described (Doyle *et al.*, 1985; Sambrook *et al.*, 1986). High-titer virus stocks were developed and used to infect fresh monolayers of CV-1 cells for analysis of the biosynthesis and enzymatic activities of the wild-type and mutant t-PAs. Conditions for growth and infection of CV-1 cells were as described previously (Doyle *et al.*, 1985).

Construction of SV40-based transient expression vectors and transfection of COS-1 cells

The *Xba*I fragment containing the wild-type and mutant t-PA cDNAs were inserted into the polylinker sequence of vector pSVT7 (Bird *et al.*, 1987). Vectors containing the t-PA sequences in the correct orientation for expression under the control of the SV40 early promoter were identified by restriction endonuclease digestion. Transfection of the vectors into COS-1 cells using DEAE-dextran was carried out as described by Bird *et al.* (1987).

Assays for t-PA

CV-1 or COS-1 monolayers producing t-PA were washed three times at 18 h post-infection or transfection with Dulbecco's minimal essential medium

Table I. Oligonucleotides utilized to generate t-PA domain deletion mutants

Domain deleted	Oligonucleotides used for mutagenesis and screening
Finger (F^-)	GCC AGA TCT TACCAA*TGC AGC GAG CCA AGG
Epidermal growth factor (E^-)	TCA GTG CCT GTCAAA*ACC AGG GCC ACG TGC
Finger and epidermal growth factor (F^-E^-)	GCC AGA TCT TACCAA*ACC AGG GCC ACG TGC
Kringle 1 (K_1^-)	GAT ACC AGG GCCACG*TGC TAC TTT GGG AAT
Kringle 1 and kringle 2 ($K_1^-K_2^-$)	AAG TGC TGT GAAATA*ACC TGC GGC CTG AGA
Heavy chain (HC^-)	GCC AGA TCT TACCAA*ACC TGC GGC CTG AGA
Kringle 2 (K_2^-)	C T C T G A G G G C (adaptor designed to insert into *Sph*I-*Xho*I sites of modified t-PA cDNA)
	G T A C G A G A C T C C C G A G C T

The 30-mer oligonucleotides shown above were designed for use in loop-out mutagenesis procedures as described in Materials and methods.
* designates the breakpoint of the deletion. The 14-mer oligonucleotides (underlined) are radiolabeled with ^{32}P by a kinase reaction and used as probes for DNA filter hybridization to identify the desired mutants. One mutant (K_2^-) was constructed using a DNA adaptor (see Materials and methods).

(DMEM) lacking serum and then incubated in DMEM for a further 46–48 h before samples of medium were collected and assayed. A solid phase radioimmunoassay was performed as previously described (Sambrook *et al.*, 1986) using a goat anti-human t-PA polyclonal antiserum to quantitate the amount of t-PA antigen present in the cell culture medium, which varied between 0.5 and 2.5 μg/ml for both wild-type and mutant recombinant t-PAs.

Metabolic labeling of CV-1 or COS-1 monolayers with [^{35}S]cysteine (NEN, DuPont) or a mixture of [^{35}S]methionine and [^{35}S]cysteine (Trans Label, ICN) was performed as previously described (Doyle *et al.*, 1985) between 42 and 45 h following infection or transfection. Immunoprecipitation of aliquots of medium and separation of labeled proteins by SDS–PAGE were also performed as described previously (Doyle *et al.*, 1985; Sambrook *et al.*, 1986). Where appropriate, labeled t-PA samples were digested with plasmin at a final concentration of 1 μg/ml on ice for 0–20 min. Proteolysis was quenched at the end of the reaction by the addition of a 10-fold excess of both aprotinin and soybean trypsin inhibitor.

The enzymatic activity of t-PA was measured using two different assays.

Direct chromogenic assay. This amidolytic assay employs the chromogenic substrate Spectrozyme tPA, which is cleaved directly by t-PA at the arginine-*p*-nitroaniline bond. The assay was performed as described by the manufacturer (American Diagnostica Inc.) and reaction progress was monitored against the appropriate blank by measuring the production of free *p*-nitroaniline spectrophotometrically at 405 nm at 2-h intervals over an 8-h period. Assays were performed at 37°C in 96-well flat-bottomed microtiter plates and and O.D. 405 nm was measured with a Bio-tek microplate autoreader. Under the conditions used, the assay was linear with time. Test reaction mixtures contained 2.5 ng of the t-PA sample to be assayed in a final volume of 200 μl. Control reactions contained equivalent volumes of media from mock-infected or mock-transfected cells.

Indirect chromogenic assay. In this assay, the chromogenic substrate Spectrozyme PL is cleaved by plasmin generated by the action of t-PA on plasminogen. The release of free *p*-nitroaniline from the chromogenic substrate is measured spectrophotometrically at 405 nm. For the standard assay, the reaction mixture (100 μl) contained the t-PA sample to be tested, Spectrozyme PL (0.4 mM), lys–plasminogen (0.1 μM), and soluble fibrin (DESAFIB, 25 μg/ml) in a buffer containing 50 mM Tris–HCl, pH 7.5, 0.1 M NaCl, 1 mM EDTA and 0.01% Tween-80. Test reaction mixtures contained 150–200 pg of the t-PA sample to be assayed. Control reactions contained equivalent volumes of media from mock-infected or mock-transfected cells. Assays were performed at 37°C in 96-well flat-bottomed microtiter plates and the optical density was measured against the appropriate blank with a Bio-tek microplate autoreader at 15- or 30-min intervals. ΔO.D. values were measured as the change in optical density between 30 and 150 min, following the lag period and complete conversion of the single-chain t-PA species to the 2-chain form (see Figure 5). Under these standard assay conditions, the soluble fibrin stimulated the activity of wild-type t-PA ~20-fold. This degree of stimulation of t-PA activation of lys–plasminogen is similar to that reported by others using the soluble fibrin DESAFIB (Karlan *et al.*, 1987) or non-polymerized fibrinogen (Tate *et al.*, 1987). It is, however, significantly less than that achieved using polymerized fibrin (Hoylaerts *et al.*, 1982; Ranby, 1982; Rijken *et al.*, 1982).

Measurement of the binding of wild-type and mutant t-PAs to lysine–Sepharose

Samples (~2.5 ng in 250 μl of DMEM) of wild-type or mutant t-PAs were mixed with 250 μl of a slurry of lysine–Sepharose in binding buffer (0.01 M $NaPO_4$ pH 7.0, containing 0.1 M NaCl and 0.01% Tween-20) and incubated on a rocker platform for 16 h at 4°C. The resin was then pelleted by centrifugation at 1000 r.p.m. for 5 min at 4°C and the supernatant solution collected. A solid phase radioimmunoassay (Sambrook *et al.*, 1986) was used to determine the amount of t-PA in the original sample and in the supernatant following incubation with lysine–Sepharose.

PAI-1

This was obtained from the conditioned medium of primary endothelial cell cultures derived from human umbilical veins and partially purified by concanavalin A–Sepharose affinity chromatography (van Mourik *et al.*, 1984), activated by guanidine hydrochloride treatment (Hekman and Loskutoff, 1985) and further purified by Sephacryl S200 gel filtration.

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