

Isolation, Identification and Pharmacokinetic Properties of Human Tissue-Type Plasminogen Activator Species: Possible Localisation of a Clearance Recognition Site

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Key words

Tissue-type plasminogen activator – Fibrinolysis – Pharmacokinetics

Summary

Purified preparations of recombinant tissue-type plasminogen activator (t-PA) from the recombinant Bowes melanoma cell line TRBM6 were shown to contain multiple species of plasminogen activator. Using a combination of chromatography on Sephadex G25, Sephadex G75 and Heparin Sepharose CL6B we have isolated two fibrinolytically active species, which, under non-reduced SDS PAGE, have apparent $M_r = 38,000$ and $56,000$. Double immunodiffusion studies indicated that both species were closely related to both the t-PA B chain and t-PA itself. N-terminal sequencing identified the $M_r = 38,000$ species as ala₁₆₀-t-PA (essentially Δ FGKI t-PA) and the $M_r = 56,000$ species as ser₁-tyr₂-gln₃-gly_x-cys₅₁ t-PA (Δ F t-PA), the latter probably produced by alternative splicing of the t-PA gene. The pharmacokinetic properties of *N,N* dimethyl-4-aminobenzoyl (DAB) derivatives of these activators and native t-PA were determined in the guinea pig. Whereas DAB \rightarrow Δ F t-PA showed a similar, rapid plasma disappearance profile to that of DAB \rightarrow t-PA, DAB \rightarrow Δ FGKI t-PA was cleared significantly slower. These results suggest that a rapid clearance recognition site resides on either the growth factor or kringle 1, or both, domains of t-PA.

Introduction

Plasminogen activators play an important role in the maintenance of haemostasis. One of these activators, tissue-type plasminogen activator (t-PA), is attracting attention as a treatment for acute myocardial infarction (1–3) and it is currently being evaluated in the clinic using infusion regimes of several hours duration. There is now interest in the development of second-generation t-PA molecules capable of bolus administration (4, 5). The apparent necessity for infusion of t-PA is a consequence of its rapid hepatic clearance from the circulation (6); therefore attention has been directed towards identification of the putative clearance site on the t-PA molecule. Rijken and Emeis (7)

showed a small difference in the clearance of the A and B chains suggesting that the recognition site of t-PA probably resided on the A-chain.

Native t-PA is a glycoprotein with $M_r = 63,000/65,000$ (8, 9), the two species differing in their extent of glycosylation (10). Other forms of t-PA have been noted including an $M_r \approx 55,000$ (11–13) and $M_r \approx 37,000$ (9, 13) but none of these have been isolated and characterised. We previously described the purification (14) of t-PA from the recombinant Bowes melanoma cell line TRBM6 (15). In some of the purified preparations we detected t-PA-like species similar to those described by other workers. It seemed possible that these species were truncated forms of native t-PA and that further analysis might provide us with some insight into the regions of the t-PA molecule responsible for its rapid clearance. This paper describes the isolation, identification and pharmacokinetic properties of these t-PA-like species.

Materials and Methods

Materials

The recombinant Bowes melanoma cell-line TRBM6 has been described before (15). Sephadex G25 (Medium grade), Sephadex G75 (Fine grade) and Heparin Sepharose CL6B were purchased from Pharmacia (Great Britain) Ltd, Milton Keynes, UK. YM10 ultrafiltration membranes were from Amicon Ltd, Stonehouse, UK.

4-amidinophenyl-*N,N*-dimethyl-4-aminobenzoate. HCl (AP-DAB) was kindly prepared by Dr R. A. G. Smith, Beecham, UK. Lysine Sepharose CL4B has been described before (14). Sodium Heparin BP was obtained from Evans Medical Ltd, Greenford, UK. S2288 was from Flow Laboratories, Irvine, UK. PAGE Blue 83 was from BDH Ltd, Eastleigh, UK.

Isolation of t-PA Fragments

Purified t-PA (14) in 0.02 M Tris/0.5 M NaCl/0.5 M L-arginine/0.01% Tween 80 pH 7.4 was concentrated by ultrafiltration using YM10 membranes. The concentrate (30 ml; 21×10^6 SU, see below) was buffer-exchanged into 0.05 M NH_4HCO_3 using Sephadex G25 (id, 5 cm; h, 20 cm) and the t-PA activity (3.7×10^6 SU) that eluted in the void volume of the column was lyophilised.

Isolation of $M_r = 38,000$ Species

Part of the lyophilisate (1.6×10^6 SU) was reconstituted in 3.0 ml 0.02 M Tris/0.05 M NaCl/0.5 M L-arginine/0.01% Tween 80, pH 7.4 buffer and chromatographed on a column (id, 2.5 cm; h, 63 cm) of Sephadex G75 equilibrated in the same buffer. The velocity of flow was 2.6 cm h^{-1} . The fractions enriched in the smallest t-PA species were pooled (total volume, 45 ml), ultrafiltered to a small volume (2.0 ml), buffer-exchanged into 0.05 M NH_4HCO_3 (3.0 ml) and lyophilised.

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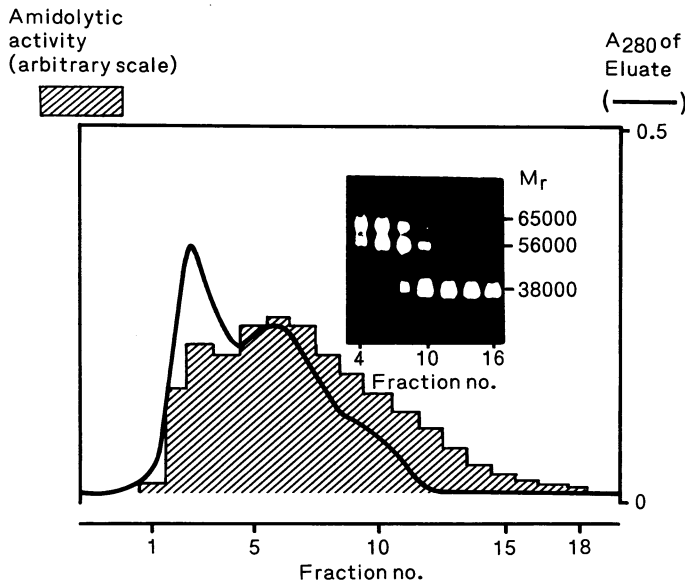


Fig. 1 Chromatography of minority t-PA species on Sephadex G75. Suitably formulated t-PA that had previously eluted in the void volume of a Sephadex G25 column was chromatographed on a column of Sephadex G75. The eluate from the column was continuously monitored at 280 nm. Fractions were analysed for S2288 activity and by SDS PAGE followed by fibrin zymography (inset). Full details are given in the Methods section

was resolved by analysing the fractions using fibrin zymography (Fig. 1 inset). This allowed the identification and subsequent preparation of first, highly purified $M_r = 38,000$ plasminogen activator and, second, an enriched mixture of the $M_r = 56,000$ and $65,000$ species alone. Chromatography on Heparin Sepharose CL6B was used to separate the two higher M_r species. The $M_r = 56,000$ species eluted at a lower ionic strength than the $M_r = 65,000$ species (Fig. 2). Preparations of both species were subsequently formulated for further studies. Analysis by SDS PAGE

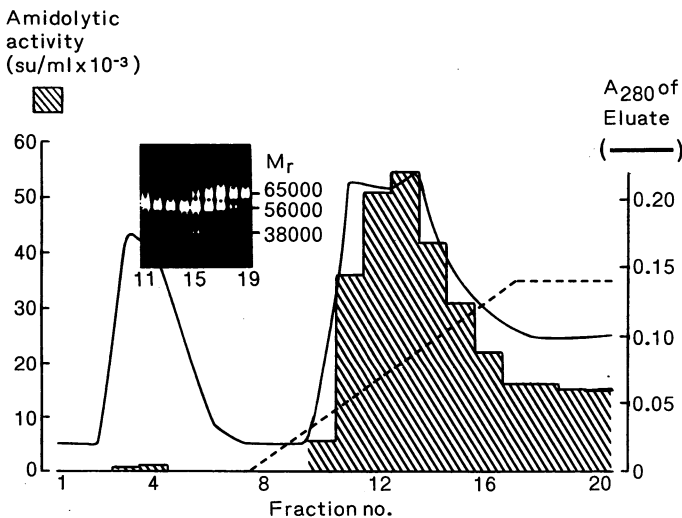


Fig. 2 Chromatography of apparent $M_r = 56,000$ and $65,000$ t-PA species on Heparin Sepharose CL6B. An enriched preparation of the higher molecular weight t-PA species from Fig. 1 was chromatographed on Heparin Sepharose CL6B. The column was developed with a linear gradient from 0.05 to 1 M NH_4HCO_3 (—) and the individual fractions were analysed by S2288 assay and by SDS PAGE followed by fibrin zymography (inset). Fractions containing the two purified t-PA species were pooled separately. Full details are given in the Methods section

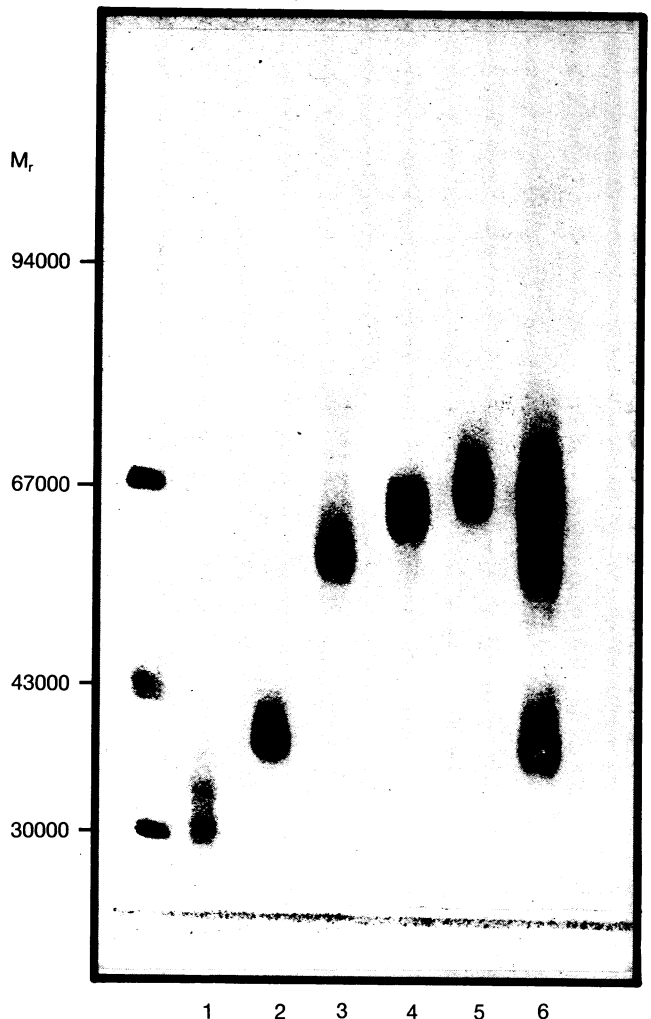


Fig. 3 SDS PAGE of purified t-PA species. Concentrated preparations of purified t-PA species were examined by SDS PAGE under non-reducing conditions followed by staining for protein using PAGE Blue 83. The individual lanes are 1. B chain, 2. $M_r = 38,000$, 3. $M_r = 56,000$, 4. $M_r = 63,000$, 5. $M_r = 65,000$ and 6. a mixture of lanes 2 to 5

under non-reducing conditions followed by staining for protein (Fig. 3) or fibrin zymography (Fig. 4) indicated that all three plasminogen activators were pure enough for further characterisation.

All three species were shown to comprise mainly two chain material by SDS PAGE under reducing conditions (results not shown). The apparent $M_r = 65,000$ species contained two major polypeptides both of $M_r \approx 36,000$. The apparent $M_r = 56,000$ species contained three major polypeptides, one at $M_r = 36,000$ – the presumed B-chain (17) – and a doublet at $M_r = 31,000$ and $33,000$. The apparent $M_r = 38,000$ species contained two major polypeptides, one at $M_r = 36,000$ and another at $M_r = 15,000$.

The interaction of all species with antibodies raised against either native t-PA (principally the $M_r = 63,000$ species) or the B-chain of native t-PA (17) was examined. Fig. 5 shows that all species cross-reacted with both antibodies. The $M_r = 65,000$ species is likely to be the Type I variant of native t-PA (9). Specific identification of the two smaller M_r species was carried out by N-terminal sequencing studies. The $M_r = 38,000$ species was sequenced without separation of the two chains (Fig. 6). By accounting for the known N-terminal sequence of the t-PA B-chain (9) it was possible to identify the $M_r = 38,000$ species as alanine₁₆₀-t-PA. It is likely that the isolated $M_r = 38,000$ species

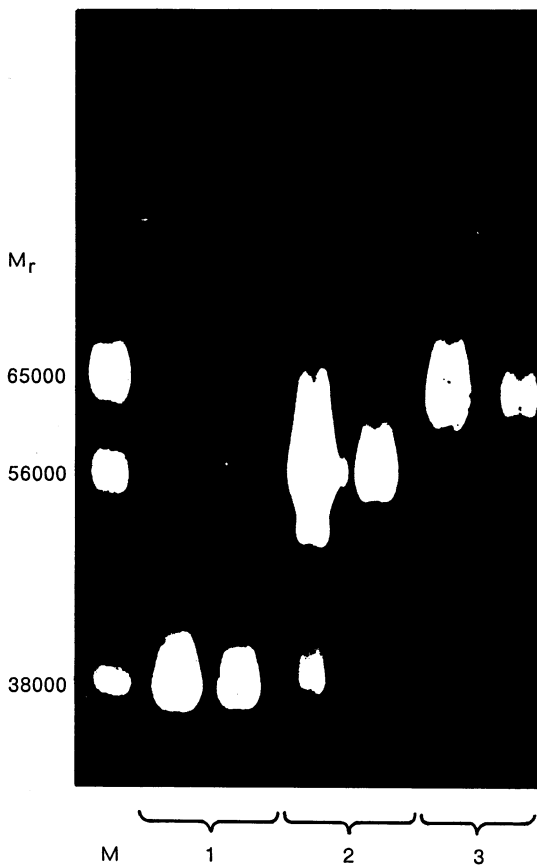


Fig. 4 SDS PAGE followed by fibrin zymography of purified t-PA species. The individual tracks are: M, markers of all three species, 1. $M_r = 38,000$ species, 2. $M_r = 56,000$ species and 3. $M_r = 65,000$ species. For lanes 1 to 3 the left-hand track contains 2 IU, the right-hand track contains 0.5 IU. The stained fibrin zymogram is shown

was the Type II equivalent variant, i.e. not glycosylated at asparagine 184 (10), because (i) evidence was obtained from other preparations for a species with apparent $M_r = 41,000$, (ii) the ratio of 38,000 to 41,000 species was the same as that of 63,000 and 65,000 ($63,000 \gg 65,000$), and (iii) the 38,000 species showed a greater affinity than the 41,000 species for lysine Sepharose, similar to native 63,000 (Type II) and 65,000 (Type I) t-PA variants (10). The deduced molecular weight of alanine₁₆₀t-PA agreed with the sum of the M_r of the individual polypeptides determined by SDS PAGE under reducing conditions, i.e. 51,000. Determination of the N-terminus of the $M_r = 56,000$ A-



Fig. 5 Double immunodiffusion studies of purified t-PA species: cross-reaction with anti-t-PA IgG (a) or anti t-PA B-chain IgG (b) (ref. 17). Antibody (500 $\mu\text{g/ml}$, 15 μl) is in the centre well (A). The outer wells contain the antigen (50 $\mu\text{g/ml}$, 15 μl): 1, 4, $M_r = 38,000$ species; 2, $M_r = 56,000$ species; 3, $M_r = 65,000$ species. The agarose gel stained for precipitin lines using PAGE Blue 83 is shown

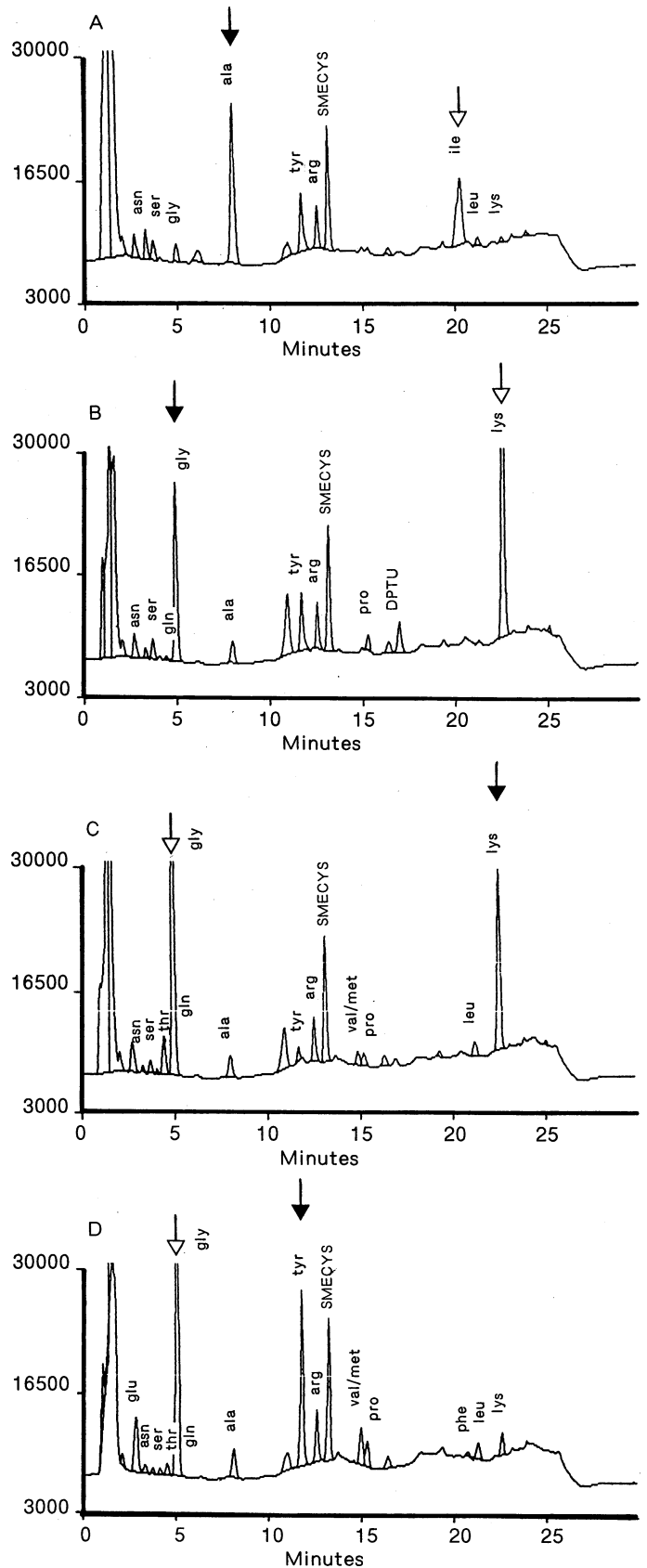


Fig. 6 N-terminal sequence analysis of the $M_r = 38,000$ species. Reduced and carboxymethylated two chain $M_r = 38,000$ t-PA was sequenced as described in the Methods section. The four panels (A to D) show the HPLC analysis of the N-terminal PTH-amino acids determined in sequence: Panel A, residue 1; Panel B, residue 2, etc. For each panel the open arrow represents the amino acid attributable to the t-PA B-chain, the filled arrow that attributable to the A-chain moiety

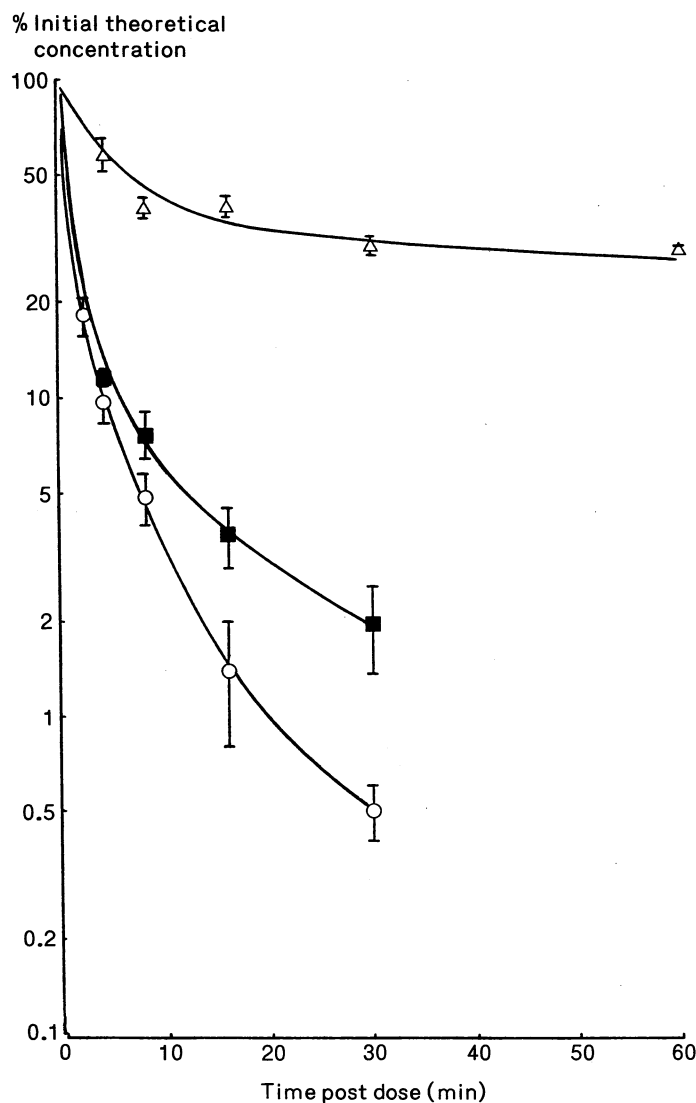


Fig. 7 Plasma disappearance profiles of acylated t-PA species in the guinea pig. Purified preparations of acylated t-PA were injected into a femoral vein of the guinea pig and samples of blood collected at intervals thereafter from a cannulated carotid artery. Euglobulin precipitates were prepared and were subsequently assayed for fibrinolytic activity. The t-PA species examined were: native t-PA, Type I, 2000 SU/kg (○), ΔF t-PA, 3500 SU/kg (■) and ΔFGKl t-PA, 3500 SU/kg (△). Full details are given in the Methods section

chain (Table 1) revealed that the species did not contain the fibronectin finger.

The three t-PA species were acylated for pharmacokinetic studies as described in Materials and Methods. The plasma disappearance results in the guinea pig are shown in Fig. 7. Both DAB → t-PA and DAB → ΔF t-PA were cleared rapidly, 80% or more being cleared within 2 to 3 min. The clearance of DAB → ΔFGKl t-PA appeared to be much slower – less than 80% was cleared within the first 30 min. The two variants of native t-PA (Type I and Type II) were cleared at approximately the same rate (results not shown).

Discussion

We have purified and identified two species of tissue-type plasminogen activator from the recombinant Bowes melanoma

cell line TRBM6. Low molecular weight forms of t-PA have been described before from mammalian cells but none have been directly purified and identified. We believed that the $M_r = 56,000$ species might be similar to enzymes described by other workers (11, 12). If this were the case it should lack the fibronectin finger – the domain in t-PA homologous with the Type I structure in fibronectin (9). This fibronectin structure appears to contain a heparin binding site (18); therefore we attempted to separate the $M_r = 56,000$ and $65,000$ species using Heparin Sepharose. This was successful. Subsequent N-terminal sequencing of the $M_r = 56,000$ species showed that the material was almost certainly identical with the fibronectin finger-lacking t-PA species identified from cDNA prepared from Detroit 562 mRNA (12). It is assumed that X_{51} in the $M_r = 56,000$ species is C_{51} of native t-PA. This species probably arises from alternative splicing of the t-PA pre-mRNA (12, 21). The origin of ΔFGKl t-PA (ala₁₆₀t-PA) is more obscure. It possibly arises from a trypsin-like cleavage of the native molecule at residues lysine₁₅₉/alanine₁₆₀. However, if the postulated tertiary structure of t-PA (9) is correct the gross conformation of t-PA should be essentially maintained due to the presence of two disulphides (cys₁₄₄-cys₁₆₈ and cys₉₂-cys₁₇₃) linking the polypeptides either side of the cleavage site. Unlike ΔF t-PA the origin of ala₁₆₀t-PA cannot be explained by alternative splicing of the pre-mRNA since no exon-intron junctions have been identified at the putative cleavage site (21). Thus the derivation of ala₁₆₀t-PA remains unknown. Both species of t-PA occurred in the harvest medium of TRBM6 and the original Bowes melanoma cell-line to approximately the same levels (results not shown). The amounts were not determined but were in the region of 5–20% of total plasminogen activator activity.

Although it is impossible to predict what effect removal of parts of the A-chain have on the overall conformation of the remainder of the molecule the double immunodiffusion studies carried out suggest it is minimal. This conclusion is supported by the observation that ala₁₆₀t-PA, in contrast to free t-PA B-chain, was adsorbed by an immobilised monoclonal antibody (ESP5; ref 22) directed at kringle 2 (results not shown). Thus ala₁₆₀t-PA is referred to as ΔFGKl t-PA as the only structural domains it contains are kringle 2 and the B-chain. Mutant, DNA-derived t-PA molecules lacking the F, G and KI domains have been described (23, 24) but pharmacokinetic studies were not carried out.

The plasma disappearance profile of reversibly inactivated (acylated) forms of the various t-PA species were determined in the guinea pig. It is known that the activity of t-PA is lost in the bloodstream because of removal by the liver rather than inactivation by plasma inhibitors (19). However, inhibition could play a major role in the neutralization of plasminogen activators that are not susceptible to fast hepatic clearance mechanisms. In order to avoid such inhibition obscuring the pharmacokinetic behaviour of the activators, each was first reversibly inactivated by acylation of the active site (20). The acyl group selected (DAB) had a deacylation half-life (in PBS-Tween at 37° C) of about 4 h, which was sufficiently long to minimize inhibition *in vivo*, yet short enough to permit regeneration of activity *in vitro* during the subsequent functional assay of the plasma samples. The possibility of artificially high apparent levels of plasminogen activator activity due to contamination of the plasma euglobulins by heparin is negated by the use of a fibrin plate assay. Fig. 7 shows that ΔFGKl t-PA had lost a major plasma disappearance recognition site whereas ΔF t-PA had not. It seems likely that the retarded clearance is not due to a conformational change elsewhere on the molecule as a result of removal of the FGKl region, because antibody cross-reactivity was not altered. Therefore the site(s) responsible for the rapid liver clearance probably resides in the G or KI domains. The biphasic nature of the clearance might

indicate that another, as yet unidentified, recognition site resides elsewhere on the molecule.

We have shown that species of t-PA identified in purified preparations from the recombinant cell line TRBM6 could be purified to near homogeneity and employed to analyse the region(s) of the molecule responsible for its rapid clearance. Further localisation of the clearance recognition site can be obtained probably only by the use of recombinant DNA techniques to dissect the molecule more specifically. The information provided here should facilitate this work and should lead to the more rapid development of a second generation t-PA molecule.

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