Common evolutionary origin of the fibrin-binding structures of fibronectin and tissue-type plasminogen activator

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Comparison of the primary structures of high-M_r urokinase and tissue-type plasminogen activator reveals a high degree of structural homology between the two proteins, except that tissue activator contains a 43 residue long amino-terminal region, which has no counterpart in urokinase. We show that this segment is homologous with the finger-domains responsible for the fibrin-affinity of fibronectin. Limited proteolysis of the amino-terminal region of plasminogen activator was found to lead to a loss of the fibrin-affinity of the enzyme. It is suggested that the finger-domains of fibronectin and tissue-types plasminogen activator have similar functions and that the finger-domains of the two proteins evolved from a common ancestral fibrin-binding domain.

Tissue-type plasminogen activator *Fibronectin* *Protein evolution* *Fibrin-binding domain*

1. INTRODUCTION

Activation of plasminogen by tissue-type plasminogen activators is known to be greatly accelerated by the presence of fibrin [1,2]. The molecular basis of the stimulatory effect of fibrin is that both plasminogen [3,4] and plasminogen activator [5,6] are bound by the fibrin polymer and fibrin facilitates plasminogen activation by aligning the enzyme with its substrate [2]. The physiological significance of this molecular mechanism is that it ensures that plasminogen activation takes place predominantly on fibrin surface and thus restricts plasmin action to fibrin [7]. Urokinase-type plasminogen activators lack this fibrin-specificity, since they are devoid of fibrin-affinity [5,6].

Recently the primary structures of human tissue-type plasminogen activator [8] and human high M_r urokinase [9,10] have been determined, and the carboxy-terminal regions of the two proteins have been shown to be homologous with the protease parts of prothrombin, plasminogen, trypsin and other trypsin-like serine proteases. The non-protease part of tissue-type plasminogen activator was shown to contain two kringle structures that are homologous to the kringles of prothrombin [11], plasminogen [12], and urokinase [9]; the remaining amino-terminal 91 amino acids of tissue-type plasminogen activator, however, were found to share little homology with kringles [8].

We here show that this region consists of a segment homologous with the growth factor-domain present in various proteases and another segment that shows homology with the finger-domains of fibronectin.

Abbreviations: t-PA, tissue-type plasminogen activator; SDS, sodium dodecyl sulfate

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2. MATERIALS AND METHODS

2.1. *Limited proteolysis of tissue-type plasminogen activator by plasmin-Sepharose 4B*

Melanoma t-PA was isolated from the serum-free harvest fluid of the Bowes human melanoma cell line [6]. t-PA, 100 Ploug units in 1 ml of 0.05

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M Tris–HCl, 0.35 M NaCl, 0.01% Tween-80 (pH 7.4) was digested with plasmin-Sepharose 4B (0.5 ml) for 15 min at 25°C.

2.2. *Adsorption of plasminogen activators to fibrin clot*

Melanoma t-PA (50 Ploug units/ml) or UK (10 Ploug units/ml), plasminogen-free fibrinogen (1.25 mg/ml) and bovine thrombin (1 NIH unit/ml) were incubated at 25°C in 0.05 M Tris–HCl, 0.15 M NaCl, 0.002% Tween-80, pH 7.4. The clot formed was continuously removed and squeezed by the aid of a glass-rod during a period of 30 min. Activator species not adsorbed to fibrin were analyzed from the liquid remaining after removal of fibrin clot. Activator species adsorbed to fibrin were extracted from the clot by 2% SDS.

2.3. *Detection of plasminogen activator species on SDS-polyacrylamide slab gels containing co-polymerized plasminogen and gelatin*

Plasminogen activators were detected on 11% SDS-polyacrylamide slab gels containing copolymerized plasminogen and gelatin [13]. The method utilizes the fact that the zymogen and gelatin incorporated into the polyacrylamide matrix at the time of casting are retained during subsequent electrophoresis of PA-samples and serve as sequential, in situ substrates for localization of PA-bands by negative staining. Failure of proteolysis to occur in plasminogen-free control gels was used as the criterion of plasminogen activator activity. SDS was removed by immersion of the slabs into 2.5% Triton X-100 for 60 min with concomitant restoration of activity. The gels were incubated in 0.1 M glycine–NaOH (pH 8.3) buffer for 180 min at 37°C and then stained with 0.1% amido-black in methanol:acetic acid:water (30:10:60) and destained in methanol:acetic acid:water (30:10:60).

3. RESULTS AND DISCUSSION

Here we show (fig.1A) that residues 44–91 of plasminogen activator display remarkable sequence homology with human and murine epidermal growth factors [14,15] and the growth factor-like regions of protein C [16], factor IX [17], factor X [18] and urokinase [9]; the degree of identity for residues 56–84 was found to be 26, 33, 36, 43 and 50%, respectively.

The amino-terminal 43 residue long segment of tissue-type plasminogen activator, however, has no counterpart in urokinase, raising the possibility that this region might be responsible for some of the properties distinguishing tissue-type plasminogen activator from urokinase. Comparison of the amino acid sequence of this segment with the sequence of fibronectin [19] revealed that it is homologous with the finger-structures of fibronectin (fig.1B) that are known to be responsible for the fibrin-affinity of fibronectin [20,21]. When the sequences of the 9 fibronectin fingers are compared, one by one, with the amino-terminal 43 residues of tissue-activator, the degree of identity for residues 6–43 is found to range from 20–27%. It is noteworthy that 6 of the 7 residues, conserved

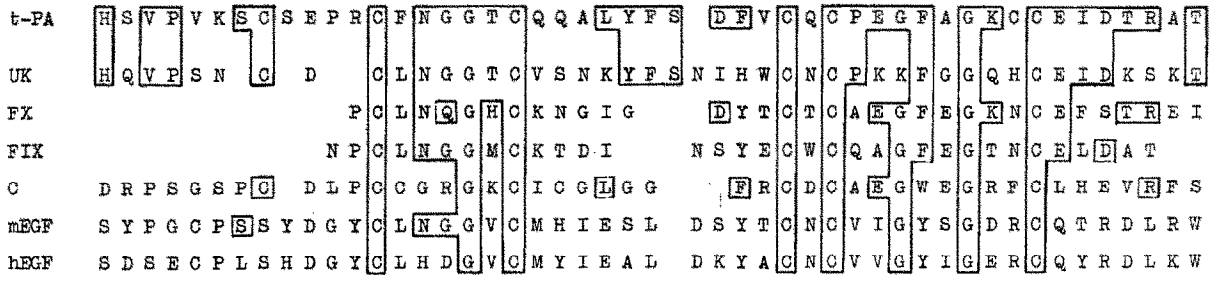

Fig.1A. Comparison of residues 44–91 of tissue-type plasminogen activator with corresponding parts of serine proteases. The sequence of human t-PA, residues 44–91, was taken from [8]; human high-M_r urokinase, residues 5–49 from [9]; bovine factor X, residues 54–88 from [18]; bovine factor IX, residues 54–87 from [17]; bovine protein C, residues 52–96 from [16]; murine epidermal growth factor, residues 2–49 from [15]; human epidermal growth factor, residues 2–49 from [14].

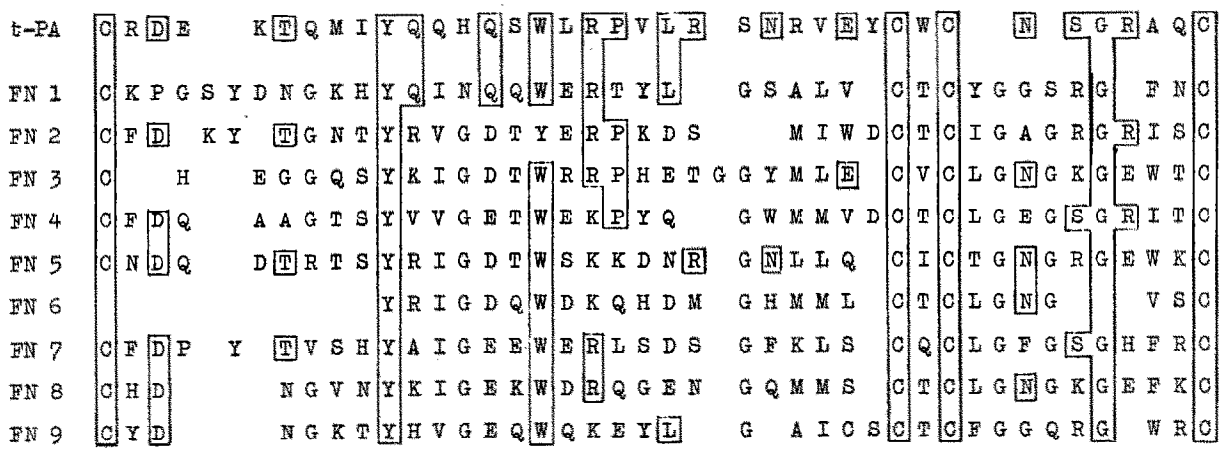

Fig.1B. Comparison of residues 6–43 of tissue-type plasminogen activator with corresponding parts of fibronectin. The sequence of human t-PA was taken from [8]; bovine fibronectin from [19]. The standard IUPAC one-letter code for amino acid residues was used. The blocks enclose residues identical with the corresponding residue in t-PA.

in all fibronectin fingers, are also conserved in plasminogen activator.

Assuming that the disulphide bond pattern of the amino-terminal region of tissue-activator is the same as in the homologous finger-domains [19] and growth factor-domains [14, 15, 22] the covalent structure of the amino-terminal 91 residue segment of tissue-activator is as depicted in fig.2.

In view of the homology of the amino-terminal region of tissue-type plasminogen activator with the fibrin-binding finger-domains of fibronectin, it seemed possible that this region plays a role in the fibrin-affinity of tissue-type plasminogen activator. To test this assumption we have subjected tissue-type plasminogen activator to limited proteolysis by immobilized plasmin, since cleavage with plasmin at Lys-49 or Arg-89, present in the inter-domain peptide regions, was expected to remove the finger-domain (fig. 2).

As shown in fig.3 proteolysis with plasmin caused the conversion of the 65 kDa activator to a ~55

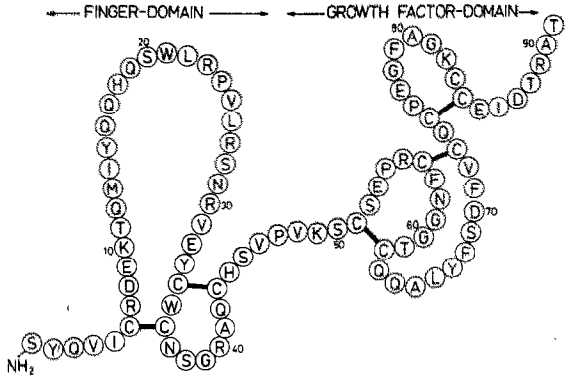

Fig.2. Potential disulphide bond pattern of the amino-terminal region of tissue-type plasminogen activator. The proposed disulphide bonds, shown by solid black bars, are based on homology with growth factor-domains of other serine proteases and finger-domains of fibronectin as shown in fig.1. The standard IUPAC one-letter code for amino acids was used.

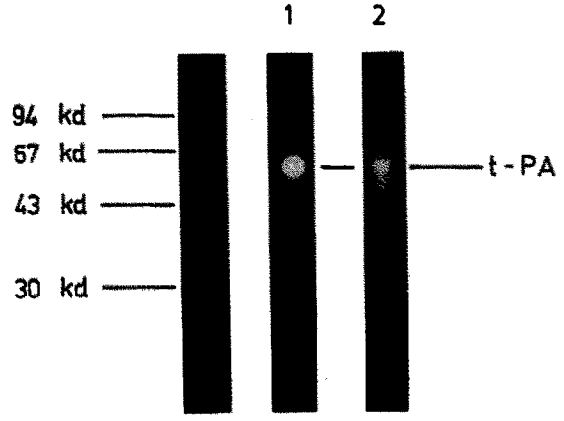

Fig.3. Limited proteolysis of tissue-type plasminogen activator by plasmin-Sepharose 4B. (1) Zymogram of melanoma t-PA; (2) Zymogram of a limited plasmic digest of melanoma t-PA. The M_r marker proteins used were: phosphorylase *b*, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa.

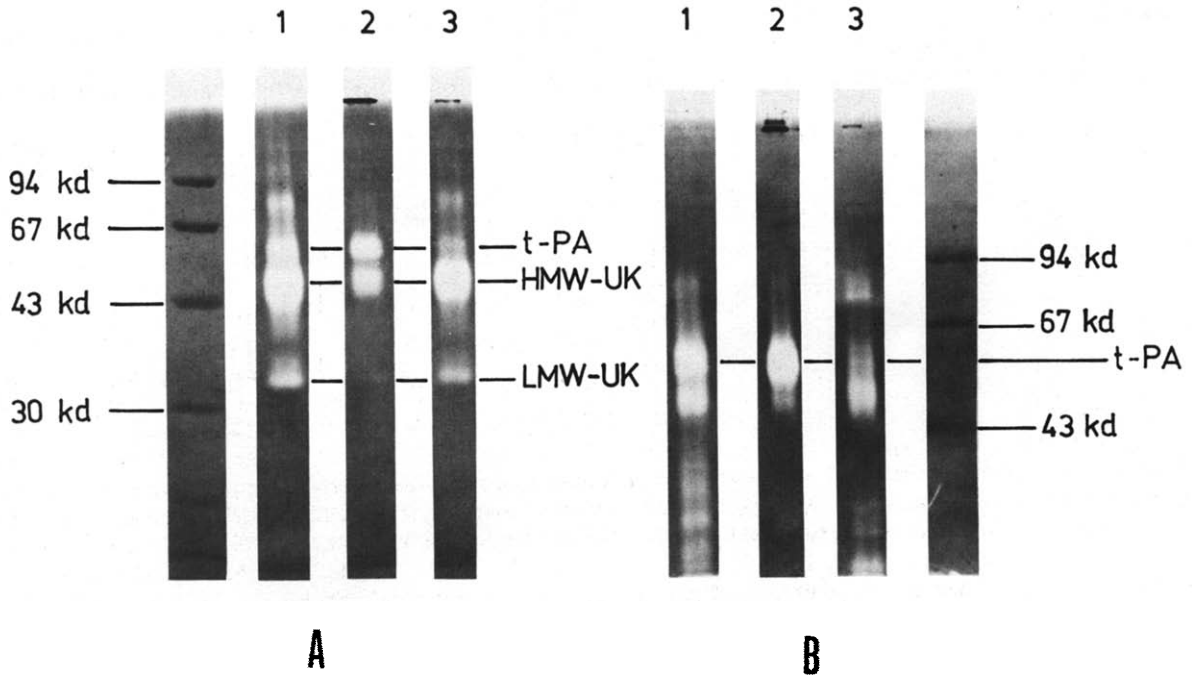

Fig. 4. Adsorption of plasminogen activators to fibrin clot. The reliability of the technique to detect differences in fibrin-affinity of activators is demonstrated by the experiment shown in A. (A) Adsorption of a mixture of tissue-type plasminogen activator and commercial urokinase to fibrin clot. Zymograms, in: (1) mixture of t-PA and UK; (2) activator species bound to the fibrin clot; (3) activator species not adsorbed to the fibrin clot. (B) Adsorption of plasminogen activator species present in the limited plasmic digest of t-PA to fibrin clot. Zymograms, in: (1) limited plasmic digest of t-PA; (2) activator species bound to the clot; (3) activator species not adsorbed to the fibrin clot. The electrophoresis time was doubled to improve separation of t-PA species formed by limited plasminolysis. The M_r marker proteins used were: phosphorylase *b*, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa.

kDa activator. Similar low-M_r tissue-type activator is present in variable amounts as a minor component in preparations obtained from the culture fluid of the melanoma cell line (Bowes) used here [13, 23]. Spontaneous appearance of this low-M_r activator in culture fluids is probably also due to limited proteolysis of the 65 kDa activator. The ~55 kDa tissue-type activator, formed either by plasminolysis of the 65 kDa activator, or present as a minor component in some of the activator preparations, was found to lack affinity for fibrin clots, whereas the 65 kDa activator was bound to fibrin clots (fig.4). This result shows that the ~10 kDa segment removed by limited proteolysis is essential for the fibrin affinity of the activator. Since limited proteolysis occurs at domain–domain boundaries [24] it seems likely that the ~10 kDa segment removed by limited proteolysis corresponds to the amino-terminal region preceding the kringle domains, in analogy with limited plasminolysis of plasminogen [25].

The homology of fibronectin to tissue-type plasminogen activator is apparently restricted to the finger-domain. It therefore seems likely that gene fusion or exon-reshuffling was the mechanism whereby these otherwise unrelated proteins acquired the same fibrin-binding domain.

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