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QUARTERLY

The epidermal growth factor-like domain from tissue plasminogen activator. Cloning in *E. coli*, purification and ESR studies of its interaction with human blood platelets*

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To examine whether the epidermal growth factor (EGF)-like domain Pro_{47}-Asp_{87} is involved in the interaction of tissue plasminogen activator (t-PA) with platelets, we have expressed this domain in *E. coli*. The peptide fragment was produced from a plasmid expression vector as a fusion protein with β-galactosidase Met_1-Val_{444} at high yield in eight clones of *E. coli*. The fusion protein was purified and subjected to mild acid hydrolysis with formic acid, then the peptide Pro_{47}-Asp_{87}, identified by immunoblotting using specific antibodies to t-PA, was isolated by HPLC. After incubation with blood platelets spin labelled with 16-doxylstearic acid or 5-doxylstearic acid, the Pro_{47}-Asp_{87} peptide fragment reduced fluidity of the membrane lipid bilayer to the same extent as did intact t-PA as indicated by ESR measurements. Our data suggest that the EGF-like domain of t-PA can directly interact with blood platelets and thus it seems to contain those sites of the t-PA molecule that bind the platelet membrane components.

The tissue plasminogen activator (t-PA)[2], similarly as other proteases of the fibrinolytic and blood coagulation system, is composed of several structural domains which appear to have evolved by genetic assembly of individual subunit with specific structural and functional properties [1]. They include the serine protease and kringle domains, the epidermal growth factor-like domain and fibronectin finger-like domain. Analysis of functional contribution of these domains includes isolation of independent structural subunits as well as the production of variant proteins which lack one or more domains. In functional studies on such variants it has been found that deletion mutants lacking the EGF-like domain show impaired activity indicating that this small domain may be involved in mediation of the in-

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[2]Abbreviations: ACD, acid-citrate dextrose; DMT, dimethoxytrityl; DS, doxylstearic acid; EGF, epidermal growth factor; β-Me, β-mercaptoethanol; PMSF, phenylmethylsulfonyl fluoride; TFA, trifluoracetic acid; t-PA and u-PA, tissue and urokinase plasminogen activator; UK, urokinase.

teraction between t-PA and fibrin or cell receptors [2, 3].

The EGF-like domain was first identified in the EGF precursor and then described in a number of functionally dissimilar proteins [4]. The consensus sequence for EGF-like domains includes six cysteine residues which form three disulphide bridges. Some of these domains (e.g. those from the vitamin K-dependent coagulation proteins, factors VII, IX, X, XII and protein C, as well as thrombomodulin and protein S), contain an additional consensus of different amino-acid residues including Asp, Asp/Asn, Asp*/Asn*, Tyr/Phe, where * denotes a β-hydroxylated residue [5].

The tissue plasminogen activator was recently found to bind directly to platelets [6]. Therefore, to study this interaction in more detail we have obtained the EGF-like domain of t-PA.

MATERIAL AND METHODS

Preparative procedures

Oligonucleotide assembly. Oligonucleotides were synthesized using phosphite triester method [7]. Phosphoramidites were obtained from dimethoxytrityl (DMT) derivatives of N^6-isopropoxyacetyladenosine, N^4-isopropoxyacetylcytidine, N^2-isopropoxyacetylguanosine and thymidine, [8]. The syntheses were performed manually on 1 μmol scale as described by Uznański *et al.* [9]. Cleavage from the solid support and both base and phosphate deblocking were accomplished by treatment with 25% aqueous ammonia for 3 h at room temperature, due to base-labile protecting groups on amino functions. Purification was achieved by two HPLC separations [10]. The first was performed with the dimethoxytrityl group still attached to 5'-hydroxyl function, and the second after removing DMT in 80% acetic acid for 20 min. HPLC fractions were concentrated *in vacuo* and the resulting lyophilized samples were used for further enzymatic treatment. A 123 bp long coding sequence of the Pro47 - Asp87 peptide fragment of the EGF-like domain of t-PA was constructed from 8 oligonucleotides (Fig. 1). In most cases, every complementary pair of nucleotides contained 3' and 5' protruding ends of 3 or 4 nucleotides. The design allowed for the simultaneous ligation of 8 separate segments into the *Eco*RI — *Hin*dIII fragment of the coding sequence. One microgram of every oligonucleotide dissolved in 5 μl of water was used. The mixtures were brought to 50 mM Tris/HCl, pH 7.6, containing 5 mM $MgCl_2$, 5 mM β-mercaptoethanol (β-Me), 2 mM ATP, then 5 units of bacteriophage T4 polynucleotide kinase were added. Phosphorylation was performed for 4 h at 37°C. Subsequently, the contents of the tubes were mixed, heated for 5 min at 80°C to inactivate the polynucleotide kinase, and cooled down to room temperature. Then the mixtures were supplemented with 1 Weiss unit of bacteriophage T4 ligase and incubated overnight at 10°C. The products of the ligation process were separated by electrophoresis in 8% polyacrylamide gels. The gels were stained with ethidium bromide and the DNA fragment coding for the Pro47-Asp87 peptide was isolated after overnight diffusion from crushed polyacrylamide [11].

Plasmid construction. The ligated synthetic coding sequence, namely, the *Eco*RI — *Hin*dIII DNA fragment, was cloned into pUC19 plasmid [12]. The insert was recovered by digestion of the pUC19 recombination plasmid with *Eco*RI and *Hin*dIII, followed by electrophoresis in polyacrylamide gels, and sequenced according to Maxam & Gilbert [11]. Then, it was transferred to the pWR450.1 expression vector [13] resulting in the expression plasmid pVtPA47-87.

Purification of the recombinant peptide fragment. Cells (*E. coli*-DH5) harboring the plasmid expression vector were grown in 300 ml of LB (Bacto Tryptone; Bacto Yeast Extract) fermentation medium containing ampicilin (100 μg/ml) for 12 h at 37°C. Cultures started the previous day from a single colony and grown in 20 ml of the same medium for 12 h at 37°C were used to inoculate 300 ml of the LB medium. Cells were harvested by centrifugation and stored at –20°C. The frozen cell paste was resuspended in 0.05 mM $NaHPO_4$ buffer, pH 7.0, containing 10 mM β-Me, 10 mM PMSF (1g of cell paste/5 ml of buffer) to which 0.2 mg/ml lysozym had been added. The cells were incubated on ice for 30 min and then frozen at –70°C. After a quick thaw, the cells were sonicated briefly, NaCl added to 0.5 mol/l, and the suspension centrifuged for 10 min at 13000 × *g* at 4°C. The precipitate was dissolved in 8 M urea buffered with 0.5 M Tris, pH 7.9, containing 0.5 M NaCl,

1 mM EDTA, 30 mM β-Me and 1mM PMSF, and centrifuged for 10 min at 13000 × *g*. The supernatant was dialysed overnight against 0.05 M Tris buffer, pH 7.9, containing 10% glycerol and 0.5 M NaCl. The fusion protein precipitated at this stage was further purified by ion-exchange chromatography on DEAE-cellulose. For this purpose the fusion protein was dissolved in 9 M urea and dialysed against 6 M urea buffered with 0.05 M Tris, pH 7.5, containing 10mM EDTA and 1 mM PMSF (buffer A). The column was equilibrated with the same buffer. The fusion protein was eluted with buffer A containing 0.1 M NaCl.

Isolation of the Pro47-Asp87 peptide fragment. To separate the Pro47-Asp87 peptide fragment from β-galactosidase, the fusion protein was dissolved in 70% formic acid and incubated for 36 h at 45°C. Under these conditions the peptide bond Asp-Pro was selectively cleaved. Cleavage products were preliminarily separated by gel filtration on Sephadex G-50, and then the peptides reacting with specific anti-t-PA antibodies were isolated by HPLC on the C_{18} column.

Isolation of blood platelets. Human blood was collected into one-sixth volume of acid-citrate dextrose (ACD) from a forearm vein through an 18 gauge needle. Platelets were isolated by differential centrifugation (20 min, at 200 × *g*). Then, the platelet-rich plasma was collected and centrifuged for 20 min at 1000 × *g* to sediment platelets. The resulting pellet was resuspended in the modified Tyrode's buffer (140 mM NaCl, 10 mM glucose and 15 mM Tris/HCl, pH 7.4), and the platelets were subsequently washed three times with the same buffer. The entire washing procedure was performed in plastic tubes and carried out at room temperature.

Analytical procedures

Protein concentration was determined by the method of Bradford [14] or by spectrophotometry at 280 nm, using the absorption coefficient at 1 mg/ml (in aqueous solution at pH 7) of 1.35 for antibodies.

SDS-polyacrylamide slab gel electrophoresis was performed according to Laemmli [15].

Western immunoblotting. Completed gels were transferred electrophoretically 96 V/cm for 15 h at 4°C) onto nitrocellulose paper for staining with a specific antibody [16]. The transfer buffer contained 25 mM Tris, 192 mM glycine (pH 8.2), 20% (v/v) methanol. For staining, completed transfers were incubated at 20°C on a rocker (Ames aliquot mixer, Miles Laboratories, Elkhart, IN, U.S.A.) as follows: 5 min in 0.15 M NaCl, 0.01 M Tris/glycine buffer (pH 7.5) which was then used throughout; 30 min in 1% bovine serum albumin in the buffer; 1–2 h in bovine serum albumin containing antiserum; 20 min in several changes of buffer containing 0.1% Triton X-100 to remove excess of the first antibody; 1 h in the buffer containing peroxidase-conjugated goat anti-rabbit IgG at 1/500 dilution; 20 min in several changes of the buffer to remove the second antibody; 5 min in substrate solution (10 mg 4-chloro-1-naphthol dissolved in 3 ml methanol and added to 47 ml of the buffer containing 50 µl of 30% H_2O_2). All volumes were 20–25 ml, the minimun to ensure uniform wetting of the paper. Sensitivity was of the order of 25 ng using the peroxidase conjugate. When dot immunoblotting was used, 2 µl aliquots of serial dilutions of peptides cross-linked to bovine serum albumin by glutaradehyde were applied onto nitrocellulose paper. Staining with specific antibody was performed as described above. Semi-quantitative determination of interaction of the antibodies with the fusion protein and its cleavage fragments was done directly on nitrocellulose using a dot technique.

ESR spectroscopy. Intact platelets (3.3×10^8 cells/ml) were incubated for 30 min at room temperature with 16-doxylstearic acid or 5-doxylstearic acid at the final spin label concentration of 5×10^{-6} mol/l. Since the concentration of the stock ethanol solutions of spin label probes was 0.1 M, the final concentration of ethanol in the platelet suspensions was 0.05% (v/v). The ordinate in the ESR spectra represent the amplitude of the ESR signal and is expressed in arbitrary units. ESR scannings were routinely recorded as the first derivative of absorption spectra. The estimated ratios were calculated from ESR graphs and the relevant amplitudes were measured as the heights of the respective peaks. ESR measurements were performed at ambient temperature (23 ± 1°C) in an SE/X-20 X-band spectrometer (Wrocław Technical University, Poland).

Binding experiments. The binding of t-PA and its EGF-like domain to resting and thrombin-stimulated platelets was performed in the

presence or absence of 1 mM Ca^{2+} ions. Usually, 10 nM aliquots of proteins were incubated for 20 min at room temperature with the suspensions containing 3×10^8 platelets per ml. Then the samples were used for ESR scannings.

All data were expressed as the relative changes of control values assumed to be 100% for each of two replicates of a given sample. The data are presented as the means of the averaged replicates ± S.D. The normal distribution of data was analyzed by Shapiro-Wilk's test. Data were also elaborated by the analysis of variance and Tuckey's test assigned for paired comparisons [17].

RESULTS

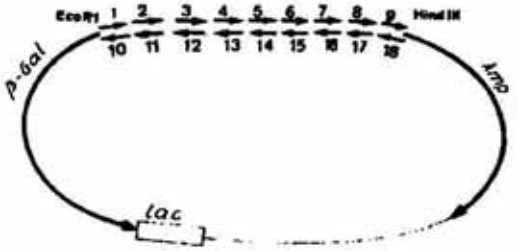
Cloning and isolation of the EGF-like domain of t-PA (Pro_{47}-Asp_{87})

To obtain the final expression vector pVtPA47-87, eight oligonucleotides shown in Fig. 1 were synthesized basing on the cDNA sequence corresponding to fragment Pro_{47}-Asp_{87} of human t-PA with the following modifications: (a) an initiating AATTCATGAGTCAGGAT sequence was included as part of the overhanging end of the 5′ *Eco*RI site, (b) a *Hin*dIII overhanging end CTGGAGAGTCAGGGATAATAAGCT was added to the 3′ end of the fragment.

Cloned isolates were characterized by DNA sequence analysis and showed the presence of that corresponding to the natural t-PA protein sequence. The fusion protein synthesized by transformed bacteria was preliminarily identified by a comparison of molecular weight of protein components of the selected clones with those of the host DH5 clone separated by SDS-polyacrylamide gel electrophoresis (Fig. 2). The Coomassie-stained gels pointed to the synthesis of a new band in the transformed clone of bacteria. The intensity of this band with relative molecular mass of 60000 was approximately the same for all selected clones (not shown). The new band was recognized by antibodies specific for t-PA.

After mechanical breakage of the bacterial cells the insoluble inclusion bodies were separated by centrifugation and the fusion protein isolated and purified as described under Methods. As judged by SDS-polyacrylamide gel electrophoresis, the fusion protein isolated by the procedure used was of 90% purity (Fig. 3). Protein blott analysis of the same samples run on a parallel gel with antibodies specific to human t-PA confirmed the presence of fragment Pro_{47}-Asp_{87} in the fusion protein (Fig. 3).

To obtain a peptide fragment of t-PA, the fusion protein was subjected to mild hydrolysis under the conditions described in methods. The peptide bond Asp-Pro connecting the peptide fragment of t-PA to β-galactosidase was selectively cleaved, as documented by SDS-PAGE (Fig. 4) of the cleavage products and subsequent fractionation by exclusion chromatography on Sephadex G-50 (Fig. 5). This pep-



5′	AATTCATGAGTCAGGATCCG	3′	(1)
	GTACTCAGTCCTAGGCCAG		(10)
	GTCAAAAGTTGCAGCGAACC		(2)
	TTTTCAACGTCGCT		(11)
	ACGATGTTTCAACG		(3)
	TGGTGCTACAAAGTTGCCTC		(12)
	GAGGAACCTGCCAGCAGGCA		(4)
	CTTGGACGGTCGTC		(13)
	CTGTACTTCTCAGA		(5)
	CGTGACATGAAGAGTCTAAA		(14)
	TTTCGTGTGCCAGTGTCCAG		(6)
	GCACACGGTCACAG		(15)
	AAGGATTTGCTGGAA		(7)
	GTCTTCCTAAACGACCTTTTA		(16)
	AATGTTGTGAAATAGATCTGG		(8)
	CAACACTTTATCTAG		(17)
	AGAGTCAGGGATAATA		(9)
	ACCTCTCAGTCCCTATTATTCGA		(18)

Fig. 1. Design of the plasmid expression vector.
The plasmid expresses the Pro_{47}-Asn_{87} fragment of t-PA corresponding to the EGF-like domain under the control of the Lac promoter.The peptide fragment is synthesized as part a fusion protein containing Met_1-Val_{444} of β-galactosidase.

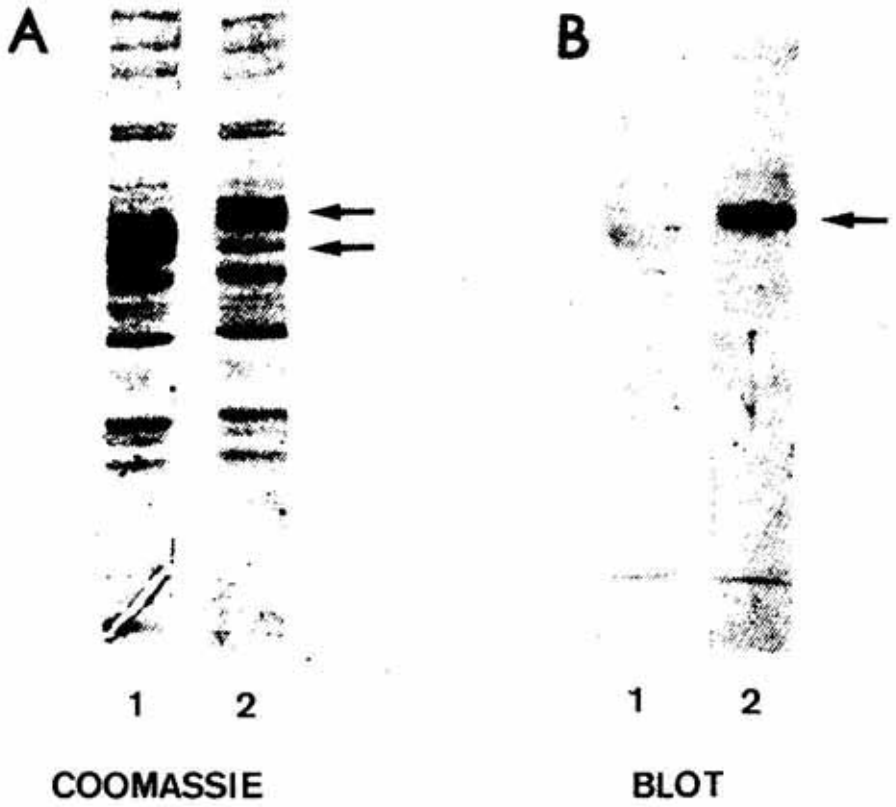


Fig. 2. The presence of a fusion protein detected by SDS-polyacrylamide gel electrophoresis in extracts of E. coli.

Cells harboring the indicated plasmid were grown as described in Material and Methods section and dissolved in Laemmli sample buffer. The arrows point to: in Coomassie-stained 15% gels (panel A) the β-galactosidase fragment Met_1-Val_{444} in a parent DH5 clone (lane 1) and a protein of M_r of 60000 (lane 2) representing the fusion protein. Panel B shows a positive reaction of the fusion protein (lane 2) with antibodies to t-PA after Western immunoblotting.

tide fragment, found to be eluted in the first peak by dot immunoblotting analysis, was further purified by HPLC on C_{18} column. As it is seen in Fig. 6, the peptide fragment was eluted from the C_{18} column after 29.90 min at 43% acetonitrile concentration. This fragment migrated as a single band during SDS-polyacrylamide gel electrophoresis in 20% gels and reacted with antibodies specific to t-PA on immunoblotting analysis (Fig. 4).

Interaction of the EGF-like domain with blood platelets

To examine the effect of the EGF-like domain of t-PA on platelets, human blood platelets were labelled with either 16-doxylstearic acid or 5-doxylstearic acid and then incubated with the peptide fragment Pro_{47}-Asp_{87} at peptide concentration of 5 ng/3 × 10^8 cells. The mobility of the spin label probes at the depth of C-16 (in 16-DS) and C-5 (in 5-DS) was measured semiquantitatively as the ratio h_{+1}/h_0 where h_{+1} and h_0 are the heights of low-field line and the middle-line of the spectra, respectively [18]. As it is seen in Fig. 7 the ratio h_{+1}/h_0 was reduced upon binding of the Pro_{47}-Asp_{87} peptide fragment indicating decreased mobility of both spin labels. The same changes were produced in resting and thrombin stimulated platelets. Though the mobilities of 16-DS and 5-DS were not significantly different, the observed changes caused by the EGF-like domain were more pronounced in 16-DS labelled platelets. Recombinant t-PA and EGF-like domain produced similar changes in the membrane fluidity as detected by ESR spectroscopy of blood platelets labelled with 16-DS (Fig. 8).

DISCUSSION

Previous studies have shown that t-PA binds in a specific, time-dependent and saturable manner to both resting and stimulated blood platelets [6]. ESR spectroscopy of blood platelets labelled with spin probes shows that such

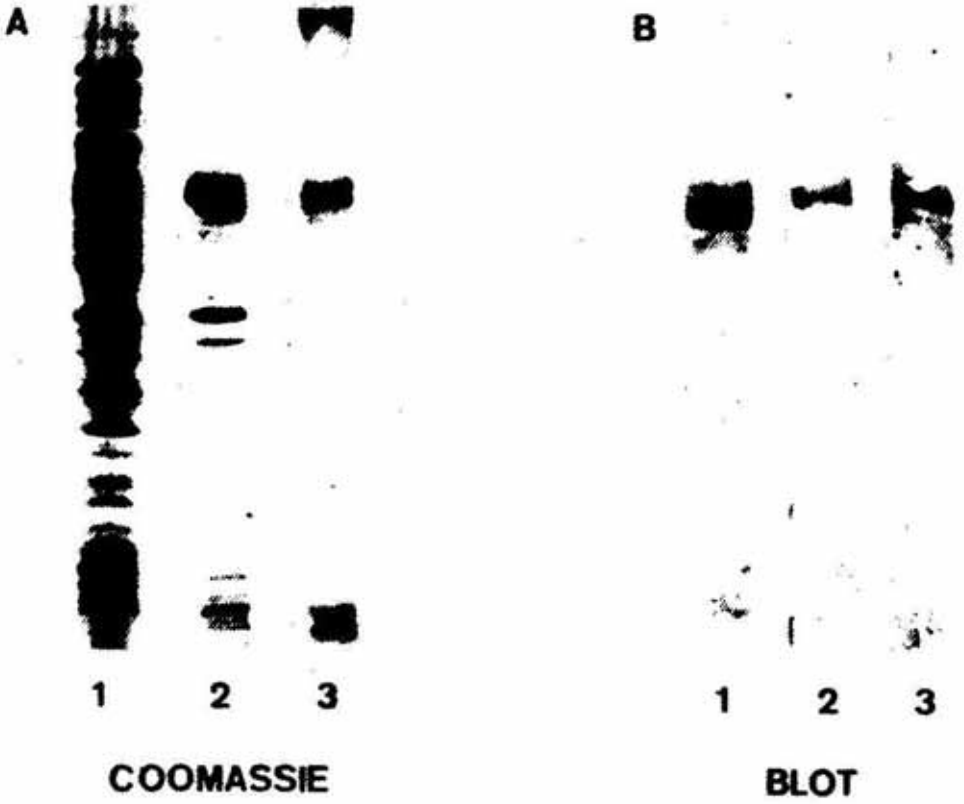


Fig. 3. SDS-polyacrylamide gel electrophoresis of the purified fusion protein detected by Coomassie blue staining (panel A) and Western immunoblotting (panel B) after incubation with antibodies specific to t-PA. Cell extract in Laemmli sample buffer containing 8 M urea (lane 1), inclusion bodies (lane 2) and the fusion protein separated by DEAE-cellulose chromatography were separated on 15% gels (lane 3).

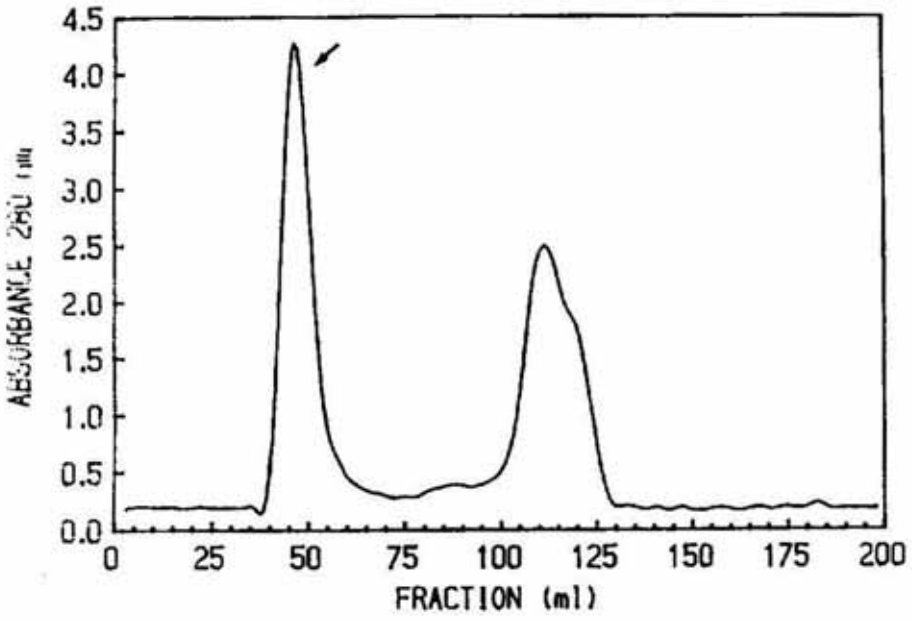


Fig. 4. Fractionation of cleavage fragments of the fusion protein by gel filtration on Sephadex G-50. Fractions eluted by 50% acetic acid were pooled within both peaks and 2 µl aliquots were applied onto nitrocellulose. After incubation with antibodies against human t-PA, the presence of Pro_{47}-Asp_{87} arrows was detected in the first peak by dot immunoblotting.

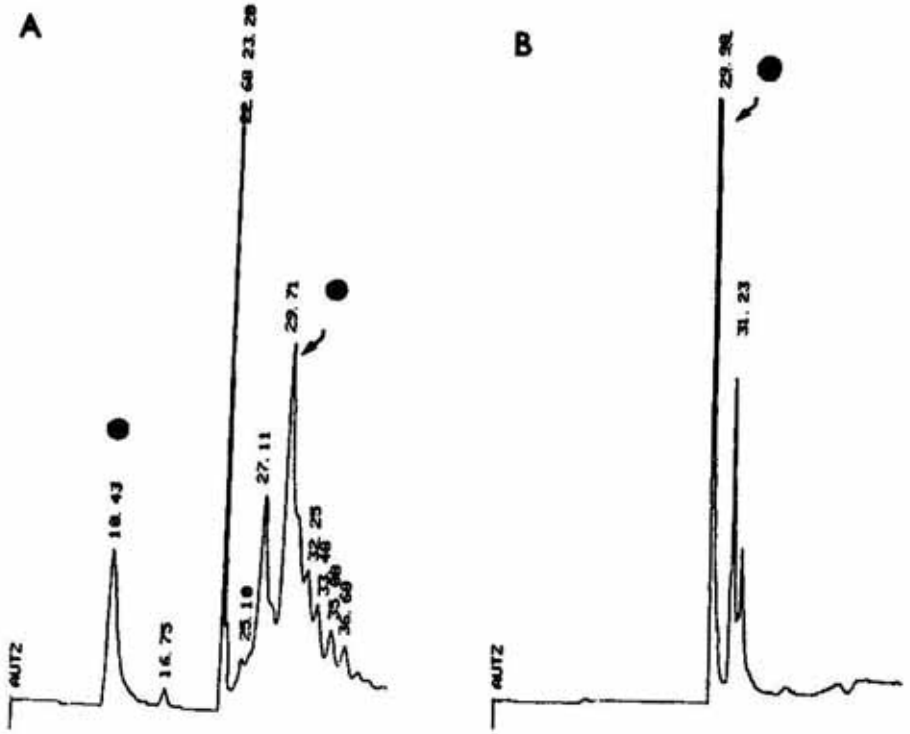


Fig. 5. Isolation of Pro47-Asp87 by HPLC on the C_{18} column using a linear gradient from 0.1% TFA to 80% acetonitrile in 0.1% TFA.
The peptide eluted in the peak at 29.71 min (panel A) reacting with antibodies specific to t-PA was further rechromatographed (panel B) and collected. "AKT Z" indicates start of HPLC run.

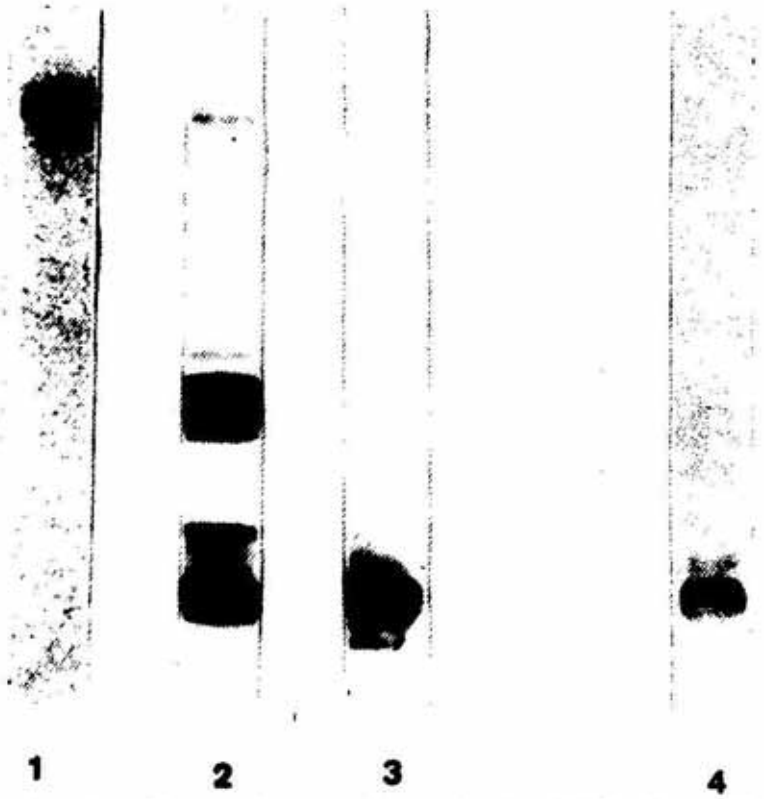


Fig. 6. SDS-polyacrylamide gel electrophoresis in 20% gels of purified fusion protein (lane 1), its cleavage products (lane 2) and the peptide Pro47-Asp87 purified by HPLC, stained with Coomassie blue (lane 3) and immunostained with antibodies to t-PA (lane 4).

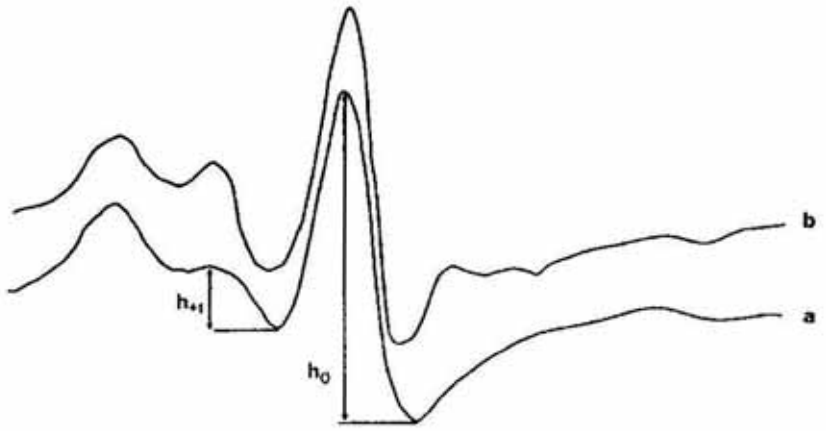


Fig. 7. Comparison of ESR spectra of 5-DS (a) and 16-DS (b) incorporated into platelet membranes of control platelets.

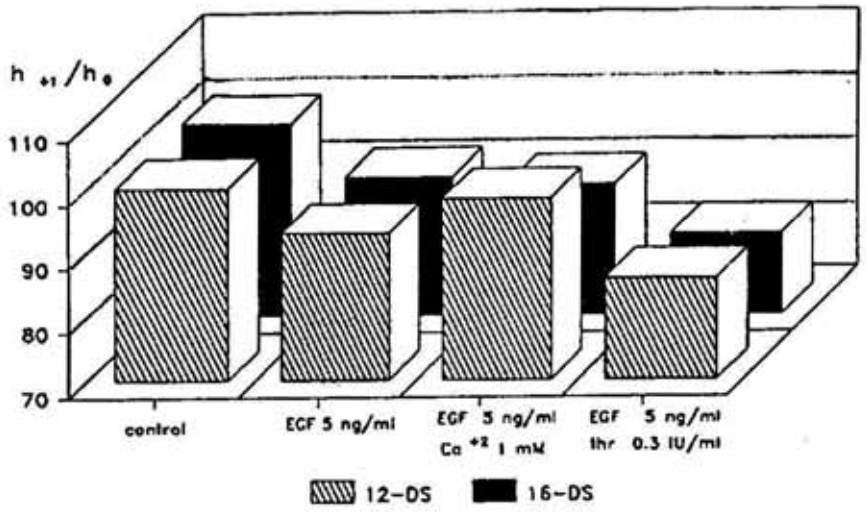


Fig. 8. Comparison of the changes induced in the ratio h_{+1}/h_0 by intact t-PA and its EGF-like domain.
The ratio h_{+1}/h_0 was calculated from ESR spectra of 5-DS and 16-DS incorporated into lipid bilayer of whole blood platelets, respectively. Platelets were incubated with 5 ng/ml of the EGF-like domain of t-PA in presence and absence of Ca^{2+} ions. In addition platelets preincubated with the same amount of the EGF-like domain were stimulated by thrombin. The changes in this ratio induced by these proteins indicate decreased fluidity of membrane lipid bilayer upon their binding to platelet membrane.

binding results in a significant rigidification of the membrane lipid bilayer [18].

The reason for selecting EGF-like domain for our studies was that this region seems to be the domain responsible for binding with cell membrane receptors both in t-PA and pro-UK [19, 20]. The deletion of EGF-like domain of u-PA and t-PA resulted in their prolonged half-life time in circulation [2, 19]. Since reduction of the half-life times of plasminogen activators *in vivo* was demonstrated to be due to hepatic clearance [21], one might assume that hepatic cells have the ability to recognize, at least partially, the EGF-like domain of pro-UK and t-PA molecules. The presence of UK receptor has been also reported for several cell lines, such as the epidermoid carcinoma cell line, A431 [22] and monocyte cell line, U-937 [19, 23], but not for hepatic cells. The EGF-like domain has been reported to be responsible for the binding of pro-UK to its receptor on U-937 cells.

To examine whether the interaction in question is mediated by the EGF-like domain of t-PA, in this study we produced in *E. coli* the

peptide fragment Pro_{47}-Asp_{87} as part of the fusion protein containing β-galactosidase because: (a) fusion of eucaryotic proteins or peptides to β-galactosidase has been reported to stabilize them in *E. coli* [20]; (b) β-galactosidase provides a rapid, quantitative measure of recombinant protein synthesis; (c) the β-galactosidase marker offers the possibility of rapid purification of the fusion protein. Our data showed that a single recombinant protein of the expected size was synthesized and that this peptide fragment was recognized by polyclonal antibodies specific to t-PA. The peptide fragment Pro_{47}-Asp_{87} of t-PA can easily be separated by HPLC on C_{18} column from the cleavage products of fusion protein produced by formic acid.

When this peptide fragment was preincubated with blood platelets labelled with spin probes, 16-doxylstearic acid or 5-doxylstearic acid, significant changes in their mobility in membrane lipid bilayer were observed. Like the intact t-PA after binding to blood platelets, the EGF-like domain caused a decrease of membrane fluidity both in resting and thrombin-stimulated platelets. Rigidization of the lipid matrix could be induced by binding of the EGF-like domain of t-PA to membrane receptor, and strongly supports the concept that this domain may be involved in the interaction of t-PA with blood platelets.

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