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Autonomous functions of structural domains on human tissue-type plasminogen activator

(exon shuffling/tissue-type plasminogen activator cDNA-deletion mutants)

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ABSTRACT **Transfected mouse Ltk$^-$ cells were employed for transient expression of recombinant human tissue-type plasminogen activator (t-PA; EC 3.4.21.31) or of recombinant-t-PA deletion proteins, encoded by SV40-pBR322-derived t-PA cDNA plasmids. The t-PA cDNA deletion mutants have two features in common, i.e., cDNA programming the signal peptide and the coding region for the light chain. Consequently, recombinant t-PA mutant proteins are efficiently secreted and display plasminogen activator activity. The gene encoding the amino-terminal heavy chain [an array of structural domains homologous to other plasma proteins (finger, epidermal growth factor, and kringle domains)] was mutated using restriction endonucleases to delete one or more structural domains. The stimulatory effect of fibrinogen fragments on the plasminogen activator activity of t-PA was demonstrated to be mediated by the kringle K2 domain and to a lesser extent by the finger/epidermal growth factor region but not by the kringle K1 domain. These data correlate well with the fibrin-binding properties of the recombinant t-PA deletion proteins, indicating that the stimulation of the activity by fibrinogen fragments is based on aligning the substrate plasminogen and t-PA on the fibrin matrix. Our results support the evolutionary concept of exon shuffling, arranging structural domains that constitute autonomous functions of the protein.**

Tissue-type plasminogen activator (t-PA; EC 3.4.21.31) is a serine protease that converts the zymogen plasminogen into plasmin, a serine protease that degrades the fibrin network (1). The activity of t-PA is substantially accelerated in the presence of fibrin, a property that has focused the attention on t-PA as a potential therapeutic anti-thrombogenic agent (2–4). The molecular mechanism of the stimulatory influence of fibrin is based on the hypothesis that the substrate plasminogen and the enzyme t-PA are bound to and aligned on the fibrin matrix, facilitating a localized generation of plasmin.

t-PA is synthesized and secreted by vascular endothelial cells as a single-chain polypeptide (5). This molecule is converted by plasmin or trypsin into a two-chain polypeptide connected by a disulfide bond. The heavy (H) chain of t-PA (M_r 39,000) is located at the amino terminus, whereas the light (L) chain (M_r 33,000) is at the carboxyl-terminal end (6). Based on the amino acid sequence homology of t-PA with the trypsin family of serine proteases, prothrombin, epidermal growth factor (EGF), and fibronectin, a model for the secondary structure of t-PA has been proposed (6, 7). In this model, different structural domains have been assembled to create a composite mosaic polypeptide. The L chain of t-PA was proposed to harbor the serine protease moiety, responsible for plasminogen activator activity. In mammalian cells separately expressed t-PA L-chain cDNA is, indeed, solely capable of converting plasminogen into plasmin, and this activity is not accelerated by fibrin (8). The H chain of t-PA contains considerable amino acid homology with several plasma proteins. Apart from a typical signal peptide and a prosequence, similar to those of serum albumin (9, 10), one can distinguish a finger domain resembling the regions on fibronectin involved in fibrin binding (11, 12) and a structure partially homologous to human and mouse EGF (13, 14). Furthermore, two kringle structures are proposed to be situated on the carboxyl-terminal part of the H chain, highly homologous to structural and functional domains on plasminogen, shown to be involved in fibrin binding of plasminogen (15).

The elucidation of the chromosomal structure of the t-PA gene and the alignment with the complete amino acid sequence revealed an intriguing observation, namely that exons or sets of exons coincide with the proposed structural domains (6, 16, 17). Those studies and others have led to the hypothesis that mosaic proteins, such as t-PA and other plasma proteins, are composed of different functional modules as a result of exon shuffling, an evolutionary rearrangement event (18, 19).

In this study, we provide evidence that structural domains in the t-PA protein, encoded by separate exons or sets of exons, harbor autonomous functions.

MATERIALS AND METHODS

Materials. Restriction endonucleases, T4 DNA ligase, T4 DNA polymerase, T4 polynucleotide kinase, and *Escherichia coli* DNA polymerase I (Klenow fragment) were purchased from New England Biolabs. Mung bean nuclease was from P.L. Biochemicals; tunicamycin was from Calbiochem; Iscove's modified medium was from Flow Laboratories; and all reagents required for the plasminogen activator assay were from Kabi Vitrum (Stockholm, Sweden).

General Methods. Plasmid DNA was isolated by a modification of the alkaline lysis procedure (20), followed by CsCl equilibrium centrifugation. Enzyme reactions were carried out using standard conditions (21). Nucleotide sequence determinations were performed using the method of Maxam and Gilbert (22).

Construction of Expression Plasmids. Plasmid ptPA8FL consists of vector pBR322 and full-length t-PA cDNA [2540 base pairs (bp)] inserted by G/C tailing into the *Pst* I site of the vector (A.J.v.Z., G. T. G. Chang, A. P. Van den Berg, T. Kooistra, J. H. Verheijen, H.P., and C. Kluft, unpublished data). Human t-PA cDNA was constructed using Bowes melanoma poly(A)$^+$ RNA. A fragment (2087 bp) of the full-length t-PA cDNA, extending from bp 78 to 2165 [numbering of the t-PA cDNA is according to Pennica *et al.* (6)]

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Abbreviations: t-PA, tissue-type plasminogen activator; r, recombinant; H, heavy; L, light; EGF, epidermal growth factor; bp, base pair(s); SV40, simian virus 40; S2251, D-Val-Leu-Lys-*p*-nitroanilide.

and containing the entire coding sequence, was subcloned between the *Hin*cII and *Bam*HI sites of the pUC9 polylinker, resulting in plasmid pUCtPA. A *Hin*dIII–*Sac* I fragment of pUCtPA was inserted together with a *Sac* I–*Bgl* II fragment of ptPA8FL into the *Hin*dIII and *Bgl* II sites of pSV2 (24) to yield pSV2tPA. This plasmid contains the origin of replication and the β-lactamase gene of pBR322, the simian virus 40 (SV40) early promoter, the t-PA-coding sequence, and SV40 splice and polyadenylylation sequences. All t-PA deletion expression plasmids were derived from pSV2tPA by constructing deletions in the coding sequence using restriction sites adjacent to the junctions of the hypothetical domains. To preserve the translation reading frame, in some of the constructions restriction fragment termini were altered using DNA-modifying enzymes (see Fig. 1). DNA-sequence determinations were performed to verify a correct in-phase ligation of all new cDNA junctions in each construct.

Tissue Culture and Transfection. Mouse Ltk$^-$ cells were maintained in Iscove's modified minimal medium, containing penicillin, streptomycin, and 10% (vol/vol) fetal calf serum. Transfection was carried out essentially as described (25). After transfection, the cells were incubated in serum-free Iscove's medium, containing penicillin, streptomycin, and acid-treated bovine serum albumin at 0.3 mg/ml. In some of the experiments, tunicamycin (1 μg/ml) was added. Five days after transfection, cell media regularly contained 1–5 μg of recombinant (r)t-PA or rt-PA mutant proteins per 8×10^6 cells.

Gelatin–Plasminogen Gel Electrophoresis. Electrophoresis on gelatin gels, containing copolymerized plasminogen at 13 μg/ml, was performed essentially as described (26). Ten percent polyacrylamide gels, containing 0.1% $NaDodSO_4$, were incubated after electrophoresis in 2.5% (vol/vol) Triton-X 100 for 2 hr to remove $NaDodSO_4$. Subsequently, the gels were incubated for 6 hr in 0.1 M glycine/NaOH (pH 8.3). Gelatin degradation, due to plasminogen activation in the gel, was visualized by contrastaining using 0.1% amido black.

Plasminogen Activator Assay. Plasminogen activator activity was determined using an indirect spectrophotometric assay (27). This assay measures the amidolytic activity of plasmin, which is generated by the plasminogen activator-catalyzed conversion of plasminogen and is based on the hydrolysis of a bond in the chromogenic substrate D-Val-Leu-Lys-*p*-nitroanilide (S2251). Assays were performed at 37°C in 250 μl of 0.1 M Tris·HCl (pH 7.5), containing 0.13 μM plasminogen, 0.1% Tween-80, CNBr-digested fibrinogen (120 μg/ml, when indicated) and 0.30 mM S2251. The absorbance at 405 nm was followed for 6 hr.

Immunoradiometric Assay for t-PA L-Chain Antigen. Goat anti-mouse IgG coupled to Sepharose beads was incubated with limiting amounts of the murine monoclonal anti-human t-PA L-chain IgG ESP2 (28) and with ^{125}I-labeled t-PA tracer in 0.01 M phosphate-buffered saline (pH 7.4), containing 1% bovine serum albumin, 0.1% Tween-80, and 10 mM EDTA. During the incubation, stoppered polystyrene tubes were rotated end-over-end for 18 hr at room temperature. Separation of bound and free ^{125}I-labeled tracer was carried out by centrifugation for 2 min at 3000 × *g*. The Sepharose beads were washed four times with 2 ml of 0.9 M NaCl, 0.1% Tween-80, 10 mM EDTA and bound radioactivity was determined using a gamma counter. Unlabeled Bowes melanoma t-PA was used as a competitor for the binding of ^{125}I-labeled t-PA. Approximately 100 ng of t-PA, in a final volume of 0.5 ml, reduced the binding of the tracer to 50% of the maximum value. Conditioned media, containing rt-PA or rt-PA mutant protein, were also used as competitor for ^{125}I-labeled t-PA binding to the monoclonal ESP2 IgG. A comparison between the percentage of competition of the conditioned media and serial dilutions of Bowes melanoma t-PA allows an estimation of the amount of L-chain antigen present.

RESULTS

Construction of t-PA Deletion Expression Plasmids. To study the biological properties of the separate hypothetical domains of t-PA, we have constructed a series of t-PA cDNA expression plasmids that systematically lack the coding sequence of one or more of the domains on the H chain. For that purpose, we have employed our cloned full-length t-PA cDNA (23), composed of a 95-bp 5′-untranslated region, a 1686-bp coding region, and a 759-bp 3′-untranslated region. Restriction enzyme analysis and partial nucleotide sequence determinations revealed identity with the DNA sequence reported by Pennica *et al.* (6). Sections of full-length t-PA cDNA were inserted into a shuttle vector (pSV2), consisting of parts of plasmid pBR322 and the eukaryotic virus SV40. In these constructs, t-PA cDNA is preceded by the SV40 early promotor and linked at its 3′ end to splice and polyadenylylation signals. All constructs have two features in common. First, they harbor the coding sequence for the t-PA signal peptide (nucleotides 85 to at least 156) to ensure secretion of the (mutant) polypeptides. Second, all constructs contain the entire coding region for the t-PA L chain (nucleotides 953–1771). The latter domain is responsible for the plasminogen activator activity (8), thereby allowing a convenient assay of the biological activity. Furthermore, the involvement of the different domains of the H chain on the stimulation of the plasminogen activator activity of t-PA by fibrin can be readily assessed. Essential junctions of the constructs were sequenced to verify the continuity of the translation reading frame. A schematic representation of the H-chain t-PA cDNA deletion mutants is shown in Fig. 1.

Characterization of the Expression Products. Mouse Ltk$^-$ cells appeared to be suitable hosts for transient expression of rt-PA programmed by t-PA cDNA-harboring plasmids, because these cells can be efficiently transfected and neither contain nor secrete plasminogen activator(s). The different expression products, present in the serum-free media 5 days after transfection, were analyzed by gelatin–plasminogen gel electrophoresis (Fig. 2*A*). This technique localizes plasminogen activator activity in $NaDodSO_4$/polyacrylamide gels. The method depends on the principle that plasminogen and gelatin, when incorporated into the polyacrylamide matrix at the time of casting, are retained during electrophoresis. *In situ* plasmin formation and consequently degradation of gelatin can be visualized by negative staining. Clearly, melanoma t-PA and rt-PA display similar mobilities in this gel system. The rt-PA-deletion mutants were also secreted, apparently due to routing directed by the signal peptide. The mobility of the deletion proteins corresponds with the expected values. Obviously, all mutant proteins exhibit a basal plasminogen activator activity in accord with our previous observations that the L chain suffices for this activity (8). To demonstrate that the heterogeneity of the products results from differential glycosylation, tunicamycin, an inhibitor of N-glycosylation, was added to the cell media during the expression of the products. The results, presented in Fig. 2*B*, show that under these conditions the proteins L, LFE, LK2, and LK1-2 display single bands on the gelatin gel, indicating that they are expressed as unique polypeptides. Our data and those of others (29) show that the carbohydrate moieties of t-PA are not involved in its biological activity. We consider the expression products to be valuable tools in studying the biological properties of the as yet hypothetical domains of the t-PA molecule.

Plasminogen Activation by the Mutant Proteins; the Influence of Fibrinogen Fragments. To investigate the effect of fibrin on the plasminogen activator activity of the deletion

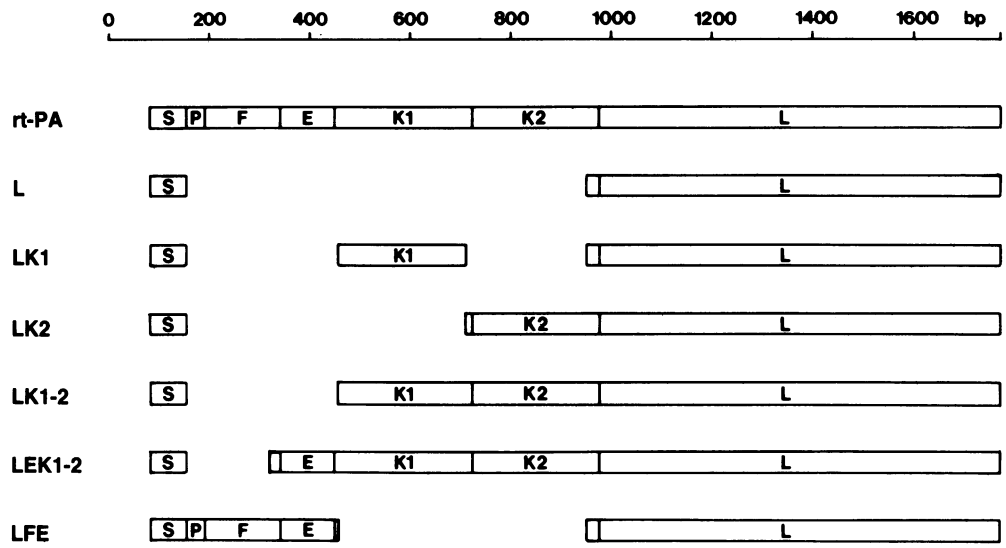

FIG. 1. Schematic representation of t-PA cDNA present in the rt-PA and rt-PA deletion expression vectors. rt-PA is coded for by the entire coding cDNA. Deletion cDNAs were constructed by fusing t-PA restriction-fragment termini that were in some cases enzymatically modified to preserve the translation reading frame. L [deletion of bp 158–953 (Δ 158–953)] was constructed by fusing the *Eco*RII terminus (position 153) [filled in by *E. coli* DNA polymerase I large fragment (Klenow)] to the *Sca* I terminus (position 950). LK2 (Δ 156–711); fusion of the *Bst*NI terminus (position 153) (Klenow) to the *Dde* I terminus (position 710) (Klenow). LK1-2 (Δ 158–458); fusion of the *Eco*RII terminus (position 153) (Klenow) to the *Hae* III terminus (position 457). LK1 (Δ 158–458/Δ 713–953); as LK1-2 with one extra deletion made by fusion of the *Dde* I terminus (position 710) [partially filled in by Klenow (TTP, dGTP) followed by a Mung bean nuclease treatment] to the *Sca* I terminus (position 950). LEK1-2 (Δ 158–326); fusion of the *Eco*RII terminus (position 153) (Klenow) to the *Dra* III terminus (position 319) (T4 DNA polymerase, adding dATP only, followed by a Mung bean nuclease treatment). LFE (Δ 455–953); fusion of the *Bst*NI terminus (position 452) (Klenow) to the *Sca* I terminus (position 950). S, signal peptide; P, propeptide; F, finger domain; E, EGF domain; K1 and K2, kringle domains; and L, light chain.

proteins, we used an amidolytic assay, relying on the chromogenic substrate S2251. We determined the activity of rt-PA and rt-PA mutant proteins in the presence and absence of CNBr-digested fibrinogen, a digest known to mimic the potentiating effect of fibrin (27). The data are given in Fig. 3. In the absence of fibrinogen fragments, rt-PA only has a basal activity, but upon adding fibrinogen fragments, the plasminogen activator activity is greatly accelerated. The mutant proteins L and LK1 display only a basal activity, and no significant stimulation of the plasminogen activator activity by fibrinogen fragments was detected. In contrast, the plasminogen activator activity of the mutant proteins LEK1-2, LK1-2, and LK2 was stimulated by fibrinogen fragments to the same magnitude as rt-PA. The plasminogen activator activity of the mutant protein LFE was also stimulated by fibrinogen fragments, but to a lesser extent than the K2-domain-containing mutant proteins. These results indicate that the K2 domain of the t-PA H chain and, to a lesser extent, the finger/EGF region mediate the stimulatory effect of fibrin. Finally, kringle K1 apparently does not contribute to the stimulatory mechanism. It is assumed that the molecular basis for the stimulation by fibrin is that both plasminogen and t-PA are bound by the fibrin polymer, hereby aligning t-PA with its substrate (3, 30). Therefore, we examined the fibrin-binding properties of the t-PA deletion proteins.

Fibrin Binding. Fibrin matrices were formed in the presence of either rt-PA or rt-PA deletion proteins and subsequently pelleted (31). Plasminogen activator activity of supernatants and of solubilized pellets were analyzed on gelatin–plasminogen gels (Fig. 4). In the case of rt-PA and L, the total amount used (input) and the nonbinding and the binding fractions are shown. Most of the rt-PA-input fraction was bound to the fibrin matrix, whereas no significant binding of the L-chain protein was detected. The input and the fraction bound to fibrin of the other proteins are also presented. LK1 does not exhibit significant binding to fibrin.

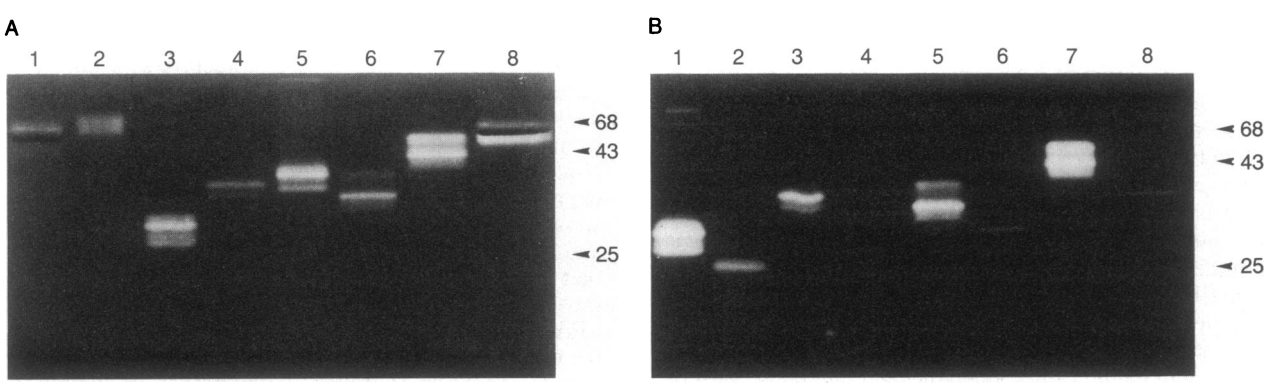

FIG. 2. Gelatin–plasminogen gel analysis of the expression products. (*A*) Melanoma t-PA (lane 1); rt-PA (lane 2); L (lane 3); LFE (lane 4); LK1 (lane 5); LK2 (lane 6); LK1-2 (lane 7); and LEK1-2 (lane 8). Molecular weight standards × 10^{-3} are indicated. (*B*) Comparison of the products expressed in the absence (−) and presence (+) of tunicamycin. L: − (lane 1), + (lane 2). LFE: − (lane 3), + (lane 4). LK2: − (lane 5), + (lane 6). LK1-2: − (lane 7), + (lane 8).

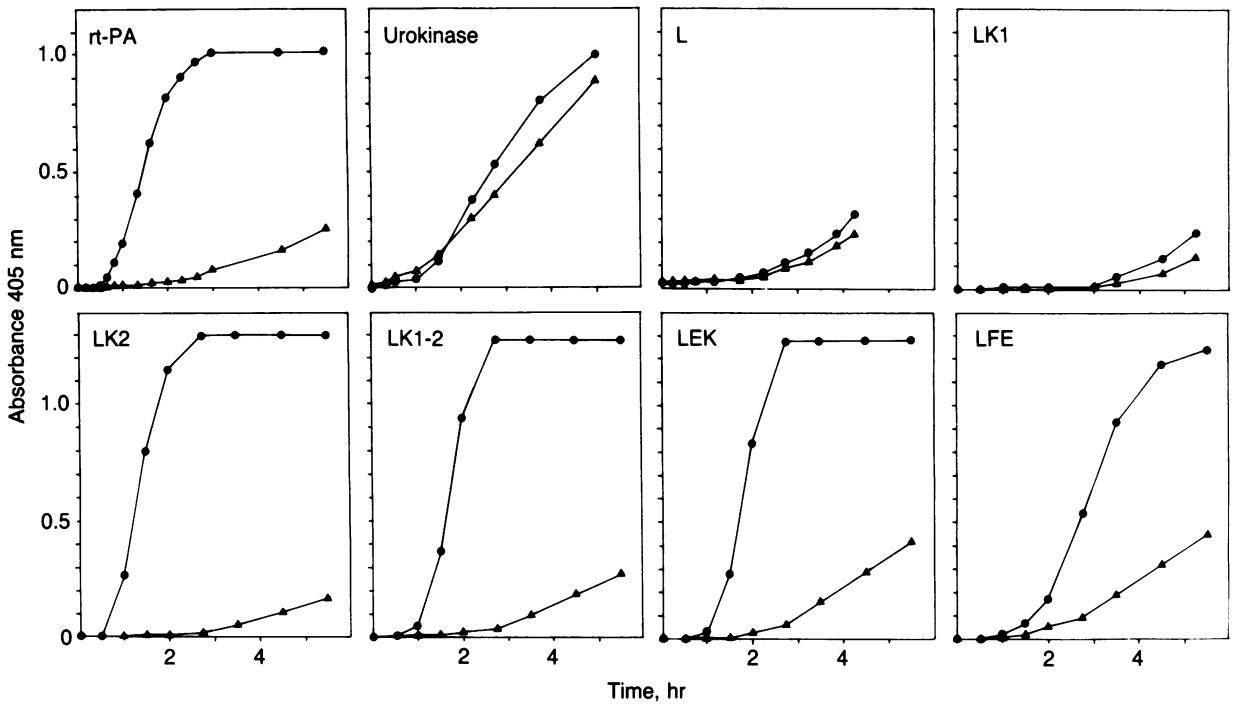

FIG. 3. The influence of fibrin (fibrinogen fragments) on the plasminogen activation by rt-PA, urokinase, L, LK1, LK2, LK1-2, LEK1-2, and LFE proteins as determined with an amidolytic assay using the chromogenic substrate S2251 (27). Serum-free medium containing about 0.15 pmol (determined by the immunoradiometric assay) of the different expression products or urokinase (2 milliunits) were used. The absorbance at 405 nm was followed for at least 4 hr and conditioned serum-free medium of Ltk$^-$ cells, which were transfected with promotorless ptPA8FL DNA, was used as a control. ●, plus fibrinogen fragments; ▲, minus fibrinogen fragments.

The K2-domain-containing mutants LEK1-2, LK1-2, and LK2 bound to fibrin to about the same extent, although not as efficient as the binding of rt-PA. The LFE mutant also bound to fibrin, but to a lesser extent than the K2-domain-containing mutant proteins. Hence, the stimulatory effect of fibrinogen fragments on the plasminogen activator activity, mediated by kringle K2 and to a lesser extent by the finger/EGF region, correlates well with the fibrin-binding characteristics of these domains. Apparently, the acceleration of the plasminogen activator activity of t-PA is intimately linked to binding to the fibrin matrix.

DISCUSSION

The elucidation of the t-PA gene structure by Ny *et al.* (16) revealed that separate exons or separate sets of exons encode structural domains, which were postulated on the basis of homology with other plasma proteins. To test whether these autonomous structural domains indeed have autonomous functions, we have expressed a series of rt-PA deletion proteins lacking one or more of the structural domains and studied the remaining biological properties. Earlier studies from this laboratory (8) showed that separately expressed L-chain molecules harbor the plasminogen activator activity, however, this activity could not be stimulated by fibrin. In those studies, the L-chain molecules were not secreted by the tissue culture cells that were used for expression of L-chain cDNA. In this paper, we have been able to show that efficient secretion of the expression products occurs, provided the signal peptide is encoded at the 5′ end of the cDNA of different deletion mutants. This result indicates that the t-PA signal peptide has an autonomous function.

The finger/EGF region exhibits a fibrin-binding function and also mediates in part the stimulatory effect of fibrin on plasminogen activator activity of t-PA. We argue that the finger domain, and not the EGF domain, is responsible for these phenomena. First, homologous finger structures (type-I homology) present in fibronectin are known to be crucial for the fibrin affinity of fibronectin (11, 12). Second, Bányai *et al.* (7) showed that limited proteolysis of the amino-terminal region of t-PA caused a loss of the fibrin affinity of the enzyme. Third, an EGF domain is also present on the closely related urokinase molecule, which does not bind to fibrin (30, 32).

Clearly, the K2 domain has an autonomous function in the binding of fibrin as well as in mediating stimulation of the plasminogen activator activity of t-PA by fibrin. Surprisingly, the kringle K1 domain does not seem to be involved in the fibrin-binding property of t-PA. Although the amino acid sequences of kringle K1 and K2 are highly homologous and the secondary structure might be similar (6, 7), apparently subtle differences may specify fibrin affinity. Such differences might explain the lack of fibrin affinity of urokinase, in spite of the presence of a kringle structure on this molecule.

We propose that the fibrin-binding characteristics of t-PA are mediated by the finger domain and the K2 domain, the latter contributing most to the binding. Additional evidence for the involvement of the kringle K2 domain in fibrin binding is that a murine monoclonal anti-human t-PA antibody (ESP5) (28), which was found to inhibit in part fibrin binding of t-PA, specifically bound to LK2, but not to the L-chain product (results not shown). The fact that this conformation-dependent monoclonal antibody reacted equally well with rt-PA as with the K2-domain-containing mutant proteins indicates that the K2 domain retains its native conformation in the deletion proteins.

Mosaic proteins (e.g., t-PA) may have evolved by exon shuffling, an evolutionary process linking different functional structures. By showing that structural domains in t-PA, encoded by separate exons or sets of exons, are autonomous functional domains, our study supports the concept of exon shuffling as a mechanism to create new genes. In our view, the approach explored here to investigate the structure–function relationships of the t-PA molecule with these and other

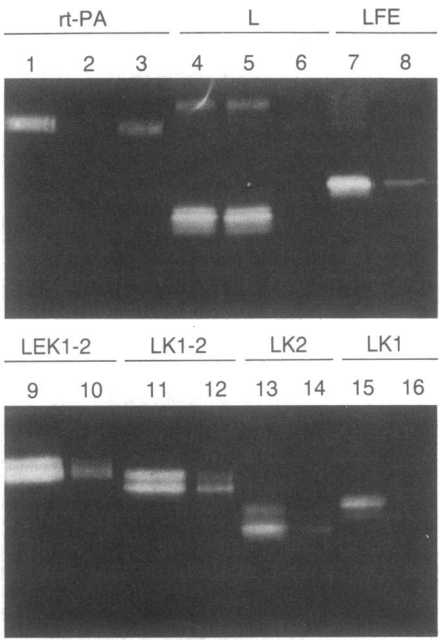

FIG. 4. Binding of rt-PA and rt-PA mutant proteins to fibrin. The assays were performed as described (31). About 0.3 pmol of expression product was mixed with 5 pmol human fibrinogen in a volume of 1 ml, containing phosphate-buffered saline (PBS), 0.01% Tween-80, 5 mM EDTA. Clotting was achieved by incubation with 1 unit of thrombin for 1 hr at 37°C, followed by centrifugation for 30 min at 40,000 × *g*. The pellets were solubilized by gentle rocking in 1 ml of 0.5% $NaDodSO_4$ in PBS for 2 hr at 37°C. Equivalent samples of the bound and nonbound fractions were electrophoresed on gelatin–plasminogen gels. rt-PA: i, input (lane 1); −, nonbinding fraction (lane 2); +, binding fraction (lane 3). L: i (lane 4), − (lane 5), + (lane 6). LFE: i (lane 7), + (lane 8). LEK1-2: i (lane 9), + (lane 10). LK1-2: i (lane 11), + (lane 12). LK2: i (lane 13), + (lane 14). LK1: i (lane 15), + (lane 16).

deletion mutants may elucidate which domains are involved in the complex formation of t-PA with plasminogen activator inhibitor(s) (33, 34) and in the binding of t-PA to the receptor(s) in the liver (35, 36) responsible for the rapid clearance of t-PA from the bloodstream.

Note. After submission of this manuscript, we learned that a "fingerless" t-PA protein (23) retained high affinity for fibrin. Those results support our conclusion that, besides the finger domain, another part of t-PA is involved in fibrin binding.

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