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Structure and Function of Human Tissue-Type Plasminogen Activator (t-PA)

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Full-length tissue-type plasminogen activator (t-PA) cDNA served to construct deletion mutants within the N-terminal "heavy" (H)-chain of the t-PA molecule. The H-chain cDNA consists of an array of structural domains homologous to domains present on other plasma proteins ("finger," "epidermal growth factor," "kringles"). These structural domains have been located on an exon or a set of exons. The endpoints of the deletions nearly coincide with exon–intron junctions of the chromosomal t-PA gene. Recombinant t-PA deletion mutant proteins were obtained after transient expression in mouse Ltk^- cells, transfected with SV40-pBR322-derived t-PA cDNA plasmids. It is demonstrated that the serine protease moiety of t-PA and its substrate specificity for plasminogen is entirely contained within the C-terminal "light" (L)-chain of the protein. The presence of cDNA, encoding the t-PA signal peptide preceding the remaining portion of t-PA, suffices to achieve secretion of (mutant) t-PA into the medium. The stimulatory effect of fibrin on the plasminogen activator activity of t-PA was shown to be mediated by the kringle K2 domain and, to a lesser extent, by the finger domain. The other domains on the H-chain, kringle K1, and the epidermal growth-factor-like domain, do not contribute to this property of t-PA. These findings correlate well with the fibrin-binding properties of the rt-PA deletion-mutant proteins, indicating that stimulation of the activity is based on aligning of the substrate plasminogen and its enzyme t-PA on the fibrin matrix. The primary target for endothelial plasminogen activator inhibitor (PAI) is located within the L-chain of t-PA. Deleting specific segments of t-PA H-chain cDNA and subsequent transient expression in mouse Ltk^- cells of t-PA deletion-mutant proteins did not affect the formation of a stable complex between mutant t-PA and PAI.

Key words: tissue-type plasminogen activator, fibrinolysis, deletion mutants

Tissue-type plasminogen activator (t-PA) is a serine protease that plays a key role in fibrinolysis, a process directed at dissolving a blood clot. t-PA converts the zymogen plasminogen into plasmin, a serine protease of broad specificity that de-

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grades the fibrin network of the thrombus [1]. t-PA is synthesized as a single-chain protein, but can be converted by plasmin into a two-chain form in which the chains are connected by a disulfide bond. The H-chain is located at the N-terminal section of the molecule, whereas the L-chain is at the C-terminal end. Based on homology with other serine proteases, it is inferred that the L-chain harbors the catalytic centre [2]. Interestingly, the activity of t-PA is greatly enhanced by fibrin, a property that causes a localized generation of plasmin. It is generally assumed that binding of both plasminogen and t-PA to fibrin leads to an alignment of the substrate and its enzyme on the fibrin matrix, resulting in an efficient formation of plasmin [3].

Apart from plasminogen and fibrin, t-PA interacts with other defined proteins as well, possibly in a competitive manner. A variety of plasminogen activator inhibitors has been encountered in different cell types that inactivate plasminogen activators by forming a complex [4–7]. Curiously, cultured vascular endothelial cells synthesize both t-PA and a plasminogen activator inhibitor (PAI), an observation that suggests a potential mechanism to regulate the activity of t-PA. The nature of the interaction between t-PA and the endothelial inhibitor (PAI) has not been clarified as yet. Furthermore, it has been demonstrated that clearance of t-PA is mediated by a receptor in the liver [8]. However, the receptor has not been isolated nor has its specificity been determined. Hence, it can be concluded that t-PA interacts with at least four different proteins: plasminogen, fibrin, plasminogen activator inhibitor(s), and a receptor.

Molecular cloning of both t-PA cDNA and of the chromosmal gene has generated new ideas about the structure and the function of the t-PA protein [2,9]. The determination of the nucleotide sequence of t-PA cDNA allowed a prediction of the amino-acid sequence of the protein that has been confirmed by amino-acid sequence analysis of the native protein [10]. Those studies, together with the exon–intron distribution of the chromosomal t-PA gene [9], has resulted in a model for the secondary structure of the t-PA protein. This model is based on homologous regions of t-PA with other plasma proteins. One can distinguish on the H-chain, respectively, a signal sequence, a prosequence, a "finger domain" homologous to type I "fingers" on fibronectin, an "epidermal growth-factor domain" homologous to both human and mouse epidermal growth factor, and two "kringle domains" similar to the kringles previously found on prothrombin [2,9,11,12]. These domains are encoded by a separate exon or a set of two adjacent exons, an observation that may implicate that structural domains constitute autonomous functions of t-PA [9,13]. This concept agrees with the hypothesis proposed by Gilbert [14] that predicts that exons encode useful portions of the protein structure, sorted independently during evolution.

In this paper, we have used molecular biological techniques to locate the domains on the t-PA protein that interact with plasminogen, fibrin, and the endothelial PAI.

INTERACTION OF t-PA WITH PLASMINOGEN

The construction of a full-length human t-PA cDNA plasmid, starting from polyA$^+$ RNA isolated from Bowes melanoma cells, has been described in detail elsewhere [15]. The nucleotide sequence of the codogenic region on this cDNA is indistinguishable from that reported by Pennica et al [2]. Our approach to localize discrete functions on the t-PA molecule has been to construct specific t-PA cDNA

deletion mutants, employing restriction enzymes and nucleic acid-modifying enzymes. The restriction enzymes that were selected for the constructions cleave t-PA at close proximity of the position of an exon–intron junction on the corresponding chromosomal t-PA gene. Junctions created during the constructions were sequenced to verify the integrity of the remaining translation reading frame [16]. A schematic representation of the structure of eight different t-PA cDNA deletion mutants and of full-length rt-PA cDNA is given in Figure 1. The nomenclature that is chosen for the t-PA cDNA deletion mutants indicates the domains still present on the cDNA. All the t-PA cDNA deletion mutants harbor the codogenic region for the signal peptide and the L-chain, except for cat/L, which lacks cDNA encoding the signal peptide.

The constructed t-PA cDNAs were inserted into the vector pSV2, a SV40-pBR322 derivative [17]. This plasmid provides the SV40 "early" promotor, preceding t-PA cDNA, and SV40 splice and polyadenylation signals located downstream of the inserted cDNA. Transfection of mouse Ltk^- cells, followed by transient expression, yielded either conditioned media or cell extracts that were assayed for their ability to activate (Glu)-plasminogen. For that purpose, an indirect amidolytic assay was employed with the chromogenic substrate S2251 measuring plasminogen activator-dependent formation of plasmin, catalyzed by either full-length t-PA (rt-PA) or by mutant t-PA. The results are presented in Table I. It is evident that the presence of the t-PA signal peptide is an essential element for the secretion of t-PA into the conditioned media. All the mutant t-PA proteins are encountered in the media at a concentration of 0.2–2 μg per ml. However, mutant cat/L, a fusion product of the 77 N-terminal amino acid residues of the bacterial protein chloramphenicol acetyltransferase (cat), providing the methionine for initiation of translation, and the complete t-PA L-chain, is only detected in cell extracts. Apparently, this property is due to the absence of the t-PA signal peptide in mutant cat/L. In the absence of components that stimulate the plasminogen activator activity of t-PA, the specific activity of the different t-PA mutants is comparable. Consequently, it can be concluded that the property of t-PA to activate plasminogen and the plasminogen specificity of the serine protease moiety is entirely contained within the L-chain segment of the molecule.

INTERACTION OF t-PA WITH FIBRIN

The plasminogen activator activity of t-PA is greatly enhanced by fibrin [3]. Soluble fragments of fibrinogen, digested with CNBr, mimic the stimulatory effect of fibrin and can be conveniently applied in the amidolytic assay to measure plasminogen activation [18]. The ability of the different t-PA deletion-mutant proteins to convert plasminogen into plasmin, in the presence or absence of fibrinogen fragments, is illustrated in Figure 2. The mutant proteins L and LK1 display only a basal activity that is not significantly stimulated by fibrinogen fragments. In contrast, the activity of LEK1–2, LK1–2 and LK2 was stimulated to the same extent as rt-PA. The plasminogen activator activity of both LFE and LF was also stimulated by soluble fibrinogen fragments; however, not to the same extent as the mutant proteins containing the kringle K2 domain. From these experiments, we further conclude that the "epidermal growth factor" E domain and the kringle K1 domain apparently do not contribute to the stimulatory effect of fibrin on the activity of t-PA.

If, an enhancement of the activity is mediated by binding of t-PA to fibrin, then the binding characteristics of the mutant proteins should coincide with their suscepti-

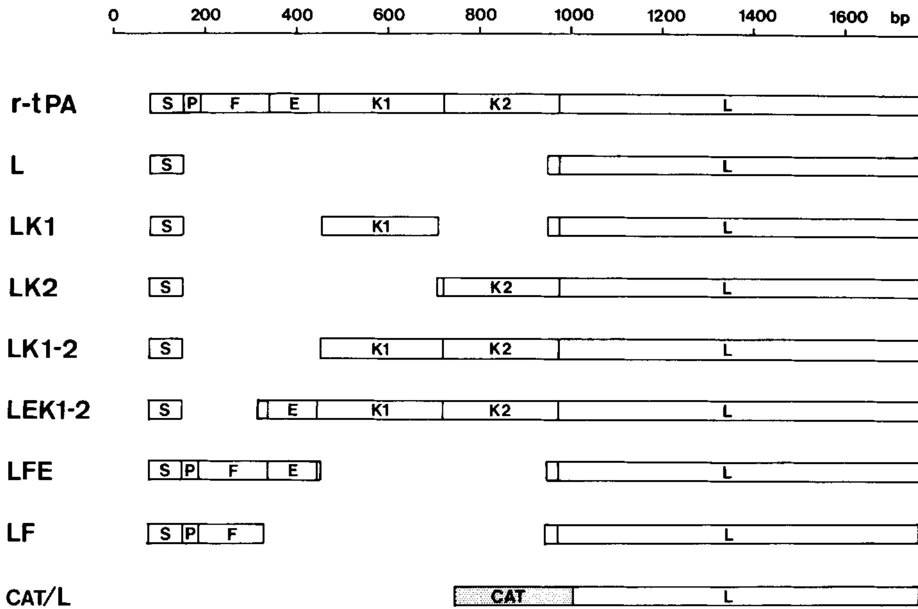

Fig. 1. Schematic representation of t-PA cDNA present in rt-PA and t-PA deletion mutants. rt-PA is encoded by full-length t-PA cDNA. The deletion-mutant cDNAs were created by joining t-PA restriction-fragment termini, altered in most cases with DNA-modifying enzymes to preserve a continuous translation reading frame. Verification of the continuity of the reading frame was indirectly determined by DNA sequencing of the junctions [14]. L [deletion of basepair 158 till 953 (Δ 158–953)] was constructed by fusing the EcoRII(153)-terminus [filled in by E coli DNA polymerase-I large fragment (Klenow)] to the ScaI(950)-terminus. LK2 (Δ 156–710); fusion of the BstNI(153)-terminus (+ Klenow) to the DdeI(710)-terminus (+ Klenow). LK1-2 (Δ 158–458); fusion of the EcoRII(153)-terminus (+ Klenow) to the HaeIII(456)-terminus. LK1(Δ 158–458/Δ 713–953); as LK1–2 with one additional deletion made by fusion of the DdeI(710)-terminus [partially filled in by Klenow (+ TTP and dGTP) followed by Mung Bean nuclease treatment] to the ScaI(950)-terminus. LEK1-2 (Δ 158–326); fusion of the EcoRII(153)-terminus (+ Klenow) to the DraIII(319)-terminus (+ T4-DNA polymerase and dATP, followed by a Mung Bean nuclease treatment). LFE (Δ 455–953); fusion of the BstNI(452)-terminus (+ Klenow) to the ScaI(950) terminus. LF (Δ 332–953) has been constructed in two steps. (a) LK1–2 was digested with NarI(517) and ScaI(950) and treated with Klenow. A BbvI fragment (252–341) was isolated from pSV2/t-PA digested with BbvI. This fragment was incubated with T4-DNA polymerase, dATP, dCTP, and TTP and subsequently with Mung Bean nuclease. This modified fragment was inserted into LK1–2, prepared as described above. (b) The resulting plasmid was digested with DraIII (at 319 and 1365); lacking 332–953). The DraIII fragment, containing the deletion, was employed to substitute for the "intact" DraIII fragment (319–1365) of pSV2/t-PA to yield LF. DNA sequencing demonstrated that either Mung Bean nuclease or T4-DNA polymerase has "nibbled" at the BbvI ends. cat/L (Δ 1–1006); fusion of a HindIII-EcoRI fragment of the chloramphenicol acetyltransferase (cat) gene of the E coli Tn9 transposon, containing the ATG initiation codon [29], and a DdeI(1005)-BlgII(2160) restriction fragment derived from pSV2/t-PA. The DdeI and EcoRI ends were treated with Klenow. After ligation, the fused segment of DNA was inserted into pRSVβ-globin [30]. The numbering of restriction sites and the created deletions in t-PA cDNA is according to the published sequence of Pennica et al [2]. S, signal peptide; P, propeptide; F, finger domain; E, epidermal growth-factor-like (EGF) domain; K1 and K2, kringle domains; and L, light chain.

TABLE I. Plasminogen Activator Activity of t-PA Deletion-Mutant Proteins

Mutant	Plasminogen activator activity	Presence in conditioned medium
rt-PA	+	+
L	+	+
cat/L	+	−

Plasminogen activator activity determinations were performed with an indirect amidolytic assay using the chromogenic substrate S2251. Similar activities were detected in the conditioned medium of transfected mouse Ltk^- cells, containing LF, LFE, LEK1-2, LK1-2, LK2 and LK1. Protein cat/L is programmed by plasmid pRSVcat/L DNA. This plasmid is based on pRSVβ-globin, a derivative of pSV2, in which the SV40-origin sequences have been replaced by the Rous sarcoma virus (RSV) long-terminal repeat [30]. In this particular case, plasminogen activator activity was demonstrated only in cell extracts of rat 3Y1 cells, containing stably integrated unrearranged copies of pRSVcat/L cDNA [30]. Integrated pRSVcat/L cDNA was detected after cotransfection with a molar excess of pRSVneo.

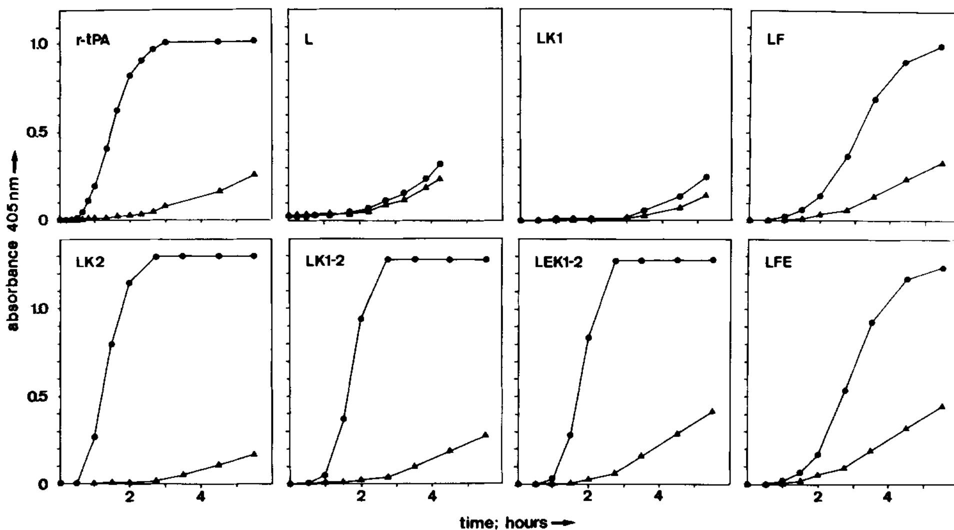

Fig. 2. The influence of "fibrin" on the plasminogen activator activity of rt-PA and t-PA deletion-mutant proteins. Instead of fibrin, soluble fragments of fibrinogen, digested with CNBr, were used which are known to mimic the effect of fibrin [16]. The activity of the proteins rt-PA, L, LK1, LK2, LK1-2, LEK1-2, LFE and LF was determined with an amidolytic assay using the chromogenic substrate S2251. We used 20 μl serum-free medium containing about 0.15 pmoles of each of the proteins in the assays. The absorbance at 405 nm was followed for at least 4 hr. A promotor-less pSV2-derived plasmid, harboring t-PA cDNA, was used as a control. ●, + Fibrinogen fragments; ▲, − fibrinogen fragments.

bility for stimulation by fibrin. This assumption was examined by determining whether rt-PA and the t-PA mutant proteins bind to fibrin matrices. An analysis of both the bound and the nonbound portion of these preparations was performed by electrophoresis on SDS–polyacrylamide gels, containing gelatin and plasminogen [19]. This technique allows a visualization of unstimulated plasminogen activator activity in situ after contrastaining of the gels. Moreover, this method generates data on the apparent molecular weights of the mutant proteins, synthesized in transfected mouse Ltk$^-$ cells. The results are presented in Figure 3. Pertinent conclusions can be drawn from this experiment. First, the apparent molecular weights of the different t-PA deletion-mutant proteins correspond roughly with the expected size, based on the length, the constructed deletion and an otherwise continuous translation reading frame of t-PA cDNA. Second, all products synthesized in the tissue-culture cells display heterogeneity. This phenomenon is caused by differential glycosylation, since, upon addition to the media of tunicamycin (an inhibitor of N-linked glycosylation), single homogeneous polypeptides are produced (results not shown). It has been reported before that the carbohydrate moieties of t-PA are not involved in plasminogen activator activity [20]. Third, it is obvious that the mutant proteins L and LK1 do not bind to the fibrin

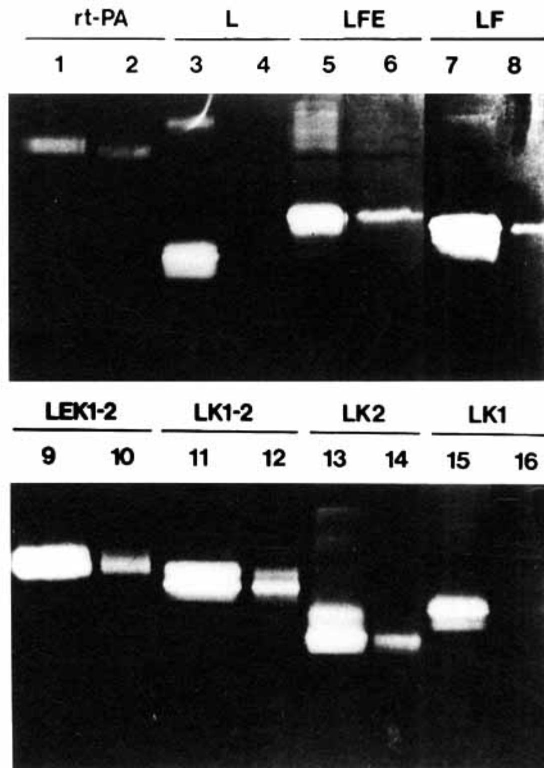

Fig. 3. Binding of rt-PA and rt-PA deletion-mutant proteins to fibrin. The assays were performed as described [31]. About 0.3 pmoles expression product was used as input material for each assay. The bound and the nonbound fractions were run on SDS-polyacrylamide gels, containing plasminogen and gelatin [17]. rt-PA, input (i) (lane 1), bound (+) (lane 2). L, i (lane 3), + (lane 4). LFE, i (lane 5), + (lane 6). LF, 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).

matrices. Substantial binding is observed for the mutant proteins LEK1-2, LK1-2, LK2, and for the full-length product rt-PA. An intermediate binding is detected, using the mutant proteins LFE and LF. Hence, it can be concluded that these data correlate well with the properties of the different mutant proteins to be stimulated by fibrin: kringle K2 contributes most to the fibrin binding and the ability to be stimulated, whereas the "finger" (F) domain provides for an additional contribution to this property. From these experiments, it became clear that the "epidermal growth factor" domain and kringle K1 are not directly involved in the interaction of t-PA with fibrin.

INTERACTION OF t-PA WITH THE ENDOTHELIAL PLASMINOGEN ACTIVATOR INHIBITOR (PAI)

A plasminogen activator inhibitor from bovine aorta endothelial cells has been purified to homogeneity and characterized by biochemical and immunological techniques [21,22]. It has been reported that this product is a glycoprotein with an apparent molecular weight of about 55,000 Daltons and displays an unusual resistance towards chemical agents, such as β-mercaptoethanol, urea, SDS, and acetic acid. Even boiling does not substantially affect the anti-activator activity. Similar results have been published for the endothelial PAI, isolated from conditioned medium of cultured human umbilical endothelial cells [7].

Serum-free conditioned medium from these human endothelial cells, containing PAI, was incubated with rt-PA or with the different t-PA deletion-mutant proteins. The complex formation between the t-PA derivatives and PAI was analyzed by the fibrin overlay technique after electrophoresis on SDS–polyacrylamide gels [23]. The results are given in Figure 4.

It is evident from this experiment that both rt-PA and the deletion-mutant proteins, which have the L-chain in common, form a complex composed of equimolar amounts of rt-PA or of t-PA derivatives and PAI. Consequently, stable interaction between t-PA and PAI is mediated by a region contained within the L-chain of t-PA. Resolving the structure and the nature of the PAI protein will provide further insight into the mechanism of action of the enzyme with the inhibitor.

DISCUSSION

The properties of the t-PA deletion-mutant proteins, programmed by recombinant DNA plasmids, demonstrate the validity of the assumption that structural domains on the t-PA gene encode autonomous functional domains. Clearly, the deletion of one or more structural domains does not grossly affect the structure of the protein and the remaining functions in an obvious manner. This conclusion is supported by our finding that 21 different monoclonal antibodies, raised against t-PA from conditioned medium of Bowes melanoma cells, form immune complexes equally well with Bowes melanoma t-PA, rt-PA, and t-PA deletion mutants, provided the relevant epitope has not been deliberately deleted (results to be published elsewhere). These observations strengthen the conclusions on the experiments reported here, stating that the autonomous functions of different domains are not affected by deleting other independent domains. Similar conclusions can be drawn from experiments with a preparation of isolated kringle 4 of plasminogen [24]. It was reported that, upon denaturation and renaturation, this peptide regained its functional lysine-binding

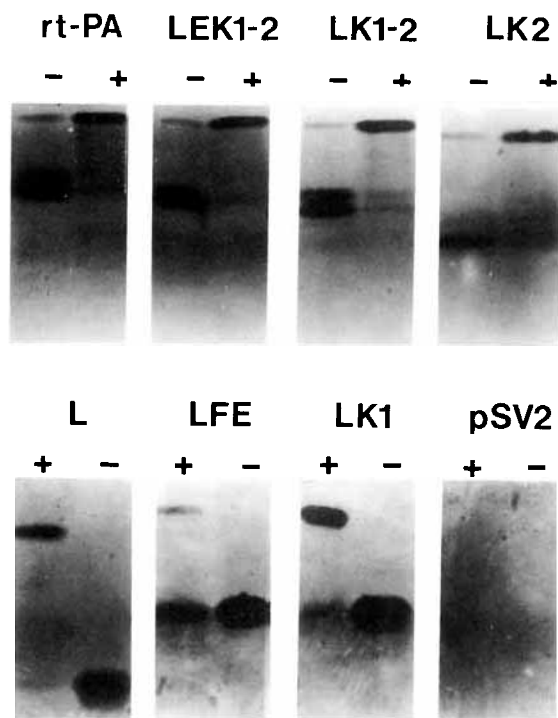

Fig. 4. Binding of rt-PA and t-PA deletion-mutant proteins to human endothelial plasminogen activator inhibitor (PAI). We depleted 5 ml of conditioned medium of cultured human endothelial cells, derived from veins of umbilical cords, of t-PA by incubating with an excess of monoclonal antihuman t-PA (PAM-2, a generous gift of Dr. Matts Ränby) coupled to Sepharose beads. Incubation was performed by "head-over-head" rotation for 2 hr at room temperature and, subsequently, the Sepharose beads were removed by centrifugation for 5 min at 3000g. We incubated 40 μl of the supernatant for 1 hr at 37°C with 40 μl of conditioned medium of each of the different transfected mouse Ltk^- cells. To the mixture, 20 μl of 12.5% (w/v) SDS, 10% (w/v) sucrose and 20 μg/ml bromophenol blue were added and the resulting volume of 100 μl was layered on a 10% polyacrylamide gel, containing 0.1% (w/v) SDS. After electrophoresis and subsequent processing of the gel, a fibrin overlay was applied as described [23], allowing a visualization of plasminogen activator activity either complexed with PAI or noncomplexed. +, with PAI, −, without PAI.

property, irrespective of the absence of the authentic, surrounding structures of plasminogen.

Based on homology of the amino-acid sequence of the L-chain of t-PA with other serine proteases, it has been proposed that the L-chain harbors the active centre [2]. This proposal is supported by the finding that diisopropyl-fluorophosphate (DFP), inactivating the protein at the serine residue within the catalytic centre, is incorporated in the L-chain [25]. Our results provide evidence for the concept that the L-chain suffices for the plasminogen activation and the substrate specificity.

Our data show that the interaction of t-PA with fibrin is mediated by two domains: kringle 2 and finger domain. These findings corroborate and extend those reported by other investigators. Limited proteolysis of the N-terminal region of t-PA was found to lead to a loss of binding to fibrin, indicating a role of the finger domain in this property of t-PA [11]. Furthermore, it has been reported that the separate finger peptide, expressed in *Escherichia coli*, acts as an effective competitor in vitro

for the binding of t-PA to fibrin [26]. The involvement of other parts of the t-PA protein in the fibrin-binding characteristics of t-PA has been demonstrated by Kagitani et al [27], who found that a finger-domain-lacking t-PA, synthesized in *Escherichia coli*, still binds with high affinity to immobilized fibrin. The properties of the t-PA deletion mutants described here clearly demonstrate that both the finger domain and kringle 2 contribute to the binding of t-PA to fibrin and apparently result in an enhancement of the plasminogen activator activity of the protein. Our data, although only partly quantitative, indicate that the major contribution to these characteristics is executed by the kringle-2 domain.

The endothelial PAI was shown in our experiments to interact with the L-chain of t-PA. This conclusion agrees with the observation of others that t-PA, inactivated by treatment with DFP, does not form complexes with a plasma plasminogen activator inhibitor that is immunologically related to PAI and possibly identical to PAI [28]. Our experiments do not exlude that PAI interacts with other regions of t-PA as well. Detailed studies on the interaction between α-2-antiplasmin and plasmin have revealed that initially a fast, reversible interaction occurs between the inhibitor and kringle 1 of the enzyme, followed by a stable, irreversible interaction with the serine protease moiety of plasmin [1]. Here, we aimed at trapping the stable interaction between PAI and rt-PA or the t-PA mutant proteins by an extended period of complex formation of the two components. We estimate that kinetic experiments are required to exclude that other parts of the protein are involved in this interaction.

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