On the Interaction of the Finger and the Kringle-2 Domain of Tissue-type Plasminogen Activator with Fibrin

Inhibition of Kringle-2 Binding to Fibrin by ε-Amino Caproic Acid

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The binding of tissue-type plasminogen activator (t-PA) to fibrin is mediated both by its finger domain and by its kringle-2 domain. In this report, we investigate the relative affinities of these domains for lysine. Human recombinant t-PA deletion-mutant proteins were prepared and their ability to bind to lysine-Sepharose was investigated. Mutants containing the kringle-2 domain bound to lysine-Sepharose, whereas mutants lacking this domain but containing the fibrin domain, the epidermal growth factor domain or the kringle-1 domain did not bind to lysine-Sepharose. Mutant proteins containing the kringle-2 domain could be specifically eluted from lysine-Sepharose with ε-amino caproic acid. This lysine derivative also abolished fibrin binding by the kringle-2 domain but had no effect on the fibrin-binding property of the finger domain. Thus, a lysine-binding site is involved in the interaction of the kringle-2 domain with fibrin but not in the interaction of the finger domain with fibrin. The implications of the nature of these two distinct interactions of t-PA with fibrin on plasminogen activation by t-PA will be discussed.

Kinetic studies indicate that in the presence of fibrin, plasminogen activation by tissue-type plasminogen activator (t-PA) is enhanced 2 to 3 orders of magnitude (1–3). The underlying principle of this fibrin dependency is that both plasminogen and t-PA specifically bind to fibrin to form a ternary complex in which activation takes place (1). Hence, the specific binding of t-PA and plasminogen to fibrin is an essential feature for the regulation of fibrinolysis.

The binding of plasminogen to fibrin has been studied extensively. This interaction is mediated by lysine-binding sites which have affinity for lysine analogs, such as ε-amino caproic acid (EACA). Two of these lysine-binding sites are located in the first and fourth kringle structures of plasminogen (4). A lysine-binding site with a different ligand specificity (AH-site, i.e. 6-aminohexyl site) also has been identified in the portion of the molecule known as miniplasminogen (Val422-Ass790) (5). This binding site prefers ligands not carrying a free carboxylate group, such as 6-aminohexyl compounds.

The high affinity of t-PA for fibrin has been known for several years (6). However, information on the nature of the interaction between these two molecules has been obtained only recently from structural studies of the t-PA molecule (7–10). Elucidation of the primary structure of t-PA (11) and comparison of this sequence to known protein sequences revealed that t-PA is organized into specific structural domains (7, 11). The COOH-terminal light chain (L-chain) displays homology with the catalytic domain of the trypsin-like serine protease family, whereas the NH2-terminal H-chain contains a finger domain, an epidermal growth factor (E) domain, and two kringle domains. Fibrin binding by t-PA is probably mediated by domains on the H-chain, since L-chain molecules, either purified from the native molecule (12) or specifically expressed using recombinant DNA techniques (13), can activate plasminogen, but this activity is not stimulated by fibrin.

Recently, we have expressed a series of t-PA deletion-mutant proenzymes in tissue-culture cells and studied the function of the individual domains of t-PA in more detail (9, 10). It was concluded that stimulation of the plasminogen activator activity by fibrin and fibrin binding were mediated both by the K2 domain and the F domain. t-PA has been shown to bind to lysine, raising the possibility that its interaction with fibrin, like that of plasminogen, could also be mediated by lysine-binding sites. In this study, we have employed these t-PA deletion-mutant proteins to localize a single lysine-binding site on the t-PA molecule and studied the involvement of both this lysine-binding site and the finger domain in fibrin binding.

Materials and Methods

Lysine-Sepharose and CNBr-activated Sepharose CL-4B were from Pharmacia P-L Biochemicals, Iscove’s modified Dulbecco’s medium and other tissue-culture reagents were from Flow Laboratories; ε-amino caproic acid, bovine serum albumin (fraction V), and human thrombin were from Sigma; and fibrinogen (plasminogen-free) and plasminogen were from Imco (Stockholm, Sweden). Two-chain mt-PA was from Biopool (Umea, Sweden). High-performance liquid chromatography (HPLC) equipment was from LKB and the HPLC mono-S column was from Pharmacia P-L Biochemicals.

Tissue Culture and Expression of Recombinant Proteins—Mouse Ltk- cells were maintained in Iscove’s modified minimal medium containing penicillin, streptomycin, and 10% fetal calf serum. Trans-
fection of the cells with t-PA deletion-mutant expression plasmids was carried out using the DEAE-dextran method, as previously described (9, 14). Five days after transfection, serum-free conditioned media, containing 0.3 mg/ml acid-treated bovine serum albumin, penicillin, and streptomycin, were harvested. Tween 80 and sodium azide were added to final concentrations of 0.01 and 0.02%, respectively. The cultures were dialyzed overnight at 4°C against PBS containing 0.1% Tween 80 and 0.02% sodium azide (PTS) and stored at -20°C until use.

**Purification of Monoclonal Antibody ESP2—**Acetate fluid (600 μl) of monoclonal anti-t-PA antibody ESP2 (15) was added to an equal volume of 50 mM sodium acetate (pH 5.2) (buffer A) and left for 1 h at room temperature. After centrifugation for 30 min at 10,000 x g, the supernatant was diluted 10-fold in buffer A and loaded on an HPLC mono-S column (HR 5/5). The column was washed with buffer A and eluted with a salt gradient of 0 to 0.5 M NaCl in buffer A. Peak fractions were dialyzed overnight against PBS and stored at -20°C for further use.

**Radioiodination of Proteins—**Purified monoclonal antibody ESP2 and mt-PA were iodinated using the chloramine-T method (16). Free label was removed by dialysis against PBS containing 0.01% Tween 80 and 0.01% KI. Specific radioactivity of mt-PA and ESP2 were 2.6 and 15.2 mCi/μg, respectively.

**Expression of t-PA Mutant Proteins—**The ability of the different conditioned media to compete for the binding of 125I-labeled mt-PA tracer protein to ESP2 Sepharose allows an estimation of the amount of expression product present in the conditioned media. Anti-t-PA IgG (ESP2) was precipitated with 50% ammonium sulfate from 500 μl of conditioned media and resuspended to CNBr-activated Sepharose CL-4B in 0.1 M NaHCO3, 0.5 M NaCl, pH 8.3, as described by the manufacturer. After coupling (6 mg of protein/1 g of Sepharose), the Sepharose was washed and diluted in PBS containing 0.02% sodium azide.

**Incubation of mixtures (0.5 ml) containing 0.12 mg of ESP2 Sepharose, PBS, 1% bovine serum albumin, 0.1% Tween 20, 15,000 cpm of 125I-labeled mt-PA tracer, and different amounts of the conditioned media was performed with end-over-end mixing for 18 h at room temperature in polypropylene tubes. Separation of bound and free radiolabeled mt-PA tracer was carried out by centrifugation for 2 min at 3000 g. The Sepharose beads were washed five times with 1.5 ml of 0.9 M NaCl, 0.1% Tween 20, 10 mM EDTA, and bound radioactivity was determined in a γ-counter. Serial dilutions of mt-PA were used as a standard. Approximately 150 ng of mt-PA/0.5 ml were required to reduce the binding of the t-PA tracer by 50%.

**Lysine-Sepharose Binding of t-PA Mutant Proteins—**Lysine Sepharose (7.5 g) was hydrated to 30 ml by incubation in 0.01 M sodium phosphate, 0.1% NaCl, 0.01% Tween 80 (binding buffer), and washed five times with 10 volumes of the same buffer. Then, 0.75 ml of the swollen gel was added to 2.0 ml of the conditioned media, each containing 5.6 pmol rt-PA or rt-PA deletion-mutant protein. Incubation (1 h) was carried out with end-over-end mixing for 18 h at room temperature. Protein-lysine-Sepharose complexes were then washed five times by centrifugation (2 min at 3,000 x g) and resuspended in 1.5 ml of binding buffer. To determine the amount of rt-PA or rt-PA deletion-mutant protein bound to the lysine-Sepharose, aliquots (100 μl) of the protein-lysine-Sepharose complexes were incubated with 15,000 cpm of 125I-labeled ESP2 IgG for 6 h at room temperature with end-over-end mixing in a final volume of 0.5 ml. After five washing steps with binding buffer, the binding of radioactive IgG was determined using a γ-counter.

**EACA Displacement Assay—**Washed protein-lysine-Sepharose complexes (rt-PA, LK1-2, LK2-1, and LK2) were split into 14 0.1-ml fractions which were incubated in 0.5 ml of binding buffer with different concentrations of EACA (0.012-100 mM). Radiolabeled monoclonal antibody ESP2 (15,000 cpm) was added to determine the amount of protein that remains bound to the lysine-Sepharose. Incubations and quantification of the bound ESP2 label were performed, as described above. EACA concentrations below 100 mM do not affect the binding efficiency of the ESP2 monoclonal antibody to the antigen. C∞ values represent the calculated concentrations of EACA required to displace 50% of the bound protein from the lysine-Sepharose.

**Fibrin Binding of t-PA Mutant Proteins—**The fibrin binding properties of different (mutant) t-PA preparations were assayed as described (17). To study fibrin binding in the presence of different concentrations of EACA (0.006-50 mM), clots were formed by adding 2 units of thrombin to a mixture (1 ml) containing 1 mg of fibrinogen, 0.1 pmol of expression product (rt-PA, LF, and LK2), 5 mM EDTA, 0.01% Tween 80 in PBS, and EACA at the concentration indicated. After 1 h at 37°C, the resulting clots and supernatants were separated by centrifugation (30 min at 40,000 x g) in an SW-60 rotor (15°C), and the clots were washed twice with 10 ml of PBS, 0.01% Tween 80. The samples were solubilized by gentle rocking in 1 ml of 0.5% SDS for 2 h at 97°C. An aliquot of each sample was then assayed for the presence of their respective expression product by gelatin-gel analysis.

**Gelatin Gel Electrophoresis—**Gelatin gel electrophoresis was performed as described (18). Samples were run overnight on 10% polyacrylamide gels, containing 0.1% SDS, 0.1% gelatin, and 18 μg/ml plasminogen. After electrophoresis, the gels were incubated for 2 h at room temperature in 2.5% Triton X-100 to remove SDS. Subsequently, the gels were incubated for 2 to 6 h in 0.1 mM glycine NaOH (pH 8.3). Gelatin degradation due to plasmin formation in the gel was visualized by contrast staining with 0.1% Amido Black and staining with 10% acetic acid, 30% methanol.

**RESULTS**

**Localization of Lysine-binding Site(s)—**We previously described both the construction of expression plasmids coding for a series of rt-PA deletion-mutant proteins and a transient expression system for the production of these mutant proteins (9). Fig. 1 shows a schematic representation of the t-PA cDNAs used to express the rt-PA derivatives. To quantify the expression proteins present in the different conditioned media, we have developed an antigen assay based on the use of monoclonal anti-t-PA antibody ESP2. The monoclonal antibody is directed against an epitope on the L-chain, which is present on all the t-PA mutant proteins. All expression product fractions were adjusted with PTS to a final concentration of 2.8 pmol antigen/ml. To localize the lysine-binding site(s) on the t-PA protein, we studied the ability of each rt-PA mutant protein to bind to lysine-Sepharose. The amount of mutant protein used to prepare protein-lysine-Sepharose complexes was below 5% of the maximal t-PA-binding capacity of the lysine-Sepharose. After incubation with the different expression products, the Sepharose fractions were washed and incubated with 125I-labeled ESP2. Antibody binding by the protein-lysine-Sepharose complex represents the amount of expression product bound to the lysine-Sepharose. The results of the binding studies are shown in Table I. While the proteins L, LF, LFE, and LK1 do not bind to lysine-Sepharose, the proteins LK2, LK1-2, LK1-2, and rt-PA are fully able to bind. Based on these results, it can be concluded that the K2 domain of t-PA mediates the affinity of t-PA for lysine-Sepharose, whereas the F domain, the E domain, the K1 domain, and the L-chain are not involved in this interaction.

**Displacement of rt-PAs from Lysine-Sepharose with EACA—**The relative affinities of the lysine analogue EACA for the lysine-binding site(s) of the proteins LK2, LK1-2, and rt-PA are as shown in Table I. The proteins L, LF, LFE, and LK1 do not bind to lysine-Sepharose, whereas the proteins LK2, LK1-2, and rt-PA are fully able to bind. Based on these results, it can be concluded that the K2 domain of t-PA mediates the affinity of t-PA for lysine-Sepharose, whereas the F domain, the E domain, the K1 domain, and the L-chain are not involved in this interaction.
LEK1-2, and rt-PA were determined by EACA-displacement experiments. Protein-lysine-Sepharose complexes were prepared and aliquots were removed from each and incubated with increasing concentrations of EACA. To measure the amount of protein that remained complexed to the lysine-Sepharose, 
\[ ^{125}I \] labeled ESP2 IgG was added. After incubation, the Sepharose fractions were washed and the bound radioactivity was determined. The resulting displacement curves (Fig. 2) and the calculated Cso values, representing the concentrations of EACA necessary to displace 50% of the bound protein from the lysine-Sepharose, are almost identical for each of the binding proteins. A Cso value of about 0.25–0.31 mM was established for all four proteins (Table I). These results support the conclusion that there is a single lysine-binding site in t-PA, localized in the K2 domain of the protein, and suggests that the interactions are specific since they can be inhibited by low concentrations of the lysine derivative.

**Fibrin Binding of rt-PAs in the Presence of EACA**—To study the involvement of the lysine-binding site in the interaction of t-PA with fibrin, we examined the influence of EACA on the fibrin-binding properties of LF, LK2, and rt-PA. Fibrin clots were formed in the presence of LF, LK2, or rt-PA, and different concentrations of EACA. The amount of LF, LK2, or rt-PA bound to fibrin was analyzed with gelatin gels (Fig. 3). As expected, fibrin binding mediated by the K2 domain, represented by LK2, was inhibited by low concentrations of EACA. The F-domain mutant (LF) lacking K2 also bound to fibrin, but this binding was still apparent even at the highest concentration of EACA employed. The fibrin binding of rt-PA was only slightly inhibited by EACA, due to fibrin binding mediated by the F domain.

Although it is difficult to estimate the amount of LK2 bound to fibrin in the presence of a low concentration of EACA, the EACA concentration required to inhibit fibrin binding by the K2 domain (0.2–0.04 mM) is in the same order of magnitude as the EACA concentrations that elute LK2 from lysine-Sepharose.

Taken together, our data indicate that the binding of the K2 domain of t-PA to fibrin is mediated by a single lysine-binding site present on this kringie. The interaction of the F domain with fibrin is not inhibited by EACA and, therefore, is of a different nature than the interaction of kringie 2 with fibrin.

**DISCUSSION**

Digestion of plasminogen with elastase results in the generation of specific degradation fragments. These fragments were used to localize and characterize lysine-binding sites on plasminogen (19). A similar approach was applied to generate specific fragments of the t-PA molecule but was unsuccessful (20). In the current study, we have employed a series of rt-PA deletion-mutant proteins to localize the lysine-binding site of t-PA. Our results clearly show that a lysine-binding site is contained within the kringie-2 domain of the molecule.

The concentration (Cso) at which 50% of the kringie-2-bound rt-PA is eluted from lysine-Sepharose with EACA was estimated to be 0.28 mM. This value is in the same order of magnitude of the EACA concentrations that elute LK2 from lysine-Sepharose with EACA.

<table>
<thead>
<tr>
<th>Expression product</th>
<th>Percentage ESP2 binding to protein-lysine-Sepharose complex</th>
<th>Cso (mM)</th>
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<tr>
<td>Control</td>
<td>1.0</td>
<td>ND²</td>
</tr>
<tr>
<td>LF</td>
<td>1.3</td>
<td>ND</td>
</tr>
<tr>
<td>LF</td>
<td>1.2</td>
<td>ND</td>
</tr>
<tr>
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<tr>
<td>LK1</td>
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</tr>
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<tr>
<td>rt-PA</td>
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*Control, conditioned medium of Ltk- cells that were transfected with pSV2 DNA (9). ND, not determined.

![Fig. 2. Elution of rt-PA, LEK1-2, LK1-2, and LK2 from lysine-Sepharose with EACA. Preformed protein-lysine-Sepharose complexes were incubated with different concentrations of EACA. The monoclonal anti-tPA antibody ESP2, radiolabeled with 
\[ ^{125}I \], was added to quantify the amount of protein that remains bound to lysine-Sepharose, as described under "Materials and Methods."](image)

![Fig. 3. Binding of rt-PA, LF, and LK2 to fibrin in the presence of EACA. The assays were performed as described under "Materials and Methods." Fibrin-binding fractions of rt-PA, LF, and LK2 in the absence (lanes 1 and 7) and in the presence of EACA were run on gelatin-plasminogen gels (lane 2, 5.0 mM; lane 3, 1.0 mM; lane 4, 0.2 mM; lane 5, 0.04 mM, and lane 6, 0.008 mM).](image)
magnitude as the \( C_{o0} \) values determined for plasminogen kringles 1 and 4 (0.9 and 1.0 mM EACA, respectively (21)). Moreover, the involvement of the lysine-binding site of the K2 domain of t-PA in the fibrin-binding property of the molecule suggests that this structure is related to the kringles in plasminogen containing lysine-binding sites (i.e., kringle 1 and 4). Several investigators have studied the structures in plasminogen kringles 1 and 4 that contribute to the lysine-binding sites. It was pointed out that the regions which display extensive amino acid sequence variations mediate the specific binding properties of the kringles (22, 23). Chemical modification studies on plasminogen kringles indicated that Asp\(^{59} \), Arg\(^{21} \) (24), and Trp\(^{25} \) present in the nonconserved regions, are involved in lysine binding. This arrangement of specific residues is also present in kringle 1 of plasminogen except that another aromatic residue appears at position 72. It is interesting to note that kringle 2 of t-PA has identical residues at similar positions, involving Asp\(^{59} \), Arg\(^{21} \), and Trp\(^{25} \), whereas kringle 1 of t-PA only has an Asp residue at position 57.

The class of lysine-binding sites present in plasminogen kringles 1 and 4 and in t-PA kringle 2, binds to lysine analogues that carry a free carboxylate group. It has been pointed out that COOH-terminal lysine residues are the likely ligands known to be present in fibrin (5). When fibrin is formed, little or no COOH-terminal lysine residues are present. It has been reported that, in circulating native fibrinogen, cleavage at the Lys\(^{444} \)-Lys\(^{446} \) bond of the Aa chain can occur (26). The presence of a small portion of fibrinogen with COOH-terminal lysine residues is therefore likely and could explain the LK2 binding in our fibrin-binding assay. However, when fibrin is degraded by plasmin, a vast number of COOH-terminal lysine residues is created (27).

Several investigators (28-31) reported that fibrin or clots, which had been partially degraded by plasmin, bound higher amounts of Glu-plasminogen than nondegraded fibrin. The induced binding sites for Glu-plasminogen were shown to have \( K_r \) values similar to the \( K_u \) value determined for the binding of Glu-plasminogen to lysine-Sepharose (31). Using the COOH-terminal lysine- and arginine-specific exopeptidase carboxypeptidase B, Chrisensens (32) directly showed that COOH-terminal lysine or arginine residues of plasmin-digested fibrinogen fragments are responsible for the interaction with Glu-plasminogen.

Norrman et al. (33) proposed a model explaining the different kinetic parameters of plasminogen activation by t-PA in the presence of intact (first phase, \( k_{cat} = 0.17 \text{s}^{-1}, K_u = 1 \text{mM} \)) and plasmin-degraded fibrin (second phase, \( k_{cat} = 0.13 \text{s}^{-1}, K_u = 0.06 \text{mM} \)). The higher rate of plasminogen activation (up to 13-fold) in the second phase was explained by the increased binding of Glu-plasminogen to plasmin-degraded fibrin.

In our view, it is conceivable that the lysine-binding site present on kringle 2 of t-PA also interacts with COOH-terminal lysine or arginine residues which accumulate upon the plasmin digestion of fibrin. Based on our observations, we propose the following mechanism for t-PA-induced fibrinolysis. In the initial phase of fibrinolysis, fibrin is intact and no COOH-terminal lysines or arginines are present; t-PA then binds specifically to fibrin by its F domain. Glu-plasminogen is bound to fibrin with a weak affinity, possibly by its so-called "AH-site" (5). A relatively weak ternary complex of t-PA, Glu-plasminogen, and fibrin is formed and Glu-plasmin is formed at a low rate. Upon degradation of fibrin by the Glu-plasmin, COOH-terminal lysines are generated, resulting in the increased binding of both Glu-plasminogen and t-PA mediated by the lysine-binding sites present in these proteins. A more stable ternary complex is formed and Glu-plasmin is generated at a high rate leading to increased fibrin degradation. In this model, the F domain is responsible for the initial specific fibrin-binding property of t-PA. Considering the limited structural requirements of the lysine-binding site ligands, the kringle-2 fibrin interaction would then play a role during the progress of plasminogen activation. Our hypothesis thus extends the model of Norrman et al. (35). It suggests that, in addition to increased binding of plasminogen, increased binding of t-PA to degraded fibrin is also responsible for the higher rate of plasminogen activation in the second phase. According to Norrman et al. (33), a possible physiological role of the kinetic transition may be to ascertain that no premature lysis of newly formed fibrin takes place, by limiting the initial rate of plasmin formation (33).

Recently, conflicting results on the structures of t-PA that are responsible for its fibrin-binding property were reported (7, 8, 34). Two groups proposed the F domain to be solely responsible for fibrin binding by t-PA (7, 34). In contrast, Kagitani et al. (8) reported that expression in Escherichia coli of a t-PA cDNA lacking the F domain yielded a t-PA protein which retained a high affinity to fibrin. Our findings that t-PA binds to fibrin via two distinct mechanisms, one of which depends on the amount of fibrin degradation, allows a possible explanation for these contradictory reports.

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REFERENCES
Fibrin Binding by Tissue-type Plasminogen Activator