Functional Properties of the Recombinant Kringle-2 Domain of Tissue Plasminogen Activator Produced in *Escherichia coli*

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The kringle-2 domain (residues 176–262) of tissue-type plasminogen activator (t-PA) was cloned and expressed in *Escherichia coli*. The recombinant peptide, which concentrated in cytoplasmic inclusion bodies, was isolated, solubilized, chemically refolded, and purified by affinity chromatography on lysine-Sepharose to apparent homogeneity. [*35S]Cysteine-methionine-labeled polypeptide was used to study the interactions of kringle-2 with lysine, fibrin, and plasminogen activator inhibitor-1. The kringle-2 domain bound to lysine-Sepharose and to preformed fibrin with a $K_d = 104 \pm 6.2 \mu M$ ($0.86 \pm 0.012$ binding site) and a $K_d = 4.2 \pm 1.05 \mu M$ ($0.80 \pm 0.081$ binding site), respectively. Competition experiments and direct binding studies showed that the kringle-2 domain is required for the formation of the ternary t-PA-plasminogen-intact fibrin complex and that the association between the t-PA kringle-2 domain and fibrin does not require plasmin degradation of fibrin and exposure of new COOH-terminal lysine residues. We also observed that kringle-2 forms a complex with highly purified guanidine-activated plasminogen activator inhibitor-1, dissociable by $0.2 M$ ε-aminoacryolic acid. The kringle-2 polypeptide significantly inhibited tissue plasminogen activator/plasminogen activator inhibitor-1 interaction. The kringle-2 domain bound to plasminogen activator inhibitor-1 in a specific and saturable manner with $K_d = 0.51 \pm 0.055 \mu M$ ($0.35 \pm 0.026$ binding site). Therefore, the t-PA kringle-2 domain is important for the interaction of t-PA not only with fibrin, but also with plasminogen activator inhibitor-1 and thus represents a key structure in the regulation of fibrinolysis.
The binding of kringle-2 to preformed fibrin was examined. The binding is specific and saturable with an apparent Kd of 4.2 ± 1.05 x 10^-4 M.

The interaction of the kringle-2 domain with immobilized lysine was studied quantitatively. Different concentrations of [35S]methionine-cysteine-labeled kringle-2 were incubated with lysine-Sepharose (200 nM immobilized lysine) at 4 °C for 16 h. Inset, Scatchard plot analysis of the same data.

The functional properties of the t-PA kringle-2 domain were derived from preformed fibrin films formed from fibrinogen fraction I-8 (open circles), and fragment X (open squares). Shown are the mean ± S.D. of three separate experiments.

The SDS-PAGE analysis of reduced samples of these fibrinogens, indicating that the t-PA kringle-2 domain is intact, while the other has lost a fragment of molecular mass about 36,000 daltons. This agrees with the accepted pattern of fibrinogen fraction I-8, in which one of the Aα-chains is intact, while the other has lost a fragment of molecular mass 36,000 by limited plasmin digestion (30). Fragment X resulted from cleavage of both Aα-chains, leading to the release of carboxyl-terminal fragments of about 40,000 daltons and leaving the N-terminal fragments of about 25,000-27,000 daltons linked to the Bβ- and γ-chains.

The binding isotherm for the interaction of the kringle-2 domain with immobilized lysine, varying concentrations of [35S]methionine-cysteine-labeled kringle-2 were incubated with lysine-Sepharose (200 nM immobilized lysine) at 4 °C for 16 h. Inset, Scatchard plot analysis of the same data.
However, it bound substantially less to preformed fibrin formed from more extensively plasmin-degraded fibrinogen, fragment X. Thus, the fibrin binding site for kringle-2 is present in intact fibrin and absent in fibrin formed from fragment X.

**Inhibition of t-PA-mediated Plasminogen Activation**—The role of the kringle-2 domain in t-PA mediated plasminogen activation was studied in the presence of different fibrin oligomers. Kringle-2 strongly inhibited when intact fibrin oligomers I-4 were used as accelerators, inhibited less when mildly plasmin-degraded fibrin oligomers I-8 were the accelerators, and did not show any inhibition when more extensively plasmin-degraded X oligomers were used as accelerators (Fig. 7). Kringle-2 had no effect on plasmin activity or the hydrolysis of the synthetic substrate S-2251, since no inhibition could be observed when X oligomers were used as the accelerators.

**Binding to Plasminogen Activator Inhibitor-1**—To study the interaction between kringle-2 and PAI-1, we used three independent experimental approaches. First, \(^{125}\)I-labeled guanidine-activated PAI-1 and \(^{35}\)S\]methionine-cysteine-labeled kringle-2 were mixed and passed over a column of polyclonal t-PA antibody immobilized on protein-A. The portion of PAI-1 which bound to kringle-2 immobilized to the t-PA antibody-Sepharose could be eluted with 0.2 M EACA, whereas kringle-2 was eluted from the column only after application of 6 M guanidine HCl which dissociates the antigen-antibody complex (Fig. 8). In control experiments, PAI-1 did not react with the antibody matrix, since the total amount of PAI-1 applied could be recovered in the flow-through fraction when kringle-2 was omitted (not shown). Secondly, the amidolytic activity of t-PA measured with the synthetic substrate S-2288 is inhibited strongly by PAI-1. In this assay system, both the isolated kringle-2 peptide and EACA interfered with the interaction of the t-PA molecule with autonomous functions, we took a different approach to clarify further the structure-function relationships of t-PA by cloning and expressing individual domains in E. coli.

**FIG. 9**. Kinetics of the inhibition of t-PA by PAI-1. t-PA activity was measured by hydrolysis of S-2288, and the change of absorbance at 405 nm was monitored over time. 16 nM t-PA, 0.2 M t-PA and 300 nM PAI-1, 16 nM t-PA, 300 nM PAI-1 and 300 nM kringle-2, , 16 nM t-PA, 300 nM PAI-1, and 10 mM EACA, .

**DISCUSSION**

Deletion mutagenesis has been used extensively to study the structure-function relationships of t-PA (9-13). However, this approach has several shortcomings; the expression of recombinant mutant proteins in different eukaryotic cell lines probably results in different post-translational modifications such as glycosylation and may lead to different molecules. Since the disulfides in the t-PA variants have not been characterized, it is not clear whether the mutated molecules are properly folded. In addition, the deletion of one or several domains and joining together of domains which are normally separated could conceivably lead to steric hindrance of one domain or another or significant conformational changes of neighboring domains. Based on the hypothesis (9) that separate exons or sets of exons encode "structural domains" in the t-PA molecule with autonomous functions, we took a different approach to clarify further the structure-function relationships of t-PA by cloning and expressing individual domains in E. coli.
The capability of the kringle-2 peptide to bind to lysine and fibrin supports the observations of van Zonneveld et al. (31) that beside the finger domain, the kringle-2 domain also contributes to the fibrin-directed properties of t-PA. They observed that a deletion derivative consisting of the kringle-2 and serine protease domains bound to immobilized lysine and to intact fibrin. These results have been substantiated by other reports (10, 13) showing the involvement of the kringle-2 domain in fibrin binding of t-PA. We extended these experiments by examining the affinities of these interactions. Our calculated dissociation constant for the interaction of kringle-2 with lysine of 104 μM is in good agreement with the 100 μM recently published (32). The significant differences of the dissociation constants of the kringle-2 domain for lysine and preformed fibrin (Kd = 4.2 μM) of more than 20-fold raise doubts about the theory that binding of kringle-2 to fibrin is exclusively mediated through a lysine binding site. Since the kringle-2 domain represents a region of high hydrophobicity within the t-PA molecule, the additional involvement of hydrophobic binding cannot be excluded.

Controversies, however, exist whether plasmin digestion of the fibrin substrate and exposure of new COOH-terminal lysine residues are required for kringle-2 binding as hypothesized by van Zonneveld et al. (9). This view is in contrast to Higgins and Vehar (33) who reported that EACA inhibited the binding of t-PA to intact fibrin, but not to plasmin-degraded fibrin. These authors concluded that the lysine binding site in t-PA is required for the interaction with intact fibrin and not with plasmin-degraded fibrin. Furthermore, Pannell and co-workers (34) demonstrated that plasmin treatment and subsequent carboxypeptidase B treatment of the fibrin substrate did not affect t-PA-mediated clot lysis. It is firmly established that plasminogen binds to COOH-terminal lysine residues upon plasmin digestion (35). Therefore, if t-PA would share the same binding sites, competition would arise between the two molecules. However, this does not appear to be the case since the presence of plasminogen increased the binding of t-PA mutants containing the kringle-2 structure (10).

Our findings that kringle-2 fails to bind significantly to polymerized fragment X and does not inhibit t-PA-mediated plasminogen activation when X oligomers were used as accelerator are supported by the observation of Niewenhuizen et al. (36) that t-PA binds to FCB-2 without fragment X, indicating that the binding site is masked in fragment X and exposed upon CNBr digestion. In fibrinogen I 4 and I 8, exposure of the binding site may be induced by conformational changes upon polymerization. Clearly, our results indicate that plasmin degradation and exposure of new COOH-terminal lysine residues are not a prerequisite of the kringle-2 domain to bind to fibrin. However, since our kringle-2 domain was produced in E. coli, it did not contain a carbohydrate side chain at position Asn-184. Therefore, it cannot be ruled out that glycosylation plays a role in fibrin binding as suggested by Hansen and co-workers (37). This is supported by the finding that a t-PA variant, lacking the carbohydrate side chain at position Asn-184, possessed a 30% to 50% greater specific fibrinolytic activity than t-PA containing this carbohydrate structure (38). Previously, we reported that the affinity of a mutant consisting of the kringle-2 domain and serine protease domain significantly decreased upon limited plasmin digestion of fibrin (39). This supports our results reported on this occasion and argues against the possibility that the lack of carbohydrate side chain attachment alters the interaction of the kringle-2 domain to intact fibrin and to limited plasmin-digested fibrin with respect to the structural requirements for binding. However, the affinities might be different.

It has been suggested that not only is the light chain of t-PA the target of PAI-1, but also that the t-PA heavy chain is involved in the complex formation. This hypothesis was based on the observation that a t-PA deletion mutant consisting of only the finger, EGF, and serine protease domains is less efficiently inhibited by PAI-1 than the parent t-PA molecule (14). Heckman and Loskutoff (40) investigated the kinetics of the t-PA/PAI-1 interactions, and their results indicate that this interaction may involve two binding sites in the t-PA molecule. Recently, de Vries et al. (41) investigated the inhibition pattern of a hybrid protein consisting of the heavy chain of t-PA and the light chain of urokinase and demonstrated that the hybrid protein is inhibited to a greater extent by PAI-1 than urokinase, suggesting the involvement of the heavy chain of t-PA in the interaction with PAI-1. The kinetics of the inhibition of t-PA by PAI-1 in the presence of fibrin showed that the inhibitor interferes with the binding of t-PA to fibrin in a competitive manner (42). It was concluded that one binding site of PAI-1 to the t-PA molecule is a sequence in or close to the fibrin binding domain of t-PA. We now present evidence that the kringle-2 domain is involved in the binding of PAI-1 to t-PA. Based on our results and the aforementioned observations, we propose the following model for the interaction of t-PA with its primary inhibitor. The reaction of t-PA with PAI-1 occurs in a two-phase reaction. The first phase is a reversible one, consisting of a reversible binding of PAI-1 to the kringle-2 domain and also a reversible interaction with the active center of t-PA. The second phase is an irreversible binding of PAI-1 with the light chain of t-PA resulting in the SDS-stable complex. This model is analogous to the plasmin/α2-antiplasmin interaction representing also a two-step reaction with a very fast reversible second order reaction followed by a slower irreversible first order transition (43).

In conclusion, the demonstration that an isolated domain in t-PA possesses biological activities supports the theory proposed by Rogers (44) and van Zonneveld et al. (45) that the t-PA molecule has evolved through exon shuffling creating individual domains with autonomous functions.

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1 FCB-2, cyanogen bromide fragment; FCB-2 of fibrinogen, which consists of fibrinogen chain fragments Aα 148–208, Bβ 191–224, 220–242, 243–305, and γ 95–265, held together by disulfide bonds.
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Expression and purification of the kringle-2 domain - Cell cultures were grown overnight in T175 in 20 ml of RPMI containing 10% FCS and 1% penicillin and streptomycin (GIBCO Laboratories, Grand Island, NY). After the 18 h incubation, the cells were subcultured and concentrated in the form of inclusion bodies. The cells were lysed by sonication and subsequently applied to a lysine-Sepharose column equilibrated with the same buffer. The column was then washed extensively with the above buffer, and the protein was eluted with 0.1 M diethylamine. EXA was removed from the eluted fractions by exhaustive dialysis against 0.1 M ammonium acetate, pH 7.0, 0.02 M Tris-Base.
Figure 1: Schematic representation of the plasmid carrying the Kringle-2 domain (pLI10/K2). The cDNA encoding the Kringle-2 domain is inserted between the Nde I and Bam HI restriction sites. Arrows indicate the approximate positions of the relevant restriction sites. The locations of the Pr promoter (Pr), the tetracycline resistant gene (TetR), the lambda repressor (λR), and the origin of replication are indicated.

Figure 5: Coomassie brilliant blue stained purified proteins analyzed by SDS-PAGE under reducing conditions. Fibrinogen I (lane 1). Fibrinogen II (lane 2), fragment A (lane 3), PAI (lane 4). The staining of marker proteins is indicated in kilodaltons and are of the same composition as described under Figure 2.