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Introduction of Lysine and Clot Binding Properties in the Kringle One Domain of Tissue-type Plasminogen Activator*

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Despite the high overall similarity in primary structure between kringle one (K1) and kringle two (K2) of tissue-type plasminogen activator (t-PA) there exists an enormous functional difference. It is thought that, in contrast to K1, K2 mediates lysine binding and fibrin binding and is involved in stimulation of plasminogen activation by fibrin or derivatives as CNBr fragments of fibrinogen. Hypothesizing that sequence differences are responsible for differences in function, we compared the amino acid sequences of K1 and K2 with a consensus kringle sequence. Six consecutive amino acids unique to K2 of t-PA were found, *i.e.* from Asn^{248} to Trp^{253}. To test whether these residues are involved in lysine binding, fibrin binding, and fibrin-dependent plasminogen activation, we constructed a set of t-PA mutant proteins containing only a kringle and the protease (P) domain: K2P, K1P, and k1P. In the latter molecule the original amino acid residues Ala^{160}–Ser^{165} from K1 were substituted by Asn^{248}–Trp^{253} from K2. As expected, K2P showed enhanced plasminogen activation in the presence of CNBr fragments of fibrinogen, bound to lysine-Sepharose and to a forming fibrin clot. K1P did not show any of these features. In contrast, k1P could be stimulated by CNBr fragments of fibrinogen and bound to lysine-Sepharose and a forming fibrin clot. These results indicate that at least a part of the functional differences between K1 and K2 of t-PA can be localized to a stretch of 6 amino acid residues from Asn^{248} to Trp^{253} present in K2.

Tissue-type plasminogen activator is a fibrin-dependent plasminogen activator (1). In the presence of fibrin, t-PA[1] efficiently converts plasminogen to plasmin, the protease responsible for the degradation of fibrin (2). Based on the primary structure, t-PA is believed to consist of structurally and functionally autonomous building blocks. From the NH_2 terminus t-PA comprises a finger domain, a growth factor domain, two kringle domains (K1 and K2), and a protease domain (P) (3, 4). Domain-deletion mutants of t-PA stressed the importance of the K2 domain in fibrin-dependent features such as the stimulation of plasminogen activation in the presence of CNBr fragments of fibrinogen (5, 6). A lysine binding site in t-PA and the involvement of this site in fibrin binding and CNBr fibrinogen fragment-dependent plasminogen activation were described and localized in the K2P domain (7–11). Amino acid residue substitution studies with t-PA (amino acid residue numbering based on the t-PA sequence according to Ref. 4) demonstrated that the negatively charged cluster in K2 involving the two Asp residues Asp^{236} and Asp^{238} mediates lysine binding. Substitution of either Asp by Asn or Ala impaired binding to lysine (12, 13). Amino acid residue substitution and nuclear magnetic resonance studies on isolated K2 (amino acid residue numbering based on the Kringle sequence according to Ref. 25) confirmed the involvement of these two Asp residues (Asp^{55} and Asp^{57}) in lysine binding (14, 15). It was further shown that Lys^{33} functions as a cationic center, and Trp^{72} mediates the hydrophobic interaction involved in binding of lysine to K2 (16–18). Despite the high overall similarity in primary structure between K2 and K1 (52%) a function of K1 is at present unknown although some authors have claimed that K1 and K2 have the same features and are exchangeable (19–21).

Generally, kringle domains are thought to function as autonomously folding modules specialized in protein-protein interaction (22). Based on homology studies of various kringle domains it was hypothesized that a kringle domain consists of conserved sequences necessary for the three-dimensional structure and variable, specific sequences involved in a specialized function, *i.e.* binding to other proteins (23). Crystallographic studies of three kringles from other kringle-containing proteins confirmed the conserved three-dimensional structure of the kringle domains (24–27). Therefore in theory it should be possible to localize these specific sequences responsible for the fibrin-dependent features of t-PA mediated by K2 and introduce these sequences into another kringle, and thus introduce a new function in this kringle. Here, we report the identification of such a K2-specific sequence Asn^{248} to Trp^{253} in K2. When this K2-specific sequence is put into K1, in the corresponding location, it endows the K1 with the expected fibrin-dependent features.

EXPERIMENTAL PROCEDURES

Primary Structure Comparison of Kringle Domains—The following primary structures were used: tissue-type plasminogen activator kringle 1 and kringle 2 (3, 4, 28), urokinase-type plasminogen activator kringle (29–31), plasminogen kringles 1–5 (32, 33), factor XII kringle (34–36), hepatocyte growth factor kringle 1–4 (37), prothrombin kringle 1 and 2 (38–40).

Amino acid residue numbering for t-PA is according to Ref. 4; amino acid residue numbering in kringles is according to Ref. 25. Sequence comparison was performed using the program PROSIS (Pharmacia LKB Biotechnology Inc.).

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[1] The abbreviations used are: t-PA, tissue-type plasminogen activator; K1, kringle 1; K2, kringle 2; P, protease, rt-PA, recombinant t-PA; KSS, kringle-specific sequence.

Construction of Mutant Proteins—The following mutants were constructed: t-PA/Δ((*S*1-*G*176) referred to as K2P and t-PA/Δ(*S*1-*R*275)ins(t-PA: *R*87-*G*176) referred to as K1P and t-PA/Δ(*S*1-*R*275)ins(K1:*R*89-*G*176(Δ(*A*160-*S*165)-ins(t-PA:*N*248-*W*253)) referred to as k1P (nomenclature of t-PA mutant proteins according to Ref. 41). The previously described expression plasmid pEV$_2$/t-PA (10) was used to construct K1P and K2P. For construction of k1P, pEV$_2$/FGK1P (42) in which primers 32 and 42 were exchanged for primers 106: 5′TACGTCTTTAAGAACCGCAGGCTGACGTGG-GAG and 107: 5′GCAGAACTCCACGTCAGCCTGCGGTTCTT was used. This molecule designated peV$_2$/FGk1P contains Asn^{248}–Trp^{253} of K2 instead of Ala^{160}–Ser^{165} of K1. Mutant DNAs were constructed using the recombinant circle polymerase chain reaction (43).

The mutant DNAs were constructed according to the exon/intron boundary of t-PA. Primers used to construct K2P were 5′GGAAACAGTGACTGCTACTT (TPA18), 5′TCTTACCAAGGA-AACAGTGACTGCTCTACTTT (TPA17), 5′TTGGTAAGATCT-GGCTCCTC (TPA7), 5′TCTGGCTCCTCTTCTGAATC (TPA8). Primers used to construct K1P and k1P were 5′GATACCAGGGCC-ACGTGCTA (TPA16), 5′TCTTACCAAGATACCAGGGCCACG-TGCTAC (TPA15), 5′CTCAGAGCAGGCAGGGGTGC (TPA14), 5′GCAGGTGGACTCAGAGCAGGCAGGGGTGC (TPA13), 5′TCC-ACCTGCGGCCTGAGACA (TPA3), 5′GGCCTGAGACAGTACAG-CCA (TPA4).

Briefly, 2 ng of CsCl-purified expression plasmid peV$_2$/t-PA or peV$_2$/FGk1P were amplified in a total volume of 100 µl containing 1–2 µM primer (Isogen, Amsterdam, The Netherlands), 10 µl of Taq amplification buffer (Amersham Corp.), 5 units of Taq polymerase (Amersham Corp.), 20 µM dNTP (Promega Corp.), and 20 µl of Nujol mineral oil (Perkin-Elmer Cetus). In total 20 amplification cycles were completed on a Perkin-Elmer Cetus thermocycler 480. One cycle consisted of 2 min of denaturation at 94 °C, 1 min reannealing at 55 °C, and 5 min extension at 72 °C. The amplification products were isolated as described (43). Denaturation-renaturation reactions were performed in a total volume of 40 µl containing 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl, and 20 µl of Nujol mineral oil. The protocol consisted of 10-min denaturation/renaturation steps at 100, 90, 80, and 70 °C, respectively, followed by a 1-h incubation at 55 °C and 2 h at room temperature. 10 µl of the denaturation/renaturation mix was used to transform *Escherichia coli* JM109 (Promega Corp.). Recombinants were screened with restriction enzyme digestion. Mutation frequencies varied between 60 and 90%. Nucleotide sequences of the mutant plasmids were checked by plasmid dideoxy sequencing (44) using the T7 sequenase kit (Promega Corp.).

LB6 Cell Transfections—t-PA expression plasmids were used to transfect mouse L cells (LB6) by calcium phosphate co-precipitation with peV$_2$/Neo, which contains the gene for aminoglycoside phosphotransferase 3′ (45). Cells that incorporated the plasmids and thus were neomycin-resistant were selected in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Boehringer Mannheim), L-glutamine (Life Technologies, Inc.), 100 units of penicillin/ml, and 100 µg/ml streptomycin (Life Technologies, Inc.) and 1.2 mg/ml of the neomycin analogue geneticin (Life Technologies, Inc.). For purification of the recombinant proteins, cells were cultured in Dulbecco's modified Eagle's medium supplemented with 100 KIU/ml (Bayer) and 10 mM ε-aminocaproic acid (Merck) to prevent plasmin activity, 0.3 g/l human serum albumin (CLB, Amsterdam, The Netherlands), L-glutamine (Life Technologies, Inc.), 100 units of penicillin/ml, 100 µg/ml streptomycin (Life Technologies, Inc.) and 1.2 mg/ml geneticin (Life Technologies, Inc.). Recombinant t-PA mutants were purified by immunoaffinity chromatography using a monoclonal antibody ESP-2 (Bioscot, Edinburgh, Scotland) against the protease domain of t-PA coupled to agarose. A 0.5-ml aliquot of anti-t-PA-Sepharose suspension was placed on a disposable PD-10 gel filtration column (Pharmacia). The tandem column was equilibrated with 0.1 M Tris-HCl, pH 7.5, 0.01% (v/v) Tween 80. Conditioned medium was loaded onto the column followed by sequential washing with the buffer mentioned above. The column was washed with 2-column volumes of a buffer containing 0.1 M Tris-HCl, pH 7.5, 1.0 M NaCl, and 0.01% (v/v) Tween 80. Subsequently the column was reequilibrated with the same buffer without NaCl. The t-PA mutant was eluted from the column with a buffer containing 0.1 M Tris-HCl, pH 7.5, 0.01% (v/v) Tween 80, and 3.0 M KSCN. Column fractions were tested for plasminogen activation activity; those fractions containing activity were pooled for further characterization.

Gel Electrophoresis—Polyacrylamide gel electrophoresis in the presence of SDS was performed under nonreducing conditions on 10% acrylamide gels with 5% stacking gels using the Laemmli system (46). After electrophoresis, gels were washed in 2.5% (v/v) Triton X-100 to remove SDS and placed on plasminogen-containing fibrin-Agarose layers (47). Upon incubation plasminogen activators appear as clear lysis zones on an opaque background.

Labeling of t-PA Analogues—For labeling of rt-PA or the t-PA analogues an active site-directed inhibitor of t-PA was used. The inhibitor 4-aminobenzoyl-Gly-Arg-CH_2Cl (a kind gift of Dr. E. Shaw) was iodinated with ^{125}I and purified as described (48). rt-PA and t-PA analogues in 0.1 M Tris-HCl, pH 7.5, and 0.1% v/v Tween 80 were incubated with 2 µM iodinated inhibitor for 4 h at room temperature. Radiolabeled t-PA and t-PA analogues were purified on a 1-ml column of zinc chelate-Sepharose and extensively washed with 0.02 M Tris-HCl, pH 7.4, 1 M NaCl, and 0.01% v/v Tween 80, and eluted with the same buffer containing 100 mM imidazole (Merck). Specific activity of the labeled t-PA or t-PA analogues was approximately 26,000 cpm/pmol.

Determination of the Relative Apparent K_d of the Lysine Binding Site—The affinity of the t-PA mutants for lysine-Sepharose was determined as described before (10). Radiolabeled rt-PA or t-PA analogues (final concentration, 0.4 nM) were incubated in a volume of 200 µl of 0.1 M Tris-HCl, pH 7.5, 0.01% (v/v) Tween 80, 100 mM NaCl with different amounts of lysine-Sepharose/Sepharose mixtures. After 3 h at 4 °C, the Sepharose was centrifuged and the amount of radioactivity in the supernatant was determined with a γ-counter. t-PA bound was expressed as the fraction of the total amount of t-PA added to the lysine-Sepharose. The data were fitted to $((\text{t-PA})_{bound}/(\text{t-PA})_{total})^{-1}$ = (apparent K_d/volume of lysine-Sepharose present) + 1. The slope and the standard deviation of the slope were determined using the linear least square regression analysis algorithm of Lotus 3.1 (Lotus Developmental Cooperation, Cambridge, United Kingdom). The apparent K_d is expressed as microliters. The relative apparent K_d = apparent K_d of the t-PA analogue/apparent K_d of rt-PA.

Binding to a Forming Fibrin Clot—Fibrin binding was performed as described before (10). Radiolabeled t-PA analogues (final concentration, 0.1 nM) were mixed with plasminogen-free fibrinogen (Kabi Diagnostica) in the presence of 500 KIE/ml Trasylol (Bayer). Clotting was performed with 2 NIH units/ml thrombin (Leo, Ballerup, Denmark). After 1 h of incubation by 37 °C, clots were centrifuged, and radioactivity in the supernatant was determined with a γ-counter. t-PA bound was expressed as the fraction of the total amount of t-PA analogue added to the fibrinogen solution.

Conversion of Single-chain t-PA to Two-chain t-PA—Conversion of the single-chain form of t-PA analogues to the two-chain form was performed as described previously (49). In short, 10 µl of plasmin-Sepharose slurry (1.95 mg of plasmin/2.2 g of Sepharose (wet weight)) was washed twice with 10 mM Tris-HCl, pH 7.5, and 0.01% (v/v) Tween 80. The buffer was removed and replaced with an aliquot of t-PA analogue in the same buffer. The reaction was carried out with constant mixing at 37 °C. Conversion of t-PA analogues from the single-chain to the two-chain form was confirmed by spectrophotometric activity determination with S-2366 (L-pyro-Glu-L-Pro-L-Arg-*p*-nitroanilide hydrochloride) and S-2288 (*H*-D-Ile-L-Pro-L-Arg-*p*-nitroanilide dihydrochloride) (KabiVitrum).

Determination of the t-PA Analogue Concentration—Spectrophotometric assays were performed as previously described (50). Briefly, the reaction mixture (250 µl of total volume) contained plasmin-treated t-PA analogues, 100 mM Tris-HCl, pH 7.4, 0.1% (v/v) Tween 80, and 1.0 mM S-2288 (*H*-D-Ile-L-Pro-L-Arg-*p*-nitroanilide) (KabiVitrum). The absorbance of the reaction mixtures was measured at 405 nm in an eight-channel microtiter plate reader against suitable blanks without termination of the reaction. The t-PA analogue sample was tested at four different dilutions. The absorbance at 405 nm was plotted against time ($\Delta A/\Delta t$) for each dilution. These slopes were plotted against four different dilutions of t-PA analogues, representing the absorbance change/time/volume of t-PA analogue added ($(\Delta A/\Delta t)/\Delta V$.

The change in absorbance at 405 nm/h for a known amount of t-PA standard ($(\Delta A/\Delta t)$/pmol = 6.87×10^{-4} h^{-1} pmol^{-1}) was compared with the $(\Delta A/\Delta t)/\Delta V$ of the t-PA analogues. Assuming that the amidolytic activity for the P domain of the t-PA standard is similar to the amidolytic activity of the P domains of the t-PA analogues, the concentration of t-PA was calculated.

Determination of the Stimulation Factor—Spectrophotometric assays were performed as previously described (51). Briefly, the reaction mixture (250 µl of total volume) contained various amounts of plasmin-treated t-PA analogues, 100 mM Tris-HCl, pH 7.4, 0.1% (v/v) Tween 80, 0.12 µM Glu-plasminogen, and 0.7 mM S-2251 (*H*-D-Val-

L-Leu-L-Lys-p-nitroanilide) (KabiVitrum). In eertain cases, 120 μg/ml CNBr-digested fibrinogen were included. The absorbance of the reaction mixtures was measured at 405 nm in an eight-channel microtiter plate reader against suitable blanks without termination of the reaction. The t-PA analogue sample was tested at 10, 20, and 40 pM (final concentration) of active enzyme both for reaction mixtures containing fibrinogen fragments and for reaction mixtures without fragments. Fibrinogen fragments were prepared as described (51). The enhancement factors were determined as follows. The change in absorbance was monitored over time for each t-PA analogue in the presence and absence of CNBr digest of fibrinogen. For each enzyme concentration a slope was calculated, representing the absorbance change over time squared ($\Delta A/\Delta t^2$). These slopes, in turn, were plotted against enzyme concentration, representing the absorbance change/time squared/molar concentration of enzyme ($(\Delta A/\Delta t^2)/\Delta M$). The ratio of the slope in the presence of fibrinogen fragments to the slope in the absence of fragments is the enhancement factor. This ratio reflects the extent to which fibrinogen fragments enhance the activity of the particular t-PA analogue preparation.

RESULTS

Determination of a Kringle Primary Structure Consensus—Previous experiments by us and others have demonstrated the involvement of the K2 domain of t-PA in fibrin-dependent plasminogen activation, lysine binding, and fibrin binding. The K1 domain has none of these features (5, 6). Comparison of 15 different kringles with alignment of the cysteine residues shows highly conserved regions probably responsible for the three-dimensional structure of the kringles (Fig. 1, *lower part*). Two regions, Trp[25]–Cys[52] designated kringle-specific sequence (KSS) 1 and Cys[64]–Cys[77] designated kringle-specific sequence 2 with less similarity, were found. As suggested before (23, 24) these are the sequences in the kringle domain, which endow the kringles with a certain function. Next we compared KSS1 and KSS2 within K1 and K2 of t-PA in more detail (Fig. 1, *upper part*). A K2-specific stretch of 6 amino acid residues Asn[248]–Trp[253] within region KSS2 was found. We hypothesized that this stretch of 6 amino acid residues is responsible for the fibrin-dependent features of K2.

Construction and Characterization of the Variant Proteins—To test whether the 6 amino acids are responsible for the functional differences between K2 and K1, we decided to substitute Ala[160]–Ser[165] of K1 for Asn[248]–Trp[253] from K2. To exclude the interference of other domains of the t-PA molecule, we constructed K2P, K1P, and k1P. Instead of the normal K1 molecule, the latter contains a modified K1-designated k1 in which Ala[160]–Ser[165] of K1 is replaced by Asn[248]–Trp[253] of K2. DNAs were constructed and expressed in LB6 cells. Zymography showed the integrity of the affinity-purified proteins (Fig. 2). K1P and k1P had a lower electrophoretic mobility than K2P. All of the mutants were secreted as single-chain molecules that could be transformed to two-chain molecules by limited plasmin digestion (results not shown).

Interaction with Lysine-Sepharose—More than 90% of the K2P molecules bound to lysine-Sepharose (Table I, column 2). K1P showed no affinity for lysine-Sepharose. In contrast, k1P, which only differs from K1P by six amino acids, showed

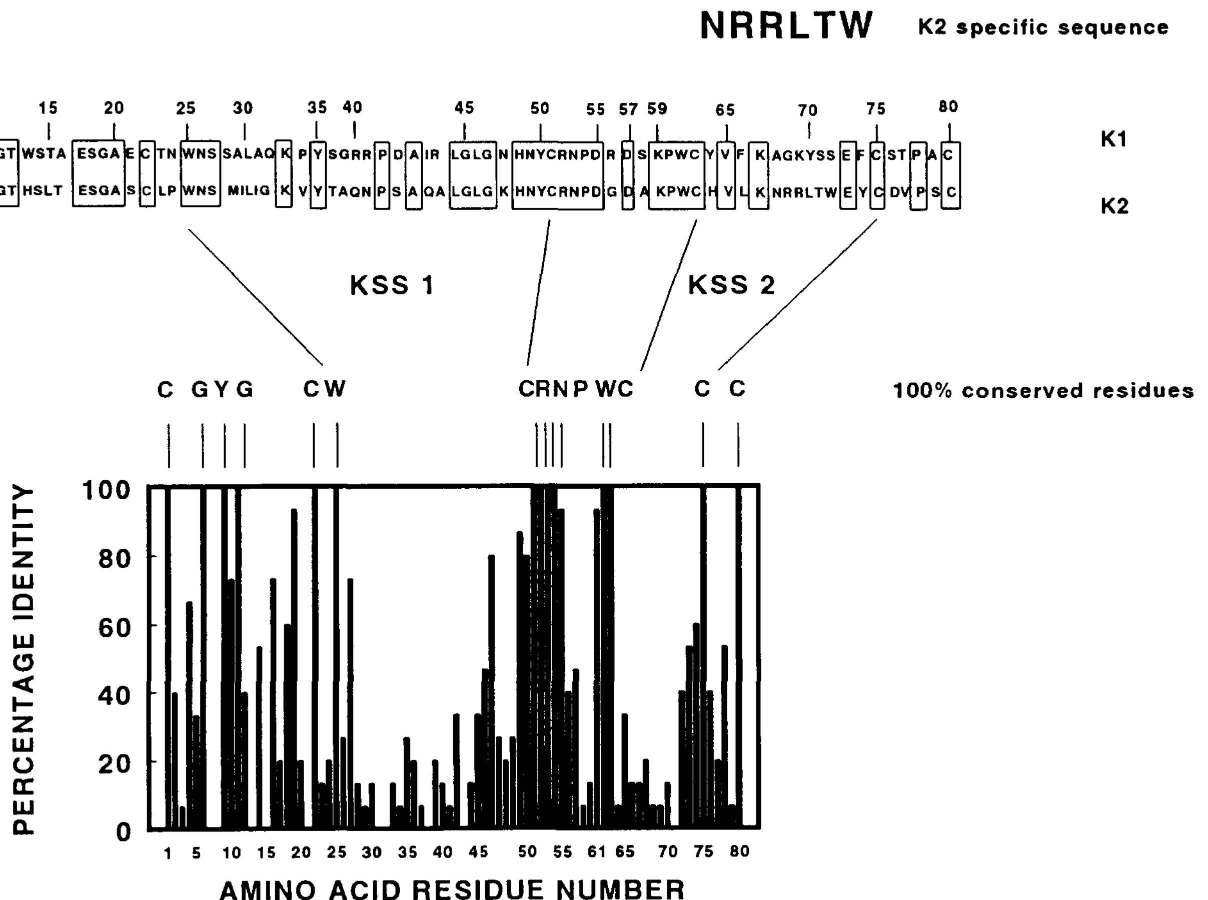

FIG. 1. **Determination of a kringle consensus sequence.** The primary structures of 15 kringles (see "Experimental Procedures") were aligned on cysteine residues. Amino acid deletions or insertions were considered as residues not identical with any of the amino acid residues. The result is plotted as the percentage identity on the *X*-axis *versus* the residue number (according to Ref. 25) on the *x*-axis. Two kringle-specific sequences (*KSS*) were found designated KSS1 and KSS2, respectively. Within KSS2, a unique amino acid sequence for K2 not present in K1 of t-PA was found.

clear affinity for lysine-Sepharose. However binding of k1P to the lysine-Sepharose column (30 ± 15%) did not reach the level of K2P, indicating that possibly a low affinity binding site was created or that only part of the k1P molecules had a lysine binding site. Furthermore, the fraction of k1P that binds to lysine-Sepharose varied for different batches of k1P. When the nonbinding fraction of k1P was applied for the second time to a lysine-Sepharose column no binding was observed. The lysine binding fraction when applied for a second time to a lysine-Sepharose column reached K2P levels of binding. This result suggests that there are two populations of k1P. Determination of the relative apparent K_d for lysine binding showed that the affinity of k1P for lysine-Sepharose is about a factor of 10 lower than that of K2P (Table I, column 3), indicating that for creation of a K2P-like lysine binding site more amino acid residue changes in K1 are required.

FIG. 2. **Fibrin zymography of t-PA analogues.** K2P, K1P, and k1P were isolated from culture media by affinity chromatography. Gel electrophoresis and zymography were performed as described under "Experimental Procedures." High molecular weight standards were run in a separate lane (not shown).

The Fibrin Binding Properties—K2P binds to a forming fibrin clot (Fig. 3). In the presence of the lysine analogue ε-aminocaproic acid, binding was disturbed suggesting that a lysine binding site is involved, supporting previous results (9–11). K1P did not have fibrin binding properties. The k1P fraction that bound to lysine-Sepharose also bound to a forming fibrin clot like K2P. ε-aminocaproic acid blocked the binding suggesting that binding occurs via the newly introduced lysine binding site in k1P. The fraction of k1P that did not bind to lysine-Sepharose did not bind to a forming fibrin clot either.

Enhancement of t-PA-mediated Plasmin Generation by CNBr Fragments of Fibrinogen—The enhancement factors for plasminogen activation in the presence of CNBr-digested fibrinogen catalyzed by K2P, K1P, and k1P were determined (Table I, columns 4–6). A stimulation factor of 30 was observed for K2P, while no stimulation was found for K1P. The fraction of k1P that bound to lysine-Sepharose showed a stimulation factor of 14. The nonlysine binding fraction of k1P was stimulated only 3-fold. The basal plasminogen activator activities of K1P, K2P, and the lysine-bound population of k1P were similar, whereas that of the nonlysine bound fraction of k1P was about a factor of 2 lower.

DISCUSSION

We set out to localize the amino acid residues in K2 of t-PA, which are involved in fibrin-dependent plasminogen activation. Based on the high similarity between K1 and K2 we reasoned that it should be possible to locate these amino acid residues by substituting individual amino acid residues or stretches of amino acids in K1. To exclude the interference of other domains we first constructed K1P and K2P. As expected K1P did not exhibit the fibrin-dependent features that are characteristic of K2P. Next we set up a strategy to localize the amino acids involved in fibrin-dependent plasminogen activation. We first compared the sequences of 15 kringles. Based on identity, we could distinguish two types of sequences in the kringle, conserved and kringle-specific. Two kringle-specific sequences were found, designated KSS1 and

TABLE I
Characteristics of rt-PA and t-PA analogues

rt-PA or t-PA analogue	Fraction bound to lysine-Sepharose[a]	Relative apparent K_d[b]	Plasminogen activator with no CNBr fragments of fibrinogen[c]	Plasminogen activator with CNBr fragments of fibrinogen[c]	Stimulation factor[d]
			activity/pmol protein $\Delta A\ h^{-2}\ pmol^{-1}$	*activity/pmol protein* $\Delta A\ h^{-2}\ pmol^{-1}$	
rt-PA	0.88 ± 0.11	1	0.079 ± 0.009	10.8 ± 0.6	135 ± 17
K2P	0.98 ± 0.11	1.3 ± 0.2	0.12 ± 0.02	4.0 ± 0.6	34 ± 8
K1P	0.2 ± 0.2	—	0.087 ± 0.009	0.055 ± 0.005	0.63 ± 0.09
K1P lysine-bound fraction	0.93 ± 0.08	11 ± 2	0.068 ± 0.006	0.9 ± 0.1	14 ± 2
K1P non-lysine-bound fraction	0.2 ± 0.2	—	0.036 ± 0.005	0.09 ± 0.01	2.7 ± 0.5

[a] Radiolabeled rt-PA or t-PA analogues were applied to 1-ml columns of lysine-Sepharose, and flow-through was collected. The columns were washed with 2.5 ml of buffer containing 50 mM of the lysine analogue ε-aminocaproic acid. The radioactivity present in the flow-through, the wash fraction, the elution fraction, and on the columns was determined. The radioactivity present in the elution fraction is expressed as percentage of the total radioactivity and was determined for three different t-PA analogue batches (for details, see "Experimental Procedures").

[b] Radiolabeled rt-PA and t-PA analogues were incubated in a volume of 200 μl of buffer with different amounts of lysine-Sepharose/Sepharose mixtures. rt-PA or t-PA bound analogue was expressed as a fraction of total rt-PA or t-PA analogue added. The apparent K_d was determined as described under "Experimental Procedures." The relative apparent K_d (apparent K_d of the t-PA analogue divided by the apparent K_d of rt-PA) was determined for three different batches of t-PA analogue. —, no binding observed (for details see "Experimental Procedures").

[c] The amount of two-chain t-PA analogue was determined using the substrate *H*-D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide-dihydrochloride (S2288) as described under "Experimental Procedures." Plasminogen activator activity of rt-PA and the t-PA analogues in the presence and absence of CNBr fragments of fibrinogen were determined as described under "Experimental Procedures" and expressed as h^{-2} $pmol^{-1}$. Three different t-PA analogue batches were tested (for details see "Experimental Procedures").

[d] Stimulation factors were calculated as follows: the $(\Delta A/\Delta t^2)$/pmol in the presence of CNBr fragments of fibrinogen divided by $(\Delta A/\Delta t^2)$/pmol in the absence of CNBr fragments of fibrinogen. Three different batches of t-PA analogue were tested (for details see "Experimental Procedures").

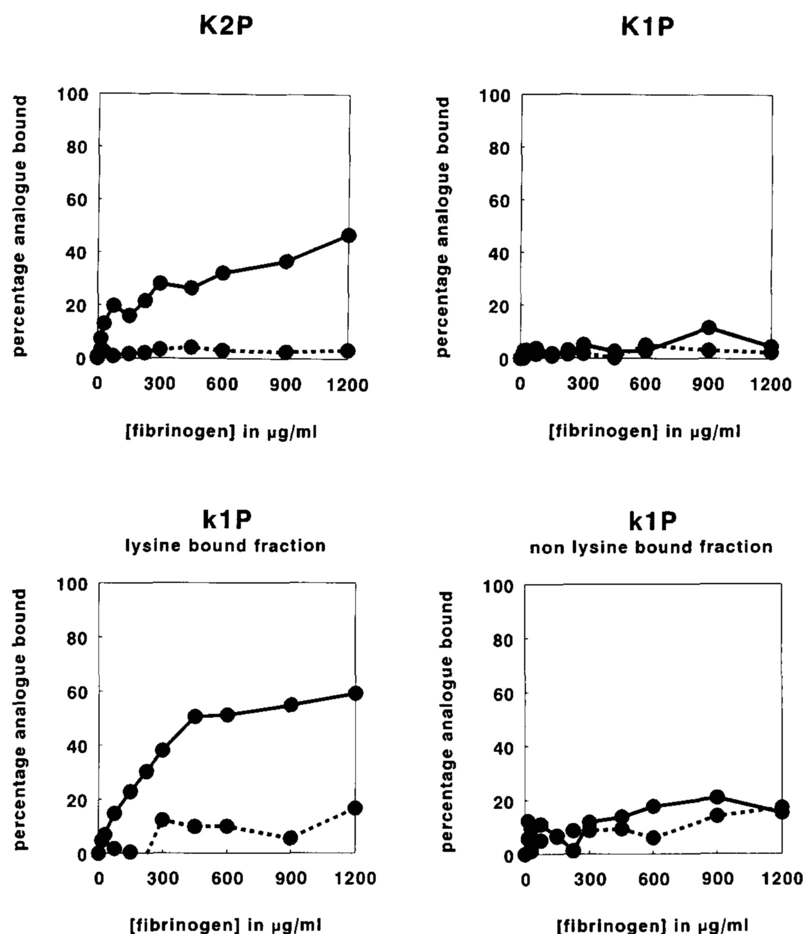

FIG. 3. **Binding of t-PA and t-PA analogues to a forming fibrin clot in the presence and absence of the lysine analogue ε-aminocaproic acid.** Radiolabeled t-PA analogues K2P, K1P, k1P lysine binding fraction, k1P nonlysine binding fraction were mixed with various amounts of fibrinogen and clotted with thrombin in the presence (- - -) or absence (——) of ε-aminocaproic acid. Clots were centrifuged, and the amount of label in the supernatant was determined using a γ-counter. Bound t-PA was then calculated on the basis of control experiments without fibrinogen or thrombin present. The fraction of bound t-PA is plotted on the y axis, and the concentration of fibrinogen is plotted on the x axis expressed as μg/ml (for details see "Experimental Procedures").

KSS2. When the primary structures of K1 and K2 of t-PA within KSS1 and KSS2 were compared, a unique stretch of six amino acid residues, Asn^{248}–Trp^{253}, in K2 was found. Introduction of Asn^{248}–Trp^{253} in K1P conferred some of the K2-specific fibrin-dependent features to K1. We further noticed that introduction of Asn^{248}–Trp^{253} resulted in two populations of k1P. The population of k1P that bound to lysine-Sepharose differed per batch. Although the plasmin inhibitor Trasylol was present during the whole culturing period it cannot be excluded that some protease activity was present and modified k1P and possibly to a lesser extent K2P. k1P that bound to lysine also bound to a forming fibrin clot and was stimulated by CNBr fragments of fibrinogen, suggesting that the six newly introduced amino acid residues are involved in these three properties.

As inferred from amino acid substitution, the amino acids involved in lysine binding are Asp^{236}, Asp^{238}, Lys^{147}, and Trp^{253} (12, 13, 16). Of these Trp^{253} in K2 is the only amino acid not present in K1. At present we do not know if Trp^{253} in the stretch Asn^{248}–Trp^{253} is sufficient or whether more residues are required for introduction of the fibrin-dependent features in K1. A reported attempt to introduce the high affinity lysine binding site of plasminogen K4 in K1 of prothrombin by substituting Asp^{57}, Gln^{34}, Trp^{72} for Ser^{57}, Glu^{34}, Arg^{72} was not successful, indicating that for prothrombin K1 more amino acid substitutions are needed for an operational lysine binding site (52). Our results strongly support the view that kringles, which can be seen as miniproteins mediating protein-protein interaction (23), consist of constant regions and kringle-specific sequences. The conserved sequences are responsible for the three-dimensional structure, whereas the kringle-specific sequences endow the kringle with a certain function. KSS2 within K2 of t-PA seems to be autonomous and transferable, at least within the t-PA molecule. Introduction of KSS2 into other kringles, for instance the kringle of urokinase-type plasminogen activator, could extend this view.

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