Identification of the Domains of Tissue-type Plasminogen Activator Involved in the Augmented Binding to Fibrin after Limited Digestion with Plasmin*

Carlie de Vries, Harry Veerman, and Hans Pannekoek‡
From the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Department of Molecular Biology, 1006 AK Amsterdam, The Netherlands

(Received for publication, January 9, 1989)

The binding of recombinant tissue-type plasminogen activator (rt-PA) to fibrin increases upon digestion of fibrin with plasmin. Optimal binding is observed following a limited plasmin digestion of fibrin, coinciding with the generation of fibrin fragment X polymers. We studied the involvement of the separate domains of the amino-terminal “heavy” (H) chain of rt-PA in this augmentation of fibrin binding. The fibrin-binding characteristics of a set of rt-PA deletion mutants, lacking either one or more of the structural domains of the H chain, were determined on intact fibrin matrices and on fibrin matrices that were subjected to limited digestion with plasmin. The augmented fibrin binding of rt-PA is independent of the presence of carboxy-terminal lysine residues in the fibrin matrix. Evidence is provided that this increase of fibrin binding is mediated by the kringle 2 (K2) domain that contains a lysine-binding site. Further increase of the fibrin binding of rt-PA is independent of the presence of carboxy-terminal lysines. It is shown that the latter increase is not mediated by the K2 domain. Based on our data, we propose that the increase in fibrin binding, unrelated to the presence of carboxy-terminal lysine residues, is mediated by the finger (F) domain, provided that this domain is correctly exposed in the remainder of the protein.

The fibrinolytic process ultimately leads to complete solubilization of the fibrin network of a thrombus (1). During this process, the serine protease plasmin gradually cleaves fibrin into distinct degradation products. Besides being a substrate for plasmin, fibrin also acts as an important cofactor in fibrinolysis. Both plasminogen, the inactive form of plasmin, and tissue-type plasminogen activator (t-PA)† can bind to fibrin. This binding is postulated to induce the formation of a so-called ternary complex that facilitates plasmin formation, thereby accelerating the degradation of fibrin (2, 3). The fibrinolytic process may be considered a dynamic process in which the cofactor function of fibrin could alter during ongoing degradation. This concept is supported by several observations. It has been shown for plasminogen that its binding to fibrin increases after limited degradation of fibrin by plasmin (4–7). Binding to intact fibrin is mediated by a so-called aminohexyl-binding site that exhibits affinity for internal lysine residues in fibrin (8, 9). Tran-Thanh et al. (7) have shown that the increased binding is due to the exposure of a new type of high affinity binding sites in fibrin. The lysine-binding sites in plasminogen are required for this interaction. Previously, we have demonstrated that the amino-terminal “heavy” (H) chain of t-PA consists of autonomous, structural, and functional domains encoded by an exon or two adjacent exons (10). Such domains can be deleted or transposed to other molecules without grossly affecting the functional properties of the remainder of such proteins (11, 12). This approach allowed us to conclude that the fibrin-binding property of t-PA and the acceleration of its activity is mediated by the “finger” (F) and the kringle 2 (K2) domains. The nature of the interaction of the F domain with fibrin is unknown, whereas the K2 domain contains a lysine-binding site (13). Recently, a lysine-binding site has also been located in the K1 domain of t-PA that may be involved in fibrin binding (14).

Based on the data, we suggested a model for the role of different t-PA domains during the process of fibrinolysis (13). Initially, binding of t-PA to intact fibrin would be mediated by the F domain. Subsequently, upon cleavage of fibrin by plasmin, carboxy-terminal lysine residues are generated. Increased binding of t-PA to fibrin would then be achieved by the lysine-binding site present in the K2 domain. In addition, it has been shown that the K2 domain also exhibits affinity for internal lysine residues, conceivably mediated by an aminohexyl-binding site (15). Consequently, the K2 domain may play a role in the initial binding to intact fibrin as well. In contrast, Higgins and Vehar (16) have shown, explicitly using plasmin-degraded fibrinogen, that increased binding of t-PA to fibrin does not involve a lysine-binding site.

To examine the fibrin binding of t-PA during ongoing fibrin degradation in further detail, we employed a fibrin binding system that has been previously described for plasminogen (7). In this assay, a matrix of intact fibrin is pre-treated with plasmin to generate discrete intermediate stages of fibrin degradation. The involvement of different t-PA domains in fibrin binding has been studied using t-PA deletion mutants lacking one or more of these domains. Furthermore, we ana-

* This study was supported by Netherlands Organization for Scientific Research (MEDIGON) Grant 900-526-070 and by a grant from Eli Lilly (Indianapolis, IN). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: § Publication Secretariat, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, P. O. Box 9406, 1006 AK Amsterdam, The Netherlands: Tel. 0-20-5123125; Fax: 0-20-5123332.

‡ The abbreviations used are: t-PA, tissue-type plasminogen activator; rt-PA, recombinant tissue-type plasminogen activator; BSA, bovine serum albumin; PBS, phosphate-buffered saline; H chain, amino-terminal “heavy” chain; L chain, carboxyl-terminal “light” chain; F, finger domain; E, epidermal growth factor-like domain; K1, kringle 1 domain; K2, kringle 2 domain; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; bp, base pairs.
analyzed the effect of the exopeptidase carboxypeptidase B in this system. Carboxypeptidase B specifically removes carboxy-terminal lysine and arginine residues from polypeptides. The application of carboxypeptidase B allows us to discriminate between a potential involvement of aminohexyl- and lysine-binding sites in fibrin binding. Consequently, this approach is an extension of our previous studies, employing the lysine-analogue e-aminocaproic acid as a competitor for fibrin binding of t-PA (13).

Here, we report that the increased binding of t-PA, formed on limited digestion of fibrin by plasmin, can be attributed in part to the lysine-binding site within F. Furthermore, we propose that the remainder of the increased binding, not dependent on carboxy-terminal lysines, is mediated by the F domain, provided this domain is correctly presented to digested fibrin.

**EXPERIMENTAL PROCEDURES**

Reagents—Iscove's modified minimal medium, minimal essential medium without leucine, Ultrasor G, fungizone, and fetal calf serum were purchased from Gibco (Paisley, Scotland). Trasylol (10,000 kallikrein inhibiting units (KIU)/ml) was obtained from Bayer (Erkelenz, Federal Republic of Germany). Synthetic oligonucleotides were made on an automated DNA synthesizer (Applied Biosystems, model 381A). Plasmid pAGO, employed for co-transfection, contains the sequence of the authentic thrombocyten-kinase gene of herpes simplex virus type 1 (16). Two matrix oligonucleotides were supplied by Sigma, and radioactive materials were obtained from the Radiochemical Centre (Amersham, United Kingdom).

**Proteins and Enzymes**—Restriction endonucleases and DNA modifying enzymes were obtained from Bethesda Research Laboratories. Purified pancreatic carboxypeptidase B (EC 3.4.17.2) was treated with diisopropylfluorophosphate by the supplier (Boehringer Mannheim, Federal Republic of Germany). Human thrombin was obtained from Sigma, whereas human fibrinogen (Grade L) was purchased from KabI Vitrum (Stockholm, Sweden). Single-chain human melanoma t-PA was from Biozool (Umea, Sweden). Fibrinogen (25 mg/ml) was treated with 1 mM diisopropylfluorophosphate at room temperature for 18 h to inactivate potential contaminating serine proteases, and subsequently dialyzed at room temperature against 50 mM Tris-HCl (pH 7.4), 0.15 M NaCl. Aliquots were frozen at -70 °C. Plasminogen was purified from fresh plasma by affinity chromatography on lysine-Sepharose, followed by gel filtration using Sephacyr S-300 superfine (Pharmacia, Upsalla, Sweden), as described before (18). Plasminogen was activated by high molecular weight urokinase (kindly provided by Dr. G. Cassani, Lepetit, Milano, Italy) that was coupled to CNBr-activated Sepharose, yielding 1.3 mg of ESP2/ml of Sepharose. This mononcoidal antibody is directed against an antigenic determinant on the t-PA L chain present on each of the deletion mutants (24). The conditioned media (70–140 ml) of different rt-PA preparations were loaded on separate columns (0.8 × 12 cm) of ESP2-Sepharose (Pharmacia). Each column was washed with 75 ml of PBS containing 0.01% (v/v) Tween 80, 50 ml of PBS with a final NaCl concentration of 1 M, 0.01% (v/v) Tween 80, and 10 ml of PBS containing Tween 80 and 0.4 M KSCN. The proteins were eluted with a buffer containing PBS, 0.01% (v/v) Tween 80, and 3 M KSCN, and fractions of 500 μl were collected. The fractions with rt-PA activity were pooled and extensively dialyzed against 50 mM sodium phosphate (pH 7.5), 0.01% (v/v) Tween 80 and subsequently stored at -70 °C until use. The reduced samples of rt-PA (variant) proteins were examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (25) followed by fluorography. The rt-PA (mutant) preparations were shown to be homogeneous in the single-chain form (Fig. 2).

**Determination of the Concentration of the rt-PA (Deletion) Proteins**—For t-PA antigen determinations, we applied two monoclonal antibodies, directed against two different epitopes on the t-PA L chain, as described before (24). Two monoclonal antibodies were directed against an antigenic determinant on the t-PA L chain present on each of the deletion mutants (24). These two antibodies were ESP2 (IgG) and MPWVPAl (IgG), kindly provided by Dr. B. R. Binder, Vienna, Austria, respectively. MPWVPAl was coupled to Sepharose and incubated in PBS, 50 ml of PBS containing 0.1% (v/v) Tween 80 with 100 μl of antibodies, or 50 ml of PBS containing 0.1% (v/v) Tween 80 with 80 μl of antibodies. This mixture was incubated at room temperature for 1 h. The Sepharose beads were washed five times with 1.5 ml of 0.15 M NaCl, 0.1% Tween 80, and resuspended in 0.01% (v/v) Tween 80 with 80 μl of antibodies. The concentration of antibodies bound to protein was determined according to the method described by Van Zonneveld et al. (24). Two monoclonal antibodies, directed against two different epitopes on the t-PA L chain, were used to determine the concentration. The concentration of antibodies bound to protein was determined according to the method described by Van Zonneveld et al. (24). These two antibodies were ESP2 (IgG) and MPWVPAl (IgM, kindly provided by Dr. B. R. Binder, Vienna, Austria), respectively. MPWVPAl was coupled to Sepharose and incubated in PBS, 50 ml of PBS containing 0.1% (v/v) Tween 80 and 100 μl of antibodies. The concentration of antibodies bound to protein was determined using antibodies directed against two different epitopes on the t-PA L chain.
in 24-well tissue culture plates was done essentially according to Tran-Thang et al. (7). To prevent nonspecific binding, the wells were coated overnight at 4°C with 20 mg/ml BSA in a 50 mM sodium carbonate buffer (pH 9.4). The “BSA coating” buffer was removed, and the wells were washed two times with PBS, 0.01% (v/v) Tween 80, 1 mg/ml BSA (buffer A). In each well, 300 μl of fibrinogen (1 mg/ml) was added to 25 μl of human thrombin (12 units/ml). A fibrin matrix was formed that was air-dried for 16 h at 37°C. It should be noted that fibrinogen preparations may contain some heterogeneity in the α-chains due to limited plasmin degradation either in plasma and/or during purification (26, 27). To eliminate potential pre-existing carboxyl-terminal lysines, in all experiments the fibrin matrices were pre-treated with 300 μl of 1 unit/ml carboxypeptidase B in buffer A for 1 h at 37°C. Fibrin-treated fibrinogen matrices in wells were prepared by adding 200 μl of buffer A, containing 6.8 mM plasmin, for 30 min at 37°C (see next paragraph). The plasmin digestion was arrested by removing the supernatant and, subsequently, the matrices were incubated for 30 min at 37°C with 300 μl of buffer A containing 0.2 M ε-aminocaproic acid and 25 KIU/ml Trasylol to elute and inactivate remaining plasmin from fibrin. The plates were then washed five times with 1.5 ml of buffer A. Fibrin matrices that were not degraded with plasmin were treated in an identical manner. Carboxypeptidase B treatments were done for 1 h at 37°C in 300 μl of buffer A containing carboxypeptidase B (1 unit/ml) and, subsequently, the matrices were washed three times with 1.5 ml of buffer A. The release of lysine residues by carboxypeptidase B treatment of plasmin-treated fibrin was shown by amino acid analysis. To that end, the fibrin matrices were treated for 1 h with carboxypeptidase B in the absence of BSA, and the supernatant was removed for amino acid analysis. As controls, fibrin matrices were incubated either with buffer alone or treated with carboxypeptidase B but not with plasmin.

**Determination of the Extent of Fibrin Degradation**—Fibrin plates were prepared as described above except that 125I-fibrinogen (approximately 150,000 counts/min/ml) was added to the unlabeled fibrinogen solution before clotting with thrombin. After the carboxypeptidase B pre-treatment (no release of counts due to the presence of carboxypeptidase B), the fibrin matrices were treated with increasing amounts of 0.2–35 nM plasmin in 200 μl of buffer A. The degradation of fibrin was expressed as the percentage of radioactivity that was released into the supernatant. The radioactivity incorporated into the fibrin matrices was taken as the 100% input value. The release of radioactivity from the fibrin matrices after plasmin incubation corresponded with the degradation of the nonlabeled fibrin, as shown by analysis of samples of the released fibrin fragments and the remaining fibrin matrix by SDS-PAGE (Fig. 1).

**Binding of 3H-Labeled rt-PA Deletion Proteins to Fibrin**—Approximately 0.15 pmol of each of the metabolically labeled rt-PA (mutant) proteins (corresponding to 1500–2000 counts/min in 200 μl of buffer A, was added to a fibrin-coated well and incubated for 1 h at 37°C. The supernatant was then quantitatively collected, diluted with 200 μl of buffer A containing 0.5% (w/v) SDS, and the radioactivity was determined by scintillation counting. The wells were rapidly washed three times with 1.5 ml of buffer A. The bound rt-PA (variants) were eluted together with the fibrin matrix from the respective well by gently shaking for 1 h at room temperature with 200 μl of buffer A, containing 0.5% SDS (w/v). The eluates were diluted with 200 μl of buffer A and the radioactivity was determined. Less than 10% of the radioactivity was lost during the washing steps. Nonspecific binding was determined using wells that were prepared identically to the fibrin-coated wells; however, the fibrinogen was omitted. The percentage of bound radioactivity was calculated and the nonspecific binding percentage was subtracted.

**RESULTS**

**Fibrin Binding of rt-PA as a Function of Plasmin Degradation of Fibrin**—To study the fibrin binding of rt-PA during ongoing fibrin degradation we devised an assay that employs immobilized noncross-linked fibrin pre-treated with increasing amounts of plasmin prior to rt-PA binding. Fibrin degradation was determined quantitatively by incorporating radiolabeled 125I-fibrinogen in the matrix. We show that an increased release of radioactivity correlates with the extent of fibrin degradation. We analyzed both the fibrin degradation products remaining in the well and the fragments released upon plasmin treatment by SDS-PAGE under reducing conditions (Fig. 1). Upon limited plasmin incubation, the α-chains of fibrin are cleaved predominantly, probably at Lys-208, Lys-221, and/or Lys-232 (28). This results in the generation of amino-terminal fragments (Mr, 25,000–27,000) of the α-chains of fibrin that remain linked by disulfide bonds to the apparently intact β- and γ-fibrin chains (Fig. 1A). It can be observed that the carboxyl-terminal fragments (Mr, 40,000–43,000), derived from the α-chains of fibrin, and several other fragments originating from the β- and γ-chains, are released from the matrix (Fig. 1B). This indicates that predominantly X-fragment polymers are present in the wells. Moreover, the fragments released from the matrix contain fragments Y, D, and E as well, as shown by SDS-PAGE under nonreducing conditions (data not shown).

To conveniently monitor the binding to fibrin of rt-PA, and subsequently of rt-PA deletion mutants, we used purified recombinant proteins that had been metabolically labeled with 125I-fibrinogen (see “Experimental Procedures”). An aliquot of SDS-solubilized, plasmin-degraded fibrin matrices were analyzed under reducing conditions on a 9% (w/v) polyacrylamide gel. Equal amounts of protein (30 μg) of the solubilized fibrin matrix were applied and protein bands were visualized by staining with Coomassie Brilliant Blue. Fgn, fibrinogen; A, intact fibrin matrix; B, fibrin matrix degraded to an extent of 1.1%; C, 3.6% degradation; D, 13.3% degradation; E, 23.3% degradation; F, 36.2% degradation; G, 54.3% degradation; H, 72.2% degradation; I, 95.3% degradation; M, molecular weight standards; 200,000, 97,000, 68,000, 43,000, 25,700, and 18,400, respectively. B, analysis of the degradation products released from the fibrin matrix upon plasmin treatment. After the various treatments, 60 μl of the 200 μl supernatant were analyzed as indicated under A. M, molecular-Weight standards as indicated above.
with \(^{[3]H}\)leucine. For that purpose, the conditioned media of mouse Ltk\(^{-}\) cells, stably transfected either with rt-PA cDNA or variants thereof (depicted in Fig. 2A), were employed and subjected to a one-step immunoaffinity chromatography procedure. This protocol yielded apparently homogeneous single-chain preparations of rt-PA and of rt-PA deletion mutants (Fig. 2B). In accord with other reports (4, 16), we observe an increase in binding of rt-PA to fibrin upon pre-incubation of the fibrin matrix with plasmin (Fig. 3). Optimal rt-PA binding is detected in a broad range between approximately 10 and 70% fibrin degradation, coinciding with the predominant appearance of the fibrin fragment X. A more extensive digestion of fibrin, causing the release of over 70% of the fibrin radioactivity, correspondingly decreases the binding of rt-PA. In our subsequent experiments we employed a plasmin treatment that induces a release of 10–16% of the incorporated radioactivity, corresponding with an initial degradation of the α-chains and an optimal rt-PA binding. We determined the binding of rt-PA to an initially plasmin-degraded fibrin matrix of a fixed amount of metabolically labeled t-PA, supplemented with an increasing concentration of unlabeled t-PA (Fig. 4). The binding increases linearly to a t-PA concentration of approximately 0.5 \(\mu\)M and subsequently reaches saturation. Consequently, all binding experiments using 0.75 nM rt-PA were performed under nonsaturable conditions. The same holds for each rt-PA variant since an identical binding percentage was found when either 0.375 or 1.5 nM of labeled rt-PA variant was used in the fibrin-binding experiments.

The Importance of Carboxyl-terminal Lysine Residues in Fibrin for the Binding of rt-PA—It has been shown before that plasminogen exhibits increased fibrin binding upon plasmin treatment of fibrin (4–7). Furthermore, evidence has been provided showing that carboxyl-terminal lysine residues are involved in the increased binding of plasminogen to fibrin (9). To study the importance of carboxyl-terminal lysine residues for t-PA binding, we analyzed the effect of the exopeptidase carboxypeptidase B on fibrin binding by rt-PA (Fig. 5, \textit{first four bars}). The binding of rt-PA to intact fibrin in this system amounts to 33.7 ± 1.1%, probably mediated by the F domain and the aminohexyl-binding site within the K2 domain, as shown previously (10, 15). The extent of binding to intact fibrin is not altered when the fibrin matrix is incubated extensively with carboxypeptidase B (34.2 ± 1.2%). Provided that carboxyl-terminal lysine residues affect the binding of rt-PA to fibrin, this observation indicates that intact fibrin is indeed devoid of carboxyl-terminal lysines. Significantly,
when the fibrin matrices were mildly digested with plasmin, an increased binding was observed up to 51.2 ± 1.4% of the applied metabolically labeled rt-PA. As expected, a subsequent incubation of the plasmin-treated fibrin matrices with carboxypeptidase B causes the release of free lysines (data not shown). The binding of rt-PA to fibrin matrices that were mildly digested with plasmin and, in addition, treated with carboxypeptidase B decreases to 41.6 ± 1.6% relative to matrices that were not incubated with carboxypeptidase B. Clearly, incubation of these plasmin-treated matrices with carboxypeptidase B does not fully reduce the binding of rt-PA to the level of the binding to intact fibrin (i.e. 33.7 ± 1.1%). Hence, we conclude that the increased binding of rt-PA to fibrin which has undergone limited plasmin proteolysis is at least partially due to the occurrence of carboxyl-terminal lysine residues in the fibrin matrix. This observation is indicative for a role of the lysine-binding site in the K2 domain, as we have postulated before (13). Moreover, our data demonstrate that part of the increased binding of rt-PA to plasmin-digested fibrin is independent of the occurrence of carboxyl-terminal lysines. It is of particular interest to establish whether distinct autonomous domains of the rt-PA protein mediate either the increased binding dependent on the presence of carboxyl-terminal lysines or the increased binding that is independent of such residues. To address this issue we have employed a series of rt-PA deletion mutant proteins, precisely lacking structural and functional domains of the H chain, and have assayed their ability to bind to fibrin that has been digested with plasmin and/or with carboxypeptidase B.

**Fibrin Binding of rt-PA Deletion Mutants**—In addition to our previous studies (10), we constructed yet another set of rt-PA deletion mutant proteins that lack one or more structural and functional domains of the H chain. Here, employing full length rt-PA cDNA, the corresponding cDNA sequences were “looped out” precisely according to the exon-intron distribution of the gene (29). Due to this strategy and based on the position of introns within the translational reading frame, it is anticipated that at the junctions of the out-looped regions novel codons are created. The resulting constructs and their subsequent mutant proteins are outlined in Table 1. All mutants were expressed and secreted by stably transfected mouse Ltk- cells and metabolically labeled using [3H]leucine. As mentioned before, immunofluorescence chromatography of these rt-PA deletion mutant proteins yields apparently homogeneous, single-chain preparations as shown by the analysis of reduced samples on SDS-PAGE (Fig. 2B).

Several groups have shown that the single-chain form of rt-PA displays a higher affinity for fibrin than the two-chain form (16, 30). Hence, to ensure that the changes in binding after pre-treatment of the fibrin matrix with plasmin are not due to conversion of the rt-PA (variants) from the single-chain to the two-chain form, we examined the composition of the rt-PA after binding by SDS-PAGE in the presence of a reducing agent. After the binding experiments, the recombinant proteins were still in the single-chain form (data not shown). Therefore, all our data concern fibrin binding by single-chain rt-PA variants. For each mutant, four different fibrin matrices were prepared: an untreated fibrin matrix and a matrix treated with plasmin (10–16% of radiolabeled [3H]fibrin released) both either followed or not followed by an extensive incubation with carboxypeptidase B. The quantitative fibrin-binding data are summarized in Fig. 5. As shown before, the deletion mutant lacking the complete H chain, del.FEK1K2, exhibits no affinity for fibrin (10). If only the K1 domain is coupled to the L chain, del.FEK2, then hardly any binding to fibrin is detected. However, all rt-PA derivatives that contain either the F or the K2 domain or both display affinity for intact fibrin in this assay system. It should be noted that for all these rt-PA variants the extent of binding to intact fibrin is not affected by treatment with carboxypeptidase B. The small rt-PA variants del.K1K2 and del.EK1K2 that contain either only the F or the F and the E domain of the H chain bind to the same extent to intact and degraded fibrin. In contrast, del.FEK1 that contains K2 exhibits increased binding to degraded fibrin. Interestingly, this increase is completely dependent on the presence of carboxyl-terminal lysine residues in fibrin, since a carboxypeptidase B treatment completely abolishes this augmentation of binding. These results demonstrate the involvement of the lysine-binding site in the K2 domain in increased binding of rt-PA to degraded fibrin. Similar observations were made for fibrin binding of the other K2 domain-containing mutants del.F and del.K1. On the other hand, the fibrin binding of del.K2, which also increases after limited degradation of the fibrin matrix, is not affected by the carboxypeptidase B treatment of plasmin-digested fibrin. Such augmented fibrin binding, independent of carboxyl-terminal residues, had been encountered in rt-PA as well. From these data, we assume that the K2 domain is not involved in the increased fibrin binding that is independent of carboxyl-terminal lysines. This particular increased binding is observed provided that the F, E, and the K1 domain are present in the same variant. In view of the basal fibrin binding of del.EK1K2 and del.K1K2 and the lack of fibrin binding of the del.FEK2 deletion mutant protein, it is conceivable that the increased carboxyl-terminal lysine-independent binding is mediated by the F domain, provided that this domain is correctly positioned with regard to plasmin-digested fibrin.

**DISCUSSION**

In the fibrinolytic process fibrin acts both as a substrate for plasmin and as an assembling surface for plasminogen and
t-PA. In the latter function, fibrin strongly potentiates the activity of t-PA and is thus considered an obligatory cofactor. It is generally believed that binding of plasminogen and t-PA to fibrin, conceivably at specific sites, results in the formation of a ternary "cyclic" complex that effectively converts plasminogen into plasmin, whereas binding is stimulated 15-30-fold upon limited proteolysis of fibrin (2). In this paper we have investigated the effect of ongoing degradation of fibrin by plasmin on the characteristics of fibrin binding by t-PA. The degradation of fibrin proceeds according to well defined, sequential proteolytic steps, resulting in a number of discrete intermediate degradation products (31). The initial degradation product, denoted fragment X, consists of apparently intact fibrin β- and γ-chains and cleaved α-chains. Clearly, fragment X polymers perform a key role in the assembly of fibrinolytic components and in the acceleration of the fibrinolytic process. Suenson and Petersen (32) have shown that, among naturally occurring intermediate degradation products, fragment X polymers are most effective in the acceleration of t-PA-mediated plasminogen activation. Furthermore, these investigators have demonstrated that the generation of polymerized fragment X coincides with maximal conversion of Glu-plasminogen into Lys-plasminogen (33). Employing the same fibrin-binding assay as reported in this paper, Tran-Thang et al. (7) show that Glu-plasminogen hardly binds to intact fibrin, whereas binding is stimulated 15-30-fold upon limited (less than 10%) plasmin digestion of fibrin. Although, these authors do not correlate the extent of fibrin digestion with the appearance of particular fibrin fragments, in our hands the indicated extent of degradation again coincides with the generation of fragment X polymers. Our data on binding of t-PA to fibrin are also in accord with the view that polymerized fragment X is a crucial determinant in assembly of fibrinolytic components. Optimal binding of t-PA to plasmin-digested fibrin again coincides with a limited digestion and the generation of polymerized fragment X. Consequently, it is conceivable that a ternary cyclic complex, required for accelerated activation of plasminogen, functions optimally if fibrin has been plasmin-digested to a limited extent.

Subsequently, we have addressed the issue of the involvement of different domains of t-PA in augmentation of binding to plasmin-digested fibrin, as well as to the nature of these interactions. A major conclusion from our results is that about half of the increased binding is due to the generation of carboxyl-terminal lysine residues, whereas the other half is independent of the presence of these chain termini. By employing a set of t-PA deletion mutants, lacking one or more of the domains of the amino-terminal H chain, we could clearly demonstrate that the increase dependent on carboxyl-terminal lysines is mediated by the lysine-binding site within the K2 domain. An apparent contradiction between this conclusion and that of Higgins and Vehar (16) can be envisaged based on the results of seemingly similar experiments. Those authors concluded that a lysine-binding site on t-PA is only involved in binding to intact fibrin and not in increased binding to fibrin upon plasmin digestion. However, it should be noted that those studies were performed with fibrinogen pre-treated with plasmin before clotting. As indicated before (7), such fibrin matrices may not reflect the physiological fibrinolytic process occurring in vivo. Moreover, it has been demonstrated that the physico-chemical properties of such matrices are quite different from those of pre-formed fibrin matrices (34). The rigidity (elasticity) of the matrices prepared from fibrinogen pre-treated with plasmin before clotting is only about 1% of those prepared from plasmin-digested fibrin. These distinct physico-chemical properties of the respective fibrin matrices may explain the observed biological discrepancies.

In agreement with our previous findings (13), the results presented in this paper indicate the presence of a single lysine-binding site within the t-PA protein, located on the K2
domain. Gething et al. (14) have claimed an additional site within the K1 domain that exhibits affinity for lysine-Sephasse. The fibrin binding data of the t-PA deletion mutants, still containing the K1 domain (del.K2 and del.FEK2), do not support the view that the K1 domain contains a lysine-binding site. In contrast to mutant proteins containing the K2 domain, these mutant proteins do not reveal increased fibrin binding upon limited plasmid digestion that is dependent on the presence of carboxyl-terminal lysines. As yet, a satisfactory explanation for the observed differences in the properties of the K1 domain is not at hand.

Both t-PA and the mutant protein del.K2 display increased binding to plasmin-digested fibrin that is independent of carboxyl-terminal lysine residues. At present, an unambiguous assignment of a particular domain responsible for this increase cannot be advanced, since several options can be considered. Actually, this effect is only observed for proteins (rt-PA and del.K2) that contain the amino-terminal domains F, E, and K1, while it is not detected with the other deletion mutant proteins. We favor the view that this increase is mediated by the F domain. For that option, we assume that the K1 domain (and possibly E) acts as an obligatory “spacer” for correct positioning of the F domain toward other parts of the molecule and, as a consequence, for its proper functioning. This assumption is in agreement with data reported by a number of groups that have constructed chimeric molecules at creating proteins that have combined the fibrin-binding features of t-PA with the catalytic activity of uroki-

[REFERENCES]
1. Collen, D. (1980) Thromb. Haemostas. 43, 77–89
2. Hoylaerts, M., Rijken, D. C., Lijnen, H. R., and Collen, D. (1982) J. Biol. Chem. 257, 2912–2919
3. Ranby, M. (1982) Biochim. Biophys. Acta 704, 461–469
4. Suenson, E., Lurzen, O., and Thorsen, S. (1984) Eur. J. Biochem. 140, 515–522
5. Harpel, P. C., Chang, T.-S., and Verderber, E. (1985) J. Biol. Chem. 260, 4432–4440
6. Bok, R. A., and Mangd, W. F. (1985) Biochemistry 24, 3279–3286
7. Tran-Thang, C., Kruchof, F. K. O., Atkinson, J., and Bachmann, F. (1986) Eur. J. Biochem. 160, 599–606
8. Gething, U. (1984) Biochem. J. 223, 413–421
9. Gething, U. (1985) FEBS Lett. 182, 43–46
10. Van Zonneveld, A. J., Veerman, H., and Pannekoek, H. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4670–4674
11. Suenson, E., Lijnen, R., Cohen, D., and Holmes, W. E. (1987) J. Biol. Chem. 262, 10855–10862
12. De Vries, C., Veerman, K., Blasi, F., and Pannekoek, H. (1988) Biochemistry 27, 2565–2572
13. van Zonneveld, A. J., Veerman, H., and Pannekoek, H. (1986) J. Biol. Chem. 261, 14214–14218
14. Gething, M. J., Adler, B., Boone, J. A., Gerard, R. D., Madison, E. L., McGookey, D., Meidell, R. S., Roman, L. M., and Sambrook, J. (1986) EMBO J. 7, 2731–2740
15. Verheijen, J. H., Caspers, M. P. M., de Munk, G. A. W., Enger-Virell, B. E., Chang, G. T. G., and Pouwels, P. H. (1987) Thrombos. Haemostasis. 58, Abstract 1814
16. Higgins, D. L., and Vehar, G. A. (1987) Biochemistry 26, 7786–7791
17. Colbere-Garapin, F., Clousterman, S., Horodniceanu, F., Kourilsky, Ph., and Garapin, A. C. (1979) Proc. Natl. Acad. Sci. U. S. A. 75, 3775–3759
18. Deutsch, D. G., and Mertz, E. T. (1970) Science 170, 1096–1096
19. Hunter, W. M., and Greenwood, P. C. (1962) Nature 194, 495–496
20. Messing, J., Crea, R., and Seeberg, P. H. (1981) Nucleic Acids Res. 9, 309–321
21. Kramer, W., Drutsa, V., Jansen, H. W., Krämer, B., Pfugfelder, M., and Fritz, H. J. (1984) Nucleic Acids Res. 12, 9441–9456
22. Suenson, E., Nicklens, S., Coll Cours, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
23. Graham, F. L., and Van der Eb, A. J. (1973) Virology 52, 456–467
24. Van Zonneveld, A. J., Veerman, H., Braekenhoff, J. J., Aarden, L. A., Cauty, J. F., and Pannekoek, H. (1986) Thrombos. Haemostas. 57, 82–86
25. Laemmli, U. K. (1970) Nature 227, 680–685
26. Pannell, R., Black, J., and Garewich, W. (1988) J. Clin. Invest. 81, 883–889
27. Cottrell, B. A., and Doolittle, R. F. (1976) Biochem. Biophys. Res. Commun. 71, 754–761
28. Doolittle, R. F., Cassetter, K. G., Cottrell, B. A., and Doolittle, R. F. (1977) Biochemistry 16, 1710–1715
29. Ny, T., Bligh, F., and Lund, B. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 5355–5359
30. Tate, K. M., Higgins, D. L., Holmes, W. E., Winkler, M. E., Heyneker, H. L., and Vehar, G. A. (1987) Biochemistry 26, 338–343
31. Doolittle, R. F. (1981) in Fibrinogen and Fibrinolysis (Bloom, A. L., and Thomas, D. P., eds) pp. 163–191, Churchill, London
32. Suenson, E., and Petersen, L. C. (1986) in Fibrinolysis: Abstracts of the 8th International Congress on Fibrinolysis, Vienna, 1986, (Davidson, Y. F., and Walker, J. D., eds) Abstr. 11, Churchill Livingstone, Edinburgh
33. Suenson, E., and Thorsen, S. (1988) Biochemistry 27, 2435–2443
34. Shen, L. L., McDonagh, R. P., McDonagh, J., and Hermans, J. (1977) J. Biol. Chem. 252, 6184–6189
35. Gheysen, D., Lijnen, H. R., Plerier, L., De Foresta, F., Demarsin, E., Jacobs, P., De Wilde, M., Bolten, A., and Collen, D. (1987) J. Biol. Chem. 262, 11779–11784
36. Erickson, L. A., Bergum, P. W., Hubert, E. V., Thieriault, N. Y., Ribberg, E. F., Palermo, D. P., de Munk, G. A. W., Verheijen, J. H., and Marotti, K. R. (1987) Thrombos. Haemostas. 58, Abstr. 1045, 289