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Fibrinogen Lysine Residue Aα157 Plays a Crucial Role in the Fibrin-induced Acceleration of Plasminogen Activation, Catalyzed by Tissue-type Plasminogen Activator*

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In previous studies, we have shown that the stretch 148–197 of the fibrinogen Aα chain plays a crucial role in the acceleration of the tissue-type plasminogen activator (t-PA)-catalyzed plasminogen activation.

In this study we have synthesized parts of Aα148–197 and analogues thereof. We found that the peptides with sequences identical with Aα148–161 and Aα149–161 of human fibrinogen accelerate the plasminogen activation by t-PA, whereas the corresponding peptides in which lysine residues Aα157 had been replaced by valine or arginine had no accelerating capacity. Furthermore, succinylation of the lysine residue(s) in the synthesized peptides Aα148–161 and Aα149–161 leads to loss of accelerating action.

These findings show that lysine residue Aα157 is crucial for the accelerating action of fibrin on the t-PA-catalyzed plasminogen activation.

Plasminogen activation, catalyzed by tissue-type plasminogen activator (t-PA),[1] is known to be accelerated in the presence of fibrin. This acceleration is relatively small in the presence of fibrinogen (1–3). Some years ago, we demonstrated that fragments derived from fibrin and also from fibrinogen can exert the same effect (4–10). Activation rate-enhancing fragments can be generated from fibrin(ogen) by digestion with plasmin or, chemically, with cyanogen bromide. In particular, we found that the soluble plasmin-generated fibrinogen fragment D_{EGTA} (7) and CNBr fragment FCB-2 (4) mimic the effect of fibrin. More detailed studies (5) revealed that an essential role is played by the Aα chain remnants of both D_{EGTA} and FCB-2, which are Aα111–197 (11) and Aα148–207 (11, 12), respectively. From these studies we concluded that the Aα chain stretch common to FCB-2 and D_{EGTA}, *i.e.* Aα148–197, plays a crucial role in the fibrin-induced acceleration of plasminogen activation by t-PA.

In the present study we have synthesized peptides analogous to parts of Aα148–197 and found that lysine residue Aα157 is essential for the acceleration to occur.

MATERIALS AND METHODS

Tissue-type Plasminogen Activator—Two-chain t-PA was purified from large scale melanoma cell culture according to Rijken *et al.* (13), as modified by Kluft *et al.* (14).

Plasminogen—Native plasminogen (Glu-plasminogen) was purified from fresh human plasma by affinity chromatography (15) on lysine-Sepharose (Pharmacia P-L Biochemicals), precipitated with ammonium sulfate, and dialyzed extensively against 0.1 M Tris·HCl, pH 7.5, containing 0.1% (v/v) Tween 80 (J. T. Baker Chemical Co.).

Peptides—The peptides were synthesized on polystyrene resin (1% cross-linking) using an automated peptide synthesizer (SAM-2, Biosearch, San Rafael, CA). The reaction sequence was performed according to the standard protocol using *tert*-butyloxycarbonyl-amino acids with the following side-chain protection: Lys-2-chlorocarbobenzoxy, 4-methoxybenzenesulfonyl, Glu-benzyl, Asp-benzyl, Ser-benzyl, Cys-*tert*-butylmercapto. Diisopropylcarbodiimide was used as the condensing reagent. Final deblocking and cleavage from the resin was performed by treatment with 1 M TFMSA, Thioanisol, *m*-cresol in trifluoroacetic acid for 1 h at 0 °C followed by filtration, precipitation from ether, and lyophilization from water. Cysteine-containing peptides were treated with 10 eq of *threo*-1,4-dimercapto-2,3-butandiol at pH 8 for 30 min and lyophilized. The crude peptides were purified by gel filtration (G-25 superfine; Pharmacia P-L Biochemicals) using 50% (v/v) acetic acid as the eluent.

Amino Acid Analyses—Peptides were hydrolyzed in 6 M HCl in evacuated sealed glass tubes for 72 h at 110 °C. The hydrolysates were lyophilized and analyzed with a Biotronik 5001 apparatus by Dr. K. D. Bos (Institute CIVO-analysis TNO, Zeist, The Netherlands).

Amino-terminal Amino Acids—Amino-terminal amino acids were determined according to Gray (16).

Assay System—In total volumes of 0.250 ml of 0.1 M Tris·HCl, containing 0.1% (v/v) Tween 80 were present 0.11 μM Glu-plasminogen, 0.3 mM S2251 (Kabi Vitrum, Stockholm, Sweden), 300 mIU t-PA, and varying concentrations of the synthetic peptide to be tested for stimulating activity. In this assay, the concentration of *p*-nitroaniline formed per time squared is proportional to the rate of plasmin formation (activation rate) (17). The ratio of the activation rate in the presence of the peptide over that in the absence of peptide was designated as stimulation factor.

Lysine Modification—Lysine-containing peptides were dissolved in 5.0 M guanidine·HCl (Aldrich, Beerse, Belgium) at pH 8.0, and solid succinic anhydride (Aldrich) was slowly added at room temperature to a final concentration of 50 mol/mol of amino groups. During the succinylation the pH was kept constant by the addition of 2 N NaOH. When the addition of succinic anhydride was completed, solid hydroxylamine (Fluka) was added to a final concentration of 1 M, and the pH was adjusted to 10 with 2 N NaOH. The reaction mixture was then incubated for 1 h at room temperature and dialyzed exhaustively in Spectrapor membrane tubing (Spectrum Medical Industries Inc., Los Angeles) with a molecular weight cutoff of 1000. Complete succinylation was confirmed by the absence of dansyl amino acids when the amino-terminal amino acids were determined.

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[1] The abbreviations used are: TFMSA, trifluoromethansulfonic acid; t-PA, tissue-type plasminogen activator; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; S2251, H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide·2HCl; FCB-2 (CNBr fragment of fibrin(ogen)) consists of chain remnants Aα148–207, Bβ191–224, 225–242, 243–305, and γ95–265, disulfide bond-linked.

RESULTS

Table I summarizes the synthetic peptide sequences used in this study. Peptides Aα148–161, Aα149–161, andAα160–172 have sequences identical with those in fibrinogen (11, 12). In the peptides (V157)Aα148–161, (V157)Aα149–161 lysine residue Aα157 has been replaced by valine, whereas this was arginine in the peptides (R157)Aα148–161 and (R157)Aα149–161.

Table II shows that the amino acid compositions and the amino-terminal amino acids of the peptides are as expected.

Table III summarizes the stimulation factors found for the individual peptides and the concentration of peptides needed for half-maximal stimulation. From Table III it is clear that the peptides Aα148–161 and Aα149–161, *i.e.* the peptides with lysine residue Aα157, have retained rate-enhancing potency, whereas the analogues in which this lysine has been replaced by valine or arginine, and peptide Aα160–172, have none. This shows that the rate-enhancing potency resides within Aα149–161 and that the lysine Aα157 is crucial for the accelerating effect on the plasminogen activation by t-PA.

Further evidence for the involvement of lysine residue(s) was obtained by the observation that succinylation of the lysine residues led to a complete loss of stimulating properties of the peptides.

DISCUSSION

From our previous work (4–10) we concluded that Aα148–197 plays an important role in the fibrin-induced acceleration of plasminogen activation by t-PA. This part of the Aα-chain is localized around the outer disulfide rings which terminate the coiled-coil regions, connecting the two fibrinogen D-domains with the central E-domain (12, 18). Fibrin, FCB-2 (4), and some plasmin degradation products, such as D_{EGTA} (7), but not intact fibrinogen, have rate-enhancing properties. From these findings we concluded (5) that the accelerating properties pre-exist in fibrinogen in a latent or buried form. The (hidden) activation rate-enhancing properties of fibrinogen can apparently be brought to expression by conversion to fibrin, by plasmin degradation, or by CNBr digestion.

TABLE I

Summary of synthetic peptides used in this study

Synthesized sequence	Designated as
K R L E V D I D I K I R S	Aα148–161
R L E V D I D I K I R S	Aα149–161
K R L E V D I D I V I R S	(V157)Aα148–161
R L E V D I D I V I R S	(V157)Aα149–161
K R L E V D I D I R I R S	(R157)Aα148–161
R L E V D I D I R I R S	(R157)Aα149–161
S C R G S C S R A L A R	Aα160–172

The sequence of Aα148–197 shows interesting charge distributions, especially from Aα148 through 161,

KRLEVDIDIKIRS . . .,
++0−0−0−0+0+0

where negative and neutral amino acids alternate (Aα150–157) and are sandwiched between two sets of positively charged amino acids. Such a charge distribution does not exist in the adjacent peptide Aα160–172. The consideration above was the main reason to synthesize the two peptides Aα148–161 and Aα160–172 in our first attempt to localize, in more detail (within Aα148–197), peptides with stimulating properties. As shown under "Results," we found that Aα148–161 enhances the rate of t-PA-catalyzed plasmin formation, whereas Aα160–172 does not.

Our own work and that of others suggested that lysine residues were involved in the rate-enhancing properties of fibrin and fibrin(ogen) degradation products. On the one hand, plasminogen has lysine binding sites, localized in the kringle I–IV domain. These are crucial to the accelerating activity of FCB-2 (9) since the activation of Val^{442}-plasminogen (mini-plasminogen) lacking the kringle I–IV domain is not accelerated by FCB-2. On the other hand, we found that 1 mM 6-aminohexanoic acid diminishes this rate-enhancement. This is in agreement with the work of others (3, 19, 20). Furthermore, succinylation of the lysines in the synthetic peptides abolished their stimulating effect completely. This latter observation agrees with those made by Radcliffe (21), who found that modification of lysines annihilates the rate-enhancing effect of fibrin.

Aα148–161, however, has two lysine residues: one at Aα148, and one at Aα157. In the next step of our study, we investigated whether both of these lysine residues are involved or only one. To answer this question we synthesized analogues of Aα148–161, which are summarized in Table I. It appeared that only those peptides which contain lysine Aα157 have stimulating properties. Thus, we conclude that lysine residue Aα157 is crucial for the stimulating effect.

Yet peptides Aα148–161 and Aα149–161 are not equally effective as fibrin. As we have reported before (5), the Aα chain remnant of FCB-2 (*i.e.* Aα148–207) enhances the activation rate of plasminogen only 20-fold, whereas fibrin and intact FCB-2 enhance it about 35-fold. Table III shows that peptides Aα148–161 and Aα149–161 enhance the reaction rate 11.3 and 9.1 times, respectively. This seems to indicate that amino acid residues other than those in Aα148–161 are also

TABLE II

Amino acid compositions of the synthetic peptides used in this study

Expected values are in parentheses.

Amino acid	Aα148–161	Aα149–161	(V157)Aα148–161	(V157)Aα149–161	(R157)Aα148–161	(R157)Aα149–161	Aα160–172
				mol/mol of peptide			
I	3.0 (3)	3.3 (3)	2.8 (3)	2.7 (3)	2.8 (3)	2.9 (3)	(0)
L	1.1 (1)	1.1 (1)	1.1 (1)	1.1 (1)	0.9 (1)	1.0 (1)	1.2 (1)
K	1.6 (2)	1.2 (1)	0.6 (1)	(0)	0.5 (1)	(0)	(0)
V	1.2 (1)	1.2 (1)	1.8 (2)	2.0 (2)	1.0 (1)	1.1 (1)	(0)
R	1.6 (2)	1.6 (2)	2.3 (2)	2.4 (2)	2.8 (3)	2.9 (3)	2.4 (3)
D	2.2 (2)	2.3 (2)	2.0 (2)	2.1 (2)	1.9 (2)	2.1 (2)	2.2 (2)
E	1.2 (1)	1.2 (1)	1.0 (1)	1.2 (1)	1.1 (1)	1.1 (1)	(0)
S[a]	0.6 (1)	0.6 (1)	0.6 (1)	0.7 (1)	0.6 (1)	0.6 (1)	3.0 (3)
G							1.1 (1)
C							1.5 (2)
Amino terminus	K (K)	R (R)	K (K)	R (R)	K (K)	R (R)	S (S)

[a] Values for S have not been corrected for losses during hydrolysis.

TABLE III

Stimulation factors of the synthetic peptides (as observed in the assay system under "Materials and Methods") and the peptide concentrations required for half-maximal stimulation

Peptide	Stimulation factor	Half-maximal stimulation at
		μM
Aα148–161	11.3 ± 3.2 (n = 31)	12
Aα149–161	9.1 ± 3.6 (n = 12)	15
(V157)Aα148–161	1.1 ± 0.1 (n = 3)	
(V157)Aα149–161	1.4 ± 0.5 (n = 3)	
(R157)Aα148–161	1.0 ± 0.1 (n = 3)	
(R157)Aα149–161	1.3 ± 0.2 (n = 3)	
Succinyl Aα148–161	1.0 (n = 2)	
Succinyl Aα149–161	1.0 (n = 2)	

involved in the rate-enhancing effect of FCB-2 and its Aα chain remnant. Moreover, the concentration of a peptide or fibrin(ogen) fragment required to obtain half-maximal rate enhancement increases with decreasing size, *i.e.* 0.02 μM for fibrin monomers (5), 0.12 μM for FCB-2 (5), 0.5 μM for Aα148–207 (5), 12 μM for Aα148–61, and 15 μM for Aα149–161 (present publication). Such a dependency of effect on concentration has also been observed for peptides and fibrinogen fragments with decreasing molecular weights and having antipolymerizing properties (22, 23).

Plasminogen activation rate-enhancing properties have been ascribed mainly to carboxyl-terminal lysines (24–26), since it is only these which seem to fulfil the requirements for binding to the lysine binding sites of plasminogen, *i.e.* an ε-amino- and an α-carboxyl function separated by a hydrophobic carbon chain. Our results seem to indicate that also non-carboxyl-terminal lysines such as Aα157 may contribute to the observed rate enhancement by fibrin. Lysine Aα157 may interact with the postulated "aminohexyl sites" (24) or with t-PA. An alternative explanation could be that the spatial conformation of *e.g.* Aα 149–161 brings a side-chain carboxyl function of one of the aspartic acid or glutamic acid residues in the peptide in a position in which it can mimic a carboxyl-terminal lysine carboxyl function. Studies to answer this question are currently in progress.

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