Identification and Characterization of Novel tPA- and Plasminogen-Binding Sites within Fibrin(ogen) αC-Domains†

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ABSTRACT: Molecular defects in the αC-domains of some abnormal fibrinogens have been associated with impaired fibrin-mediated activation of plasminogen (Pg) by its activator tPA, suggesting the involvement of these domains in fibrinolysis. To test this suggestion, we expressed in *E. coli* the αC-fragment (residues Aα221−610) corresponding to the entire αC-domain as well as its NH_2- and COOH-terminal halves (residues Aα221−391 and Aα392−610) and tested their effects on activation of Pg and their interaction with Pg and tPA. When the activation was monitored by cleavage of a chromogenic substrate with newly formed plasmin, the reaction was much more efficient in the presence of the αC-fragment. This stimulation was abolished upon digestion of the αC-fragment with plasmin. In surface plasmon resonance experiments, both tPA and Pg bound to the immobilized αC-fragment with K_ds of 33 and 32 nM, respectively. Similar results were obtained by ELISA. This binding occurred via independent sites since saturating amounts of Pg did not prevent binding of tPA and vice versa. Both sites were localized in the COOH-terminal half of the αC-domain since the Aα392−610 fragment bound both tPA and Pg and was an effective stimulator whereas Aα221−391 was inactive. These results indicate that the fibrinogen αC-domains contain novel high-affinity tPA- and Pg-binding sites that play an important role in the regulation of fibrinolysis.

The plasma protein fibrinogen is involved in a number of important physiological and pathological processes including blood coagulation and fibrinolysis. Thrombin-mediated conversion of fibrinogen to fibrin results in spontaneous polymerization of the latter and formation of an insoluble fibrin clot that prevents the loss of blood upon vascular injury and serves as a provisional matrix in subsequent tissue repair. When the hemostatic role of the clot is accomplished, it is dissolved by degradation with a specific fibrinolytic enzyme plasmin. Although plasmin exhibits proteolytic activity toward many proteins, its activity in vivo is restricted mainly to places of fibrin deposition, allowing effective dissolution of the latter. This important natural adaptation occurs through a number of orchestrated interactions between fibrin, plasminogen, and its activator, tPA,[1] resulting in localization of fibrinolysis.

The tPA-mediated activation of plasminogen is slow in solution, and fibrinogen does not stimulate it; however, it is accelerated dramatically in the presence of fibrin (*1*−*3*). Conversion of fibrinogen into fibrin is accompanied by conformational changes that result in the exposure of multiple binding sites and modulation of various activities. The exposure of tPA- and plasminogen-binding sites provides effective activation of plasminogen on the fibrin surface. Both plasminogen and tPA contain a number of domains which mediate their interaction with different proteins and receptors. Interaction of plasminogen with fibrin is mediated by several kringle domains through their Lys-binding sites (*4*−*7*). Interaction of tPA with fibrin is mediated primarily by its finger domain and one of its two kringle domains in a Lys-independent and Lys-dependent manner, respectively (*8*−*11*). Fibrinogen also contains multiple domains that are grouped into three major structural regions: the central E and two identical terminal D regions (*12*−*15*). Cryptic tPA- and plasminogen-binding sites have been localized in the fibrinogen D region. Namely, a portion of its Aα chain including residues 148−160 binds both plasminogen and tPA (*16*−*18*) while its γ chain residues 312−324 are involved in binding of tPA only (*19*−*21*).

Besides its D and E regions, fibrinogen also contains two self-interacting αC-domains formed by the COOH-terminal two-thirds of the two Aα chains (residues Aα220−610) (*22*, *23*). It was reported that degradation of fibrin with neutrophil elastase, which seems to cleave the αC-domains at the early stage of proteolysis, is associated with decreased stimulation of tPA-catalyzed plasminogen activation (*24*). Studies with the congenitally abnormal fibrinogens Dusart and Marburg revealed that the molecular defects in their αC-domains are associated with the impaired fibrin-stimulated activation of plasminogen by tPA (*25*−*28*). It was suggested that this

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[1] Abbreviations: tPA, tissue-type plasminogen activator; Pg, plasminogen; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; S-2251, H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide; TBS, 0.02 M Tris, pH 7.4, buffer with 0.15 M NaCl; ϵ-ACA, ϵ-aminocaproic acid; ELISA, enzyme-linked immunosorbent assay; SPR, surface plasmon resonance; k_{obs}, observed rate constant; k_{diss}, dissociation rate constant; k_{ass}, association rate constant; K_d, equilibrium dissociation constant.

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effect may be connected either with the reduced binding of plasminogen (*29*, *30*) or with the impaired αC-domain-dependent lateral aggregation of fibrin protofibrils and formation of an abnormal plasmin-resistant fibrin clot (*28*, *30*, *31*).

Although the above-mentioned studies suggest the involvement of αC-domains in fibrinolysis, their exact role in this process is still unclear. To clarify this role, we expressed three fragments corresponding to the entire αC-domain and its NH_2- and COOH-terminal halves and tested their stimulating effect and ability to bind tPA and plasminogen. The results indicate that the isolated αC-domain and its COOH-terminal half both bind tPA and plasminogen with high affinity and stimulate effectively the activation of the latter. The stimulating effect is abolished upon digestion of these domains with plasmin, suggesting their participation in regulation of fibrinolysis.

EXPERIMENTAL PROCEDURES

Proteins. Plasminogen-depleted human fibrinogen and bovine serum albumin (BSA) were purchased from Calbiochem. Recombinant single-chain tPA was a Genentech product. Human Glu-plasminogen (form II) was prepared from citrated human plasma by affinity chromatography on Lys-Sepharose 4B (*32*) and further purified by size exclusion chromatography on Superdex 200 (Pharmacea). Human plasmin and bovine α-thrombin were from Sigma. Recombinant factor XIII (*33*) was obtained from Dr. P. Bishop of ZymoGenetics. The $D-D:E_1$ complex was prepared as described in (*34*).

Antibodies. The peroxidase-conjugated anti-sheep and anti-goat polyclonal antibodies were purchased from Sigma. The sheep anti-tPA polyclonal antibodies and the goat anti-plasminogen polyclonal antibodies were from Chemicon.

Expression of Recombinant αC-Fragments. A recombinant αC-fragment corresponding to the human fibrinogen αC-domain (residues Aα221−610) was produced in *E. coli* using pET-20b expression vector as described earlier (*35*). This vector was used as a template to produce truncated variants of the αC-domain, the NH_2- and COOH-terminal halves including residues Aα221−391 and Aα392−610, respectively. A cDNA encoding the Aα221−391 and Aα392−610 regions was produced by polymerase chain reaction using the following primers: 5′-AGAGACATATGCAGCTTCAGAAGGTACCC-3′ (forward) and 5′-AGAGAAAGCTTTTACCAGTCTGGGTTGTTAG-3′ (reverse) for the Aα221−391 fragment, and 5′-AGAGACATATGGGCACATTTGAAGAGG-3′ (forward) and 5′-AGAGAAAGCTTTTAGACAGGGCGAGATTTAG-3′ (reverse) for the Aα392−610 fragment. The forward primers incorporated the *Nde*I restriction site immediately before the coding region; the final three bases of the *Nde*I site, ATG, code for the fMet residue that initiates translation. The reverse primers included a TAA stop codon immediately after the coding segment, followed by a *Hin*dIII site. The amplified cDNA fragments were purified by electrophoresis in agarose gel, digested with *Nde*I and *Hin*dIII restriction enzymes, and ligated into the pET-20b expression vector. The resulting plasmids were used for transformation of DH5α and then BL21(DE3) pLysS *E. coli* host cells. Both cDNA fragments were sequenced in both directions to confirm the integrity of the coding sequences.

Purification of Recombinant Fragments. The αC-fragment was purified from the insoluble pellet of the bacterial lysate as described in (*35*). The Aα221−391 fragment was prepared from the soluble fraction of the bacterial lysate. After precipitation with 30% ammonium sulfate, the pellet was dissolved in 20 mM Tris, pH 8.0, containing 0.1 mM PMSF, and applied on a Q-Sepharose column (Pharmacia) equilibrated with the same buffer. The nonbound material represented mainly the Aα221−391 fragment; the contaminants were removed by size exclusion chromatography on a Superdex 75 column (Pharmacia) equilibrated with TBS. The Aα392−610 fragment was purified from the insoluble fraction of the bacterial lysate. The pellet was washed 3−5 times with TBS containing 5 mM EDTA and 0.5% Triton X-100, resulting in significant purification from *E. coli* membrane proteins. The pellet was then solubilized in 8 M urea, and the soluble protein was further purified and urea was removed by size exclusion chromatography on a Superdex 75 column equilibrated with TBS.

Cross-Linking with Factor XIIIa. Cross-linking of the αC-fragment was performed as described earlier (*35*). The reaction mixture contained 1 mg/mL αC-fragment, 30 μg/mL factor XIII, and 1 unit/mL α-thrombin in TBS with 10 mM Ca^{2+}. The mixture was incubated for 6 h at 25 °C, centrifuged to remove nonsoluble material, and then subjected to size exclusion chromatography on Superdex 75 to separate non-cross-linked αC-fragment from its soluble cross-linked oligomers.

Digestion with Plasmin. The digestion was initiated by the addition of 0.05 unit/mL plasmin to a solution containing 1.5 mg/mL αC-fragment in TBS. The 250 μL aliquots were withdrawn at different times, treated with diisopropyl fluorophosphate to inactivate plasmin, dialyzed overnight against TBS to remove the inhibitor, and assayed for stimulating activity in a chromogenic substrate assay.

Chromogenic Substrate Assay. The stimulating effect of the αC-fragment and its truncated variants on the tPA-catalyzed conversion of plasminogen into plasmin was evaluated by determination of the amidolytic activity of the newly formed plasmin with the chromogenic substrate S-2251 (H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide) (Chromogenix) as described in (*36*). The assay system contained 0.2 μM Glu-plasminogen, 0.14 nM tPA, 0.3 mM S-2251, and 1 μM fibrinogen or its fragments in TBS with 0.05% Tween 80. The assay was performed in the wells of a microtiter plate at 37 °C. The amidolytic activity was determined by measuring an absorbance at 405 nm using the TERMOmax 96-well plate reader (Molecular Devices).

Solid-Phase Binding Assay. Solid-phase binding was performed in plastic microtiter plates using an enzyme-linked immunosorbent assay (ELISA). Microtiter plate wells (Fisher) were coated overnight with 100 μL/well of 5 μg/mL αC-fragment or its truncated variants in 0.1 M Na_2CO_3, pH 9.5 (coating buffer). The wells were then blocked with 1% BSA in TBS. Following washing with TBS containing 0.05% Tween 20, the indicated concentrations of plasminogen and tPA in the same buffer were added to the wells and also to control wells coated with just BSA and incubated for 1 h. Bound plasminogen was measured by the reaction with the goat anti-plasminogen polyclonal antibodies and the peroxidase-conjugated anti-goat polyclonal antibodies. Bound tPA was measured by the reaction with the sheep anti-tPA

polyclonal antibodies and the peroxidase-conjugated anti-sheep polyclonal antibodies. A TMB Microwell Peroxide Substrate (Kirkegaard & Perry Laboratories Inc.) was added to the wells, and the amount of bound ligand was measured spectrophotometrically at 450 nm. Data were analyzed by nonlinear regression analysis using eq 1:

$$B = B_{max}/(1 + K_d/[L]) \quad (1)$$

where B represents the amount of ligand bound, B_{max} is the amount of ligand bound at saturation, [L] is the molar concentration of free ligand, and K_d is the dissociation constant.

Biosensor Assay. The interaction of plasminogen and tPA with the αC-fragment and its variants was studied by surface plasmon resonance (SPR) using the IAsys biosensor (Fisons, Cambridge, U.K.) which measures association/dissociation of proteins in real time (*37*). The αC-fragment or its variants were covalently coupled to the activated carboxymethyldextran-coated biosensor chip at a coupling density 9−15 ng/mm^2 by the procedure recommended by the manufacturer. Binding experiments were performed in 50 mM Tris buffer, pH 7.4, containing 50 mM NaCl, 0.1 mM PMSF, and 0.05% Tween 20 (binding buffer). The association between the immobilized fragments and the added proteins was monitored as the change in the SPR response. The dissociation of the complex was initiated by substitution with the same buffer lacking ligand and monitored in the same manner. To regenerate the chip, complete dissociation of the complex was achieved by adding 10 mM HCl for 1 min following reequilibration with binding buffer. The traces of the association and dissociation processes were recorded, and the data were analyzed using the FASTfit kinetic analysis software supplied with the instrument as described in detail (*38*). Briefly, the association curves at each concentration of ligand were fitted to the pseudo-first-order equation to derive the observed rate constant, k_{obs} (termed on-rate constant in FASTfit). Then the concentration dependence of k_{obs} was fitted to eq 2:

$$k_{obs} = k_{diss} + k_{ass}[\text{ligand}] \quad (2)$$

to find the association rate constant (k_{ass}) from the slope and the dissociation rate constant (k_{diss}) from the intercept. The dissociation equilibrium constant (K_d) was calculated as $K_d = k_{diss}/k_{ass}$. The values were examined for self-consistency of the data as described (*39*).

RESULTS

Stimulating Effect of the αC-Fragment on tPA-Catalyzed Plasminogen Activation. It is well established that fibrin binds tPA and plasminogen and accelerates dramatically activation of the latter by the former promoting fibrinolysis. Such a stimulating effect is preserved in some fibrin(ogen) fragments derived from the D region that contain tPA- and/or plasminogen-binding sites (*16, 20, 40, 41*). To test a possible involvement of the αC-domains in fibrinolysis, we first studied the effect of the recombinant αC-fragment (Aα221−610) corresponding to this domain on the activation of plasminogen by tPA. When the activation was monitored by cleavage of the chromogenic substrate S-2251 with newly formed plasmin, the reaction was much more efficient in the presence of the αC-fragment (Figure 1A); its truncated variant Aα392−610 also exhibited a high stimulation effect (Figure 1B). The stimulation was comparable with that of the D−D:E$_1$ complex, a well-known stimulator (*42, 43*), and much higher than that of fibrinogen, which in fact exhibited very low stimulating activity despite the presence of αC-domains within it. These results indicate that the isolated αC-domain is an effective stimulator of tPA-catalyzed plasminogen activation. They also imply that this domain interacts with tPA and/or plasminogen. To test this suggestion, we studied directly its interaction with both proteins by ELISA and surface plasmon resonance (SPR).

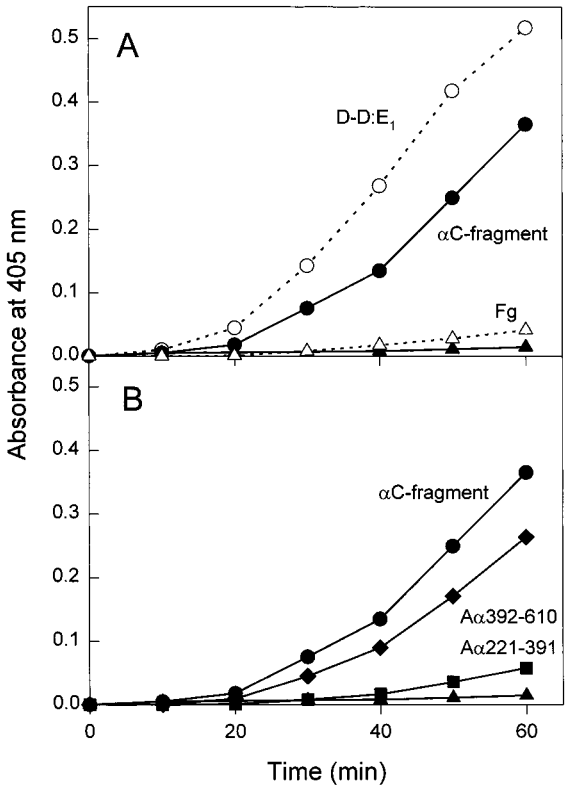

FIGURE 1: Stimulating effect of the αC-fragments on activation of plasminogen by tPA. Panel A: stimulating effect of the αC-fragment (closed circles) in comparison with that of the D−D:E$_1$ complex (open circles) and fibrinogen (Fg, open triangles). Panel B: stimulating effect of the αC-fragment (circles) in comparison with its Aα221−391 fragment (squares) and Aα392−610 fragment (diamonds). The activation of plasminogen in the absence of stimulators is shown by closed triangles in both panels. The stimulating effect was measured by hydrolysis of chromogenic substrate S-2251 with newly formed plasmin as described under Experimental Procedures.

Interaction of tPA and Plasminogen with the Isolated αC-Fragment. When the αC-fragment was immobilized on a sensor chip, it bound both plasminogen and tPA in a dose-dependent manner (Figure 2). The values of K_d calculated from the association data for plasminogen and tPA were found to be 32 and 33 nM, respectively (Table 1 and insets in Figure 2). The addition of 50 mM ϵ-aminocaproic acid (ϵ-ACA) completely abolished their binding, suggesting a Lys-dependent mechanism for this interaction. In separate SPR experiments (not shown), neither plasminogen nor tPA, when immobilized, bound soluble fibrinogen, in agreement with its very low stimulating effect observed in a chromogenic substrate assay (Figure 1A). These findings were confirmed in ELISA experiments, in which both plasminogen

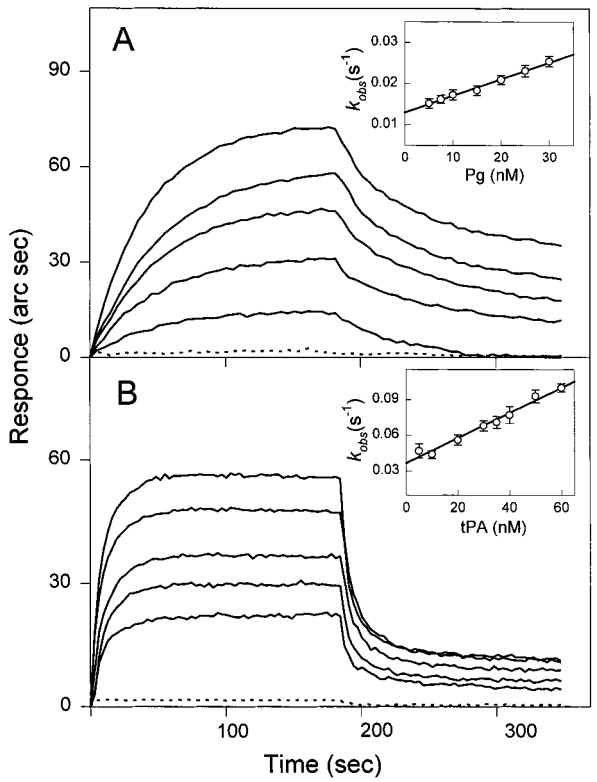

FIGURE 2: Analysis of binding of plasminogen and tPA to the immobilized αC-fragment by surface plasmon resonance. Increasing concentrations of plasminogen (panel A) or tPA (panel B) were added to the immobilized αC-fragment, and their association/dissociation was monitored in real time while registering the resonance signal (response). The concentrations were 5, 10, 15, 20, and 25 nM for plasminogen and 10, 15, 20, 30, and 40 nM for tPA. The inset in each panel shows a plot of the values of k_{obs} determined for each association curve versus ligand concentration to derive k_{ass}, k_{diss} and thus determine the dissociation equilibrium constants, K_d, presented in Table 1. Error bars in each inset represent ±SD obtained from three independent experiments for each concentration of ligand. Dotted lines in both panels represent binding curves for plasminogen and tPA in the presence of 50 mM ϵACA. Here and in other figures a signal of 200 arc s corresponds to 1 ng of protein bound/mm^2 of the sensor chip surface.

Table 1: Dissociation Constants for the Interaction of tPA and Plasminogen with the αC-Fragments[a]

	plasminogen K_d (nM)		tPA K_d (nM)	
fragments[b]	SPR	ELISA	SPR	ELISA
αC-fragment (221−610)	32 ± 2[c]	16 ± 2[d]	33 ± 5[c]	29 ± 8[d]
Aα392−610 fragment	27 ± 2[c]	13 ± 1[d]	47 ± 6[c]	30 ± 7[d]

[a] Values are means ± SD of three independent experiments. [b] No binding was observed with the Aα221−391 fragment in all cases. [c] Obtained by SPR. [d] Obtained by ELISA.

and tPA also bound to the immobilized αC-fragment in a dose-dependent manner (Figure 3). The calculated K_d was 16 nM for plasminogen and 29 nM for tPA (Table 1). This binding was also blocked by 50 mM ϵ-ACA. All these results clearly indicate that the isolated αC-domain contains tPA- and plasminogen-binding sites that are not accessible in fibrinogen.

Localization of tPA- and Plasminogen-Binding Sites in the αC-Domain. To clarify which region(s) of the αC-domain participate(s) in the interaction with plasminogen and tPA, we tested the binding of both to the recombinant Aα221−391 and Aα392−610 fragments corresponding to the NH_2-

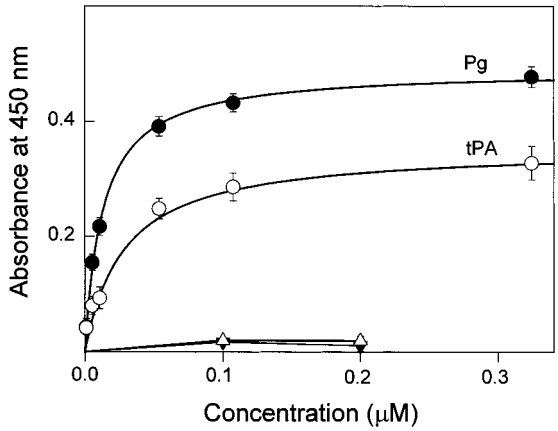

FIGURE 3: Analysis of the binding of plasminogen and tPA to the immobilized αC-fragment by ELISA. Increasing concentrations of plasminogen (closed circles) or tPA (open circles) were incubated with microtiter wells coated with the αC-fragment. Bound species were detected with polyclonal antibodies against plasminogen or tPA. Results are mean ± SD of three independent determinations. The curves represent the best fit of the data to eq 1. The results obtained with both proteins in the presence of 50 mM ϵACA are presented by triangles.

and COOH-terminal halves of the αC-domain, respectively. In SPR experiments, both plasminogen and tPA bound to the immobilized Aα392−610 fragment in a dose-dependent manner while very little binding to the immobilized Aα221−391 fragment was observed (Figure 4). The values of K_d calculated from the association data for plasminogen and tPA were found to be 27 and 47 nM, respectively, close to those determined for the full-length αC-fragment (Table 1 and insets in Figure 4). This finding was confirmed in ELISA experiments, in which both plasminogen and tPA bound to the immobilized Aα392−610 fragment in a dose-dependent manner with K_ds of 13 and 30 nM, respectively (Table 1), while no binding was observed with the Aα221−391 fragment (Figure 5). Thus, SPR and ELISA data indicate that the tPA- and plasminogen-binding sites are located in the COOH-terminal half of the αC-domain. In agreement, the Aα392−610 fragment exhibited high stimulating activity in a chromogenic substrate assay while that of the Aα221−391 fragment was very low (Figure 1B).

Competition Experiments. Since both plasminogen and tPA bind to the αC-fragment in a Lys-dependent manner with similar affinity, one might suspect that both proteins bind to the same site(s) through their kringle domains. Alternatively, they may bind to different binding sites. To select between these alternatives, we performed competition binding experiments. In ELISA, when increasing concentrations of plasminogen were added to the immobilized αC-fragment, the binding was unaffected by the presence of a high excess of tPA (1 μM) (Figure 6A). A value of 13 nM was obtained for K_d, close to the value of 16 nM obtained for plasminogen alone (Table 1). In another experiment, when increasing concentrations of tPA were added to wells containing 0.2 μM plasminogen, no inhibition of plasminogen binding to immobilized αC-fragment was observed even at 10-fold molar excess of tPA (Figure 6B). In SPR experiments, in which the response signal is proportional to the bound mass, the amount of bound tPA and plasminogen was additive even at saturating concentrations of both components (0.2 μM) (Figure 7A). In further experiments, when tPA or plasmi-

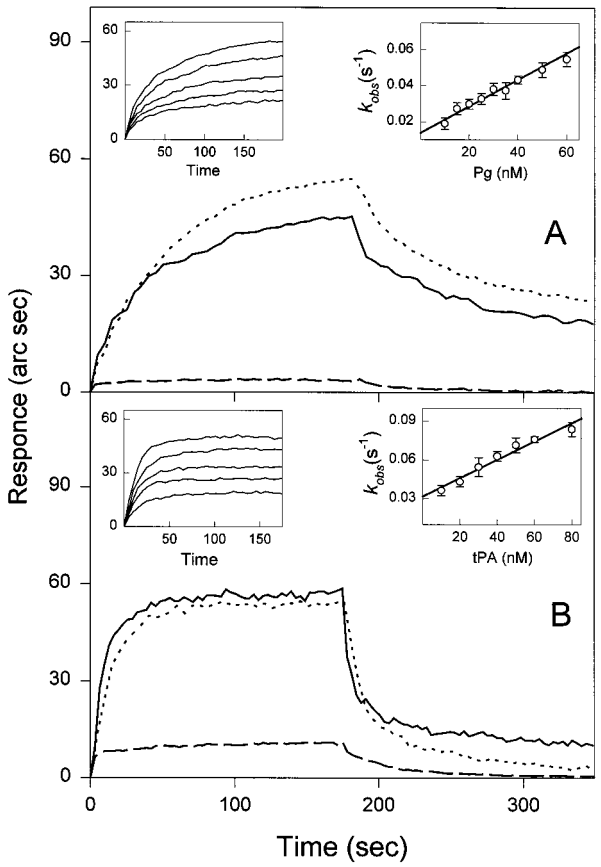

FIGURE 4: Analysis of the binding of plasminogen and tPA to the NH_2- and COOH-terminal halves of the αC-fragment by surface plasmon resonance. Association/dissociation of plasminogen at 20 nM (panel A) and tPA at 40 nM (panel B) with the immobilized αC-fragment (dotted curves) and its subfragments, Aα221−391 (dashed curves) and Aα392−610 (solid curves), was monitored in real time while registering the resonance signal. The left inset in each panel shows an example of dose-dependent binding of plasminogen (panel A) and tPA (panel B) to Aα392−610; the right insets show plots of the values of k_{obs} versus ligand concentration to derive k_{ass}, k_{diss} and thus determine the dissociation equilibrium constants, K_d, presented in Table 1. Error bars in each right panel represent the ±SD obtained from three independent experiments for each concentration of ligand.

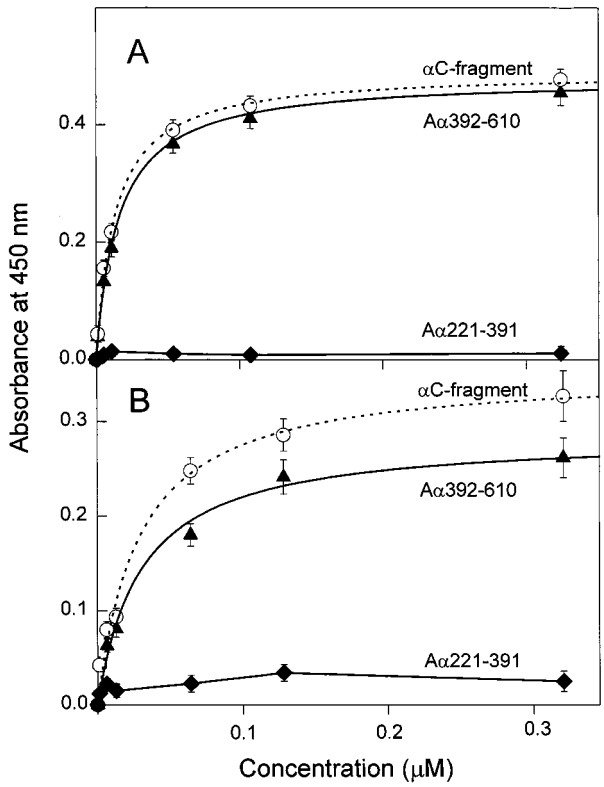

FIGURE 5: Analysis of the binding of plasminogen and tPA to the NH_2- and COOH-terminal halves of the αC-fragment by ELISA. Increasing concentrations of plasminogen (panel A) and tPA (panel B) were incubated with microtiter wells coated with the Aα221−391 fragment (diamonds) or with the Aα392−610 fragment (triangles). The results for the αC-fragment (open circles) that are the same as in Figure 2 are presented for comparison. Bound species were detected with polyclonal antibodies against plasminogen or tPA. Results are the mean ± SD of three independent determinations. The curves for the αC-fragment and the Aα392−610 fragment represent the best fit of the data to eq 1.

nogen at saturating concentration was added to the immobilized αC-fragment followed by the addition of their mixture, the increase in signal was again additive (Figure 7B). Taken together, these results indicate that plasminogen and tPA bind to the isolated αC-domain via independent binding sites.

Effect of FXIIIa-Catalyzed Cross-Linking and Plasmin Cleavage on the Stimulating Activity of the αC-Fragments. It is well established that the αC-domains in fibrin form factor XIIIa-cross-linked polymers. The αC-domains are also sensitive to proteolysis (*44*) and are the first portions of fibrin to be removed upon fibrinolysis. To test the effect of cross-linking and proteolytic degradation on the stimulating activity of the αC-domains, we compared the stimulating effects of the cross-linked versus non-cross-linked αC-fragments and the intact versus plasmin-treated αC-fragments. Incubation of the αC-fragment with FXIIIa resulted in the appearance of dimers, trimers, and oligomers (Figure 8A, lane 2 in the inset). The cross-linked species were separated from the non-cross-linked one by size-exclusion chromatography (lanes 3 and 4), and both fractions were tested for their stimulating effect. The results presented in Figure 8A indicate that cross-linking increases the stimulating activity of the αC-fragments almost 2-fold. In other experiments, we monitored the changes of the stimulating activity of the αC-fragment upon its treatment with plasmin. Samples were withdrawn from the digest at the indicated times (Figure 8B, inset) and assayed for their stimulating effect, after inhibiting the plasmin in each sample to prevent its interference with the assay (see Experimental Procedures). The results presented in Figure 8B indicate that proteolytic degradation of the αC-domain results in the loss of its stimulating activity.

DISCUSSION

Although studies with the congenitally abnormal fibrinogens Dusart and Marburg suggested the involvement of the αC-domains in fibrinolysis, the exact mechanisms remained unclear. Indeed, in both cases the defect is connected with the appearance in the αC-domain of a free Cys that reacts with plasma albumin, resulting in a disulfide-linked fibrinogen−albumin complex (*26, 27*). The presence of albumin interferes with polymerization of both fibrinogens, resulting in abnormal fibrin assembly (*27, 28, 31*). Thus, it is not clear whether the reduced stimulating effect of fibrin prepared from both fibrinogens is connected with the reduced binding of plasminogen or with the formation of the abnormal clots that could be more resistant to plasmin (*27*−*31*). To clarify the

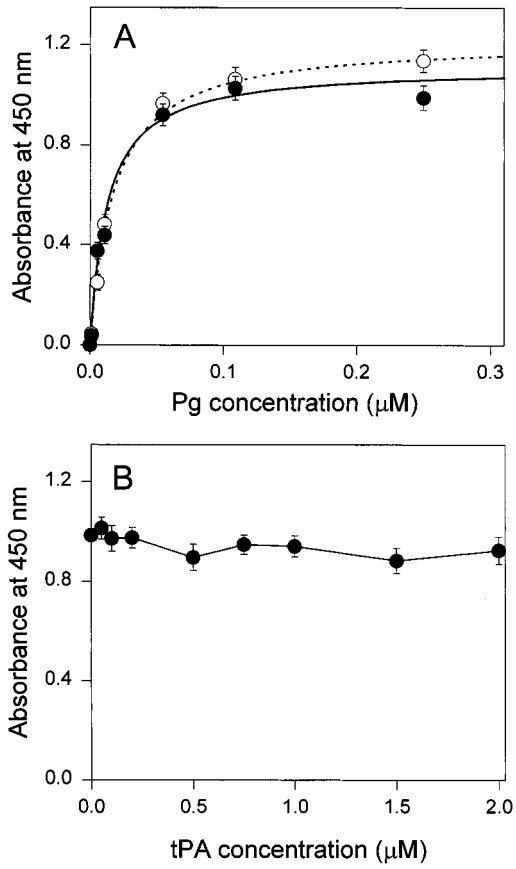

FIGURE 6: Competition experiments on the binding of plasminogen and tPA to the immobilized αC-fragment performed by ELISA. Panel A: increasing concentrations of plasminogen in the absence (open circles) or presence (closed circles) of 1 μM tPA were incubated with microtiter wells coated with the αC-fragment. Bound species were detected with polyclonal antibodies against plasminogen. Both curves represent the best fit of the data to eq 1. Panel B: plasminogen at 0.2 μM in the presence of increasing concentrations of tPA was incubated with microtiter wells coated with the αC-fragment. Bound protein was detected with polyclonal antibodies against plasminogen. Results in both panels are the mean ± SD of three independent determinations.

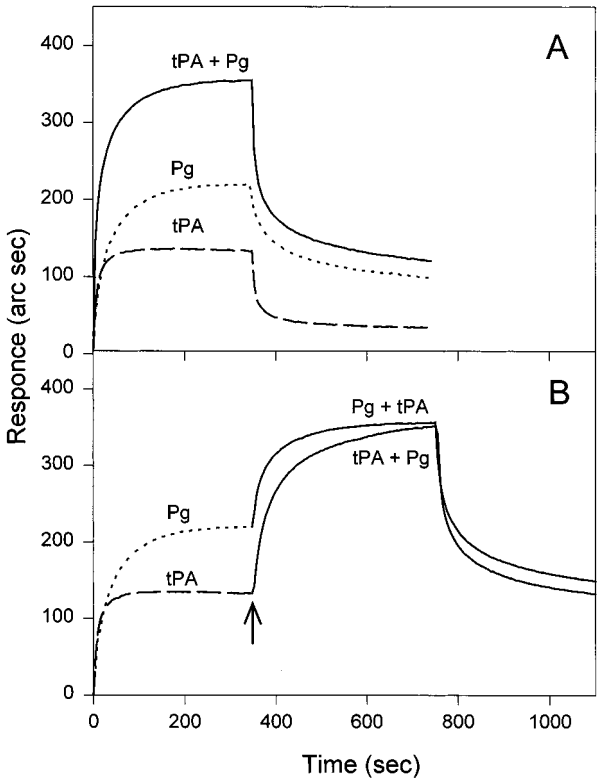

FIGURE 7: Competition experiments on the binding of plasminogen and tPA to the immobilized αC-fragment performed by surface plasmon resonance. Panel A: plasminogen (dotted curve) or tPA (broken curve) at saturating concentration (0.2 μM each) or their mixture at the same concentration of each component (solid curve) was added to the immobilized αC-fragment, and their association/dissociation was monitored in real time while registering the resonance signal. Panel B: plasminogen (dotted curve) or tPA (broken curve) at saturating concentration (0.2 μM each) was added to the immobilized αC-fragment followed by the addition of their mixture at the same concentrations (solid curve) at a time indicated by the arrow.

role of the αC-domains in fibrinolysis, we prepared the isolated αC-domain and its truncated variants by a recombinant method and tested directly their effect on the activation of plasminogen by tPA and their interaction with these proteins.

It is well established that each fibrin(ogen) D region contains two cryptic sites that include sequences Aα148−160 and γ312−324 and are associated with the stimulating properties of fibrin and its fragments (*17, 19*−*21, 40*). Binding of plasminogen and tPA to these sites results in generation of plasmin and initiation of fibrinolysis, while subsequent cleavage of fibrin with newly formed plasmin results in generation of COOH-terminal Lys residues that are involved in propagation of fibrinolysis (*45*−*49*). In this study, we found that the αC-domain also contains cryptic tPA- and plasminogen-binding sites in its COOH-terminal half and can stimulate effectively the activation of plasminogen by tPA. Binding of both tPA and plasminogen to the isolated αC-domain occurs with high affinity with K_ds in the range of 13−47 nM (Table 1). The reported affinities for tPA- and plasminogen-binding sites of the D region vary depending on the conditions and methods employed (*16, 21, 34*); our measurements using SPR, a technique employed in this study, resulted in K_ds of 0.6−1.2 μM for both plasminogen and tPA (*34*); i.e., their affinity is much lower than that determined here for the novel sites in the αC-domains. This indicates that the tPA- and plasminogen-binding sites in the αC-domains may contribute substantially to the initial stage of fibrinolysis.

Another important finding of this study is that degradation of the isolated αC-domain with plasmin abolishes its stimulating effect. The loss of stimulating activity may be due to the loss of one or both binding sites or due to their separation and may play an important physiological role. It is well established that the αC-domains are very sensitive to proteolysis. In fact, they are removed from fibrinogen or fibrin at the early stage of fibrinolysis before fibrin(ogen) is split into soluble D (or cross-linked D−D) and E fragments. However, in contrast to these fragments which are resistant to further digestion, the αC-domains are degraded into a smaller fragments. The first point of plasmin attack occurs after Lys583 and then after Lys206 and Lys230, resulting in a 40 kDa fragment that then is quickly degraded into fragments of half the size (*44*, and references cited therein). The time-course of digestion presented in the inset in Figure 8B is in good agreement with this scheme. Interestingly, the largest plasmin-resistant fragment comprising residues

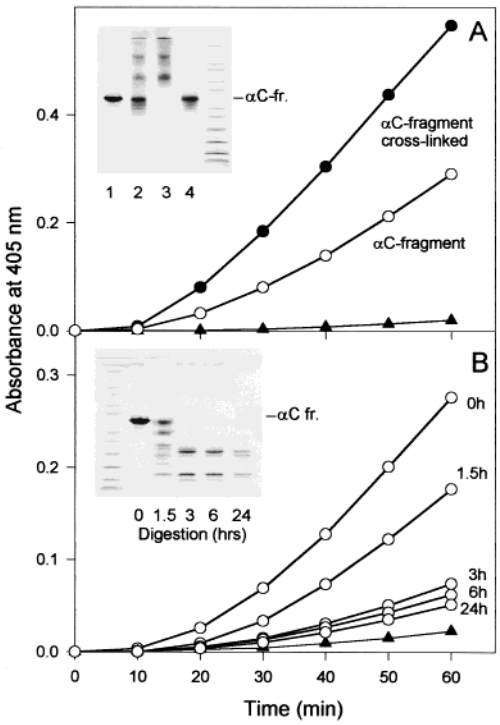

FIGURE 8: Effect of factor XIIIa-catalyzed cross-linking and plasmin digestion on the stimulating activity of the αC-fragment. Panel A represents the stimulating effect of the non-cross-linked (open circles) and cross-linked by factor XIIIa (closed circles) αC-fragment. The SDS−PAGE patterns of the αC-fragment (lane 1), the reaction mixture after 6 h of incubation with factor XIIIa (lane 2), and the separated cross-linked (lane 3) and non-cross-linked (lane 4) αC-fragments are presented in the inset. Panel B represents stimulating effects of the intact αC-fragment (0 h) and its plasmin digest at 1.5, 3, 6, and 24 h (open circles). The inset shows the time-course of digestion of the αC-fragment with plasmin. The outer lanes in each inset contain molecular mass markers of 3.5, 6, 14.4, 21.5, 31, 36.6, 55.4, 66.3, 97.4, 116.3, and 200 kDa. The stimulating effect was measured by hydrolysis of chromogenic substrate S-2251 with newly formed plasmin as described under Experimental Procedures. The activation of plasminogen in the absence of stimulators is shown by triangles in both panels.

Aα254−425 includes most of the NH_2-terminal half of the αC-domain which in our experiments did not bind plasminogen or tPA and did not exhibit the stimulating activity, while the COOH-terminal half which contains these sites is degraded into smaller fragments. Our finding that the αC-domains stimulate activation of plasminogen and that their degradation with plasmin abolishes this function seems to explain the physiological significance of their high sensitivity to proteolysis. First, it suggests that the proteolytic removal of the αC-domains may reduce the stimulating activity of fibrin, thus contributing to the regulation of the early stages of fibrinolysis. Second, further degradation of the liberated 40 kDa αC-fragment seems to be required to inactivate its stimulating effect and to prevent further generation of plasmin in plasma that could cause fibrinogenolysis.

Both of these novel sites are cryptic in fibrinogen and are exposed in the isolated αC-domains and presumably in fibrin. The previously characterized tPA- and plasminogen-binding sites of the D regions are also cryptic, and their exposure is triggered by the DD:E interactions that result in fibrin formation (*17*, *19*, *34*, *50*). The mechanism of exposure of the tPA- and plasminogen-binding sites may be different. It was suggested that in fibrinogen two αC-domains interact with each other and that they switch from intra- to intermolecular interaction in fibrin (*51*−*53*), resulting in the formation of factor XIIIa-cross-linked αC-polymers (*35*, *44*). This mechanism may provide the exposure of the tPA- and plasminogen-binding sites in the αC-domains and generation of their high stimulating activity. One can speculate that in fibrinogen both sites may be located on or near the interacting interfaces and that they become exposed when the αC-domains are isolated or when they dissociate in fibrin. However, it is still unclear whether dissociation of the αC-domains alone is sufficient for the exposure of these sites and generation of the stimulating activity or if formation of the αC-polymers is also required. In this respect, it should be noted that the cross-linked αC-fragments tested in this study exhibited almost 2-fold higher stimulating activity than the non-cross-linked ones (Figure 8A). More studies are required to clarify the relationship between the conformational changes in the αC-domains upon conversion of fibrinogen to fibrin and the exposure of their function-related cryptic binding sites.

In summary, in this study we identified and characterized novel tPA- and plasminogen-binding sites in the fibrinogen αC-domains. That these sites are cryptic in fibrinogen, that the αC-domains bind tPA and plasminogen with high affinity and effectively stimulate activation of the latter, and that this stimulating effect is abolished upon their degradation with plasmin suggest that these domains play an important role in regulation of fibrinolysis.

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