Regulation of Plasmin-dependent Fibrin Clot Lysis by Annexin II Heterotetramer*

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In a previous report we showed that plasmin-dependent lysis of a fibrin polymer, produced from purified components, was totally blocked if annexin II heterotetramer (AIIt) was present during fibrin polymer formation. Here, we show that AIIt inhibits fibrin clot lysis by stimulation of plasmin autodegradation, which results in a loss of plasmin activity. Furthermore, the C-terminal lysine residues of its p11 subunit play an essential role in the inhibition of fibrin clot lysis by AIIt. We also found that AIIt binds to fibrin with a $K_d$ of 436 nM and a stoichiometry of about 0.28 mol of AIIt/mol of fibrin monomer. The binding of AIIt to fibrin was not dependent on the C-terminal lysines of the p11 subunit. Furthermore, in the presence of plasminogen, the binding of AIIt to fibrin was increased to about 1.3 mol of AIIt/mol of fibrin monomer, suggesting that AIIt and plasminogen do not compete for identical sites on fibrin. Immunohistochemical identification of p36 and p11 subunits of AIIt in a pathological clot provides important evidence for its role as a physiological fibrinolytic regulator. These results suggest that AIIt may play a key role in the regulation of plasmin activity on the fibrin clot surface.

The formation of a fibrin polymer from its soluble precursor, fibrinogen, results from the hydrolytic catalysis of fibrinogen by thrombin, the terminal proteolytic enzyme in the coagulation cascade (1–3). The (patho-)physiological existence of fibrin is linked to its homeostatic roles, such as temporary matrix formation in wound healing and the formation of a hemostatic plug. Accumulation of fibrin is a hallmark of a variety of diseases, such as cancer and arteriosclerosis (4). The polymerization of the fibrin monomer and the degradation of the fibrin polymer are physiologically balanced by the coagulation and fibrinolytic systems, respectively (5–9). Dissolution of a fibrin clot is mainly conducted by plasmin, the terminal enzyme of the fibrinolytic cascade. Plasmin, an 85-kDa serine protease, is involved in a variety of physiological and pathological processes, including fibrinolysis, wound healing, tissue remodeling, embryogenesis, and the invasion and spread of transformed tumor cells (5, 8, 10–16). Active plasmin is produced from two serial activation processes. First, [Glu]plasminogen is converted to the more reactive [Lys]plasminogen by plasmin itself through proteolytic removal of the N-terminal 77 amino acids. Second, plasmin also activates the plasminogen activators, tissue-type plasminogen activator (tPA)1 and urokinase-type plasminogen activator, which, in turn, convert [Lys]- or [Glu]plasminogen to plasmin (13).

It is well established that fibrin stimulates the rate of tPA-dependent plasminogen activation by at least two orders of magnitude due to the localization of plasminogen and tPA to the fibrin surface via their lysine-binding kringle domains (17, 18). Other proteins, such as the histidine-proline rich glycoproteins or certain extracellular matrix proteins that interact with the kringle domains of plasminogen, have also been shown to stimulate plasminogen activation (19–21). Typically, these interactions involve the binding of the kringle domains with the C-terminal lysine residues of the plasminogen-binding protein. Hajjar’s group recently reported that the Ca2+-binding protein, annexin II, stimulated the tPA-dependent formation of plasmin from [Glu]plasminogen or [Lys]plasminogen 20-fold or 14-fold, respectively, in vitro. They also reported that plasminogen activation was inhibited on human umbilical vein endothelial cell surfaces after transfection of these cells with antisense oligonucleotides directed against annexin II mRNA, suggesting that annexin II may function as a fibrinolytic receptor for plasminogen on the surface of endothelial cells (23–27).

Annexin II was originally described as an intracellular Ca2+- and phospholipid-binding protein, and subsequent studies suggested that this protein could be involved in regulating membrane trafficking events such as exocytosis or endocytosis (28). Surprisingly, annexin II does not contain a C-terminal lysine residue, and the mechanism by which this protein interacted with plasminogen was unclear. Annexin II also exists in cells as a heterotetramer, called annexin II heterotetramer (AIIt), which consists of two annexin II molecules referred to as the p36 subunit and two molecules of an 11-kDa regulatory subunit referred to as the p11 light chain. Importantly, p11 contains two lysine residues at its C terminus. Ninety to ninety-five percent of the total cellular annexin II is present in the heterotetrameric form in many cells such as Madin-Darby canine kidney cells, bovine intestinal epithelial cells, and calf pulmonary arterial endothelial cells (29, 30).

Recently, it was established that AIIt binds tPA, plasminogen, and plasmin and stimulates tPA-dependent plasminogen activation about 300-fold in vitro (31). This activation was mediated by binding of plasminogen kringle domains to the

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2The abbreviations used are: tPA, tissue-type plasminogen activator; AIIt, annexin II heterotetramer; e-ACA, e-aminoacaproyic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; p-NA, p-nitroanilide.
C-terminal lysine residue of the p11 subunit, suggesting that the p11 subunit facilitates the majority of the AIIt-mediated stimulation of tPA-dependent plasminogen activation. Furthermore, immunofluorescence and coimmunoprecipitation experiments established the colocalization of p11 and annexin II on the surface of human umbilical vein endothelial cells (31), BT20 human breast carcinoma cells, U87 human glioblastoma cells (32, 33), and fibrosarcoma cells. ² Taken together, these data provided evidence that AIIt played a key role in plasminogen activation on the cell surface.

We recently reported that AIIt inhibited the fibrinolytic ac-

² K.-S. Choi et al., manuscript in preparation.
tivity of plasmin (34). These experiments were conducted using fibrin clots generated from purified components in a cell-free system (34). We also demonstrated that AIIt associated with the fibrin clot in vitro (34). In a separate series of experiments, we showed that preincubation of AIIt and plasmin resulted in the stimulation of the autocatalytic digestion of the plasmin heavy and light chains, resulting in a decrease in plasmin activity (31). It was therefore unclear from our results if the antifibrinolytic activity of AIIt was due to its stimulation of plasmin autodegradation or due to its ability to associate with fibrin thereby sterically hindering the interaction of plasmin with fibrin.

In the present report, we demonstrate that the antifibrinolytic activity of AIIt is due to its stimulation of plasmin autodegradation by a mechanism that requires the C-terminal lysine residues of the p11 subunit.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Bovine intestinal mucosa heparin (3 kDa, 50 USP units/mg), e-aminocaproic acid (e-ACA), and thrombin (2300 NIH units/mg) were obtained from Sigma Chemical Co. Human recombinant tPA was obtained from Genentech, was purified by chromatography on benzamidine-Sepharose (35), and was an 80–90% single chain as determined by SDS-PAGE. [Glu]plasminogen, [Lys]plasminogen, plasmin, the amidolytic plasmin specific substrate, Spectrozyme 251 (H-n-norleucyl-hexahydrotroxylysyl-lysine-p-nitroanilide), and the tPA amidolytic substrate Spectrozyme 444 (methyl-D-cyclohexanyl-glycyl-arginine paranitroaniline acetate) were obtained from American Diagnostica. Fibrinogen was obtained from Sigma and further purified by chromatography on Superose 12 (Amersham Pharmacia Biotech) to remove contaminating plasminogen.

**Annexin II tetramer (AIIt)** was prepared from bovine lung as previously described (36). Recombinant annexin II monomer and p11 subunits were expressed in Escherichia coli as described previously (37, 38). Purification of recombinant annexin II from bacterial lysates involved chromatography on hydroxyapatite (Bio-Rad), affinity chromatography on heparin-Sepharose, and gel permeation chromatography on Superose 12. Recombinant AIIt was formed by mixing equimolar annexin II and p11 followed by gel permeation chromatography on Superose 12. All proteins were stored at –70 °C in 40 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 0.1 mM EGTA, and 150 mM NaCl. Anti-p11 and annexin II monoclonal antibodies were purchased from Transduction Laboratories.

**Mutagenesis of Annexin II**—The bacterial expression vector (pDS10) encoding the cDNA of human total calcium mutant annexin II was kindly provided by Dr. Volker Gerke. This vector has all five Ca$^{2+}$-
binding sites of annexin II inactivated and has the mutation A65E in the annexin II sequence to provide an epitope for antibody recognition.

Production of the pl1 Subunit Deletion Mutant—The cDNA of the pl1 subunit deletion mutant (lacking the two C-terminal lysine residues) was produced by PCR mutagenesis as previously described (38). The cDNA expression construct, pAED4.91-pl1del KNK, was confirmed by DNA sequencing to lack Lys-95 and Lys-96. The expression of the pl1del KNK in BL21(DE3) E. coli and the subsequent purification were performed as previously described (37).

Fibrin Polymer Lysis Assay—Fibrin polymer lysis experiments were performed in a volume of 400 μl at 25 °C using a Beckman DU 640 spectrophotometer to monitor changes in turbidity at 450 nm (34). Initially, fibrin clots were formed by incubation of 8 μM fibrinogen with 0.83 NIH units/ml thrombin in buffer A (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 5 mM CaCl2) at room temperature. After turbidity had reached a stable level, fibrin clot lysis was triggered by gently layering 200 nM radioiodinated plasminogen and 25 nM tPA with or without 1 μM AIIt. After 12 h of incubation at 25 °C, 10 μl of each reaction was subjected to SDS-PAGE, and the pattern of plasminogen or plasmin degradation was detected by autoradiography. The autoradiographs were quantitated by densitometric analysis using a Hewlett Packard ScanJet 4c flatbed scanner and Adobe Photoshop 5.5.

Fibrin Binding Assay—Fibrin polymerization was initiated by addition of thrombin (5 NIH units/ml) to a series of Eppendorf tubes, preheated to 37 °C, containing 5 mM 125I-labeled AIIt, and various concentrations of unlabeled AIIt in buffer A. After incubation at 37 °C for 1 h, fibrin clots were pelleted by centrifugation at 10,000 rpm for 30 min at 4 °C. After three washes with buffer A, AIIt bound to the fibrin clot was calculated from radioactivity of clot. Inset, Scatchard plot analysis of the binding isotherm.

A inset binding to fibrin. AIIt was radioiodinated as described “Experimental Procedures.” Fibrin polymerization was initiated by addition of thrombin (5 NIH units/ml) to a series of Eppendorf tubes, preheated to 37 °C, which contained 648 nM fibrinogen, 4.2 nM 125I-labeled AIIt, and various concentrations of non-labeled AIIt in buffer A. After incubation at 37 °C for 1 h, fibrin clots were pelleted by centrifugation at 10,000 rpm for 30 min at 4 °C. After three washes with buffer A, AIIt bound to the fibrin clot was calculated from radioactivity of clot. Inset, Scatchard plot analysis of the binding isotherm.
tPA-mediated plasminogen activation in the absence or presence of AIIt on fibrin was determined by measuring amidolytic activity of the plasmin generated during activation of plasminogen. The reaction was performed with the substrate H-D-norleucyl-hexahydrotyrosyl-lysine-p-nitroanilide (Spectrozyme 251) at a final concentration of 100 μM, and in buffer A containing 2 nM tPA in the absence or presence of 1 μM AIIt. Alternatively, AIIt-stimulated plasmin formation on the fibrin clot was examined in the presence of various concentrations of AIIt. The reaction was initiated by the addition of 0.1 μM [Glu]plasminogen and was monitored at 405 nm in a PerkinElmer Life Sciences HTS 7000 Bioassay reader.

Data Analysis—Initial rates of plasmin generation were calculated using linear regression analysis of plots of A_{405 nm} versus time squared as previously outlined (43). Linear regression was performed with the

FIG. 5. Analysis of the interaction of AIIt with fibrin. Fibrin was polymerized by incubation of 648 nM fibrinogen with thrombin (5 NIH units/ml) in the presence of 4.2 nM 125I-labeled AIIt and various concentrations of heparin (A), TCM AIIt (B), and AIIt(K-K)^− (C). The binding was assessed as described under “Experimental Procedures.”
Role of AIIt in Fibrinolysis

Effects of Plasminogen on AIIt Binding to Fibrin in the Absence or Presence of e-ACA—Fibrinogen was polymerized by incubation of 648 nm fibrinogen with thrombin (5 NIH units/ml) in buffer A containing 4.2 nm 125I-labeled AIIt, 2 μM unlabeled AIIt, and various concentrations of plasminogen in the absence (filled circles) or presence (open circles) of 10 mM e-ACA. The binding was assessed as described under “Experimental Procedures.”

As shown in Fig. 1, the two respective processes of fibrin polymerization (clotting) and fibrinolysis are indicated by an initial rapid increase in turbidity to a plateau value (polymer formation) and, after addition of plasmin to the fibrin polymer, a subsequent return to the value of nonpolymerized material (fibrinolysis). Lysis of the fibrin clot was completely abolished in the presence of AIIt. Interestingly, the AIIt-mediated inhibition of plasmin fibrinolytic activity was blocked by glycerol in a dose-dependent manner (Fig. 1). Glycerol has been shown to inhibit plasmin autodegradation but not plasmin-dependent clot lysis (44). Our data therefore suggested that AIIt blocked fibrin clot lysis by accelerating plasmin autodegradation, thereby reducing the availability of plasmin for fibrin clot lysis.

Effects of AIIt on Plasmin Degradation during Fibrinolysis—It has been documented that plasmin autodegradation results in a loss in plasmin activity and degradation of the plasmin heavy (A) and light (B) chains. To establish if AIIt blocked fibrin clot lysis via stimulation of plasmin autodegradation, we analyzed the plasmin reaction products during fibrinolysis. Fibrinogen was incubated with thrombin, and, after 30 min, a stable fibrin clot was formed. Radiolabeled plasminogen was added to the clot, and fibrinolysis was initiated by addition of tPA. After 12 h, the reaction products were analyzed by SDS-PAGE. As shown in Fig. 2, in the presence of fibrin, tPA converted plasminogen into plasmin as illustrated by the appearance of the 55-kDa A chain and 25-kDa B chain of plasmin. In contrast, when AIIt was added to the reaction, degradation of both the plasmin A (H-Pm) and B (L-Pm) chain was apparent (Fig. 2). Densitometric quantitation of the autoradiogram showed that 11.8% of the A chain and 43.6% of the B chain of plasmin remained after incubation with 1 μM AIIt. Furthermore, in the presence of 5 μM AIIt, only 5.4% of the A chain and 35.1% of the B chain of plasmin remained (Fig. 2, inset). These results therefore establish that the inhibition of fibrin clot lysis by AIIt is due to its acceleration of plasmin autodegradation.

Domains of AIIt Responsible for the Inhibition of Plasmin-dependent Fibrin Clot Lysis—AIIt is a Ca^2+−binding protein, and binding of Ca^2+ to the p36 subunit regulates many of its biological activities (28, 46). The p36 subunit of AIIt contains five established Ca^2+−binding sites. We utilized a recombinant AIIt mutant to establish if the Ca^2+−binding sites of AIIt played a role in the inhibition of fibrin clot lysis. TCM AIIt, which has all five Ca^2+−binding sites of the p36 subunit inactivated, significantly blocked plasmin-dependent fibrin clot lysis in a similar fashion to wild-type AIIt (Fig. 3A). This suggested that the five Ca^2+−binding sites in the p36 subunit of AIIt do not play a role in the inhibitory effect of AIIt on fibrin clot lysis.
Fig. 7. Stimulation of tPA-dependent plasminogen activation by AIIt on fibrin surface. A, time course of AIIt-dependent stimulation of plasmin formation on the fibrin clot. After a stable fibrin polymer was formed, tPA-dependent plasminogen (Pg) activation was examined in the absence (open circles) or presence (triangles) of 1 μM AIIt as described under “Experimental Procedures.” Potential plasmin contamination in the plasminogen preparation was also measured (closed circles). Inset, the rate constant for the plasminogen activation was calculated from the slope.
AllIt binds plasminogen via the C-terminal lysine residues of its p11 subunit (38). Removal of the C-terminal lysines blocks the ability of AllIt to accelerate tPA-dependent plasminogen conversion to plasmin (38). To examine if the C-terminal lysines of the p11 subunit of AllIt participate in the antifibrinolytic activity of AllIt, we carried out plasmin-dependent fibrin clot lysis in the presence of recombinant mutant AllIt (AllIt(K-K)) whose two p11 C-terminal lysine residues were deleted (Fig. 3B). The results show that, unlike the wild-type and TCM AllIt, AllIt(K-K) did not block plasmin-dependent fibrin clot lysis. Therefore, these data suggested that the C-terminal lysine residues of AllIt play an important role in the inhibitory effect of AllIt on fibrin clot lysis.

Fibrin Binding Properties of AllIt—We previously demonstrated that AllIt pelleted with a fibrin clot (34). This result prompted us to characterize the binding of AllIt to the fibrin polymer. A fibrin polymer was formed by incubation of fibrinogen and thrombin with radiolabeled AllIt. The fibrin clot was centrifuged, and the AllIt binding to the fibrin clot was quantitated. Fig. 4 shows the binding isotherm for the binding of AllIt to fibrin. AllIt bound fibrin polymer in a specific and saturable manner with a $K_d$ of 269.4 ± 60.0 nM (mean ± S.D., $n = 3$). The binding approached a plateau at about 1.6 μM AllIt, which corresponded to about 0.28 mol of AllIt/mol of fibrin monomer. Scatchard analysis (Fig. 4, inset) established that the binding of AllIt to fibrin was mediated by a single, moderate affinity-binding site. The low stoichiometric binding of AllIt to fibrin could be due to steric hindrance. For example, it is possible that the binding sites in the dense areas of the clot may not be accessible to AllIt.

It was previously reported that heparin binds AllIt (47) and that it inhibits the association of AllIt with the fibrin polymer (34). As shown in Fig. 5A, heparin blocks the binding of AllIt to fibrin polymer. Inhibition of AllIt binding to fibrin by heparin was dose-dependent, and half-maximal inhibition occurred at 1.1 ± 0.2 μM heparin (mean ± S.D., $n = 3$).

To determine if the Ca$^{2+}$-binding sites of the p36 subunit or the C-terminal lysines of the p11 subunit of AllIt played a role in fibrin binding, we investigated the ability of the AllIt mutants, TCM and AllIt(K-K), to dissociate radiolabeled wild-type AllIt from the fibrin clot. It was therefore expected that the recombinant mutant AllIt would compete with the radiolabeled wild-type AllIt, unless the mutation rendered the protein unable to bind to fibrin. Fig. 5, B and C, shows that both TCM AllIt and AllIt(K-K) competitively inhibited wild-type AllIt binding to fibrin polymer with an IC$_{50}$ values of about 260 or 330 nM, respectively. Therefore, neither the Ca$^{2+}$-binding sites of AllIt nor the C-terminal lysine residues of the p11 subunit of AllIt mediate the binding of AllIt to the fibrin polymer.

Effects of Plasminogen on AllIt Binding to the Fibrin Polymer—Plasminogen exists at micromolar concentration in blood. Because plasminogen binds to both fibrin and AllIt, we examined the binding of AllIt to the fibrin clot in the presence of plasminogen (Fig. 6). The binding of AllIt to fibrin polymer was increased by addition of plasminogen in a dose-dependent manner and approached a plateau at about 1 μM plasminogen. Half-maximal binding was obtained at ~200 nM plasminogen (Fig. 6, filled circles). This binding event was also examined in the presence of a lysine analogue, ε-ACA, which inhibits the interaction between AllIt and plasminogen. The addition of 10 mM ε-ACA blocked the increased AllIt binding to fibrin polymer.

The interaction of AllIt with the fibrin polymer may therefore physiologically occur in at least one of two ways; its intrinsic binding ability (independent of the C-terminal lysine as shown in Fig. 5C), or through plasminogen-mediated additional binding capacity, which is governed by its p11 C-terminal lysine residues.

Acceleration of Plasminogen Activation by AllIt on Fibrin Surface—AllIt has been reported to stimulate both the tPA-dependent conversion of plasminogen to plasmin as well as plasmin autodigestion in vitro (39). Although our data shows that, in the presence of fibrin stimulation of plasmin autodigestion is the predominant reaction, we could not rule out the possibility that AllIt might also stimulate tPA-dependent plasmin formation. Fig. 7A shows that fibrin accelerated tPA-dependent plasmin formation 7.8-fold. This fibrin-accelerated tPA-dependent plasmin formation was increased further 6.4 times by AllIt (Fig. 7A, inset). Analysis of the dose dependence of the stimulation of tPA-dependent plasmin formation by AllIt showed that about 0.58 ± 0.10 μM AllIt was required for half-maximal stimulation of plasmin formation (Fig. 7B). This concentration range of AllIt was similar to that required for half-maximal inhibition of plasmin-dependent fibrin polymer lysis. Collectively, our data suggest that AllIt may support the turnover of plasmin at the fibrin clot surface by stimulating both plasmin formation and destruction, although under our experimental conditions the latter reaction predominates.

Immunohistochemical Identification of AllIt in a Physiological Blood Clot—AllIt or annexin II has been shown by several groups to localize to the extracellular surface of variety of cells (31–33). However, whether or not AllIt associates with fibrin under physiological conditions has not been established. Accordingly, lung tissue obtained from a patient with pulmonary thromboembolism was stained for annexin II and p11 (Fig. 8). The results show that both annexin II and p11 localize at contact areas of the fibrin clot and cell components. Thus, the association of AllIt with fibrin observed in vitro appears to be of physiological relevance.

DISCUSSION

The formation of a fibrin polymer subsequent to blood vessel injury not only conserves blood constituents but also provides temporary matrices for blood vessel growth. However, the accumulation of a fibrin clot due to unbalanced hemostasis may lead to a variety of pathological disorders, including inflammation, arteriosclerosis, or stroke. During the formation of a solid blood clot, plasmin-mediated fibrinolysis is triggered by the actions of blood vessel endothelial cells growing on the fibrin substratum during the wound healing process. This process relies on the spatial and temporal regulation of plasmin activity within the fibrin clots.

Recently, annexin II, the 36-kDa subunit of AllIt, was shown to function as a receptor for both plasminogen and tPA on the surface of endothelial cells (22). Furthermore, it has recently been shown that AllIt, but not annexin II, inhibits plasmin-dependent fibrinolysis (34) as well as accelerates both tPA-dependent plasminogen activation (31) and subsequent plasmin degradation.
Role of AII\(\text{t}\) in Fibrinolysis

autodegradation (45). In this report, we demonstrate that AII\(\text{t}\) can bind to the fibrin clot \emph{in vitro} and associates with the fibrin clot \emph{in vivo}. Furthermore, we show that AII\(\text{t}\) inhibits plasmin-dependent fibrin clot lysis by stimulation of plasmin autodegradation \emph{in vitro}.

The ability of AII\(\text{t}\) to inhibit the fibrinolytic activity of plasmin severely diminishes the concentration of active plasmin that is normally targeted for fibrin clot dissolution. This notion is supported by the finding that glycerol, an inhibitor of the plasmin autodegradation, inhibits the antifibrinolytic activity of AII\(\text{t}\) (Fig. 1). The inhibition of plasmin-dependent fibrinolysis by AII\(\text{t}\) also corresponded with plasmin heavy and light chain degradation (Fig. 2), suggesting a relationship between the antifibrinolytic activity of AII\(\text{t}\) and AII\(\text{t}\)-stimulated plasmin autodegradation. Although the stimulation of plasmin breakdown is the predominant reaction observed under our experimental condition, we have also shown that, in the presence of the fibrin clot, AII\(\text{t}\) stimulates tPA-dependent plasmin formation. This suggests that the physiological role of AII\(\text{t}\) may be to provide a transient pulse of plasmin activity on the fibrin surface.

We also examined two AII\(\text{t}\) mutants to determine the potential role of the Ca\(^{2+}\)-binding sites of the p36 subunit of AII\(\text{t}\) or of the C-terminal lysine residues of p11 subunit of AII\(\text{t}\) in its antifibrinolytic activity (Fig. 3). We first examined a mutant AII\(\text{t}\) in which all five Ca\(^{2+}\)-binding sites of the p36 subunit were inactivated (TCM AII\(\text{t}\)). This mutant was chosen because Ca\(^{2+}\) is an essential cofactor in the coagulation cascade, and it was, therefore, reasonable to suspect that the Ca\(^{2+}\)-binding sites of AII\(\text{t}\) might play a role in its inhibition of fibrin clot lysis. However, as observed in Fig. 3A, the TCM AII\(\text{t}\) also inhibited plasmin-dependent fibrinolysis. Thus, the Ca\(^{2+}\)-binding sites of AII\(\text{t}\) do not play a direct role in mediating its antifibrinolytic activity.

The second mutational analysis of AII\(\text{t}\) involved the removal of the C-terminal lysine residues of the p11 subunit (AII\(\text{t}(K)\)). We have previously shown that removal of these key residues disables the ability of AII\(\text{t}\) to stimulate tPA-dependent plasminogen activation (38). These residues are believed to be involved in the binding of kringle domains of plasminogen, plasmin, and tPA. As shown in Fig. 3B, this mutant AII\(\text{t}\) does not inhibit plasmin-dependent fibrin clot lysis. This suggests that the C-terminal lysine residues of the p11 subunit of AII\(\text{t}\) participate in both the stimulation of plasmin formation and destruction by AII\(\text{t}\). In the absence of fibrin the former reaction is favored, whereas, in the presence of fibrin, the latter reaction is favored.

The profound effect of fibrin on the regulatory properties of AII\(\text{t}\) led us to examine in detail the interaction of AII\(\text{t}\) with fibrin. First we found that AII\(\text{t}\) bound fibrin with a \(K_d\) of 269.4 ± 60.0 nM (S.D., \(n = 1\)) (Fig. 4). Scatchard analysis reveals that a single saturable binding site is involved in this interaction (Fig. 4, inset). The low stoichiometry (0.28 mol of AII\(\text{t}\)/mol of fibrin monomer) probably indicates that all of the AII\(\text{t}\) binding sites in the artificial fibrin polymer formed in test tubes are not accessible to AII\(\text{t}\). Second, we found that the interaction of AII\(\text{t}\) with fibrin was inhibited by heparin as an IC\textsubscript{50} of 1.1 ± 0.2 \(\mu\)M (S.D., \(n = 1\)). The heparin binding region is located at residues 306–313 of the p36 subunit and may, therefore, be involved in the interaction of AII\(\text{t}\) with fibrin (Fig. 5A). Third, using the TCM AII\(\text{t}\) and the AII\(\text{t}(K)\) mutants, we found that the binding of AII\(\text{t}\) to fibrin does not involve the Ca\(^{2+}\)-binding sites of the p36 subunit nor the C-terminal lysine residue of the p11 subunit (Fig. 5, B and C). Fourth, we found that in the presence of plasminogen the binding of AII\(\text{t}\) to fibrin was actually increased (Fig. 6). The simplest explanation for this observation was that AII\(\text{t}\) bound to fibrin and to fibrin-associated plasminogen. This was an important finding, because it ruled out the possibility that AII\(\text{t}\) and plasminogen competed for similar sites on the fibrin polymer. Fifth, we demonstrated the localization of AII\(\text{t}\) to a thrombus \emph{in vivo}.
This established that under physiological conditions AIIt associated with fibrin (Fig. 8).

The binding of these components to the p11 subunit of AIIt localizes tPA, plasminogen, plasmin, and AIIt to the fibrin surface. It is therefore reasonable to assume that the mechanism by which the C-terminal lysines of the p11 subunit of AIIt stimulates plasmin autodegradation is due to the spatial localization of plasmin to the C-terminal lysine binding sites of AIIt thus resulting in a localized concentration of plasmin at these sites. It is also possible that the interaction of plasmin with the C-terminal lysines of the p11 subunit of AIIt may induce a conformational change in plasmin resulting in stimulation of plasmin autodegradation.

Our results suggest that the inhibition of plasmin-dependent fibrinolytic activity by AIIt requires both the interaction of AIIt with the plasminogen activation system (enzyme system) and with the fibrin polymer (substrate components). These binding events appear to localize tPA, plasminogen, plasmin, and AIIt to the fibrin surface. The demonstration of AIIt-stimulated tPA-dependent plasminogen activation on the fibrin surface suggests that AIIt may transiently increase plasmin activity on the fibrin surface (Fig. 7). It is clear that, in the presence of fibrin, AIIt stimulates both plasmin formation and destruction. However, under our experimental conditions, the AIIt-mediated destruction of plasmin predominates. We cannot, however, rule out the possibility that other regulators of AIIt may be present at or in the vicinity of the fibrin clot. These putative regulators could influence whether or not plasmin formation or destruction is the predominant reaction stimulated by AIIt.

The above results, along with previous findings, form the basis for a model of the mechanism of plasmin regulation by AIIt (Fig. 9). AIIt bound to fibrin is capable of accelerating the tPA-dependent formation of plasmin from plasminogen bound either to fibrin or AIIt itself. The ability of AIIt to bind fibrin, plasminogen, and tPA allows recruitment of plasminogen and tPA to the fibrin surface and thus spatially regulates plasmin production. Moreover, AIIt also accelerates the autodegradation of plasmin subsequent to its activation. The result is a tight regulation of plasmin activity on the fibrin surface. It is possible that the dual role of AIIt in plasmin formation and destruction provides the fibrin surface with a transient pulse of proteolytic activity. This allows for dissolution of the fibrin clot and prevents constitutive activation of plasminogen, which is also detrimental to hemostatic balance.

In summary, the present report establishes that the ability of AIIt to inhibit plasmin-dependent fibrin clot lysis is due to its acceleration of plasmin autocatalytic activity and therefore the destruction of plasmin. Additionally, AIIt accelerates tPA-dependent plasmin formation, although under our experimental conditions plasmin destruction is the predominant reaction stimulated by AIIt. Furthermore, AIIt and plasminogen form a complex on the fibrin polymer, in vitro. The data also establish for the first time that AIIt is present on the surface of a fibrin clot in vivo. These novel findings suggest that AIIt may play a central role in regulation of plasmin activity on the fibrin surface.