The Role of Annexin II Tetramer in the Activation of Plasminogen*

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Annexin II tetramer (AIIt) is a major Ca²⁺-binding protein of endothelial cells which has been shown to exist on both the intracellular and extracellular surfaces of the plasma membrane. In this report, we demonstrate that AIIt stimulates the activation of plasminogen by facilitating the tissue plasminogen activator (t-PA)-dependent conversion of plasminogen to plasmin. Fluid-phase AIIt stimulated the rate of activation of [Glu]plasminogen about 341-fold compared with an approximate 6-fold stimulation by annexin II. AIIt bound to [Glu]plasminogen(S741C-fluorescein) with a $K_d$ of 1.26 ± 0.04 μM (mean ± S.D., n = 3) and this interaction resulted in a large conformational change in [Glu]plasminogen. Kinetic analysis established that AIIt produces a large increase of about 190-fold in the $k_{cat, app}$ and a small increase in the $K_{app}$, which resulted in a 90-fold increase in the catalytic efficiency ($k_{cat}/K_m$) of t-PA for [Glu]plasminogen. AIIt also stimulated the t-PA-dependent activation of [Lys]plasminogen about 28-fold. Furthermore, other annexins such as annexin I, V, or VI did not produce comparable activation of t-PA-dependent conversion of [Glu]plasminogen to plasmin. The stimulation of the activation of [Glu]plasminogen by AIIt was Ca²⁺-independent and inhibited by ε-aminocaproic acid. AIIt bound to human 293 cells potentiated t-PA-dependent plasminogen activation. AIIt that was bound to phospholipid vesicles or heparin also stimulated the activation of [Glu]plasminogen 5- or 11-fold, respectively. Furthermore, immunofluorescence labeling of nonpermeabilized HUVEC revealed a punctated distribution of AIIt subunits on the cell surface. These results therefore identify AIIt as a potent in vitro activator of plasminogen.

One of the major physiological functions of the proteolytic enzyme plasmin is the degradation and solubilization of fibrin, the major constituent of blood clots. Plasmin has a broad trypsin-like specificity and the production of plasmin from its precursor plasminogen is precisely regulated (reviewed in Refs. 1–5). One way in which plasmin activity is localized to the fibrin clot involves a fibrin-specific mechanism for the conversion of plasminogen to plasmin by tissue-type plasminogen activator (t-PA).1 For example, recent studies have shown that by virtue of its ability to bind both t-PA and plasminogen, fibrin acts as a template that promotes the formation of a t-PA-fibrin-plasminogen ternary complex. The catalytic efficiency of t-PA-dependent conversion of plasminogen to plasmin is determined by the stability of the ternary complex (6). Thus, fibrin is both a substrate of plasmin and a template for plasmin production. Fibrin also plays a role in the plasmin-dependent stimulation of plasmin formation. For example, the partial proteolysis of fibrin results in the transient generation of new carboxy-terminal lysine residues that act as high affinity binding sites for the lysine-binding sites of plasminogen (7, 8). The partially proteolyzed fibrin, but not intact fibrin, also stimulates the plasmin-dependent conversion of [Glu]plasminogen to [Lys]plasminogen (9). Since [Lys]plasminogen is more rapidly converted by t-PA to plasmin than [Glu]plasminogen, this results in a substantial enhancement in plasmin formation. Other proteins that interact with the lysine-binding sites of plasminogen such as the histidine-proline-rich glycoprotein or certain proteins of the extracellular matrix have also been shown to stimulate plasminogen activation (10–12).

Recently, the endothelial cell-surface Ca²⁺-binding protein, annexin II, has also been shown to stimulate the t-PA-dependent formation of plasmin from plasminogen (13, 14). Annexin II was originally identified as an intracellular Ca²⁺- and phospholipid-binding protein and subsequent studies suggested that this protein was potentially involved in the regulation of membrane trafficking events such as exocytosis or endocytosis (reviewed in Ref. 15). Annexin II can exist in cells as both a monomer or as a heterotetramer. The heterotetramer, called annexin II tetramer (AIIt) consists of two annexin II molecules and two molecules of an 11-kDa regulatory subunit referred to as the p11 light chain. The binding of the p11 light chain regulates many of the in vitro activities of annexin II and the biochemical properties of AIIt are distinct from the annexin II monomer (16, 17). In many cells such as Madin-Darby canine kidney cells, bovine intestinal epithelial cells, and calf pulmonary arterial endothelial cells, 90–95% of the total cellular annexin II is present in the heterotetrameric form (18, 19). Annexin II and AIIt have been shown to exist on the extracellular surface of many cells although the relative extracellular distribution of the two forms of the protein has not been quantified (13, 20–23).

In the present report, we have compared the kinetics of annexin II and AIIt-dependent activation of t-PA-mediated plasminogen activation. These experiments establish the presence of AIIt on the HUVEC surface and that AIIt is a potent in vitro activator of t-PA-mediated plasminogen activation.

EXPERIMENTAL PROCEDURES

Materials—Fibrinogen was obtained from Sigma and further purified by gel permeation chromatography on Superose 12 to remove PAGE, polyacrylamide gel electrophoresis; HUVEC, human umbilical vein endothelial cells; PBS, phosphate-buffered saline.
containing plasminogen. Human recombinant t-PA was obtained from Genentech and was 80–90% single chain as determined by SDS-PAGE. [Gluplasminogen, [Lys]plasminogen, plasmin, the amidoctylic plasmin-specific substrate, Spectrozyme 251 (H-o- norleucyl-hexahydro-tyrosyl-lysine-p-nitroanilide), and the t-PA amidoctylic substrate Spectrozyme 444 were obtained from Calbiochem and had an average molecular mass of 17 kDa and an activity of 149 USP units/mg. Cyanogen bromide-digested fibrinogen was prepared as described (25). Recombinant human plasminogen (S741C-fluorescein) was a generous gift from Dr. Michael E. Nesheim (Departments of Biochemistry and Medicine, Queen's University, Kingston, Ontario, Canada).

**Plasminogen Activation Assay**—The kinetics of t-PA-mediated plasminogen activation were determined by measuring amidoctylic activity of the plasmin generated during activation of plasminogen. The reaction was performed with the substrate Spectrozyme 251 at a concentration of 104 μM, and in a buffer consisting of 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM CaCl2, and 5.6 mM t-PA. The reaction was conducted at 25 °C in a final volume of 0.6 ml. The reaction was initiated by the addition of 0.11 μM [Gluplasminogen and the reaction progress was monitored at 405 nm. In the absence of t-PA, plasmin generation did not occur regardless of the presence or absence of annexin II or AIIt (data not shown). Since phospholipid was found to affect plasmin activity, the rate of plasminogen activation in the presence of phospholipid was corrected according to the equation: 

\[ R(405 \text{ nm} / 10 \text{ min}) = R0(405 \text{ nm} / 10 \text{ min}) - \frac{A_0}{A_{\text{Hill}}(405 \text{ nm} / 10 \text{ min})} \]

where \( R_0 \) is the rate of plasminogen activation, and \( A_0 \) refers to the plasmin activity measured in the absence or presence (\( A \)) of phospholipid.

Alternatively, the t-PA-dependent activation of plasminogen was measured with recombinant human plasminogen, in which the serine of the active site of plasmin had been replaced by cysteine and labeled with fluorescein plasminogen (S741C-fluorescein). The reaction was conducted at 20 °C in a reaction mixture consisting of 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 1 mM CaCl2.

**Amidolytic Activity Assay**—Plasmin activity was directly measured with the substrate Spectrozyme 251 at a concentration of 104 μM t-PA amidoctylic substrate, Spectrozyme 444 under identical conditions to that of plasmin amidoctylic activity assay.

**Data Analysis**—Initial rates of plasminogen generation were calculated using linear regression analysis of plots of A_{max} versus t as outlined in Ref. 26. Linear regression was performed with the computer program SigmaPlot (Jandel Scientific) and time course data were analyzed according to the equation describing the rate of p-nitroanilide production from a mixture of t-PA, plasminogen, and H-o-norleucyl-hexahydro-tyrosyl-lysine-p-nitroanilide: \( \frac{dA_{\text{meas}}}{dt} = k \times [\text{t-PA}] \times [\text{plasminogen}] \times \frac{B}{K + [\text{plasminogen}]} \), where \( B \) is the rate constant for the acceleration of p-nitroanilide generation and \( K \) is the y intercept. Under our experimental conditions, \( B \) was proportional to the initial rate of plasmin formation from plasminogen. Typically, the initial rates of plasmin generation were reported in units of A_{max} nm/min^2 × 10^10.

Nonlinear least-squares fitting was performed with the computer program SigmaPlot. Titration data were analyzed as detailed in the SigmaPlot reference manual with the Hill equation \( k = k_0 \times (1 + [B])/K + [B] \), where \( k_0 \) is the t-PA turnover number and \( K_0 \) is the Hill coefficient. The nonlinear least squares curve fitting was then iterated by allowing the three fitting parameters to float while utilizing the Marquardt method for the minimization of the sum of the squared residuals. Typically results are representative of at least three experiments. The value for \( K \) was fixed as described according to Ref. 26 following the equation: \( B/2 = 0.5 \Delta A_{\text{max}}(1 + [\text{t-PA}]) \), where \( \Delta A_{\text{max}} = 10,500 \) (28), [t-PA] = 5.6 mM, and \( k_0 \), the plasmin turnover number was calculated from a standard curve of plasmin amidoctylic activity as 7.29 s⁻¹.

**Fluorescence binding data** were analyzed with the four parameter logistic equation: \( f = (a-d)/(1 + (w/xc)^d) + d \), where \( a \) asymptotic maximum, \( n \) is Hill coefficient, \( c = \) value at inflection point \( (K_0) \), \( d \) parameter, and \( w \) was asymptotic minimum. The nonlinear least squares curve fitting was then iterated by allowing the four fitting parameters to float while utilizing the Marquardt method for the minimization of the sum of the squared residuals.

**Immunocytochemistry**—HUVEC (ECV304) were obtained from ATCC (CRL-1998) and cultured as per the supplier’s instructions. The cells were seeded on fibronectin-coated glass slides. Cells were rinsed three times with PBS (137 mM NaCl, 8 mM NaHPO4, 1.4 mM KH2PO4, 2.7 mM KCl, pH 8.0) and fixed with 4% formaldehyde for 10 min on ice. Alternatively, washed cells were incubated with primary antibody for 2 h prior to fixation. Fixed cells were blocked with 3% bovine serum albumin in PBS at 4 °C overnight. Typically, antibodies were applied to cells for 2 h at a concentration of 1 μg/ml in PBS and 3% bovine serum albumin at room temperature. For colocalization experiments, rabbit anti-p11 antibody and mouse monoclonal anti-annnexin II antibody were incubated with cells. Cells were washed three times with PBS and incubated with secondary antibodies. For p11 detection, Cy3-conjugated goat anti-rabbit antibody was used as secondary antibody. For p38 detection, cells were incubated with biotin-conjugated goat anti-mouse antibody followed by rhodamine green-conjugated monoclonal anti-biotin antibody. Cells prepared in parallel were also stained with mouse anti-vimentin antibody. Coverslips were mounted in a solution of Prolong Antifade (Molecular Probes) and visualized using a Zeiss Axioskop microscope and a Kodak Megaplus (KAF-1800) digital camera.

**Immunoprecipitation**—Cells were plated in 24-well plates, and the supernatant was removed, quenched with 4 mM sodium acetate, pH 3.8, and the immunoprecipitation was performed as described in Ref. 30. Immunoprecipitation data were analyzed with the four parameter logistic equation: 

\[ f = (a-d)/(1 + (w/xc)^d) + d \]

where \( a \) asymptotic maximum, \( c = \) value at inflection point \( (K_0) \), \( d \) parameter, and \( w \) was asymptotic minimum. The nonlinear least squares curve fitting was then iterated by allowing the four fitting parameters to float while utilizing the Marquardt method for the minimization of the sum of the squared residuals.

**Immunoprecipitation and Western Blotting**—Immunoprecipitation of HUVEC extracts was preceded by desalting the total cellular extract (Sephadex G-25) with 20 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 0.25 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml antipain. The desalted extract (2 mg) was precipitated with 20 μl of protein A Plus-agarose beads (Santa Cruz Biotechnology) and then incubated with 4 μg of mouse monoclonal anti-annnexin II antibody (Calbiochem) for 2 h on ice. The mixture was then incubated at 4 °C with 50 μl of protein A Plus-agarose beads overnight on a platform rotator, centrifuged, and the pellet washed five times with PBS containing 0.5% Tween 20. The pellets were washed twice in PBS and resuspended in 10 ml of CD buffer (40 mM Tris, pH 7.5, 150 mM NaCl, 3% Triton X-100, 60 mM N-octyl-β-D-glucopyranoside, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 0.2 mM PMSF, and 0.2 mM EDTA). Lysates were preclreated and subjected to immunoprecipitation.

**Molecular fractionation—**AIIIT concentration was determined spectrophotometrically using an extinction coefficient A_{280 nm} = 0.68 for 1 mg/ml AIIIT. Phospholipid liposomes were prepared daily according to Ref. 26 for 40 μg/ml plasminogen and the reaction was conducted at 25 °C in buffer consisting of 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 5 mM CaCl2 containing 44 mM (Glu)plasminogen and 75 μM amidoctylic substrate. The reaction was initiated by the addition of 8.5 mM t-PA and at timed intervals aliquots were removed, quenched with 4 mM sodium acetate, pH 3.8, and the absorbance at 405 nm determined.

**RESULTS**

**Stimulation of t-PA-dependent Conversion of Plasminogen to Plasmin by Annexin II and AIIIT**—Annexin II has been shown to accelerate the t-PA-dependent conversion of plasminogen to plasmin. Whether or not the heterotetrameric form of annexin II, AIIIT has a similar function is unclear (13). Fig. 1 compares...
the rates of activation of [Glu]plasminogen by annexin II and AIIt. As shown in this figure, the \(A_{405\text{ nm}}\) versus \(t^2\) plot of the AIIt-dependent activation of [Glu]plasminogen was linear. In contrast to the 6-fold stimulation of [Glu]plasminogen activation by 5 \(\mu M\) annexin II, we found that 2 \(\mu M\) AIIt stimulated the activation of [Glu]plasminogen about 196-fold. The decrease in initial rates observed when the \(A_{405\text{ nm}}\) exceeded 0.8 was due to depletion of the plasmin amidolytic substrate. Increasing the amidolytic substrate concentration delayed the decrease in initial rates. Furthermore, preincubation of 2 \(\mu M\) AIIt with 50 \(\mu M\) plasmin for 30 min did not affect the potency of AIIt (data not shown).

We also examined the possibility that AIIt might directly affect either the t-PA activity or the plasmin activity. Since the t-PA amidolytic activity was minimally stimulated by 2 \(\mu M\) AIIt (20.6 \(\pm\) 4.6%; mean \(\pm\) S.D., \(n = 3\)) and the plasmin amidolytic activity was minimally inhibited by 2 \(\mu M\) AIIt (22.3 \(\pm\) 7.4%; mean \(\pm\) S.D., \(n = 5\)) these results establish that AIIt directly stimulates the t-PA-dependent activation of [Glu]plasminogen without affecting either the t-PA or plasmin activity.

Since the \(NH_2\)-terminal domain of annexin II plays an important role in the regulation of many of the in vitro activities of annexin II (reviewed in Ref. 15), we examined the possible involvement of this domain in the annexin II-dependent activation of [Glu]plasminogen. For this analysis we used recombinant annexin II, recombinant annexin II which was subjected to chymotryptic digestion to remove the first 29 residues of the \(NH_2\)-terminal domain (31) and recombinant annexin II which had six amino acids added to the \(NH_2\) terminus (17). As shown in Fig. 1, inset A-E, \(NH_2\)-terminal deleted annexin II or \(NH_2\)-terminal extended annexin II stimulates the initial rate of t-PA-dependent activation of [Glu]plasminogen 8- or 4-fold, respectively, by the protein. These data suggest that removal or extension of the \(NH_2\) terminus of annexin II has modest but significant effects on the ability of annexin II to stimulate the basal initial rate of t-PA-dependent activation of [Glu]plasminogen.

[Lys]Plasminogen has been shown to be a significant, although not essential, intermediate in the activation of [Glu]plasminogen during in vitro fibrinolysis (32). As shown in Fig. 1, inset F-H, annexin II and AIIt enhance the production of plasmin from [Lys]plasminogen 2- and 28-fold, respectively. Therefore, compared with the AIIt-dependent activation of [Glu]plasminogen, the extent of activation of plasmin production is reduced when [Lys]plasminogen is used as substrate. This decreased stimulatory effect of AIIt can be partly explained by the more rapid rate of t-PA-dependent production of plasmin from [Lys]plasminogen compared with [Glu]plasminogen.

The stimulation of [Glu]plasminogen activation by AIIt was concentration-dependent. As little as 0.1 \(\mu M\) AIIt produced a 4.5-fold stimulation of [Glu]plasminogen activation (Fig. 2). At the maximum concentration of AIIt, 10 \(\mu M\), AIIt stimulated the activation of [Glu]plasminogen about 473-fold. At concentrations greater than 10 \(\mu M\), we found that AIIt began to precipitate out of solution and therefore the AIIt concentration could not be accurately maintained.

The kinetic parameters for the activation of [Glu]plasminogen by t-PA were determined in the presence or absence of AIIt. Fig. 3 presents the results from a typical experiment. We found that the t-PA-dependent activation of plasminogen in the pres-
ence of AIIt did not obey Michaelis-Menten kinetics as evidenced by the sigmoidal plot of the activation rate versus the [Glu]plasminogen concentration. From these plots, values for the $k_{\text{cat,app}}$, $K_m$, and Hill coefficient were determined as 16.8 ± 1.75 (mean ± S.D., n = 3), 51.0 ± 6.5 nM (mean ± S.D., n = 3), and 2.52 ± 0.86 (mean ± S.D., n = 3). As expected, the activation of [Glu]plasminogen by t-PA in the absence of AIIt obeyed Michaelis-Menten kinetics. The kinetics constants obtained were 25 ± 10 nM for the $K_m$ and 0.090 s$^{-1}$ for the $k_{\text{cat,app}}$. Therefore, AIIt stimulates the t-PA-dependent activation of [Glu]plasminogen by producing a large increase of about 190-fold in the $k_{\text{cat,app}}$ and a small increase in the $A_{0.5}$. Overall, 2 μM AIIt increases the catalytic efficiency ($k_{\text{cat}}/A_{0.5}$) of t-PA for [Glu]plasminogen about 90-fold.

Characterization of the Activation of [Glu]Plasminogen by AIIt—Fig. 4A compares the rates of the activation of [Glu]plasminogen by AIIt in the presence of buffer containing Ca$^{2+}$ with the rates of the activation of [Glu]plasminogen by AIIt in the presence of Ca$^{2+}$-free buffer containing EGTA. Since the rates of [Glu] activation in the presence or absence of Ca$^{2+}$ are virtually identical, we have concluded that the activation of [Glu]plasminogen by AIIt is Ca$^{2+}$-independent.
We have also investigated the possibility that the AIIt-dependent stimulation of t-PA activity might be due to the interaction of the lysine-binding sites of t-PA or [Glu]plasminogen with lysine residues of AIIt. As shown in Fig. 4B, the lysine analogue, e-amino-n-caproic acid, stimulated the rate of t-PA-dependent activation of [Glu]plasminogen about 10-fold. In contrast, e-amino-n-caproic acid dramatically inhibited the AIIt-dependent stimulation of [Glu]plasminogen about 18-fold. Furthermore, in the presence of e-amino-n-caproic acid the rate of [Glu]plasminogen activation was only slightly stimulated by AIIt. These data therefore suggest that the stimulation of the rate of activation of [Glu]plasminogen by AIIt occurs via lysine-binding site-dependent interactions.

The stimulating effect of CNBr-digested fibrinogen on t-PA-dependent activation of plasminogen has been established by many laboratories and is thought to involve in part the interactions of newly exposed lysine residues of the CNBr-digested fibrinogen with the lysine-binding sites of plasminogen (33–35). We therefore compared the rate of CNBr-digested fibrinogen-dependent activation of plasminogen alone or in combination with AIIt. As shown in Fig. 4C, CNBr-digested fibrinogen or AIIt produced a substantial activation of plasminogen. However, when incubations contained both CNBr-digested fibrinogen and AIIt, the rate of activation of plasminogen was similar to the combined rates of plasminogen activation observed with each activator.

AIIt binds heparin and formation of the heparin-AIIt complex results in a large change in the secondary structure of AIIt (36). We therefore examined the effects of AIIt-heparin complex on t-PA-dependent plasminogen activation. As shown in Fig. 4D, heparin stimulates the rate of plasminogen activation by t-PA about 20-fold. When the incubation mixture contained both AIIt and heparin, the enhancement of plasminogen activation was similar to the combined rates of plasminogen activation observed for AIIt and heparin individually. We also observed that heparin did not affect the amidolytic activity of plasmin (data not shown). Therefore, when AIIt is present as an AIIt-heparin complex, it can accelerate t-PA-dependent plasminogen activation.

**Specificity of the Activation of [Glu]Plasminogen by AIIt**—To assess whether or not the activation of [Glu]plasminogen was specific to AIIt, we tested several annexins for a potential involvement in the regulation of t-PA-dependent conversion of [Glu]plasminogen to plasmin. As shown in Table I, when assayed at 5 μM concentration, annexins V and VI only slightly increased the rate of activation of [Glu]plasminogen. However, annexin I produced about a 15-fold stimulation of the rate of [Glu]plasminogen activation. Although the rate enhancement of [Glu]plasminogen activation produced by annexin I is small compared with the 341-fold enhancement produced by a similar concentration of AIIt, this rate enhancement is comparable to the 7-fold rate enhancement observed for the activation of [Glu]plasminogen by annexin II.

**Activation of [Glu]Plasminogen by Phospholipid-bound AIIt**—We also examined whether AIIt that was associated with phospholipid could participate in the activation of plasminogen. Our experimental conditions were chosen in such a way that 90–95% of the AIIt present in the reaction mixture was bound to phosphatidylinositol phospholipid (17, 36). Fig. 5 examines the effect of phospholipid liposomes on the stimulation of plasminogen activation by AIIt. In the presence of phosphatidylinositol liposomes, the basal rate of t-PA-dependent activation of plasminogen was stimulated about 100-fold by the addition of AIIt. In contrast, in the presence of phosphatidylcholine liposomes, the basal rate of t-PA-dependent activation of plasminogen was stimulated about 21-fold by the addition of AIIt. However, the rate of AIIt-stimulated plasminogen activation was not stimulated by the addition of phosphatidylcholine liposomes to the reaction mixture but was stimulated about 5-fold by the addition of phosphatidylinositol liposomes. Since AIIt binds to phosphatidylinositol liposomes but not to phosphatidylcholine liposomes, these results suggest that the binding of AIIt to phosphatidylinositol liposomes stimulates the ability of AIIt to activate plasminogen.

Recent work from our laboratory established that AIIt bound heparin with high affinity and specificity (36). We have also reported that the AIIt-heparin complex could be harvested by centrifugation. We therefore decided to properly examine the association of t-PA, [Glu]plasminogen, and plasmin with the AIIt-heparin complex. In the first experiment, AIIt, heparin,
t-PA, and [Glu]plasminogen were incubated together and after the [Glu]plasminogen had been converted to plasmin, the mixture was centrifuged. The t-PA and plasmin amidolytic activity present in the pellet (associated with the AIIt-heparin complex) and supernatant was quantified. As shown in Table II, under these experimental conditions, about 25% of the total t-PA amidolytic activity and 59% of the total plasmin activity were bound to the AIIt-heparin complex. In contrast, when [Glu]plasminogen was incubated with AIIt and heparin, we found that 96% of the plasminogen was bound to the AIIt-heparin complex. Furthermore, when t-PA was incubated with AIIt and heparin only about 50% was bound to the AIIt-heparin complex. In subsequent experiments, we found that under our experimental conditions, neither t-PA nor [Glu]plasminogen bound to heparin (data not shown). These results therefore demonstrate that the AIIt present in the AIIt-heparin complex binds t-PA, [Glu]plasminogen, and plasmin.

**Interaction of AIIt with Human Recombinant [Glu]Plasminogen(S741C-fluorescein)—** Recently Nesheim's group described the characterization of a variant of plasminogen referred to as [Glu]plasminogen(S741C-fluorescein) in which the serine of the plasmin catalytic site was replaced by cysteine and this cysteine labeled with fluorescein (37). The activation of this form of plasminogen by t-PA or u-PA was accompanied by a 50% quench of the fluorescence intensity of the fluorescein reporter group, indicating a substantial change in the microenvironment of the fluorescein reporter group upon cleavage of the plasminogen. An additional advantage of the [Glu]plasminogen(S741C-fluorescein) was that once cleaved to plasmin the resulting protein retained only about 1% of plasmin activity.

We originally intended to use the [Glu]plasminogen(S741C-fluorescein) as a tool to measure the AIIt-dependent activation of [Glu]plasminogen in the presence of fibrin. We expected that the addition of t-PA to a mixture containing AIIt and [Glu]plasminogen(S741C-fluorescein) would result in a rapid decrease in fluorescence compared with a mixture in which AIIt was not present. Although this rapid decrease in the fluorescence of [Glu]plasminogen(S741C-fluorescein) was observed (data not shown), quite surprisingly, we found that in the absence of t-PA, addition of AIIt directly to [Glu]plasminogen(S741C-fluorescein) resulted in a rapid decrease in [Glu]plasminogen(S741C-fluorescein) fluorescence (Fig. 6A). Furthermore, addition of t-PA to the AIIt-[Glu]plasminogen(S741C-fluorescein) complex did not further decrease the fluorescence quenching. This suggested that both the cleavage of plasminogen by t-PA and the binding of AIIt to plasminogen induced a conformational change in the microenvironment in the vicinity of the active site of plasminogen.

Analysis of the binding of AIIt to [Glu]plasminogen(S741C-fluorescein) is presented in Fig. 6B. AIIt bound to [Glu]plasminogen(S741C-fluorescein) with a $K_d$ of 1.26 ± 0.04 μM (mean ± S.D., n = 3). The interaction of the proteins appeared to be cooperative as suggested by the n of 1.74 ± 0.09 (mean ± S.D., n = 3).

**AIIt-dependent Cell Surface Plasminogen Activation—** We also examined the possibility that cell surface-bound AIIt might bind plasminogen and stimulate the activation of the enzyme. Renal epithelial 293 cells were chosen for these experiments because these cells have been demonstrated to have a low expression of extracellular annexin II (14). Furthermore, immunofluorescence of these cells confirmed that incubation of the 293 cells with AIIt results in enhanced appearance of extracellular AIIt (data not shown). As shown in Fig. 7, cells incubated with AIIt show an approximate 3-fold enhanced rate of plasmin generation. These data suggest that cell surface-bound AIIt accelerates plasminogen activation.

**Identification of AIIt on the Surface of Endothelial Cells—** Nonpermeabilized human endothelial cells were double-la-

**TABLE II**  

| Mixture | t-PA | [Glu]Plasminogen | Plasmin |
|---------|------|-----------------|--------|
| A       | Supernatant 75 | 11 |
|         | Pellet 25 | 89 |
| B       | Supernatant 4 | |
|         | Pellet 96 | |
| C       | Supernatant 51 | |
|         | Pellet 49 | |

**FIG. 5.** Phospholipid-bound AIIt stimulates plasmin generation. T-PA (5.6 nM) was incubated at 25 °C in buffer A, phospholipid vesicles and Spectrozyme 251 substrate (104 μM) in the absence (open circles) or presence of 2 μM AIIt. After 10 min the reaction was terminated by addition of a 10-fold dilution of 4 mM sodium acetate, pH 3.8, centrifuged at 14,500 × g for 10 min, and the amidolytic activity of plasmin was monitored at 405 nm as described under “Experimental Procedures.”**
FIG. 6. Interaction of AIIt with recombinant [Glu]plasminogen(S741C-fluorescein). A, [Glu]plasminogen(S741C-fluorescein) (0.11 μM) was incubated at 25 °C in buffer A and the fluorescence intensity was measured at excitation and emission wavelengths of 495 and 535 nm, respectively. After about 60 min, 2 μM AIIt (2 μM) or annexin II (5 μM) was added to the reaction. B, various concentrations of [Glu]plasminogen(S741C-fluorescein) were incubated at 25 °C in buffer A with 2 μM AIIt and after 10 min, the fluorescence intensity was measured. Fluorescence data were analyzed as described under "Experimental Procedures." The results are representative of four separate experiments.

FIG. 7. AIIt-dependent cell-surface plasminogen activation. Human embryonal kidney 293 cells were incubated in PBSC in the presence (filled circles) or absence (open circles) of 0.5 μM AIIt for 1 h. Subsequently, the cells were washed twice with PBSC and adjusted to 44 nM [Glu]plasminogen and 75 μM plasmin amidolytic substrate. The reaction was initiated by addition of 8.5 nM t-PA and the absorbance at 405 nm was determined.
beled with antibodies to annexin II and p11. Immunostaining of these cells demonstrated that both proteins were present on the surface of the HUVEC (Fig. 8, A and B). Bright punctuated clusters of antibody were observed predominately in the cell periphery. Double immunofluorescence established that the majority of annexin II co-localized with p11. Furthermore, positive staining of cells for vimentin required prior permeabilization, therefore suggesting that the cells were not lysed (data not shown). Western blotting analysis of HUVEC extracts confirmed the specificity of the anti-annexin II and anti-p11 antibodies (Fig. 8C). These data suggest that much of the extracellular annexin II was bound to p11 and therefore present as AIIt on the surface of HUVEC.

HUVEC were also surface biotinylated, lysed in CD buffer, and immunoprecipitated with mouse monoclonal anti-annexin II antibody (Fig. 8D). The anti-annexin II antibody immunoprecipitated biotinylated annexin II and p11 complex, suggesting that extracellular annexin II is present as a complex with p11.

**DISCUSSION**

The most dramatic changes in the structure and function of annexin II occur upon the binding of the p11 light chains to annexin II and hence the formation of the heterotetrameric form of the protein, AIIt. For example, the formation of the heterotetramer redistributes the protein from the cytosol to the plasma membrane and also decreases the $K_d$ (Ca$^{2+}$) for the binding of annexin II to biological membranes (reviewed in Ref. 15). Although both annexin II and AIIt have been shown to exist on the extracellular surface of many cells and AIIt has been shown to comprise 90–95% of the total pool of annexin II present in endothelial, epithelial, and Madin-Darby canine kidney cells (18, 19), the extracellular distribution of the two forms of the protein has not been extensively investigated. Furthermore, detailed characterization of the various *in vitro* activities of these proteins has established that annexin II and AIIt are functionally distinct (17). As shown in Fig. 8, A and B, annexin II and p11 co-localize to the surface of endothelial cells. Furthermore, after surface biotinylation of intact living HUVEC, anti-annexin II antibody coprecipitated p11, therefore confirming the presence of AIIt on the surface of HUVEC (Fig. 8D). These observations differ with a previous report suggesting that p11 is not present on the extracellular surface of HUVEC (38). However, this conclusion was based on the observation that Western blots of a cellular extract prepared by the washing of HUVEC with EGTA failed to stain for p11. However, as shown in Fig. 8B, cells washed with EGTA stain positive for annexin II and p11. This suggests, as has been reported by others that a portion of the binding of annexin II to membranes is Ca$^{2+}$-independent (39, 40).

The annexin II present on the extracellular surface of endothelial cells has been shown to bind t-PA, plasminogen, and plasmin and to stimulate the activation of plasminogen (13). As much as 40% of the total plasminogen binding capacity of HUVEC has been suggested to be due to annexin II (14). Considering our observation that a significant amount of annexin II is present on the extracellular surface of HUVEC as AIIt we were interested in establishing whether or not AIIt could also activate plasminogen. We found that compared with

**FIG. 8.** Identification of AIIt on the surface of human endothelial cells. Annexin II (p36) and p11 were visualized on the surface of nonpermeabilized cells by double immunocytological staining of HUVEC with rabbit anti-p11 antibody and mouse monoclonal anti-annexin II antibody ("Experimental Procedures"). A, cells were washed with PBS, fixed, then stained for annexin II and p11. The arrow illustrates a representative region of the cell that demonstrates the co-localization of annexin II and p11 immunofluorescence. B, cells were washed with PBS containing 1 mM EGTA, fixed, then stained for annexin II and p11. C, Western blot analysis of HUVEC extracts with anti-annexin II (a) and anti-p11 antibodies (b). D and E, HUVEC were surface biotinylated, lysed with CD buffer, separated by SDS-PAGE and transferred to nitrocellulose. a, immunoprecipitations were conducted using a mouse monoclonal anti-annexin II antibody. b, immunoprecipitations were conducted omitting the primary antibody. D, the nitrocellulose was incubated with horseradish peroxidase-avidin and cell surface proteins were visualized by horseradish peroxidase activity. E, the nitrocellulose was stained for p11 using mouse monoclonal anti-p11 antibody and followed by horseradish peroxidase-goat anti-mouse antibody. Immunoprecipitations were specific since incubations in the absence of primary antibody showed no signal (b). HC, heavy chain of immunoglobulin; LC, light chain of immunoglobulin.
annexin II, AIIt was an extremely potent activator of plasminogen. Therefore, in the present study we have characterized the features of the AIIt-dependent activation of plasminogen in detail. The major findings of this study are summarized as follows.

First, the plots of $A_{405\,\text{nm}}$ versus time squared for the activation of plasminogen by annexin II and AIIt are linear for the initial rates of plasminogen activation. This establishes that annexin II and AIIt have an immediate, intrinsic stimulatory effect on plasminogen activation and do not require processing by either t-PA or plasmin. Many other plasminogen activators such as fibrin or fibrinogen fragments exhibit a lag period during which the internal lysine residues are exposed by plasmin to form new plasminogen-binding sites that accelerate the plasminogen activation process (7–9). Alternatively, the stimulation of plasminogen activation by the histidine-proline-rich glycoprotein does not require processing since this protein contains the prerequisite carboxyl-terminal lysine residue (41). Considering that annexin II does not possess a carboxyl-terminal lysine residue but activates plasminogen by a lysine-dependent mechanism (13), this suggests that annexin II or the annexin II subunit of AIIt may utilize internal lysine residues to activate plasminogen.

Second, AIIt stimulates plasminogen activation by increasing the apparent $k_{\text{cat}}$. Previous studies have shown that annexin II enhances plasminogen activation by decreasing the $K_m$. Other activators of plasminogen such as fibrin, or the histidine-proline-rich glycoprotein also have been shown to decrease the $K_m$. However, several extracellular matrix proteins such as laminin and type IV collagen activate plasminogen by a lysine-dependent mechanism (13), this suggests that annexin II or the annexin II subunit of AIIt may utilize internal lysine residues to activate plasminogen.

Third, the AIIt-dependent stimulation of plasminogen activation is Ca$^{2+}$-independent and inhibited by $\varepsilon$-amino-caproic acid. Our observation that the enhancement of the rate of [Glu]plasminogen activation by AIIt was Ca$^{2+}$-independent was unexpected. To date all of the in vitro activities reported for AIIt have been shown to be Ca$^{2+}$-dependent (15). For example, AIIt bundles F-actin, binds heparin, and aggregates chromaffin granules at physiological Ca$^{2+}$ concentrations in vitro. Furthermore, the activation of catecholamine secretion in chromaffin cells by AIIt has also been shown to be Ca$^{2+}$-dependent (42–46). Our observation that $\varepsilon$-amino-$n$-caproic acid inhibits the AIIt-dependent stimulation of [Glu]plasminogen activation (Fig. 4B) suggests that the lysine residues involved in the interaction of AIIt with t-PA and [Glu]plasminogen are fully exposed and are not influenced by Ca$^{2+}$-dependent conformational changes in AIIt.

Fourth, both fluid-phase and solid-phase AIIt activate plasminogen. Although our examination of the activation of plasminogen by fluid-phase AIIt has allowed us to establish the mechanism for activation of plasminogen, it is important to stress that in vivo, AIIt is bound to the extracellular surface of the plasma membrane. It has been shown that the annexin II bound to the extracellular surface of the endothelial plasma membrane participates in the enhancement rate of [Glu]plasminogen activation (13), however, it has not been established how annexin II or AIIt binds to the surface of cells. As shown in Fig. 7, the binding of AIIt to 293 cells results in the potentiation of t-PA-dependent plasminogen activation. Conceivably, AIIt could bind to plasma membrane phospholipid, glycosaminoglycan, or specific protein receptors. Since the biochemical properties of fluid-phase AIIt could be distinct from those of plasma membrane-bound AIIt, we examined the ability of either phospholipid-bound or heparin-bound AIIt to enhance the rate of [Glu]plasminogen activation. Our observation that AIIt complexed with either phospholipid (Fig. 5) or heparin (Fig. 4D) activates [Glu]plasminogen, establishes that fluid-phase and solid-phase AIIt are both capable of the activation of [Glu]plasminogen. Whether or not the kinetics of plasminogen activation by fluid-phase or heparin-bound AIIt are similar remains to be established.

Our observation that under our assay conditions (5.6 nM t-PA and 0.11 $\mu$M [Glu]plasminogen) 94% of [Glu]plasminogen and 49% of t-PA bound to the AIIt-heparin complex (Table II) suggests that these proteins bind to AIIt-heparin with nanomolar dissociation constants. Since we measured a $K_d$ of 1.26 $\mu$M for the interaction of AIIt with [Glu]plasminogen (Fig. 6B), and also observed that 0.11 $\mu$M [Glu]plasminogen was almost totally bound to AIIt-heparin (Table II) it is reasonable to propose that [Glu]plasminogen binds to AIIt-heparin with much higher affinity than to AIIt alone.

Fifth, the binding of AIIt to [Glu]plasminogen (741C-fluorescein) results in a conformational change in the microenvironment of the active site of thezymogen. The $K_d$ of 1.26 $\mu$M for the interaction of AIIt with [Glu]plasminogen was stronger than the $K_d$ of 30 $\mu$M estimated for the binding of [Glu]plasminogen to fibrin but similar to the $K_d$ of 1.2 $\mu$M estimated for the binding of [Lys]plasminogen to fibrin (47). Native [Glu] plasminogen exhibits a tight spiral structure which results in the occlusion of the activation cleavage site (Arg$^{561}$-Val$^{562}$) from attack by t-PA. It is therefore possible that the AIIt-dependent conformational change in [Glu]plasminogen results in exposure of the activation cleavage site of [Glu]plasminogen to t-PA.

Last, of the five annexins tested, AIIt is by far the most potent activator of plasminogen (Table I). For example, 5 $\mu$M annexin II stimulated the rate of [Glu]plasminogen conversion about 7-fold compared with 341-fold for AIIt. Furthermore, although annexin I, V, and VI have been visualized on the extracellular surface of various cells (23, 48), the low enhancement of the rate of [Glu]plasminogen activation by these proteins, compared with AIIt suggests that a functional domain unique to AIIt is probably involved in the activation of [Glu]plasminogen.

At present the physiological significance of our observations must remain speculative. Although AIIt is present on the surface of endothelial cells, the nature of the AIIt-binding sites on the cellular surface are not known. Considering the high affinity of AIIt for heparin it is tempting to speculate that AIIt may associate with heparan sulfate glycosaminoglycan on the cell surface. One popular theory is that the interactions of plasminogen with cell surface heparan sulfate glycosaminoglycan functions to elevate local plasminogen concentrations on the cell surface. It is therefore reasonable to suspect that cell surface heparan sulfate glycosaminoglycan binding may be a mechanism for colocalizing and concentrating both plasminogen and AIIt on the endothelial cell surface.

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