Annexin II Tetramer Inhibits Plasmin-Dependent Fibrinolysis†

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ABSTRACT: In this paper, we have characterized the regulation of plasmin activity by annexin II tetramer (AIIt). Plasmin activity was measured by a fibrin lysis assay in which a fibrin polymer was produced from purified components and the extent of polymer lysis was determined by following changes in turbidity. Extrinsic lysis of the fibrin polymer, initiated by addition of tissue plasminogen activator (t-PA), was totally blocked if AIIt was present during fibrin polymer formation. Furthermore, fibrin polymer formed in the presence of AIIt was resistant to extrinsic lysis initiated by addition of plasmin. AIIt bound to fibrin polymer under conditions in which polymer lysis was inhibited. Plasmin-dependent extrinsic lysis of the fibrin polymer was also blocked if AIIt was present in the incubation medium, and under these conditions the amidolytic activity of plasmin, measured with an artificial substrate, was inhibited about 5-fold. In contrast, in the absence of fibrin, and at an AIIt/plasmin molar ratio of 526, the amidolytic activity of plasmin was inhibited by only 22.3% ± 7.4% (mean ± SD, $n = 5$) by AIIt. Plasmin-dependent fibrinolysis was only slightly inhibited if fibrin polymer was formed in the presence of annexins I, II, V, or VI. These results identify AIIt as an *in vitro* regulator of plasmin activity.

Cleavage and removal of fibrinopeptides A and B from soluble fibrinogen is catalyzed by thrombin, the terminal proteolytic enzyme of the coagulation cascade. The cleaved fibrinogen, or fibrin monomers, spontaneously polymerizes, forming a fibrin polymer that constitutes the major proteinaceous component of the hemostatic plug (Tracy, 1988; Stump & Mann, 1988). The hemostatic plug, referred to as the fibrin clot, is in addition to fibrin composed of platelets, erythrocytes, and other blood cells as well as other accessory factors. Subsequent removal of a fibrin clot occurs by solubilization of the fibrin polymer to fibrin degradation products by proteolysis catalyzed by plasmin, the terminal enzyme of the fibrinolytic cascade. Whereas both coagulation and fibrinolysis, respectively, are necessary to prevent loss of blood and to maintain blood fluidity, regulation of these two antagonistic cascades is required to minimize or prevent the futile cycle of indiscriminate clot formation and degradation. Although plasmin displays a broad trypsin-like substrate specificity, fibrin-specific proteolysis is accomplished by formation of plasmin within a fibrin clot.

Recently, a protein capable of binding both t-PA and plasminogen was identified on the endothelial cell surface (Hajjar & Hamel, 1990; Hajjar, 1991). This protein was subsequently identified as the Ca^{2+}-binding protein annexin II (Hajjar et al., 1994; Cesarman et al., 1994).

Annexin II is a Ca^{2+}-and phospholipid-binding protein that can exist as a monomer or as a heterotetramer. The heterotetramer referred to as annexin II tetramer (AIIt)[1] is composed of two annexin II subunits and two p11 subunits [reviewed in Waisman (1995)]. Annexin II has been identified as a 40 kDa endothelial cell surface protein that specifically and independently binds both t-PA and plasminogen with high affinity [K_d(t-PA) 25 nM, K_d(plasminogen) 161 nM, and K_d(plasmin) 75 nM] (Cesarman et al., 1994). The binding of these proteins to annexin II resulted in a 20-fold increase in the rate of t-PA-dependent [Glu]plasminogen activation and a 14-fold increase in the t-PA-dependent activation of [Lys]plasminogen. Transient transfection of renal epithelial 293 cells with annexin II cDNA resulted in a 4−6-fold greater expression of extracellular annexin II and a 6-fold greater generation of plasmin by the transfected cells. On the basis of these observations of the effect of annexin II on the kinetics of t-PA-dependent conversion of plasminogen to plasmin, it has been suggested that annexin II bound to the extracellular membrane accelerates the t-PA-dependent conversion of plasminogen to plasmin.

Fibrin binds t-PA, plasminogen, and plasmin and therefore functions not only as the fibrinolytic substrate but also as an allosteric activator of fibrinolysis. For example, fibrin structure plays a key role in the t-PA-dependent conversion of plasminogen to plasmin (Carr & Alving, 1995). Since

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[1] Abbreviations: BSA, bovine serum albumin; AIIt, annexin II tetramer; p11, p11 light chain of annexin II tetramer; t-PA, tissue plasminogen activator; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; buffer A, 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 5 mM $CaCl_2$.

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our preliminary results suggested that AIIt bound to fibrin polymer (Choi et al., manuscript in preparation) and another group had suggested that annexin II bound plasminogen and plasmin but did not affect the activity of plasmin directed against an artificial amidolytic substrate (Cesarman et al., 1994), we were interested in the possibility that AIIt might regulate plasmin fibrinolytic activity. We have therefore examined the potential role of AIIt in the regulation of plasmin activity using a fibrin polymer lysis assay system and measuring plasmin fibrinolytic activity by turbidity. Our results suggest that although AIIt modestly inhibits plasmin amidolytic activity, AIIt dramatically inhibits plasmin fibrinolytic activity.

EXPERIMENTAL PROCEDURES

Materials. Annexin II tetramer (AIIt) was prepared from bovine lung (Khanna et al., 1990). As determined by SDS−PAGE and electrospray ionization mass spectrometry (ESI-MS), the subunits of the purified bovine AIIt were not proteolyzed (Kang et al., 1997). Recombinant annexin II monomer, p11 subunit, and AIIt were expressed in *Escherichia coli* (Kang et al., 1997). Final purification of recombinant annexin II from bacterial lysates involved ion-exchange chromatography on Fast-S, affinity chromatography on heparin−Sepharose, and gel-permeation chromatography on Superose 12. Typically, we obtained about 27 mg of pure recombinant annexin II from 1 L of bacterial culture. Amino acid sequencing of the recombinant annexin II identified the N-terminus of the protein as STVHEIL. The M_r of human recombinant annexin II was determined to be 38 480.84 ± 7.24 by ESI-MS, which was similar to the M_r 38 472 deduced from the cDNA sequence of initiating Met deleted, nonacetylated human annexin II. Final purification of recombinant p11 involved ion-exchange chromatography on Fast-Q and Fast-S and gel-permeation chromatography on Superose 12. About 30 mg of pure recombinant p11 was purified from 1 L of bacterial culture. Gel-permeation chromatography of recombinant p11 on Superose 12 columns established that the recombinant protein existed as a dimer. Amino acid sequencing of the purified recombinant p11 confirmed that the N-terminal sequence (PSQME) of the recombinant protein was identical to that of the native p11. The M_r of recombinant p11 was determined by ESI-MS as 11 068.28 ± 0.23 by ESI-MS, which was very similar to the M_r 11 069.38 ± 1.59 determined for the native p11. All proteins were stored at −70 °C in 40 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.1 mM EGTA, and 150 mM NaCl.

Bovine intestinal mucosa heparin (3 kDa; 50 USP units/mg) and thrombin (2300 NIH units/mg) were obtained from Sigma. Human recombinant t-PA was obtained from Genentech and was 80−90% single chain as determined by SDS−PAGE. [Glu]plasminogen, plasmin, and the plasmin amidolytic substrate, Spectrozyme 251 (H-D-norleucyl-hexahydrotyrosyl-lysine *p*-nitroanilide) were obtained from American Diagnostica. Fibrinogen was obtained from Sigma and further purified by chromatography on Superose 12 to remove contaminating plasminogen. Frozen fresh plasma was thawed and dialyzed against 50 mM Tris-HCl, pH 7.4, and 100 mM NaCl to remove anticoagulant. After centrifugation at 30000*g* for 30 min at 4 °C, the plasma was aliquoted and stored frozen at −70 °C. Fluorescein-conjugated collagen type IV (CD-12052) was obtained from Molecular Probes.

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. To analyze intrinsic lysis, reaction mixtures contained 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 8 μM fibrinogen, 42 nM plasmin, 0.83 NIH unit/mL thrombin, and 5 mM $CaCl_2$. Extrinsic lysis of the fibrin polymer was measured as above except that [Glu]plasminogen (200 nM) was included in the reaction mixture. After turbidity had reached a stable level, 100 μL of 25 nM t-PA (25 nM) was gently layered on top of the fibrin polymer reaction mixture and turbidity was measured as described above. Lysis of fibrin polymer, produced in the presence of plasma, was performed by incubating thrombin (0.83 NIH unit/mL), fibrinogen (5 μM), plasmin (500 nM), and dialyzed human plasma (4-fold diluted) in buffer A at 25 °C. Lysis time ($A_{0.5}$) in all cases was determined by measuring the time at which the turbidity was decreased to 50% of the maximum value.

Plasmin Activity Assay. Plasmin amidolytic activity was measured with the substrate H-D-norleucyl-hexahydrotyrosyl-lysine *p*-nitroanilide (Spectrozyme 251) at 25 °C in a final volume of 0.6 mL, in a buffer consisting of 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 5 mM $CaCl_2$. Typically the reaction was initiated by addition of plasmin (3.8 nM) and the reaction progress was monitored at 405 nm.

Data Analysis. Nonlinear least-squares fitting was performed with the computer program SigmaPlot (Jandel Scientific). Titration data were analyzed as detailed in the SigmaPlot reference manual with the four-parameter logistic equation $f = (a - d)/[1 + (x/c)^b] + d$, where a = plateau value for the polymerization process, b = slope parameter, c = value at the transition midpoint ($A_{0.5}$), and d = baseline value for complete fibrinolysis. 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. Typically results are representative of at least three experiments.

Miscellaneous Techniques. AIIt concentration was determined spectrophotometrically using an extinction coefficient $A_{280nm} = 0.68$ for 1 mg/mL AIIt. Collagenase activity was measured at 37 °C in a final volume of 100 μL in a buffer consisting of 50 mM Tris (pH 7.6), 150 mM NaCl, 5 mM $CaCl_2$, 0.8 μM fluorescein-conjugated collagen type IV, and 0.12 μM plasmin. Substrate hydrolysis was measured continuously as relative fluorescence units at an excitation of 494 nm, emission of 515 nm, and slit widths of 2 and 10 nm in a Model 650 Perkin-Elmer spectrofluorometer.

RESULTS

Extrinsic Lysis of Fibrin Polymer. The presence of annexin II on the outer surface of the endothelial cell and the reported activation of the t-PA-dependent conversion of plasminogen to plasmin by plasma-membrane associated annexin II have suggested that annexin II functions to increase the local plasmin concentration on the endothelial cell surface (Cesarman et al., 1994). We have therefore examined the possible role of annexin II and AIIt in the

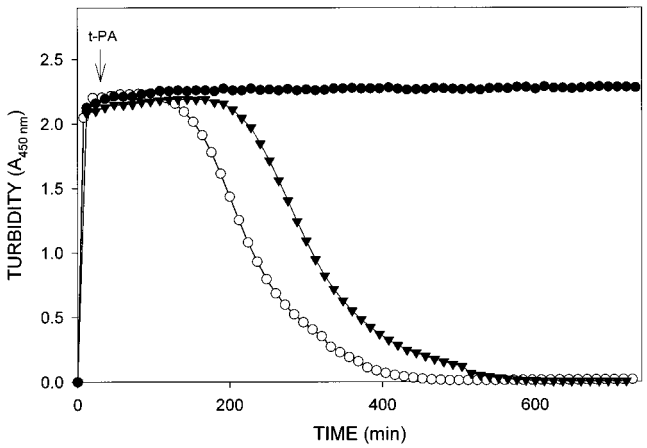

FIGURE 1: Inhibition of extrinsic t-PA-dependent fibrin polymer lysis by AIIt. Thrombin (0.83 NIH unit/mL), fibrinogen (8 μM), and plasminogen (200 nM) were incubated at 25 °C in buffer A (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 5 mM $CaCl_2$) in the absence (○) or presence of annexin II (5 μM) (▼) or annexin II tetramer (1 μM) (●). After a fibrin polymerization was complete (30 min), 100 μL of 25 nM t-PA was layered on top of the fibrin polymer and turbidity was measured as described in Experimental Procedures.

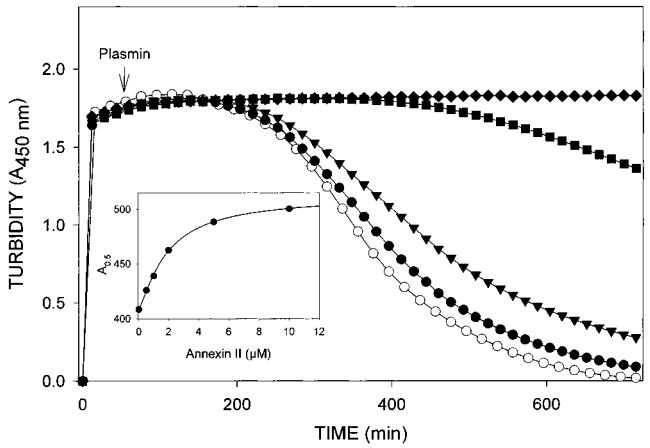

FIGURE 2: Inhibition of extrinsic plasmin-dependent fibrin polymer lysis by annexin II and AIIt. Thrombin (0.83 NIH unit/mL) and fibrinogen (8 μM) were incubated at 25 °C in buffer A (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 5 mM $CaCl_2$) in the absence (○) or presence of 0.3 μM (●), 0.5 μM (▼), 0.7 μM (■) and 1 μM (◆) of AIIt. Fibrin polymer lysis was initiated by the addition of 1.25 μM plasmin after 30 min and monitored by turbidity. Inset: Effect of annexin II on extrinsic plasmin-dependent fibrin polymer lysis. The time required for the turbidity to decrease to half the difference between the plateau and baseline values is plotted against the annexin II concentration.

regulation of plasmin activity using an *in vitro* fibrin polymer lysis assay. In this assay, fibrinogen, thrombin, and plasminogen were incubated in the presence or absence of annexin II or AIIt and fibrin polymer dissolution was activated by addition of t-PA. Polymerization and subsequent lysis were measured by turbidity.

As shown in Figure 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 t-PA to the fibrin polymer, a subsequent return to the value of nonpolymerized material (fibrinolysis). The time required for the turbidity to decrease to half the difference between the plateau and baseline values was designated as the transition midpoint ($A_{0.5}$) and was taken as a measure of plasmin fibrinolytic activity. Fibrin polymer formed in the presence of 5 μM annexin II yielded an $A_{0.5}$ of about 301 min compared to an $A_{0.5}$ of about 224 min for fibrin polymer formed in the absence of annexin II. This suggested that the plasmin fibrinolytic activity is only slightly inhibited by annexin II. In contrast, when fibrin polymer was formed in the presence of 1 μM AIIt, fibrin polymer lysis was completely abolished and an $A_{0.5}$ value could not be determined. Even after 48 h, significant lysis of the fibrin polymer was not observed (data not shown). These results therefore establish that AIIt substantially inhibits t-PA-dependent extrinsic lysis of fibrin polymer. However, mechanistically the inhibitory effect of AIIt could be due to inhibition of the t-PA-dependent conversion of plasminogen to plasmin or due to a direct effect on plasmin activity.

We therefore examined the effect of AIIt on the plasmin-dependent extrinsic lysis of fibrin polymer. In this experiment, fibrin was polymerized by incubation of thrombin and fibrinogen in the presence or absence of AIIt. After a stable fibrin polymer was formed, plasmin was added to initiate fibrinolysis. As shown in Figure 2, fibrin polymers that formed in the presence of AIIt were resistant to plasmin-dependent fibrinolysis. Furthermore, SDS−PAGE analysis of the fibrin polymer suggested that less than 10% of the

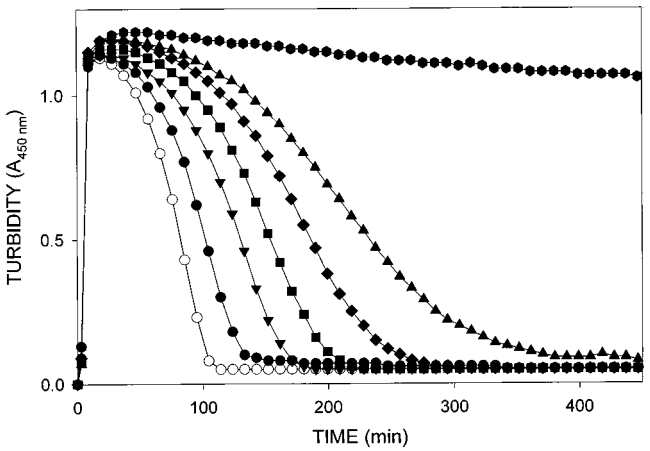

FIGURE 3: Inhibition of plasmin-dependent intrinsic fibrin polymer lysis by AIIt. Thrombin (0.83 NIH unit/mL), fibrinogen (8 μM), and plasmin (42 nM) were incubated at 25 °C in buffer A in the absence (○) or presence of annexin II tetramer (closed symbols), and turbidity was measured as described in Experimental Procedures. The concentrations of AIIt were 0.03 μM (●), 0.063 μM (▼), 0.125 μM (■), 0.25 μM (♦), 0.5 μM (▲), and 1 μM (⬢).

AIIt was proteolyzed over a period of 2−3 h (data not shown). The inhibitory effect of AIIt was concentration-dependent and about 1 μM AIIt was required to completely inhibit plasmin-dependent fibrinolysis. In contrast, high concentrations of annexin II produced only a modest inhibition of extrinsic plasmin-dependent fibrin polymer lysis (Figure 2, inset). These results therefore establish that AIIt inhibits plasmin activity directly.

Intrinsic Lysis of Fibrin Polymer. We also examined the effect of AIIt on plasmin-dependent intrinsic lysis of fibrin polymer. Fibrin polymer was prepared by incubation of fibrinogen, thrombin, and plasmin in the presence or absence of AIIt and the rates of fibrin polymer lysis were compared. As shown in Figure 3, AIIt inhibits fibrinolysis under these conditions. The inhibition of plasmin activity by AIIt was concentration-dependent and as little as 0.03 μM AIIt

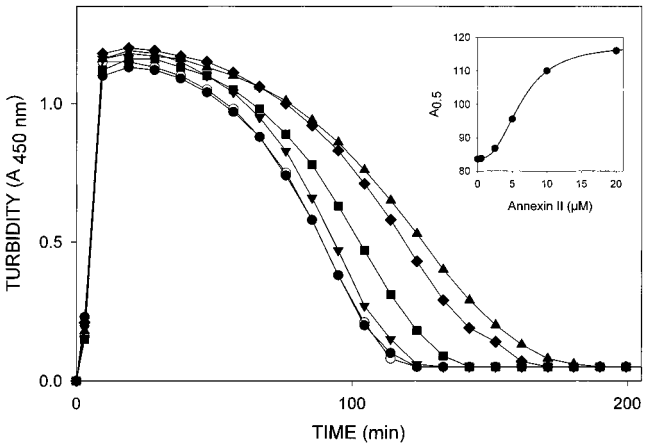

FIGURE 4: Inhibition of intrinsic plasmin-dependent fibrin polymer lysis by annexin II. Intrinsic plasmin-dependent in vitro fibrin polymer lysis was measured by turbidity as described in the legend of Figure 3 except that annexin II was substituted for AIIt. The concentrations of annexin II present during fibrin polymerization were 0 (○), 0.5 μM (●), 2.5 μM (▼), 5 μM (■), 10 μM (♦) and 20 μM (▲). Inset shows $A_{0.5}$ versus various concentrations of annexin II.

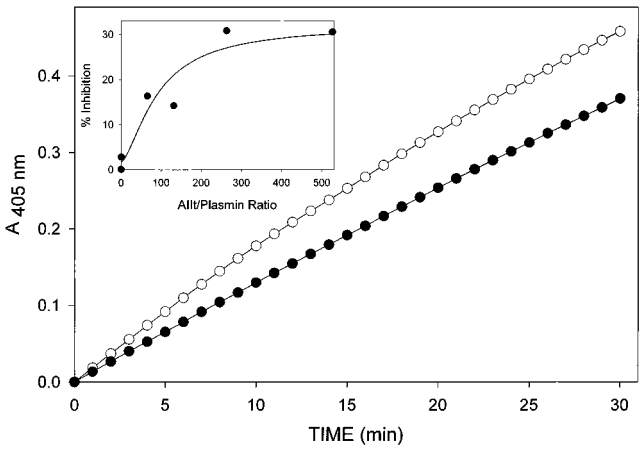

FIGURE 5: Effect of AIIt on the amidolytic activity of plasmin. The amidolytic activity of plasmin was assayed in buffer A at 25 °C with Spectrozyme 251 substrate (104 μM) in the absence (○) or presence (●) of 2 μM AIIt. The reaction was initiated by the addition of 3.8 nM plasmin. Inset: Inhibition of plasmin amidolytic activity (expressed as the percentage of plasmin amidolytic activity in the absence of AIIt) is plotted as a function of the AIIt/plasmin molar ratio. Plasmin amidolytic activity was measured as described above except that the reaction was conducted at room temperature in a final volume of 200 μL and the plasmin amidolytic rate was measured in a microplate reader.

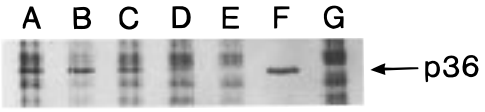

FIGURE 6: Interaction of AIIt with fibrin. Fibrin polymer was formed by incubation of thrombin (0.83 NIH unit/mL) and fibrinogen (8 μM) at 25 °C in buffer A. After a polymerization was complete, 10 μM AIIt was added without (A) or with 0.5 μM (B), 1 μM (C), 2 μM (D), or 4 μM heparin (E) and the fibrin polymer was incubated for 30 min at 25 °C. The fibrin polymer was washed with buffer A and then subjected to SDS−PAGE. (F) Annexin II standard; (G) fibrin polymer incubated in the absence of AIIt.

produced a significant inhibition of fibrinolysis. Furthermore, as observed for the extrinsic lysis of fibrin polymer, 1 μM AIIt completely inhibited the intrinsic lysis of fibrin polymer.

We also found that annexin II produced a concentration-dependent inhibition of plasmin activity in the intrinsic fibrin polymer lysis assay. As shown in Figure 4, measurable inhibition of intrinsic fibrin polymer lysis required 2.5 μM annexin II and a half-maximal change in $A_{0.5}$ required 6.3 μM annexin II (Figure 4, inset). Interestingly, unlike the results observed with AIIt (Figure 3), high concentrations of annexin II did not totally block fibrin polymer lysis. Saturating concentrations of annexin II delayed fibrin polymer lysis from an $A_{0.5}$ of about 84 min to an $A_{0.5}$ of about 118 min.

Effect of AIIt on the Plasmin Amidolytic Activity. The results presented in Figures 1−3 establish that plasmin activity, assayed in the presence of its physiological substrate fibrin, is substantially inhibited by AIIt. For example, in the case of plasmin-dependent fibrin polymer lysis, we observed that 1 μM AIIt totally inhibited the fibrinolytic activity of 1.25 μM plasmin (Figure 2). However, as shown in Figure 5, when plasmin activity (3.8 nM) was assayed with the artificial amidolytic substrate D-norleucyl-hexahydrotyrosyl-lysine *p*-nitroanilide, only a 22.3% ± 7.4% (mean ± SD, $n = 5$) inhibition of plasmin activity was observed in the presence of 2 μM AIIt. The half-maximal inhibition of plasmin amidolytic activity required about 0.35 μM AIIt and was maximal in the presence of about 1 μM AIIt (Figure 5, inset). The inhibition of the amidolytic activity of plasmin by AIIt was observed regardless of the order of addition of plasmin, AIIt, or substrate (data not shown).

AIIt-Dependent Inhibition of Fibrin-Associated Plasmin Activity. We have also examined the possibility that AIIt binds to fibrin. As shown in Figure 6, AIIt binds to fibrin and this binding is reversed by heparin. The binding of AIIt to heparin did not result in the dissociation of the p11 subunit (data not shown). Considering that both AIIt and fibrin bind heparin (Kassam *et al.*, 1997), it is unclear if the inhibitory activity of heparin on the interaction of AIIt and fibrin is due to the interaction of heparin with either or both of these proteins. We are currently examining in detail the kinetics of binding of AIIt to fibrin and the mechanism by which heparin reverses this binding (Choi et al., manuscript in preparation). Considering our observation that AIIt binds to fibrin (Figure 6), it is possible that AIIt inhibits the lysis of fibrin polymer by binding to fibrin and inducing a conformational change in fibrin that blocks the interaction of plasmin with fibrin. We have therefore examined the possible mechanism by which AIIt inhibits plasmin activity. Fibrin polymer was formed by incubation of thrombin and fibrinogen, and after the fibrin polymerization was complete, extrinsic lysis of the fibrin polymer was initiated by addition of 1 μM plasmin. As shown in Figure 7, the simultaneous addition of AIIt and plasmin resulted in significant inhibition of extrinsic fibrin polymer lysis. Fibrin polymer lysis was completely blocked by about 5 μM AIIt. After 720 min, the artificial amidolytic substrate D-norleucyl-hexahydrotyrosyl-lysine *p*-nitroanilide was added to control (no AIIt) or reaction mixtures that contained AIIt. As shown in Figure 7, inset, the amidolytic activity of plasmin was also substantially inhibited in the presence of AIIt. These results demonstrate that the interaction of plasmin, fibrin, and AIIt results in the inactivation of both plasmin fibrinolytic (Figure

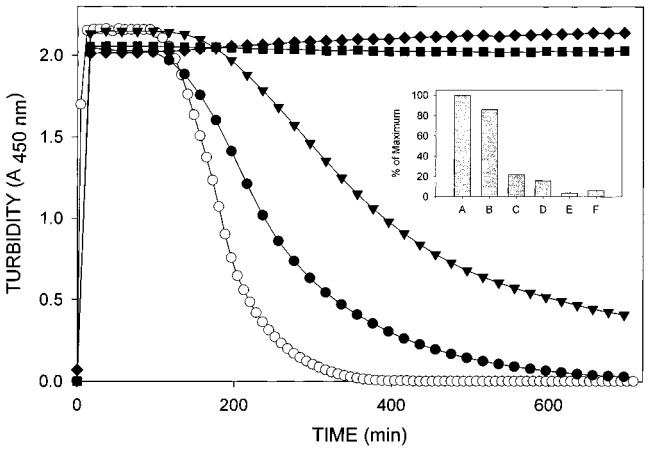

FIGURE 7: Amidolytic activity of plasmin during AIIt-dependent inhibition of in vitro fibrin polymer lysis. Fibrin polymerization was initiated by incubation of fibrinogen (8 μM), and thrombin (0.83 NIH unit/mL) in a 400 μL reaction mixture. After 30 min, 100 μL of plasmin (1 μM) was added without (○) or with (filled symbols) various concentrations of AIIt, and the turbidity was measured at 450 nm. After 720 min, the whole reaction mixture excluding the fibrin polymer was added to 3 mL of buffer A containing 200 μM Spectrozyme 251 amidolytic substrate, and after 5 min, the reaction was quenched with 5.5% TCA. After centrifugation (14500*g*, 15 min), the supernatant was removed and the optical density of the supernatant was measured at 405 nm (presented in the inset). The concentrations of AIIt used were 0 (○, A), 1 μM (●, C), 2 μM (▼, D), 5 μM (■, E), and 10 μM (♦, F). Inset: Amidolytic activity of plasmin in the presence of AIIt is plotted as a percentage ratio of the amidolytic activity of plasmin measured in the absence of AIIt. To compensate for the small inhibitory effect of AIIt on the amidolytic activity of plasmin, a duplicate sample taken at 720 min corresponding to the reaction conducted in the absence of AIIt (○) was adjusted to 2 μM AIIt before addition of amidolytic substrate (inset, B).

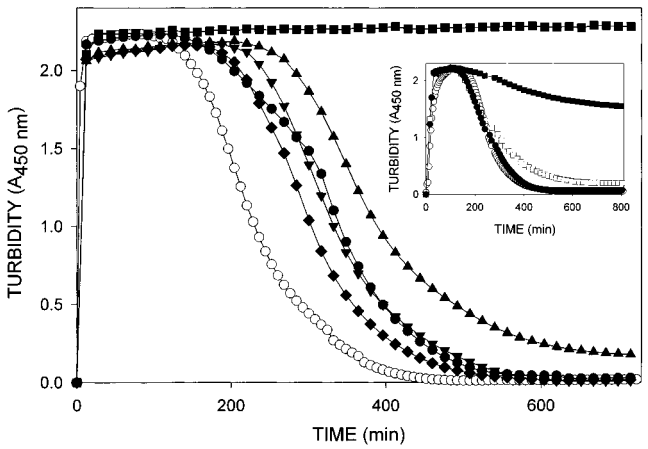

FIGURE 8: Inhibition of t-PA-dependent extrinsic lysis of fibrin polymer by other annexins. Fibrin polymerization was initiated by incubation of thrombin (0.83 NIH unit/mL), fibrinogen (8 μM), and plasminogen (200 nM) without any annexin (○) and with 1 μM AIIt (■), 5 μM annexin I (●), 5 μM annexin II (▼), 5 μM annexin V (♦) or 5 μM annexin VI (▲). Thirty minutes after initiation of fibrin polymerization, fibrinolysis was initiated by addition of 25 nM t-PA. Inset: Effect of the recombinant proteins on extrinsic plasmin-dependent fibrin polymer lysis. The concentrations of recombinant proteins used were 0 (○), 10 μM p11 (□), 10 μM p36 (●), or 7 μM AIIt (■). Plasmin (1.25 μM) and recombinant proteins were added to the fibrin clot at 30 min.

7) and amidolytic activity (Figure 7, inset). Considering that AIIt produces only a slight inhibition of plasmin amidolytic activity in the absence of fibrin (Figure 5), the results suggest that the formation of a complex between AIIt, fibrin, and plasmin results in a conformational change in AIIt or plasmin or both of these proteins. As a result of this(these) conformational change(s), both the amidolytic and fibrinolytic activity of plasmin is inhibited.

Specificity of the Regulation of Plasmin Activity. AIIt is a member of a large family of Ca^{2+}-binding proteins. In order to assess whether or not the regulation of plasmin activity was specific to AIIt, we tested several annexins as potential antifibrinolytic proteins. Fibrin polymer was formed by incubation of thrombin, fibrinogen, plasminogen, and an annexin protein. After the fibrin polymerization was complete, extrinsic fibrin polymer lysis was initiated by addition of t-PA. As shown in Figure 8, at 5 times the concentration of AIIt, annexins I ($A_{0.5}$ = 327 min), II ($A_{0.5}$ = 310 min), V ($A_{0.5}$ = 332 min), and VI ($A_{0.5}$ = 372 min) had only a slight inhibitory effect on plasmin activity when compared to fibrin polymer prepared in the absence of annexin ($A_{0.5}$ = 221 min).

Recently our laboratory produced prokaryotic cDNA expression constructs for both annexin II and the p11 subunit and demonstrated that the recombinant subunits were structurally identical to the native proteins isolated from bovine lung (Kang et al., 1997). This has allowed analysis of the role of the AIIt subunits in the AIIt-dependent inhibition of fibrinolysis. As shown in the Figure 8 inset, neither the p11 subunit of AIIt nor annexin II inhibits intrinsic plasmin-dependent fibrin polymer lysis. In contrast, recombinant AIIt substantially inhibits plasmin-dependent fibrin polymer lysis. These results therefore suggest that the antifibrinolytic activity of AIIt requires the participation of both subunits of the protein.

Intrinsic Lysis of Fibrin Polymer Produced in Plasma. Plasma contains a variety of proteins that are involved in the regulation of thrombosis and fibrinolysis. Furthermore, the formation of fibrin polymers occurs on the surface of the endothelial cells that are bathed in plasma. Our previous results established that AIIt inhibited the fibrinolytic activity of plasmin. We also examined the effect of AIIt on plasmin activity using a fibrin polymer formed in the presence of plasma. As shown in Figure 9, in the presence of plasma and absence of AIIt, fibrin polymerizes and is dissolved rapidly. However, if fibrin polymer is formed in the presence of plasma and AIIt, subsequent dissolution is dramatically inhibited. Furthermore, the similar potency of AIIt for inhibition of the lysis of both the fibrin polymer formed in plasma and the fibrin polymer formed with purified components suggests that plasma does not contain factors that inactivate the inhibitory activity of AIIt.

AIIt Does Not Inhibit the Collagenolytic Activity of Plasmin. Several annexins, including annexin II, have been shown to bind to collagen (Genge et al., 1992; Wirl & Schwartz-Albiez, 1990; Pfaffle et al., 1990; Wu et al., 1991). We have also observed the binding of AIIt to type IV collagen (data not shown). Since plasmin has been recently shown to degrade collagen type IV (Mackay et al., 1990; Akeli et al., 1991), we examined the effects of AIIt on plasmin collagenolytic activity. As shown in Figure 10, AIIt did not affect the plasmin collagenolytic activity. This suggests that the inhibition of plasmin fibrinolytic activity by AIIt is not a general effect of AIIt on plasmin proteolytic activity.

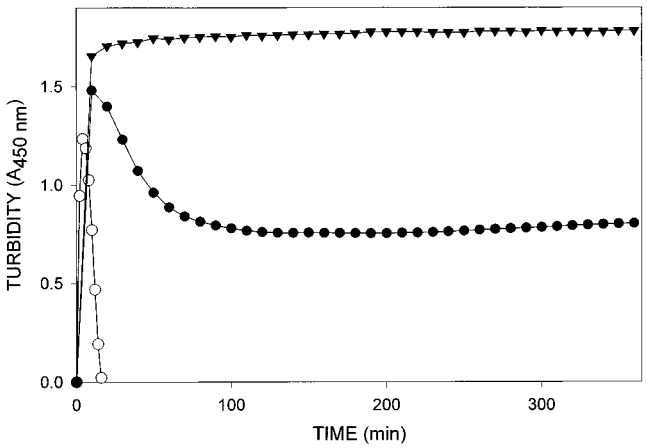

FIGURE 9: Inhibition by AIIt of plasmin-dependent intrinsic lysis of fibrin polymerized in plasma. Dialyzed human plasma was diluted 4-fold into buffer A and incubated at 25 °C with thrombin (0.83 NIH unit/mL) fibrinogen (5 μM), and plasmin (500 nM) in the absence (○) or presence of either 1 μM AIIt (●) or 2 μM AIIt (▼). Turbidity was measured as described in Experimental Procedures.

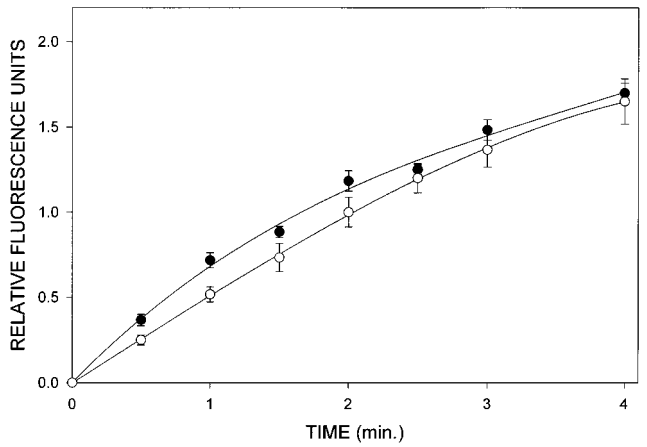

FIGURE 10: AIIt does not inhibit the collagenolytic activity of plasmin. Collagen type IV was preincubated at 37 °C for 10 min in the absence (●) or presence (○) of 2 μM AIIt in a buffer containing 50 mM Tris (pH 7.5), 100 mM NaCl, 5 mM $CaCl_2$, and 0.8 μM fluorescein-conjugated collagen type IV. The reaction was initiated by addition of 0.12 μM plasmin, and the relative fluorescence was continuously monitored. Results are expressed as the mean ±SD, $n = 3$.

DISCUSSION

These studies were undertaken to investigate the role of annexin II and annexin II tetramer in the regulation of plasmin fibrinolytic activity. Earlier reports demonstrated that annexin II was present on the outer surface of endothelial cells and that extracellular annexin II bound t-PA, plasminogen, and plasmin, with high affinity. Since the binding of t-PA and plasminogen to annexin II resulted in an increase in plasmin amidolytic activity, it was concluded that annexin II acts as a fibrinolytic receptor by facilitating the t-PA-dependent conversion of plasminogen to plasmin (Hajjar et al., 1994; Cesarman et al., 1994). Since this laboratory also reported that the amidolytic activity of plasmin was unaffected by annexin II, their results predicted that endothelial cell annexin II would participate in the constitutive production of large amounts of fully active plasmin.

In the present paper we have demonstrated that AIIt, but not other annexins such as annexin I, II, V, and VI, is a potent regulator of plasmin activity *in vitro*. Furthermore, when analyzed at similar concentrations, recombinant AIIt but not recombinant annexin II or p11 subunit inhibited the plasmin-dependent fibrin polymer clot lysis. The inhibition of plasmin activity by AIIt was observed under a variety of experimental conditions including t-PA-dependent extrinsic lysis of AIIt-containing fibrin polymer (Figure 1), plasmin-dependent extrinsic lysis of AIIt-containing fibrin polymer (Figure 2), plasmin-dependent intrinsic lysis of AIIt-containing fibrin polymer (Figure 3), and plasmin-dependent extrinsic lysis of fibrin polymer (Figure 7). Collectively, these results suggest that AIIt is a specific and potent regulator of plasmin fibrinolytic activity *in vitro*.

Under experimental conditions in which AIIt blocked the plasmin-dependent lysis of fibrin polymer, plasmin activity measured with an artificial amidolytic substrate was also attenuated (Figure 7, inset). In contrast, the amidolytic activity of plasmin in the absence of fibrin polymer was only slightly inhibited by AIIt (Figure 5). These results establish three important points. First, AIIt substantially inhibits the activity of plasmin directed toward its physiological substrate, fibrin, but does not inhibit the plasmin activity directed toward the artificial amidolytic substrate. Second, since in the presence of fibrin both the fibrinolytic and amidolytic activities of plasmin are inhibited by AIIt (Figure 7), it is unlikely that AIIt binding to fibrin (Figure 6) either sterically blocks the plasmin−fibrin interaction or induces a conformational change in fibrin resulting in inhibition of the plasmin−fibrin interaction. If the binding of AIIt to fibrin did inhibit the subsequent binding of plasmin to fibrin, it would be expected that the plasmin would retain full amidolytic activity under these conditions. Therefore, it is possible that fibrin colocalizes AIIt and plasmin to the fibrin filament, which results in the formation of a fibrin−AIIt−plasmin ternary complex in which plasmin is inactive. Our observation that both the amidolytic and the fibrinolytic activities of plasmin are inhibited in the presence of both fibrin and AIIt but not in the presence of AIIt and the absence of fibrin (Figure 7) is consistent with this proposed mechanism. Third, AIIt does not inhibit the collagenolytic activity of plasmin (Figure 10). This result suggests that AIIt is not a general inhibitor of plasmin proteolytic activity. Finally, we have observed that the amidolytic activity of t-PA measured in the presence of fibrin was not inhibited by AIIt (data not shown). This result establishes that AIIt is not a general serine protease inhibitor.

It is not surprising that the inhibitory effect of AIIt on plasmin activity is dependent on the presence of fibrin. Plasmin displays a broad trypsin-like substrate specificity. Fibrin-specific proteolysis is accomplished by formation of plasmin within the fibrin polymer. The binding of plasmin to the fibrin polymer is mediated by one or more of the five N-terminal heavy-chain lysine-binding sites or kringle domains (of plasmin) that recognize lysine residues in fibrinogen (Fleury et al., 1993; Liu & Gurewich, 1992; Nesheim et al., 1990; Hoylaerts et al., 1982). Activation of plasminogen is also rendered fibrin-specific by virtue of the affinity of t-PA for fibrin and the resulting fibrin-dependent, t-PA-catalyzed plasminogen activation. Fibrin thus binds both t-PA and plasminogen and thereby functions not only as the fibrinolytic substrate but also as an allosteric activator of fibrinolysis. Our data demonstrate that fibrin can also

interact with AIIt, which results in the inhibition of plasmin activity.

It is also interesting that AIIt, but not the annexin II subunit or p11 subunit of AIIt, is a potent inhibitor of plasmin activity. Although AIIt consists of two copies of the annexin II subunit and two copies of a p11 subunit, the association of the p11 subunit with the annexin II subunit modulates many of the biochemical properties of the protein [reviewed in Kang et al. (1997) and Waisman (1995)]. For example, AIIt, but not annexin II, bundles F-actin (Ikebuchi & Waisman, 1990), aggregates chromaffin granules at physiological Ca^{2+} concentrations (Drust & Creutz, 1988; Jones et al., 1994), modulates Cl^- channels (Nilius et al., 1996), and participates in exocytosis (Chasserot-Golaz et al., 1996). The binding of the p11 subunit to the annexin II subunit also results in the movement of the protein from the cytoplasm to the cytoplasmic surface of the plasma membrane, where it associates with the submembranous cytoskeleton (Harder et al., 1993). Consistent with the distinct subcellular localization of annexin II and AIIt is the finding that these proteins turn over with different half-lives of 15 h and 40−50 h, respectively (Zokas & Glenney, 1987).

Considering our results that demonstrate that AIIt, but not annexin II, is a potent regulator of plasmin activity, it is important to establish which protein is dominant on the extracellular surface of the plasma membrane. Antibodies to annexin II have been used to establish the existence of annexin II on the extracellular surface of a variety of cells (Yeatman et al., 1993; Ma et al., 1994; Chung & Erickson, 1994; Wright et al., 1995); however, since annexin II antibodies react to both annexin II and AIIt, it has been difficult to determine whether annexin II or AIIt is dominant. Western blotting with both annexin II and p11 subunit antibodies has established that the total cellular annexin II/AIIt ratio can vary from cell to cell. For example, although thymus cells contain predominantly annexin II (Saris et al., 1987), cultured fibroblasts have been shown to have an annexin II/AIIt ratio of 1 (Erikson et al., 1984). Furthermore, MDCK cells, bovine intestinal epithelial cells, and calf pulmonary artery endothelial cells have been shown to have annexin II/AIIt ratios of less than 0.1 (Nilius et al., 1996; Gerke & Weber, 1984). One study that examined the expression of both annexin II and p11 subunit found the presence of both proteins on the extracellular surface of the plasma membrane at similar concentrations in a variety of cells, suggesting that in certain cells AIIt may be dominant on the extracellular surface of the plasma membrane (Yeatman et al., 1993). The demonstration that 90−95% of total endothelial annexin II exists as AIIt (Nilius et al., 1996) and our demonstration of p11 immunoreactivity on the endothelial cell surface (data not shown) suggest that AIIt may also be the dominant form of annexin II on the extracellular surface of the endothelial cell membrane. However, at the present time neither the concentration of annexin II nor the relative distributions of annexin II or AIIt on the extracellular surface of the endothelial cell are known.

Intravascular fibrin formation can occur in response to injury or pathological thrombosis and results in the intimate contact of fibrin with the vascular endothelium. Cellular interactions and responses to the fibrin polymer are probably critical for the activation and regulation of wound healing and revascularization. The adhesion, spreading, and migration of endothelial cells is thought to involve the recognition and binding of these cells to specific domains of the fibrin molecule. Our results therefore present the possibility that the AIIt that is present on the extracellular surface of the endothelial cell may act to block plasmin-dependent lysis of cell-surface associated fibrin. AIIt is unique among the annexins in that this protein inhibits the fibrinolytic activity of plasmin. This presents the possibility that certain stimuli, such as possibly the phosphorylation of AIIt, may act as a regulatory mechanism by affecting the conformation of AIIt and causing AIIt to release its inhibitory constraints on plasmin activity.

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