Regulation of Plasmin Activity by Annexin II Tetramer†

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ABSTRACT: Annexin II tetramer (AIIt) is a major Ca^{2+}-binding protein of the endothelial cell surface which has been shown to stimulate the tissue plasminogen activator (t-PA)-dependent conversion of plasminogen to plasmin. In the present report, we have examined the regulation of plasmin activity by AIIt. The incubation of plasmin with AIIt resulted in a 95% loss in plasmin activity. SDS−PAGE analysis established that AIIt stimulated the autoproteolytic digestion of plasmin heavy and light chains. The kinetics of AIIt-stimulated plasmin autoproteolysis were first-order, suggesting that binding of plasmin to AIIt resulted in the spontaneous autoproteolysis of the bound plasmin. AIIt did not affect the activity of other serine proteases such as t-PA or urokinase-type plasminogen activator. Furthermore, other annexins such as annexin I, II, V, or VI did not stimulate plasmin autoproteolysis. Increasing the concentration of AIIt on the surface of human 293 epithelial cells increased cell-mediated plasmin autoproteolysis. Thus, in addition to stimulating the formation of plasmin, AIIt also promotes plasmin inactivation. These results therefore suggest that AIIt may function to provide the cell surface with a transient pulse of plasmin activity.

Native plasminogen is a zymogen that consists of a single polypeptide of approximate M_r 92 000. The protein is converted to the active two-chain form, plasmin, by the cleavage of an argininyl−valine bond by tissue plasminogen activator (t-PA)[1] or urokinase-type plasminogen activator (u-PA). The larger of the two chains of plasmin, the A chain, consists of the five lysine-binding sites and is connected to the active site-containing B chain by two disulfide bridges (reviewed by refs *1*−*3*). Plasmin, a serine protease with a relatively low substrate specificity, has been shown to be 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 (reviewed in refs *1* and *4*−*8*). Plasminogen is present in the serum at a concentration of about 2 μM and is concentrated at the cell surface by virtue of its binding to its cell surface receptor (reviewed in ref *9*). Because of this high concentration of plasminogen on the cell surface, the release of minute amounts of t-PA or u-PA has the potential to generate high local concentrations of plasmin. Therefore, the regulation of both plasmin activity and the conversion of plasminogen to plasmin are critical to avoid inappropriate tissue damage and proteolysis of the extracellular matrix.

Recent work from our laboratory has established that plasmin formation is regulated by the Ca^{2+}-binding protein annexin II tetramer (AIIt) in vitro (*10*, *11*). Specifically, we have shown that AIIt binds t-PA, plasminogen, and plasmin and dramatically stimulates the t-PA- and u-PA-dependent conversion of plasminogen to plasmin in vitro (reviewed in ref *9*). Furthermore, the binding of plasmin to AIIt protected plasmin from inactivation by α_2-antiplasmin. We have also reported that the recombinant p11 subunit of AIIt stimulated the rate of t-PA-dependent activation of [Glu]-plasminogen about 46-fold compared to approximately 2-fold by the recombinant p36 subunit and 77-fold by recombinant AIIt. This established that the p11 subunit of AIIt participated in the stimulation of t-PA-dependent plasminogen activation. Last, we have shown that AIIt is present on the surface of many cells, including HUVEC, and that the loss of AIIt from the cell surface results in a decrease in the cellular conversion of plasminogen to plasmin (*9*).

Three separate mechanisms are involved in the regulation of plasmin. First, α_2-antiplasmin, the physiological inhibitor of plasmin, rapidly inactivates fluid-phase plasmin by forming a tight complex with the enzyme (*12*−*14*). Second, plasmin can activate metalloproteinases which can digest and inactivate plasmin (*15*). Third, plasmin is capable of autoproteolysis. This process is a bimolecular reaction in which

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[1] Abbreviations: BSA, bovine serum albumin; AIIt, annexin II tetramer; AIItdel, annexin II tetramer deletion mutant composed of wild-type p36 subunit and mutated p11 subunit (last two C-terminal residues deleted); p11, p11 light chain of annexin II tetramer; t-PA, tissue plasminogen activator; scu-PA, single-chain urokinase-type plasminogen activator; tcu-PA, two-chain urokinase-type 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; *p*NA, *p*-nitroaniline; U, units for expression of the initial rates of plasmin generation with the amidolytic peptide substrate ($A_{405\ nm}$/min × 10^3); buffer A, 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 5 mM $CaCl_2$; PBS, phosphate-buffered saline (137 mM NaCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 2.7 mM KCl, pH 8.0); AMCHA, *trans*-4-aminomethylcyclohexanecarboxylic acid.

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one plasmin molecule attacks another (*16*). The autoproteolysis reaction is retarded by fibrinogen, by ϵ-aminocaproic acid, by increasing ionic strength, and by glycerol. Although the autoproteolysis reaction involves the breakdown of both the A and B chains of plasmin, it is the breakdown of the B chain that has been suggested to contribute to the irreversible inactivation of the enzyme (*16*−*22*).

In the present study, we have examined the role of AIIt in the regulation of plasmin activity. Our results establish that AIIt regulates plasmin activity by stimulating plasmin autoproteolysis.

EXPERIMENTAL PROCEDURES

Materials. [Glu]-Plasminogen, [Lys]-plasminogen, plasmin, the amidolytic plasmin-specific substrate Spectrozyme #251 (H-D-norleucylhexahydrotyrosyllysine-*p*-nitroanilide), the u-PA amidolytic substrate Spectrozyme #244L, and the t-PA amidolytic substrate Spectrozyme #444 (methyl-D-cyclohexatyrosylglycylarginine-*p*-nitroaniline acetate) were obtained from American Diagnostica. Fluorescein-conjugated DQ gelatin was obtained from Molecular Probes. Human recombinant t-PA was obtained from Genentech and was 80−90% single-chain as determined by SDS−PAGE. Scu-PA and tcu-PA were generously provided by Abbott Laboratories. Annexin II tetramer was prepared from bovine lung (*23*), and recombinant wild-type annexin II monomer, p11 subunit, and annexin II tetramer were prepared from *Escherichia coli* (*24*) and stored at −70 °C in 40 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.1 mM EGTA, and 150 mM NaCl. The p11 subunit deletion mutant, lacking the last two lysine residues of the C-terminus, was produced by PCR mutagenesis according to ref *10*.

Plasmin Activity Assay. Plasmin activity was measured with the amidolytic peptide substrate H-D-norleucylhexahydrotyrosyllysine-*p*-nitroanilide (Spectrozyme #251) at a concentration of 104 μM. The reaction was conducted at 37 °C in a final volume of 0.6 mL, in a buffer consisting of 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM $CaCl_2$, 0.01% Tween 80, and 10 nM plasmin in the presence or absence of 1 μM AIIt. Typically, the reaction was preincubated at 37 °C for various times and then initiated by addition of plasmin amidolytic substrate. The reaction progress was monitored at 405 nm. The initial rates of plasmin activity were expressed in units of as units (U) of $A_{405\ nm}/min \times 10^3$. Alternatively, plasmin activity was measured as described above except that the reaction was initiated with the fluorogenic substrate DQ-gelatin (1.39 μM). Fluorescence was monitored in a Perkin-Elmer HTS 7000 Bioassay Reader (excitation wavelength of 429 nm and emission wavelength of 535 nm) and expressed as relative fluorescence units. Plasmin activity was also measured with scu-PA as the substrate. After preincubation of plasmin in the presence or absence of AIIt, the reaction was adjusted to 104 μM tcu-PA amidolytic peptide substrate (Spectrozyme #244L) and initiated by addition of scu-PA (50 nM). The plasmin-dependent conversion of scu-PA to tcu-PA was determined by analysis of the initial rate curves.

T-PA amidolytic activity was measured with 104 μM t-PA amidolytic peptide substrate, Spectrozyme #444, under identical conditions to those of the plasmin amidolytic activity assay. Tcu-PA activity was measured under identical conditions to those used for determination of the plasmin amidolytic activity except that 104 μM tcu-PA amidolytic peptide substrate (Spectrozyme #244L) was used.

Data Analysis. Plasmin activity was quantitated by measuring the increase in *p*NA concentration following the cleavage of the plasmin amidolytic peptide substrate. Initial rates of plasmin activity were calculated using linear regression analysis of plots of $A_{405\ nm}$ versus time, and plasmin activity was expressed as $A_{405\ nm}/min \times 10^3$. Typically, the initial rates of plasmin activity were reported in units, (U) of $A_{405\ nm}/min \times 10^3$.

Titration data were analyzed with the four-parameter logistic equation $f = (a - d)/[1 + (x/c)^n] + d$ where a = asymptotic maximum, n = slope factor, c = value at inflection point (IC_{50}), and d = 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.

To establish the mechanism of inactivation of plasmin by AIIt, data were analyzed according to the first-order kinetic model and the bimolecular kinetic model. The first-order mode of reaction is defined by the equation: $\ln(P_0/P_t) = kt$, where k is a first-order rate constant. A plot of $\ln(P_0/P_t)$ vs time yields a straight line of slope k. The bimolecular mode of reaction is defined by the equation: $1/P_t = 1/P_0 + kt/K_d$, where P_t refers to the plasmin activity at time t, P_0 refers to the plasmin activity at time = 0, and k is a rate constant. Therefore, a plot of $1/P_t$ vs time yields a straight line with slope k/K_d.

AIIt-Dependent Cell Surface Plasmin Inactivation. Human embryonic kidney 293 cells (ATCC) were grown in 24-well dishes in DMEM media containing 1 mM sodium pyruvate and 10% heat-inactivated fetal calf serum at 37 °C and 5% CO_2. The cells were washed twice in PBS containing 1 mM $CaCl_2$ (PBSC) and incubated with PBSC containing various concentrations of AIIt for 60 min at 37 °C. The cells were detached from the well by washing with PBSC. The cells were then extensively washed and incubated at 37 °C in buffer A containing 10 nM plasmin for 60 min. The reaction was initiated by the addition of 104 μM plasmin amidolytic substrate to the cell suspension and the absorbance at 405 nm determined.

Miscellaneous Techniques. AIIt concentration was determined spectrophotometrically using an extinction coefficient $A_{280\ nm} = 0.68$ for 1 mg/mL AIIt.

RESULTS

Inhibition of Plasmin Activity by Annexin II Tetramer. AIIt binds t-PA, plasminogen, and plasmin and dramatically accelerates the t-PA-dependent conversion of plasminogen to plasmin (*11*). However, the question of whether the binding of plasmin to AIIt influences plasmin activity has not been addressed. As shown in Figure 1A, when plasmin activity was directly measured by monitoring the hydrolysis of the plasmin amidolytic substrate, the inclusion of AIIt in the reaction mixture did not affect the plasmin activity. Since plasmin is known to be destroyed by autoproteolysis (*16*, *19*, *20*), we examined the possible effects of AIIt on this process. Accordingly, plasmin or plasmin and AIIt were preincubated at 37 °C for 15 or 30 min, and the plasmin

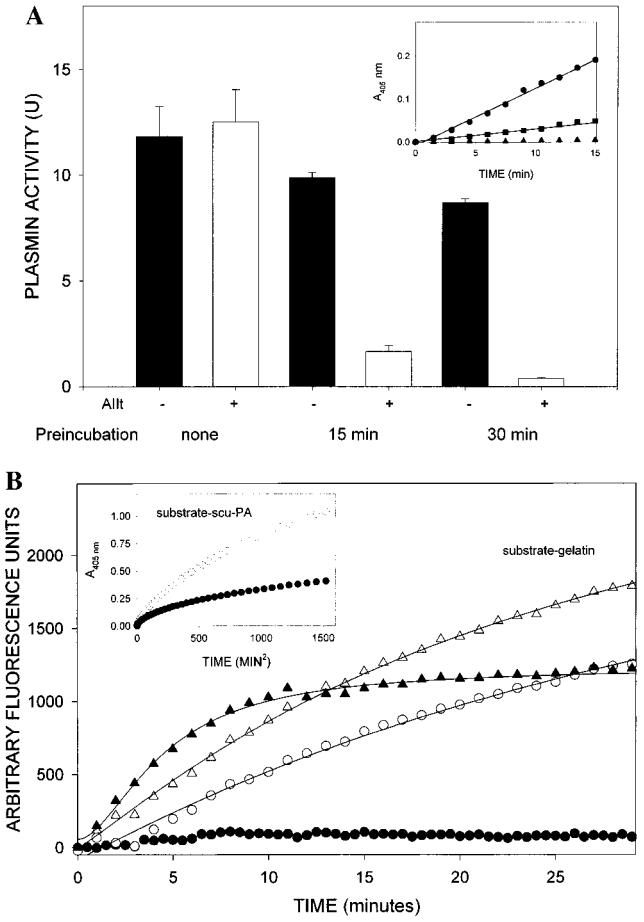

FIGURE 1: Inhibition of plasmin activity by AIIt. (A) Plasmin (10 nM) was incubated in the presence or absence of 1 μM AIIt at 37 °C in a final volume of 0.6 mL, in a buffer consisting of 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM $CaCl_2$, 0.01% Tween 80. At the indicated times, the reaction was initiated by addition of the plasmin amidolytic substrate H-D-norleucylhexahydrotyrosyllysine-*p*-nitroanilide (104 μM). The reaction progress was monitored at 405 nm as described under Experimental Procedures. Inset: Time-course of plasmin activity measured after preincubation of 10 nM plasmin with 1 μM AIIt for 0 (closed circles), 15 (closed squares), or 30 min (closed triangles). (B) Plasmin gelatinolytic activity was determined before (triangles) or after (circles) a 30 min preincubation with (filled symbols) or without (open symbols) AIIt as described above, and the reaction was conducted in a buffer consisting of 50 mM Tris, pH 7.6, 150 mM NaCl, 5 mM $CaCl_2$, and 0.2 μM sodium azide and was initiated with the fluorescence substrate DQ-gelatin (1.39 μM). The reaction progress was monitored in a fluorescence plate reader as described under Experimental Procedures. Inset: The plasmin activity was measured with scu-PA as substrate. The reaction was conducted with 10 nM plasmin, 104 μM urokinase amidolytic substrate in the absence (open circles) or presence (closed circles) of 1 μM AIIt. The reaction was initiated with scu-PA (50 nM).

activity was determined after addition of the plasmin amidolytic substrate. As shown in Figure 1A, a 30 min preincubation of plasmin alone resulted in a loss of about 25% of the plasmin activity. However, when plasmin was preincubated in the presence of AIIt, a dramatic loss in plasmin activity occurred, and after 30 min of preincubation with AIIt, only about 3−5% of plasmin activity remained.

The observation that the preincubation of AIIt with plasmin resulted in a loss of plasmin activity suggested that time courses of plasmin activity measured in the presence of AIIt would not be linear and would decrease over time as AIIt stimulated plasmin autoproteolysis. However, as shown in Figure 1A, inset, the initial rate of plasmin activity was linear in the presence of AIIt. This suggested that the plasmin peptide substrate might protect plasmin against inactivation.

We also utilized two other plasmin substrates to verify that preincubation of plasmin with AIIt caused a dramatic loss in plasmin activity. As shown in Figure 1B or Figure 1B, inset, plasmin activity measured with gelatin or scu-PA as substrate was also reduced after preincubation with AIIt. In contrast to the results obtained with the plasmin peptide substrate, even in the absence of preincubation, the initial rates of plasmin-dependent proteolysis of gelatin or scu-PA were inhibited by AIIt. As shown in Figure 1B, the time-course of plasmin-dependent hydrolysis of gelatin proceeded at a constant velocity for about 15 min and was followed by a slow decline in the plasmin gelatinolytic activity. In the presence of AIIt, a steady rate of gelatinolytic activity was observed for 10 min followed by a rapid and complete loss of gelatinolytic activity. Similarly, when scu-PA was used as a plasmin substrate, the plasmin activity was rapidly diminished in the presence of AIIt compared to the plasmin activity in the absence of AIIt (Figure 1B, inset).

Plasmin is a member of the family of serine proteases which includes other proteases such as t-PA and u-PA. The addition of AIIt to a reaction mixture composed of tcu-PA and tcu-PA amidolytic substrate did not affect the amidolytic activity of tcu-PA (data not shown). Furthermore, preincubation of tcu-PA with AIIt did not result in a subsequent loss in tcu-PA amidolytic activity. Similarly, t-PA, a serine protease that binds to AIIt (*11*), was unaffected by preincubation with AIIt. However, in the absence of a preincubation step, the t-PA amidolytic activity was slightly stimulated by AIIt. These results suggest that the AIIt-dependent loss in plasmin activity is specific to plasmin and not shared by other serine proteases.

Characterization of the AIIt-Dependent Loss in Plasmin Activity. We also examined the dependency of the AIIt-dependent loss in plasmin activity on the AIIt concentration. Plasmin was preincubated with various concentrations of AIIt and the plasmin activity determined with the plasmin amidolytic substrate. As shown in Figure 2, the loss in plasmin activity was dependent on the concentration of AIIt present during preincubation. The half-maximal loss in plasmin activity required about 0.14 μM AIIt or a molar ratio of 14 AIIt/plasmin. However, when the experiment was repeated at fixed AIIt concentration and variable concentrations of plasmin, it was observed that the AIIt-dependent loss in plasmin amidolytic activity was relatively insensitive to the plasmin concentration (Figure 2, inset). At a plasmin concentration of 0.5 μM (2 mol of AIIt/mol of plasmin), the plasmin activity was reduced to about 0.5% of the original activity, compared to 1.2% of the plasmin activity remaining after incubation of AIIt with 10 nM plasmin.

Mechanism of Plasmin Inactivation by AIIt. Figure 4 presents the time-course of AIIt-dependent plasmin inactivation. Under our assay conditions, half-maximal inactivation of 10 nM plasmin by 1 μM AIIt required about 5 min. The time-course data were initially fit to the equation: $1/P_t = 1/P_0 + kt/K_d$, where P_t refers to the plasmin activity at time t, P_0 refers to the plasmin activity at time = 0, and k is a rate constant. If the AIIt-dependent inhibition of plasmin activity follows a bimolecular mechanism, then a plot of

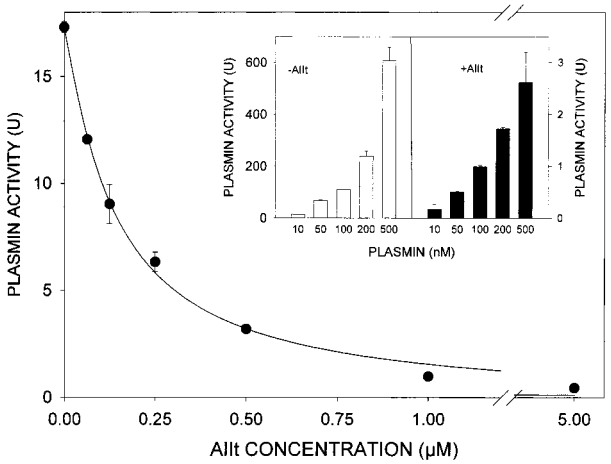

FIGURE 2: Characterization of AIIt-dependent plasmin inhibition. Plasmin (10 nM) was preincubated with various concentrations of AIIt for 30 min at 37 °C. The reaction was then initiated by addition of plasmin amidolytic substrate (104 μM), and the reaction progress was monitored at 405 nm as described under Experimental Procedures. Data shown are mean ± SD ($n = 3$). The line through the points is a nonlinear least-squares curve-fit of the data points calculated from computer modeling of data to the four-parameter logistic equation (see Experimental Procedures). The four fitting parameters were *a* (asymptotic maximum), *b* (slope parameter), *c* (value at inflection point, $A_{0.5}$), and *d* (asymptotic minimum). Inset: Various concentrations of plasmin were preincubated with 1 μM AIIt for 30 min at 37 °C, and aliquots of the reaction mixture were removed and diluted to obtain a final plasmin concentration of 10 nM. The reaction was initiated with the plasmin amidolytic substrate and activity determined as described under Experimental Procedures.

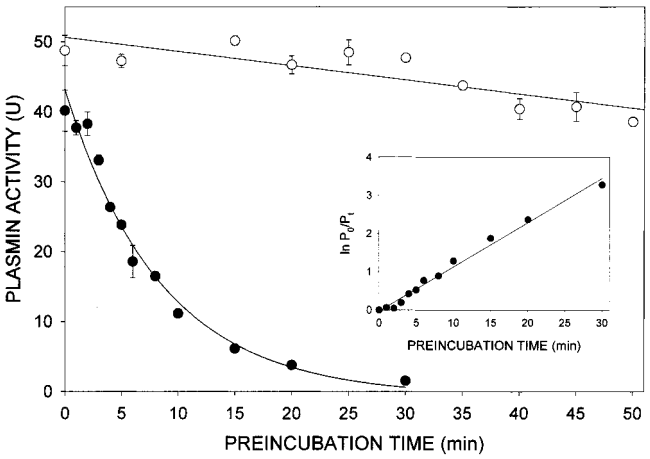

FIGURE 3: Mechanism of inhibition of plasmin activity by AIIt. Plasmin (10 nM) was incubated in the absence (open circles) or presence of 1 μM AIIt (closed circles), and aliquots were removed at the indicated times and assayed for plasmin activity as described in the legend to Figure 1. The line through the points is a nonlinear least-squares curve-fit of the data points calculated from computer modeling of data to the first-order rate equation: plasmin activity $= a - [b[1 - \exp(-kt)]]$. The three fitting parameters, *a* (plasmin activity at time 0), *b* (maximum change in plasmin activity), and *k* (apparent first-order rate constant), were allowed to float during the computer iterations. Inset: Data were plotted according to the linearized first-order rate equation: $\ln(P_0/P_t) = kt$ where P_0 is the plasmin activity at time 0, P_t is the plasmin activity at time *t*, and *k* is the apparent first-order rate constant.

$1/P_t$ vs time is expected to yield a straight line with slope k/K_d . Since a regression coefficient of 0.88 was obtained by this curve-fitting procedure it is unlikely that the reaction follows a bimolecular mechanism. However, the data fit extremely well to the first-order rate equation (correlation coefficient 0.99) (Figure 3, inset). These data fitting procedures allowed determination of an apparent first-order rate constant of 0.12 ± 0.02 min^{-1} (mean ± SD, $n = 3$) for the AIIt-dependent inactivation of plasmin amidolytic activity.

The stimulation of t-PA-dependent plasminogen activation by AIIt is inhibited by lysine analogues such as ϵ-ACA (*11*). However, the AIIt-dependent loss of plasmin amidolytic activity was only slightly inhibited by lysine analogues (data not shown). Furthermore, the AIIt-dependent loss of plasmin amidolytic activity occurred in the presence or absence of Ca^{2+}, establishing that the Ca^{2+}-binding sites of AIIt do not play a role in the interaction of plasmin with AIIt.

Plasmin autoproteolysis has also been shown to occur over a broad range of pHs and to be temperature-dependent. Furthermore, the loss in plasmin amidolytic activity due to autoproteolysis correlates with the proteolytic degradation of the plasmin light chain (*20*) or the heavy chain. As shown in Figure 4A, the AIIt-dependent loss in plasmin amidolytic activity occurs over a broad pH range. Furthermore, the AIIt-dependent loss in plasmin amidolytic activity is strongly temperature-dependent with a sharp increase in the AIIt-dependent loss in plasmin amidolytic activity between 20 and 30 °C (Figure 4B). However, the AIIt-stimulated loss in plasmin amidolytic activity corresponds to the proteolytic degradation of the plasmin heavy chain although a slight proteolysis of the plasmin light chain was observed (Figure 4B, inset). These results establish that the inactivation of plasmin amidolytic activity by AIIt is due to the AIIt-dependent acceleration of plasmin autoproteolysis and not due to inhibitory allosteric interactions between AIIt and plasmin.

Regulation of Plasminogen-Derived Plasmin by AIIt. Physiologically, plasmin is produced by the t-PA- or u-PA-dependent conversion of plasminogen to plasmin. AIIt binds t-PA, plasminogen, and plasmin and accelerates the t-PA-dependent conversion of [Glu]-plasminogen or [Lys]-plasminogen to plasmin on the extracellular surface. It was therefore possible that the interaction of t-PA and plasminogen with AIIt could affect the stimulation of plasmin autoproteolysis by AIIt. This was tested by incubating t-PA and [Lys]-plasminogen in the presence or absence of AIIt and comparing the plasmin amidolytic activity at various times during the reaction. As shown in Figure 5, AIIt dramatically inhibited the plasmin amidolytic activity produced from the t-PA-dependent cleavage of plasminogen. Furthermore, SDS−PAGE analysis of the reaction established that AIIt stimulated the autoproteolysis of both the plasmin heavy and light chains (Figure 5, inset).

Specificity of the Inactivation of Plasmin by AIIt. To assess whether the stimulation of plasmin autoproteolysis was specific to AIIt, we tested several annexins for a potential involvement in the regulation of plasmin amidolytic activity. As shown in Figure 6, the preincubation of plasmin with high concentrations of several annexins did not result in a significant loss in plasmin amidolytic activity. Interestingly, the p36 subunit of AIIt (annexin II) also failed to stimulate a loss in plasmin amidolytic activity whereas the p11 subunit inhibited plasmin amidolytic activity by about 50%. Last, we had previously shown that an AIIt deletion mutant, which consists of a wild-type p36 subunit and a p11 subunit in

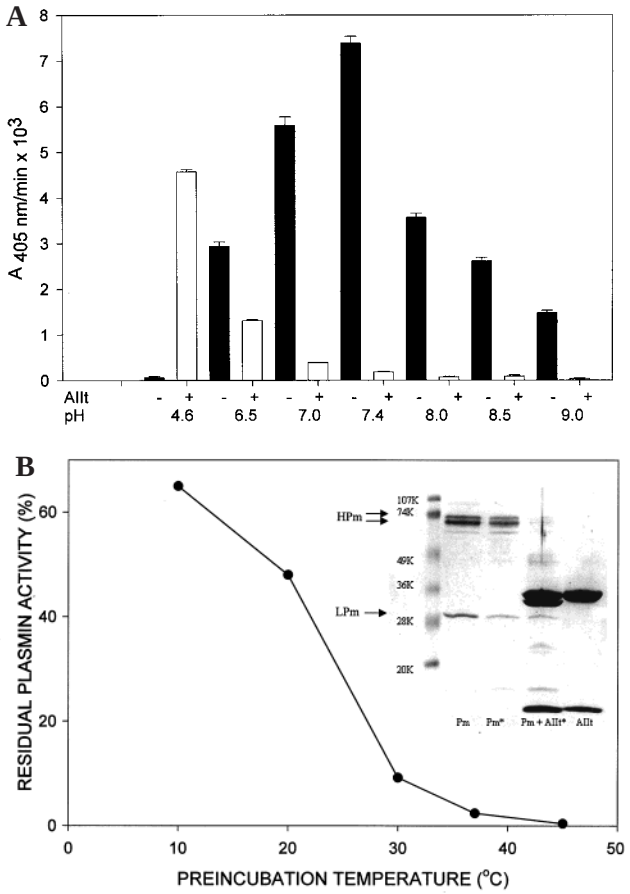

FIGURE 4: Attenuation of the AIIt-dependent loss in plasmin activity. (A) pH profile of AIIt-stimulated loss in plasmin activity. Plasmin was preincubated with μM AIIt in a buffer consisting of 50 mM Tris, 50 mM MES, 100 mM NaCl, 5 mM $CaCl_2$, and 0.01% Tween-100 at several different pHs. The reaction was conducted at 37 °C for 30 min. The reaction was diluted 3-fold and assayed as described in the Figure 1 legend. (B) Plasmin (10 nM) was incubated in the presence or absence of AIIt (1 μM) at various temperatures for 30 min. The reaction mixture was rapidly equilibrated to 37 °C and the plasmin activity determined. The results are expressed as a percentage of the plasmin activity assayed in the presence of AIIt compared to the plasmin activity assayed in the absence of AIIt. The inset shows the SDS−PAGE analysis of reactions in which 600 nM plasmin was incubated in the absence (Pm*) or presence (Pm+AIIt*) of 1 μM AIIt for 30 min at 37 °C. The standards include plasmin (Pm) and AIIt (AIIt). HPm and LPm refer to the heavy and light chains of plasmin, respectively.

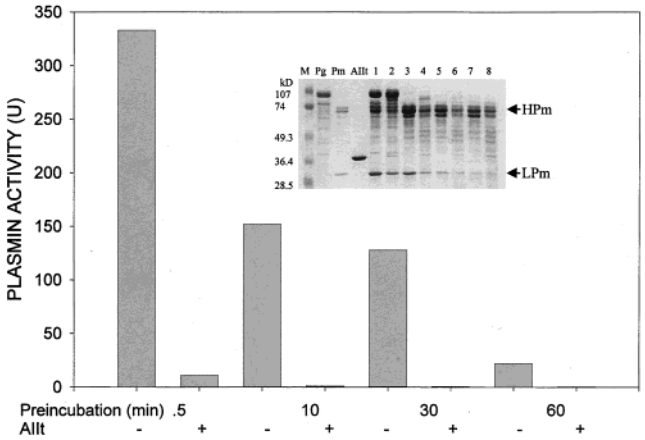

FIGURE 5: Effect of AIIt on the plasmin generated by t-PA-dependent plasminogen cleavage. [Lys]-Plasminogen (92 μM) was incubated with t-PA (0.39 μM) at 37 °C in the absence or presence of AIIt (2 μM). At various timed intervals, an aliquot of the reaction mixture was removed and either subjected to SDS−PAGE (inset) or assayed for plasmin activity after dilution in plasmin assay buffer. Inset: The reaction was conducted in the absence (odd numbers) or presence of AIIt (even numbers) for 0.5 min (1, 2), 10 min (3, 4), 30 min (5, 6) or 60 min (7, 8). HPm, LPm, Pg, and Pm refer to the plasmin heavy chain, the plasmin light chain, [Glu]-plasminogen, and plasmin, respectively.

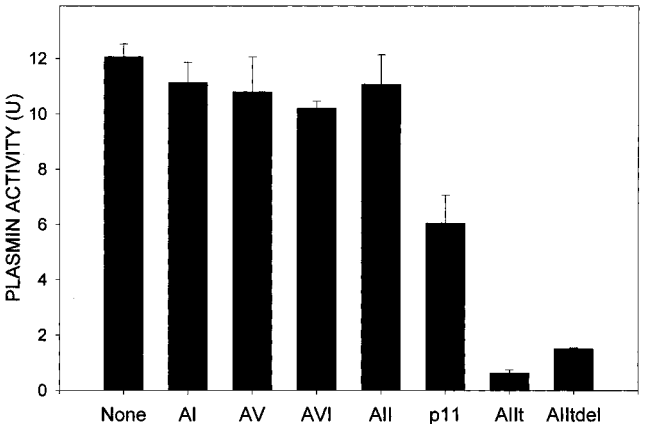

FIGURE 6: Specificity of the annexin-dependent loss in plasmin activity. Plasmin (10 nM) was incubated with 1 μM AIIt or 2 μM test protein for 30 min at 37 °C. The reaction was initiated by the addition of plasmin amidolytic substrate. Plasmin activity was determined as described in the legend to Figure 1.

which the last two C-terminal lysines have been deleted, failed to bind to plasminogen and stimulate t-PA-dependent plasminogen activation (*10*). As shown in Figure 6, the AIIt deletion mutant inhibited plasmin amidolytic activity almost as potently as wild-type AIIt.

Regulation of Membrane-Bound Plasmin by AIIt. AIIt is present on the surface of a variety of cultured cells (reviewed in ref *9*). We therefore examined the possibility that AIIt, bound to the extracellular surface of the plasma membrane, might also stimulate a loss in plasmin amidolytic activity. Human embryonic kidney 293 cells were used in these experiments because these cells do not have appreciable amounts of AIIt on their extracellular surface but can bind exogenous AIIt (*25*). Therefore, the 293 cells were incubated with several concentrations of AIIt and extensively washed to remove free AIIt. FACS analysis established that the concentration of cell surface-associated AIIt was increased (data not shown). As shown in Figure 7, increasing the concentration of AIIt on the extracellular surface of human embryonic kidney 293 cells results in an enhanced capability of these cells to decrease plasmin amidolytic activity.

DISCUSSION

The low substrate specificity of plasmin requires that cells localize its formation in order to prevent concomitant tissue damage. Furthermore, once concentrated at the cell surface, effective mechanisms exist to inactivate membrane-associated plasmin in order to avoid cellular damage. For example, macrophages regulate membrane-bound plasmin by stimulation of plasmin autoproteolysis (*26*). Our current model for extracellular plasminogen regulation involves a key role for AIIt in the binding of t-PA, plasminogen, and plasmin and the activation of plasminogen on the cell surface (*9*). However, our model does not provide a mechanism for the eventual inactivation of AIIt-associated plasmin.

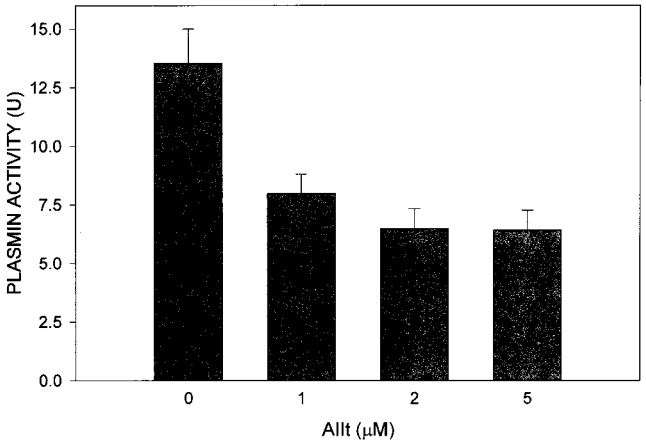

FIGURE 7: AIIt-dependent cell surface plasmin inactivation. Human embryonic kidney 293 cells were incubated in PBSC in the presence or absence of several concentrations of AIIt for 1 h at 37 °C. Subsequently, the cells were washed twice with PBSC and incubated with 10 nM plasmin for 1 h at 37 °C. The plasmin activity assay was then initiated by addition of plasmin amidolytic substrate (104 μM), and the absorbance at 405 nm was determined.

We report here that AIIt dramatically stimulates the loss of plasmin activity toward three distinct substrates, namely, scu-PA, casein, and an amidolytic peptide substrate. Since AIIt binds plasmin, the effect of AIIt on plasmin activity could be due to two possible mechanisms. First, the binding of AIIt to plasmin could induce a conformational change in plasmin resulting in the inhibition of plasmin activity. This allosteric mechanism could result in a reversible loss in plasmin activity. Second, AIIt could stimulate plasmin autoproteolysis, resulting in an irreversible loss in plasmin activity. Plasmin autoproteolysis results in the autoproteolysis of the plasmin heavy and light chains. Since the enhanced loss of plasmin activity observed in the presence of AIIt corresponds with an enhanced digestion of the plasmin heavy and light chains, we have concluded that AIIt stimulates plasmin autoproteolysis.

Several laboratories have suggested that autoproteolysis of plasmin results in the digestion of the plasmin heavy and light chains in vitro (*16*−*20*). The digestion of the plasmin light chain has also been suggested to best correlate with the loss in plasmin activity in vitro (*22*). Other laboratories have shown that cleavage of the plasmin heavy chain can result in a loss in plasmin activity (*27*, *28*). Studies with macrophages have also shown that cell surface plasmin autoproteolysis results in the cleavage of the plasmin heavy chain (*26*). Similarly, our data establish that AIIt stimulates the autoproteolysis of both plasmin heavy and light chains; the plasmin heavy chain was the most extensively digested in the presence of AIIt. As shown in the inset of Figure 4B, the AIIt-stimulated loss of plasmin activity occurs when the plasmin heavy chain is completely digested but only slight proteolysis of the plasmin light has occurred. Our data are consistent with a model in which the stimulation of plasmin autoproteolysis best correlates with the digestion of the plasmin heavy chain.

Although our data establish that AIIt stimulates plasmin autoproteolysis, it was also interesting that the patterns of fragmentation of plasmin in the presence or absence of AIIt were not identical (Figure 5, inset). The simplest explanation for this observation is that the binding of plasmin to AIIt induces a conformational change in plasmin, which results in the exposure of new proteolytic sites. This suggestion is consistent with our previous observation that the binding of plasminogen to AIIt results in a conformational change in plasminogen (*11*).

To establish the mechanism by which AIIt stimulates plasmin autoproteolysis, we examined two possible experimental models: the bimolecular mode of reaction and the first-order mode of reaction. The bimolecular mechanism predicts that the AIIt−plasmin complex cleaves free plasmin and the free plasmin therefore acts as a substrate. In contrast, the first-order mechanism predicts that plasmin bound to AIIt catalyzes its own proteolysis. We attempted to fit the time-course of AIIt-dependent inactivation of plasmin data (Figure 3) to both experimental models, and the data were found to be consistent with the first-order model. This suggested that the binding of plasmin to AIIt accelerated the autoproteolysis of AIIt-bound plasmin. We also observed that in the absence of preincubation of plasmin and AIIt, the plasmin-dependent proteolysis of the plasmin amidolytic substrate, or gelatin, or scu-PA was not stimulated by AIIt. However, if the bimolecular mechanism were correct, then it would be expected that the AIIt−plasmin complex would have accelerated activity toward free plasmin or against these substrates. Last, we have also observed that the half-time of the rate of plasmin inactivation by AIIt is insensitive to the concentration of plasmin (data not shown). Again, the simplest explanation for these data is that AIIt induces a conformational change in plasmin and in this new conformation the plasmin catalytic site is positioned to allow the intramolecular proteolysis of the plasmin heavy and light chains.

We cannot, however, rule out the possibility that the stimulation of plasmin autoproteolysis is second-order. The second-order rate equation is relevant when the catalytic rate is small enough to allow the reactants to form the enzyme−substrate complex under equilibrium conditions. If AIIt greatly stimulates the catalytic rate, then the enzyme−substrate complex will not reach equilibrium, and the data will not fit the second-order rate equation. However, since AIIt does not stimulate the initial rates of plasmin activity toward the amidolytic peptide substrate (*11*) or other substrates such as uPA or collagen (Figure 1B), this possibility seems unlikely.

Previous work from our laboratory established that the C-terminal lysines of the p11 subunit of AIIt play a key role in the AIIt-dependent stimulation of t-PA-dependent plasminogen conversion to plasmin. For example, we observed that lysine analogues such as ϵACA inhibited the AIIt-dependent stimulation of t-PA-dependent plasminogen conversion to plasmin. In addition, an AIIt deletion mutant consisting of the wild-type p36 subunit and a mutant p11 subunit that was missing the C-terminal lysine residues failed to stimulate t-PA-dependent plasminogen conversion to plasmin. We also reported that the C-terminal lysines of p11 were required for the binding of plasminogen to AIIt and for the AIIt-dependent conformational change in AIIt (*10*). In contrast to these observations, we have observed that lysine analogues do not block the stimulation of plasmin autoproteolysis by AIIt and the AIIt deletion mutant is almost as potent a stimulator of plasmin autoproteolysis as the

wild-type AIIt. These results suggest that the plasminogen and plasmin binding sites occupy distinct regions on AIIt. The C-terminal lysines of the p11 subunit play a key role in the AIIt-dependent stimulation of t-PA-dependent plasminogen conversion to plasmin and in plasminogen binding but do not appear to play a role in the AIIt-dependent regulation of plasmin activity.

We also tested several annexins as potential regulators of plasmin activity. However, of the annexins tested, only AIIt regulated plasmin activity. Since the p36 subunit of AIIt (annexin II) failed to inhibit plasmin activity and the p11 subunit had a limited ability to inhibit plasmin activity, it is likely that the domain of AIIt responsible for plasmin regulation comprises regions of both subunits. We also observed that of the three serine proteases tested, namely, t-PA, u-PA, and plasmin, only plasmin activity was regulated by AIIt. Although tPA binds to AIIt, it was interesting that AIIt did not affect the serine protease activity of t-PA.

Our observation that AIIt inhibited plasmin activity by stimulating plasmin autoproteolysis was based on experiments that examined the direct interaction of plasmin and AIIt. However, plasmin is generated on the cell surface by the t-PA- or u-PA-dependent conversion of plasminogen to plasmin. We therefore examined the effect of AIIt on plasmin activity during the AIIt-stimulated t-PA-dependent conversion of plasmin to plasminogen. Under these conditions, we observed that AIIt also inhibited plasmin activity by stimulating plasmin autoproteolysis.

Previous work from our laboratory has utilized both immunofluorescence colocalization and immunoprecipitation of AIIt from surface-biotinylated cells to establish the presence of AIIt on the cell surface (*11*). We have also shown that decreasing the extracellular AIIt concentration of RAW 117 cells by transfection with a dominant-negative mutant of AIIt results in a significant loss in cellular tPA-dependent conversion of plasminogen to plasmin (*9*). To investigate the potential physiological significance of the stimulation of plasmin autoproteolysis by AIIt, we added AIIt exogenously to the cell surface of human embryonic kidney 293 cells. These cells are normally devoid of extracellular annexin II. It was interesting that AIIt added exogenously to the cell surface only decreased plasmin activity by 53% compared to the complete loss of plasmin activity observed by incubation of AIIt and plasmin in vitro. One possible explanation is that under our experimental conditions, plasmin binds to the 293 cells at sites distinct from AIIt and this membrane-bound plasmin is inaccessible to AIIt. The binding of plasmin to 293 cells has been reported, so it is reasonable to suspect the presence of plasmin binding sites on these cells that are distinct from AIIt. However, it is not clear if in the presence of physiological inhibitors of plasmin such as α_2-antiplasmin these sites will protect plasmin from inactivation. In contrast, the binding of plasmin to AIIt protects plasmin from inactivation by α_2-antiplasmin in vitro (*10*).

AIIt appears to have a dual function in the regulation of plasminogen. In the presence of t-PA or u-PA, AIIt stimulates the production of plasmin from plasminogen. On the other hand, once the plasmin is produced, it is rapidly degraded due to the AIIt-dependent stimulation of plasmin autoproteolysis. In this respect, AIIt is the consummate cellular plasminogen receptor since it regulates both the formation and the destruction of plasmin. In contrast, endothelial cell surface annexin II has been shown to stimulate the constitutive production of plasmin from plasminogen. Specifically it was suggested that annexin II was cleaved by an unidentified cell surface protease at Lys^{307} and this resulted in the exposure of a new C-terminal lysine. Isolation and characterization of this novel, proteolyzed form of annexin II have been elusive. Furthermore, cell surface annexin II did not protect tPA or plasmin from inhibition by their physiological inhibitors. The inability of annexin II to protect plasmin from inactivation by its physiological inhibitor, α_2-antiplasmin, coupled with the low rates of stimulation of plasmin formation suggests that annexin II may have a limited role in terms of plasminogen regulation (*29*, *30*). It is therefore tempting to speculate that AIIt functions to localize plasmin production to the cell surface where the competition between the stimulation of plasmin production and destruction by AIIt ensures that only a transient pulse of plasmin activity is produced at the cell surface.

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