Identification of annexin II heterotetramer as a plasmin reductase

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Running title

Role of annexin II tetramer in plasmin reduction
Summary

Annexin II heterotetramer (AIIt) is a Ca$^{2+}$- and phospholipid-binding protein that consists of two copies of a p36 and p11 subunit. AIIt regulates the production and autoproteolysis of plasmin at the cell surface. In addition to its role as a key cellular protease, plasmin also plays a role in angiogenesis as the precursor for antiangiogenic proteins. Recently we demonstrated that the primary antiangiogenic plasmin fragment, called A$_{61}$ (Lys$^{78}$-Lys$^{468}$) was released from cultured cells. In the present study we report for the first time that AIIt possesses an intrinsic plasmin reductase activity. AIIt stimulated the reduction of the plasmin Cys$^{462}$-Cys$^{541}$ bond in a time- and concentration-dependent manner which resulted in the release of A$_{61}$ from plasmin. Mutagenesis of p36 Cys$^{334}$-Ser, and either p11 Cys$^{61}$-Ser or p11 Cys$^{82}$-Ser inactivated the plasmin reductase activity of the isolated subunits, suggesting that specific cysteinyl residues participated in the plasmin reductase activity of each subunit. Furthermore, we demonstrated that the loss of AIIt from the cell surface of HT1080 cells transduced with a retroviral vector encoding p11 antisense, dramatically reduced the cellular production of A$_{61}$ from plasminogen. This is the first demonstration that AIIt regulates the cellular production of the antiangiogenic plasminogen fragment, A$_{61}$.
Introduction

Angiostatin was originally identified in the urine of mice bearing Lewis lung carcinoma (LLC) as a 38 kDa proteolytically-derived fragment of plasminogen which encompassed the first four kringle domains (Lys^{78}-Ala^{440}). Angiostatin was shown to be a potent antiangiogenic protein that inhibited the growth of human and murine carcinomas and also induced dormancy in their metastases. Angiostatin was also characterized as a specific antiangiogenic protein that blocked microvascular endothelial cell proliferation but not the proliferation of nonendothelial cells (1).

It is now apparent that angiostatin is a member of a family of anti-angiogenic plasminogen fragments (AAPFs). Physiologically relevant AAPFs include a 38 kDa AAPF isolated from the conditioned media of tumor-infiltrating macrophages (2), a 43 kDa and 38 kDa AAPF identified in the conditioned media of Chinese hamster ovary and HT1080 fibrosarcoma cells and a 48 kDa AAPF present in macrophage conditioned media (3). Other AAPFs include a 43 kDa and a 38 kDa AAPF isolated from the conditioned media of human prostate carcinoma PC-3 cells (4;5) and AAPFs of 66, 60 and 57 kDa detected in the conditioned media of HT1080 and Chinese hamster ovary cells (6). Since the carboxyl-terminus of most of these AAPFs was not determined, the exact primary sequence of most of the AAPFs is not known.

Two distinct pathways have been identified for the formation of AAPFs. First, certain proteinases can directly cleave plasminogen into AAPFs. These proteinases include metalloelastase, gelatinase B (MMP-9), stromelysin-1 (MMP-3), matrilysin (MMP-7), cathepsin D and prostate-specific antigen (7-11). The source of these proteinases may be tumor-infiltrating macrophages (2) or the cancer cells themselves. For example, the conversion of
plasminogen to angiostatin by macrophages is dependent on the release of metalloelastase from these cells. In comparison, Lewis lung carcinoma cells release MMP-2 which also cleaves plasminogen to angiostatin (12). Second, AAPFs are also generated by a three step mechanism which involves the conversion of plasminogen to plasmin by urokinase type plasminogen activator (u-PA), the autoproteolytic cleavage of plasmin and the release of the resultant plasmin fragment by cleavage of disulfide bonds. The cleavage of the plasmin disulfide bonds can be accomplished by free sulfhydryl group donors (FSD) such as glutathione or by hydroxyl ions at alkaline pH (4;5;13;14). Alternatively, the plasmin disulfide bonds can be cleaved enzymatically by a plasmin reductase such as phosphoglycerate kinase (15;16).

Our laboratory has shown that the primary AAPF present in mouse and human blood has a molecular weight of 61 kDa (14). We have produced this AAPF, called A61, in a cell-free system consisting of u-PA and plasminogen. A61 was shown to be a novel four-kringle containing plasminogen fragment consisting of the amino acid sequence, Lys78-Lys468 (14). The release of A61 from plasmin required cleavage of the Lys468-Gly469 bond by plasmin autoproteolysis and also cleavage of the Cys462-Cys541 disulfide. Since A61 was generated in our cell-free system from plasmin at alkaline pH in the absence of sulfhydryl donors, we concluded that cleavage of the Cys462-Cys541 disulfide was catalyzed by hydroxyl ions in vitro. In contrast, at physiological pH, we observed that the conversion of plasminogen to A61 was very slow. These results contrasted with our observation that at physiological pH, HT1080 fibrosarcoma and bovine capillary endothelial (BCE) cells stimulated the rapid formation of A61. The mechanism
by which these cells stimulated plasmin reduction and the release of $A_61$ from plasmin was unclear, however it was reasonable to suspect that the cells utilized a plasminogen-binding protein on the cell surface to facilitate the formation of $A_61$.

Annexin II tetramer (AIIt) is a Ca$^{2+}$-binding protein that binds plasminogen and plasmin and stimulates both the formation and autoproteolysis of plasmin at the cell surface (17-19)(reviewed in (20)). The protein consists of two copies of a 36 kDa subunit (p36) called annexin II and two copies of a 11 kDa subunit (p11) called S100A10. Previous work from our laboratory has shown that the carboxyl-terminal lysines of the p11 subunit play a key role in plasminogen binding and activation (18).

In the present report we show that AIIt stimulates the conversion of plasminogen to $A_61$ in vitro. We also report for the first time that AIIt possesses an intrinsic plasmin reductase activity, and that the cysteinyl residues of both subunits of AIIt participate in the reduction of plasmin. Among the seven annexins tested, only annexin II (p36) and AIIt act as plasmin reductase. Additionally, the antisense nucleotide-directed down-regulation of AIIt on the surface of HT1080 fibrosarcoma cells, greatly diminishes $A_61$ production by these cancer cells. Our results therefore show for the first time that AIIt may play a role in angiogenesis by regulating the cellular release of an antiangiogenic protein.
EXPERIMENTAL PROCEDURES

Materials - Two-chain urokinase-type plasminogen activator (u-PA) was a generous gift from Dr. H. Stack (Abbott Laboratories). [Glu]-plasminogen and plasmin were purchased from American Diagnostica. Antiangiogenic plasminogen fragment (A61) was purified as outlined previously (14). Annexin II tetramer (AIIt) and other annexins were purified from bovine lung as described in (21). Monoclonal anti-human plasminogen kringle 1-3 antibody was purchased from Enzyme Research Laboratories Inc. Monoclonal anti-annexin II and anti-annexin II light chain antibodies were purchased from Transduction Laboratories. Anti-mouse horseradish peroxidase-conjugated secondary antibody was purchased from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated streptavidin and protein disulfide isomerase were purchased from Calbiochem. Anti-mouse R-phycoerythrin-conjugated secondary antibody was purchased from Caltag Laboratories Inc. Nα-(3-maleimidylpropionyl)biocytin (MPB) was purchased from Molecular Probes. Reduced glutathione, iodoacetamide, iodoacetic acid, diisopropylfluorophosphate (DIFP), and thioredoxin were purchased from Sigma. L-lysine-Sepharose was purchased from Amersham Pharmacia Biotech. Iodobeads were purchased from Pierce. Phosphoglycerate kinase (PGK) was a generous gift from Dr. P. J. Hogg (Center for Thrombosis and Vascular Research, University of New South Wales, Sydney, Australia). Stably transfected HeLa cells expressing p11 antisense or sense mRNA were a generous gift from Dr. J. H. Shelhamer (Critical Care Medicine Department, National Institutes of Health, USA). HT1080 fibrosarcoma cells were obtained from American Type Culture Collection. Dulbecco’s modified eagle medium (DMEM) was purchased from Life Technologies.

Mutagenesis of Annexin II and p11 - Bacterial expression vectors containing the wild-
type sequence for annexin II (pAED4.91-annexin II) and p11 (pAED4.91-p11) were mutated using the QuikChange" Site-Directed Mutagenesis Kit (Stratagene). Briefly, mutagenic primers were synthesized that introduced Cys à Ser mutations at positions 8 and 334 of annexin II, as well as positions 61 and 82 of p11. All of the mutations introduced were verified by DNA sequence analysis. These various plasmids were then transformed into *E. coli* BL21 (DE3) and grown as previously described (22).

**Purification of Wild-type and Mutant Annexin II** - After 4 hours of induction with IPTG, bacteria were collected by low speed centrifugation. The cells were subsequently sonicated in lysis buffer (10 mM imidazole, pH 7.5, 150 mM NaCl, 2 mM EGTA, 1 mM DTT + protease inhibitors) and centrifuged at 100,000 x g for 1 hour at 4 °C. Both mutant annexin II proteins were purified in the same manner as wild-type annexin II via hydroxyapatite, heparin-Sepharose affinity and gel permeation chromatography as reported previously (22). The elution profiles of the recombinant wild-type and mutant annexin II on hydroxyapatite, heparin affinity and gel permeation chromatography were indistinguishable. In addition, the circular dichroism spectra of each of the proteins were very similar, indicating little secondary structure perturbation.

**Purification of Wild-type and Mutant p11** - After 4 hours of induction with IPTG, bacteria were collected by low speed centrifugation. The cells were subsequently sonicated in lysis buffer (100 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10 mM MgCl₂, 2 mM DTT + protease inhibitors) and centrifuged at 100,000 x g for 1 h at 4 °C. Both p11 mutants were purified in the same manner as wild-type p11 (23). Briefly, the cell lysis supernatant was precipitated with 50% (NH₄)₂SO₄, and the supernatant was applied to a Butyl-Sepharose column equilibrated in lysis
buffer containing 50% (NH₄)₂SO₄. The p11 was eluted with a linear gradient of (NH₄)₂SO₄ from 50% to 0%, and peak fractions containing p11 were pooled and dialyzed against 10 mM imidazole, pH 7.4, 1 mM EGTA, 0.5 mM DTT, and 0.1 mM EDTA. The dialyzed fractions were subsequently applied to a DEAE-Sepharose column equilibrated in the same buffer. The p11 was eluted with a linear NaCl gradient, concentrated to 4 mL, and applied to a Sephacryl S-100 column equilibrated in 40 mM Tris-HCl, pH 7.4, 0.1 mM EGTA, and 0.1 mM DTT. A single protein peak was recovered at the expected molecular weight based on gel filtration standards.

**Plasmid Construction and Transfection of HT1080 Cells** - Sense and antisense p11 expression vector were produced by cloning the full-length human p11 cDNA into the pLin retroviral vector in the sense (pLin-p11S) or antisense orientation (pLin-p11AS) as reported (24). Control cells transduced with the vector alone (pLin-V) were also established. The pLin vector carries the Moloney murine leukemia virus 5’ LTR enhancer/promoter region to promote strong, constitutive expression of the cloned p11 inserts and neomycin phosphotransferase gene in mammalian cells. The pLin constructs were propagated in a PA317 retroviral packaging cell line. Packaging cells were selected in 300 µg/ml neomycin and conditioned media that contained high titers of the virus were used to transduce the HT1080 fibrosarcoma cells. After viral transduction the neomycin resistant HT1080 fibrosarcoma cells were cloned and permanent cell lines established (Choi, K-S et al., manuscript in preparation).

**Dialysis of Candidate Plasmin Reductase Proteins** – After purification or reconstitution, AlIt, p11, p36 (annexin II), other annexins, thioredoxin, protein disulfide isomerase, and phosphoglycerate kinase were dialyzed against 20 mM Tris (pH 7.5) and 140 mM NaCl under argon gas to prevent possible oxidation.
**Plasmin Reductase Assay** – [Glu]-plasminogen (4 µM) was incubated with 0.075 µM u-PA and a candidate plasmin reductase protein (4 µM, unless described) in a buffer containing 20 mM Tris (pH 7.5) and 140 mM NaCl at 37 °C for 2h. A portion of reaction mixture was diluted with SDS-PAGE sample buffer and subjected to non-reduced SDS-PAGE followed by Coomassie blue staining. To label any free thiol groups of produced protein(s), the reaction mixture was incubated with 100 µM MPB at room temperature for 30 min. The reaction mixture was then treated with 200 µM reduced glutathione at room temperature for 10 min to quench the unreacted MPB. The unreacted glutathione and other free thiols in the reaction mixture were blocked with 400 µM of iodoacetamide at room temperature for 10 min. Then the reaction mixture was incubated with 1-lysine-Sepharose at room temperature for 30 min to purify the kringle-containing, plasminogen-derived proteins. The matrix was extensively washed with PBS and the bound proteins were eluted by boiling the resin with SDS-PAGE sample buffer. Each sample was subjected to non-reduced SDS-PAGE followed by Western blot with horseradish peroxidase-conjugated streptavidin (streptavidin-HRP) as indicated below.

**Detection of free thiols in AIIt** – 2 µM AIIt, p11, or p36 was incubated with 100 µM MPB in a buffer containing 20 mM Tris (pH 7.5) and 140 mM NaCl at room temperature for 30 min. After incubation, 200 µM reduced glutathione and 400 µM iodoacetamide were added sequentially, and the reaction mixture was subjected to reduced SDS-PAGE followed by either Coomassie blue staining or Western blot with streptavidin-HRP as indicated below.

**Cell-Mediated Generation of A61** – Transduced HT1080 cells were maintained in DMEM supplemented with 10 % heat-inactivated fetal bovine serum, 2 mM L-glutamine, 10 units/ml penicillin G, 10 µM streptomycin sulfate, and 300 µg/ml neomycin. Approximately
1x10^5 cells were added to each well of 24-well tissue culture plates and incubated at 37 °C for 24 h. The cell monolayers were then washed three times with DMEM and 2 µM [Glu]-plasminogen, plasmin, or diisopropylfluorophosphate (DIFP)-treated plasmin in DMEM was added to each well. The conditioned medium was removed at indicated times, diluted with SDS-PAGE sample buffer with or without β-mercaptoethanol and subjected to SDS-PAGE followed by Western blot with monoclonal anti-human plasminogen kringle 1-3 antibody (anti-K1-3) as indicated below.

_Electrophoresis and Western Blot -_ Samples were diluted with SDS-PAGE sample buffer and subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose membrane (0.45 µm pore size) at 4 °C for 1 h. The membrane was blocked in TPBS (phosphate buffered saline containing 0.1 % Tween-20) with 5 % skim milk at room temperature for 1 h and incubated at 4 °C overnight with a 0.4 µg/ml monoclonal anti-human plasminogen kringle 1-3 antibody in TPBS with 5 % skim milk. The blot was extensively washed with TPBS at room temperature and then incubated at room temperature for 1 h with a 0.16 µg/ml horseradish peroxidase-conjugated goat anti-mouse secondary antibody in TPBS with 5 % skim milk. In the case of MPB-reacted protein samples, the membrane was blocked and incubated at room temperature for 1 h with a 0.1 µg/ml streptavidin-HRP in TPBS with 5 % skim milk. The membrane was extensively washed with TPBS and visualized by enhanced chemiluminescence (Pierce).

_Binding of A61 to AIIt –_ The purified A61 was iodinated according to manufacturer’s procedures. Iodinated A61 retained biological activity as determined by the endothelial cell
proliferation assay (14). 96-well Immulon-1 Removawell strips (Dynex Technologies) were coated with phospholipid mixture containing 3:1 ratio of phosphatidylserine to phosphatidylcholine and air-dried. The coated strips were blocked with 1 % fatty acid-free bovine serum albumin (BSA) in a buffer containing 20 mM Hepes (pH 7.4), 140 mM NaCl, and 2 mM CaCl$_2$ (buffer A) at room temperature for 2 h. The strips were washed with buffer A and incubated with 1 µM AIIIt in buffer A at room temperature for 4 h. The strips were then washed and incubated with 0.008-5 µM iodinated A$_{61}$ with or without 30-fold molar excess of cold A$_{61}$ or BSA at 4°C. After overnight incubation, the strips were washed five times with PBS, and individual wells were detached and measured for radioactivity with a $\gamma$-counter. The data shown (n=6) are the average of two separate experiments.

Flow Cytometric Analysis of Transduced HT 1080 Cells – Transduced HT 1080 cells were harvested and 1 x 10$^6$ cells in PBS were divided into each tube. The cells were fixed with 4 % paraformaldehyde in PBS at room temperature for 20 min and washed twice with PBS. The cells were then incubated with 1 µg of monoclonal anti-annexin II or anti-annexin II light chain antibody at room temperature for 30 min. For the control staining, 1 µg of mouse Ig G was used. The cells were washed and incubated with 2 µg/ml anti-mouse R-phycoerythrin-conjugated secondary antibody at room temperature for 30 min. The cells were washed and subjected to flow cytometric analysis using FACSscan (Beckton Dickinson) and analyzed by the FlowJo program. The data shown are a representative of three separate experiments.
Results

**AIIt stimulates the conversion of plasminogen to A₆₁** - A₆₁ is an internal fragment of plasminogen that encompasses the sequence Lys₇₈-Lys₄⁶₈. In order for A₆₁ to be released from plasmin, the Cys⁴₆₂-Cys⁵₄₁ disulfide of plasmin must be reduced. The release of A₆₁ will therefore result in the generation of a free sulfhydryl residue at Cys⁴₆₂. Since plasminogen and plasmin contain only disulfides, A₆₁ can be discriminated from these proteins on the basis of its reactivity with free sulfhydryl-reactive reagents such as 3-(N-maleimidylpropionyl)biocytin (MPB). The reaction of free-sulfhydryl-containing proteins with MPB results in the biotinylation of the protein which allows easy detection with streptavadin-HRP.

As shown in Fig. 1A (lane 2), the incubation of u-PA with plasminogen resulted in the generation of plasmin. As expected, the plasmin generated by this reaction did not contain a free cysteiny1 residue and therefore did not react with MPB (Fig. 1B, lane 2). However, the addition of AIIt to the u-PA-plasminogen reaction resulted in the appearance of A₆₁ (which displays a
single major band and two minor bands of about Mr 50 K on non-reduced SDS-PAGE) and disappearance of plasmin (Fig. 1A). Furthermore, the A₆₁ generated in these reactions reacted with MPB, confirming the presence of a free sulphydryl in A₆₁ (Fig. 1B).

AIIt stimulated the dose- and time-dependent conversion of plasminogen to A₆₁ (Fig. 1A-D). The maximal conversion of plasminogen to A₆₁ occurred at approximately equimolar concentrations of AIIt and plasminogen (Fig. 1A,B). At equimolar concentrations the half-maximal conversion of plasminogen to A₆₁ occurred between 30-60 min (Fig. 1C,D). Since AIIt stimulated the generation of A₆₁ in the absence of sulphydryl donors, our data suggest that AIIt promoted the cleavage of a plasmin disulfide, presumably the Cys462-Cys541 disulfide, resulting in the release of A₆₁ from plasmin and the generation of a free cysteine (Cys462) in A₆₁. Interestingly, since AIIt looses reactivity to MPB during the reaction (data not shown) it is likely that AIIt becomes oxidized during plasmin reduction.

Role of the p36 and p11 subunits in the plasmin reductase activity of AIIt – AIIt is composed of two copies of a p36 and two copies of a p11 subunit. The p11 subunit plays a key role in plasminogen binding and stimulation of the plasminogen activator-catalyzed conversion of plasminogen to plasmin (18). We therefore attempted to identify which subunit of AIIt was responsible for the plasmin reductase activity of AIIt. As shown in Fig. 2A,B the incubation of either the p36 or p11 subunit with u-PA and plasminogen stimulated the formation of A₆₁. However, AIIt was a more potent plasmin reductase than either subunit, suggesting that the interaction of the subunits potentiated the plasmin reductase activity of either subunit.
Theoretically, either the disulfides or cysteiny1 residues of AIIt could participate in the reduction of plasmin. It is possible that upon plasmin(ogen) binding the disulfide of annexin II is reduced by the thiols of annexin II and the newly formed thiols participate in reduction of plasmin. Since the p11 subunit does not contain disulfides we suspected that the thiols of this subunit were important for its plasmin reductase activity. p11 contains two cysteiny1 residues: Cys61 which plays a critical role in the binding of p36 and Cys82 which is a free thiol (25). We prepared two recombinant forms of this subunit in which individual cysteiny1 residues were mutated to serine. This conservative mutation results in the substitution of a thiol group for a hydroxyl group. As shown in Fig. 2B, substitution of either of these cysteine residues resulted in a loss of plasmin reductase activity of the p11 subunit. This suggests that both cysteiny1 residues of p11 are required to sustain the plasmin reductase activity of the protein.

Human p36 contains two thiol-containing cysteines, Cys8 and Cys334. As shown in Fig. 2B, substitution of Cys334, but not Cys8, with serine blocked the plasmin reductase activity of p36. This result suggests that the Cys334 thiol is critical for plasmin reductase activity of the p36 subunit.

**Thiol reactivity of AIIt and its subunits** – In order to identify the reactive thiols of AIIt we incubated AIIt and its individual subunits with MPB and resolved the mixture with SDS-PAGE followed by Coomassie blue staining (Fig. 3A) or Western blot with streptavidin-HRP (Fig. 3B). As shown in Fig. 3B, the p11 subunit and both Cys61Ser and Cys82Ser p11 mutants were labeled with MBP. In contrast, although p36 and the Cys334Ser p36 mutant were labeled with MPB, the Cys8Ser mutant was not labeled. This suggests that the thiols of the isolated p11 are
accessible to MPB whereas the Cys\textsuperscript{334} of p36 is not accessible. Interestingly, both the p36 and p11 subunits within AIIt were labeled with MPB. However, preincubation of AIIt with iodoacetic acid or MPB resulted in only a small decrease in the plasmin reductase activity of AIIt (Fig. 3C). This suggests that the Cys\textsuperscript{334} thiol of the p36 subunit of AIIt is inaccessible to the solvent.

**Specificity of the plasmin reductase activity of AIIt** – The Cys\textsuperscript{334} residue is highly conserved among many of the annexins. Since our data suggested that this residue participated in the plasmin reductase activity of p36, we suspected that other annexins which possess this cysteine might also have plasmin reductase activity. We therefore examined seven different annexins for plasmin reductase activity. As shown in Fig. 4, only annexin II (p36) and AIIt possessed plasmin reductase activity. This result establishes that plasmin reductase activity is not a common feature of the annexins.

**A\textsubscript{61} binds to AIIt** – Previous data from our laboratory have established that AIIt binds both plasminogen and plasmin and is present at discrete regions of the extracellular surface (17;20;26). This suggested that AIIt could act as a scaffolding protein and focus the proteolytic activity of plasmin to the plasma membrane. It was unclear if the A\textsubscript{61} generated by cells could remain bound to AIIt at the cell surface or be immediately released into the media. However, as shown in Fig. 5, A\textsubscript{61} bound to AIIt with a $K_d$ of 1.0 ± 0.05 μM (n=6). Interestingly, we observed that A\textsubscript{61} did not block the stimulation of tPA-catalyzed conversion of plasminogen to plasmin (data not shown), suggesting that the binding sites on AIIt for A\textsubscript{61} are distinct from those for plasminogen or...
plasmin. This suggests that some of the A$_61$ produced by the AIIt’s plasmin reductase reaction may remain bound to AIIt at the cell surface.

**Comparison of AIIt with other disulfide reductases** – Protein disulfide isomerase, thioredoxin and phosphoglycerate kinase are three protein disulfide reductases that are secreted by cultured cells (16;27-29). The three reductases have been shown to act as plasmin reductases (16). As shown in Fig. 6, under the assay conditions AIIt was a more potent plasmin reductase than the other reductases *in vitro*.

Thioredoxin and protein disulfide isomerase share a common sequence, Trp-Cys-Gly-Pro-Cys-Lys which participates in the cleavage, formation and reshuffling of disulfide bonds. This sequence is not present in phosphoglycerate kinase or AIIt, suggesting that these reductases have distinct catalytic mechanisms. Typically, the disulfide reductase activity of thioredoxin or protein disulfide isomerase is measured by determination of their rates of reduction of insulin disulfide (30;31). Interestingly, although protein disulfide isomerase and thioredoxin exhibit potent insulin reductase activity, AIIt failed to exhibit insulin reductase activity (data not shown). This further confirms that the catalytic mechanism of AIIt is distinct from that of the traditional protein reductases.

**Down-regulation of AIIt blocks A$_61$ generation by HT1080 fibrosarcoma cells** – Recently, we successfully utilized retroviral-mediated gene transfer to stably transduce HT1080 fibrosarcoma cells with a pLin retroviral vector encoding a p11 gene in the sense (pLin-p11S) or antisense (pLin-p11AS) orientation (Choi, K.-S. *et al.*, manuscript in preparation). We have cloned the transduced cells and begun to characterize three cloned HT1080 cell lines: pLin-p11S, pLin-p11AS and pLin-V. Interestingly, the pLin-p11AS cells showed decreased both p11
and p36 subunits on the cell surface whereas pLin-p11S cells showed increased both p11 and p36 subunits (Fig. 7A). As shown in Fig. 7B, incubation of the pLin-p11S cells with plasminogen resulted in enhanced A₆₁ formation compared to the pLin-V control cells. In contrast, the pLin-p11AS cells failed to produce A₆₁. Additionally, we confirmed that A₆₁ produced by HT 1080 cells was reduced since it reacted with MPB (data not shown). We found that HeLa cells transfected with p11 antisense also failed to convert plasminogen to A₆₁ (data not shown).

The data in Fig. 7B establishes a role for AIIt in A₆₁ formation in HT 1080 fibrosarcoma cells. However, it is unclear if plasmin could be directly reduced or if plasmin autoproteolysis preceded plasmin reduction. As shown in Fig. 7C, the incubation of plasmin with the pLin-p11S cells also resulted in the accelerated disappearance of plasmin and concomitant enhanced appearance of A₆₁ compared with pLin-p11V cells. In contrast, the pLin-p11AS cells showed reduced plasmin loss and concomitant A₆₁ formation compared to the pLin-p11V cells. In another series of experiments, plasmin was inactivated by prior treatment of the serine protease inhibitor, DIFP and then incubated with the HT1080 cells. However, under these conditions we were unable to detect A₆₁ production by the cells (data not shown). Furthermore, the catalytically inactive plasmin that was incubated with the cells was not reduced since it did not react with MPB (data not shown). This result establishes that plasmin autoproteolysis is required before plasmin reduction can occur. Collectively, our results suggest that the mechanism of A₆₁ formation involves the u-PA-dependent conversion of plasminogen to plasmin followed by
plasmin autoproteolysis and reduction of cleaved plasmin (Fig. 8).

Discussion

In this report we have demonstrated for the first time that the extracellular protein, AIIt, contains an intrinsic plasmin reductase activity. Our results suggest that AIIt regulates the formation of the antiangiogenic plasminogen fragment, \( A_{61} \) by catalyzing the reduction of plasmin disulfides which allows the release of \( A_{61} \) from plasmin. Our observation that HT1080 fibrosarcoma cells transduced with an antisense p11 vector show both dramatically reduced extracellular AIIt and \( A_{61} \) production further indicates that AIIt is the predominant regulatory protein for \( A_{61} \) generation on the surface of HT1080 fibrosarcoma cells. Furthermore, the ability of AIIt to bind \( A_{61} \) suggests that AIIt may localize \( A_{61} \) to the cell surface.

Previously, we demonstrated that AIIt was localized to the extracellular surface of a variety of cultured cells including human umbilical vein endothelial cells, BT20 breast carcinoma cells, U87 glioma and HT1080 fibrosarcoma cells (17;20;26). Our \textit{in vitro} studies established that AIIt and specifically its p11 subunit stimulated tPA- and u-PA-catalyzed conversion of plasminogen to plasmin (17;18;20). Additional studies showed that AIIt stimulated plasmin autoproteolysis (19). Most recently we demonstrated that HT1080 fibrosarcoma or bovine capillary endothelial cells converted plasminogen into the antiangiogenic plasminogen fragment, \( A_{61} \) (14). The current study has consolidated our previous studies by demonstrating that AIIt stimulates the formation of \( A_{61} \) from plasminogen.

Our results also allow the development of a model to define the mechanism by which
HT1080 cells generate A$_{61}$ from plasminogen. Our model postulates that HT1080 fibrosarcoma cells convert plasminogen to A$_{61}$ in a three-step process (Fig. 8). First, u-PA cleaves the Arg$_{561}$-Val$_{562}$ of plasminogen resulting in the formation of plasmin. Second, plasmin autoproteolysis results in the cleavage of the Lys$_{77}$-Lys$_{78}$ and Lys$_{468}$-Gly$_{469}$ bond. However, the presence of a Cys$_{462}$-Cys$_{541}$ disulfide prevents release of A$_{61}$ (Lys$_{78}$-Lys$_{468}$). Third, AIIt catalyzes the reduction of the Cys$_{462}$-Cys$_{541}$ disulfide which allows the release of A$_{61}$ from the rest of the molecule. Since AIIt does not reduce catalytically inactive plasmin we have concluded that plasmin autoproteolysis must occur before plasmin reduction. This suggests that autoproteolyzed plasmin is the substrate for AIIt’s plasmin reductase activity.

Our model of A$_{61}$ generation differs from earlier reports. Soff’s group reported that the mechanism for AAPF formation involved plasmin autoproteolysis and reduction by sulfhydryl donors such as reduced glutathione (5). In contrast, Hogg’s group postulated that AAPFs are formed by the reduction of plasmin by phosphoglycerate kinase followed by the serine protease-dependent release of the fragment from plasmin (16). Our mechanism is unique in that we show that plasmin autoproteolysis must precede plasmin reduction and that plasmin reduction can be accelerated by AIIt. The AIIt present at the surface of HT1080 fibrosarcoma cells is required to convert plasmin(ogen) to A$_{61}$ and loss of extracellular AIIt results in the inhibition of cell-generated A$_{61}$. Since large amounts of plasmin(ogen) are converted to A$_{61}$ by these cells, AIIt must act as a catalyst. This suggests that in order for AIIt to continually reduce plasmin it must proceed through cycles of oxidation by plasmin and reduction by unknown reducing equivalents.
The mechanism by which oxidized extracellular AIIt is reduced is unknown at this time.

Our data indicate that both the p36 and p11 subunits of AIIt possess plasmin reductase activity. In the case of the p36 subunit the Cys\textsuperscript{334} residue is essential for plasmin reductase activity. In the case of the p11 subunit both Cys\textsuperscript{61} and Cys\textsuperscript{82} are capable of participating in plasmin reduction. Although speculative, the simplest explanation for these results is that plasmin and autoproteolyzed plasmin can bind to AIIt but the unique conformational change induced by the binding of autoproteolyzed plasmin to AIIt results in increased accessibility of cysteinyl residues of AIIt which participate in reduction of autoproteolyzed plasmin. Since Cys\textsuperscript{334} of p36 is not labeled with the thiol specific reagent MPB it is likely shielded from the solvent. Furthermore, the p36 Cys\textsuperscript{334}-Ser mutant is inactive in terms of plasmin reductase activity. It is therefore reasonable to suspect that Cys\textsuperscript{334} may be shielded and only accessible for reduction of plasmin upon binding of autoproteolyzed plasmin to AIIt.

Protein disulfide reductases typically contain the Cys-X-X-Cys motif in their active sites. Members of this family of proteins include thioredoxin, protein disulfide isomerase, fibronectin, von Willebrand factor and platelet integrin α\textsubscript{IIb}β\textsubscript{3} (28;29;32-36). Typically these proteins share the general property of catalyzing the reduction of insulin disulfides. In contrast, AIIt does not contain the Cys-X-X-Cys motif and does not catalyze the reduction of insulin disulfides. This suggests that the intrinsic plasmin reductase activity of AIIt is due to a novel mechanism of disulfide reduction.

Future studies will be directed towards defining the interactions that regulate the plasmin reductase activity of AIIt and determining how the subunits participate in this activity.
Acknowledgments

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References


Footnotes

Abbreviations: AIIt, annexin II heterotetramer; A_61, plasminogen fragment defined by the amino acid sequence Lys^{78}-Lys^{468}; AAPF, antiangiogenic plasminogen fragment; tPA, tissue-type plasminogen activator; u-PA, two chain urokinase-type plasminogen activator; DTT, dithiothreitol; MPB, 3-[N-maleimidylpropionyl]biocytin; streptavidin-HRP, horseradish peroxidase-conjugated streptavidin; PGK, phosphoglycerate kinase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified eagle medium; 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); TPBS, phosphate buffered saline containing Tween-20 (137 mM NaCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 2.7 mM KCl, pH 8.0 and 0.1% Tween-20).
Figure Legends

**Figure 1.** Stimulation of conversion of plasminogen to A₆₁ by AIIt. *(A,B)* AIIt dose-dependent generation of A₆₁. [Glu]-plasminogen (4 µM) was incubated with u-PA (0.075 µM) and the following concentration of AIIt in a buffer containing 20 mM Tris (pH 7.5) and 140 mM NaCl at 37 °C for 2 h; 0.5 µM *(lane 3)*; 1 µM *(lane 4)*; 2 µM *(lane 5)*; 4 µM *(lane 6)*; 6 µM *(lane 7).* As a control, [Glu]-plasminogen was incubated with u-PA *(lane 2).* After incubation, a portion of reaction mixture was subjected to non-reduced SDS-PAGE followed by Coomassie blue staining *(A).* The remaining reaction mixture was incubated with MPB (100 µM) at room temperature for 30 min, further incubated with reduced glutathione (200 µM) at room temperature for 10 min, then incubated with iodoacetamide (400 µM) at room temperature for 10 min. Then the reaction mixture was incubated with l-lysine-Sepharose at room temperature for 30 min. The matrix was extensively washed with PBS and the bound proteins were eluted by boiling the resin with SDS-PAGE sample buffer. Each sample was subjected to non-reduced SDS-PAGE followed by Western blot with streptavidin-HRP *(B).* A₆₁ standard is also shown *(lane 1).* *(C,D)* Time-course generation of A₆₁. [Glu]-plasminogen (4 µM) was incubated with u-PA (0.075 µM) and AIIt (4 µM) in a buffer containing 20 mM Tris (pH 7.5) and 140 mM NaCl at 37 °C for the indicated time; 0.5 h *(lane 3)*; 1.0 h *(lane 4)*; 1.5 h *(lane 5)*; 2.0 h *(lane 6)*; 2.5 h *(lane 7).* As a control, [Glu]-plasminogen was incubated with u-PA at 37 °C for 2.5 h *(lane 2).* The other experimental procedures are the same as described in Figure 1A,B. The results are shown as Coomassie blue staining *(C)* and Western blot with streptavidin-HRP *(D).*
Figure 2. Identification of the role of p11 and p36 subunits in the plasmin reductase activity of AIIt. [Glu]-plasminogen (4 µM) was incubated with u-PA (0.075 µM) and the following subunit of AIIt or AIIt (4 µM) in a buffer containing 20 mM Tris (pH 7.5) and 140 mM NaCl at 37 °C for 2 h; wild-type p11 (lane 2); p11-C61S mutant (lane 3); p11-C82S mutant (lane 4); wild-type p36 (lane 5); p36-C8S mutant (lane 6); p36-C334S mutant (lane 7); AIIt (lane 8). As a control, [Glu]-plasminogen was incubated with u-PA at 37 °C for 2 h (lane 1). The other experimental procedures are the same as described in Figure 1A, B. The results are shown as Coomassie blue staining (A) and Western blot with streptavidin-HRP (B).

Figure 3. Identification of thiol reactivity of AIIt and its subunits. AIIt or its subunits (2 µM) was incubated with MPB (100 µM) in a buffer containing 20 mM Tris (pH 7.5) and 140 mM NaCl at room temperature for 30 min; wild-type p11 (lane 1); p11-C61S mutant (lane 2); p11-C82S mutant (lane 3); wild-type p36 (lane 4); p36-C8S mutant (lane 5); p36-C334S mutant (lane 6); AIIt (lane 7). After incubation, reduced glutathione and iodoacetamide was added sequentially, and the reaction mixture was subjected to reduced SDS-PAGE followed by either Coomassie blue staining (A) or Western blot with streptavidin-HRP (B). (C) AIIt (18 µM) was incubated with iodoacetic acid (IAA, 10 mM) or MPB (200 µM) in a buffer containing 20 mM Tris (pH 7.5) and 140 mM NaCl at room temperature for 30 min. After incubation the reaction mixture was dialyzed against 20 mM Tris (pH 7.5) and 140 mM NaCl under argon gas. Then, [Glu]-plasminogen (4 µM) was incubated with u-PA (0.075 µM) and the indicated AIIt (4 µM) in a buffer containing 20 mM Tris (pH 7.5) and 140 mM NaCl at 37 °C for 2 h; AIIt (lane 2); IAA-treated AIIt (lane 3); MPB-treated AIIt (lane 4). As a control, [Glu]-plasminogen was incubated
with u-PA at 37 °C for 2 h (lane 1). The other experimental procedures are the same as described in Figure 1A, B. The result shown is a Western blot with streptavidin-HRP.

Figure 4. Specificity of the plasmin reductase activity of AIIt. [Glu]-plasminogen (4 µM) was incubated with u-PA (0.075 µM) and the indicated annexin proteins (4 µM) in a buffer containing 20 mM Tris (pH 7.5) and 140 mM NaCl at 37 °C for 2 h; annexin 1 (lane 2); annexin 2 (lane 3); annexin 3 (lane 4); annexin 4 (lane 5); annexin 5 (lane 6); annexin 6 (lane 7); AIIt (lane 8). As a control, [Glu]-plasminogen was incubated with u-PA at 37 °C for 2 h (lane 1). The other experimental procedures are the same as described in Figure 1A, B. The result shown is a Western blot with streptavidin-HRP.

Figure 5. Binding of A61 to AIIt. The wells of Removawell strips were coated with phospholipid, blocked with bovine serum albumin, incubated with AIIt, and extensively washed. Various concentrations of iodinated A61 were added into the AIIt-coated wells in the absence (filled circles) or presence of 30-fold molar excess of bovine serum albumin (filled triangles) or cold A61 (open circles). After overnight incubation, the strips were washed five times with PBS, and individual wells were detached and measured for radioactivity with γ-counter. The data shown (n=6) were the average of two separate experiments.

Figure 6. Comparison of AIIt with other disulfide reductases. [Glu]-plasminogen (4 µM) was incubated with u-PA (0.075 µM) and the following proteins in a buffer containing 20 mM Tris (pH 7.5) and 140 mM NaCl at 37 °C for 2 h; 2 µM AIIt (lane 2); 4 µM AIIt (lane 3); 2 µM thioredoxin (lane 4); 4 µM thioredoxin (lane 5); 2 µM protein disulfide isomerase (lane 6); 4 µM protein disulfide isomerase (lane 7); 2 µM phosphoglycerate kinase (lane 8); 4 µM
phosphoglycerate kinase (lane 9). As a control, [Glu]-plasminogen was incubated with u-PA at 37 °C for 2 h (lane 1). The other experimental procedures are the same as described in Figure 1A,B. The result shown is a Western blot with streptavidin-HRP.

**Figure 7. Down-regulation of AIIt blocks A₆₁ generation by HT1080 cells.** (A) Flow cytometric analysis of transduced HT 1080 cells. The staining procedures were performed as indicated under “Experimental Procedures”. Control staining (dotted line) indicates that mouse Ig G was used instead of anti-p11 or anti-p36 antibody. The result of p11 or p36 staining for p11 antisense-transduced cells (solid line), vector-transduced cells (dashed line) and p11 sense-transduced cells (bold line) is shown. (B,C) Comparison of the generation of A₆₁ by transduced HT 1080 cells. The generation of stable HT 1080 clones were performed as indicated under “Experimental Procedures”. Transduced HT 1080 cells were incubated with DMEM containing 2 µM [Glu]-plasminogen (B) or plasmin (C). After the indicated time of incubation, the medium was analyzed by reduced SDS-PAGE (B) or non-reduced SDS-PAGE (C) followed by Western blot with monoclonal anti-human plasminogen kringle 1-3 antibody. As a control, each protein was incubated with DMEM in the absence of cells (lane 1,5,9). The following HT 1080 clones were used; p11 sense-transduced cells (lane 2,6,10); p11 antisense-transduced cells (lane 3,7,11); control vector-transduced cells (lane 4,8,12). A₆₁ standard is also shown (lane 13).

**Figure 8. Diagrammatic illustration of the mechanism of A₆₁ formation.** K represents the kringle domain of plasminogen. S-S indicates the disulfide bond and SH represents the free thiol generated. Plasmin catalyzes the cleavage of the Lys⁷⁷-Lys⁷⁸ and Lys⁴⁶⁸-Gly⁴⁶⁹ bonds of plasminogen.
Fig. 4
Fig. 5
Fig. 6

Streptavidin-HP blots

Pg + uPA
Pg + uPA + AIIt (2 μM)
Pg + uPA + AIIt (4 μM)
Pg + uPA + Thioredoxin (2 μM)
Pg + uPA + Thioredoxin (4 μM)
Pg + uPA + PDI (2 μM)
Pg + uPA + PDI (4 μM)
Pg + uPA + PGK (2 μM)
Pg + uPA + PGK (4 μM)
Plasminogen \textit{inactive} \xrightarrow{\text{AIIt}} \text{uPA} \rightarrow \text{Plasmin \textit{active}} \xrightarrow{\text{AIIt}} \text{Plasmin \textit{autoproteolyzed}} \xrightarrow{\text{AIIt}} \text{Plasmin \textit{reduced}} \xrightarrow{\text{dissociation}} \text{A}_{61} \text{antiangiogenic}

\begin{center}
\textbf{Fig. 8}
\end{center}
Identification of annexin II heterotetramer as a plasmin reductase
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