The p11 Subunit of Annexin II Heterotetramer Is Regulated by Basic Carboxypeptidase[†]

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ABSTRACT: The Ca^{2+}-dependent phospholipid-binding protein annexin II heterotetramer (AIIt) is composed of two copies of annexin II and a p11 dimer. The interaction of the carboxyl-terminal lysine residues of the p11 subunit of AIIt with the lysine-binding kringle domains of plasminogen is believed to play a key role in plasminogen binding and stimulation of the tPA-catalyzed cleavage of plasminogen to plasmin. In the current report, we show that AIIt-stimulated plasminogen activation is regulated by basic carboxypeptidases, in vitro. The incubation of AIIt with a 1/400 molar ratio of carboxypeptidase B for periods as short as 2 min resulted in a significant loss in AIIt-stimulated plasminogen activation. Carboxypeptidase B (CpB) as well as thrombin-activated fibrinolysis inhibitor (TAFIa) and carboxypeptidase N (CpN) rapidly reduced AIIt-stimulated plasminogen activation by 80%. The molar ratio of carboxypeptidase/AIIt for half-maximal inhibition of AIIt was 1/4700, 1/700, and 1/500 for CpB, TAFIa, and CpN, respectively. Treatment of AIIt with carboxypeptidase resulted in loss of both carboxyl-terminal lysine residues from the p11 subunit, which correlated with a decrease in the k_{cat} and an increase in the K_m for plasminogen activation. The data reveal a novel mechanism for the regulation of AIIt-stimulated plasminogen activation.

Plasminogen is present in the blood as an inactive zymogen at a concentration of about 2 μM. The activation of plasminogen results from the cleavage of the $Arg^{561}-Val^{562}$ bond by tissue-type (tPA)[1] or urokinase (uPA) plasminogen activator. The resultant broad substrate protease, plasmin, degrades a variety of substrates including fibrin and extracellular matrix proteins and participates in a host of physiological and pathological processes including fibrinolysis during blood clot dissolution, wound healing, and tumor growth and metastasis (*1, 2*). Plasminogen activation is a tightly regulated process since overproduction of plasmin leads to widespread cellular destruction and excessive degradation of the extracellular matrix (*3*).

Cell surface plasminogen receptors are widely distributed and play a key role in regulating plasminogen activation (*4*−*8*). Although several extracellular proteins have been shown to bind plasminogen and stimulate plasminogen activation in vitro, the key regulator(s) of cellular plasminogen activation has (have) not been identified in vivo. The specific binding and accelerated activation of plasminogen on cells is dependent on interaction of the kringle domains of plasminogen with the carboxyl-terminal lysine residues of the plasminogen receptors. This interaction can be abrogated with the carboxyl-terminal lysine analogue ϵ-aminocaproic acid (ϵ-ACA) or by treatment of cells with carboxypeptidase B (CpB) which removes basic residues from the carboxyl termini of proteins (*8*).

The Ca^{2+}- and phospholipid-binding protein annexin II has been suggested to be a specific plasminogen receptor on endothelial cells (*9*) and immune cells (*10*); however, monomeric annexin II lacks the carboxyl-terminal lysine residue required for specific plasminogen binding. We have shown that the calmodulin-related protein S100A10 is present on the surface of many cells and is a potent stimulator of tPA-catalyzed plasminogen activation. The majority of extracellular S100A10 forms a tight complex with annexin II (*11*). This heterotetrameric complex, referred to as annexin II heterotetramer (AIIt), is composed of two subunits of annexin II (p36 subunit) and two subunits of S100A10 (p11 subunit). In vitro, recombinant AIIt stimulates tPA-catalyzed plasminogen activation about 77-fold compared to about 2-fold or 46-fold for recombinant annexin II or S100A10, respectively. However, the plasminogen activator activity of AIIt can be reconstituted by a complex of S100A10 and a peptide consisting of the 14 amino acids of the S100A10-binding site of annexin II. Therefore, we have proposed that the p11 subunit of AIIt plays a key role in the regulation of

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[1] Abbreviations: tPA, tissue-type plasminogen activator; AIIt, annexin II heterotetramer; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MGTA, DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid; CpB, carboxypeptidase B; CpN, carboxypeptidase N; TAFIa, active thrombin-activated fibrinolysis inhibitor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; GEMSA, 2-guanidinoethylmercaptosuccinic acid.

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plasminogen activation and that annexin II may serve to target S100A10 to the plasma membrane.

The ultimate and penultimate carboxyl-terminal residues of p11 are lysine, which suggests that these residues may play a role in plasminogen binding and activation. Moreover, when the carboxyl-terminal lysine residues of p11 were deleted by site-directed mutagenesis, the ability of the protein to accelerate plasminogen activation was decreased by 80% (*12*). This suggested that the carboxyl-terminal lysine residues of the p11 subunit played a key role in the regulation of plasminogen activation. However, whether removal of the lysine residues from the C-terminus of p11 represented an actual regulatory mechanism was not established. The demonstration that carboxypeptidase B could remove the carboxyl-terminal lysines of extracellular plasminogen receptors and thereby block cellular plasminogen activation suggested that if AIIt was a bone fide plasminogen regulatory protein then its carboxyl-terminal lysines would be cleaved by carboxypeptidase B.

In the present study, we have investigated the effect of carboxypeptidase B on AIIt-stimulated plasminogen activation. Treatment of AIIt with CpB resulted in release of approximately 4 mol of lysine/mol of AIIt and abrogated AIIt-stimulated plasminogen activation. The removal of the carboxyl-terminal lysines resulted in an increased mobility of p11 in SDS−PAGE. Other carboxypeptidases such as carboxypeptidase N (CpN) or the thrombin-activated fibrinolysis inhibitor (TAFI) also cleaved the carboxyl-terminal lysines of AIIt and blocked AIIt-stimulated plasminogen activation.

EXPERIMENTAL PROCEDURES

Materials. Human recombinant tPA was obtained from Genentech and was 80−90% single chain as determined by SDS−PAGE. Native glutamic acid plasminogen (Glu-plasminogen), plasmin, Mab Anti-Mini-plasminogen (α-plasminogen), and the amidolytic plasmin substrate Spectrozyme 251 (H-D-norleucyl-hexahydrotyrosyl-lysine-*p*-nitroanilide) were obtained from American Diagnostica. Human recombinant wild-type annexin II, p11, and annexin II tetramer were prepared from *Escherichia coli* (*13*) and stored at −70 °C in buffer A [40 mM Tris-HCl, pH 7.5, 0.1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 150 mM NaCl] containing 1 mM DTT. AIIt was prepared from bovine lung as described (*14*). Before use, all proteins were dialyzed into buffer A containing 0.1 mM DTT. The p11 mutant lacking the last two lysine residues from the carboxy terminus was produced as described previously (*12*). Mouse monoclonal antibodies to annexin II monomer and p11 were obtained from Transduction Laboratories (Lexington, KY). Horseradish peroxidase-conjugated goat anti-mouse secondary antibody was obtained from Santa Cruz (Santa Cruz, CA). Porcine pancreatic CpB was obtained from Worthington Biochemical (Lakewood, NJ). CpN was purified from outdated human plasma according to (*15*) with some modifications. Briefly, CpN was purified using lysine-Sepharose affinity chromatography, followed by precipitation in 65% $(NH_4)_2SO_4$. The resuspended pellet was dialyzed against 50 mM HEPES, pH 7.4, and applied to Fast Q and eluted with 50 mM HEPES, pH 7.4, and a linear NaCl gradient (0−400 mM). The eluted CpN was then dialyzed against 50 mM HEPES, pH 7.4, overnight, followed by gel permeation chromatography on Sephacryl S-300. The resultant single peak of protein had an apparent molecular mass of 280 kDa and had activity against carboxypeptidase substrates (see below). Recombinant human TAFI (rTAFI) was produced in bovine hamster kidney (BHK) cells and purified as described previously (*16*). The synthetic carboxypeptidase substrates hippuryl-lysine-OH (Hipp-K-OH) and hippuryl-arginine-OH (Hipp-R-OH) were obtained from Bachem. *N*-[3-(2-Furylacryloyl)]-L-alanyl-L-lysine (FAAL), *N*-[3-(2-furylacryloyl)]-L-alanyl-L-arginine (FAAR), ϵ-aminocaproic acid (ϵ-ACA), DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (MGTA), and 2-guanidinoethylmercaptosuccinic acid (GEMSA) were obtained from Sigma (St. Louis, MO).

Construction of p11 Carboxyl-Terminal Lysine Deletion Mutants. Production and purification of recombinant p11 in which the two carboxyl-terminal lysine residues were deleted ($p11_{des-KK}$) were previously described (*12*). The QuickChange Kit (Stratagene, La Jolla, CA) was used to produce a mutant p11 construct in which only the lysine at position 96 was deleted ($p11_{del-K96}$). This construct was transformed into BL21(DE3) *E. coli*, expressed as described previously (*13*), and purified according to Ayala-Sanmartin (*17*) with modifications. After cell lysis in lysis buffer (100 mM Tris, pH 7.5, 200 mM NaCl, 10 mM $MgCl_2$, 2 mM DTT) and centrifugation at 40 000 rpm for 40 min, the supernatant was precipitated with 50% $(NH_4)_2SO_4$ and centrifuged at 15 000 rpm for 20 min. This supernatant was then applied to a Butyl-Sepharose column (Pharmacia) equilibrated with the same buffer. The p11 was eluted with a linear gradient from 50% to 0% $(NH_4)_2SO_4$ in the same buffer. This was then dialyzed against 10 mM imidazole, pH 7.4, 1 mM EGTA, 0.1 mM EDTA, 0.5 mM DTT and applied to a DEAE-Sepharose column (Pharmacia). The flow through was subjected to gel permeation chromatography on high-resolution Sephacryl S-200 (Pharmacia) to obtain highly pure mutant p11.

Activation of TAFI. Recombinant human TAFI (rTAFI) was activated for 10 min at 25 °C with 20 nM thrombin and 500 nM thrombomodulin in 20 mM HEPES, pH 7.5, 10 mM $CaCl_2$ and was used immediately.

Plasminogen Activation Assay. Rates of tPA-dependent plasminogen activation were determined by measurement of the amidolytic activity of the plasmin generated from Glu-plasminogen by tPA as described previously (*18*). Reactions were performed in triplicate in 200 μL volumes of reaction buffer containing 50 mM Tris, pH 7.4, 3 mM $CaCl_2$, 100 mM NaCl with 10 nM tPA and 2 μM AIIt, p11, or annexin II before or after treatment with various carboxypeptidases. The basal rate of tPA-catalyzed plasminogen activation was determined by substituting an equal volume of buffer A containing 0.1 mM DTT for the added proteins. The reaction was initiated by addition of 100 nM Glu-plasminogen for time-dependence studies, or increasing concentrations of Glu-plasminogen for dose-dependence studies, into wells of 96-well plates. Liberation of *p*-nitroanilide from Spectrozyme 251 by plasmin was monitored by the change in absorbance at 405 nm in a BioTek El_x 808 platereader.

Treatment of AIIt with Carboxypeptidase. For carboxypeptidase concentration dependence studies, wild-type or mutant AIIt (40 μM), or annexin II was treated for 40 min with various concentrations of CpB, CpN, or TAFIa in a 37

°C circulating water bath. For time-dependence studies, 0.1 μM CpB, 0.5 μM CpN, or 0.1 μM TAFIa was used, and aliquots of the reaction mixture were removed at various time points. For determination of the kinetic parameters of plasminogen activation, 40 μM AIIt was treated with 0.1 μM CpB, 0.2 μM CpN, or 0.1 μM TAFIa, and various concentrations of Glu-plasminogen were used. The reactions were terminated with 10 μM MGTA for activity assays, or by immediate boiling in SDS−PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, 0.025% bromophenol blue, with 0.5% β-mercaptoethanol) for 4 min for PAGE analysis.

Lysine Determination. AIIt, annexin II, or p11 (35 μM) was treated with 44 nM CpB for various times, and the reaction was terminated by the addition of 10 μM MGTA. The reaction mixture was then divided into two aliquots; one aliquot was used for analysis of the effect of CpB on the stimulation of tPA-dependent plasminogen activation as described above. The second aliquot was used to determine whether treatment with CpB results in release of lysine from each protein using the method described by Nakatani (*19*). The protein was precipitated with perchloric acid and removed by centrifugation at 14 000 rpm for 5 min. The supernatant was then neutralized with KOH and incubated on ice for 5 min, and insoluble potassium perchlorate was removed by centrifugation. NADH and α-ketoglutarate (Sigma) were added to the supernatants to a final concentration of 0.5 and 1 mM, respectively, and the reaction mixture was adjusted to a volume of 190 μL with HBS. The reaction was initiated by the addition of 0.33 unit of saccharopine dehydrogenase (Sigma), and oxidation of NADH to NAD^+ was monitored as the decrease in absorbance at 340 nm over a 25 min time course at room temperature in a Perkin-Elmer HTS 7000 fluorescent plate reader. The decrease in absorbance was converted to the amount of lysine liberated by interpolation of a standard curve generated with known concentrations of free L-lysine (Sigma).

Ligand Blotting. Purified protein samples were denatured by boiling in SDS sample buffer in the absence of β-mercaptoethanol and resolved on a 15% polyacrylamide gel. The gel was then incubated for 4 h at 25 °C in 50 mM Tris-HCl, pH 7.5, with 2.5% Triton X-100, to promote protein renaturation, and the Triton X-100 was subsequently removed by incubation in 50 mM Tris-HCl, pH 7.5, for 4 h at 25 °C. Proteins were transferred to nitrocellulose membranes (0.22 μm pore size) for 90 min at 100 V at 4 °C (*20*). Membranes were air-dried on filter paper and blocked with 5% skim milk powder (BioRad) in Tris-buffered saline (TBS: 10 mM Tris, pH 7.4, 150 mM NaCl) for 30 min at room temperature and incubated with 1% skim milk solution containing 1 μM Glu-plasminogen at room temperature for 45 min. The membrane was then washed twice with TBS containing 0.05% Tween-20 (TTBS), fixed for 10 min with 2% paraformaldehyde, washed twice in TBS, and incubated at 4 °C overnight with mouse monoclonal α-mini-plasminogen antibody in 2.5% skim milk in TBS. Membranes were washed 5 times (3 min per wash) with TTBS and incubated for 1 h at room temperature with anti-mouse IgG conjugated to horseradish peroxidase in 1% skim milk in TBS. After washing as above, membranes were then developed with Supersignal chemiluminescent substrate (Pierce), and exposed to X-ray film.

Data Analysis. Initial rates of plasmin formation were calculated using linear regression analysis of plots of $A_{405\ nm}$ versus t^2 as outlined previously (*21*) using SigmaPlot (Jandel Scientific). Titration data were analyzed using 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. The value for the tPA turnover number, k, was calculated according to (*22*) using the equation: $A_{405\ nm} = 0.5\Delta\epsilon_{405}k_1k[tPA]t^2$, where $\epsilon_{405} = 6026.4$, [tPA] = 10 nM, and k_1, the plasmin turnover number, was calculated from a standard curve of plasmin amidolytic activity as 4.9 s^{-1}.

RESULTS

Treatment of AIIt with Carboxypeptidase Abrogates Its Ability To Stimulate Plasminogen Activation. We have previously shown that the carboxyl-terminal lysines of the p11 subunit of AIIt are required for the AIIt-dependent stimulation of tPA-catalyzed plasminogen activation (*18*). Carboxypeptidase B is known to inactivate extracellular plasminogen-binding proteins or block fibrin-dependent plasminogen activation by removal of carboxyl-terminal lysines from these proteins. It was therefore reasonable to suspect that the removal of the carboxyl-terminal lysines from AIIt could represent a potential regulatory mechanism. To determine if CpB treatment of AIIt modulates its ability to stimulate tPA-catalyzed plasminogen activation, AIIt was incubated with CpB for intervals up to 40 min. As shown in Figure 1, the incubation of AIIt with a 1/400 molar ratio of CpB for periods as short as 2 min resulted in a significant loss in AIIt-stimulated plasminogen activation. A 40 min exposure to CpB reduced the activity of AIIt to 20% of untreated AIIt, and longer exposures of AIIt to CpB failed to enhance the inactivation of AIIt (data not shown). Furthermore, the CpB treatment of AIIt reduced its plasminogen activator activity to a similar level as the activity of recombinant AIIt formed with wild-type recombinant p36 and a mutant recombinant form of p11 lacking both carboxyl-terminal lysine residues ($rp11_{des-KK}$) [reference (*12*) and data not shown].

We observed that the recombinant $p11_{des-KK}$ mutant had an increased mobility on SDS−PAGE compared to either native or wild-type recombinant p11 (Figure 2, inset). Consistent with that observation, we also observed that the loss in stimulatory activity of p11 after carboxypeptidase-dependent cleavage of the C-terminal lysines of p11 occurred concomitant with a small but reproducible increase in mobility of the p11 subunit on SDS−PAGE (Figure 2, inset). Inclusion of the carboxypeptidase inhibitor MGTA prevented this CpB-induced mobility shift. Since the incubation of AIIt and CpB in the presence of MGTA blocked the inactivation of AIIt by CpB, it was unlikely that the p11 subunit was proteolyzed by a contaminating protease in the CpB preparation. This suggested that CpB acted on the carboxyl-terminal residues of the p11 subunit of AIIt. However, the loss of one or two lysine residues from the p11 subunit would not be predicted to result in a noticeable shift in mobility on SDS−PAGE. This suggested that the treatment of AIIt with

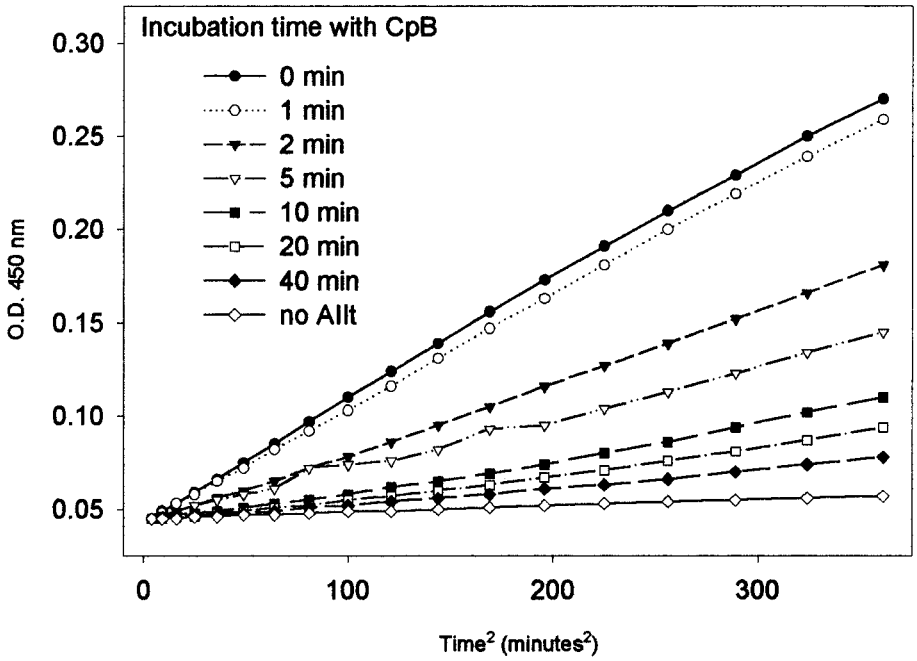

FIGURE 1: Time course of inactivation of AIIt by carboxypeptidase B. AIIt (40 μM) was incubated at 37 °C with 0.10 μM CpB. Aliquots were removed at the indicated time intervals, and carboxypeptidase was inactivated with 10 μM MGTA. The CpB-exposed AIIt was incubated with 0.1 μM Glu-plasminogen and 10 nM tPA, and plasmin formation was monitored at 405 nm using the plasmin-specific substrate S-2251 (American Diagnostica) at a concentration of 105 nM. The rate of plasmin generation was calculated from the equation: $A_{405\ nm} = B + K^*t^2$, where K, the slope, is the rate constant for the acceleration of plasmin generation and B is the y-intercept.

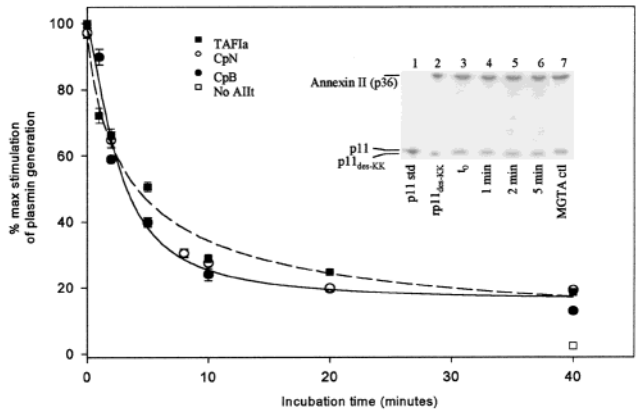

FIGURE 2: Inactivation of AIIt by plasma carboxypeptidases. AIIt was treated with 0.1 μM TAFIa (filled squares), 0.5 μM CpN (open circles), or 0.1 μM CpB (filled circles), and aliquots were removed at the indicated time points, and the reaction was quenched with 10 μM MGTA. The rate of plasmin formation by CpB-, CpN-, or TAFIa-exposed AIIt (2 μM) was then determined as indicated in the legend to Figure 1. The curves are nonlinear least-squares curve-fits of the data points calculated from computer modeling of data to the four-parameter logistic equation described under Experimental Procedures. The open square represents the rate of tPA-dependent plasminogen activation in the absence of AIIt, expressed as a percentage of the rate of plasminogen activation in the presence of untreated AIIt. Data points represent mean ± standard error for at least three experiments. The inset shows the increase in mobility of p11 on reduced SDS−PAGE upon CpB treatment. Lane 1, wild-type recombinant p11; lane 2, AIIt formed from wild-type annexin II (p36) and recombinant deletion mutant p11 ($p11_{des-KK}$); lanes 3−6, time course for CpB treatment of AIIt at time 0, 1, 2, and 5 min, respectively; lane 7, CpB treated AIIt in the presence of the carboxypeptidase inhibitor MGTA (10 μM).

CpB resulted in a conformational change in the p11 subunit. Analysis of AIIt secondary structure using circular dichroism, however, did not reveal a measurable conformational change in the protein (data not shown). Therefore, the overall conformation of AIIt is probably not affected grossly by CpB treatment. Notably, the electrophoretic mobilities of calmodulin (*23*) and creatine kinase (*24*) are also altered upon removal of carboxyl-terminal lysine residues by carboxypeptidases.

CpB shares some similarities to two plasma carboxypeptidases, namely, TAFIa and CpN. We therefore examined if these plasma carboxypeptidases could also inactivate AIIt. As shown in Figure 2, short-term exposures of AIIt to these carboxypeptidases also resulted in a loss in AIIt-stimulated plasminogen activation. As can be seen from the figure, exposure of AIIt to carboxypeptidase led to approximately an 80% loss in stimulatory activity. It is unknown at this time whether the remaining activity is derived from the annexin II subunit, or whether the internal lysine residues near the carboxyl-terminus of the p11 subunit contribute to AIIt activity. We are currently investigating these possibilities. These observations establish that the ability of AIIt to stimulate tPA-dependent plasminogen activation is dramatically diminished by the carboxypeptidases CpB, TAFIa, and CpN.

Dose-Dependent Inactivation of AIIt by Carboxypeptidases. The data presented in Figure 1 suggested that very low molar ratios of CpB/AIIt were sufficient to inactivate AIIt. We therefore examined the concentration-dependency of the carboxypeptidase-mediated inactivation of AIIt. As shown in Figure 3, the treatment of AIIt with pancreatic CpB, activated TAFI (TAFIa), or plasma CpN resulted in a dose-dependent decrease in AIIt-stimulated plasminogen activation. CpB was the most potent of the carboxypeptidases tested, followed by TAFIa and then CpN. Nanomolar concentrations of the carboxypeptidases were required to half-maximally reduce the stimulation of plasminogen activation by AIIt. The molar ratio of carboxypeptidase/AIIt for half-maximal inhibition of AIIt was determined to be

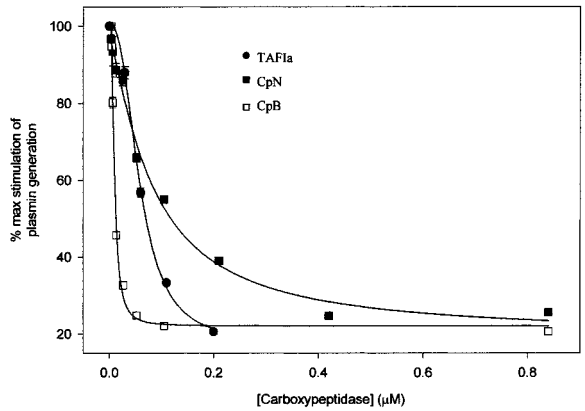

FIGURE 3: Dose-dependency of carboxypeptidase-dependent inactivation of AIIt. 40 μM AIIt was treated for 40 min at 37 °C with various concentrations of activated TAFIa (filled circles), CpN (filled squares), or CpB (open squares), and the carboxypeptidase was inactivated by addition of 10 μM MGTA. The rates of plasmin formation were determined for CpB-, CpN-, or TAFIa-exposed AIIt (2 μM) according to the legend for Figure 1. Data represent the mean ± standard error of at least three experiments. The curves shown are nonlinear least-squares curve-fits of the data points calculated from computer modeling of data to the four-parameter logistic equation described under Experimental Procedures.

Table 1: Kinetic Parameters for tPA-Dependent Plasminogen Activation in the Absence of AIIt, or in the Presence of Untreated or Cp-Treated AIIt

cofactor	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})	stimulation (fold)[a]
no cofactor			2	1
untreated AIIt	17.27 ± 0.68	0.06 ± 0.01	300.35	150.18
CpB-treated AIIt	8.79 ± 0.68	0.22 ± 0.05	40.51	20.08
CpN-treated AIIt	5.50 ± 0.52	0.126 ± 0.03	43.65	21.80
TAFIa-treated AIIt	7.45 ± 0.42	0.195 ± 0.32	38.21	19.1

[a] Fold stimulation is defined as the ratio of the catalytic efficiency (k_{cat}/K_m) of tPA-dependent plasminogen activation in the presence of untreated AIIt to that in its absence (tPA alone). All data are expressed as mean ± SE of at least three experiments.

1/4700, 1/700, and 1/500 for CpB, TAFIa and CpN, respectively.

Effect of Carboxypeptidase Treatment of AIIt on the Kinetics of Plasminogen Activation. The kinetic parameters for AIIt-stimulated plasminogen activation were measured in the absence or presence of AIIt and compared with the values obtained with carboxypeptidase-treated AIIt. Treatment of AIIt with CpB, TAFIa, or CpN resulted in both a decrease in the k_{cat} and a marked increase in the K_m for the reaction (Figure 4A). As summarized in Table 1, the catalytic efficiency, k_{cat}/K_m, of AIIt-stimulated plasminogen activation decreased from 300.35 μM^{-1} s^{-1} in the absence of carboxypeptidase to 40.51, 43.65, or 38.21 μM^{-1} s^{-1} after treatment for 40 min with 0.1 μM CpB, 0.2 μM CpN, or 0.1 μM TAFIa, respectively.

To determine the effects of carboxypeptidase treatment on the individual subunits of AIIt, we examined the kinetics of plasminogen activation using recombinant p11 or annexin II before and after treatment with CpB. Figure 4B shows that treatment of p11 with CpB results in a decrease in k_{cat} and an increase in K_m for p11 stimulation of plasminogen

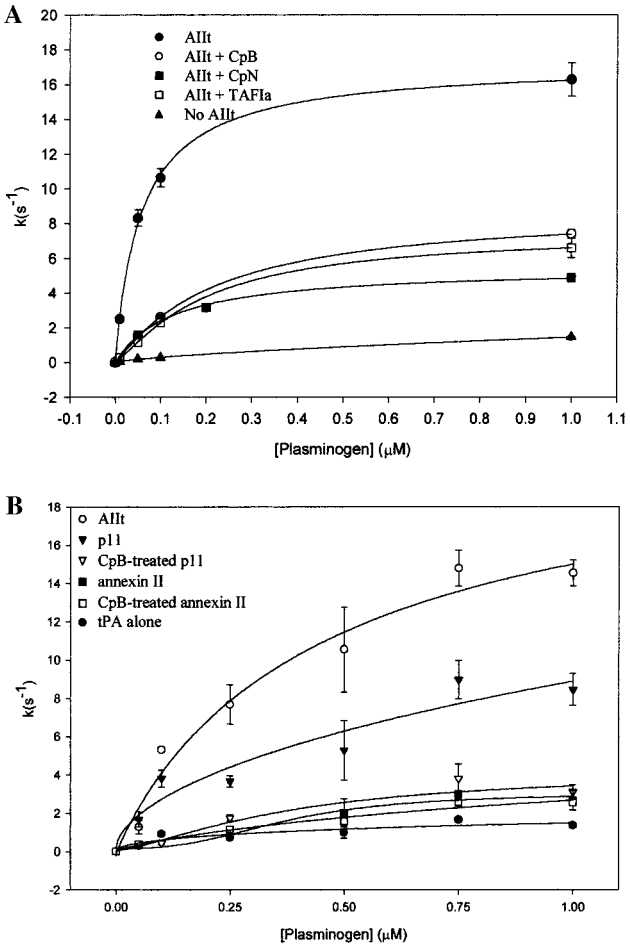

FIGURE 4: Kinetics of plasminogen activation in the presence of carboxypeptidase-treated AIIt or p11. (A) AIIt (40 μM) was incubated for 40 min at 37 °C in the absence (filled circles) or presence of 0.1 μM TAFIa (open squares), 0.4 μM CpN (filled squares), or 0.1 μM CpB (open circles). Filled triangles represent the rates of plasminogen activation in the absence of AIIt. (B) Recombinant wild-type p11 (triangles) or annexin II (squares) was incubated for 40 min at 37 °C in the absence (filled symbols) or presence of 0.1 μM CpB (open symbols). The circles represent the rates of plasminogen activation in the absence (filled circles) or presence (open circles) of 2 μM recombinant AIIt. In each case, carboxypeptidase was inactivated by the addition of 10 μM MGTA, and 2 μM each of carboxypeptidase-exposed AIIt was incubated with the indicated concentration of Glu-Pg, and the rates of plasmin production were determined as indicated in the legend to Figure 1. Data points represent the mean ± standard error of at least three experiments. The curves shown are nonlinear least-squares curve-fits of the data points calculated from computer modeling of data to the four-parameter logistic equation described under Experimental Procedures.

activation. The rates of plasminogen activation by carboxypeptidase-treated AIIt or p11 were similar to those observed for untreated annexin II. Treatment of annexin II with CpB did not significantly affect its ability to stimulate plasminogen activation.

Loss of Plasminogen Binding upon Treatment of AIIt with Carboxypeptidase. To determine whether carboxypeptidase treatment of AIIt affected the binding of plasminogen to AIIt, we analyzed the interaction of plasminogen with wild-type or mutant p11 using far-western immunoblotting. Wild-type p11 or mutant AIIt composed of wild-type annexin II and the deletion mutant of p11 ($p11_{des-KK}$) were resolved by SDS−PAGE, transferred to nitrocellulose, and incubated

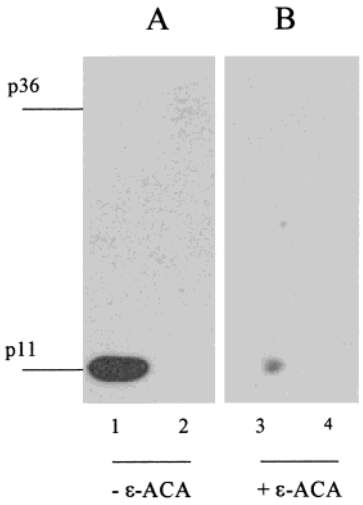

FIGURE 5: Far Western immunoblot for plasminogen binding to wild-type and mutant p11. (A) 2 μg of wild-type p11 (lanes 1, 3) or 17 μg of mutant AIIt composed of wild-type annexin II and mutated p11 ($p11_{des-KK}$) (lanes 2, 4) was loaded into wells of a 15% polyacrylamide gel and subjected to SDS−PAGE. The proteins were renatured, transferred onto nitrocellulose membranes, and probed with 1 μM Glu-plasminogen as described under Experimental Procedures. After washing, the nitrocellulose membrane was incubated with anti-plasminogen antibody and goat anti-mouse second antibody. (B) In parallel experiments, 5 mM ϵ-ACA was included during the Glu-Pg incubation step.

with 1 μM Glu-plasminogen. The membranes were then probed with anti-plasminogen antibodies. As shown in Figure 5 plasminogen bound to wild-type p11 but not $p11_{des-KK}$, indicating that the carboxyl-terminal lysine residues of p11 were required for the interaction of plasminogen with AIIt. The interaction between plasminogen and p11 detected by this procedure was dependent on p11 lysine residues since binding was abrogated by inclusion of the lysine analogue ϵ-ACA. Interestingly, we were unable to detect the binding of plasminogen to the annexin II subunit AIIt.

Release of Lysine from p11 Is Responsible for the Decrease in the Stimulation of Plasminogen Activation. To directly determine if carboxypeptidase treatment of AIIt caused the release of lysine from the protein, we treated recombinant p11 with CpB, and measured the lysine concentration in the supernatants. As can be seen in Figure 6A, the release of lysine by CpB corresponds exactly with the decrease in stimulation of plasminogen activation by p11. Half-maximal inactivation of p11 corresponded to the release of approximately 2.12 mol of lysine/mol of p11 dimer. Carboxypeptidase treatment resulted in a maximal loss in p11-stimulated plasminogen activation of about 20−30% of untreated p11, and this corresponded to the liberation of approximately 4 mol of lysine/mol of p11 dimer. Therefore, the ultimate and penultimate lysine residues of each of the two subunits of p11 in the dimer are susceptible to cleavage by carboxypeptidase.

We also investigated the possibility that the loss of only 70−80% of the p11 activity could be due to stimulation of the rate of tPA-dependent plasminogen activation due to the presence of free lysine in solution. Under our assay conditions, we expect about 4 μM free lysine assuming complete removal of C-terminal lysines of the p11 subunit. Half-maximal activation of tPA-dependent conversion of plasminogen to plasmin occurs at about 10 mM lysine or 1 mM

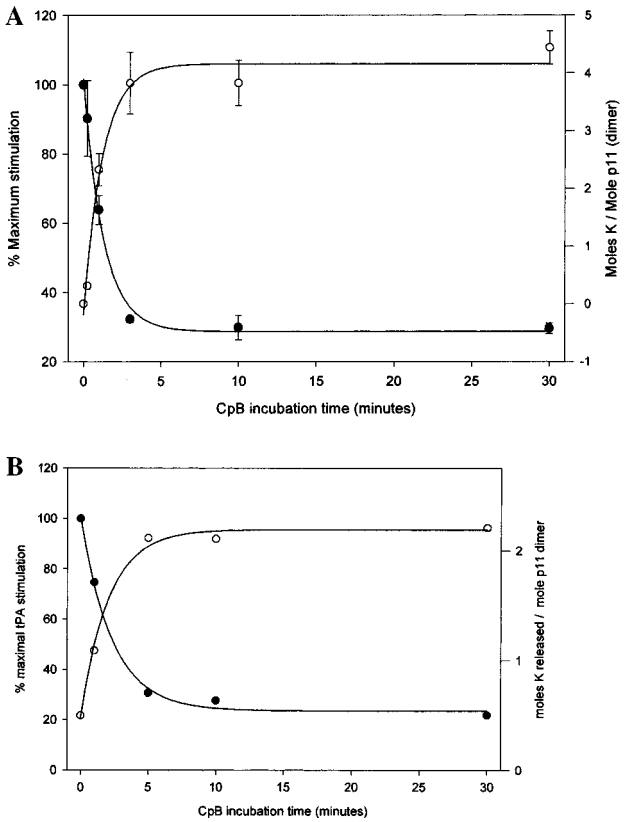

FIGURE 6: Loss in stimulation of plasminogen activation corresponds with release of lysine from p11. 35 μM wild-type recombinant p11 (A) or recombinant $p11_{des-K96}$ (B) was incubated with 43 nM CpB at 37 °C. At the indicated time points, the carboxypeptidase was inactivated with MGTA, and aliquots were removed and assayed for plasmin production according to the legend to Figure 1 (closed circles). The amount of free lysine in solution was determined by monitoring dehydration of NADH using saccharopine dehydrogenase as described under Experimental Procedures (open circles). Data shown in (A) represent the mean ± standard error for three independent experiments.

ϵACA. Although unlikely, it was possible that p11 was completely inactivated by carboxypeptidase digestion but that the free lysine or possibly di-lysine, liberated by carboxypeptidase action on p11, stimulated the tPA-dependent plasminogen activation (*25*). To investigate this possibility, we treated p11 with carboxypeptidase as above, and removed the liberated lysine by dialysis. Removal of free lysine or di-lysine from the CpB-treated p11 did not significantly alter the stimulation of plasminogen activation (data not shown).

A Single Carboxyl-Terminal Lysine Is Sufficient for p11-Dependent Plasminogen Activation Stimulation and Is Susceptible to Carboxypeptidase Cleavage and Inactivation. The carboxyl-terminal lysine of the plasminogen receptor is thought to be critical for plasminogen binding and activation. AIIt is unusual because the p11 subunit contains both ultimate and penultimate lysine residues. Although carboxypeptidases cleaved both lysine residues and this resulted in a maximal loss in AIIt-stimulated plasminogen activation, it was unclear if the carboxyl-terminal di-lysines were both required for plasminogen binding and activation. We therefore constructed a mutant p11 protein in which the carboxyl-terminal lysine was deleted ($p11_{del-K96}$) and a single lysine, lysine-95, became the carboxyl-terminal amino acid. The ability of this p11 mutant to stimulate tPA-catalyzed plasminogen activation was then tested. Somewhat surprisingly,

the stimulatory activity of $p11_{del-K96}$ in tPA-dependent plasminogen activation was similar to that of the wild-type protein (not shown).

As shown in Figure 6B, treatment of $p11_{del-K96}$ with CpB caused both the rapid release of lysine and the inactivation of p11 activity. The release of 2 mol of lysine/mol of $p11_{del-K96}$ dimer corresponded with the maximal loss of p11 activity. The $p11_{del-K96}$ mutant was nearly as effective in stimulating plasminogen activation as the wild-type protein, and treatment with CpB reduced its stimulatory activity to a similar extent (around 30% of maximum). This result suggests that p11 does not require both lysine residues at its carboxyl terminus for the stimulation of plasminogen activation or for digestion by carboxypeptidase B. Furthermore, the observation that the $p11_{del-K96}$ mutant had similar activity to the wild-type p11 further confirmed our observation that removal of ultimate and penultimate carboxyl-terminal lysines was required to inactivate p11.

DISCUSSION

In the present paper, we have shown for the first time that AIIt and its p11 subunit, which are known to stimulate extracellular plasminogen activation, are inactivated upon treatment with carboxypeptidase B and physiological concentrations of the plasma carboxypeptidases TAFIa and CpN. Treatment with carboxypeptidase resulted in the release of both the ultimate and the penultimate carboxyl-terminal lysine residues from p11 and AIIt. We observed that CpB was the most effective carboxypeptidase in terms of inactivation of AIIt, followed by TAFI and then CpN (CpB > TAFI > CpN). However, it is possible that, due to its instablility (*16*, *26*, *27*), the actual concentration of active TAFI was lower than calculated and thus its potency may have been underestimated. Still, all carboxypeptidases tested were effective at abrogating the stimulation of plasminogen activation by AIIt at molar ratios of Cp/AIIt of less than 1/400. Furthermore, incubation of the carboxypeptidases for as short an interval as 2 min was sufficient to inhibit AIIt activity. The carboxypeptidases reduced the AIIt activity by about 80%. A similar loss in activity was reported for mutants of AIIt that consisted of wild-type p36 subunit and a mutant p11 subunit that lacked the last two lysine residues of the carboxyl terminus. It was also interesting to note that the mutant p11 that possessed a single carboxyl-terminal lysine was fully active, and removal of this lysine was required for inactivation (Figure 6B). This suggests that if carboxypeptidase removes carboxyl-terminal lysines sequentially then the loss of a single lysine from the carboxyl terminus will not affect p11 activity; i.e., both lysines must be removed from a carboxyl terminus before a loss in activity occurs.

The role of carboxypeptidases in the regulation of peptide hormone activity and processing has also been well characterized [reviewed in (*28*, *29*)]. It has also been well established that the carboxyl-terminal lysines on fibrin and on plasminogen receptors on the extracellular surface of the cell can serve as plasminogen-binding sites by interacting with the lysine-binding sites of plasminogen. The basic carboxypeptidases are a family of enzymes that regulate this interaction by cleaving carboxyl-terminal lysine or arginine residues from the plasminogen-binding sites on fibrin clots (*16*, *30*) or cell surface proteins (*7*, *31*). Cellular plasminogen binding, which is mediated by both proteins and nonproteins such as gangliosides, is only partially sensitive to carboxypeptidase treatment. In contrast, the plasminogen receptors that play a role in acceleration of cell-associated plasmin are extremely sensitive to carboxypeptidase treatment and therefore contain lysine at the carboxyl terminus (*7*, *32*). The plasma carboxypeptidases TAFIa and CpN eliminate plasminogen-binding sites and serve to dampen fibrinolysis and cell-associated proteolysis. CpB is primarily a digestive enzyme produced by the pancreas (*33*), while CpN is constitutively active in plasma and has been implicated in peptide hormone processing (*34*), protection against anaphylatoxins (*28*), and binding of plasminogen to cells (*31*). TAFI has been primarily implicated in fibrinolysis (*16*, *30*, *31*, *35*, *36*) but also appears to play a role in regulating cellular plasminogen binding (*31*).

We have previously observed that AIIt and the p11 subunit of AIIt bind tPA, plasminogen, and plasmin and are important stimulators of the tPA-catalyzed cleavage of plasminogen to plasmin (*12*, *18*). AIIt is a particularly potent activator and is capable of stimulating tPA-catalyzed conversion of plasminogen to plasmin by 190-fold in vitro. While the activation of plasmin is critical for fibrinolysis (*25*), cell migration during such physiological processes as inflammation (*37*, *38*), and the pathological conditions of tumor metastasis and angiogenesis [reviewed in references (*39*−*42*)], its unregulated production would be deleterious to these processes, causing massive destruction of the extracellular matrix and detachment of cells from the substratum (*3*). Therefore, mechanisms must exist which limit the generation of plasmin, providing a means of spatially and temporally regulating pericellular proteolysis. Our observation that AIIt is regulated by carboxypeptidases suggests that a physiological mechanism exists for the regulation of AIIt-mediated proteolysis.

Several cell surface associated proteins have been shown to interact with plasminogen and to possess carboxyl-terminal lysine residues, and thus are considered candidate regulators of cell surface plasmin activity. α-Enolase, present on the surface of a variety of hematopoietic, epithelial, and endothelial cells [reviewed in reference (*43*)], is one such candidate plasminogen receptor. α-Enolase was shown to bind plasminogen (*8*, *44*), interact with tPA (*45*), and stimulate plasmin generation (*44*). However, increased expression of α-enolase on transformed rat fibroblasts did not lead to an increase in plasmin activity (*46*). More recently Hawley et al. (*47*) have identified a protein, called TIP49a, on the surface of U937 cells that binds plasminogen in a carboxyl-terminal lysine-dependent manner. The authors showed that plasmin generation was stimulated when plasminogen was bound to immobilized TIP49a, in comparison to soluble plasminogen alone. However, no direct comparison was made between intact and CpB-treated TIP49a in terms of plasmin generation (*47*). Therefore, AIIt remains an attractive candidate as an important plasmin regulator.

Typically, basic carboxypeptidases prefer substrates with alanine or glycine as the penultimate residue (*15*, *48*), so their ability to cleave the di-lysine C-terminus of p11 was somewhat surprising. However, several substrates of basic carboxypeptidases also have di- or tribasic carboxyl termini. For example, complement protein C4α and β chains have

-Arg-Arg-Arg and -Lys-Lys-Arg, respectively, at their carboxyl termini, and complement factor Ba has -Gln-Lys-Arg as its C-terminus (*28*). Because of the rapid kinetics of the reaction, it could not be determined whether the lysine residues from p11 were being removed synchronously or sequentially. However, since both the native (-Gly^{94}-Lys^{95}-Lys^{96}) and the mutant (-Gly^{94}-Lys^{95}) carboxyl termini of p11 are suitable substrates for basic carboxypeptidases, it is reasonable to speculate that the carboxyl-terminal lysines are removed sequentially.

It is now clear that the plasminogen receptor(s) involved in stimulation of plasminogen activation is (are) sensitive to ϵ-ACA and treatment with carboxypeptidase; i.e., they contain carboxyl-terminal lysine residues that interact with plasminogen. Our data clearly indicate that the p11 subunit of AIIt or p11 itself stimulates tPA-dependent plasminogen activation via interaction between the carboxyl-terminal lysines of p11 and kringle domains of plasminogen. Carboxypeptidases represent potential regulators of AIIt via their ability to remove carboxyl-terminal lysine residues from the p11 subunit. As TAFI circulates at approximately 70−270 nM and CpN at approximately 100 nM (*33*), it is possible that these enzymes are involved in regulation of plasminogen activation by inhibiting the stimulation of plasmin formation by AIIt. Given its established role in the regulation of plasminogen activation along with these novel findings regarding inactivation by basic carboxypeptidase, it is clear that AIIt may not be a constitutive stimulator of plasminogen activation.

It is presently unclear how AIIt avoids carboxypeptidase action in vivo. Under physiological conditions, cells are exposed to high concentrations of plasminogen (about 2 μM) and nanomolar concentration of carboxypeptidases. It is reasonable to suspect that AIIt is fully saturated with plasminogen in vivo and protected from carboxypeptidase attack. However, when the cell is activated and plasmin generation stimulated, it is possible that the increased plasmin activity results in the activation of membrane-associated carboxypeptidases such as carboxypeptidase M, which results in the loss of the C-terminal lysines of p11 and loss of AIIt-dependent plasmin generation. Once the cellular mechanisms for regulation of carboxypeptidases are better understood, it will be possible to elucidate the physiological mechanisms for the regulation of AIIt by the carboxypeptidases.

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