Degradation of Tau Protein by Puromycin-Sensitive Aminopeptidase in Vitro†

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ABSTRACT: Tau, a microtubule associated protein, aggregates into intracellular paired helical filaments (PHFs) by an unknown mechanism in Alzheimer's disease (AD) and other tauopathies. A contributing factor may be a failure to metabolize free cytosolic tau within the neuron. The buildup of tau may then drive the aggregation process through mass action. Therefore, proteases that normally degrade tau are of great interest. A recent genetic screen identified puromycin-sensitive aminopeptidase (PSA) as a potent modifier of tau-induced pathology and suggested PSA as a possible tau-degrading enzyme. Here we have extended these observations using human recombinant PSA purified from *Escherichia coli*. The enzymatic activity and characteristics of the purified PSA were verified using chromogenic substrates, metal ions, and several specific and nonspecific protease inhibitors, including puromycin. PSA was shown to digest recombinant human full-length tau in vitro, and this activity was hindered by puromycin. The mechanism of amino terminal degradation of tau was confirmed using a novel N-terminal cleavage-specific tau antibody (Tau-C6g, specific for cleavage between residues 13−14) and a C-terminal cleavage-specific tau antibody (Tau-C3). Additionally, PSA was able to digest soluble tau purified from normal human brain to a greater extent than either soluble or PHF tau purified from AD brain, indicating that post-translational modifications and/or polymerization of tau may affect its digestion by PSA. These results are consistent with observations that PSA modulates tau levels in vivo and suggest that this enzyme may be involved in tau degradation in human brain.

Intraneuronal aggregation of the hyperphosphorylated microtubule associated protein, tau, is one of the pathological hallmarks of Alzheimer's disease (AD)[1] (*1*−*3*). Tau pathology is also involved in a number of other neurodegenerative disorders, collectively known as tauopathies (*4*−*6*). One contributing factor in development of tau pathology may be impaired proteolysis of normal or abnormally modified tau, leading to increased levels of cytosolic tau available for aggregation (*7*−*9*). In vitro, tau has been reported to be a substrate for a wide variety of proteases (*10*−*17*); however, the proteases that actually degrade tau or affect its function in the brain are still unknown.

One promising family of enzymes is the neutral aminopeptidases, which includes puromycin-sensitive aminopeptidase (PSA or EC 3.4.11.14), a 100-kDa enzyme of the M1 class of metallopeptidases (*18*, *19*), which was recently identified in a Drosophila model of AD (*20*). Functionally, PSA is closely related to aminopeptidase A, aminopeptidase N, and leukotriene A_4 hydrolase, but PSA can be distinguished from these enzymes by its sensitivity to the protein synthesis inhibitor puromycin (*21*, *22*). PSA is found in neurons but not in surrounding glial cells or in blood vessels (*23*) and is present and active in both cytosolic and membrane-bound fractions (*24*−*27*). The broad tissue distributions of PSA and other neutral aminopeptidases, as well as their high homology and expression in a variety of species (*23*, *28*−*30*), suggest important physiological roles for such enzymes. PSA, in particular, is responsible for over 90% of the aminopeptidase activity in the brain (*26*).

PSA digests a broad range of substrates, preferring hydrophobic and basic amino acids (*27*, *31*, *32*). It is involved in a number of physiological processes, including normal cellular protein turnover (*33*, *34*), cell cycle regulation (*29*), processing of antigenic peptides for display on class I MHC molecules (*35*), and degradation of neuropeptides (*30*, *36*). Along with aminopeptidase N and the neutral endopeptidase, PSA has been implicated in the degradation of enkephalins (*22*, *31*, *37*−*41*) and has also been shown to degrade dynorphins, cholecystokinin, and somatostatin (*25*, *42*, *43*). The wide range of PSA substrates already described suggests that it may act on many as yet unidentified substrates. Furthermore, the tissue distribution of PSA makes it an ideal candidate for normal neuronal degradation of tau. In fact, recent experiments in a Drosophila model of Alzheimer's

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[1] Abbreviations: AD: Alzheimer's disease; FTD: fronto-temporal dementia; MAP: microtubule-associated protein; NA: nitroanilide; PHF: paired helical filament; PSA: puromycin-sensitive aminopeptidase; TEV: tobacco etch virus.

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disease identified the *Psa* gene as a potent regulator of the development of tau pathology and neurodegeneration (*20*). These data point to the possibility that tau is a potential target of PSA.

In this study, we characterized the digestion of tau by PSA in vitro. We expressed and purified human recombinant full-length PSA from *Escherichia coli* and verified its purity electrophoretically. The aminopeptidase activity of this novel recombinant PSA preparation was confirmed by detailed characterization using chromogenic substrates, metal ions, and various specific and nonspecific protease inhibitors. PSA degraded human recombinant full-length tau in vitro, and this process was inhibited by puromycin and bestatin but was not inhibited by PMSF. The aminopeptidase degradation of tau was verified using a novel antibody specific for an N-terminal truncation site on tau (Tau-C6g) and an antibody specific for a previously described C-terminal cleavage site (Tau-C3). To determine whether PSA could also digest tau isolated from more physiological sources, we performed the digestion of tau purified from normal human brain, AD brain, and porcine brain. Here we show that PSA effectively digests soluble tau purified from human and porcine brain and also partially degrades soluble or PHF tau purified from AD brain. These results identify a role for PSA in the modulation of tau levels and tau pathology in the brain.

EXPERIMENTAL PROCEDURES

Cloning of Human PSA in a Bacterial System. Recombinant human PSA was produced by cloning the PSA gene into a bacterial expression system (*20*). The cDNA corresponding to human PSA was generated by PCR from the Large Insert Human Brain cDNA library (Clontech) using the Advantage cDNA PCR kit (BD Biosciences). The following primers were used: forward - 5′ ATG CCG GAG AAG AGG CCC TTC GAG C 3′ and reverse - 5′ TCA CAC TGT GGG TGG TGA GGC CTT C 3′. Using this PCR product as a template, another PCR product was made by using vent DNA polymerase (NEB) where the TEV protease cleavage site was placed upstream of the vector specific sequence in the forward primer. This PCR product was then ligated into the pET-41 Ek/LIC vector (Novagen) having an N-terminal glutathione *S*-transferase (GST) fusion tag according to the manufacturer's protocol. The pET-41-GST-PSA construct was then transformed into *E. coli* BL-21 (DE3*) cells (Novagen). The sequence of PSA was confirmed by di-deoxy sequencing.

Expression and Purification of Recombinant PSA. The pET-41-GST-PSA clone was grown at 37 °C and IPTG (Calbiochem) was then added to a final concentration of 1 mM. Induction was allowed to proceed for 16−17 h at 18 °C, after which cells were harvested, resuspended in phosphate buffer (pH 7.3), and disrupted with a French press. The lysate was centrifuged at 15000*g* for 30 min. The clarified supernatant was applied to a glutathione sepharose (Amersham Biosciences) column. Recombinant PSA was eluted from the resin with 50 mM Tris-HCl (pH 8.0) containing 10 mM reduced glutathione. The active fractions were pooled and dialyzed against 10 mM Tris-HCl (pH 7.4) containing 200 mM NaCl. The protein was then applied to a Superdex-200 (Amersham Biosciences) column. The eluted active fractions were then dialyzed against 10 mM Tris-HCl, pH 7.4, and concentrated. The soluble, purified GST−PSA migrated largely as a single band on SDS−PAGE (Figure 1). Using TEV protease, the enzyme was separated from the fusion tags, but no significant difference in the activity was found between the native protein and the fusion protein (data not shown). Therefore, the GST fusion protein was used for all subsequent studies.

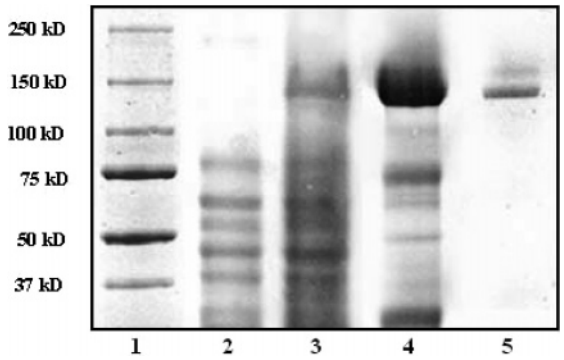

FIGURE 1: Purification of human PSA from *E. coli*. PSA at various stages of purification was analyzed by SDS−PAGE and Coomassie blue staining. Lane 1: molecular-weight standards. Lane 2: crude cell extract before IPTG induction. Lane 3: crude cell extract after 16−17 h of induction showing overexpressed GST−PSA. Lane 4: pooled active fractions following glutathione sepharose column elution. Lane 5: purified recombinant PSA after Superdex-200 column size exclusion chromatography.

Assay of Aminopeptidase Activity. Enzyme activity was assayed using amino acyl *p*-nitroanilides (*p*-NAs) of Lys, Ala, Met, Leu, Val, and Pro (Sigma) on a Beckman DU640 spectrophotometer employing a colorimetric assay modified from a previous report (*29*). Reaction mixtures (500 μL) contained 5 μg of enzyme and 1 mM DTT in 10 mM Tris-HCl, pH 7.4, and reactions were initiated by adding amino acyl *p*-NA at a final concentration of 0.2 mM. After incubation at 37 °C for 15 min, reactions were stopped by adding 500 μL of 0.1 M sodium acetate, pH 4, and the absorbance of the liberated *p*-nitroaniline was measured spectrophotometrically at 405 nm. Because of weak activity toward *p*-nitroanilides of Val and Pro, incubation was carried out for more than 15 min for these compounds. Activities were calculated assuming that 1 unit of enzyme liberates *p*-nitroaniline at a rate of 1 nmol min^{-1} (ϵ_{405} = 9500 L mol^{-1} cm^{-1}). Bar diagrams represent average ± one standard deviation for triplicate determinations. The K_m and k_{cat} for different amino acyl *p*-nitroanilides were calculated by Lineweaver−Burk plot where variable concentrations of each substrate were used for this analysis.

Assay of Carboxypeptidase Activity. The lack of carboxypeptidase activity in preparations of PSA and tau was verified using the substrates hippuryl-L-phenylalanine and hippuryl-L-arginine (Sigma). For carboxypeptidase assays using hippuryl-L-phenylalanine, reaction mixtures contained 0.4 units of PSA, 0.3 μg of tau, or 0.4 units of carboxypeptidase A (E.C. 3.4.17.1, used as a positive control) per mL, with 1 mM substrate in 25 mM Tris-HCl buffer with 500 Mm NaCl, pH 7.5, and were carried out at 25 °C for 10 min. For carboxypeptidase assays using hippuryl-L-arginine, reaction mixtures contained 0.27 units of PSA, 0.2 μg of tau, or 0.27 units of carboxypeptidase B (E.C. 3.4.17.2, used as a positive control) per mL, with 1 mM substrate in 25 mM Tris-HCl buffer with 100 mM NaCl, pH 7.65, and were

Table 1: Characteristics of the Antibodies Used in This Study[a]

antibody	epitope (amino acids)	concentration	ref
anti-PSA	polyclonal	1:500 dilution	commercial
tau-12	9−18	4 ng/mL	*14, 61*
tau-5	210−230	20 ng/mL	*46*
tau-7	430−441	40 ng/mL	*50*
tau-C3	C-truncation after D421	400 ng/mL	*13, 48*
tau-C6g	N-truncation after D13	1:100 dilution of tissue culture supernatant	this report

[a] The numbering of amino acids is based on the longest isoform of tau in the central nervous system (hTau40), with 441 amino acids, including two amino terminal inserts and four microtubule binding repeat regions.

carried out at 25 °C for 10 min. The liberated hippuric acid was measured at 254 nm. Activities were calculated assuming that one unit of enzyme hydrolyzes 1.0 μmol min^{-1} substrate at 25 °C ($\epsilon_{405} = 0.36$ L mol^{-1} cm^{-1}).

Effects of Metal Ions and Inhibitors on Enzyme Activity. To assess the effects of metal ions on enzyme activity, 5 μg of purified enzyme was incubated with 1 mM $MgCl_2$, 5 mM $CaCl_2$, or 0.01 mM $ZnCl_2$ for 15 min at 37 °C prior to the addition of the substrate Lys-*p*-NA. Inhibition assays were carried out with 5 μg of purified enzyme incubated for 5 min at room temperature with the indicated inhibitor (Sigma) in the assay buffer containing 1 mM DTT, followed by the addition of Lys-*p*-NA. The k_i values of puromycin and bestatin were determined at a fixed concentration (10μM) of each inhibitor by varying the concentration of L-Met-*p*-NA.

Tau Preparations. The full-length tau used in this study was the recombinant htau40, 441 residues in length, which corresponds to the longest tau isoform found in the central nervous system (*44*). To accurately study aminopeptidase activity on tau, we used a tau construct in which the poly histidine tag (used for purification) was placed at the C-terminus (a gift from Dr. T. Christopher Gamblin). This construct was expressed and purified as previously described (*45*, *46*). Arachidonic acid-induced polymerization of this protein was indistinguishable from that of N-terminally poly histidine tagged tau (data not shown). The C-terminally poly histidine tagged hTau40 Δ2−13 mutation was generated by PCR using two 5′-phosphorylated primers flanking the desired deletion, and purified as described previously. The purification of N-terminally poly histidine tagged hTau40 Δ422−441 has been described previously (*13*). The purification of soluble tau from human and porcine brains, as well as soluble and PHF tau from AD brain, has been described previously (*47*).

Digestion of Tau Proteins with PSA and Inhibition of PSA. Digestion of recombinant tau proteins (hTau40 and tauΔ2−13) was carried out at 37 °C in 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM DTT, with 5 μg tau and 1.4 units of PSA. Aliquots were taken at various time points, and the reaction was terminated by adding Laemmli SDS sample buffer and boiling for 5 min. Samples of tau incubated without PSA and removed at the indicated time points were used as negative controls. Samples were analyzed by SDS−PAGE and Western blotting with the primary antibodies listed in Table 1. Horseradish peroxidase (HRP)-conjugated antispecies specific secondary antibodies (Vector Laboratories) and enhanced chemiluminescence (Amersham Biosciences) were used to visualize the bands. Inhibition experiments were performed by using PSA preincubated 15 min in the presence of 1 or 2 mM puromycin, 2 mM bestatin, or 2 mM PMSF. Digestion was carried out at 37 °C for 20 h, and samples were analyzed as above. The digestions of human brain soluble tau, porcine soluble tau, and soluble and PHF tau from AD brains were carried out at 37 °C for 18 h in the digestion buffer using 5 μg of tau and 7 units of PSA, and samples were analyzed as described above. Band intensities were quantified in Adobe Photoshop 7.0, using a marquee rectangle of fixed size centered on the band and the Histogram function. Graphs represent the average and one standard error of the mean from seven independent experiments. Statistical significance was determined by one-way ANOVA followed by Tukey's test.

Generation of Monoclonal Antibodies to N-Terminally Cleaved Tau. Since we have previously shown that caspase-6 can cleave hTau40 N-terminally in vitro at residue D13 (*14*), we sought to generate antibodies specific for this cleavage. The immunization strategy employed was similar to that used to generate the Tau-C3 antibody, which is specific for C-terminal truncation at residue D421 by caspase-3 (*13*). Briefly, a peptide was generated corresponding to residues 14−28 of tau plus a C-terminal cysteine (HAGTYGLGDRKDQGGC, Cell Essentials, Boston, MA). This peptide was conjugated to maleimide-activated keyhole limpet hemocyanin (KLH), and suspended 1:1 in Freund's Incomplete or Complete adjuvant. Five female Balb/c mice were immunized every 3 weeks until serum titers were positive by ELISA at 1:20 000. The immune spleens were removed, dissociated, and fused to SP2/o myeloma cells to form hybridomas. Clones were HAT-selected and grown for 2 weeks. Clonal supernatants were screened for binding to the peptide antigen and subcloned three times (until stably expressing antibody). ELISA titers for substrate specificity of Tau-C6g, an IgG1 antibody generated from this fusion, were performed as previously described (*48*), using hTau40 and caspase-6 cleaved tau (*14*).

RESULTS

As full-length recombinant PSA purified from a bacterial source has not previously been characterized (*20*), it was necessary to compare the enzymatic properties of the PSA in this preparation to those previously described for PSA purified from other sources, including rat and bovine brain tissue. We therefore investigated the activity of bacterially purified PSA with respect to substrate specificity and behavior in the presence of selected metal ions and protease inhibitors.

Comparison of Specific Activity. PSA purified from rat and bovine brain has been reported to cleave beta-naphthylamides and acyl-*p*-NAs with preference for basic and neutral amino acids (*27*, *31*, *32*). The specific activity of our PSA was determined using a number of amino acyl-*p*-NAs. The order of specific activity of recombinant PSA against amino acyl-*p*-NAs was Lys, Ala, Met > Leu (Figure 2A). No activity against Val-*p*-NA and Pro-*p*-NA could be detected under the conditions used in our assay within 15 min, but on increasing the incubation time, some activity against Val-*p*

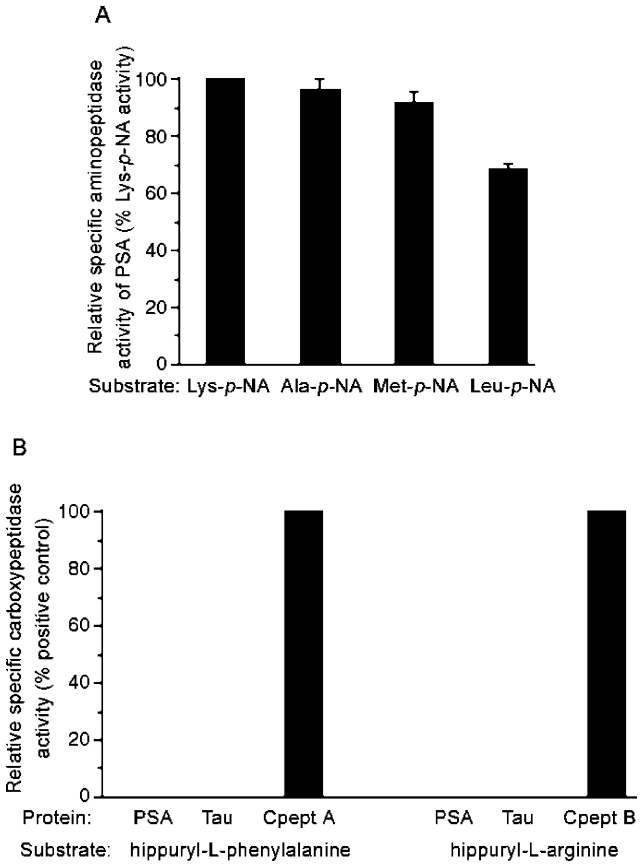

FIGURE 2: Relative substrate specificity of purified GST-PSA. (A) The specific activities (nmol min^{-1} mg^{-1}) of PSA against Lys-, Ala-, Met-, and Leu-*p*-nitroanilides were compared and expressed as a percentage of Lys-*p*-nitroanilide cleavage. (B) The carboxypeptidase substrates hippuryl-L-phenylalanine and hippuryl-L-arginine were not cleaved by the purified PSA, indicating the absence of contaminating carboxypeptidases. Carboxypeptidases A (Cpept A) and B (Cpept B) were used as positive controls for specific cleavage (nmol/min/mg) of substrates hippuryl-L-phenylalanine and hippuryl-L-arginine, respectively, in these experiments.

Table 2: Substrate Specificity of Puromycin-Sensitive Aminopeptidase

substrate	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
Lys-*p*-NA	2.20	5.34×10^3	2.42×10^3
Leu-*p*-NA	0.25	1.19×10^3	4.76×10^3
Ala-*p*-NA	0.27	1.25×10^3	4.60×10^3
Met-*p*-NA	0.80	1.80×10^3	2.30×10^3
Pro-*p*-NA	0.47	1.39×10^2	2.95×10^2
Val-*p*-NA	0.21	85.90	4.09×10^2

NA and Pro-*p*-NA was noted (data not shown). These results show the preference of recombinant PSA for basic and neutral amino acids. The proteolysis would be slowed greatly upon encountering amino acid sequences rich in proline and/or valine.

The K_m and k_{cat} for the six amino acyl-*p*-nitroanilides used in this study were also measured (Table 2). There was no large difference in K_m among the *p*-nitroanilides except for Lys-*p*-NA, which had a very high K_m of 2.2. The k_{cat}/K_m for Leu-*p*-NA and Ala-*p*-NA were almost 2-fold higher than that for Lys-*p*-NA and Met-*p*-NA, and 10-fold higher than that for Pro-*p*-NA and Val-*p*-NA. These values indicate that PSA prefers Leu-*p*-NA most among the six *p*-nitroanilides tested in this study and this k_{cat}/K_m value of Leu-*p*-NA is comparable to the value reported for rat brain tissue (*32*).

Table 3: Effects of Divalent Metal Cations and EDTA on PSA Activity

metal ion or chelator	concentration (mM)	relative activity (%)
none		100
Mg^{2+}	1	126.0 ± 0.15
Ca^{2+}	5	109.8 ± 0.20
Zn^{2+}	0.01	79.4 ± 0.52
EDTA	0.5	44.00 ± 0.20

Table 4: Effects of Various Inhibitors on Aminopeptidase Activity, Measured Using the Substrate Lys-*p*-nitroanilide

inhibitor	concentration	activity remaining (%)
none		100
puromycin	100 μM	3.12 ± 0.02
bestatin	100 μM	2.57 ± 0.08
leupeptin	100 μg/mL	83.00 ± 0.10
aprotinin	100 μg/mL	82.00 ± 0.09
PMSF	1 mM	100.10 ± 0.03
anti-PSA IgG	100 μg/mL	53.40 ± 0.17

By using bovine brain tissue, Hersh et al. (*22*) showed that pro-β-naphthylamide has the lowest k_{cat}/K_m value, which is in agreement with our result showing the lowest k_{cat}/K_m value for Pro-*p*-NA.

To rule out the presence of any contaminating carboxypeptidase in the PSA and tau preparations and to confirm that our recombinant PSA was acting as an aminopeptidase, control experiments were conducted using the carboxypeptidase substrates, hippuryl-L-phenylalanine and hippuryl-L-arginine. Neither PSA nor tau preparations digested these substrates (Figure 2B), confirming the absence of any contaminating carboxypeptidase in both protein preparations.

Enzyme Activity in the Presence of Metal Ions and Inhibitors. As PSA is an M1 class metallopeptidase and contains a zinc-binding consensus motif, we tested the effect of three divalent cations on enzyme activity: Mg^{2+}, Ca^{2+}, and Zn^{2+} (Table 3). Previously, it was reported that 1 mM $MgCl_2$ and 5 mM $CaCl_2$ increased the specific activity of PSA derived from human and bovine brain tissue (*35*, *49*) and that 0.01 mM $ZnCl_2$ has an inhibitory effect (*25*). Similarly, we also noted that 1 mM $MgCl_2$ and 5 mM $CaCl_2$ increased the specific activity of PSA by about 26 and 10%, respectively, and the specific activity was decreased by about 20% in the presence of 0.01 mM $ZnCl_2$. The activity of PSA was also greatly reduced by 0.5 mM of the divalent cation chelator EDTA, as has been previously reported (*27*, *32*).

PSA purified from tissue has been reported to be inhibited by puromycin and bestatin but is not inhibited by the covalent serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (*21*, *25*, *29*, *31*−*33*, *40*, *49*). Similarly, our recombinant PSA was almost completely inhibited by puromycin and bestatin (k_i values: 2 and 1.5 μM, respectively; Table 4). PSA was not inhibited by PMSF and was only slightly inhibited by leupeptin and aprotinin. The enzyme was also inhibited about 50% by 100 μg/mL anti-PSA IgG (Table 4), as has been previously reported (*29*).

Digestion of Tau by PSA and Inhibition by Puromycin. To determine if PSA could degrade tau in vitro, we incubated PSA with human recombinant purified tau protein (hTau40) harboring a C-terminal poly histidine tag. PSA caused a time-dependent loss of tau in the samples, as detected by Western blotting with a C-terminal tau antibody, Tau-7 (*50*) (Figure

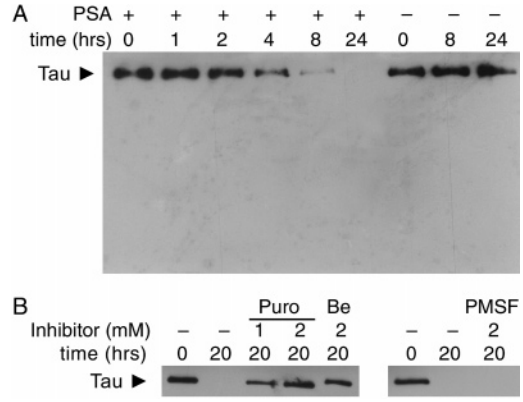

FIGURE 3: PSA digests tau in vitro. (A) Kinetics of PSA digestion of C-terminally poly histidine tagged hTau40 in the presence of PSA. In the absence of PSA (−), tau is not degraded, but incubation of tau with PSA (+) causes time-dependent loss of tau from the samples. No specific cleavage products (lower molecular weight bands) were observed in any of these experiments. (B) Tau digestion is inhibited at 20 h by the specific PSA inhibitor puromycin (1 or 2 mM) and by a potent nonspecific PSA inhibitor, bestatin (2 mM). However, in the presence of PMSF (2 mM), which does not inhibit PSA, tau digestion proceeded to completion. The Tau-7 antibody was used in all three blots.

3A). No lower molecular weight bands were detected with Tau-7 (Figure 3A), or by using two antibodies to other regions of tau (Tau-12 and Tau-5, data not shown), suggesting the absence of endopeptidase activity in these samples. Tau incubated without PSA remained intact, indicating that in the absence of PSA, the tau protein is stable at this temperature. The degradation of tau by PSA was inhibited by the specific inhibitor puromycin, as well as bestatin, another potent inhibitor of PSA. In the presence of PMSF, however, tau digestion proceeded to completion (Figure 3B).

Truncation-Specific Tau Antibodies Confirm N-Terminal Degradation of Tau. While the data above indicated tau is degraded in these samples, we wished to verify that the degradation was in fact occurring from the amino terminus of tau. Since no distinct bands of partially degraded tau were visualized by Western blotting, another set of antibodies was employed in order to identify specifically the ephemeral N- or C-terminally cleaved tau species in these samples. Truncation-specific tau antibodies have already been reported (Tau-C3 and MN423), which detect C-terminal truncation sites of tau and do not cross-react with full-length tau (Table 1).

Specific detection of N-terminal truncation required the generation and use of a novel antibody. As we have previously reported, caspase-6 can cleave tau N-terminally in vitro at residue D13 (*14*). Therefore, we sought to make antibodies that specifically detect cleavage at this site. Mice were immunized with a KLH-conjugated peptide corresponding to the free amino terminus of caspase-6 cleaved tau (residues 14−28), and clones were selected for production of antibodies specifically detecting D13-cleaved tau and not full-length hTau40. Tau-C6g, an IgG antibody that met these criteria, shows remarkable selectivity for N-terminally truncated tau (tau residues 14−441) over full-length tau (Figure 4A). Tau-C6g also does not bind efficiently to the recombinant protein tauΔ2−13, which contains an initial methionine residue immediately N-terminal to the 14−28 epitope (Figure 4B).

We hypothesized that aminopeptidase degradation of tauΔ2−13 would cause rapid removal of the single initial methionine residue, and therefore the appearance of the Tau-C6g truncation-specific epitope. This would show removal of an individual N-terminal amino acid, verifying aminopeptidase activity. At later time points, this epitope would be lost as the remaining tau becomes completely degraded. Furthermore, N-terminal degradation of tau would not cause appearance of the C-terminal truncation-specific epitope of the antibody Tau-C3 (cleavage at residue D421).

Western blotting with these antibodies clearly shows the rapid appearance and gradual disappearance of the Tau-C6g epitope in PSA-treated but not in untreated tauΔ2−13 (Figure 4B). While the Tau-C3 C-terminal truncation-specific antibody labeled the positive control (tau Δ422−441), it did not label any of the PSA-treated or untreated samples, indicating that the truncation of tau is due to aminopeptidase activity, rather than another contaminating exopeptidase.

PSA Digests Normal Human Brain Tau, But Digests Tau from AD Brain Less Efficiently. Next, the ability of PSA to digest tau from brain tissue sources was investigated. PSA was incubated with soluble tau purified from non-pathological human or porcine brain as well as soluble and insoluble (PHF) tau purified from AD brain (Figure 5A), and the extent of tau digestion was quantified (Figure 5B). After 18 h of incubation, the non-pathological human brain soluble tau was over 75% digested. In contrast, both soluble and insoluble (PHF) tau samples from AD brain tissue were relatively resistant to PSA digestion for this extended time period ($p < 0.05$ and $p < 0.01$, respectively). Interestingly, soluble tau purified from porcine brain was degraded to a similar extent as normal human soluble tau. No lower molecular weight bands were observed in any of these samples, suggesting the absence of endopeptidase activity in the purified tau samples. These results indicate that, while abnormal post-translational modifications of tau may hinder its degradation by PSA in vivo, tau from AD brain tissue is still susceptible to PSA digestion.

DISCUSSION

While the initial causative factors leading to tau polymerization and tangle formation in Alzheimer's disease are still undefined, one potential contributing factor is the buildup of normal or abnormal tau within the neuronal cytoplasm. Therefore, the proteases that degrade tau under normal and disease conditions are of great interest. Puromycin-sensitive aminopeptidase, originally described as an enkephalin-degrading enzyme (*21*, *51*), has recently been shown to influence the development of tau pathology in a Drosophila model of Alzheimer's disease (*20*). Karsten et al. (*20*) demonstrated that altered levels of PSA expression had a remarkable effect on tau-induced neurodegeneration in vivo, i.e., increased PSA expression was associated with decreased tau-induced neurodegeneration, and vice versa. PSA may therefore be important in the N-terminal processing and degradation of normal and pathologic tau in AD.

Previous studies of N-terminal tau processing have used tau constructs containing an amino terminal poly histidine tag (*14*, *20*). To study more accurately the N-terminal digestion of tau by PSA, we chose as our substrate the longest isoform of tau expressed in human brain, containing instead

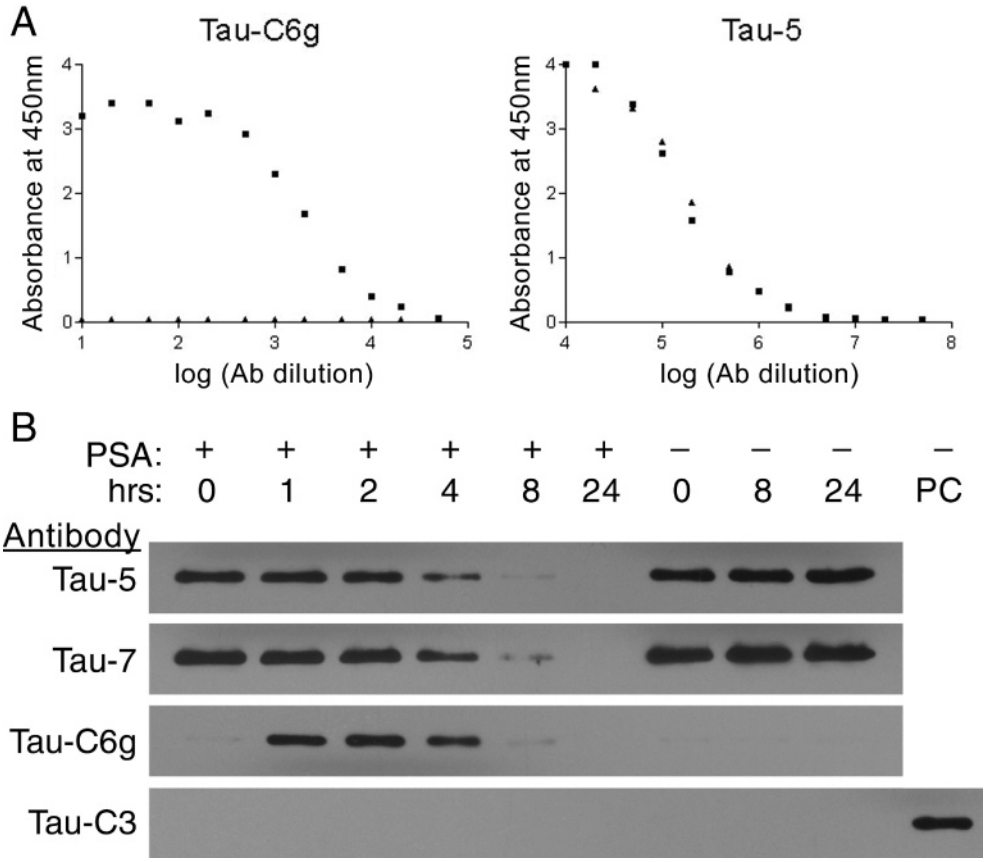

FIGURE 4: Truncation-specific antibodies confirm that PSA degrades tau as an aminopeptidase. (A) Tau-C6g is a novel monoclonal antibody specific for tau cleaved N-terminally at the caspase-6 site after residue D13 (■), which does not react with full-length tau (▲). Tau-5 is a non-cleavage specific control. Tau-C6g was used in serial dilutions of a tissue culture supernatant, while Tau-5 was diluted from a purified (1 mg/mL) stock. (B) Western blotting with truncation-specific tau antibodies shows N-terminal, but not C-terminal, digestion of tauΔ2−13. Tau-5 and Tau-7 show all of the tauΔ2−13 on the membrane. The binding of the Tau-C6g antibody to tauΔ2−13 is impaired due to the initial methionine residue just N-terminal to residue 14. Addition of PSA causes the rapid appearance and gradual disappearance of the Tau-C6g truncation-specific epitope, indicating N-terminal digestion of the first methionine, followed by the remainder of the molecule. Tau-C3 recognizes a C-terminal truncation-specific epitope (cleavage after D421), and does not blot PSA-treated tauΔ2−13, although it labels tauΔ422−441, a positive control (PC).

a carboxy-terminal poly histidine tag. This resulted in a tau molecule with a more physiologically relevant N-terminus, as well as a tau preparation that was relatively more pure electrophoretically than N-terminally poly histidine tagged protein preparations.

Next, as PSA was not available commercially, we produced recombinant PSA purified from bacteria. Since this preparation of PSA has not been adequately characterized in the literature (*20*), we first verified that it was biochemically similar to previously published reports in all tests of substrate specificity and enzyme inhibition. Furthermore, a number of controls were used to verify that PSA, and not another contaminating protease, was digesting tau. Both the purified PSA and tau preparations were devoid of carboxypeptidase activity. Additionally, the digestion of tau by PSA was inhibited by the specific protease inhibitor puromycin as well as another known potent aminopeptidase inhibitor, bestatin, but was unaffected by PMSF, which does not inhibit PSA. Furthermore, blotting with a variety of monoclonal tau antibodies showed no distinct lower molecular weight bands or smears in any of the tau samples treated with PSA, indicating the absence of endopeptidase activity. Finally, the digestion of tauΔ2−13 by PSA led to the formation of a transient N-terminal truncation-specific epitope by cleavage of a single methionine residue (as recognized by the novel Tau-C6g antibody) but did not result in C-terminal digestion as measured by a truncation-specific antibody at the other end of the molecule (Tau-C3). Collectively, these results show that the bacterially purified recombinant human PSA is digesting tau as an aminopeptidase.

While PSA has been reported to cleave a number of small peptide substrates (e.g., neurotransmitters), the preferred physiological substrates of PSA have not been identified, and the role of PSA in degrading larger proteins has scarcely been addressed. However, here we have demonstrated that full-length catalytically active human PSA expressed in bacteria readily cleaves human recombinant tau protein. The digestion also shows some specificity, as soluble tau purified from non-pathological human and porcine brain is digested to a greater extent than similarly prepared soluble tau from AD brain. Insoluble tau from AD brain was also relatively resistant to PSA treatment, suggesting that post-translational modifications and/or polymerization of tau can impair the ability of PSA to degrade tau. However, the partial digestion of tau samples from AD brain suggests that PSA may still be able to degrade abnormal tau from pathological lesions.

In AD, tau is known to be heavily phosphorylated and N-terminally nitrated (*52*, *53*). Furthermore, AD-associated conformations of PHF tau (*46*, *54*) may impart tau with protease resistance: precedent for this may be found in the

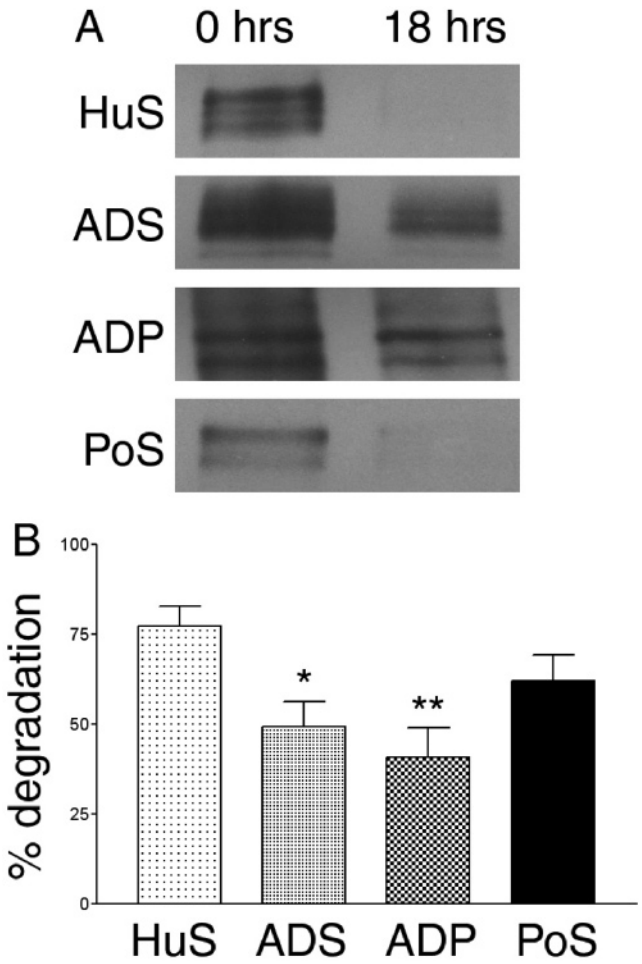

FIGURE 5: Western blot analysis (with Tau-7) illustrating the proteolysis of tau purified from various brain sources. (A) PSA degrades soluble tau purified from non-pathological human brain (HuS) following 18 h incubation. Soluble AD brain tau (ADS) and insoluble PHF AD brain tau (ADP) fractions are relatively more resistant to PSA treatment. Tau from porcine brain soluble fractions (PoS) is also degraded by PSA. (B) Quantitative analysis of Western blotting data indicating the extent of tau digestion. Human brain soluble tau was degraded to a greater extent than AD brain soluble tau (*$p < 0.05$) and AD brain PHF tau (**$p < 0.01$).

prion disease literature, where an abnormal conformation of the prion protein causes resistance to digestion by proteinase K in vitro (*55*). It is possible that PSA prefers "unstructured" protein and peptide substrates, and the transition of tau from its soluble state to the PHF form heavily populated by beta-sheet structure reduces the accessibility of PHF tau. The effects of these myriad post-translational modifications of tau on its digestion by PSA may be complex, and they merit further research.

The amino terminal degradation of tau, in particular, has several implications regarding the prevention of tau pathology formation in vivo. The interactions of the N-terminus with distal portions of tau are complex and are known to regulate the solubility and polymerization of tau (*46*, *50*). The conformational change by which tau transitions from soluble monomers to filamentous AD pathology requires the extreme N-terminus of tau coming into contact with other regions of the molecule, most notably the third microtubule binding repeat (*46*). Furthermore, removal of the first 18 residues of tau greatly inhibits tau polymerization in vitro (*56*). Thus, even if PSA were to degrade only the extreme N-terminus of tau in vivo, a significant decrease in tau polymerization would be expected.

The association of tau and PSA biochemically is particularly interesting in light of the similarities in the sequences of these two proteins. Tau and PSA are located in nearby regions of chromosome 17q (*57*, *58*), and besides its catalytic domains, PSA contains two motifs that show significant similarity to the microtubule-binding sites of MAP-2, MAP-4 and tau (*29*). As tau is known to self-associate via its microtubule repeat region, the structure of PSA suggests a possible targeting mechanism of the protein for members of the MAP-4 superfamily. Alternatively, the MTBR-like domains could also target PSA to the microtubule, bringing it into close apposition with tau and other MAPs. While these two proteins can be linked theoretically based on their microtubule binding domains, they have already been linked genetically: genetic screens in both mice and Drosophila showed that PSA expression was correlated with rescue of FTD-like tau pathology, while loss of function in Drosophila led to accelerated neurodegeneration (*20*).

It has been shown that PSA expression is relatively high in human cerebellar neurons than cerebral cortex, and this observation supports the hypothesis that PSA digestion of tau might prevent tau accumulation, as cerebellum is relatively spared in AD and FTDs compared to cerebral cortex (*20*). In addition, it is also known that PSA co-immunoprecipitates with amyloid precursor protein (*59*) and is localized around senile plaques in the cerebral cortex and hippocampus of AD brain (*60*). This relationship between PSA and beta amyloid is intriguing, and one might speculate whether beta amyloid alters the activity of PSA in vivo. Furthermore, our results suggest that in vivo degradation of tau by PSA may be affected by post-translational modifications of tau or other unknown mechanisms, and that this disruption of tau degradation contributes to the development of tau pathology in AD. These findings, along with our previously published results describing the prevention of tau-induced neurodegeneration with expression of PSA, have identified a role for PSA in the modulation of different tau levels and tau pathology in the brain.

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