Aminopeptidases do not directly degrade tau protein

K. Martin Chow
*University of Kentucky*, mchow1@uky.edu

Hanjun Guan
*University of Kentucky*, hguan2@uky.edu

Louis B. Hersh
*University of Kentucky*, lhersh@uky.edu

Follow this and additional works at: https://uknowledge.uky.edu/biochem_facpub

Part of the Biochemistry, Biophysics, and Structural Biology Commons

Right click to open a feedback form in a new tab to let us know how this document benefits you.

Repository Citation

Chow, K. Martin; Guan, Hanjun; and Hersh, Louis B., "Aminopeptidases do not directly degrade tau protein" (2010). *Molecular and Cellular Biochemistry Faculty Publications*. 28.
https://uknowledge.uky.edu/biochem_facpub/28

This Article is brought to you for free and open access by the Molecular and Cellular Biochemistry at UKnowledge. It has been accepted for inclusion in Molecular and Cellular Biochemistry Faculty Publications by an authorized administrator of UKnowledge. For more information, please contact UKnowledge@lsv.uky.edu.
Aminopeptidases do not directly degrade tau protein

Digital Object Identifier (DOI)
http://dx.doi.org/10.1186/1750-1326-5-48

Notes/Citation Information
Published in Molecular Neurodegeneration, v. 5, 48.

© 2010 Chow et al; licensee BioMed Central Ltd.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

This article is available at UKnowledge: https://uknowledge.uky.edu/biochem_facpub/28
Aminopeptidases do not directly degrade tau protein

K Martin Chow*, Hanjun Guan, Louis B Hersh

Abstract

Background: Tau hyperphosphorylation and aggregation to form intracellular neurofibrillar tangles is prevalent in a number of tauopathies. Thus there is current interest in the mechanisms involved in Tau clearance. It was recently reported that Tau can be degraded by an aminopeptidase known as the puromycin sensitive aminopeptidase (PSA). Until now PSA has been reported to only cleave peptides, with the largest reported substrates having 30-50 amino acids. We have studied this unique PSA cleavage reaction using a number of different PSA preparations.

Results: An N-terminally His tagged-PSA was expressed and purified from Sf9 insect cells. Although this PSA preparation cleaved Tau, product analysis with N and C terminal Tau antibodies coupled with mass spectrometry showed an endoproteolytic cleavage atypical for an aminopeptidase. Furthermore, the reaction was not blocked by the general aminopeptidase inhibitor bestatin or the specific PSA inhibitor puromycin. In order to test whether Tau hydrolysis might be caused by a protease contaminant the enzyme was expressed in E. coli as glutathione S-transferase and maltose binding protein fusion proteins or in Sf9 cells as a C-terminally His-tagged protein. After purification to near homogeneity none of these other recombinant forms of PSA cleaved Tau. Further, Tau-cleaving activity and aminopeptidase activities derived from the Sf9 cell expression system were separable by molecular sieve chromatography. When tested in a cellular context we again failed to see a PSA dependent cleavage of Tau. A commercial preparation of a related aminopeptidase, aminopeptidase N, also exhibited Tau cleaving activity, but this activity could also be separated from aminopeptidase activity.

Conclusion: It is concluded that PSA does not directly cleave Tau.

Introduction

The microtubule-associated protein tau (Tau) is located primarily in the central nervous system (CNS) and regulates the stability of microtubules. Tau is normally phosphorylated in cells with its state of phosphorylation related to developmental state [1]. Under abnormal conditions Tau becomes hyperphosphorylated, dissociates from microtubules and forms aggregates [2,3]. There are a number of neurodegenerative diseases caused by Tau aggregation collectively termed tauopathies. Among these is Alzheimer’s disease in which intracellular Tau aggregates known as tangles are found in the brain and are believed to contribute to the etiology of the disease [4-6].

Like other microtubule-associated proteins (MAPs), a tandem microtubule-binding motif GSxxNxxHxPGGG is found at the C-terminus of Tau. Isoforms of Tau [7] have either 3 or 4 of these binding repeats due to alternative mRNA splicing of exon 10, which contains the fourth repeat. Additional variants of Tau are derived by an N-terminal insertion of exons 2 and 3, by insertion of only exon 3, and a form with no insertion. Together these variants result in six Tau isoforms in brain. The longest isoform (2N4R) contains exons 2 and 3 and 4 binding repeats (441 amino acids), while the shortest isoform (0N3R) has no N-terminal insert and 3 repeats (352 amino acids).

Due to its importance in neurodegenerative diseases, there have been a number of studies of Tau degradation by proteases such as the proteosome [8], caspase [9], and thrombin [10]. Recent reports suggest that the puromycin sensitive aminopeptidase (PSA, EC 3.4.11.14) may regulate Tau levels in vivo [11], and is able to hydrolyze Tau in vitro [12]. PSA has been characterized [13-15] as a zinc containing exopeptidase that...
sequentially cleaves the N-terminal amino acid from small peptides [16]. It is uniquely sensitive to micromolar concentrations of puromycin, hence its name. PSA is inhibited by the classical aminopeptidase inhibitor bestatin and its analogs. Until now PSA was thought to only cleave peptides containing no more than 30-50 amino acids. Thus Tau would be a novel substrate for PSA being considerably larger than any previously known substrate. The present study was designed to further investigate how PSA cleaves Tau; however, the results of these studies led us to conclude that Tau is not directly cleaved by PSA or by the closely related aminopeptidase, aminopeptidase N.

Experimental Procedures

Materials

PMSF, EDTA, bestatin, and o-phenanthroline were purchased from Sigma-Aldrich. Puromycin was from Invitrogen (Life Technologies). Monoclonal antibody (5A6) directed against N-terminal Tau residues 16 to 46 [17] was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology. A C-terminal Tau antibody A0024, which is directed against the C-terminal ~1/3 of Tau, was obtained from DAKO.

Cloning and Expression of Tau forms

Tau cDNAs in the pcDNA3.1 vector were generously provided by Dr. M. Hutton (Mayo Clinic, Jacksonville, FL). Two isoforms of Tau were used in this study: the longest isoform (Tau 2N4R) containing exons 2 and 3 as well as 4 microtubule binding repeats and Tau 0N4R containing 4 microtubule binding repeats without an N-terminal insert. For expression in E. coli, the 0N4R isoform-containing plasmid was amplified by PCR with the following primers:

Forward primer (5'-AATAACATATGGCTGACGCCG), which contains an Nde 1 site (underlined). Reverse primer (5'-GAATCTGAGTTACTACAAACCTGCTT), which removes the native stop codon and introduces an Xho 1 site (underlined) that fuses a C-terminal His6 tag.

PCR products were first subcloned into the pZero2 vector according to the manufacturer’s instructions. Three days after infection with baculovirus, Sf9 cells containing sequential baculovirus amplification were performed according to the manufacturer’s instructions. Three days after infection with baculovirus, Sf9 cells containing recombinant enzymes were collected by centrifugation at 2,000g for 10 min., and frozen at ~80°C until use. The procedure for purifying N-His6-PSA is similar to that previously described [21]. Briefly, a cell paste was suspended in 10 volumes of lysis buffer (20 mM Tris-HCl, pH 7.5, 1 mM DTT using a Millipore centricon. Both heat treated Tau [18] and His tagged Tau [12] have been shown to retain the ability to polymerize tubulin.

A stable tetracycline-inducible human Tau (2N4R) expressing cell line was obtained from Dr. Jeff Kuret (Ohio State University).

Cloning and Expression of PSA

A full length human PSA cDNA [19] was subcloned into the pFastbac vector for expression as an N-terminal hexahistidine PSA (N-His6-PSA) in Sf9 insect cells [20]. To generate a C-terminal hexahistidine PSA (C-His6-PSA), a C-terminal fragment containing six His residues was amplified using PSA as a template with the following primers:

Forward (5’-TGCCCCCTGTGGATCGACTTGG-3’), preceding an internal Xho1 site, and reverse (5’-CGA-TAAGCTTCAGTGTTGTCGTGTCACGTG-3’), containing a HindIII site (underlined).

This amplified 901 bp product and the 5’ 1745 bp fragment were cloned into pFastbac1 using HindIII/ Xho1 and Xho1/BamH1 sites, respectively. The procedures of transposing pFastbac-PSA to bacmid and subsequent baculovirus amplification were performed according to the manufacturer’s instructions. Three days after infection with baculovirus, Sf9 cells containing recombinant enzymes were collected by centrifugation at 2,000g for 10 min., and frozen at ~80°C until use. The procedure for purifying N-His6-PSA was similar to that as previously described [21]. Briefly, a cell paste was suspended in 10 volumes of lysis buffer (20 mM Tris buffer, pH 7.5, 1 mM PMSF and 10 μM E-64) and sonicated with a Branson Sonifier 450 (Output control at 4, Duty cycle 30%, 10 sec for 3 times). The homogenate was centrifuged 30 min at 30,000g and the supernatant applied to a Waters Acell Plus QMA anion exchange column (15 mL) in 20 mM Tris buffer, pH 7.5. Proteins were eluted with a 0 to 0.5 M linear NaCl gradient with PSA eluting at ~0.11 M NaCl. The eluted enzyme was concentrated and applied to a Pharmacia Superdex 200 column (1.6 cm × 60 cm) equilibrated and run with 20 mM Tris buffer, pH 7.5. The active PSA fractions were pooled, and concentrated with a
Millipore Centricron concentrator and stored in 25% glycerol at -80°C.

For purifying C-His₆-PSA, cell pellets were sonicated as described above with 300 mM NaCl and 5 mM imidazole included in the buffer. The supernatant was passed through a 5-ml His-Select Nickel affinity column and eluted with 100 mM imidazole. After concentration and buffer exchange the purified enzyme was made 20% glycerol and stored at -80°C.

PSA was also expressed in E. coli as GST and MBP fusion proteins. The expression vectors pET41, which contains a GST sequence and a TEV protease cleavage site and pETa-MBP [22], which contains a His₈-Maltose binding protein and a TEV cleavage site were used. These plasmids were transformed into E. coli BL-21 (DE3)-RP and the bacteria were allowed to grow until an OD₆₀₀ of 0.5 to 0.7 was reached. PSA expression was induced by the addition of IPTG to a final concentration of 0.5 mM, and carried out overnight at 16°C, after which time cells were harvested, washed with PBS and stored at -20°C. A GST Prep FF 16/10 column (GE Health Science) was used to purify GST-PSA as previously described [21]. Purified GST-PSA was treated overnight with TEV protease at 4°C to remove GST and further purified by chromatography on an S-200 (GE Health Science) gel filtration column. GST-PSA, without being treated with TEV protease, was also chromatographed on the same column. His₈-MBP-PSA was purified by chromatography on Ni resin as described above followed by S-200 chromatography. The active fractions were concentrated and treated with TEV protease to remove His₈-MPB. Lastly the enzyme was purified on a 1-ml 15Q anionic exchange resin (GE Health Science).

**Lentivirus production**

cDNAs for human PSA and an inactive E309A point mutant (PSAx) [20] were subcloned into the lentiviral vector pCSC-SP-PW. Lentiviruses were then produced using a four-plasmid transfection system as described previously [23].

**Aminopeptidase activity assays**

Aminopeptidases were routinely assayed using 20 μM alanine 4-methoxy-2-naphthylamine (Ala-4-MNA) as substrate in 25 mM Tris-HCl buffer, pH 7.5. Free 4-MNA released by PSA was monitored on a fluorescent plate reader (GX, Molecular Device) at an excitation of 340 nm and an emission of 425 nm. Hydrolysis of Leu-enkephalin (YGGFL) was determined by reverse phase HPLC on a Phenomenex C₁₈ column (5.0 × 4.6 mm) by analyzing Tau products. For unknown proteins, MS/MS spectra were used to search against the Uniprot_sprot_20100125+ contams.fasta database through Protein Pilot (Applied Biosystems) Ver. 2.0, Rev. 50861.

**Protein assay**

Protein concentration was determined using Coomassie Plus (Pierce Chemical Company). Bovine serum albumin was used as the protein standard.

**Identification of protein fragments by Mass Spectrometry**

Protein bands were identified by mass spectrometry at the University of Kentucky Proteomics core within the Center of Structure Biology. This facility is supported in part by grant P20RR020171 from the NIH NCRR. Briefly, gel bands were excised from SDS-PAGE, digested with Sigma proteases grade trypsin, and alkylated with iodoacetamide. Samples were then analyzed with an Applied Biosystems 4800 MALDI TOF/TOF Proteomics Analyzer. MALDI MS spectra were used for analyzing Tau products. For unknown proteins, MS/MS spectra were used to search against the Uniprot_ -sprot_20100125+ contams.fasta database through Protein Pilot (Applied Biosystems) Ver. 2.0, Rev. 50861.

**Results**

We initially tested the reaction of purified recombinant human PSA expressed in Sf9 insect cells with Tau. This
form of PSA was expressed as a fusion protein containing an N-terminal hexahistidine affinity tag (N-His6-hPSA) as previously described [20]. The purity of N-His6-hPSA was estimated to be ~90% as judged from a Coomassie stained SDS-PAGE gel, figure 1a. After removal of the affinity tag this purified hPSA (hPSA6) was tested for its ability to hydrolyze Tau by incubation with recombinant Tau 0N4R for 16 hours at 37°C at a molar ratio of Tau to PSA of 15:1 as described by Karsten et al. [11]. Cleavage of Tau was apparent as assessed by Coomassie blue protein staining of the reaction subjected to SDS-PAGE, figure 1b. To further analyze the reaction, major products shown in figure 1b were analyzed by peptide mass spectral fingerprinting. The identified peptides from these products are listed in Table 1. Additionally, degradation products were reacted with antibodies specific to the N- and C-terminal regions of Tau (figure 1c). Since hPSA6 is an exopeptidase that cleaves amino acids sequentially from the N-terminus, we were surprised to find that reaction products, N1 to N3, which were decreased in size by apparent molecular weights of greater than 15 kDa, still retained the epitopes for the N-terminally directed antibody 5A6. The identified peptides (Table 1) agree with that of Western analysis in that the N-terminus of these cleavage products is intact. This finding shows that Tau cleavage was internal, not expected for an aminopeptidase.

We also identified four C-terminal peptides derived from the PSA cleavage reaction (C1 through C4). Although these fragments could result from sequential amino acid release from the N-terminus, the number and size of the bands detected by the C-terminal Tau antibody A0024 makes this unlikely. This follows since one would expect a series or ladder of products detected by the C-terminal antibody rather than discreet peptides. figure 2 schematically superimposes the identified peptide masses.

| Table 1 Identification of Tau degradation products by mass spectral peptide fingerprinting |
|-----------------------------------------------|----------------|-----------------|------------------------------|
| Protein Band | Calc’d | Expt’d | Peptides identified |
|---------------|--------|--------|---------------------|
| N1            | 2069.89 | 2069.67 | 6QEFEVMDHAGTYGLGDR |
|               | 2181.90 | 2181.74 | 25DQGGYTMHQDQEGTDAGLKL |
|               | 2424.13 | 2424.89 | 4AEAGIGDTPSLEDEAAGHVTQAR |
|               | 1393.63 | 1393.50 | 137SGYSSPGSGPTPGSR |
|               | 842.55  | 842.43  | 213VQINHK |
|               | 1980.09 | 1979.87 | 241HPGGGSGVQIVKPVDSLK |
| N3            | 2069.89 | 2069.81 | 6QEFEVMDHAGTYGLGDR |
|               | 2424.13 | 2424.05 | 4AEAGIGDTPSLEDEAAGHVTQAR |
|               | 1393.63 | 1393.61 | 137SGYSSPGSGPTPGSR |
|               | 1066.59 | 1066.55 | 154TPSLPTPFPTR |
|               | 842.55  | 842.50  | 213VQINHK |
| C1            | 1420.78 | 1420.64 | 154TPSLPTPFPTR |
|               | 842.55  | 842.48  | 213VQINHK |
|               | 1974.00 | 1973.90 | 254CGSSLGNIHHKPGGGQVEVK |
|               | 1578.82 | 1578.76 | 296IGSLDNITHVPGGGGNK |
|               | 978.54  | 978.51  | 318LTFRENAK |
|               | 1101.55 | 1101.52 | 338SPVVSGDTSPR |
|               | 1381.66 | 1381.61 | 381QGLLEHIIIIII |
| C3            | 842.55  | 842.44  | 213VQINHK |
|               | 1578.82 | 1578.67 | 296IGSLDNITHVPGGGGNK |
|               | 978.54  | 978.46  | 318LTFRENAK |
|               | 1101.55 | 1101.45 | 338SPVVSGDTSPR |
|               | 1381.66 | 1381.54 | 381QGLLEHIIIIII |

**Figure 1 Human PSA**

Human PSA preparation cleaves Tau. (a) Purity of hPSA6 (5 μg). (b) Degradation of Tau by hPSA6. Tau was incubated with PSA at a molar ratio of 15:1 (Tau:PSA) in 10 mM Tris buffer, pH 7.5, 1 mM DTT at 37°C for 16 h. Reactions containing 1 μg Tau were analyzed by SDS-PAGE on a 12.5% gel. Labeled products and intact Tau were further analyzed by mass spectrometry and the result is shown in Table 1. (c) Reaction products immunoblotted with N-terminal Tau antibody 5A6 (red) or C-terminal Tau antibody A0024 (green). Twenty percent of the reaction mix was loaded in each lane. Intact Tau shows a yellow color due to the red/green overlap. N1 also reacts to A0024, but was overshadowed by higher intensity signal of 5A6. Molecular weight markers in kDa are shown at the left.
peptides on the Western analysis and shows that the cleavage sites are located in the vicinity of the four microtubule-binding motif repeats.

We found that the hPSA<sub>SF</sub> dependent cleavage of Tau was not blocked by the aminopeptidase inhibitors bestatin and puromycin, but was inhibited by the metal chelator o-phenanthroline (figure 3). Puromycin rather than inhibit had an activation effect on the reaction. As a control we showed that the reaction of hPSA<sub>SF</sub> with two known PSA substrates, alanine 4-methoxy-2-naphthalamide (Ala-4-MNA) and Leu-enkephalin, was completely inhibited by bestatin and puromycin under the same conditions in which these inhibitors failed to inhibit Tau cleavage. (Table 2). The finding of endopeptidase-like cleavage of Tau, the insensitivity of the reaction to PSA inhibitors, together with the sensitivity of the reaction to the metal chelator o-phenanthroline suggest either contamination of the hPSA<sub>SF</sub> preparation with a metalloendopeptidase that cleaves Tau or that the cleavage of Tau by hPSA<sub>SF</sub> proceeds through a novel mechanism.

In an attempt to resolve this issue we expressed hPSA in <i>E. coli</i> assuming that if the cleavage of Tau was due to a contaminating protein derived from Sf9 cells it would be highly unlikely that the same or similar contaminant would be present in enzyme preparations derived from a totally different system. We thus expressed hPSA as two different fusion proteins, a glutathione S-transferase fusion protein (GST-hPSA) and a-maltose binding protein fusion protein (MBP-hPSA). Since N-His<sub>6</sub>-hPSA did not bind well to a nickel resin and was purified conventionally, an additional form of PSA containing a C-terminal His<sub>6</sub> tag (C-His<sub>6</sub>-PSA) was expressed in Sf9 insect cells and purified on a

![Figure 2 Diagrammatic presentation of Tau (ON4R) degradation and the possible cleavage regions.](http://www.molecularneurodegeneration.com/content/5/1/48)

![Figure 3 Hydrolysis of Tau by hPSA<sub>SF</sub> in the presence of protease inhibitors.](http://www.molecularneurodegeneration.com/content/5/1/48)
nickel resin. The specific activities of all of the PSA preparations derived from Sf9 cells or E. coli were essentially the same, ~6 μmol of 4-MNA released from Ala-4-MNA per min per mg protein. Equal amounts of PSA activity from these different preparations was tested for their ability to cleave full-length Tau 2N4R, since it has been previously reported [11,12] that the 2N4R isoform is cleaved by hPSA<sup>GST</sup> with or without the GST removed. We used the same reaction conditions as described [11], which included 10 mM Tris buffer, pH 7.5 and 1 mM DTT. When tested, with the exception of the original hPSA<sup>N</sup> (N-His<sub>6</sub>-hPSA with His tag removed), none of these other PSA preparations cleaved Tau 2N4R, figure 4. An identical result was obtained when Tau 0N4R was used as a substrate, figure 4b. Thus, it is very likely that the N-His<sub>6</sub>-hPSA preparation, being the only one of four different PSA preparations that cleaved Tau, contains a contaminating protease responsible for Tau cleavage.

Next, we attempted to resolve PSA activity from Tau cleaving activity in the N-His<sub>6</sub>-hPSA Sf preparation. A preparation of N-His<sub>6</sub>-hPSA<sup>Sf</sup> was purified from an Sf9 cell extract following the purification scheme described in Methods. At the final molecular sieve purification step PSA activity toward the synthetic substrate Ala-4-MNA and Tau cleaving activity were assayed in fractions collected from the S200 column. As shown in figure 5a, Tau activity was eluted in a broad peak with maximal activity at fraction 27. In contrast true PSA activity as measured with Ala-4-MNA peaked at fraction 29. Despite the fact that elution of Tau cleaving activity exhibits a broad peak that overlaps with PSA activity, these activities are clearly distinguishable. For example, Tau cleaving activity is approximately the same in fractions 27 and 29, but aminopeptidase activity in fraction 29 was ~35 times higher than that of fraction 27.

As a further control we examined Tau cleaving activity in extracts from untransfected Sf9 cells taken through the same purification scheme. As shown in figure 5b (upper panel) Tau activity was eluted in a broad peak with maximal activity at fraction 27. In contrast true PSA activity as measured with Ala-4-MNA peaked at fraction 29. Despite the fact that elution of Tau cleaving activity exhibits a broad peak that overlaps with PSA activity, these activities are clearly distinguishable. For example, Tau cleaving activity is approximately the same in fractions 27 and 29, but aminopeptidase activity in fraction 29 was ~35 times higher than that of fraction 27.

As a further control we examined Tau cleaving activity in extracts from untransfected Sf9 cells taken through the same purification scheme. As shown in figure 5b (upper panel) Tau activity was eluted in a broad peak with maximal activity at fraction 27. In contrast true PSA activity as measured with Ala-4-MNA peaked at fraction 29. Despite the fact that elution of Tau cleaving activity exhibits a broad peak that overlaps with PSA activity, these activities are clearly distinguishable. For example, Tau cleaving activity is approximately the same in fractions 27 and 29, but aminopeptidase activity in fraction 29 was ~35 times higher than that of fraction 27.

As a further control we examined Tau cleaving activity in extracts from untransfected Sf9 cells taken through the same purification scheme. As shown in figure 5b (upper panel) Tau activity was eluted in a broad peak with maximal activity at fraction 27. In contrast true PSA activity as measured with Ala-4-MNA peaked at fraction 29. Despite the fact that elution of Tau cleaving activity exhibits a broad peak that overlaps with PSA activity, these activities are clearly distinguishable. For example, Tau cleaving activity is approximately the same in fractions 27 and 29, but aminopeptidase activity in fraction 29 was ~35 times higher than that of fraction 27.
**Figure 5** Separation of aminopeptidase and Tau-cleaving activities on an S-200 column. (a). PSA derived from Sf9 cells was purified as described in Methods. Following an anionic QMA column, the enzyme was chromatographed on an S-200 column and fractions were assayed for both Tau and aminopeptidase activity. The arrows show the eluted PSA activity detected on the gel. Tau activity is shown as the % hydrolysis (solid square), while aminopeptidase activity toward alanine 4-MNA is shown by solid triangles. (b). An Sf9 cell extract from non-infected cells was fractionated the same way as the above. Upper panel: Fractions assayed for Tau-cleaving activity. Lower panel: Fractions assayed for Tau-cleaving activity in the presence of exogenous C-His$_6$-PSA with a molar ratio of Tau:PSA (5:1).
To determine whether PSA might facilitate cleavage of Tau in a cellular context, we transduced HEK 293 cells expressing a tetracycline-inducible human 2N4R Tau with a lentivirus expressing PSA. As controls lentviruses expressing GFP or an inactive point mutant of PSA (PSAx) were used. The expressed PSA had no effect of the intracellular level of Tau under steady-state expression conditions (figure 6A) or when Tau expression was induced (figure 6B).

We tested the reactivity of another aminopeptidase, aminopeptidase N (ApN) with Tau because it is a related protein with very similar kinetics and substrate specificity to PSA [16]. Surprisingly, we found that a commercial purified porcine ApN preparation cleaved Tau. To determine whether the Tau cleaving activity is caused by ApN, we fractionated this ApN preparation by hydrophobic chromatography on a Tosohas butyl-650 column and tested the reactivity of the eluted fractions toward Tau and toward the aminopeptidase substrate Ala-4-MNA. The results showed that aminopeptidase activity and Tau-cleaving activity are clearly resolved (figure 7), demonstrating that aminopeptidase N does not hydrolyze Tau. Attempts to identify the Tau-cleaving enzyme by mass spectral fingerprinting also met the same difficulty as with the insect protein, namely a lack of an adequate database. Unfortunately only ~300 porcine DNA sequences are currently deposited in the NCBI database, and none of those showed up in our analysis. Thus the identification of the Tau cleaving enzymes derived both Sf9 cells and from porcine kidney remains unknown, but is clearly not PSA.

Discussion

In this study several lines of evidence have been obtained which show that the puromycin sensitive aminopeptidase does not directly cleave Tau. (i) The cleavage pattern obtained with Sf9 derived N-His₆-hPSA is inconsistent with aminopeptidase cleavage. (ii) Several PSA preparations derived using different expression systems and purification methods do not cleave Tau. Only the preparation obtained from Sf9 cells containing an N-His₆-affinity tag cleaves Tau. The N-His₆ affinity tag containing PSA was the only form that was not purified by affinity chromatography. (iii) Aminopeptidase and Tau-cleaving activity can be separated by gel filtration chromatography for PSA and by hydrophobic chromatography for the related aminopeptidase N. (iv) Within a cellular context increasing PSA expression did not affect Tau steady-state levels. Thus, we are forced to conclude that PSA does not cleave Tau and that the Tau-cleaving activity we observed in our PSA preparation is caused by an as yet unidentified contaminating protease. Since this activity was inhibited by α-phenantridine this contaminating protease is likely a metalloprotease.

Our finding that PSA does not cleave Tau appears at variance with several published reports [11,12,28]. Although our purified PSA preparations, including the

---

**Figure 6 PSA does not cleave Tau in cells**. HEK293 cells expressing a tetracycline-inducible 2N4R Tau were transduced with lentivirus expressing GFP, PSA or its inactive mutant form (PSAx) and cultured for 48 hrs. Tetracycline (1 μg/ml [Tet(+)] or vehicle (1:1 vol/vol water/ethanol) [Tet(-)] was added and cells were collected 12 hrs later. Cell extracts were subjected to SDS-PAGE, and analyzed by Western blot analysis with goat anti-hPSA (Chemicon) at 1:2000, and mouse anti-Tau antibody 5A6, at 1:4000. This was followed by incubation with peroxidase conjugated secondary antibodies and ECL Western Blotting detection.

---

**Figure 7 Separation of aminopeptidase N from Tau-cleaving activity**. A commercial preparation of aminopeptidase N was fractionated on a butyl S-650 hydrophobic chromatography. (a) Tau-cleaving activity was estimated by the intensity of the remaining Tau band as analyzed by SDS-PAGE. T = Tau with buffer only. (b) Aminopeptidase N activity as determined with alanine 4MNA as substrate.
same GST fusion protein previously studied [11] did not cleave Tau, it is still possible that PSA regulates Tau cleavage by an indirect mechanism. Studies in TauP301L transgenic mice in conjunction with a functional analysis in *Drosophila* associate Tau as being regulated by PSA [11] and a recent report showed that using an interference RNA against PSA increased Tau levels in SH-SY5Y cells [28]. However as shown in this study over expression of PSA in HEK cells did not change Tau levels. Since we conclude that PSA does not directly cleave Tau, the regulation of Tau levels by PSA might work through its interaction with other proteins or by regulating the level of a peptide that facilitates Tau cleavage. For example it has been shown that PSA works in conjunction with the proteosome dependent degradation of polyglutamine containing proteins by cleaving the released polyglutamine peptides [29]. Understanding the mechanism by which PSA affects Tau levels will clearly require additional studies.

Abbreviations

PSA: Purumycin-sensitive aminopeptidase; GST: Glutathione S-transferase; MBP: maltose binding protein; E-64: trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane, DTT: dithiothreitol, MNA: 4-methoxy-2-naphthylamide; PMSF: phenylmethylsulfonylfluoride.

Acknowledgements

This research was supported by grants from the National Institute on Drug Abuse, grant DA02243 and from the National Center for Research Resources (NCRR), grant P20RR02017. We would like to thank Dr. Jeff Kuret (Ohio State University) for supplying the Tau expressing HEK 293 cells and Dr. Mike Hutton (Mayo Clinic, Jacksonville, FL) for generously providing us Tau clones. We thank Dr. Carol Beach for conducting the mass spectrometry analysis at the University of Kentucky Proteomics Core facility, which is supported in part by National Center for Research Resources grant P20RR02017.

Authors' contributions

HG and KMC performed all the experiments. KMC and LBH participated in the experimental design, data analysis and manuscript drafting. All authors read and approved the manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 29 July 2010 Accepted: 5 November 2010

Published: 5 November 2010

References

1. Avila J, Lucas JJ, Perez M, Hernandez F: Role of tau protein in both physiological and pathological conditions. *Physiol Rev* 2004, 84:361-84.

2. Grunke-Iqbal I, Iqbal K, Quinlan M, Tung YC, Zaidi MS, Wisniewski HM. Microtubule-associated protein tau. A component of Alzheimer paired helical filaments. *J Biol Chem* 1986, 261:6084-9.

3. Grunke-Iqbal I, Iqbal K, Tung YC, Quinlan M, Wisniewski HM, Binder LI. Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. *Proc Natl Acad Sci USA* 1986, 83:4013-7.

4. Tolnay M, Pobst A. Tau protein pathology in Alzheimer's disease, and related disorders. *Neuroat & Applied Neurobiol* 1999, 25:171-187.

5. Goedert M. Tau protein and neurodegeneration. *Semin Cell Dev Biol* 2004, 15:45-9.

6. Goedert M, Spillantini MG. A century of Alzheimer's disease. *Science* 2006, 314:777-781.

7. Liu F, Gong CX. Tau exon 10 alternative splicing and tauopathies. *Mol Neurodegener* 2008, 3:B.

8. David DC, Layfield SR, Serpell LI, Narain Y, Goedert M, Spillantini MG. Proteasomal degradation of tau protein. *J Neurochem* 2002, 82:176-85.

9. Gambulin TC, Chin F, Zambrano A, Abraha A, Lagalwar S, Guillotiz AL, Lu M, Fu Y, Garcia-Sierra F, LafiPonte N, Miller R, Berry RW, Binder LI, Cryns VL. Caspase cleavage of tau: linking amyloid and neurofilibrillary tangles in Alzheimer's disease. *Proc Natl Acad Sci USA* 2003, 100:10332-7.

10. Ari A, Guo JP, McGeer PL. Proteolysis of non-phosphorylated and phosphorylated tau by thrombin. *J Biol Chem* 2005, 280:5145-53.

11. Karsten SL, Sang TK, Gehman LT, Chatterjee S, Liu J, Lawless GM, Sengupta S, Berry RW, Pompian J, Oh HS, Schulz C, Hui KS, Wiedau-Pazos M, Vinters HV, Binder LI, Geschwind DH, Jackson GR. A genomic screen for modifiers of tauopathy identifies purumycin-sensitive aminopeptidase as an inhibitor of tau-induced neurodegeneration. *Neuron* 2006, 51:549-60.

12. Sengupta S, Horowitz PM, Karsten SL, Jackson GR, Geschwind DH, Fu Y, Berry RW, Binder LI. Degradation of tau protein by purumycin-sensitive aminopeptidase in vitro. *Biochemistry* 2006, 45:15111-9.

13. Hersh LB. Inhibition of aminopeptidase and acetylcholinesterase by purumycin and purumycin analogs. *J Neurochem* 1981, 36:1594-6.

14. Johnson GD, Hersh LB. Studies on the substrate specificity of the rat brain purumycin-sensitive aminopeptidase. *Arch Biochem Biophys* 1990, 276:305-9.

15. Constam DB, Tobler AR, Rensing-EIN A, Kemler I, Hersh LB, Fontana A. Purumycin-sensitive aminopeptidase. Sequence analysis, expression, and functional characterization. *J Biol Chem* 1995, 270:26931-9.

16. Safavi A, Hersh LB. Degradation of dynorphin-related peptides by the purumycin-sensitive aminopeptidase and aminopeptidase M. *J Neurochem* 1995, 65:389-95.

17. Johnson GC, Seubert P, Cox TM, Motter R, Brown JP, Galasko D. The tau protein in human cerebrospinal fluid in Alzheimer’s disease consists of proteolytically derived fragments. *J Neurochem* 1997, 68:430-3.

18. Weinigarten MD, Lockwood AH, Hvo SY, Kissmeyer MW. A Protein Factor Essential for Microtubule Assembly. *Proc Natl Acad Sci USA* 1975, 72:1858-1862.

19. Tobler AR, Constam DB, Schmitt-Gräff A, Malipiero U, Schlapbach R, Fontana A. Cloning of the human purumycin-sensitive aminopeptidase and evidence for expression in neurons. *J Neurochem* 1997, 68:889-97.

20. Thompson MW, Govindaswami M, Hersh LB. Mutation of active site residues of the purumycin-sensitive aminopeptidase: conversion of the enzyme into a catalytically inactive binding protein. *Arch Biochem Biophys* 2003, 413:236-42.

21. Chow KM, Galh Q, Payne IC, Juliano MA, Juliano L, Isaya G, Hersh LB. Mammalian pitrilysin: substrate specificity and mitochondrial targeting. *Arch Biochemistry* 2009, 48:768-77.

22. Lu P, Rha GB, Melkischki M, Wu G, Addkins BC, Fried MG, Chi Yi. Structural basis of natural promoter recognition by a unique nuclear receptor, HNF-4alpha. *Diabetes gene product.* *J Biol Chem* 2008, 283:33685-97.

23. Guan H, Liu Y, Daily A, police S, Kim MH, Odbb S, Laferla FM, Pauly JR, Murphy MP, Hersh LB. Peripherally expressed neprilysin reduces brain amyloid burden: a novel approach for treating Alzheimer’s disease. *J Neurosci Res* 2009, 87:1462-1473.

24. Bandyopadhyay B, Li G, Yin H, Kuret J. Tau aggregation and toxicity in a cell culture model of tauopathy. *J Biol Chem* 2007, 282:16434-16464.

25. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, 227:680-685.

26. Rasband WS. *ImageJ.* U. S. National Institutes of Health, Bethesda, Maryland, USA. 1997 [http://rspbinfo.ih.gov/l/]

27. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979, 76:4350-4354.

28. Yanagi K, Tanaka T, Kato K, Sadik F, Morihara T, Kudo T, Takeda M. Involvement of purumycin-sensitive aminopeptidase in proteolysis of tau protein in cultured cells, and attenuated proteolysis of
frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) mutant tau. Psychogeriatrics 2009, 9:157-66.

29. Bhutani N, Venkatraman P, Goldberg AL. Puromycin-sensitive aminopeptidase is the major peptidase responsible for digesting polyglutamine sequences released by proteasomes during protein degradation. EMBO J 2007, 26:1385-96.

doi:10.1186/1750-1326-5-48
Cite this article as: Chow et al. Aminopeptidases do not directly degrade tau protein. Molecular Neurodegeneration 2010 5:48.