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Tau protein degradation is catalyzed by the ATP/ubiquitin-independent 20S proteasome under normal cell conditions

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Abstract

Tau is the major protein exhibiting intracellular accumulation in Alzheimer disease. The mechanisms leading to its accumulation are not fully understood. It has been proposed that the proteasome is responsible for degrading tau but, since proteasomal inhibitors block both the ubiquitin-dependent 26S proteasome and the ubiquitin-independent 20S proteasome pathways, it is not clear which of these pathways is involved in tau degradation. Some involvement of the ubiquitin ligase, CHIP in tau degradation has also been postulated during stress. In the current studies, we utilized HT22 cells and tau-transfected E36 cells in order to test the relative importance or possible requirement of the ubiquitin-dependent 26S proteasomal system *versus* the ubiquitin-independent 20S proteasome, in tau degradation. By means of ATP-depletion, ubiquitylation-deficient E36ts20 cells, a 19S proteasomal regulator subunit MSS1-siRNA approaches, and *in vitro* ubiquitylation studies, we were able to demonstrate that ubiquitylation is not required for normal tau degradation.

Keywords

protein degradation; tau protein; proteasome; ubiquitination; lactacystin

Introduction

The tau-protein is abundant in both neurons and astrocytes of the brain, where its normal function is the stabilization of microtubules. In a complex process, orchestrated by numerous kinases and phosphatases, both the quality and quantity of tau phosphorylation are modulated to effect tau binding to microtubules which, in turn, controls microtubule stabilization/destabilization. In several neurodegenerative diseases, such as Alzheimer disease however, for reasons which are not entirely clear the tau protein accumulates in a hyperphosphorylated form.[1]. Such hyperphosphorylated tau accumulates in insoluble aggregates, known as paired helical filaments (PHF's) [2–4].

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Although, the mechanisms leading to this tau accumulation are not yet fully understood, it does seem likely that impaired degradation of the hyperphosphorylated tau protein is involved. Evidence exists, that the activity of the proteasome is downregulated or inhibited in Alzheimer disease [5–7]. It now seems clear that the tau protein is largely degraded by the proteasomal system [8–10] but that hyperphosphorylation of tau diminishes its recognition by the proteasome [11]. Despite these advances, it is not at all clear which form(s) of the proteasome is/are responsible for tau degradation

Various pathways of proteasomal degradation exist, including the classical ATP/ubiquitin-dependent 26S proteasome pathway, and a 20S proteasome pathway not requiring ubiquitin or ATP. The 20S proteasome pathway plays a major role in the degradation of oxidized proteins, as we [12–18] and others [19–21] have demonstrated. There is also mounting evidence that several non-oxidized proteins, including ornithine decarboxylase [22,23], p21/Cip1 [24,25], TCR α [26], I κ B α [27], cJun [28], calmodulin [29] and thymidylate synthase [30] are degraded without ubiquitin. In the case of ornithine decarboxylase [31] and p53 [32] the involvement of the 20S proteasome was clearly shown.

Since tau is an unusual intracellular protein in that it exhibits little or no tertiary structure [33], the question arises whether tau is degraded by an ATP/ubiquitin-dependent proteasomal pathway or an ATP/ubiquitin-independent pathway, or perhaps by both. Interestingly, it was proposed by Hoyet et al. [34] that the tau protein might be degraded *in vivo* by the 20S proteasome without ubiquitinylation, and we have certainly shown that the ATP/ubiquitin-independent 20S proteasome can degrade tau *in vitro* [11]. On the other hand evidence exists that, under certain conditions, the tau protein is poly-ubiquitinated via the CHIP E3-Ligase, forming a complex together with Hsc70/Hsp40 in a phosphorylation dependent manner [8,10]. Shimura et al. [8] concluded that, by ubiquitinating tau, CHIP could rescue phospho-tau-induced cell death. Involvement of the heat shock protein system, however, may suggest that this pathway might be stress-related and not account for normal tau turnover. Furthermore, CHIP is required for protection against environmental stress-induced apoptosis [35]. On the other hand in an earlier publication we demonstrated that, in HT22 neuronal cells, phosphorylated tau is a very poor substrate for degradation [11].

Due to the presence of such (apparently conflicting reports in the literature, where many studies have not focused on the actual mechanism of tau turnover, we decided to test the turnover of tau for proteasome dependency, for ubiquitinylation and ATP dependency, and for involvement of either the 26S proteasome or the 20S proteasome. Finally we tested the possibility of tau ubiquitination in *in vitro* assays.

Materials and Methods

Materials

Tissue culture media and supplements were purchased from Invitrogen or Gibco BRL; sera from Biochrom KG; cell culture materials from PAA. The specific proteasome inhibitor lactacystin was obtained from Sigma. Sepharoses were purchased from Affiniti (S5a-sepharose) and Amersham (GSH-sepharose), and siRNA was from Dharmacon. Routine chemicals were purchased from Sigma, Merck, Calbiochem, Bachem and Amersham. The various antibodies used were obtained from Zymed (anti-tau), DacoCytomation (anti-ubiquitin, anti-tau), Calbiochem (anti-GAPDH), Affiniti (anti-MSS1), Oncogen (anti-GST), and secondary antibodies were from Amersham, or Jackson ImmunoResearch (anti-mouse-FITC-labeled, and anti-mouse-TRITC-labelled antibodies). TG-5 was the kind gift of Dr. P. Davies, Albert Einstein College, NY, USA. The E1-, E2-, CHIP- enzymes, HSP40 and

HSC70 were a kind gift of from Prof. Dr. J. Höhfeld, Institute for Cell Biology, University of Bonn, Germany.

Cell Culture

CH E36/ts20, U87, HT22 cells were maintained in 75 cm² flasks (T75). HT22 cells were grown in Dulbecco's Modified Eagles Medium (DMEM) high glucose, U87 in DMEM low glucose and CH E36/ts20 cells in MEM alpha Medium. All Media were supplemented with 10 % fetal calf serum, 1% penicillin/streptomycin and 1 % glutamine (HT22 and E36/ts20 cells) or 1 % glutamax (U87). Additionally the media of HT22 cells was supplemented with 0.35 % glucose. All cell lines were grown under an atmosphere of 5 % CO₂ at 37°C, except for E36/ts20 cells which were grown at 30.5°C. Cells were sub-cultivated before reaching confluence and media were changed two or three times per week. 24h before experiments commenced, the cells were dissociated and seeded into 75 cm² flasks (T75), for immunocytochemistry, and siRNA transfections in Petri dishes (Ø 30 mm).

Proteasome inhibition was performed by incubating cells with the proteasome inhibitor lactacystin (12 µM) for 20h. Protein synthesis inhibition was achieved by adding cycloheximide (40 µg/ml) to the tissue culture medium for 20h.

Isolation of recombinant tau from bacterial cells

Recombinant tau pETH40 (the tau gene was kindly provided by Prof. E. and E.M. Mandelkow) was expressed in E. coli BLN21 (DE3) pLysS. Bacteria grew overnight at 37°C in LB-Medium. The next day, 0.4 mM IPTG was added for 4h incubation. Cells were then centrifuged and the pellets re-suspended in 1–3 ml PBS containing a protease inhibitor cocktail. For cell lysis, 2 mg/ml lysozyme were added for 30 min on ice. After 30 min boiling, the mixture was centrifuged and recombinant tau was concentrated in Centricon tubes (Amicon, 10 000 MW).

Immunoblot analyses

Cells were lysed by repeated freeze-thawing cycles in lysis buffer (250 mM sucrose, 25 mM HEPES, 10 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 0.4 mM PMSF, pH 7.8), and suspended by pipetting several times. This procedure was followed by centrifugation at 14,000 × g for 10 min at 4°C. Supernatants were used for protein determination by the Bradford assay. Identical amounts of total proteins were boiled in Laemmli loading buffer, separated by electrophoresis (SDS-PAGE according to Laemmli), and transferred onto a PVDF membrane (Amersham). Afterwards immunoblot analysis was performed using the indicated antibodies: anti-Tau (Zymed), anti-ubiquitin (DakoCytomation), anti-GST (Oncogen), TG-5 (Dr. Peter Davies) and anti-E1 (Calbiochem). Anti-mouse or anti-rabbit antibodies were used as secondary antibodies. Gel development was performed using a POD chemiluminescence kit. Pre-stained precision markers (Bio-Rad) were used as molecular weight standards. Optical densities were quantified using an AlphaEaseFC's "FluorChem 8900"-software.

In vitro ubiquitination

GST-ubiquitin fusion protein was used together with the isolated recombinant hTau-protein. The reaction was performed using U87 cell lysate (as a source of the E1-, E2-, and E3-ubiquitination enzymes) in 50 mM Tris-HCl, 5 mM MgCl₂, 1 mM DTT, pH 7.5. Reactions were conducted in the presence of either an ATP-degrading system (hexokinase and deoxyglucose), or an ATP-regenerating system (ATP, phosphoenolpyruvate, pyruvate kinase, and inorganic pyrophosphatase) system. Lactacystin was used to inhibit the proteasome. Ubiquitin-aldehyde was used in some assays to prevent the action of the

ubiquitin-hydrolases. The reaction was performed for 45–120 min at 30°C. For some assays instead of the U87 cell lysate the ubiquitin-enzymes were added separately: E1; E2: UbcH5b, 0.3 μ M; E3; CHIP, 3 or 6 μ M, (all of them were a special kind of gift of Dr. J. Höhfeld). These assays were performed in the presence of 0.3 μ M Hsp40 [36,37] and 3 μ M Hsc70 [8,10], in a buffer containing: 25 mM MOPS, 100 mM KCl, 5 mM ATP, 10 mM DTT, 0.5% PMSF. After the ubiquitination assay either an SDS-PAGE or a separation with GSH- or S5a-sepharose was performed.

Sepharose separation

After ubiquitinylation assays, proteins were incubated with either sepharose coupled to the proteasome subunit S5a (Affiniti, to bind poly-ubiquitin) or with GSH-sepharose (Amersham, to bind GST-ubiquitin) in order to separate ubiquitinated proteins from non-ubiquitinated proteins. These incubations were conducted in standard binding buffer (TBS, 20mM Tris HCl, pH 7.5, 0.15M NaCl, 5% (v/v) glycerol) for several hours at 4.0°C with gentle shaking, and samples were then centrifuged for 2min (4,000 rpm, 4°C). Sepharoses were carefully washed three times with binding-buffer (TBS, 20mM Tris HCl, pH 7.5, 0.15 M NaCl). The bound proteins were detached through boiling with SDS-loading buffer.

Ubiquitin-hydrolase treatment

After performing *in vitro* ubiquitinylation assays and either of the sepharose-separations, a buffer exchange was performed for the poly-ubiquitinated protein fraction (final buffer: 50mM HEPES, pH 7.5, 1mM DTT) using Microcon YM10-tubes (Millipore). For ubiquitin-hydrolase treatment the probes were incubated with 0.19 μ M ubiquitin-hydrolase (Affiniti) for 6h at 37°C, and then analyzed by immunoblotting.

ATP-depletion and measurement

HT22 cells were plated as above. In some experiments, 0.5 mM KCN and 3 mM deoxyglucose were added to the tissue culture medium for 20h to deplete ATP. Cells were harvested, centrifuged and 6% (v/v) perchloric acid was added to precipitate proteins. After protein precipitation, samples were centrifuged (1,200 $\times$ g, 5 min) and immediately neutralized with K₂CO₃. To remove the potassium perchlorate the samples were incubated for 1h at 4°C followed by centrifugation (1,200 $\times$ g, 5 min). The supernatant was stored at –20°C and analyzed for ATP by HPLC according to Grune T. et al, [38].

Transfection of hTau into CH E36/ts20 cells

The tau plasmid (pIJhT40/GFP) (Tau gene provided by Prof. E. and E.M. Mandelkow) was transfected into CH E36/ts20 using TransFectin for 8h (BioRad). One day before transfection, 1×10^6 cells were seeded into 4-well plates. Transfection was performed at 30.5°C, for 8h, and cells were grown overnight at the same temperature. The tau transfection efficiency was monitored by immunoblotting using anti-tau antibody.

siRNA transfection of HT22 cells

An siRNA pool (siGENOMS SMARTpool reagents M-042584-00-0020, mouse PSMC2, NM-011188) was used for the experiments. The transfection optimum and optimal cell concentration was tested employing a siTox according to the standard protocols of Dharmacon. For all following transfections Dharmafect4 (Dharmacon) was used in a concentration of v/v 1:2, and OptiMEM (Invitrogen), 100 μ M siRNA, with 5×10^3 cells per well in 96-well plates (seeded in medium without penicillin/streptomycin). For some experiments Petri dishes ($\varnothing$ 30mm, 10cm²) were used. In these experiments 2 ml of 8.35×10^5 cells/ml were seeded (medium without penicillin/streptomycin). After 5 days, the

transfection procedure was repeated under the same conditions. After 4 more days (altogether 9days), the cells were analyzed.

Immunofluorescence

Tissue culture medium was removed, cells were washed using PBS with 1% FBS, and fixed using a diethylether:ethanol (1:1) mixture for 10min at 4°C. This solution was removed and exhaustive washing with washing buffer was performed (at 4°C) and blocked for 30min at 4°C with PBS with 1% FBS. Cells were incubated, using the first antibody diluted 1:100 in PBS, with 1% FBS for 1h at 4°C, then washed and incubated using the secondary antibody (FITC/TRIC-labelled anti-mouse diluted 1:100 in PBS with 1% FBS) for 1h at 4°C. After an extensive final washing, cells were analyzed by fluorescence microscopy using an 'Olympus BX-60' transmission fluorescence microscope running standard software. Quantification was achieved with AlphaEaseFC's "FluorChem 8900"-software.

In situ proteasome activity

The analysis was performed according to Brégégère et al. [39]. The artificial fluorogenic peptide suc-LLVY-AMC was used. Cells were washed gently with PBS and fixed with 1% paraformaldehyd for 15min at room temperature, washed again, and treated for 15min with 100 µl 25mM Tris-HCl (pH 7.6), 6 mM KCl, 0.01% triton X-100, ATP (4 mM) and suc-LLVY-AMC (50 µM, 2 mM stock solution in DMSO). This solution was incubated for 3h at 37°C. Fluorescence determinations were carried out at an excitation wavelength of 380 nm, and an emission wavelength of 440 nm, using free AMC as a standard. The background fluorescence, without suc-LLVY-MCA, was subtracted. The protein concentration was determined directly after the activity assay. Cells were washed gently with PBS, then 200 µl of Bradford reagent was added and cells were lysed by pipetting. Bradford protein measurements were conducted at 620nm.

Results

Tau turnover is proteasome dependent in HT22-cells

We utilized the HT22 murine neuronal cell line to test the turnover of the tau protein. This cell line endogenously expresses the tau protein as demonstrated in Fig. 1A. Addition of the proteasomal inhibitor lactacystin caused an accumulation of the tau protein. This is in agreement with data published previously by us [7,11] and others [40–42] indicating that tau turnover is proteasome-dependent. The efficiency of proteasome inhibition was monitored by the accumulation of polyubiquitinated proteasomal substrates (Fig. 1A). However, since lactacystin inhibits all proteasomal forms and our major goal was to discriminate between the ATP/ubiquitin-dependent and –independent proteasomal pathways, we also performed ATP-depletion experiments in HT22 cells.

ATP-depletion does not lead to tau accumulation

ATP-depletion was performed as described in Materials & Methods, using KCN and deoxyglucose [43]. This method blocks both mitochondrial ATP synthesis (by preventing respiration), and glycolysis. As judged by HPLC, the intracellular ATP level declined, resulting in a low intracellular ATP/ADP-ratio (Fig. 1B). Despite this partial energy depletion, however, there was no resultant accumulation of the tau protein (Fig. 1B). In fact, the intracellular tau protein level actually continued to decline (by some 80%) as ATP levels dropped. Furthermore, the concentration of GAPDH, a cytosolic enzyme degraded by the 26S proteasome is not declining. Importantly, partial ATP depletion did not cause a loss of the 19S proteasomal regulator, as judged by antibody studies of the MSS1 subunit, and the

efficiency of the ATP depletion is clearly visible by the almost 60% loss of polyubiquitinated proteins (Fig 1B), due to the fact that ubiquitinylation requires ATP.

Unfortunately, the ATP-depletion approach inhibits numerous cellular processes including, protein synthesis, ATP-dependent proteasomal degradation, and ubiquitinylation. Since it is unknown at what precise cytosolic ATP levels each of these processes is affected, we next explored a ubiquitin-activating enzyme E1 temperature-sensitive cell line.

Tau-degradation is not ubiquitin-dependent in ts20 cells

We explored the CH E36/ts20 cell line which harbors a temperature-sensitive mutation in the E1 ubiquitin-activating-enzyme – a key enzyme of the ubiquitination pathway. It was previously demonstrated, that a temperature shift to 39.5°C leads to a decline of E1 levels in these cells and, therefore, to a non-functioning ubiquitinylation system [44]. As shown in Fig. 2A, incubation at 39.5°C resulted in an almost complete loss of E1 ubiquitin activating enzyme, only in the ts20 mutants. Importantly, as previously reported [44], this loss of E1 also resulted in loss of ubiquitinylation (confirmatory data not shown). Our goal was to study tau turnover in these cells but, CH E36 Chinese hamster fibroblasts do not express the tau protein in detectable amounts. Therefore, we transfected both CH E36 parent cells and E36ts20 mutants with the hTau40-protein and then transferred all the cells to the restrictive (39.5°C) temperature. To stop the synthesis of new tau protein we blocked all protein synthesis with cycloheximide. As demonstrated in Fig. 2B, tau transfection into CH E36 and E36ts20 was successful with different efficiencies. However, after adding cycloheximide, it is clear that tau levels declined in both cell lines, irrespective of the presence or absence of an efficient ubiquitinylation system (Fig. 2B). These results indicate that tau might be degraded by an ubiquitin-independent pathway. However, as treatment with lactacystin reveals the ubiquitin-dependent pathway is proteasome dependent. We next focused our attention on the possible involvement of other components of the ubiquitin/proteasome system in tau degradation (such as the 19S proteasome) regulator, and on testing the relative importance of the 20S proteasome versus the 26S proteasome.

MSS1 ‘knock-down’(with siRNA) does not affect tau turnover in HT22-cells

In order to test for a possible requirement of the 19S regulator of the 26S proteasome for the degradation of tau protein, we tested the effects of siRNA to the MSS1 subunit (one of the ATPases) of the 19S regulator base. After development of an siRNA transfection strategy, we determined the MSS1 content by immunofluorescence microscopy (Fig. 3A) and by immunoblotting (Fig. 3B). A dramatic decline of the MSS1 subunit expression took place following siRNA treatment, such that the signal intensity was suppressed by approximately 50%. This decline in MSS1 content was accompanied by more than a 90% decline in the in the ATP-stimulable degradation of the fluorogenic peptide suc-LLVY-MCA, a widely used substrate of the chymotrypsin-like proteasomal activity (Fig. 3C). In the MSS1-siRNA treated cells, a dramatic accumulation of poly-ubiquitinated proteins took place, as judged by immunofluorescence using an anti-ubiquitin antibody (Fig. 3A). In addition to that also proteins accumulate which are degraded by the ubiquitin-26S proteasome pathway as judged by the accumulation of GAPDH (Fig. 3A). An increase in polyubiquitinated proteins could be detected by immunoblotting. Taken together, these results indicate that the treatment of HT22 cells with MSS1-siRNA produced a malfunctioning 26S proteasome. When we tested the levels of tau under these conditions, we failed to observe any accumulation of the tau protein, either by immunofluorescence (Fig. 3A) or by immunoblotting (Fig. 3B). The results of Fig. 3 suggest that the 26S proteasome, which is required for degradation of ubiquitinated substrates, is not required for tau degradation.

No detectable tau ubiquitinylation *in vitro*

The results of Figs. 1 – 3 demonstrate that the tau protein is degraded in HT22 and E36ts20 cells by some form of the proteasome, even if the 26S proteasome or the ubiquitinylation system is not working, or if the ATP level is too low to support ubiquitin-dependent proteolysis. One simple interpretation of these results is that tau might be degraded by the 20S proteasome, which requires neither ATP nor a ubiquitinylation system for proteolysis. Although this interpretation fits our hypothesis, it was certainly possible that the 20S proteasome takes-over tau degradation only under conditions of a failure of the 26S-proteasome-ubiquitin-system, and turnover of the tau protein normally involves poly-ubiquitinylation and 26S proteasome-dependent degradation. If true, this would mean that the tau ubiquitinylation machinery is present in tau expressing cells and should be able to poly-ubiquitinylate tau. In order to test this possibility we decided to analyze in *in vitro* assays for the presence of tau ubiquitination systems.

In a first approach we examined the possibility of tau ubiquitinylation in a simple ubiquitinylation assay, in which we first tested the efficiency of ubiquitinylation using anti-ubiquitin- and anti-GST-antibodies. For these experiments, we utilized cell lysates of U87 cells, a human astrocytoma cell line, which expresses the tau protein endogenously. As demonstrated in Fig. 4A the *in vitro* ubiquitinylation assay resulted in enhanced formation of multiple poly-ubiquitinated proteins, but there was no detectable formation of poly-ubiquitinylated tau protein (Fig. 4A). Even longer periods of incubation time (up to 120 min) for the *in vitro* ubiquitinylation assay did not lead to any formation of poly-ubiquitinylated-tau conjugates.

Of course, it was important to test whether our *in vitro* ubiquitinylation system was really working effectively. Therefore, we utilized a protein that is well-known to be ubiquitinylated: glyceraldehyde 3-phosphate dehydrogenase (GAPDH). In Fig. 4B the ubiquitinylation of GAPDH in our *in vitro* ubiquitinylation assay is shown. It demonstrates an active ubiquitinylation in the presence of ATP, accompanied by an accumulation of ubiquitinylated proteins.

Since it was previously shown by Shimura et al, [8] and Petrucelli et al. [10], that tau might be polyubiquitinylated by the CHIP (E3) ubiquitin ligase in the presence of HSP70 proteins and the ubiquitin conjugating enzyme Ubc5b (E2), we tested the same system for the human tau40 protein and the human tau352 protein. Petrucelli et al. [8] suggested that Hsp40 is unlikely to be necessary for the reduction of steady-state tau levels and but others [36,37] showed an enhancement of CHIP ubiquitinylation activities in the presence of HSP40, so we added HSP40 in our assays. Despite extensive testing, we failed to find any evidence of the formation of poly-ubiquitinated tau with these enzymes and proteins (Fig. 4C) even though, in positive control experiments, other proteins could be ubiquitinylated by the system (data not shown).

Concentration of poly-ubiquitin conjugates after *in vitro* ubiquitinylation

One possible reason for our failure to detect poly-ubiquitinylated-tau conjugates might be the sensitivity of detection. Therefore, we decided to concentrate proteins in the reaction mixtures to increase the likelihood of detecting any poly-ubiquitinylated conjugates that might be formed. To concentrate and separate potential poly-ubiquitinylated protein conjugates, we repeated the *in vitro* ubiquitination assay of Fig. 4B, followed by a sepharose-mediated separation and concentration. We tested both S5a- (Fig. 5A) and GSH-sepharose (data not shown) for this procedure. As demonstrated in Fig. 5 we were unable to detect any poly-ubiquitinated tau protein in the bound fraction of either of the separations,

although a clear concentration of other poly-ubiquitinated proteins took place, as evidenced by anti-ubiquitin antibody.

Ubiquitin hydrolase treatment does not reveal *in vitro* ubiquitinylation of tau

The results of Fig. 5A suggest that lack of sufficient sensitivity of antibody detection is not the reason for our failure to detect a tau poly-ubiquitinylation. As a last possibility, we wondered if the antibodies used were unable to detect poly-ubiquitinylated tau.

To test whether our antibodies were unable to recognize poly-ubiquitinylated tau protein, we performed the same experimental set-up of Figs. 4B and 5, including *in vitro* ubiquitinylation, separation and concentration of tau, but followed these measures by treatment with ubiquitin hydrolase. Fig. 5B shows immunoblots of the S5a-sepharose-bound fractions, with, and without, ubiquitin hydrolase treatment. No antibody reactivity was detectable after the whole procedure (Fig. 5B). To test the effect of ubiquitin hydrolase treatment we determined the amount of ubiquitinylated proteins. As demonstrated in Fig. 5B (right panel) the bulk of poly-ubiquitination conjugates were degraded by ubiquitin hydrolase treatment. In the next step we performed the same experiments using the conformation-specific Tau antibody, TG-5. Also in this case we were unable to detect any tau reactivity among the S5a-sepharose bound fraction (Fig. 5C).

From our *in vitro* experiments we conclude that tau is not regularly ubiquitinylated under normal conditions. From both our *in vitro* and cellular experiments we conclude that the tau protein is normally degraded in an ubiquitin-, ATP- and 26S-proteasome-independent manner.

Discussion

The discussion about which proteins are degraded by ATP/ubiquitin-**dependent** proteasomal pathways *versus* ATP/ubiquitin-**independent** proteasomal pathways has gone on for many years. We [45–49] and Rivett [50,51] proposed as early as 1985 that oxidatively modified proteins could be degraded in mammalian cells by an ATP-independent pathway. Starting in 1995, we published a series of papers showing that the 20S proteasome recognizes and degrades the partially unfolded forms of proteins that have been partially denatured by oxidation, in an ATP and ubiquitin-independent mechanism [12–18]. Other researchers have demonstrated that, although the bulk of 26S proteasomal proteolysis requires ATP, ubiquitin, and multiple enzymes involved in ubiquitin activation/elongation/ligation, certain proteins can be degraded by either the 20S proteasome or the 26S proteasome with no requirement for either ATP or ubiquitin. For example, Hoyt et al. [34] reviewed a number of proteins which are degraded in an ubiquitin-independent manner, including ornithine decarboxylase [22,23], p21/Cip1 [24,25], TCR α [26], I κ B α [27], cJun [28], calmodulin [29] and thymidylate synthase [30].

The most probable reason that the proteasome may ‘recognize’ certain protein substrates that are not bound to polyubiquitin is because it binds to their unfolded, or partially unfolded structures: for example, p21/Cip1 which is not completely folded [52]. We felt that the same reasoning should also apply for the tau protein, which is also essentially unfolded [33]. The importance of a partially unfolded state on the degradation of protein substrates by the 20S proteasome was previously demonstrated for oxidized proteins [53].

Recently more examples of ubiquitin-independent proteasome-mediated degradation have been reported, including the retinoblastoma protein (Rb) [54] and p53 [55,56]. The ubiquitin-independent degradation of Rb is promoted by MDM2. Interestingly MDM2 is responsible for ubiquitinylation of p53. Another E3-Ligase, CHIP, was found to be responsible

for ubiquitinylation of p53, by forming a complex with the chaperones Hsp40 and Hsc70/Hsp70 [56]. CHIP was also postulated to be involved in the ubiquitinylation of tau [8,10]. On the other hand it was shown that p53 can be degraded in an ubiquitin-independent manner by the 20S proteasome [31,32]. It seems likely (to us) that CHIP may ubiquitinylate proteins such as tau, only (or mostly) under certain stress conditions. This would fit very well with our data, since we failed to show any ubiquitinylation of tau under normal conditions, whereas Shimura et al. [8] and Petrucelli et al. [10] demonstrated ubiquitinylation of tau during stress.

It seems clear that some proteins can be degraded either by an ubiquitin-dependent or an ubiquitin-independent pathway, depending on the cellular conditions and the state of the protein. A major question for future studies will be quantifying the participation of each pathway, and the conditions that may alter or regulate the prominence of each pathway.

Interestingly, tau aggregates under in vivo conditions as found in Alzheimers disease are heavily ubiquitinated [57]. This might have two reasons: (i) that there is a ubiquitination of the already aggregated protein or (ii) that under in vivo conditions additional factors might lead to an tau ubiquitination.

Our current results indicate that the normal turnover of the tau protein is catalyzed by the proteasome in an ATP/ubiquitin-independent manner. Our studies of cellular ATP-depletion and (MSS1) 19S regulator knock-down further suggest that the 20S proteasome is more important for normal tau turnover than is the 26S proteasome. This interpretation also seems reasonable, since the tau protein is largely unfolded and, therefore, should not require ATP for unfolding prior to degradation. Under other conditions, including certain stress situations, where CHIP regulates protein quality control and transcriptional activation of stress response signaling [35], the ATP/ubiquitin pathway and the 26S proteasome may be more important.

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Abbreviations

CLMS	confocal laser scanning microscopy
NFTs	neurofibrillary tangles
PHFs	paired helical filaments
tau-GFP	tau-green fluorescent protein fusion protein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase

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Fig. 1. Tau-degradation in HT22 cells is proteasome, but not ATP-dependent

HT22 cells were seeded one day before experiments as described in 'Materials & Methods'. Cells were incubated in the presence or absence of the proteasome inhibitor, lactacystin (12 μ M) for 20h (**Panel A**). Cells were then lysed and analyzed by immunoblotting using anti-tau and anti-ubiquitin antibodies. One representative out of three experiments is shown. Quantification was performed by using Alpha Ease FC's "FluorChem 8900"-software. **Panel B** shows the effect of partial cellular ATP-depletion by KCN and deoxyglucose addition (0.5 mM KCN; 3mM deoxyglucose) on tau levels. The ATP/ADP ratio was calculated from a nucleotide analysis by ion-pair reversed phase HPLC. At the same time point, the content of the 19S proteasomal regulator subunit MSS1, of the tau protein, of GAPDH, and of polyubiquitin conjugates was determined using the corresponding antibodies after immunoblotting. The amounts of human tau and ubiquitin were quantified using AlphaEaseFC's "FluorChem 8900"-software. Representative results of four independent experiments are shown.

A. Ubiquitinylation enzyme E1 is temperature-sensitive in ts20 cells

B. Tau degradation does not require intact ubiquitinylation

Fig. 2. Tau degradation does not require ubiquitin in transfected ts20 fibroblasts

Human tau40 was transfected into CH E36 and ts20 fibroblasts as described in 'Materials & Methods.' The cells were grown at 30.5°C or 39.5°C (restrictive temperature) over night.

The content of the E1-enzyme was measured by immunoblot analyses (**Panel A**).

Cycloheximide ('CHX') was added at $40 \mu\text{g} \times \text{ml}^{-1}$ and cells were incubated for another 20h.

For some experiments cells were incubated in the presence of the proteasome inhibitor, lactacystin (12 μM) for 20h. The tau content was determined by immunoblotting (**Panel B**).

Tau was quantified using AlphaEaseFC's "FluorChem 8900"-software. Representative results of four independent experiments are shown.

A. MSS1-siRNA does not increase tau accumulation: Fluorescence microscopy

B. MSS1-siRNA does not increase tau accumulation: immunoblotting

C. ATP-stimulated Proteasome activity after MSS1-siRNA

Fig. 3. Tau degradation does not require the complete 26S proteasome: Effects of MSS1-siRNA
HT22 cells were either treated, or not treated, with siRNA directed towards the MSS1 subunit of the 19S regulator: a constitutive component of the 26S proteasome. The exact treatment is extensively described in 'Materials & Methods.' **Panel A** demonstrates the content of MSS1, tau, ubiquitin and GAPDH analyzed by immunofluorescence studies. In parallel a transmitted light and the corresponding immunostained microscopic image are shown. The staining intensity was determined using AlphaEaseFC's "FluorChem 8900"-software (see right portion of panel A). After the same treatment cells were lysed and analyzed by immunoblotting using anti-MSS1, anti-tau and anti-ubiquitin antibodies (**Panel B**). Some of the cells were used to determine proteasomal activity. In **Panel C** the 26S proteasomal activity is shown calculated from the difference of the suc-LLVY-MCA degradation in the presence minus the suc-LLVY-MCA degradation in the absence of ATP. The data presented are Means $\pm$ S.E., n=4.

Recombinant human tau40 was prepared and isolated as described in 'Materials & Methods.' Ubiquitinylation assays were also performed as described in 'Materials & Methods,' using a GST-ubiquitin fusion protein as described previously [11,44]. In order to test the electrophoretic and immunoblotting procedure we used recombinant human tau40 as a loading control. **Panel A** demonstrates the immunoblots of ubiquitinylation assays using lysates of human U87 cells and monitoring either anti-GST-, anti-ubiquitin- or anti-tau-antibodies. In **Panel B** the same ubiquitinylation assay was performed, with the exception that GAPDH was used as an ubiquitinylation substrate. Consequently an anti-GAPDH-antibody and an anti-ubiquitin antibody were used for detection. **Panel C** demonstrates immunoblots of the same ubiquitinylation assay with the exception that the ubiquitin activating enzyme E1, the E2 enzyme UbcH5b, the E3-ligase CHIP, and HSC70/HSP40 were used. Both hT40 and htau352 were tested. Analysis was by immunoblotting with anti-GST, anti-tau and anti-TG-5 antibodies. Representative results of four independent experiments are shown.

Fig. 5. Concentration of ubiquitin-conjugates after *in vitro* ubiquitinylation

The cell lysate mediated ubiquitinylation assay was performed as described in Fig. 4. The resulting protein mix was separated by a S5a-sepharose- (**Panel A**). The reaction mix of the ubiquitinylation assay was divided into polyubiquitinated proteins (bound fractions) and non-ubiquitinated proteins (unbound fractions). Analysis was performed by immunoblotting using anti-tau and anti-ubiquitin antibodies (**Panel A**). In further studies the sepharose bound fractions (polyubiquitinated proteins) were either exposed, or not exposed, to ubiquitin hydrolase (0.19 μ M, for 6h, in 50mM HEPES plus 1mM DTT, at pH 7.5.). The resulting protein mixture was analyzed with anti-tau or anti-ubiquitin antibodies (**Panel B**) or with anti-TG-5 antibody (**Panel C**). Isolated hT40 was again used as a loading control to test immunoblot efficiency. An ATP-degrading system, consisting of hexokinase and deoxyglucose; or an ATP-regenerating system, with ATP, phosphoenolpyruvate, pyruvate kinase, and inorganic pyrophosphatase, was used in the ubiquitinylation assay. Representative results of four independent experiments are shown.