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Proteasomal degradation of tau protein

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Abstract

Filamentous inclusions composed of the microtubule-associated protein tau are a defining characteristic of a large number of neurodegenerative diseases. Here we show that tau degradation in stably transfected and non-transfected SH-SY5Y cells is blocked by the irreversible proteasome inhibitor lactacystin. Further, we find that *in vitro*, natively unfolded tau can be directly processed by the 20S proteasome without a requirement for ubiquitylation, and that a highly reproducible pattern of degradation intermediates is readily detectable during this process. Analysis of these intermediates shows that 20S proteasomal processing of tau is bi-directional, proceeding from both N- and C-termini, and that populations of relatively stable intermediates arise probably because of less efficient digestion of the C-terminal repeat region. Our results are consistent with an *in vivo* role for the proteasome in tau degradation and support the existence of ubiquitin-independent pathways for the proteasomal degradation of unfolded proteins.

Keywords: Alzheimer's disease, paired helical filament, proteasome, tau, ubiquitin.

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Alzheimer's disease (AD) is characterized by two major neuropathological hallmarks, the extracellular deposits of the amyloid beta (Aβ) peptide (Glenner and Wong 1984) and the intraneuronal inclusions of fibrillar hyperphosphorylated tau protein in the form of straight and paired helical filaments (SFs and PHFs; Goedert 1993). Tau pathology also characterizes a large group of other neurodegenerative disorders collectively known as the tauopathies (Goedert *et al*. 1998). Tau is a microtubule-binding protein that has limited ordered structure in solution (Schweers *et al*. 1994) and thus belongs to the family of 'natively unfolded' proteins. In the adult human brain, six tau isoforms are expressed, generated by alternative mRNA splicing from a single gene (Goedert *et al*. 1989). The finding that mutations in the tau gene cause frontotemporal dementia shows that dysfunction of tau protein can directly lead to neurodegeneration (reviewed in Lee *et al*. 2001).

Active protein degradation systems are necessary to avoid the accumulation of misfolded or damaged proteins, as well as to maintain the rapid turnover of short-lived proteins. One such extra-lysosomal system utilizes proteasomes, multisubunit protease complexes, to catalyse the selective degradation of proteins (reviewed in Voges *et al*. 1999). Proteasomes contain a catalytic 20S core particle, comprised of four-stacked heptameric rings containing protease active sites with trypsin-like, chymotrypsin-like and peptidyl-glutamyl-hydrolysing activities (Orlowski 1990). This 20S core can be complexed at each end with different regulators; association with two 19S 'caps' results in the formation of a 26S proteasome particle, or with two 11S regulators gives rise to an 'immunoproteasome'. Proteasomes cleave their substrates in a highly processive manner to short peptides, with mean lengths between six and 10 amino acids (Kisselev *et al*. 1998, 1999), which can then be further processed to free amino acids by other proteases. As the substrate is thought not to dissociate from the enzyme during this process, degradation intermediates are generally not detected (Akopian *et al*. 1997; Kisselev *et al*. 1998). To date, the only

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Abbreviations used: Aβ, amyloid beta; AD, Alzheimer's disease; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PHF, paired helical filament; SDS, sodium dodecyl sulphate; SF, straight filament.

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notable exception to this is the NFκB precursor p105, which undergoes limited proteasomal proteolysis, giving rise to the p50 protein (Palombella *et al*. 1994).

The best characterized role for proteasomes is in ubiquitin-dependent proteolysis. The post-translational conjugation of isopeptide-linked chains of multiple copies of the ubiquitin protein to a cellular target (ubiquitylation) signals degradation by the 26S proteasome (reviewed in Pickart 2000). Subunits of the 19S regulator confer substrate recognition through the binding of poly-ubiquitin chains containing at least four ubiquitins (Thrower *et al*. 2000), and subsequent substrate unfolding prior to entry into the narrow axial passageway of the 20S core is catalysed by ATPase subunits of the 19S regulator which possess chaperone activity (Navon and Goldberg 2001). The observation that mutations in enzymes involved in the ubiquitin-dependent proteasomal degradative pathway cause some familial neurodegenerative diseases (Leroy *et al*. 1998; Saigoh *et al*. 1999; Shimura *et al*. 2000) strongly suggests that altered function of this pathway could be one of the mechanisms leading to neurodegeneration (reviewed in Layfield *et al*. 2001). However, there is also increasing evidence for the participation of proteasomes in parallel pathways of ubiquitin-independent proteolysis *in vivo*, with the 26S particle being able to degrade some substrates without prior ubiquitylation (Kisselev *et al*. 1999; Benaroudj *et al*. 2001), as can the 20S core alone (Kisselev *et al*. 1999; Nunan *et al*. 2001; Tofaris *et al*. 2001; Touitou *et al*. 2001).

The protein degradation systems which are responsible for tau metabolism *in vivo* are not well characterized, although tau has previously been shown to be processed by various proteases *in vitro,* including caspases (Canu *et al*. 1998), calpains (Yang and Ksiezak-Reding 1995), cathepsins (Kenessey *et al*. 1997) and thrombin (Olesen 1994). Some PHFs contain ubiquitylated N-terminally truncated tau (Morishima-Kawashima *et al*. 1993); however, a role for the 26S proteasome in tau degradation has not been demonstrated. The increasing number of unstructured or relatively unfolded proteins found to be directly degraded by proteasomes in a ubiquitin-independent manner (Kisselev *et al*. 1998, 1999; Benaroudj *et al*. 2001; Tofaris *et al*. 2001; Touitou *et al*. 2001) suggests that tau could also be processed in a similar way. Here we show that lactacystin, a specific inhibitor of the 20S proteasome catalytic core, inhibits the degradation of tau in stably transfected and non-transfected SH-SY5Y cells. Additionally, we report that unfolded recombinant tau can be directly degraded by the 20S proteasome *in vitro* in an ubiquitin-independent bi-directional manner, and that the formation of stable intermediates can occur during this degradation process. We also show that conformational changes in tau protein induced by sodium dodecyl sulphate (SDS) are sufficient to prevent its degradation by the 20S proteasome *in vitro*. Our results are consistent with an *in vivo* role for proteasomes in tau degradation, via a pathway that does not have an absolute requirement for ubiquitylation.

Materials and methods

Recombinant tau proteins

Two human tau isoforms, tau43 and tau46, were used for this study. They correspond to the 383 and 412 amino acid isoforms of human brain tau. Recombinant tau43 and tau46 proteins were expressed and purified as described (Goedert and Jakes 1990).

Tau degradation and proteasome inhibition in cells

Human *tau43* cDNA was subcloned into the *Eco*RI site of pcDNA3 + (Invitrogen, Groningen, the Netherlands) and the resulting construct was verified by sequencing. Human dopaminergic neuroblastoma SH-SY5Y cells were grown at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Paisley, UK) containing 1% (v/v) penicillin/streptomycin and 10% (v/v) fetal calf serum (FCS). For transfection, cells were grown in 6-well plates to 50% confluency and treated with a mixture of 10 μL of Lipofectin Reagent (Life Technologies) containing 2 μg of plasmid DNA in serum-free medium. After 4 h, cells were returned to culture medium with serum and 48 h after transfection G-418 (300 μg/mL; Life Technologies) was added for selection of stably transfected cells. For proteasome inhibition, stably transfected or non-transfected cells were grown to 60–80% confluency in 5-cm diameter dishes and incubated in fresh medium containing 10% (v/v) FCS supplemented with 50 μg/mL cycloheximide [5 mg/mL sterile stock solution in phosphate-buffered saline (PBS), Sigma-Aldrich, Poole, UK] and 10 μM lactacystin (Affiniti Research Products, Exeter, UK; controls received cycloheximide but no lactacystin). Cells were lysed at the desired time points in 70 μL of RIPA buffer [50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.25% (w/v) sodium deoxycholate, 0.1% (v/v) Nonidet P-40, 100 μM sodium orthovanadate, 1 mM sodium fluoride], supplemented with 0.2% (v/v) mammalian protease inhibitor cocktail (Sigma-Aldrich) and centrifuged for 7 min at 13 400 *g* to remove cellular debris. Protein concentrations in the supernatant were measured using the BCA Protein Assay Kit (Pierce, Cheshire, UK) and equal amounts of protein were analysed by western blotting. Samples were resolved by SDS polyacrylamide gel electrophoresis (PAGE) using 5–20% acrylamide gradient gels and proteins were transferred onto nitrocellulose membrane (Hybond C-Super, Amersham Pharmacia Biotech, Amersham, UK). Blots were blocked in 4% (w/v) milk powder in TBS (10 mM Tris–HCl, 150 mM NaCl, pH 7.5) and probed with the 5A6 anti-human tau monoclonal (Developmental Studies Hybridoma Bank, University of Iowa, IA, USA) for 1 h at room temperature or overnight at 4°C (1 : 7000 dilution for transfected or 1 : 2000 for non-transfected cells). Blots were developed with peroxidase-conjugated rabbit anti-mouse serum (Dako Ltd, Ely, UK) at 1 : 3000 for transfected or 1 : 2000 for non-transfected cells, and visualized by enhanced chemiluminescence (Renaissance ECL reagent, NEN Life Science Products, Boston, MA, USA).

In vitro 20S proteasomal degradation of tau

A specific *in vitro* degradation assay was established using purified recombinant human tau isoforms, at physiological pH, under conditions where aggregation does not occur, and a highly purified

commercial preparation of 20S proteasomes (from human erythrocytes). This proteasome preparation was shown by silver-staining and also by western blotting with well characterized antibodies against 19S components to contain only 20S subunits (data not shown). Only minor variabilities in the activities of different batches of 20S proteasomes, as judged by different overall rates of tau degradation, were noted. For degradation, recombinant tau was diluted to a final concentration of 275 nM in 100 μL of reaction mixture containing 50 mM Tris–HCl, pH 7.5, 0.02% (v/v) Tween-20, 14 nM purified human erythrocyte 20S proteasome (Affiniti Research Products). When required, proteasome activity was inhibited by the addition of 10 μM lactacystin. Parallel 20S proteasome degradation assays were performed using tau43 in the presence of 0.01% (w/v) SDS, a treatment known to activate 20S activity *in vitro* (Coux *et al*. 1996). Reactions were incubated at 37°C and at the desired time points, 10 μL of the mixture was removed and immediately mixed with an equal volume of SDS–PAGE sample buffer to stop the reaction. Tau degradation was revealed by western blotting as described above. Blots were probed with a panel of anti-human tau antibodies (see Fig. 1): TAU-5 at 1 : 2000 (Biosource International, Camarillo, CA, USA); 5A6 at 1 : 7000; BR134 at 1 : 3000, and developed for 1 h at room temperature with the appropriate peroxidase-conjugated secondary antibody: rabbit anti-mouse at 1 : 2000 for TAU-5 or at 1 : 3000 for 5A6 and swine anti-rabbit (Dako Ltd) at 1 : 2000 for BR134. If required, blots were stripped (Restore Western Blot Stripping Buffer, Pierce) for 30 min at 37°C and re-probed. As a negative control for 20S proteasomal degradation, human α-lactalbumin (275 nM, Sigma-Aldrich), which has a folded structure, was used instead of tau. The assay was conducted under identical conditions with 14 nM 20S proteasome and samples were separated by SDS–PAGE. Western blotting was carried out essentially as above, except that the blot was blocked in 0.1% (w/v) bovine serum albumin, probed with anti-human α-lactalbumin serum (1 : 10000, Sigma-Aldrich), and revealed with peroxidase-conjugated swine anti-rabbit serum (1 : 10000).

Circular dichroism

Circular dichroism spectra from tau43 (0.1 mg/mL) in water with and without SDS (3 mM, tau : SDS molar ratio equivalent to that used in 20S proteasome degradation assay with SDS) were taken using a Jasco J-720 spectrapolarimeter using a 0.02-cm pathlength quartz cuvette. The data were collected from 185 nm to 250 nm and accumulated over 20 runs, the presented data being the average.

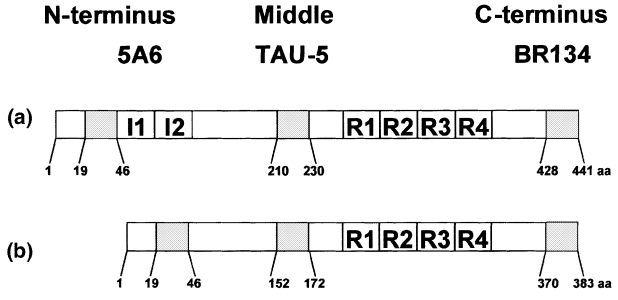

Fig. 1 Recognition sites of the different tau antibodies used. Epitopes (shaded) are shown relative to (a) the longest tau isoform containing two N-terminal inserts (I1 and I2) and four microtubule binding repeat motifs (R1–R4) and (b) the tau43 isoform, lacking I1 and I2 but containing R1-R4.

Results

Proteasomal degradation of tau43 in SH-SY5Y cells

To determine if proteasomes contribute to tau degradation in cells, stably tau-transfected SH-SY5Y human dopaminergic neuroblastoma cells were produced. These cells expressed high levels of tau43 compared to non-transfected controls, as assessed by western blotting (data not shown). *De novo* protein synthesis in the transfected cells was blocked with cycloheximide, and degradation of residual tau43 in the absence or presence of 10 μM lactacystin, a specific inhibitor of the 20S proteasome catalytic core (Dick *et al*. 1997), was monitored by western blotting with the 5A6 anti-human tau antibody. The experiment was replicated on three separate occasions and a representative example is shown (Fig. 2). An obvious decrease in full-length tau43 immunoreactivity from cells not treated with lactacystin was noted, compared to no decrease in tau43 from cells with lactacystin. The observation that lactacystin was able to completely stabilize tau levels suggests that in this cellular model the proteasomal pathway of degradation is a major contributor to tau turnover. No lower molecular weight tau degradation intermediates were detected at any of the time points (data not shown). Although accurate measurements could not be made using this semiquantitative method, densitometric analyses of western blots allowed estimations that the half-life of transfected tau in this cellular model was of the order of 12 h.

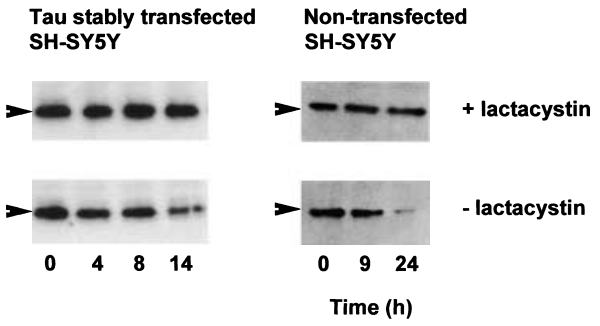

Fig. 2 Proteasome-dependent degradation of tau in stably (tau43) transfected and non-transfected SH-SY5Y cells. Protein synthesis was inhibited with cycloheximide (50 μg/mL) at time zero then cells were grown for the indicated times in the presence or absence of lactacystin (10 μM). The full-length tau protein remaining at each time point (arrowed) was detected by western blotting with the 5A6 antibody (37 μg of transfected or 30 μg non-transfected cell lysate per. lane). Tau was degraded with a half-life of approximately 12–14 h and degradation was completely inhibited with lactacystin treatment.

To exclude the possibility that tau overexpression itself may activate the proteasome system, we performed identical analyses in non-transfected cells which express low levels of fetal tau (the shortest tau isoform). Again, a decrease in full-length tau protein immunoreactivity from cells not treated with lactacystin was seen, compared to no decrease in tau protein from the cells with lactacystin (Fig. 2). Lower molecular weight tau degradation intermediates were not detected, and the estimated half-life of endogenous tau was similar to that of transfected tau (approximately 14 h).

In vitro ubiquitin-independent degradation of unfolded tau by the 20S proteasome

To extend our observation of proteasome-dependent tau degradation in SH-SY5Y cells, we sought to reconstitute proteasomal degradation of tau *in vitro*. Because tau is natively unfolded when not bound to microtubules, we hypothesized that it may bypass the cellular ubiquitylation machinery and recognition elements of the 19S regulator complex, and be directly degraded by the 20S proteasome core. Accordingly, we found that purified recombinant tau43 could be directly and rapidly degraded by the 20S proteasome *in vitro* (Fig. 3a). Surprisingly, the disappearance of full-length tau43 was accompanied by the time-dependent appearance of a series of lower molecular weight degradation products, initially detected by the TAU-5 antibody raised against the middle region of the protein (see Fig. 1). Upon longer incubation (> 2 h), all tau43-immunoreactive bands disappeared, suggesting that the protein could be completely degraded (data not shown). *In vitro* degradation of tau43 was significantly inhibited by the addition of lactacystin (10 μM; Fig. 3b). Under these conditions, some limited tau43 degradation still occurred which was accompanied by the appearance of a range of degradation products (over 25 kDa in size) with time, probably because lactacystin is directed only against the chymotrypsin- and trypsin-like activities of the 20S core. Additionally, prolonged incubation of tau protein alone did not give rise to any degradation products, confirming the absence of contaminating protease activity in the purified tau sample (data not shown). The specificity of the 20S proteasome in the degradation of unfolded tau was demonstrated using folded α-lactalbumin as a negative control. In identical *in vitro* assays run in parallel with those for tau43 and at the same molar ratio of substrate : enzyme, no significant degradation of α-lactalbumin by the 20S proteasome was detected by western blotting (Fig. 3c).

Whilst supplementing the tau degradation assays with concentrations of SDS routinely used to activate the 20S proteasome *in vitro* (0.01% w/v), we noted that this treatment strongly inhibited degradation of tau43, even more potently than using 10 μM lactacystin (Figs 4a–c). Fluorogenic peptide cleavage assays confirmed that SDS treatment did not impair 20S proteasome catalytic activity, and in fact resulted in an approximately 20% increase in chymotryptic activity

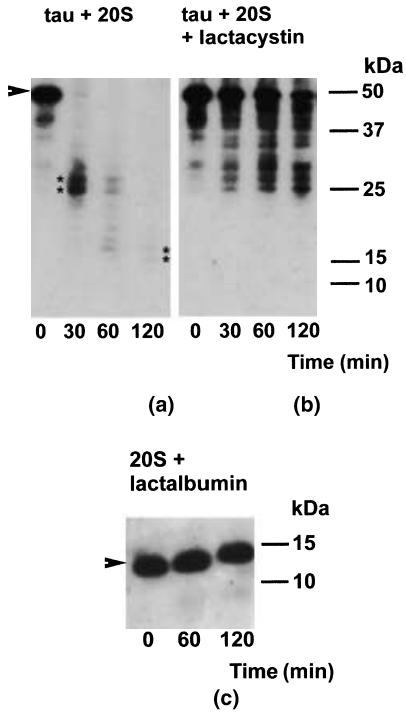

Fig. 3 *In vitro* ubiquitin-independent degradation of recombinant tau43 by purified 20S proteasomes. Tau43 (275 nM) was incubated with 20S proteasome (14 nM) for the indicated times in the absence (a) or presence (b) of lactacystin (10 μM) and tau degradation was detected by western blotting with the TAU-5 antibody. The conversion of full-length tau43 (arrowed) to relatively stable populations of degradation intermediates (asterisks) was significantly inhibited by the addition of lactacystin. The asterisks highlight species of apparent molecular weights 27.6, 25.9, 17.4 and 16.2 kDa. (c) Folded α-lactalbumin (arrowed), detected by western blotting with anti α-lactalbumin, was completely resistant to ubiquitin-independent degradation by purified 20S proteasomes under identical conditions.

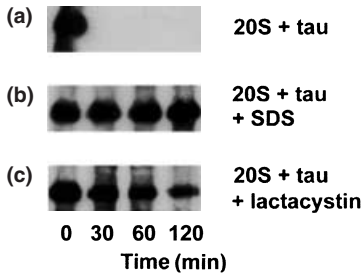

Fig. 4 Inhibition of *in vitro* 20S proteasomal degradation of tau by SDS. Tau43 (275 nM) was incubated with 20S proteasome (14 nM) for the indicated times in the absence (a) or presence of (b) SDS (0.01% w/v) or (c) lactacystin (10 μM). Tau degradation was detected by monitoring the disappearance of full-length protein by western blotting with the TAU-5 antibody. SDS treatment inhibited the 20S proteasome-catalysed degradation more potently than lactacystin.

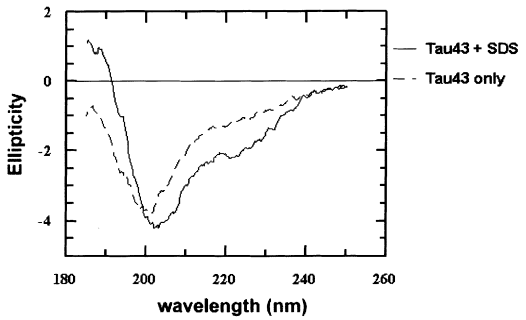

Fig. 5 SDS induces secondary structure in tau43. Circular dichroism spectra of tau43 (0.1 mg/mL) in water with (solid line) or without (dashed line) the addition of SDS (3 mM). The data were collected from 185 nm to 250 nm, accumulated over 20 runs, and averaged. The spectrum of tau alone is representative of predominantly random coil conformation. Upon, addition of SDS, the spectrum is consistent with the partial folding of the tau protein.

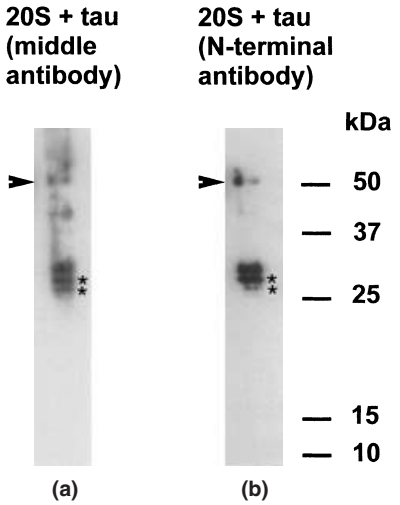

Fig. 6 *In vitro* 20S proteasomal degradation of tau43 gives rise to C-terminally truncated intermediates. Tau43 (275 nM) was incubated with 20S proteasome (14 nM) for 20 mins and tau degradation was detected by Western blotting, probing replicate membranes with (a) TAU-5 or (b) 5A6 antibodies. The arrowed band represents residual full-length tau. Of the three bands highlighted with asterisks, the two lower bands are equivalent to the 27.6 and 25.9 kDa bands noted in Fig. 1; these species contain an intact N-terminus and also the middle tau epitope and must therefore be C-terminally truncated.

(data not shown). Because SDS has previously been shown to induce some structural changes in tau (Yanagawa *et al.* 1998), circular dichroism spectroscopy was carried out on tau43 with and without SDS at the same molar ratio of SDS : tau used in the degradation assays (Fig. 5). Tau43 alone gave a spectrum with a negative maximum at around 198 nm, representative of predominantly random coil conformation, confirming the material used in our *in vitro* assays to represent natively unfolded tau. Upon addition of SDS, the negative maximum was shifted to 205 nm and the spectrum showed an increase in negative maximum at around 220 nm. This would be consistent with the partial folding of the tau43 protein into an α-helical or β-sheet conformation with some random coil conformation remaining, presumably a sufficient change to prevent substrate entry into the 20S axial chamber. Other detergents such as Tween-20, which was routinely included in the *in vitro* degradation assays, did not affect tau conformation or its degradation by the proteasome (data not shown).

Immunochemical characterization of tau degradation products generated by the 20S proteasome *in vitro*

We found that the 20S proteasome was always able to generate a highly reproducible pattern of lower molecular weight degradation products for each individual tau isoform used, suggesting a high specificity of the proteolytic mechanism. During 20S proteasomal processing of tau43, the TAU-5 antibody detected the appearance of two relatively stable populations of degradation intermediates (see Fig. 3a). The 'upper' population, highlighted by the two asterisks at the 30-min time point, comprised two bands of apparent molecular mass 27.6 ± 0.2 kDa and 25.9 ± 0.5 kDa (in each case bands from four independent blots were analysed). The 'lower' population consisted of two bands of apparent molecular mass 17.4 ± 0.1 kDa and 16.2 ± 0.3 kDa, highlighted by the two asterisks at the 120-min time point. Between these times (i.e. at the 60-min time point), both populations were just visible, along with other minor species of molecular mass between 26 and 17 kDa, suggesting that the upper population gives rise to the lower population in a processive manner. The tau degradation products were regularly spaced and invariably separated by 1–2 kDa, consistent with them representing species in which approximately six to 10 amino acids were being processively removed by the 20S proteasome.

To further investigate these intermediates, we used additional antibodies (see Fig. 1) directed against the N-terminus (5A6) and the C-terminus (BR134) of tau. Probing tau43 degradation intermediates with 5A6 (Fig. 6b) and in parallel with TAU-5 (Fig. 6a) revealed that both antibodies recognized the upper population of degradation intermediates (the two bands highlighted with asterisks in the upper population in Fig. 3a are equivalent to the two bands highlighted in Figs 6a and b), demonstrating that these species contain both the N-terminus and the middle region of the tau protein and therefore must be truncated only at the C-terminus. At this 20-min time point we also noted a third, higher molecular mass intermediate in the upper population. Interestingly, probing tau43 degradation intermediates with BR134 (Fig. 7b) revealed that a fraction of the tau protein is also rapidly degraded in a processive manner through the N-terminus to a relatively stable intermediate population with

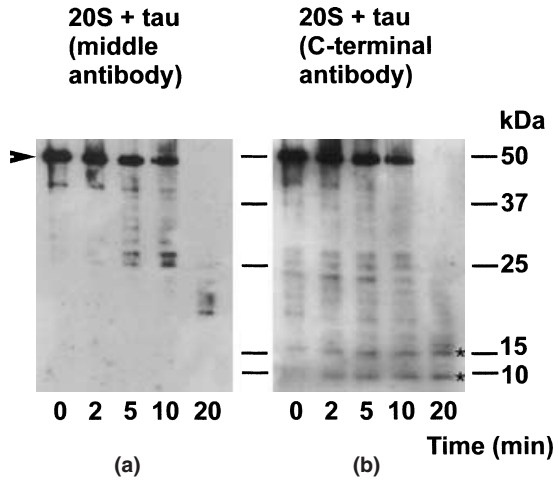

Fig. 7 *In vitro* 20S proteasomal degradation of tau43 gives rise to N-terminally truncated intermediates. Tau43 (275 nM) was incubated with 20S proteasome (14 nM) for the indicated times and tau degradation was detected by Western blotting with (a) TAU-5 antibody. The blot was then stripped and re-probed with (b) BR134 antibody. The arrowed band represents residual full-length tau The asterisks highlight species of apparent molecular mass 15.2 and 10.3 kDa, which contain only the C-terminal tau epitope and therefore represent N-terminally truncated tau.

major species of 15.2 ± 0.1 kDa and 10.3 ± 0.2 kDa (bands highlighted with asterisks). In this case the blot was first probed with TAU-5 (Fig. 7a), before being stripped and re-probed with BR134 (Fig. 7b), thus allowing direct comparison of intermediate patterns; these lower C-terminal tau fragments clearly did not contain the epitope recognized by the 'middle' TAU-5 antibody. Therefore, taken together, these results suggest that the 20S proteasome can process tau in a bi-directional manner through both N- and C-termini.

Bi-directional degradation by the 20S proteasome of a different tau isoform, tau46, is demonstrated in Fig. 8, where replicate degradation assays probed with 5A6, TAU-5 and BR134 are shown. In this case detailed analyses of the sizes of degradation intermediates were not made; however, proteolysis clearly proceeded from both the N- and C-termini. Again, regularly spaced degradation products separated by 1–2 kDa were apparent (Fig. 8d). Although both N-terminally truncated degradation intermediates were not clearly obvious for this tau isoform, in fact both were present, with the higher molecular weight band being only weakly immunoreactive. Intriguingly, some of the intermediates recognized by the TAU-5 antibody (boxed in Fig. 8c) were not recognized by either the 5A6 (N-terminal; Fig. 8a) or the BR134 (C-terminal; Fig. 8b) antibody. This suggests that some tau molecules were being degraded from both ends, although whether this processing occurred simultaneously is currently unclear.

Discussion

Degradation of cellular tau by the proteasome

Using the proteasome inhibitor lactacystin at levels known to be non-toxic in SH-SY5Y cells (Tofaris *et al*. 2001), we demonstrate the proteasomal degradation of tau in a cellular model. An important caveat to the use of the currently available chemical inhibitors of the proteasome, including lactacystin, is that they are directed against the 20S catalytic core and thus inhibit both 20S- and 26S-dependent proteolysis. Consequently, ubiquitin-dependent and ubiquitin-independent proteasomal proteolysis cannot be readily distinguished. The relatively long half-life that we measured for the proteasomal degradation of tau in our cells (approximately 12–14 h) is in contrast to the generally shorter half-lives of proteins known to be degraded by the 26S proteasome in a ubiquitin-dependent manner (presumably the process of ubiquitylation and recognition by the 19S machinery has evolved to improve the efficiency of regulated intracellular proteolysis). This observation, taken together with the knowledge that tau is a structureless protein which therefore does not require an 'unfoldase' activity to allow entry into the proteasomal catalytic chamber, indicated the

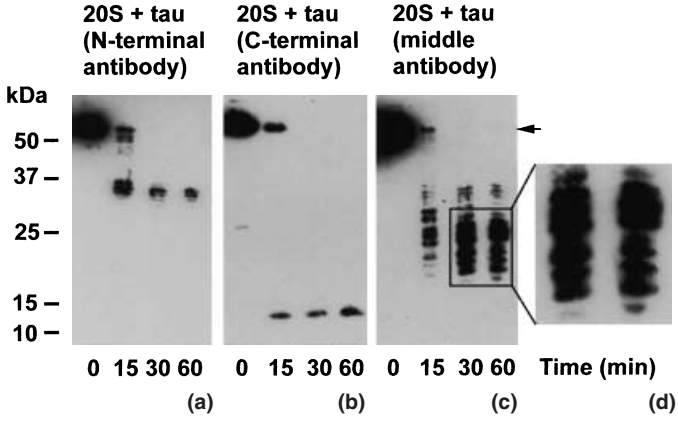

Fig. 8 Bi-directional *in vitro* 20S proteasomal degradation of tau46. Tau46 (275 nM) was incubated with 20S proteasome (14 nM) for the indicated times and tau degradation was detected by Western blotting, probing replicate membranes with (a) 5A6 (b) BR134 and (c) TAU-5 antibodies. C-terminally and N-terminally truncated intermediates are noted in panels (a) and (b), respectively. Boxed species in panel (c) are enlarged in (d) and represent proteasomal degradation products separated by 1–2 kDa lacking both N- and C-termini.

possibility of a ubiquitin-independent proteasomal mechanism of tau degradation.

Unfolded tau can be directly degraded by the 20S proteasome *in vitro* in a ubiquitin-independent manner

We have found that unfolded human tau can be directly degraded by the 20S proteasome *in vitro* without prior ubiquitylation. To date, only a small number of proteins has been found to be similarly degraded. They include $p21^{WAF1/CIP1}$ (Touitou *et al*. 2001), casein (Kisselev *et al*. 1999; Davies 2001), the β-secretase-derived C-terminal fragment of the amyloid precursor protein (Nunan *et al*. 2001) and α-synuclein (Tofaris *et al*. 2001). The latter also belongs to the family of natively unfolded proteins. In contrast, a more extensive list of proteins with secondary structure can be specifically processed by the 26S proteasome, also in a ubiquitin-independent manner, including the transcription factor c-Jun (Jariel-Encontre *et al*. 1995), ornithine decarboxylase (Murakami *et al*. 1992) and apocalmodulin (Tarcsa *et al*. 2000). In these cases, the unfolding activity of the 19S particle, coupled with ATP hydrolysis, is still required to allow entry of the polypeptide chain into the proteasome catalytic core.

Inducing unfolded tau to gain secondary structure through the addition of SDS to a concentration which activates 20S activity was sufficient to completely inhibit tau degradation by the 20S proteasome, presumably by preventing substrate access to the proteasome axial catalytic chamber. Thus, our results support the proposal that unstructured forms of some proteins, including members of natively unfolded family of proteins, may be capable of bypassing the ubiquitin-conjugation and targeting system whilst still being subjected to proteasome-dependent degradation.

Mechanism of tau degradation by the 20S proteasome *in vitro*

Kisselev *et al*. (1999) showed that both 20S and 26S proteasomes were able to degrade some unstructured substrates in a highly processive manner, generating a range of small peptides with mean lengths between six and 10 amino acids residues. The 20S proteasome catalytic core, which can accommodate a stretch of approximately 50 amino acids (Yewdell 2001), was found to cleave peptide bonds of a substrate until the peptides generated are small enough to diffuse out of the core (Kisselev *et al*. 1999). In contrast to other proteases, during this process the substrate is thought not to dissociate from the enzyme, and consequently degradation intermediates have generally not been detected. Indeed, previous studies have shown that the rate of disappearance of protein substrates of the 20S proteasome paralleled the rate of appearance of small peptides (Akopian *et al*. 1997), providing evidence for a processive mechanism of degradation. The only notable exception to this is in the degradation of the NF-κB precursor p105, which gives rise to the p50 protein through limited proteolysis catalysed by the 26S proteasome in a ubiquitin-dependent manner (Palombella *et al*. 1994). It is therefore very interesting that we were able to detect numerous intermediates, including some which were relatively stable, during the 20S proteasomal degradation of tau *in vitro*; we currently do not know if this property is unique to tau, although to our knowledge similar proteasome degradation intermediates have not been seen before. It is possible that in our *in vitro* assays the molar ratio of tau : proteasome was higher than that typically occuring in cells and that this may account for the occurrence of degradation intermediates. Alternatively, other cellular factors (e.g. molecular chaperones) could participate in the proteasomal degradation of tau, perhaps making the process proceed more efficiently so that intermediates do not accumulate in cells. We also cannot exclude the possibility that the tau degradation intermediates serve as substrates for other cellular proteases which assist in preventing the accumulation of these polypeptides, or that cellular post-translational modifications of tau protein, e.g. phosphorylation, in some way facilitate *in vivo* proteasome-catalysed tau degradation.

Regardless, because we were able to routinely visualize tau degradation intermediates *in vitro* we have been able to make new observations about the mechanism of 20S proteasomal proteolysis. In our study we found that 20S proteasome-catalysed degradation of tau *in vitro* is bi-directional, i.e. the isolated 20S proteasome is capable of processing tau from either C- or N-terminus. It is noteworthy that in the case of p105, degradation by the 26S proteasome occurs directionally from the C-terminus (Palombella *et al*. 1994) and that a glycine-rich region located near the C-terminus of the p50 sequence within the p105 sequence enhances the stability of the p50 protein, preventing further proteolysis (Orian *et al*. 1999). Additionally, the ClpP protease of *Escherichia coli* (a prokaryotic protease complex related to the proteasome) also catalyses directional processing of some substrates, again proceeding through the C-terminus (Reid *et al*. 2001). However, the demonstration that N-end-rule substrates can be degraded cotranslationally in both yeast and mammalian cells suggests that proteasomes must also be able to process proteins through their N-terminus (Turner and Varshavsky 2000). Thus, our *in vitro* observations of bi-directional proteasomal processing reflect processes that occur *in vivo*, and may have important implications in understanding the proteolytic mechanisms of other proteasomal complexes, e.g. 26S proteasomes or immunoproteasomes, as these structures utilize the 20S core particle.

Analysis and significance of tau43 degradation intermediates

By extrapolating percentage differences between the actual and apparent denatured molecular masses of the full-length tau43 protein (i.e. tau43 has an actual denatured molecular

mass of 40 kDa, but runs on SDS–PAGE with an apparent molecular mass of 50 kDa; these differences arise because basic residues are over-represented, but evenly distributed, in tau protein), the actual denatured molecular masses of the principal N-terminally and C-terminally truncated tau43 degradation intermediates generated by the 20S proteasome *in vitro* could be estimated. These estimates, combined with empirical observations of the cross-reactivity of the intermediates with different tau antibodies, allowed predictions of their primary sequences to be made. The two C-terminally truncated species (27.6 and 25.9 kDa) in the 'upper' population of intermediates (see Fig. 6) were predicted to approximately correspond to amino acid residues 1–213 and 1–200, respectively. The two N-terminally truncated intermediates (15.2 and 10.3 kDa; see Fig. 7) were predicted to approximately correspond to amino acid residues 269–383 and 305–383, respectively. The observation that the TAU-5 antibody, which is directed against amino acid residues 152–172 in the tau43 isoform (equivalent to residues 210–230 in the longest tau isoform; Carmel *et al*. 1996) recognized C-terminally but not N-terminally truncated intermediates, agrees with these predictions. In each case, the sites of 'final' proteasome cleavage which would give rise to the intermediates were within four amino acid residues of theoretical proteasome cleavage sites predicted using a neural network predictor (data not shown). Significantly, each of the proteasome cleavage sites which would generate the 27.6, 15.2 and 10.3 kDa fragments of tau43, is also located close to glycine-rich regions of the tau microtubule-binding domain (within repeats R1, R2 and R4, respectively). This suggests that, as for p105 (Orian *et al*. 1999), tri-glycine repeats in the C-terminal repeat region of tau may indeed share some functional similarities and enhance the stability of the degradation intermediates.

However, the *in vivo* relevance of the tau degradation intermediates that we noted *in vitro*, and in particular any relationship to the pathogenesis of tauopathies, remains unclear. Certainly, some PHFs contain ubiquitylated N-terminally truncated tau (Morishima-Kawashima *et al*. 1993), although the protease(s) that catalyse this truncation have not been identified. Interestingly, ubiquitylated PHF-tau is found mainly in a mono-ubiquitylated form (as opposed to poly-ubiquitylated), a modification which does not constitute a degradation signal for the 26S proteasome (Thrower *et al*. 2000). Additionally, forms of PHF-tau which are less processed at the N-terminus are generally ubiquitin-negative, indicating that this proteolytic processing of tau in PHFs occurs before ubiquitylation (Morishima-Kawashima *et al*. 1993), and tau is the main component of PHFs, whereas ubiquitin becomes associated later with mature PHF-containing lesions (Bancher *et al*. 1991) and is not found in all tau-positive PHFs. Furthermore, tau deposits are not consistently ubiquitylated in FTDP-17 and other tauopathies. These results are consistent with a model in which tau fibrillization and N-terminal truncation occur before ubiquitylation; indeed, tau is capable of forming filaments *in vitro* without ubiquitylation (Goedert *et al*. 1996; Perez *et al*. 1996). Ubiquitylation of tau may therefore represent a late cytoprotective event in neurofibrillary tangle formation. Gray (2001) proposed that the mono-ubiquitylation of aggregates may be a mechanism by which cells limit the damage resulting from the accumulation of deposits that could otherwise impair the ubiquitin-proteasome system (Bence *et al*. 2001). Whilst it is possible that the N-terminal truncation of tau prior to PHF formation is catalysed by ubiquitin-independent proteasomal proteolysis, this seems unlikely. In fact, our results indicate that ubiquitin-independent proteolysis would avoid generating the sequences which constitute the core of the tau filament (Novak *et al*. 1993).

It may be noteworthy that other studies have reported the presence of lower molecular weight tau degradation products in human cerebrospinal fluid (CSF). CSF from normal and AD patients was found to contain non-phosphorylated C-terminally truncated 26–28 kDa tau fragments (Johnson *et al*. 1997), as well as a 32-kDa partially phosphorylated C-terminally truncated fragment with elevated levels in diseased patients compared to controls (Ishiguro *et al*. 1999). It is possible that these species are equivalent or related to the proteasome-derived C-terminally truncated tau43 intermediates we have observed, although the significance of the presence of these species in the CSF is not known. In cases of head injury, 30–50 kDa tau fragments truncated at both N- and C-termini have been described in the CSF (Zemlan *et al*. 1999). Again, these species may be related to the N- and C-terminally truncated tau46 fragments we noted, although exact comparisons cannot be made, as we used only two of the six adult brain tau isoforms for our studies, and as our recombinant proteins were not phosphorylated.

Relevance of the 20S proteasome *in vivo* and its role in disease

The relatively long half-life of tau in cells, together with our *in vitro* observation that the 20S proteasome alone can efficiently process tau, suggests that the proteasome-dependent tau degradation is at least in part ubiquitin-independent. Research into proteasome-mediated protein degradation has in the main concentrated on 26S proteasome-catalysed, ubiquitin-dependent proteolysis. Only more recently has ubiquitin-independent processing by the 26S or 20S proteasome come to the fore. In some cell types, the 'free' 20S particle is the predominant form of the proteasome and is found in a three- to fourfold molar excess over the 19S cap of the 26S proteasome or the 11S regulator of the immunoproteasome (Brooks *et al*. 2000; Davies 2001). The 20S proteasome has been implicated in the degradation of mildly oxidized proteins that arise through oxidative stress (Davies 2001). Moreover, ATP/ubiquitin-dependent proteolysis is inactivated under oxidative conditions as a result of the

glutathiolation of ubiquitin-activating (E1) and -conjugating (E2) enzymes (Jahngen-Hodge *et al*. 1997). Thus, the 20S proteasome may play an important role under conditions where oxidative damage occurs and aggregates of oxidized proteins accumulate (Giasson *et al*. 2000).

Although we have demonstrated that tau can be directly degraded by the 20S proteasome *in vitro*, we have currently no direct evidence that the free 20S proteasome performs this role *in vivo*. A logical next step of investigations into tau degradation *in vivo* would be the demonstration of proteasome-dependent tau degradation in primary neurones in culture or in brain. It is possible that other cellular factors may be required for the 'gating' of the 20S proteasome, the axial chamber of which is predicted to be closed (Groll *et al*. 1997). Presumably, the 20S proteasomes we used in our *in vitro* assays represent species that had become 'activated' during the purification process. Certainly the 19S cap or the 11S regulator is capable of opening the axial chamber, but whether these or other cofactors participate in tau degradation *in vivo* remains to be seen. Furthermore, other factors may be involved in the targetting of tau to the proteasome, in a similar way that antizyme specifically targets ornithine decarboxylase to the 26S proteasome (Murakami *et al*. 1992), or a degradation signal in $p21^{WAF1/CIP1}$ specifically targets the protein to the C8 subunit of the 20S proteasome (Touitou *et al*. 2001). Possibly hydrophobic peptide sequences, which have recently been shown to bind to the 20S proteasome and promote peptide hydrolysis (Kisselev *et al*. 2002) and also occur in the tau protein, are the motifs recognised by the 20S proteasome *in vivo*.

In summary, our results suggest that proteasomes directly contribute to tau turnover in a pathway that bypasses the requirement for ubiquitylation. Such a pathway may also be of relevance for other natively unfolded proteins. Impairment of proteasome function in the diseased state, as has been reported in AD (Keller *et al*. 2000), may be a mechanism contributing to the accumulation of aggregation-prone tau protein in sporadic tauopathies.

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