Phosphorylation of tau by glycogen synthase kinase 3β affects the ability of tau to promote microtubule self-assembly

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To study the effects of phosphorylation by glycogen synthase kinase-3β (GSK-3β) on the ability of the microtubule-associated protein tau to promote microtubule self-assembly, tau isoform 1 (foetal tau) and three mutant forms of this tau isoform were investigated. The three mutant forms of tau had the following serine residues, known to be phosphorylated by GSK-3, replaced with alanine residues so as to preclude their phosphorylation: (1) Ser-199 and Ser-202 (Ser-199/202 → Ala), (2) Ser-235 (Ser-235 → Ala) and (3) Ser-396 and Ser-404 (Ser-396/404 → Ala). Wild-type tau and the mutant forms of tau were phosphorylated with GSK-3β, and their ability to promote microtubule self-assembly was compared with the corresponding non-phosphorylated tau species. In the non-phosphorylated form, wild-type tau and all of the mutants affected the mean microtubule length and number concentrations of assembled microtubules in a manner consistant with enhanced microtubule nucleation. Phosphorylation of these tau species with GSK-3β consistently reduced the ability of a given tau species to promote microtubule self-assembly, although the affinity of the tau for the microtubules was not greatly affected by phosphorylation since the tau species remained largely associated with the microtubules. This suggests that the regulation of microtubule assembly can be controlled by phosphorylation of tau at sites accessible to GSK-3β by a mechanism that does not necessarily involve the dissociation of tau from the microtubules.

INTRODUCTION

The microtubule-associated protein (MAP) tau is principally a neuronal MAP and is involved in the regulation of many aspects of microtubule behaviour. The neuronal microtubule network is central to the functioning of neurons, and investigation into the role of MAPs within these cells is crucial to the understanding of how the complex morphology of neurons is established and maintained.

Many studies *in vitro* have been performed on the ability of tau to regulate aspects of microtubule behaviour, including growth and shortening of microtubules [1–4], dynamic instability of microtubules [5,6] and the promotion of microtubule nucleation [6–9]. All of the described microtubule behaviours can be examined *in vitro* [10]. This study has focused on how the phosphorylation of tau affects its ability to promote microtubule self-assembly, where microtubule number and length after a period of tubulin self-assembly give an indication of microtubule nucleation [7].

Nucleation of microtubules is important in neurons because when nucleation is inhibited, neurite growth is severely disrupted, indicating that a constant source of microtubules is needed for axonal growth [11]. The ability of tau to promote microtubule nucleation seems to lie in the properties of residues 154–307 (numbering in accordance with the longest isoform [12]) of the tau protein from human brain, a sequence that is conserved in all isoforms of tau; a similar region can be found in MAP2 [6,8,13], although the exact mechanism by which this domain functions in nucleating microtubules is unknown. This region of tau is particularly interesting as it contains many potential phosphorylation sites and because phosphorylation has been shown to affect the ability of tau to promote microtubule assembly *in vitro*. Foetal tau is known to be hyperphosphorylated [14,15] and it is now recognized that much of the tau isolated from adult brain is highly phosphorylated. However, to observe this fraction it is necessary to isolate the tau rapidly, indicating that tau phosphatase activity is greater in adult brain than in foetal brain [16,17]. Thus phosphorylation of tau might have a significant role in regulating the dynamics of microtubule assembly/disassembly in neurons.

One of the pathological hallmarks of Alzheimer's disease is the neurofibrillary tangles, which are composed of paired helical filaments (PHF) of which the main component is hyperphosphorylated tau [15,18–21]. Many of the sites that are phosphorylated in PHF-tau are also phosphorylated in the more heavily phosphorylated fractions of foetal and adult brain tau, although there are some additional sites phosphorylated in PHF-tau. The transition from normal soluble tau to insoluble PHF is not understood; neither is it known whether phosphorylation has a major role in this progression. However, the hyperphosphorylated PHF-tau after solubilization has been shown to be incompetent at promoting microtubule assembly [22] and because phosphorylation alters the ability of tau to bind and stabilize microtubules [2,23–27], an increase in the proportion of tau that is hyperphosphorylated could be crucial in leading to the known total loss of the cytoskeleton in neurons containing PHF, eventually leading to cell death [28,29].

Several protein kinases have been shown to phosphorylate tau *in vitro* but because many of the sites phosphorylated in PHF-tau and the more phosphorylated fractions of foetal and adult brain

Abbreviations used: C_n, microtubule number concentration; C_p, microtubule polymer mass; C_s, tubulin concentration in solution; DTT, dithiothreitol; GSK-3, glycogen synthase kinase 3; $\langle L \rangle$, average microtubule length; PEM, microtubule assembly buffer; PHF, paired helical filaments; MAP, microtubule-associated protein.

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tau are either serine or threonine residues immediately followed in the sequence by proline, most attention has focused on proline-directed kinases. Of this class of protein kinases, cdk5, p42 and p44 MAP kinases, and glycogen synthase kinase-3 (GSK-3)α and GSK-3β, which are expressed in post-mitotic neurons, have all been demonstrated to phosphorylate tau *in vitro* at many of the sites phosphorylated *in vivo* [30]. However, in living cells, MAP kinase does not seem to phosphorylate tau at these sites and cdk5 has not yet been reported to phosphorylate tau in cells, whereas both GSK-3α and GSK-3β do so [31–33]; thus the two GSK-3 enzymes are stronger candidates as tau kinases.

Many of the phosphorylation sites on tau generated *in vitro* by GSK-3β have been identified [34–38] including Ser-199, Ser-202, Ser-235, Ser-396 and Ser-404. Recently the ability of GSK-3β to phosphorylate Ser-262 under certain conditions has been discovered; this is a site that might significantly influence tau–microtubule binding [26].

Because the GSK-3 enzymes are likely to be protein kinases acting on tau *in vivo* and no previous study of these kinases has been made to alter *in vitro* tau properties, other than effects on microtubule-binding affinity [36], it is of interest to investigate the effects of phosphorylation of tau by these kinases on microtubule properties and to study the roles of individual phosphorylation sites. Here the effect of phosphorylation of tau by GSK-3β on the ability of tau to promote microtubule self-assembly was investigated. The following three mutant forms of tau were also constructed, in which serine phosphorylation sites were replaced by alanine residues so as to prevent phosphorylation: Ser-199 and Ser-202 → Ala, Ser-235 → Ala and Ser-396 and Ser-404 → Ala. By phosphorylating wild-type tau and the mutant forms of tau, a comparison could be made of the ability of each tau species to promote the self-assembly of microtubules, and hence the contribution to this function of these sites could be inferred. The results suggest that the phosphorylation of tau by GSK-3β alters the ability of tau to promote microtubule self-assembly.

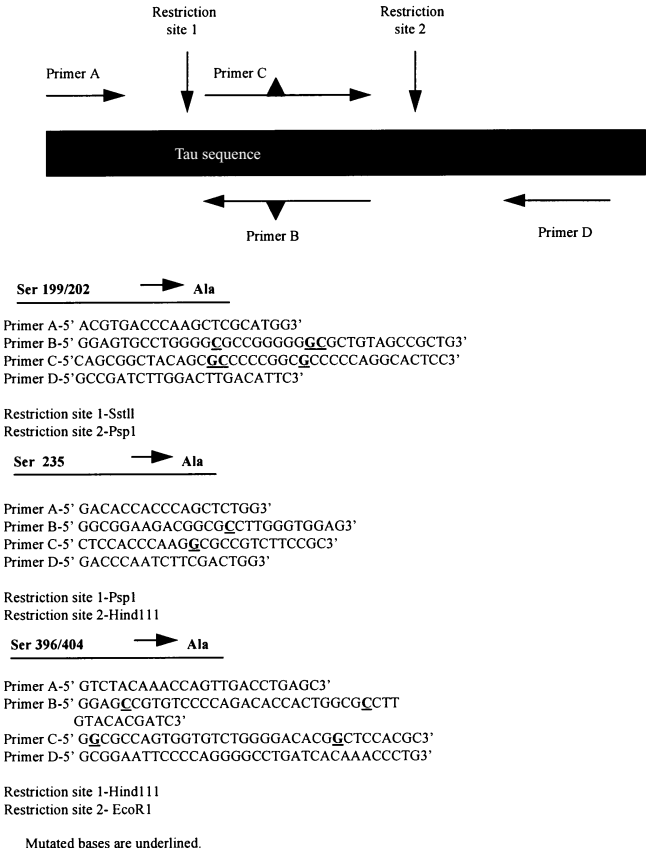

Figure 1 Tau mutants generated by PCR mutagenesis

See the Materials and methods section for details.

MATERIALS AND METHODS

Purification of human recombinant tau isoform one

Tau isoform one (no N-terminal inserts, three C-terminal repeats) in the expression plasmid prk172 (kindly donated by M. Goedert, MRC, Cambridge, U.K.) was transformed by electroporation into *Escherichia coli* BL21(DE3). The following purification procedure was based that described in [39]. Bacterial pellets were washed in 50 mM Mes buffer, pH 6.5, and recentrifuged at 12000 ***g*** (Sorvall RC5B, rotor SS34) for 20 min at 4 °C. Pellets were resuspended on ice in 50 mM MES (pH 6.5)/1 mM dithiothreitol (DTT)/1 mM PMSF (approx. 0.15 ml of buffer/ml of culture). The suspension was sonicated on ice for 10 min in 30 s bursts with 30 s intervals (Virsonic 475; Virtis) and pelleted at 20400 ***g*** (Sorvall RC5B, rotor SS34) for 30 min at 4 °C. The supernatant was subjected to a 0–45% satd. $(NH_4)_2SO_4$ precipitation. The pellet was resuspended in 50 mM Mes/1 mM DTT/1 mM PMSF and centrifuged at 20400 ***g*** (Sorvall RC5B, rotor SS34) for 30 min at 4 °C. The supernatant was dialysed overnight at 4 °C against 50 mM Mes (pH 6.5)/5 mM DTT/1 mM PMSF. The dialysed mixture was applied to a Resource-S column (Pharmacia Biotech.) [equilibrated in 50 mM Mes (pH 6.5)/5 mM DTT] fitted to an FPLC system loaded at a flow rate of 0.2 ml/min. A separation gradient was run of 0–0.5 M NaCl over 60 min at a flow rate of 1 ml/min. The relevant fractions containing tau were dialysed overnight at 4 °C against 50 mM NH_4HCO_3 and then freeze-dried (SpeedVac SC100; Savant). Samples were dissolved in microtubule assembly buffer [PEM; 100 mM Pipes (pH 6.5)/0.1 mM EGTA/1.7 mM $MgCl_2$]. Protein concentrations were determined by using the Bradford protein assay (Bio-Rad), with BSA as the standard.

Generation of mutant forms of tau

Human tau (isoform 1) was originally excised from p19tau cloned into pBR322 [kindly donated by K. Kosik, Department of Neurology (Neuroscience), Harvard Medical School and Center for Neurological diseases, Brigham and Women's Hospital, Boston, MA, U.S.A.] and subcloned into the vector puc8Δ*Hin*dIII. PCR mutagenesis was performed by overlap-extension [40] to generate the following three mutant forms of tau isoform 1: (1) Ser-199 and Ser-202 replaced by Ala residues (Ser-199/202 → Ala); (2) Ser-235 replaced by Ala (Ser-235 → Ala); (3) Ser-396 and Ser-404 replaced by Ala residues (Ser-396/404 → Ala) (see Figure 1 for primers used). All initial PCR reactions with primers A and B together, and primers C and D together, were performed with Exo$^-$*Pfu* DNA polymerase (Stratagene), and the final PCR reactions with primers A and D were performed with *Taq* polymerase (Promega).

The AD fragments generated were cloned directly into a TA cloning vector with the TA cloning system (Invitrogen Corporation). Sequencing of the whole fragment to be cloned back into the expression vector was performed with the Exo$^-$*Pfu* Cyclist DNA sequencing kit (Stratagene) except for the Ser-199/202 mutation where the Sequenase kit, Version 2.0, with 7-

deaza-dGTP was used (United States Biochemicals). The mutated tau fragment was excised from the TA cloning vector with the restriction enzymes indicated in Figure 1, cloned into prk172tau and used to transform *E. coli* BL21(DE3). All clones were sequenced after transformation to verify the presence of the desired mutation and to confirm that the rest of the tau sequence was identical with the published human cDNA sequence [18]. Expression and purification of the mutant tau proteins were performed as for the wild-type protein.

Baculovirus-mediated expression of GSK-3β

Spodoptera frugiperda (*Sf*9) insect cells were routinely grown in stirrer flasks on a microcarrier stirrer MC5-1045 (Techne) at 18–21 °C in TC100 media supplemented with 10 % (v/v) foetal calf serum (heat-inactivated), 1 % (v/v) glutamax (Gibco BRL), 1 % (v/v) antibiotic/antimycotic (Gibco BRL) and 0.02 mg/ml gentamycin. Working stocks of cells were kept at approx. $(0.5–1) \times 10^6$ cells/ml. Amplification of viral stocks was performed by the method of [41].

The method used for the expression and purification of GSK-3β (rat GSK-3β cloned into the baculovirus expression vector pAcC5) was based on that of [42]. Exponentially growing *Sf*9 cells were plated at a density of 5×10^5/ml and incubated at 27 °C overnight or at a density of 10^6/ml on the day of infection. Approx. 1 litre of cell suspension, added to twenty 175 mm² flasks to form monolayers, was used for each protein preparation. The infection of cell cultures for the purpose of protein production was performed at a multiplicity of infection of 20 plaque-forming units per cell. The appropriate quantity of virus was mixed with medium to a final volume of 5 ml per 175 mm² flask (containing 2.5×10^8 cells) and added to the cell monolayers. The cells were incubated for 1 h at 27 °C with gentle shaking at regular intervals to ensure complete coverage of the monolayer with the virus. A 45 ml sample of medium was then added to each flask and incubated for 48 h at 27 °C. Cells were then dislodged by gentle tapping of the flask and centrifuged at 4 °C for 5 min at 1000 ***g*** (Mistral 3000i, rotor 43122-105). All subsequent stages were performed at 4 °C. All cells were lysed on ice with 10 strokes of a homogenizer in 80 ml of 10 mM potassium phosphate (pH 7.05)/1 mM EDTA/5 mM EGTA/50 mM sodium glycerophosphate/1 mM DTT/0.1 % (v/v) 2-mercaptoethanol/10 mM $MgCl_2$/1 mM sodium orthovanadate/40 μg/ml PMSF/10 μg/ml pepstatin A. The suspension was centrifuged at 20400 ***g*** (Sorvall RC5B, rotor SS34) for 30 min. The supernatant was applied to a 10 ml column of phosphocellulose (Whatman P-11, activated in accordance with the manufacturer's instructions) equilibrated with 25 mM Tris/HCl (pH 7.5)/1 mM EDTA/1 mM DTT/0.1 % (v/v) 2-mercaptoethanol/5 % (v/v) glycerol/50 mM NaCl/1 mM sodium orthovanadate. The column was washed with approx. 10 column volumes of P-11 equilibration buffer containing 100 mM NaCl. Subsequent elution of the bound protein was made by increasing the NaCl concentration to 200 mM. Fractions containing the highest specific activity for GSK-3 (assay detailed in [31]) were pooled and added directly to a phenyl-Sepharose column. The phenyl-Sepharose column (0.5 ml) (Phenyl-Sepharose CL-4B; Whatman) was equilibrated with 25 mM Tris/HCl (pH 7.5)/0.1 mM EDTA. After being loaded, the column was washed with 5 column volumes of 25 mM Tris/HCl (pH 7.5)/2 mM EGTA/40 mM *p*-nitrophenyl phosphate/1 mM sodium orthovanadate/1 mM benzamidine/5 μg/ml leupeptin/2 μg/ml aprotinin/1 μg/ml pepstatin A/0.1 mM PMSF/35 % (v/v) ethylene glycol. Portions (0.5 ml) of the wash buffer containing 40 %, 45 %, 50 %, 55 %, 60 % and 65 % (v/v) ethylene glycol were passed individually through the column and each fraction was collected, after which it was assayed for protein content, with the Coomassie protein assay (Bio-Rad), and for GSK-3 activity.

Phosphorylation of wild-type and mutant forms of tau for microtubule dynamics studies with GSK-3β from baculovirus-infected *Sf*9 cells

The following kinase reaction was assembled: 20 mM Hepes (pH 7.5)/1 mM $MgCl_2$/0.5 mM DTT/3 mM ATP/1 mM PMSF/15 μM aprotinin/10 μM leupeptin/7 μg of tau protein (final tau concentration approx. 3 μM)/10 μl of GSK-3β {10 μl of the 0.5 ml fraction from the phenyl–Sepharose column eluted with 50 % (v/v) ethylene glycol was used (stock GSK-3 0.08 mg/ml, specific activity 153 nM phosphate incorporated into GS1 peptide/min per mg; assayed as described in [31])} in a final volume of 50 μl. The reaction mixtures were incubated at 30 °C for approx. 20 h. Reactions were stopped by incubation at 100 °C for 5 min.

The wild-type tau and each of the three mutants were phosphorylated under the same conditions. Control phosphorylation reactions (serving as controls in the microtubule dynamics studies) were also prepared containing all the reaction mixture but with inactive GSK-3β (enzyme inactivated by incubation at 100 °C for 10 min). An additional control sample of the reaction mixture, inactive kinase and buffer to replace the tau protein was also prepared to serve as a control sample to add to tubulin in the microtubule dynamics studies.

All phosphorylation reactions were analysed by SDS/PAGE [43] and Western blotting with a phosphorylation-independent antibody, TP70 [44], and phosphorylation-dependent antibodies AT8 [45,46] and 8D8 [44].

Analysis of the effect of phosphorylation on the properties of wild-type and mutant forms of tau: microtubule length distribution and microtubule polymer mass determination

MAP-free pig tubulin was prepared as described [47]. To measure the ability of tau and phosphorylated tau to promote tubulin self-assembly, the following mixtures were prepared on ice for each of the prepared tau species: 15 μM purified tubulin, 1.5 mM GTP and 1.2 μM tau. (Each mixture contained 26 μl of the phosphorylation reaction and 34 μl of PEM buffer to a final volume of 60 μl.) These mixtures were incubated at 37 °C for 60 min. Aliquots were taken and diluted in PEM buffer containing 0.25 % glutaraldehyde to fix the assembled microtubules. The length distribution within each population was determined by dark-field microscopy as described previously [48,49].

The microtubule polymer mass, C_p, in each sample was determined by ultracentrifugation of the microtubules at 100000 ***g*** for 15 min at 37 °C (Beckman TLA 100). The resulting pellets and supernatants were analysed by SDS/PAGE and Western blotting; the probe for tau was polyclonal antibody TP70 [44]. The protein concentration of the supernatants was determined with the Bradford protein assay. [Known concentrations of tubulin were used as standards. The contribution of added tau to the total protein assayed with the Coomassie protein assay reagent (Bio-Rad) was negligible.] C_p was determined as $C_p = C_t - C_s$, where C_t is the total tubulin concentration (15 μM) and C_s is the tubulin concentration of the supernatant as determined. The same values of C_p were observed regardless of whether the tau was present at the start of microtubule assembly or whether pre-assembled microtubules were diluted to a total concentration $C_t = 15$ μM into tau-containing buffers and incubated for 60 min at 37 °C. This indicates that in the experiments described in this work micro-

tubules have always been sampled at a steady state of assembly. The microtubule number concentration, C_n, is calculated as $C_n = C_p/\langle L \rangle \times 1625$, where $\langle L \rangle$ is the average microtubule length and 1625 is the number of tubulin subunits per μm of microtubule.

RESULTS AND DISCUSSION

The possible biological significance of the phosphorylation of tau by GSK-3 [36,50] is that so far this is the only kinase to have been shown to produce a electrophoretic pattern closely similar to that of PHF-tau found in Alzheimer's disease when it is used to phosphorylate tau isoforms *in vitro* [51]. In cultured cells, phosphorylation by GSK-3β has also been shown to generate a PHF-like state of tau [31–33,52,53] and to affect microtubule bundling [33,53]. The present work aims at extending the previous observations by investigating not only whether GSK-3β has the ability to phosphorylate tau, but also how the resultant

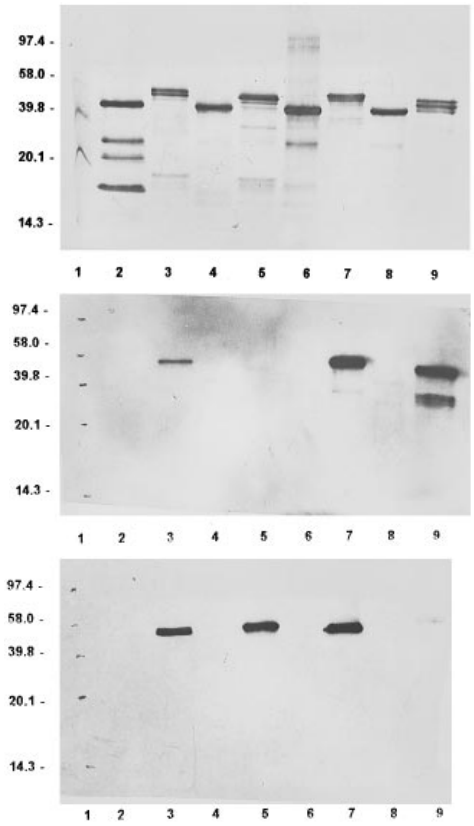

Figure 2 Phosphorylation of wild-type and mutant forms of tau with GSK-3β

Phosphorylation of wild-type tau isoform 1 and the three mutants of tau was performed with GSK-3β purified from *Sf*9 cells. An aliquot of the kinase reaction was stopped by the addition of SDS sample buffer and incubation at 100 °C for 5 min. The phosphorylated tau samples were analysed by running on an SDS/10% (w/v) polyacrylamide gel alongside a non-phosphorylated tau sample. Proteins were transferred to nitrocellulose from the SDS/polyacrylamide gel; the presence of tau was determined by probing with a range of antibodies. Top panel, detection of tau with polyclonal antibody TP70 (dilution 1:20000); middle panel, detection of tau with monoclonal antibody AT8 (dilution 1:200); bottom panel, detection of tau with monoclonal antibody 8D8 (dilution 1:100). Lanes in all panels: lane 1, molecular mass markers (values in kDa indicated at the left); lane 2, wild-type tau isoform 1; lane 3, phosphorylated wild-type tau; lane 4, Ser-199/202 → Ala mutant form of tau; lane 5, phosphorylated Ser-199/202 → Ala mutant form of tau; lane 6, Ser-235 → Ala mutant form of tau; lane 7, phosphorylated Ser-235 → Ala mutant form of tau; lane 8, Ser-396/404 → Ala mutant form of tau; lane 9, phosphorylated Ser-396/404 → Ala mutant form of tau. Loadings in the top panel correspond to 2 μl of the kinase reaction; loadings in the middle and bottom panels correspond to 4 μl of the kinase reaction. The faster-migrating species below the main tau band in lane 2 in the upper panel are degradation products that are accentuated on antibody staining with TP70; these bands are minimal components compared with intact tau when stained with Coomassie Brilliant Blue (results not shown).

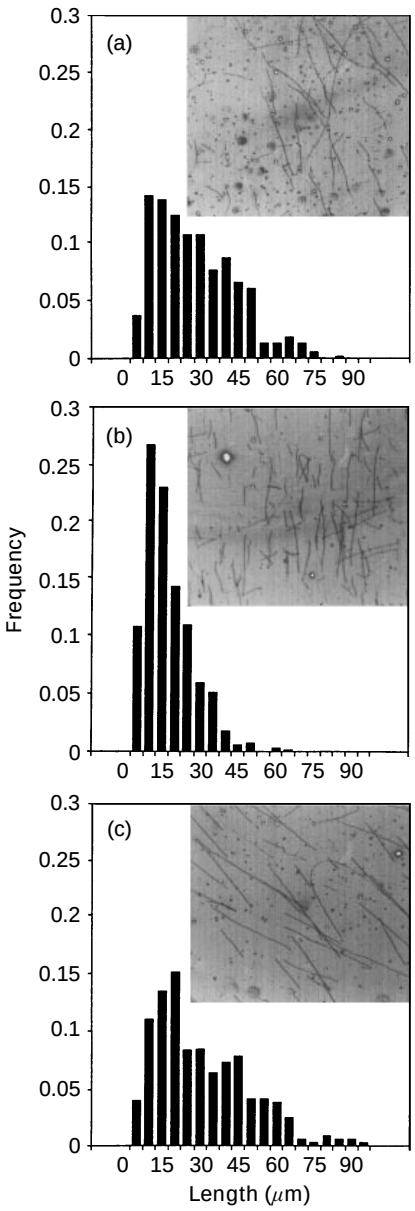

Figure 3 The effect of various tau species on microtubule length

The various tau species (1.2 μM) within the GSK-3β kinase reaction mixture were added to tubulin (15 μM) and incubated at 37 °C for 60 min to allow tubulin self-assembly. All samples including the control contained all the components of the kinase reaction (non-phosphorylated samples contained inactivated kinase). Microtubule populations were fixed and microtubule lengths measured. The frequencies of microtubules of a given length in each population are given in the histograms; Frequency = (number of microtubules of a given length)/(total microtubules in population). (**a**) Tubulin assembled in the absence of tau, 567 microtubules, $\langle L \rangle = 28.59$ μm; (**b**) tubulin assembled in the presence of wild-type tau, 673 microtubules, $\langle L \rangle = 15.03$ μm; (**c**) tubulin assembled in the presence of GSK-3β-phosphorylated wild-type tau, 672 microtubules, $\langle L \rangle = 28.40$ μm. The insets show typical dark-field images of the corresponding microtubule populations. (Histograms and pictures of microtubules assembled in the presence of the tau mutants are not shown). Magnification of insets: 87 μm × 80 μm.

Table 1 The effect of tau species on $\langle L \rangle$, C_p and C_n

$\langle L \rangle$ was measured as shown in Figure 3; the experimental variation was given by $\langle L \rangle \pm \sqrt{\langle L \rangle}$, assuming the observed length distributions conform approximately to a Poisson distribution. C_p, was determined by ultracentrifugation of the microtubule samples and C_s was measured. The polymer mass was calculated as $C_p = C_t - C_s$, where C_t is the total tubulin concentration. The experimental variation is given as the standard deviation, S.D.(C_p), for at least five measurements. C_n was calculated as $C_n = C_p/\langle L \rangle \times 1625$. The experimental variation on C_n was calculated as: $[C_n^2\ (a^2 + b^2)]^{\frac{1}{2}}$ with $a = \sqrt{\langle L \rangle}/\langle L \rangle$ and $b =$ S.D.(C_p)/C_p; N is the number of microtubules measured.

	Unphosphorylated tau species			Phosphorylated tau species		
Tau species	$\langle L \rangle$ (μm) (N)	C_p (μM)	C_n (nM)	$\langle L \rangle$ (μm) (N)	C_p (μM)	C_n (nM)
Control (no tau)	25.6 ± 5.1 (567)	7.7 ± 0.7	0.19 ± 0.04	–	–	–
Wild-type tau	15.0 ± 3.9 (671)	7.4 ± 1.4	0.30 ± 0.10	28.4 ± 5.3 (672)	7.6 ± 1.2	0.16 ± 0.04
Ser-199/202 → Ala tau	13.9 ± 3.7 (891)	5.8 ± 2.0	0.26 ± 0.11	26.4 ± 5.1 (97)	5.4 ± 1.3	0.13 ± 0.04
Ser-235 → Ala tau	13.8 ± 3.7 (891)	10.2 ± 0.9	0.45 ± 0.13	26.1 ± 5.1 (525)	9.9 ± 1.0	0.23 ± 0.05
Ser-396/404 → Ala tau	13.0 ± 3.6 (701)	10.3 ± 0.7	0.49 ± 0.14	20.8 ± 4.6 (691)	10.0 ± 0.9	0.30 ± 0.07

phosphorylation alters the properties of tau with respect to microtubule assembly in a system *in vitro*.

Tau isoform 1 and three mutant forms of tau were phosphorylated with GSK-3β; the Western blot analysis is shown in Figure 2. Phosphorylated epitopes recognized by antibodies AT8 (phosphorylated Ser-202 and Thr-205 [45,46]) and 8D8 (phosphorylated Ser-396 [44]) were generated on phosphorylation of wild-type tau isoform 1 with GSK-3β. To investigate the contribution of particular phosphorylation sites to the properties of tau, certain sites were mutated to alanine residues so as to prevent their phosphorylation. The sites were Ser-199 and Ser-202 as a pair, Ser-235, and Ser-396 and Ser-404 as a pair. After phosphorylation of these mutants with GSK-3β, the absence of AT8 staining of the phosphorylated Ser-199/202 → Ala mutant and absence of 8D8 staining of the phosphorylated Ser-396/404 → Ala mutant was observed, as expected in view of the specificity of these antibodies for the phosphorylation sites removed by mutagenesis.

The effect of tau and each of the tau mutants, non-phosphorylated and phosphorylated, on the self-assembly of tubulin at 37 °C was investigated. Care was taken to ensure that all components of each microtubule assembly reaction were identical apart from the tau species present. At 60 min from the start of self-assembly, the resulting microtubule populations were sampled for analysis by dark-field microscopy and the average length of the microtubules was determined (Figure 3). This dark-field assay, rather than bulk assembly in a spectrophotometer, was used to determine the effect of tau on microtubule self-assembly owing to a limited supply of GSK-3β. Table 1 shows the experimentally determined average length as well as the measured C_{ps} and the derived C_n for all the microtubule assembly conditions. It is clear that there are significant differences in the populations of microtubules assembled in the presence of the various tau species. In all cases, microtubules assembled in the presence of unphosphorylated tau are shorter than in the absence of tau, and this effect is abolished partly or entirely by phosphorylation.

Microtubules assembled in the presence of wild-type tau isoform 1 were much shorter and more numerous (average microtubule length $\langle L \rangle = 15.0\ \mu$m, $C_n = 0.30$ nM) than those assembled in the absence of tau ($\langle L \rangle = 25.6\ \mu$m, $C_n = 0.19$ nM). In a cuvette experiment, the steady state of microtubule assembly was achieved at approx. 15 min in the presence of wild-type tau and at approx. 30 min in the absence of tau. The lag phase of the self-assembly was shortened in the presence of the wild-type tau. These results indicate that the nucleation of microtubule assembly is promoted by wild-type tau. Because microtubules show dynamic instability under these assembly conditions and in the absence of tau, the question arises of whether tau is able to affect this property. In control experiments it was found that $\langle L \rangle$ for tubulin alone increased by 11 μm from 30 to 60 min at steady-state, whereas at 2.5 % wild-type tau (molar percentage relative to tubulin concentration), $\langle L \rangle$ increased by only 5.4 μm. At 5 % tau (molar percentage relative to tubulin concentration) no effect on $\langle L \rangle$ was observed. In principle, dynamic instability can be a major contributor to the length redistribution of microtubules under conditions where the fast shortening phase causes the disappearance of microtubules from the population, resulting in a decrease in C_n. However, the effect of wild-type tau is clearly to increase C_n. In addition, direct microscopic observations showed that length excursions of individual microtubules were not suppressed by wild-type tau (in agreement with reports by Trinczek et al. [27]) under conditions where the dynamic length redistribution is greatly decreased. Thus the major effect of wild-type tau does not seem to be on microtubule dynamic instability: an alternative explanation for changes in $\langle L \rangle$ and C_n is therefore required. In the presence of an agent (such as tau) that can enhance nucleation, it is likely that when microtubules shorten to the limit under conditions of dynamic instability, nucleating structures will either persist or reform. Hence changes in C_n at steady state would be much less and the changes in $\langle L \rangle$ would also be decreased. Thus it seems quite feasible for microtubules to retain dynamic instability in the presence of tau and yet show tau-dependent effects on microtubule nucleation and stability.

The interpretation of the observations is somewhat more complicated with the individual mutant forms of tau. However, in qualitative terms the effects on C_n are similar to the effects observed with wild-type tau. In fact, the presence of either the Ser-235 → Ala mutant or the Ser-396/404 → Ala mutant results in an even greater C_n value. However, in the presence of the Ser-199/202 → Ala mutant, a slight but significant decrease in C_p is observed, from 7.7 ± 0.7 μM in the control assembly to 5.8 ± 2.0 μM, indicating that this isoform has a destabilizing effect on the microtubule lattice [54]. This does not exclude, however, the possibility that this tau should not also be a promotor of microtubule nucleation because, in principle, these two properties are not necessarily related to one another. The fact that the microtubules are shorter in the presence of Ser-199/202 → Ala tau than tubulin alone might have resulted from the stimulation of nucleation or from the inhibition of growth (compare [48]), or both. Although there is some difference in C_n between this case (0.26 nM) and for tubulin in the presence of wild-type tau (0.30 nM), C_n is still higher than in the control (0.19 nM), suggesting that increased microtubule nucleation is

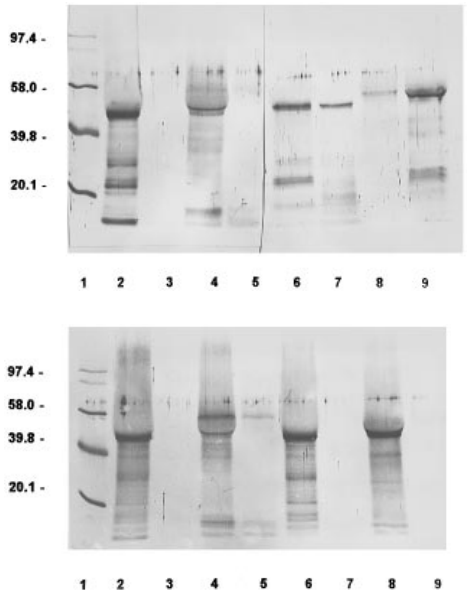

Figure 4 The binding of wild-type tau, mutant forms of tau and phosphorylated tau to microtubules

Microtubules were assembled in the presence of the tau species as indicated in the Materials and methods section. Samples were centrifuged and the pelleted microtubules were dissolved in PEM buffer. Supernatants and resuspended pellets were analysed by separating proteins on SDS/10% (w/v) polyacrylamide gels, transferring them to nitrocellulose and probing with the tau polyclonal antibody TP70. Upper panel: lane 1, molecular mass markers (values in kDa indicated at the left); lane 2, wild-type tau, pellet; lane 3, wild-type tau, supernatant; lane 4, GSK-3β-phosphorylated wild-type tau, pellet; lane 5, GSK-3β-phosphorylated wild-type tau, supernatant; lane 6, Ser-199/202 → Ala mutant tau, pellet; lane 7, Ser-199/202 → Ala mutant tau, supernatant; lane 8, GSK-3β-phosphorylated Ser-199/202 → Ala mutant tau, pellet; lane 9, GSK-3β-phosphorylated Ser-199/202 → Ala mutant tau, supernatant. Lower panel: lane 1, molecular mass markers (values in kDa indicated at the left); lane 2, Ser-235 → Ala mutant tau, pellet; lane 3, Ser-235 → Ala mutant tau, supernatant; lane 4, GSK-3β-phosphorylated Ser-235 → Ala mutant tau, pellet; lane 5, GSK-3β-phosphorylated Ser-235 → Ala mutant tau, supernatant; lane 6, Ser-396/404 → Ala mutant tau, pellet; lane 7, Ser-396/404 → Ala mutant tau, supernatant; lane 8, GSK-3β-phosphorylated Ser-396/404 → Ala mutant tau, pellet; lane 9, GSK-3β-phosphorylated Ser-396/404 → Ala mutant tau, supernatant.

probably the predominant effect. Interestingly, the distribution of unphosphorylated Ser-199/202 → Ala tau between the pellet (microtubules) and the supernatant (solution) after ultracentrifugation was markedly different from that observed with the wild-type tau and the other mutants (Figure 4). In particular, a large fraction of this mutant was recovered in the supernatant, indicating that it was not bound to microtubules. This suggests that the Ser-199/202 → Ala mutation results in a reduced affinity of tau for the microtubules; however, the small decrease in C_p might indicate a preferential interaction of Ser-199/202 → Ala tau with tubulin in solution, thereby displacing the steady-state balance towards the disassembled state. At 22 μM tubulin and 2.2 μM wild-type tau, it was observed that wild-type tau isoform 1 can form aggregates with tubulin that appear amorphous in negative-stain electron microscopy and are not pelleted under the described conditions for ultracentrifugation. The concentration conditions of the experiments in this work were chosen to avoid this complication, but it is possible that Ser-199/202 → Ala tau has a higher propensity for such aggregation and that this might be further enhanced by its phosphorylation (Figure 4).

For the other mutant tau proteins, Table 1 shows that the effect of the mutations Ser-235 → Ala and Ser-396/404 → Ala is to cause an increase in C_p to approx. 10 μM, indicating that the microtubule lattice is stabilized compared with control microtubules in the absence of tau. The increase in the C_n values indicates that microtubule nucleation is efficient in the presence of these mutants, and this can be due in part to their stabilizing effect on the microtubule lattice (compare the differential effects on nucleation and stabilization observed with MAP1B and MAP2 [54]). In principle it is possible that all the mutations studied could result in differences in the ability of tau to nucleate microtubule assembly, because the region of tau associated with the ability to promote microtubule nucleation seems to be residues 154–307 [6,8,13].

The effect of phosphorylation of tau and the mutants on the parameters of microtubule assembly are also shown in Table 1. In all cases studied here, the phosphorylation of a given tau species has little effect on C_p but has significant effects on $\langle L \rangle$ and hence on C_n. With the exception of the Ser-199/202 → Ala mutant, the phosphorylated tau is still mainly pelleted with the polymer (Figure 4), indicating that the affinity of tau for microtubules is largely unaffected by phosphorylation with GSK-3β. The ability of GSK-3α and GSK-3β to phosphorylate tau and to have only a small influence on the binding affinity of tau for microtubules *in vitro* is well established [36,50]. The absence of a significant effect on C_p also suggests that the phosphorylation of a given tau has little or no effect on its ability to stabilize the microtubule lattice. However, for wild-type tau and the three mutants the effect of phophorylation is a substantial increase in $\langle L \rangle$, similar in fact to that of the control in the absence of tau. At the same time, for a given tau the effect of the phosphorylation is also to decrease the observed C_n value, again towards the levels observed with microtubules in the absence of tau. Given that, for any given tau, C_p remains unaffected by its phosphorylation, the marked effects of phosphorylation observed on $\langle L \rangle$ and C_n are unlikely to be due to marked effects of the phosphorylation on the dynamic behaviour of microtubules [54] but are more likely to be due to a decreased ability of the phosphorylated tau to nucleate microtubule assembly.

Phosphorylation of Ser-262 located within a microtubule-binding repeat of tau seems to promote the dissociation of tau from microtubules [55]. Under the phosphorylation conditions used here it is highly unlikely that GSK-3β would phosphorylate this site, as this has been shown to occur only under stimulatory conditions in the presence of heparin [38]. From our results it seems that phosphorylation of residues that are only adjacent to the microtubule-binding repeats might not have the more marked effect of dissociating tau from the microtubules but might have a more subtle regulatory effect on microtubule assembly exerted by tau molecules that remain bound to the microtubules.

Closer inspection of the effect of the phosphorylated mutant isoforms of tau shows that preventing the phosphorylation of residues 199 and 202 or residue 235 does not alter the overall effect of phosphorylation on microtubule assembly compared with the phosphorylation of wild-type tau. However, when residues 396 and 404 are not phosphorylated, the ability of GSK-3β phosphorylated Ser-396/404 → Ala tau to promote microtubule assembly seems to be partly preserved: in the presence of phosphorylated Ser-396/404 → Ala tau, $\langle L \rangle = 20.8 \pm 4.6$ μm and $C_n = 0.30 \pm 0.09$ nM, whereas in the presence of phosphorylated wild-type tau $\langle L \rangle = 28.4 \pm 5.3$ μm and $C_n = 0.16 \pm 0.06$ nM. This suggests that one or both of these sites could be important in regulating the function of tau by phosphorylation. However, because the five serine residues investigated here might not be the only residues to be phosphorylated by GSK-3β, the possibility cannot be excluded that the phosphorylation of other residues not considered here might also be important.

In conclusion, it seems that the main effect of the phosphorylation of tau isoform 1 is to decrease or even abolish its ability to nucleate microtubule assembly, rather than to alter the dynamic properties of the resulting microtubules. As many of the sites investigated in the present study can be found in a phosphorylated state *in vivo* [16,17], the ability of phosphorylation of tau to regulate microtubule assembly *in vivo* could occur as a result of phosphorylation at one or more of a number of sites in a manner similar to that shown here.

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