Published in final edited form as:

Neurobiol Dis. 2008 September ; 31(3): 368–377. doi:10.1016/j.nbd.2008.05.011.

Pre-assembled tau filaments phosphorylated by GSK-3 β form large tangle-like structures

Carolyn A. Rankin, Qian Sun, and T. Chris Gamblin*

Department of Molecular Biosciences, University of Kansas, Lawrence, KS 66045

Abstract

Hyperphosphorylated tau protein is a major component of neurofibrillary tangles, a prominent intracellular hallmark of Alzheimer's disease. Both hyperphosphorylated tau and neurofibrillary tangles have been shown to correlate with dementia in Alzheimer's disease, but the relationship between hyperphosphorylation and tangle formation is not clear. Using a cell-free in vitro model system, in which tau polymerization is induced by arachidonic acid, we show that GSK-3 β phosphorylation of pre-assembled tau filaments makes those filaments prone to coalesce into large neurofibrillary tangle-like structures. Five phosphorylation sites, S199, T205, T231, S396 and S404, were identified in the phosphorylated filaments; many of the five are within epitopes recognized by Alzheimer's disease-associated antibodies. These tangle-like structures are optically visible and are similar to those formed by polymerization of GSK-3 β phosphorylated tau monomer and to those isolated from Alzheimer's disease tissue. We conclude that the phosphorylation of tau by GSK-3 β either prior to or following polymerization promotes polymer/polymer interactions that result in stable clusters of tau filaments.

Keywords

Tau; Alzheimer's disease; GSK-3 β ; phosphorylation; hyperphosphorylation; neurofibrillary tangle; polymerization

Introduction

Neurofibrillary tangles (NFT), a prominent intracellular hallmark of Alzheimer's disease (AD), are composed primarily of hyperphosphorylated tau protein that has polymerized into straight and paired-helical filaments (reviewed in (Buee et al., 2000; Lee et al., 2001; Stoothoff and Johnson, 2005)). Tau protein is normally very soluble and functions to regulate neuronal microtubule dynamics. Although tau's function is normally modulated via controlled phosphorylation and de-phosphorylation, the pathological deregulation of tau phosphorylation correlates with dementia in AD (Holzer et al., 1994) and appears to precede polymerization and NFT formation (Baner et al., 1989; Vincent et al., 1998). The kinase(s) responsible for the hyperphosphorylation of tau in AD has not been identified, although tau is readily phosphorylated by numerous kinases (reviewed in (Buee et al., 2000; Lee et al., 2001; Stoothoff

*To whom correspondence should be addressed, Department of Molecular Biosciences, University of Kansas, 1200 Sunnyside Ave, Lawrence, KS 66045, 785-864-5065, Fax: 785-864-5321, gamblin@ku.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

and Johnson, 2005)). One leading candidate for this role in AD is glycogen synthase kinase-3 β (GSK-3 β).

GSK-3 β is an attractive candidate for modulating tau phosphorylation levels in AD since GSK-3 β is expressed in neurons, GSK-3 β activity levels are increased in AD brain (Leroy et al., 2007), and GSK-3 β phosphorylates many of the same sites on tau that are phosphorylated in AD (Balaraman et al., 2006; Illenberger et al., 1998; Ishiguro et al., 1993; Song and Yang, 1995). Additionally, many of the sites phosphorylated by GSK-3 β are proline directed sites which were demonstrated to be critical for tau neurotoxicity in a transgenic *Drosophila* model (Steinhilb et al., 2007). Lastly, GSK-3 β greatly increased the severity of disease in transgenic animal models of AD when expressed in conjunction with tau (Engel et al., 2006; Jackson et al., 2002).

Pathological tau polymerization can be modeled in vitro using a well established cell-free system to induce the formation of tau filaments (Abraha et al., 2000; Barghorn and Mandelkow, 2002; Carlson et al., 2007; Chirita et al., 2003; DeTure et al., 2006; King et al., 1999; King et al., 2000; Rankin et al., 2007). We have previously employed this system to investigate the effects of GSK-3 β phosphorylation on the polymerization of tau (Rankin et al., 2007). We found that phosphorylation of monomeric tau by GSK-3 β resulted in arachidonic acid-induced filaments clustering into tangle-like structures similar to those isolated from AD brain (Rankin et al., 2007). In the current study, we are testing the hypothesis that the polymerization of tau can precede phosphorylation by GSK-3 β and the subsequent clustering of tau filaments into NFT-like structures. We have found that GSK-3 β phosphorylates pre-assembled tau filaments at a minimum of five sites causing the filaments to coalesce into optically visible tangle-like structures similar to those formed by pre-phosphorylated tau and those isolated from AD brain. These results suggest that tau phosphorylation, regardless of whether it precedes or follows polymerization, causes the formation of neurofibrillary tangle-like structures and that the formation of these structures could be responsible for the appearance of optically visible tau pathology in AD brain.

Materials and Methods

Reagents and supplies

Arachidonic acid (ARA) was obtained from Cayman Chemicals (Ann Arbor, MI), thioflavine S (ThS) and recombinant glycogen synthase kinase (GSK-3 β) from Sigma (St. Louis, MO), uranyl acetate and formvar carbon coated grids from Electron Microscopy Sciences (Hatfield, PA). SDS-PAGE markers are Precision Plus Protein Standards from Bio-Rad (Hercules, CA). Rabbit polyclonal anti-tau phosphorylation-specific antibodies (sites S199, S199/202, T205, T212, S214, T217, T231, S262, S356, S396, S400, S404, S409, and S422) were obtained from Biosource (now a division of Invitrogen, Carlsbad, CA). The accompanying phosphorylated and non-phosphorylated peptides for each antibody site were also obtained from Biosource.

Recombinant htau40 protein

Full length tau protein (441 amino acids, containing exons 2, 3, and 10) was expressed in and purified from *E. coli* as described previously (Rankin et al., 2005). Protein concentration was determined using the Pierce BCA assay (Pierce Biotechnology, Rockford, IL). The purity of protein was assessed by SDS-PAGE.

GSK-3 β phosphorylation of tau monomer

Twenty micrograms of tau protein at a final concentration of 16 μ M (20 μ g tau/25 μ l reaction) was incubated with 0.018 U GSK-3 β /pmol of tau in phosphorylation buffer containing 40 mM HEPES, pH 7.64, 5 mM EGTA, 3 mM MgCl₂, and 2 mM ATP for 20 h at 30 °C. One unit of

GSK-3 β is defined as the amount of enzyme that will transfer one pmol phosphate from ATP to phosphatase inhibitor 2 per minute at pH 7.5 at 30 °C using a heated lid or in complete submersion in a water bath to avoid condensation. Phosphorylated monomeric tau could be prepared in advance and stored up to a week at -20 °C prior to polymerization with the same results as with freshly phosphorylated samples.

Polymerization of tau monomer

Tau, at a concentration of 2 μ M (20 μ g tau/200 μ l reaction), was polymerized in a buffer containing 10 mM HEPES, pH 7.64, 100 mM NaCl, 5 mM EGTA, and 5 mM DTT. ARA inducer was added to a final concentration of 25 μ M (3.75% ethanol carrier) and the polymerization reaction incubated overnight at room temperature (25 °C).

Polymerization of phosphorylated tau monomer

20 μ g phosphorylated tau in a 25 μ l phosphorylation reaction (prepared as described above), was diluted into a 200 μ l polymerization reaction, such that the final concentrations were 10 mM HEPES, pH 7.64, 100 mM NaCl, 5 mM EGTA, 5 mM DTT, 0.375 mM MgCl₂ and 0.25 mM ATP. The GSK-3 β , MgCl₂ and ATP that carried over from the phosphorylation reaction did not appear to significantly affect polymerization. Polymerization was induced by the addition of ARA at a final concentration of 25 μ M (3.75% ethanol carrier). The polymerization reaction was incubated overnight at room temperature (25 °C).

GSK-3 β phosphorylation of tau polymer

Non-phosphorylated tau protein at a concentration of 2.3 μ M was induced to polymerize in the presence of 29 μ M ARA as described above for 20 h at room temperature (25 °C). Following the incubation, phosphorylation reagents were added to the tau polymerization reaction to a final concentration of 3 mM MgCl₂, 2 mM ATP, and 0.018 U GSK-3 β /pmol tau, bringing the final concentration of tau to 2 μ M and the final concentration of ARA to 25 μ M. The reaction was incubated overnight (16–20 h) at 30 °C.

Determination of phosphate incorporation for tau monomer

Phosphate incorporation was determined as previously described (Rankin et al., 2007) except that three repetitions were performed for each sample and all samples were incubated for 20 h at 30 °C. The final concentration of tau in these phosphorylation reactions was 16 μ M (20 μ g tau/25 μ l reaction). Three microliters of the reaction was diluted into 100 μ l of polymerization buffer (10 mM HEPES pH 7.64, 100 mM NaCl, 0.1 mM EDTA and 5 mM DTT), and filtered through a BIOMAX-10 Ultrafree-MC, 10,000 NMWL filter unit (Millipore, Bedford, MA) to remove unincorporated phosphate. Samples were washed with two-250 μ l volumes polymerization buffer and spun to dryness. The filters were placed in scintillation fluid and counted in a Packard 1600TR liquid scintillation counter.

Determination of phosphate incorporation for tau polymer

Non-phosphorylated tau monomer at a final concentration of 2 μ M was polymerized in the presence of 25 μ M ARA as described above. Following a 20 h incubation at room temperature (25 °C), phosphorylation reagents were added to a final concentration of 3 mM MgCl₂, 0.018 U GSK-3 β /pmol tau, and 2 mM ATP containing 10 μ Ci [γ -³²P] labeled ATP (Specific activity: 3000 Ci/mmol) (Perkin-Elmer, Boston, MA). Reactions were then incubated for 20 h at 30 °C. Since the concentration of tau in the monomer reaction was 16 μ M, but 2 μ M in the pre-assembled filament sample, eight times as much (24 μ l) pre-assembled filament sample was diluted onto BIOMAX-10 Ultrafree-MC, 10,000 NMWL filter units (Millipore, Bedford, MA), then washed and counted as described above. The amount of phosphate incorporated was calculated using the specific activity of the γ -³²P and the molecular concentration of tau.

Parallel reactions minus GSK-3 β were used to determine background counts. Background was subtracted prior to phosphate incorporation calculations.

SDS-PAGE and dot blot analysis of tau protein phosphorylation

Tau polymers (prepared as described above) were sedimented via centrifugation at 80,000 rpm for 30 min at 25 °C in a TLA 100 rotor using a Beckman Optima TLX Ultracentrifuge. No polymer could be detected in the supernatant by ThS fluorescence (as described below). Pelleted filaments were resuspended in 8 M guanidine hydrochloride and incubated for one hour at room temperature (25 °C) to unmask potentially hidden epitopes. The guanidine hydrochloride was removed from the samples using BIOMAX-10 Ultrafree-MC, 10,000 NMWL filter units (Millipore, Bedford, MA) according to manufacturer's protocols. The samples were reconstituted in 100 μ l polymerization buffer (minus ARA). Ten microliters was removed and analyzed by 10% SDS-PAGE. Protein concentrations from the gel (Figure 1A, lanes 2 and 3) were determined using Adobe Photoshop (Adobe Systems, Incorporated, Palo Alto, CA) as previously described (Rankin et al., 2007). The remaining samples were diluted in TBS (20 mM Tris pH 7.5, 150 mM NaCl) and spotted on to Immobilon P membrane (Millipore, Bedford, MA) at 25 ng/spot. Dot blot preparation and analysis was performed as previously described (Rankin et al., 2007).

Dot blots of tau peptides

Two different non-phosphorylated peptides, (approximately 12–22 amino acids in length, Biosource) and containing tau phosphorylation sites, S199 and S396, were phosphorylated by GSK-3 β under the same conditions as for phosphorylation of pre-assembled filaments as described above. In addition, a control reaction containing ethanol carrier but without ARA was run in parallel. Duplicate dot blots were prepared by spotting 50 and 20 ng onto Immobilon P membrane (Millipore, Bedford, MA) and probed with anti-pS199 or anti-pS396 polyclonal antibodies (Biosource). Preparation and analysis of dot blots was as previously described (Rankin et al., 2007).

Electron microscopy of tau filaments and tangle-like structures

Grids were prepared and viewed as previously described (Rankin et al., 2007). Measurements of individual tangle-like structures were performed utilizing the Optimas analytical imaging software (Media Cybernetics, Silver Spring, MD) and GraphPad Prism software (Graphpad Software, San Diego, CA) as previously described (Rankin et al., 2007). Tangle-like structures were measured for both pre-phosphorylated tau filaments and for phosphorylated pre-assembled tau filaments. Measurements were made for three separate trials of each condition. The following parameters were measured for each tangle-like structure: average filament length within a tangle, number of filaments within a tangle, total length of filaments within a tangle (filament "mass"), the area occupied by each tangle, and the total length of filaments per area occupied by a tangle.

Statistical tools for analyzing filament data

Since many statistical analyses require the assumption that the data being compared are taken from a Gaussian distribution, the normality of the different distributions of tangle measurements (described above) was tested using the D'Agostino and Pearson omnibus normality test in GraphPad Prism. This test determines whether the data is likely to be Gaussian based on the skewness and kurtosis of the data. Since most of the data failed the normality test and were not repeated measures, the data was compared using the Kruskal-Wallis test (a one-way ANOVA nonparametric test that does not assume Gaussian distributions) and Dunns post test to compare all pairs of columns using GraphPad Prism.

Kinetic measurements of tau polymer levels by ThS fluorescence

Tau polymerization reactions were performed as described above for 20 h at room temperature. Phosphorylation reagents were added as described above resulting in a final concentration of 2 μ M tau and 25 μ M ARA. ThS was added to a final concentration of 20 μ M. The levels of polymer during the phosphorylation reaction were monitored in a time dependent fashion using a FlexStation II fluorometer microplate reader (Molecular Devices Corporation, Sunnyvale, CA). Settings included: Excitation λ = 440, Emission λ = 520, PMT = high. Readings were taken every 30 min for 20 h.

Sucrose gradient analysis

Tangle-like structures were analyzed by sucrose gradient centrifugation as previously described (Rankin et al., 2007).

Epifluorescence microscopy of tangle-like structures

Non-phosphorylated tau polymers, GSK-3 β pre-phosphorylated tau polymers and pre-assembled tau polymers phosphorylated by GSK-3 β were prepared as described above. ThS was added to each sample at a final concentration of 20 μ M and 150 of the 200 μ l sample was loaded into glass bottomed 14 mm microwell 35 mm petri dishes (Matek, Ashland, MA), covered with a #1.5 cover slip (Fisher, Pittsburgh, PA) and sealed with silicone grease. The total time for sample preparation was approximately 48 h as phosphorylation and polymerization reactions were run sequentially, each for approximately 20 h.

Samples were viewed with an Olympus IX81 optical microscope using a 60 $\times$ planapochromat NA 1.45 n.a. epi-fluorescence objective and were excited using a 100W mercury bulb. Excitation was performed at λ = 458 $\pm$ 20 nm and fluorescent emission was detected over a range of 515 $\pm$ 15 nm. Exposure of labeled samples to excitation wave-lengths was limited to 200 milliseconds. Fluorescence was detected using an electron-multiplying back-thinned cooled coupled detector (EMBTCCD). Intensification of camera signal was kept constant, but exposure times varied with the density of fluorescence across the fields in each sample in order to avoid pixel saturation. Images were captured on a Hamamatsu 14 bit C9100 camera using the Slidebook software package (Intelligent Imaging Innovations, Inc., Denver, CO).

Results

GSK-3 β phosphorylates pre-assembled tau filaments

We sought to determine whether tau filaments, formed in the presence of arachidonic acid (ARA), could be phosphorylated by GSK-3 β . Monomeric tau protein phosphorylated by GSK-3 β demonstrates a characteristic upward mobility due to SDS-resistant conformational changes caused by phosphorylation (Goux, 2002) (Figure 1A, lane 1). GSK-3 β phosphorylated tau monomer that is induced to polymerize in the presence of arachidonic acid (ARA) also shows the characteristic band shift (Figure 1A, lane 2), although approximately 90% of the material is lost during the process of pelleting the filaments, denaturing them with guanidine hydrochloride, exchanging buffer and resuspending in SDS-sample buffer. Non-phosphorylated tau monomer that is induced to polymerize in the presence of ARA before being phosphorylated with GSK-3 β did not show an upward shift (Figure 1A, lane 3) and was more similar to non-phosphorylated monomer (Figure 1A, lane 4); the polymerized sample also had a significant loss of material during sample preparation. These results suggested that pre-formed tau filaments are either not phosphorylated by GSK-3 β or are not phosphorylated at sites that produce guanidine hydrochloride- and SDS-resistant monomer conformation. To distinguish these possibilities, we utilized phosphorylation-specific antibodies to analyze dot blots of the samples (Figure 1B). In phosphorylated monomeric tau, eleven of the twelve

previously identified sites were identified (Figure 1B, Column 1 – phos mono.) (Rankin et al., 2007). The same eleven sites were detected in denatured samples of monomer phosphorylated prior to filament formation (Figure 1B, Column 2 – pre-poly.). The polyclonal anti-pS262 used for this study did not detect tau phosphorylation at S262. Following phosphorylation of pre-assembled tau filaments (Figure 1B, Column 3 – post poly), at least five of the eleven sites showed signal greater than background (Figure 1B, Column 4 – mono.). These five sites included S199, T205, T231, S396 and S404.

A comparison of dot blot intensity of monomeric protein and pre-assembled filaments suggested the possibility that pre-assembled filaments are not a good substrate for GSK-3 β . We therefore tested the amount of phosphate incorporation following GSK-3 β phosphorylation under three conditions. The first was at high protein concentrations, equivalent to conditions used to pre-phosphorylate tau. The second was at low protein concentrations, equivalent to conditions used to phosphorylate “pre-assembled tau” but without ARA. The last was at low protein concentrations in the presence of ARA, equivalent to conditions used to phosphorylate pre-assembled tau. The amounts of phosphate incorporation under these three conditions were 6.0 ± 0.7 , 2.8 ± 0.6 and 1.7 ± 0.7 mol phosphate / mol tau respectively ($n = 3$ for each condition). These results suggested that either tau polymer is a less suitable substrate for GSK-3 β or that GSK-3 β is inhibited by ARA. To eliminate the effect of polymerization on phosphorylation efficiency, two tau peptides containing phosphorylation sites S199 and S396 were phosphorylated and assayed by dot blot analysis (Figure 2A). Under conditions comparable to phosphorylation of pre-assembled filaments, the presence of ARA reduced the amount of peptide phosphorylation by approximately 40% (Figure 2B); a similar reduction in phosphate incorporation was also observed for full length tau in the presence of ARA. We conclude that tau filaments are a good substrate for GSK-3 β and phosphorylation can be detected at a minimum of five sites. However, these sites or levels of phosphorylation are insufficient to produce an SDS- and guanidine hydrochloride-resistant SDS-PAGE band shift. The reduction of phosphorylation levels is due to lower protein concentrations and a partial inhibition of GSK-3 β by ARA.

GSK-3 β phosphorylation of pre-assembled tau filaments results in the formation of neurofibrillary tangle-like structures

To test the effects of GSK-3 β phosphorylation on pre-assembled tau filaments, samples were analyzed by electron microscopy. The filaments were localized into discrete tangle-like clusters (Figure 3A). If any component of the phosphorylation reaction was not included (ATP, MgCl₂ or GSK-3 β) the filaments did not cluster together (Figures 2B, 2C and 2D respectively). These results indicate that phosphorylation of tau is a primary requirement for the formation of neurofibrillary tangle-like structures.

Pre-assembled filaments coalesce slowly over time

The formation of neurofibrillary tangle-like structures was monitored by electron microscopy in a time dependent fashion following the addition of GSK-3 β , ATP and MgCl₂. At time zero, immediately following the addition of the kinase, the tau filaments were evenly distributed on the grid surface with no indication of local accumulations of filaments (Figure 4A). After 2 h of incubation in the presence of kinase, some examples of small accumulations of tau filament were observed (Figure 4B). The number and size of accumulations increased after 4 h of incubation (Figure 4C). By 20 h the majority of filaments had coalesced into large discrete accumulations with few unassociated filaments remaining (Figure 4D). Parallel reactions were monitored by ThS fluorescence (Figure 4E). Only a gradual and modest decline in the intensity of ThS fluorescence was detected over the 20 h incubation period. The data suggests that pre-assembled filaments aggregate into neurofibrillary tangle-like structures following GSK-3 β

phosphorylation. However, a more complicated alternate pathway of disassembly and reassembly cannot be entirely ruled out by this data.

Neurofibrillary tangle-like structures formed from pre-phosphorylated tau monomer and from phosphorylation of pre-assembled tau filaments are similar

Samples of filaments formed using GSK-3 β phosphorylated tau monomer were viewed by electron microscopy (Figure 5A, pre-phosphorylation) and compared to pre-assembled filaments that were subsequently phosphorylated by GSK-3 β (Figure 5B and 5C, post phosphorylation). In both cases there was a predominance of clustered filaments. At higher magnification it was apparent that the clusters shared structural similarities with neurofibrillary tangle-like structures generated from GSK-3 β pre-phosphorylated tau as previously described (Rankin et al., 2007). These included filaments that are in close proximity to the cluster but without apparent direct contact (Δ), branching filaments (*) and tendril-like fibers bridging the lateral gap between larger filaments ($\blacktriangle$) (Figure 5C).

The properties of tangle-like structures are similar regardless of how they are produced

Digital electron micrographs of tangle-like structures generated from the polymerization of pre-phosphorylated tau and from the phosphorylation of pre-assembled filaments were analyzed to measure A) the number of filaments in each tangle, B) the total length of filaments in each tangle, C) the area covered by each tangle, and D) the density of filaments in each tangle (total filament length/area). The experiments were performed in triplicate and the resulting measurements are shown in Figure 6 A-D. There was considerable overlap between the two conditions for tangle formation (Figure 6 A-D), suggesting that the tangles were quite similar regardless of how they were produced. The general trend in the data was that tangles produced by the GSK-3 β phosphorylation of pre-assembled filaments (post 1, 2 and 3) had fewer filaments, decreased mass (sum of all filament lengths), smaller size, and had greater density than tangles produced from pre-phosphorylated tau protein. However, statistical analyses were difficult due to the large biological variability within individual trials and due to the unlikelihood that the data was Gaussian. Moreover, the significance in variation between repeated trials was not consistent (Supplementary Table). The data show that while the order of phosphorylation may alter the properties of tangle-like structures somewhat, it is not a major determinant of tangle size, shape and density.

The tangle-like structures are similar to those isolated from human AD brain as analyzed by sucrose gradient centrifugation

Tangles generated from pre-phosphorylated tau and those generated by phosphorylation of pre-assembled filaments were subjected to discontinuous sucrose density gradient centrifugation through 1.0, 1.5 and 2.0 M sucrose. The fractions were analyzed by electron microscopy (Figure 7). Tangle-like structures were found at the 1.0/1.5 M and/or 1.5/2.0 M interfaces (AL2 or AL3 per Ksiezak-Reding), the same positions to which tangle-like structures isolated from human AD brain had migrated (Ksiezak-Reding et al., 1994). The tangles formed from pre-phosphorylation conditions migrated to the AL2 region (Figure 7B), the position to which the majority of tangle-like structures isolated from human brain had migrated. And, tangles formed by phosphorylated filaments migrated to both the AL2 and AL3 regions (Figure 7 E, F).

The sucrose gradient centrifugation experiment shows not only that the in vitro tangle-like structures are similar to those isolated from AD brain, but also that they are quite stable. The tangles remained intact through gradient centrifugation and multiple washes to remove the sucrose during grid preparation. These tangle structures are not cross-linked with glutaraldehyde, but prepared for TEM without fixative.

Cell-free in vitro tangles are visible by light microscopy

Given that approximately 60% of the neurofibrillary tangle-like structures are larger than $0.5 \mu\text{m}^2$ and ThS reactive, we investigated whether the structures would be optically visible. Polymerization reactions were stained with ThS and viewed by epifluorescence on an inverted optical microscope with a 60×1.45 n.a. oil immersion objective. Individual tau filaments without GSK-3 β phosphorylation did not produce detectable fluorescence using this technique (Figure 8A). Discrete points of fluorescence approximately $0.75 \mu\text{m}^2$ in area were observed in samples containing tangle-like structures induced by GSK-3 β phosphorylation of tau monomer or of pre-assembled tau filaments (Figure 8B and C, respectively). These results suggest the possibility that neurofibrillary tangle-like structures induced by GSK-3 β are optically visible and that the order of phosphorylation with respect to polymerization is not critical to the process.

Discussion

Previously, in an in vitro cell-free model system we demonstrated that formation of NFT-like structures was closely tied to GSK-3 β phosphorylation of tau (Rankin et al., 2007). In that study, pre-phosphorylated tau monomer was polymerized, resulting in tau filaments that interacted to form tangle-like structures. In this study, we tested the hypothesis that tau polymerization could precede phosphorylation in the formation of NFT-like structures. We found that pre-assembled tau filaments can be phosphorylated by GSK-3 β and that these filaments then cluster into NFT-like tangles. The tangles form as filaments slowly coalesce into localized clusters as they are phosphorylated. A comparison of tangles formed from phosphorylated filaments with those formed from pre-phosphorylated tau monomer showed that the tangles were similar to each other and to NFT-like tangles isolated from AD brain. Finally, the tangles formed in these cell-free, in vitro experiments are optically visible.

In our previous study we identified twelve sites phosphorylated by GSK-3 β in tau monomer (Rankin et al., 2007); here we identified a subset of those sites, S199, T205, T231, S396, and S404, phosphorylated by GSK-3 β in pre-assembled filaments. These five sites are not new to the field of tau AD research; and are recognized by several AD-associated antibodies. S199 and T205 are part of the AT8 epitope (found very early in AD) and the AT8 cross reaction sites (Biernat et al., 1992; Braak et al., 1994; Braak, 1996; Goedert et al., 1995; Mercken et al., 1992; Porzig et al., 2007). Another of the five sites, pT231, is where the prolyl isomerase, Pin1, binds to tau. The Pin1 knock-out mouse model (Liou et al., 2003; Lu et al., 1999) was important in demonstrating a functional relationship between phosphorylated tau and reduced tau-microtubule binding as well as a causal relationship between phosphorylated tau and filament/NFT formation. Phosphorylated S396 and S404 are recognized by two carboxy terminal AD-associated antibodies, PHF-1 and AD2 (Otvos et al., 1994). The carboxy terminal region inhibits ARA induced tau polymerization in vitro (Abraha et al., 2000; Berry et al., 2003) and it has been hypothesized that phosphorylation of S396 and S404 may alter the inhibitory effect of the carboxy terminal region (Abraha et al., 2000).

Although five sites were positively identified as phosphorylated in the pre-assembled filaments, our data does not exclude the possibility that other sites are also phosphorylated. However, since an upward band shift, resistant to both SDS and 8M guanidine hydrochloride, appeared on SDS-PAGE only in the phosphorylated monomer sample, there is a conformational difference between the two samples. That conformational difference may be due to different sites phosphorylated or to a difference in total number of phosphates incorporated, since phosphate incorporation and phosphorylated peptide dot blot studies suggested that arachidonic acid reduced overall phosphorylation by approximately 40%. However, these differences appeared to have little effect on the properties of tangle-like filament clusters. The number of filaments within each tangle, the filament "mass", the area

covered by the tangle, and the density of the tangle were very similar regardless of whether monomer or pre-assembled filaments were phosphorylated.

Historically, NFT structures have been closely associated with AD toxicity, although recent experimental work has suggested that polymerization and NFT formation may reverse rather than cause toxicity. Much work has been undertaken in an attempt to understand the mechanisms of tau filament formation; however, the *in vivo* polymerizing agent for tau in AD has not been identified, although arachidonic acid, the inducer used in our model system, may have a role in the process (Farooqui and Horrocks, 2006). Our studies are the first to clarify the role of phosphorylation in NFT formation. We have learned that although phosphorylation appears to have only a modest effect on tau polymerization (Rankin et al., 2005; Rankin et al., 2007), it plays a significant role in formation of NFT-like structures. Further, formation of these NFT-like structures does not depend on tau conformational changes resistant to SDS or chaotropic agents. It is noteworthy that five sites identified as phosphorylated in pre-assembled filaments are used to identify AD tau pathology. Significantly, phosphorylation sites that produce NFT-like tangles in our *in vitro* model appear to overlap with sites appearing very early in AD (S199, T205), with sites involved in loss of tau-microtubule binding (T231), and with sites hypothesized to reduce carboxy-terminal inhibition of polymerization (S396, S404); therefore it is not surprising that these particular characteristics of tau pathology either presage or are closely associated with the formation of tau neurofibrillary tangles. It is also significant that protective cellular mechanisms (such as the binding of Pin1 to pT231 with the subsequent increase in the efficiency of phosphatase, PP2A (Liou et al., 2003; Lu et al., 1999), or the CHIP-Hsc70 ubiquitination that clears phosphorylated tau through the proteosome pathway or induces tau structures that are less toxic (Shimura et al., 2004)) are triggered by GSK-3 β phosphorylation at sites we identified in phosphorylated tau monomer and phosphorylated pre-assembled tau filaments.

In conclusion, we have demonstrated that the formation of neurofibrillary tangle-like structures similar to those purified from Alzheimer's disease brain can be generated by GSK-3 β phosphorylation of pre-assembled tau filaments. This suggests that tau polymerization can precede phosphorylation in tangle formation. We have also demonstrated that the neurofibrillary tangle-like structures can be detected optically. A large part of the tau literature consists of observations on tissue or cell samples viewed by light microscopy; therefore optically visible NFTs are a major feature of the accumulated data and conclusions. Further, the *in vitro* NFT-like structures are phosphorylated at sites within epitopes recognized by antibodies AT8, TG3, AT180, PHF1 and AD2 that also recognize AD pathology. Therefore, we conclude that the hyperphosphorylation implicated in tau toxicity also renders tau filaments prone to coalesce into neurofibrillary-tangle like structures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank David Moore and Heather Shinogle, Director and technician, respectively, of Microscopy and Analysis Imaging Laboratory, University of Kansas, for their help in obtaining the epifluorescence data. We thank Kellen Voss and Mike Branden for their critical reading of the manuscript. This work was supported by AG022428 (TCG) and AG025898 (TCG).

References

- Abraham A, et al. C-terminal inhibition of tau assembly *in vitro* and in Alzheimer's disease. *J Cell Sci* 2000;113(Pt 21):3737–3745. [PubMed: 11034902]

- Balaraman Y, et al. Glycogen synthase kinase 3 β and Alzheimer's disease: pathophysiological and therapeutic significance. *Cell Mol Life Sci* 2006;63:1226–1235. [PubMed: 16568235]
- Bancher C, et al. Accumulation of abnormally phosphorylated tau precedes the formation of neurofibrillary tangles in Alzheimer's disease. *Brain Res* 1989;477:90–99. [PubMed: 2495152]
- Barghorn S, Mandelkow E. Toward a unified scheme for the aggregation of tau into Alzheimer paired helical filaments. *Biochemistry* 2002;41:14885–14896. [PubMed: 12475237]
- Berry RW, et al. Inhibition of tau polymerization by its carboxy-terminal caspase cleavage fragment. *Biochemistry* 2003;42:8325–8331. [PubMed: 12846581]
- Biernat J, et al. The switch of tau protein to an Alzheimer-like state includes the phosphorylation of two serine-proline motifs upstream of the microtubule binding region. *Embo J* 1992;11:1593–1597. [PubMed: 1563356]
- Braak E, et al. A sequence of cytoskeleton changes related to the formation of neurofibrillary tangles and neuropil threads. *Acta Neuropathol (Berl)* 1994;87:554–567. [PubMed: 7522386]
- Braak, H. Evolution of Alzheimer's disease related intraneuronal changes. Fifth International Conference: On Alzheimer's Disease And Related Disorders; Osaka, Japan. 1996.
- Buee L, et al. Tau protein isoforms, phosphorylation and the role in neurodegenerative disorders. *Brain Res Brain Res Rev* 2000;33:95–130. [PubMed: 10967355]
- Carlson SW, et al. A complex mechanism for inducer mediated tau polymerization. *Biochemistry*. 2007In Press
- Chirita CN, et al. Anionic micelles and vesicles induce tau fibrillization in vitro. *J Biol Chem* 2003;278:25644–25650. [PubMed: 12730214]
- DeTure M, et al. Evidence for independent mechanisms and a multiple-hit model of tau assembly. *Biochem Biophys Res Commun* 2006;339:858–864. [PubMed: 16325769]
- Engel T, et al. Coexpression of FTDP-17 tau and GSK-3 β in transgenic mice induce tau polymerization and neurodegeneration. *Neurobiol Aging* 2006;27:1258–1268. [PubMed: 16054268]
- Farooqui AA, Horrocks LA. Phospholipase A2-generated lipid mediators in the brain: the good, the bad, and the ugly. *Neuroscientist* 2006;12:245–260. [PubMed: 16684969]
- Goedert M, et al. Tau protein in Alzheimer's disease. *Biochem Soc Trans* 1995;23:80–85. [PubMed: 7758799]
- Goux WJ. The conformations of filamentous and soluble tau associated with Alzheimer paired helical filaments. *Biochemistry* 2002;41:13798–13806. [PubMed: 12427043]
- Holzer M, et al. Abnormally phosphorylated tau protein in Alzheimer's disease: heterogeneity of individual regional distribution and relationship to clinical severity. *Neuroscience* 1994;63:499–516. [PubMed: 7891861]
- Illenberger S, et al. The endogenous and cell cycle-dependent phosphorylation of tau protein in living cells: implications for Alzheimer's disease. *Mol Biol Cell* 1998;9:1495–1512. [PubMed: 9614189]
- Ishiguro K, et al. Glycogen synthase kinase 3 β is identical to tau protein kinase I generating several epitopes of paired helical filaments. *FEBS Lett* 1993;325:167–172. [PubMed: 7686508]
- Jackson GR, et al. Human wild-type tau interacts with wingless pathway components and produces neurofibrillary pathology in *Drosophila*. *Neuron* 2002;34:509–519. [PubMed: 12062036]
- King ME, et al. Ligand-dependent tau filament formation: implications for Alzheimer's disease progression. *Biochemistry* 1999;38:14851–14859. [PubMed: 10555967]
- King ME, et al. Differential assembly of human tau isoforms in the presence of arachidonic acid. *J Neurochem* 2000;74:1749–1757. [PubMed: 10737634]
- Ksiezak-Reding H, et al. Tau immunoreactivity and SDS solubility of two populations of paired helical filaments that differ in morphology. *Brain Res* 1994;649:185–196. [PubMed: 7953631]
- Lee VM, et al. Neurodegenerative tauopathies. *Annu Rev Neurosci* 2001;24:1121–1159. [PubMed: 11520930]
- Leroy K, et al. Increased level of active GSK-3 β in Alzheimer's disease and accumulation in argyrophilic grains and in neurones at different stages of neurofibrillary degeneration. *Neuropathol Appl Neurobiol* 2007;33:43–55. [PubMed: 17239007]
- Liou YC, et al. Role of the prolyl isomerase Pin1 in protecting against age-dependent neurodegeneration. *Nature* 2003;424:556–561. [PubMed: 12891359]

- Lu PJ, et al. The prolyl isomerase Pin1 restores the function of Alzheimer-associated phosphorylated tau protein. *Nature* 1999;399:784–788. [PubMed: 10391244]
- Mercken M, et al. Monoclonal antibodies with selective specificity for Alzheimer tau are directed against phosphatase-sensitive epitopes. *Acta Neuropathol* 1992;84:265–272. [PubMed: 1384266]
- Otvos L Jr, et al. Monoclonal antibody PHF-1 recognizes tau protein phosphorylated at serine residues 396 and 404. *J Neurosci Res* 1994;39:669–673. [PubMed: 7534834]
- Porzig R, et al. Epitope mapping of mAbs AT8 and Tau5 directed against hyperphosphorylated regions of the human tau protein. *Biochem Biophys Res Commun* 2007;358:644–649. [PubMed: 17499212]
- Rankin CA, et al. Pseudo-phosphorylation of tau at Ser202 and Thr205 affects tau filament formation. *Brain Res Mol Brain Res*. 2005
- Rankin CA, et al. Tau phosphorylation by GSK-3 β promotes tangle-like filament morphology. *Mol Neurodegener* 2007;2:12. [PubMed: 17598919]
- Shimura H, et al. CHIP-Hsc70 complex ubiquitinates phosphorylated tau and enhances cell survival. *J Biol Chem* 2004;279:4869–4876. [PubMed: 14612456]
- Song JS, Yang SD. Tau protein kinase I/GSK-3 β /kinase FA in heparin phosphorylates tau on Ser199, Thr231, Ser235, Ser262, Ser369, and Ser400 sites phosphorylated in Alzheimer disease brain. *J Protein Chem* 1995;14:95–105. [PubMed: 7786411]
- Steinhilb ML, et al. S/P and T/P phosphorylation is critical for tau neurotoxicity in *Drosophila*. *J Neurosci Res* 2007;85:1271–1278. [PubMed: 17335084]
- Stoothoff WH, Johnson GV. Tau phosphorylation: physiological and pathological consequences. *Biochim Biophys Acta* 2005;1739:280–297. [PubMed: 15615646]
- Vincent I, et al. Mitotic phosphoepitopes precede paired helical filaments in Alzheimer's disease. *Neurobiol Aging* 1998;19:287–296. [PubMed: 9733160]

Figure 1. Tau monomer and polymer are phosphorylated by GSK-3 β

Panel A: Coomassie stained 10% SDS-PAGE was used to compare products from reactions in which either tau monomer or polymer was phosphorylated. Lane 1: 1 μ g phosphorylated tau protein. Lane 2: denatured filaments formed with GSK-3 β phosphorylated monomer. Lane 3: denatured GSK-3 β phosphorylated pre-assembled filaments. Lane 4: 1 μ g non-phosphorylated tau protein. Lane 5: molecular weight markers. Filaments for lanes 2 and 3 were pelleted by centrifugation, denatured with 8 M guanidine hydrochloride, and boiled in Laemmli SDS sample buffer prior to electrophoresis, resulting in a loss of material. Panel B: 25 ng of the samples analyzed by coomassie stained SDS-PAGE in Panel A were dotted onto Immobilon P membrane. Column 1: Phos Mono. = phosphorylated tau monomer; Column 2: Pre-poly. =

phosphorylation occurred prior to polymerization; Column 3: Post-poly. = phosphorylation occurred after polymerization; Column 4: Mono. = non-phosphorylated monomer. The protein concentration of the denatured filaments (Columns 2 and 3) was determined from the coomassie stained gel in Panel A. Each row was probed with a polyclonal tau antibody specific for the phosphorylated serine or threonine listed at the right. Sites phosphorylated in tau filaments (Column 3) at levels greater than the background of non-phosphorylated monomer (Column 4) are indicated by asterisks.

Figure 2. Inhibition of GSK-3 β activity by arachidonic acid

Panel A is a dot blot analysis of GSK-3 β phosphorylation reactions using small tau peptides incapable of polymerization. The peptide on the left hand panel contains the S199 phosphorylation site and the peptide on the right hand panel contains the S396 phosphorylation site. 50 ng and 20 ng dots were made in triplicate of non-phosphorylated control peptide (top row, non-phos control), peptides phosphorylated in the absence of ARA (middle row, no ARA) and peptides phosphorylated in the presence of 25 μ M ARA (bottom row, ARA). The peptide samples were probed with their paired antibodies. The extent of antibody staining was determined for the No ARA sample and the ARA containing sample and is graphed in Panel B. Anti-Ser199 staining are white bars and anti-Ser396 are black bars. The bars are an average

of the comparison of all six spots and are shown $\pm$ s.e.m. The presence of ARA reduced phosphorylation by 30–40%, when standardized to the No ARA sample.

Figure 3. Electron micrographs of GSK-3β phosphorylated tau filaments

Panel A: The three reagents required for GSK-3β phosphorylation (ATP, Mg^{2+} , and GSK-3β) were added to pre-assembled tau polymer/filaments resulting in the formation of tangle-like structures. Panel B-D: Omission of any one of the three reagents, ATP, Mg^{2+} , or GSK-3β, respectively, produced only non-interacting filaments. Images were recorded at 20,000 ×. The scale bar in panel D represents 500 nm and is applicable to all four images.

Figure 4. Phosphorylated filaments gradually coalesce into clusters

To analyze formation of tangle-like structures, duplicate GSK-3 β phosphorylation reactions of pre-assembled tau filaments were followed over time. Filament samples for TEM analysis were removed from the phosphorylation reaction at time zero, immediately following addition of GSK-3 β , as well as at 2, 4, and 20 h after GSK-3 β addition. TEM micrographs of these filament samples (Panels A-D) show that over time, tangle-like structures increase in size and number. By 20 h very few independent filaments are observed. Images were recorded at 20,000 $\times$. The scale bar in panel D represents 500 nm and applicable to all four images. ThS kinetics was used to follow the total filament mass over time (Panel E) and showed no dramatic change

in filament mass as tangle-like structures were formed. Measurements were taken every 30 minutes and compared to a control sample with no ThS.

Figure 5. TEM comparison of tangle-like structures

TEM micrographs of tangle-like structures formed by GSK-3 β phosphorylated monomer (Panel A) were compared to those formed by GSK-3 β phosphorylated filaments (Panel B and C). Images in Panel A and Panel B were recorded at 20,000 $\times$. The scale bar in Panel A represents 500 nm and applicable to Panels A and B. The image in Panel C is the same field of view as Panel B but was recorded at 50,000 $\times$ and the scale bar represents 250 nm. Tangle characteristics, such as non-touching filaments (Δ), branching filaments (*) and tendril-like fibers between longer filaments ($\blacktriangle$) are identified.

Figure 6. Properties of tangle-like structures are similar regardless of whether the tangles are formed from phosphorylated tau monomer or polymer

TEM images of tangle-like structures were analyzed with the Optimas analytical imaging software and GraphPad Prism software. A total of two hundred thirty tangle-like structures were analyzed in three separate experiments. Each of the three experiments contained two samples, one in which phosphorylation preceded polymerization (Pre1, Pre2, and Pre3) and one in which pre-assembled filaments were phosphorylated, i.e. "post polymerization" (Post1, Post2, Post3). Number of tangles analyzed for each sample: Pre1 = 37; Pre2 = 41; Pre3 = 37; Post1 = 37; Post2 = 39; Post3 = 39. Parameters analyzed included the number of filaments per tangle (panel A), the total filament length in each tangle (panel B), the area occupied by each

tangle (panel C) and the density of filaments within the tangle area (panel D). The mean for each sample is represented by a horizontal line.

Figure 7. Sucrose gradient analysis of tangle-like structures

Tangled filaments formed by pre-phosphorylated monomer and by phosphorylation of pre-assembled filaments were subjected to discontinuous sucrose gradient analysis (2.0, 1.5 and 1.0 M sucrose). Tangle-like structures were found at the 1.0/1.5 M interface (Panel B and E) and the 1.5/2.0 M interface (Panel F). Panels A-C show tangles formed from pre-phosphorylated tau monomer (Pre-Poly.); panels D-F, tangles from phosphorylation of pre-assembled filaments (Post-Poly.). Images were recorded at 20,000 $\times$. The scale bar in Panel F represents 500 nm and is applicable to all six images.

Figure 8. Tangle-like structures are optically visible

A control sample with non-phosphorylated tau produced individual non-interacting filaments that are not visible by optical fluorescence microscopy (No phosphorylation, Panel A). Products produced by polymerization of pre-phosphorylated tau monomer (Pre-phosphorylation, Panel B) and phosphorylation of pre-assembled tau filaments (Post-Phosphorylation, Panel C) were stained with ThS and viewed by Epifluorescence on an inverted optical microscope with a $60\times$ oil immersion objective. Excitation $\lambda = 458 \pm 20$ nm and emission $\lambda = 515 \pm 15$ nm. Exposure times = 44 ms, 98 ms, and 402 ms, respectively.