Glycogen Synthase Kinase-3β Is Complexed with Tau Protein in Brain Microtubules*

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In Alzheimer’s disease, microtubule-associated protein tau is hyperphosphorylated by an unknown mechanism and is aggregated into paired helical filaments. Hyperphosphorylation causes loss of tau function, microtubule instability, and neurodegeneration. Glycogen synthase kinase-3β (GSK3β) has been implicated in the phosphorylation of tau in normal and Alzheimer’s disease brain. The molecular mechanism of GSK3β-tau interaction has not been clarified. In this study, we find that when microtubules are disassembled, microtubule-associated GSK3β dissociates from microtubules. From a gel filtration column, the dissociated GSK3β elutes as an ∼400-kDa complex. When fractions containing the ∼400-kDa complex are chromatographed through an anti-GSK3β immunofinity column, tau co-elutes with GSK3β. From fractions containing the ∼400-kDa complex, both tau and GSK3β co-immunoprecipitate with each other. GSK3β binds to nonphosphorylated tau, and the GSK3β-binding region is located within the N-terminal projection domain of tau. In vitro, GSK3β associates with microtubules only in the presence of tau. From brain extract, ∼6-fold more GSK3β co-immunoprecipitates with tau than with GSK3α. These data indicate that, in brain, GSK3β is bound to tau within a ∼400-kDa microtubule-associated complex, and GSK3β associates with microtubules via tau.

In Alzheimer’s disease (AD), microtubule-associated protein tau becomes hyperphosphorylated and aggregates into paired helical filaments (PHFs) (reviewed in Refs. 1 and 2). PHF-tau (tau isolated from PHFs) is highly insoluble, displays a reduction of tau phosphorylation, disrupted microtubules, and promotes microtubule assembly (3, 4). Abnormal phosphorylation has been suggested to cause the loss of tau function, microtubule instability, and neurodegeneration in AD brain (1, 2). Thus, abnormal phosphorylation of tau is an important pathological process during AD ontogeny. The identification of tau kinases and the elucidation of the mechanism of tau phosphorylation are essential to determine how tau becomes abnormally phosphorylated in AD brain.

There are six tau isoforms generated through mRNA alternative splicing (5). On an SDS gel, tau isolated from brain extract displays several bands with sizes 50–68 kDa representing various tau isoforms with different phosphorylation states (5, 6). In normal brain, tau binds to microtubules and stabilizes microtubule structure. Tau phosphorylation reduces the affinity of tau for microtubules, destabilizes microtubules, and regulates microtubule dynamics (1, 7). Normal adult tau is phosphorylated on four sites, whereas juvenile tau is phosphorylated on 10 sites (8). PHF-tau on the other hand, is phosphorylated on 19 sites (9). Most of these sites are situated at an (S/T)P motif recognized by a proline-directed kinase. Among proline-directed kinases, MAP kinase, Cdc2 kinase, Cdk5, and glycogen synthase kinase-3β (GSK3β) phosphorylate tau in vitro (2). In addition, many non-proline-directed kinases such as CAMP-dependent protein kinase (PKA), protein kinase C, calmodulin-dependent protein kinase II, and phosphorylase kinase also phosphorylate tau in vitro (for a list of tau kinases, see Ref. 10).

GSK3 is widely expressed in various mammalian tissues and cell lines (11). It phosphorylates many proteins such as glycogen synthase, eukaryotic initiation factor 2B, ATP citrate lyase, the regulatory subunit of PKA, c-Jun, c-Myc, CAMP-response element-binding protein, inhibitor-2, β-catenin, neurofilaments, synapsin, and adenomatous polyposi coli gene product (12–22) and regulates glycogen metabolism, lipid synthesis, cytoskeletal arrangement, gene expression, apoptosis, and cell fate specification (see Refs. 12 and 13 for reviews). There are two GSK3 isoforms: ∼51-kDa GSK3α and ∼47-kDa GSK3β. These two isoforms are products of two distinct genes and are abundantly expressed in the brain (11).

In vitro, GSK3β phosphorylates tau on Ser139, Thr214, Ser396, and Ser403 (numbered according to the longest tau isoform) (23), and these sites are phosphorylated in PHF-tau (9). In mammalian cells, co-transfection of tau with GSK3β leads to tau phosphorylation and microtubule rearrangement (24, 25). Transgenic mice overexpressing GSK3β display tau hyperphosphorylation, disrupted microtubules, and apoptotic neurons (26, 27). The GSK3β inhibitor LiCl suppresses tau phosphorylation, enhances tau-microtubule binding, and promotes micro-

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1 The abbreviations used are: AD, Alzheimer’s disease; Cdk5, cyclin-dependent protein kinase 5; GST, glutathione S-transferase; MAP, microtubule-associated protein; Mops, 4-morpholinepropanesulfonic acid; PKA, cAMP-dependent protein kinase; R-tau, recombinant tau; DTT, dithiothreitol; BSA, bovine serum albumin; IB, immunoblot.
tubule assembly in cultured neurons (28, 29). These in vivo observations indicate that GSK3β is a tau kinase and may be involved in pathological tau phosphorylation in AD brain.

Ishiguro et al. isolated tau kinase 1 from bovine brain microtubules (30). Biochemical and molecular cloning studies later determined that tau kinase 1 is GSK3β (31). Subsequent studies have confirmed that GSK3β is tightly associated with neuronal microtubules (32). As discussed above, tau is a microtubule-associated protein and regulates microtubule dynamics by binding to microtubules and stabilizing microtubule structure (7). These studies therefore suggest that GSK3β-tau interaction may occur within microtubules, and this interaction may be important in the regulation of microtubule dynamics. Elucidation of the biochemical nature of tau-GSK3β interaction may also provide a clue as to the cause of abnormal tau phosphorylation in AD brain. However, very little information is available about microtubule-associated GSK3β. In this study, we examined GSK3β within bovine brain microtubules. We now demonstrate that microtubule-associated GSK3β is a ~400-kDa species complexed with tau. We also provide evidence for the binding of GSK3β to microtubules via tau.

**MATERIALS AND METHODS**

cDNA Cloning—Pfu DNA polymerase-catalyzed PCR was carried out in a reaction mixture supplemented with 10% MnSO by using a forward primer (5′-TCC CCG GGG ATG GGG CCG-3′) containing a Smal site (italicized), a reverse primer (5′-CTC GAG TCA GGT GGA TTG ACA GCG-3′) containing a PstI site (italicized), and a pBluescript plasmid template containing human brain GSK3β cDNA (a gift from J. R. Woodgett, University of Toronto). PCR conditions were as follows: one cycle of 94°C for 1 min; 25 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 2 min; and one cycle of 72°C for 10 min. Adenine overhangs were added to the PCR product by incubating the product with Adenine-HindIII in Exonuclease I/T4 DNA polymerase at 72°C for 10 min. Then, 10 μl of a final PCR product was purified and ligated into a pGEX-T Easy TA vector (Promega, Madison, WI) and amplified. GSK3β cDNA was excised by SmaI/XhoI from the pGEX-T Easy TA vector and ligated into the Smal/XhoI site of a pGEX-6P-2 vector (Amerham Biosciences). The recombinant plasmid was transfected into E. coli BL21(DE3) cells. The cloning was verified by DNA sequencing. Construction of various tau deletion mutant plasmids has been described previously (33).

**Proteins—Glutathione S-transferase (GST)-GSK3β was purified from bacterial lysates. The overnight bacterial culture was diluted 20-fold in a fresh medium and incubated at 37°C with vigorous shaking. After ~4 h, protein expression was induced by adding isopropyl-β-D-thiogalactoside to 1 mM, and the incubation and shaking was continued for another 4 h. The incubated medium was centrifuged at 16,000 × g for 30 min, and the pellet was suspended in ice-cold lysis buffer (50 mM Tris-HCl (pH 7.5), 0.2 mM NaCl, 1 mM DT, and 1 mM EDTA) containing 1% Tween 20 and protease inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 5 mM mg/ml of benzamidine, and 1 μg/ml each of leupeptin, aprotinin, and pepstatin). The suspended pellet was sonicated twice for 30 s each and centrifuged at 27,000 × g for 30 min at 4°C. The supernatant was dialyzed against ice-cold buffer for 4 h and then mixed with glutathione-agarose beads (Sigma) previously equilibrated with lysis buffer. The beads were shaken end-over-end overnight and then packed in a glass column. The column was washed with ~30 ml of lysis buffer and eluted with 10 ml reduced glutathione in lysis buffer. Fractons containing GST-GSK3β were combined, concentrated to ~1 ml by dialysis against Aquacide III (Calbiochem), and dialyzed against lysis buffer at 4°C. After dialyzing for 2 h, 10 units of precision protease (Sigma) were added, and the sample was incubated with end-over-end shaking at 4°C for 16 h to remove the GST tag. The incubated sample was loaded onto a glutathione-agarose column equilibrated in lysis buffer. GST remained bound to the column, whereas flow-through fractions containing GSK3β were combined and dialyzed against PEM buffer (0.1 M Pipes (pH 6.8), 1 mM EGTA, 1 mM MgSO4, and 1 mM β-mercaptoethanol) for 4 h at 4°C and used to generate Fig. 7.

Tubulin was purified from bovine brain microtubules through phosphocellulose chromatography (33). Purification of recombinant tau (R-tau) and R-tau deletion mutants has been described previously (33). A polyclonal antibody against the C-terminal region of GSK3β (QT-NNAASASASNSD) was prepared at Zymed Laboratories Inc. (San Francisco, CA). Monoclonal antibodies against tau, GSK3β, GSK3βδ, and tubulin were from NeoMarkers (Fremont, CA), Transduction Laboratories (Lexington, KY), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and Sigma, respectively. A polyclonal anti-GSK3α antibody was purchased from Santa Cruz Biotechnology. Production of anti-tau polyclonal antibodies has been described previously (34).

**Protein and Peptide Concentrations**—The concentration of R-tau was determined spectrophotometrically (35). Concentrations of R-tau deletion mutants and phosphorylated R-tau were determined by a Bio-Rad protein assay using R-tau as the standard. All other protein concentrations were determined by the Bio-Rad protein assay using bovine serum albumin (BSA) as the standard. The concentration of GSK3β substrate was based on amino acid analysis.

**Anti-GSK3β Immunoaffinity Column—**Anti-GSK3β rabbit serum (~0.5 ml) was diluted with an equal volume of coupling buffer (0.1 mM NaHCO3 (pH 8.3), 0.5 mM NaCl) and mixed with ~3 ml of CNBr-activated Sepharose 4B gel (Amersham Biosciences) previously washed with 1 ml HCl and equilibrated in coupling buffer. The mixture was shaken end-over-end overnight at 4°C. After shaking, the gel was recovered by centrifugation and washed with ~20 ml of coupling buffer. The washed gel was blocked with 0.1 ml Tris-HCl (pH 8.0) for 2 h at room temperature and washed with 0.1 mM CH2COONa (pH 4.0), 0.5 mM NaCl followed by 0.1 ml Tris-HCl (pH 8.0), 0.5 mM NaCl. The washed gel was then equilibrated in 25 mM Tris-HCl (pH 7.5), 0.2 mM NaCl, 0.1 mM EDTA, and 0.1 mM DTT.

**GSK3 Activity Assay—**A synthetic peptide (KRREILRSPRSYR), derived from the cAMP response element-binding protein that becomes a specific substrate for GSK3 upon PKA phosphorylation (18), was synthesized at the peptide synthesis facility of the University of Calgary. This peptide was phosphorylated by PKA, and the phosphopeptide was purified by high pressure liquid chromatography. Purified phosphopeptide was used to assay GSK3 activity. The assay was initiated by adding 10 μl of the sample to 20 μl of the reaction mixture containing the rest of the assay components. The final concentrations of assay components were 50 mM HEPES (pH 7.2), 0.1 mM EDTA, 0.1 mM DTT, 0.3 mM [γ32P]ATP, 10 mM MgCl2, and 50 μM PKA-phosphorylated peptide substrate. After 30 min at 30°C, 10 μl of trichloroacetic acid was added to the assay mixture to stop the reaction, and the mixture was incubated at 4°C for 10 min. Following incubation, the assay mixture was centrifuged for 5 min using a bench top centrifuge, and 20 μl of the supernatant was analyzed by phosphocellulose filter paper assay to determine the amount of radioactivity incorporated into the substrate peptide (34). One unit of GSK3 activity transfers 1 pmol of phosphate/min to substrate peptide under the above described standard assay conditions.

**Microtubule Sedimentation Assay—**Microtubule sedimentation assay was carried out as described previously (33) with some modifications. Microtubules were assembled at 37°C in an assay mixture (50 μl each) containing 0.1 mM Pipes (pH 7.0), 1 mM EGTA, 1 mM MgSO4, 1 mM β-mercaptoethanol, 0.4 mg/ml tubulin, 24 μg/ml microtubule-stabilizing drug taxol (Sigma), and 1 mM GTP. When included, the concentrations of taxol were 10, 40, and 40 μg/ml, respectively. After mixing all of the assay components in various combinations, each mixture was incubated at 37°C for 30 min and centrifuged at 50,000 × g for 5 min at 37°C. The supernatant was withdrawn, and the pellet was dissolved in 30 μl of sample buffer for SDS-PAGE. The pellet and the supernatant (15 μl each) were subjected to SDS-PAGE. The gels were either immunoblotted using anti-GSK3β antibody or stained with Coomassie Brilliant Blue (Bio-Rad).

**Purification of GSK3β from the Microtubule Fraction—**All procedures were performed at 4°C. Microtubules were purified from a fresh bovine brain extract by three cycles of temperature-induced microtubule assembly and disassembly as described previously (33). The purified microtubule preparation containing ~5 mg/ml protein was incubated at 0°C for 30 min to disassemble microtubules. The incubated sample was centrifuged at 27,000 × g for 30 min. The supernatant (~25 ml) was loaded onto a phosphocellulose (Whatman, Fairfield, NJ) column (30 × 5 cm) equilibrated in PEM buffer containing 0.1 mM GTP. Flow-through fractions containing tubulin, whereas GSK3 activity remained bound to the column. The column was washed with ~75 ml of PEM buffer and eluted with 200 ml of NaCl gradient (0.1 M microtubule-stabilizing drug taxol, and 1 mM EDTA in PEM buffer). Fractions containing GSK3β activity were combined (~50 ml) and dialyzed against Mops buffer (25 mM Mops (pH 7.4), 50 mM β-glycerophosphate, 0.2 mM NaCl, 10 mM NaF, 15 mM MgCl2, 1 mM EDTA, and 1 mM DTT). The dialyzed sample (labeled MAP-fraction in Figs. 64A and 9) was concentrated to ~7 ml by dialysis against Aquacide III. The concentrated sample was dialyzed against Mops buffer for 2 h and centrifuged at 27,000 × g for 30 min. The supernatant was chro-
RESULTS

Gel Filtration Analysis of Microtubule-associated GSK3β—

In a previous study, ammonium sulfate precipitation was utilized during purification of GSK3β from microtubule fraction (30). This treatment can dissociate a protein complex (33). We therefore excluded the ammonium sulfate precipitation step in our purification procedure and disassembled purified microtubules by incubating them at 0°C for 30 min. Disassembled microtubules were then chromatographed through a phosphocellulose column. Tubulin was recovered within the flow-through fractions, and column-bound GSK3β was eluted with a NaCl gradient (data not shown). Efluent MAP fraction containing GSK3β was analyzed by an FPLC Superose 12 gel filtration column (Fig. 1A). The activity assay (Fig. 1A) and immunoblot analysis (Fig. 1B) showed that GSK3β elutes as a relatively large species from the gel filtration column. The size of GSK3β in Fig. 1A (fraction 52) was estimated to be ~400 kDa. Since GSK3β is a globular protein with size ~47 kDa (11), our data indicate that microtubule-associated GSK3β is part of a ~400-kDa complex.

Identification of Protein Complexed with Microtubule-associated GSK3β—To identify the protein complexed with microtubule-associated GSK3β, we analyzed several gel filtration column fractions in Fig. 1A by immunoblot analysis using antibodies against MAP1, MAP2, tau, and tubulin (data not shown). MAP1 and MAP2 eluted within fractions 32–52. Tau was present within fractions 34–72, whereas tubulin was undetectable in all of the column fractions analyzed. A protein-stained SDS gel (Fig. 1C) showed many protein bands with various sizes in fractions enriched with GSK3β (fractions 44–64).

Since the above described SDS gel (Fig. 1C) and immunoblots did not give us any indication as to the identification of protein(s) complexed with GSK3β in the microtubule fraction, we combined fractions 44–64 from Fig. 1A (designated as the gel filtration fraction) and chromatographed the gel filtration fraction through an anti-GSK3β immunofluorescence column. A silver-stained SDS gel showed a prominent band and three or four faint bands (Fig. 2A) in fraction 5 (the peak effluent fraction). The major ~47-kDa band was identified as GSK3β by immunoblot analysis using anti-GSK3β antibody (data not shown). Faint protein bands migrated with sizes ~50–68 kDa on an SDS gel. Since the various tau isoforms also migrate as ~50–68-kDa bands on a SDS gel, we immunoblotted various anti-GSK3β immunofluorescence column fractions with anti-tau antibody (Fig. 2B). Tau was indeed present in anti-GSK3β immunofluorescence column effluent fractions. These data demonstrate that tau co-elutes with GSK3β from an anti-GSK3β immunofluorescence column and suggest that GSK3β is complexed with tau in the gel filtration fraction.

To gain more evidence in support of the above suggestion, we immunoprecipitated GSK3β from the gel filtration fraction by using an anti-GSK3β antibody and then immunoblotted the anti-GSK3β immunoprecipitate with an anti-tau antibody. In a similar manner, we immunoprecipitated tau from the gel filtration fraction and immunoblotted the anti-tau immunocomplex with an anti-GSK3β antibody. Tau-co-immunoprecipitated with GSK3β (Fig. 3A) and GSK3β co-immunoprecipitated with tau (Fig. 3B). Based on these data, we conclude that tau is complexed with GSK3β in the gel filtration fraction.
GSK3β Directly Binds to Tau—GSK3β may directly bind to tau within a GSK3β-tau complex. Alternatively, GSK3β may associate with tau via another adapter molecule within the complex. To test these possibilities, we performed a GST pull-down assay. Glutathione-agarose beads coated with GST or GST-GSK3β were mixed with R-tau, washed, and then immunoblotted with anti-tau antibody. As shown in Fig. 4, R-tau bound to GST-GSK3β but not GST. These data indicate that GSK3β directly binds to tau.

Location of GSK3β-binding Region within Tau—Tau has three well defined structural domains (Fig. 5A): an N-terminal projection domain, a microtubule-binding region, and a C-terminal tail. To identify the GSK3β-binding region within tau, we constructed three tau deletion mutants: R-tau-(1–244) containing the N-terminal projection domain, R-tau-(245–369) containing the microtubule-binding region, and R-tau-(245–441) containing both the microtubule-binding region and the C-terminal tail. We evaluated the binding of these mutants and wild-type R-tau with GST-GSK3β by the GST pull-down assay. GSK3β bound to wild type (Fig. 5B) and R-tau-(1–244) (Fig. 5C) but not R-tau-(245–369) (Fig. 5D) and R-tau-(245–441) (Fig. 5E). These data demonstrate that GSK3β binds to the N-terminal projection domain of tau.

Effect of Phosphorylation on the Binding of GSK3β to Tau—Tau is a phosphoprotein and binds to many proteins in a phosphorylation-dependent manner (33, 37). To examine if tau phosphorylation influences GSK3β-tau interaction, we examined the phosphorylation state of tau that eluted from an anti-GSK3β immunoaffinity column by immunoblot analysis using various tau phosphorylation-sensitive monoclonal antibodies. Tau-1 cross-reacts with nonphosphorylated tau, whereas 12E8, PHF-1, and AD-2 recognize phosphorylated tau (10, 38–41). As shown in Fig. 6A, we detected Tau-1-, 12E8-, PHF-1-, and AD-2-immunoreactive tau in brain extract, indicating that the brain contains a mixture of phosphorylated and nonphosphorylated tau. However, tau within the MAP fraction cross-reacted with Tau-1 and AD-2 but not with PHF-1 or 12E8, which indicated that AD-2-specific sites are phosphorylated in microtubule-associated tau. Tau in the anti-GSK3β immunoaffinity fraction cross-reacted with Tau-1 but not with 12E8, PHF-1, and AD-2 (Fig. 6A), demonstrating that tau within the GSK3β-tau complex is not phosphorylated on sites recognized by the aforementioned antibodies. These data suggest that GSK3β may bind to tau that has not been phosphorylated on Tau-1, 12E8, PHF-1, and AD-2-specific sites.

To substantiate the above suggestion, we performed a GST pull-down assay. Glutathione-agarose beads coated with
**DISCUSSION**

To investigate the biochemical nature of microtubule-associate GSK3β and to study its interaction with tau, we dissociated microtubules by cold incubation and removed tubulin from the MAP fraction containing GSK3β by phosphocellulose chromatography. When the MAP fraction was subjected to an FPLC gel filtration analysis, GSK3β was eluted as a 6-fold more than GSK3α. Thus 6-fold more tau is complexes with GSK3β than with GSK3α in MAP fraction and brain extract. These data indicate that GSK3α and GSK3β are present in brain extract and various microtubule pellets in almost equal amounts.

In Figs. 6 and 7, we demonstrated that GSK3β binds to tau. Since we found that GSK3α is also present in microtubule fractions, we wished to examine how tau interacts with GSK3α as compared with GSK3β. To do this, we immunoprecipitated tau from brain extract and MAP fraction using an anti-tau antibody. The anti-tau immune complex was then immunoblotted using an anti-GSK3β antibody. As shown in Fig. 9A, GSK3α and GSK3β were detected in the anti-tau immune complex from both the MAP fraction and brain extract. Blot band intensity quantitation revealed that the ratio of GSK3β to GSK3α ratio in MAP fraction and brain extract was 1.05 and 0.85, respectively (Fig. 9B). However, the GSK3β/GSK3α ratio within the anti-tau immune complex from MAP fraction and brain extract was 6.3 and 5.5, respectively. These data indicate that GSK3β co-immunoprecipitates with tau from the MAP fraction and brain extract 6-fold more than GSK3α. Thus 6-fold more tau is complexes with GSK3β than with GSK3α in MAP fraction and brain extract.

**FIG. 7. Microtubule sedimentation assay.** Microtubules were assembled at 37°C in the presence of the indicated proteins for 30 min and centrifuged. After centrifugation, the pellet (P) and the supernatant (S) were separated by SDS-PAGE. The gel was either protein-stained by using Coomassie Brilliant Blue or immunoblotted by using anti-GSK3β monoclonal antibody. A, immunoblot stained gel; B, immunoblotted gel. Similar results were obtained in two independent experiments.
kDa complex (Fig. 3). In vitro, GSK3β bound to R-tau (Fig. 4). Taken together, these data indicate that GSK3β and tau are the components of a ~400-kDa microtubule-associated complex, and these two proteins are directly bound to each other within the complex.

The sum of the molecular masses of GSK3β and tau is ~97-kDa, whereas the gel filtration size of the complex containing GSK3β and tau is ~400 kDa (Fig. 1A). The protein-stained SDS gel (Fig. 2A) indicates that the fractions eluting from the anti-GSK3β immunoaffinity column contain 2–3-fold more GSK3β than tau. It is possible that there may be multiple GSK3β-binding regions within the projection domain of tau, and a single tau molecule may bind 2–3 GSK3β molecules. The 400-kDa complex may therefore contain a tau molecule bound to several GSK3β molecules. Alternatively, from a gel filtration column, GSK3β elutes within fractions 44–64 (Fig. 1A), with sizes ranging from 50 to 500 kDa. These data indicate that bovine brain extract contains free GSK3β as well as GSK3β complexed with tau. Since we used gel filtration fractions 44–64 to generate Fig. 2, the relatively higher amount of GSK3β compared with tau in fractions eluting from an anti-GSK3β immunoaffinity column may be due to the presence of the GSK3β-tau complex along with free GSK3β in these fractions. The 400-kDa complex may therefore contain tau and GSK3β with a 1:1 stoichiometry. The 400-kDa size of the GSK3β-tau complex may be attributed to the asymmetric nature of tau within the complex, which is known to elute as a relatively large size species from a gel filtration column (35). Finally, a third possibility that cannot be excluded is that the 400-kDa complex may also contain protein(s) other than tau and GSK3β that may be below the detection limit of silver staining as shown in Fig. 2A. It has been reported that Cdk5 and protein phosphatase 1, which are also present in neuronal microtubules, bind to tau and associate with microtubules via tau in a manner similar to GSK3β (33, 42). Therefore, besides tau and GSK3β, the 400-kDa complex may also contain Cdk5, protein phosphatase 1, and/or any other tau-binding protein(s).

GSK3β co-purifies with microtubules during temperature-induced microtubule assembly/disassembly and is an integral part of neuronal microtubules (30, 32). The biochemical basis of the association of GSK3β to microtubules was unknown. In this study by microtubule sedimentation assay, we demonstrated that GSK3β binds to microtubules through tau. We also determined that GSK3β binds to the projection domain of tau. Since the microtubule-binding region of tau does not overlap with the projection domain (43), it is likely that tau can simultaneously bind to microtubules and GSK3β in vitro (Fig. 7) and perhaps in vivo.

T Tau isolated from brain extract is heterogeneous due to the presence of several tau isoforms, which are endogenously phosphorylated on various sites (5, 6). We find that tau within the 400-kDa complex is also heterogeneous (Figs. 2, 3A, and 6A). We analyzed the phosphorylation state of tau within the 400-kDa complex by using four anti-tau monoclonal antibodies sensitive to tau phosphorylation: Tau-1, 12E8, PHF-1, and AD-2. Tau-1 cross-reacts with tau that is not phosphorylated on residues Ser199, Ser198, and Ser202 (38). 12E8 recognizes tau phosphorylated on Ser198, Ser199, Ser202, Ser204, Ser262, Ser398, Ser400, and Ser404 and suggest that...
GSK3β-bound tau isoforms may be in nonphosphorylated states. However in vivo, tau is also phosphorylated on several other sites (8) for which antibodies are not available, precluding more detailed analysis. Thus, we conclude that within the GSK3β-tau complex, tau isoforms are not phosphorylated on above indicated sites but may or may not be phosphorylated on any additional sites.

Interestingly, some of the target sites of GSK3β are generated by phosphorylation through the action of another kinase. For example, the phosphorylation of glycogen synthase by GSK3β requires prior phosphorylation of glycogen synthase by casein kinase 2 (44, 45). The phosphorylation of glycogen synthase by casein kinase 2 generates a recognition sequence for GSK3. Subsequently, GSK3 phosphorylates and inactivates glycogen synthase (44, 45). Likewise, tau purified from bovine brain extract containing endogenous phosphate is efficiently phosphorylated by GSK3β. However, purified brain extract tau that has been dephosphorylated by a phosphatase is a poor GSK3β substrate (46). This dephosphorylated tau is then robustly phosphorylated by GSK3β after being previously phosphorylated by Cdk5 (46). It has been suggested that Cdk5 primes tau for GSK3β action (23, 46).

The presence of tau not phosphorylated on Ser198, Ser199, Ser202, Ser235, Ser398, and Ser404 within the tau-GSK3β complex (Fig. 6A) suggests that GSK3β-tau interaction may be regulated by phosphorylation of tau on these sites. Since Cdk5 phosphorylates Ser198, Ser199, Ser202, Ser235, Ser398, and Ser404 in vitro (34, 47), we evaluated the binding of Cdk5-phosphorylated R-tau with GSK3β and find that GSK3β binds to R-tau but not Cdk5-phosphorylated R-tau (Fig. 6B). Previous studies have shown that Cdk5 is also a component of neuronal microtubules, binds to tau, and associates with microtubules via tau (33) in a manner similar to GSK3β (this study). Taken together, these observations suggest that GSK3β-tau binding may be regulated by phosphorylation of tau by Cdk5. This in turn may suggest that Cdk5 may not only prime tau for GSK3β action (23, 46) but may also regulate tau-GSK3β binding in vitro and possibly in vivo. It should also be noted that Cdk5-phosphorylated tau does not bind to microtubules (37). GSK3β-tau and tau-microtubule interactions may therefore be regulated by phosphorylation on the same tau sites. It is possible that, in the brain, nonphosphorylated tau simultaneously binds to GSK3β and microtubules, bridges GSK3β to microtubules, and stabilizes microtubule structure. Upon phosphorylation, tau dissociates from both microtubules and GSK3β.

Although GSK3β has been implicated to be an in vivo tau kinase (23, 23–30), mammalian brain contains GSK3α and GSK3β in almost equal amounts (Ref. 11; Fig. 8). From brain extract, both GSK3α and GSK3β co-puriﬁy with microtubules during temperature-induced microtubule assembly/disassembly (Ref. 32; Fig. 8). GSK3α and GSK3β have ~98% sequence identity within their kinase domains, display very similar substrate speciﬁcity, and are regulated in a very similar manner (11). In vitro, both GSK3α and GSK3β phosphorylate tau (48, 49). These observations suggest that both GSK3α and GSK3β may phosphorylate tau in mammalian brain.

As reported previously (32), we observed that GSK3α and GSK3β are stably associated with microtubules in bovine brain extract (Fig. 8A). Despite the amounts of GSK3α and GSK3β in brain extract and MAP fraction being almost equal, ~6-fold more GSK3β co-immunoprecipitates with tau from brain extract and MAP fraction than GSK3α (Fig. 9B). These data indicate that ~6-fold more tau is complexed with GSK3β than with GSK3α in brain. With such a profound difference between the amounts of each kinase complexed with tau, it is very likely that most of tau in the brain will be phosphorylated by GSK3β rather than by GSK3α.

Full elucidation of the biochemical basis and physiological significance of the presence of a significantly higher amount of tau complexed with GSK3β compared with GSK3α in the brain extract will require further investigation. It is known that both GSK3β and GSK3α contain a N-terminal regulatory region, a central kinase domain and a C-terminal tail (44). The central kinase domains of these two kinases are ~98% identical, whereas N-terminal regulatory regions and C-terminal tails are quite different (11). It is possible that the tau-binding region may be located within either the N-terminal regulatory region or the C-terminal tail of GSK3β and therefore tau may bind to GSK3β with greater affinity than to GSK3α.

GSK3β has been regarded as one of the kinases that phosphorylate tau in AD brain (2, 22–30). Although GSK3β is involved in a number of distinct signaling pathways and phosphorylates such diverse cellular proteins as glycogen synthase, ATP citrate lyase, PKA regulatory subunit, c-Jun, c-Myc, cAMP-response element-binding protein, inhibitor-2, β-catenin, neurofilaments, synapsin, and adenosomatous polyposi coli gene product (12–21), only tau in AD brain becomes significantly hyperphosphorylated (2). An important question raised from these observations is how GSK3β specifically phosphorylates tau in normal and diseased brain. In this study, we demonstrated that GSK3β and tau are parts of a ~400-kDa complex located within the microtubule fraction. Within the GSK3β-tau complex, GSK3β will phosphorylate tau preferentially with respect to other cellular targets. Consistent with this model, GSK3β specifically phosphorylates β-catenin within the β-catenin destruction complex containing GSK3β and β-catenin (12, 19, 20). Our study provides a biochemical mechanism of specific tau phosphorylation by GSK3β in normal and possibly in AD brain.

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