Articles

Glycogen Synthase Kinase 3β Phosphorylates Alzheimer's Disease-Specific Ser^{396} of Microtubule-Associated Protein Tau by a Sequential Mechanism†

Tong Li‡ and Hemant K. Paudel*,‡,§

Bloomfield Center for Research in Aging, Lady Davis Institute for Medical Research, Sir Mortimer B. Davis-Jewish General Hospital, and Department of Neurology and Neurosurgery, McGill University, 3755 Cote Ste-Catherine Road, Montreal, Quebec H3T 1E2, Canada

Received August 16, 2005; Revised Manuscript Received November 29, 2005

ABSTRACT: Phosphorylation of tau on S^{396} was suggested to be a key step in the development of neurofibrillary pathology in Alzheimer's disease brain [Bramblett, G. T., Goedert, M., Jacks, R., Merrick, S. E., Trojanowski, J. Q., and Lee, V. M.-Y. (1993) *Neuron 10*, 1089−1099]. GSK3β phosphorylates Ser^{396} of tau in the brain by a mechanism which is not clear. In this study, when HEK-293 cells were cotransfected with tau and GSK3β, GSK3β co-immunoprecipitated with tau and phosphorylated tau on S^{202}, T^{231}, S^{396}, and S^{400} but not on S^{262}, S^{235}, and S^{404}. Blocking phosphorylation on T^{231}, S^{235}, S^{396}, S^{400}, or S^{404} did not prevent the subsequent phosphorylation on S^{202} by GSK3β. These data suggest that GSK3β directly phosphorylates tau on S^{202} (without requiring prephosphorylation). However, preventing phosphorylation on S^{235}, S^{400}, and S^{404} prevented GSK3β-dependent phosphorylation of T^{231}, S^{396}, and S^{400}, respectively. This indicates that phosphorylation of T^{231}, S^{396}, and S^{400} by GSK3β depends on a previous phosphorylation of S^{235}, S^{400}, and S^{404}, respectively. To examine S^{396} phosphorylation, we analyzed phosphorylation of S^{396}, S^{400}, and S^{404}. Blocking phosphorylation of S^{404} prevented the subsequent GSK3β-dependent phosphorylation of both S^{400} and S^{396}. When phosphorylation of S^{404} was allowed but S^{400} blocked, GSK3β failed to phosphorylate S^{396}. Thus, GSK3β phosphorylates S^{396} by a two-step mechanism. In the first step, GSK3β phosphorylates S^{400} of previously S^{404}-phosphorylated tau. This event primes tau for second-step phosphorylation of S^{396} by GSK3β. We conclude that GSK3β phosphorylates tau directly at S^{202} but requires the previous phosphorylation on S^{235} to phosphorylate T^{231}. Phosphorylation of S^{396}, on the other hand, occurs sequentially. Once a priming kinase phosphorylates S^{404}, GSK3β sequentially phosphorylates S^{400} and then S^{396}.

Neurofibrillary tangles (NTs) are one of the two neuropathological hallmarks of Alzheimer's disease (AD)[1] (reviewed in ref *1*). Paired helical filaments (PHFs) are the major structural component of NTs and are mainly composed of microtubule-associated protein tau (*2*−*4*). PHF-tau (tau isolated from PHFs) is highly insoluble, displays a retarded mobility on a SDS gel, and is incapable of binding to microtubules because it is abnormally phosphorylated (phosphorylated on more sites than normal tau) (*1*−*4*). Upon dephosphorylation, PHF-tau becomes soluble and becomes as capable as normal tau in binding and promoting microtubule assembly (*5*−*7*). Abnormal tau phosphorylation is believed to cause tau dysfunction, microtubule instability, axonal transport loss, neurodegeneration, and dementia associated with AD (*8*). Therefore, preventing and/or reducing the level of abnormal tau phosphorylation to restore the normal tau function in the brain is one of the current therapeutic strategies for AD.

PHF-tau is phosphorylated at ≤21 sites (*9*). It is not clear which of these sites are involved in converting tau to PHFs, but some studies suggest that S^{396} plays a key role. Ser^{396}-phosphorylated tau migrates with a reduced mobility on a SDS gel and has a weakened ability to bind and promote microtubule assembly and microtubule structure stabilization (*7*). Dephosphorylation of PHF by protein phosphatase causes dissociation of tau from PHFs with concomitant removal of a phosphate group from Ser^{396} of tau. The dissociated tau displays normal mobility on a SDS gel and full activity in binding and promoting microtubule assembly (*5*−*7*). It has recently been suggested that phosphorylation of tau on S^{396} causes a conformational change in tau which accelerates tau

† This work was supported by grants from the Canadian Institute of Health Research, the American Health Assistance Foundation, and the Alzheimer Society of Canada.

* To whom correspondence should be addressed: Bloomfield Center for Research in Aging, Lady Davis Institute for Medical Research, 3755 Cote Ste-Catherine, Montreal, Quebec H3T 1E2, Canada. Telephone: (514) 340-8222, ext. 4866. Fax: (514) 340-8295. E-mail: hemant.paudel@mcgill.ca.

‡ Sir Mortimer B. Davis-Jewish General Hospital.

§ McGill University.

[1] Abbreviations: AD, Alzheimer's disease; Cdk5, cyclin-dependent protein kinase 5; GSK3β, glycogen synthase kinase 3β; PHF, paired helical filament.

10.1021/bi051634r CCC: $33.50
Published on Web 02/14/2006

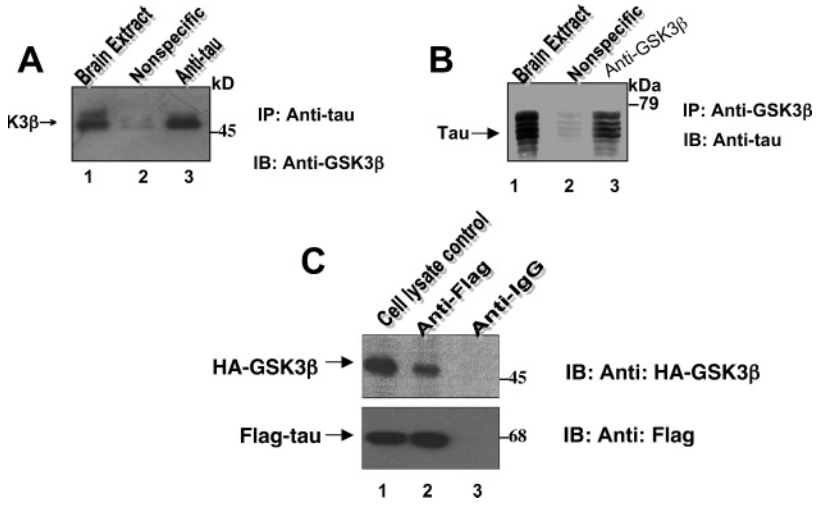

FIGURE 1: Co-immunoprecipitation. Tau and GSK3β were immunoprecipitated from a fresh bovine brain extract (A and B) or from lysates of HEK-293 cells transfected with Flag-tau and HA-GSK3β (C) using the indicated antibodies. Each resulting immune complex was then immunoblotted against the indicated antibody. This experiment was conducted at least four times with similar results.

aggregation in AD brain (*10*). Phosphorylation of tau at Ser^{396} has been suggested to be a critical event in AD pathogenesis (*6*).

The level of Ser^{396}-phosphorylated tau relative to the total level of tau is elevated in the cerebrospinal fluid of AD patients and measures the severity of the disease (*11*). This elevation is not observed in the cerebrospinal fluid of normal brains or in brains of patients suffering from vascular dementia or non-AD neurological diseases (*11*). Therefore, the CSF level of Ser^{396}-phosphorylated tau has been suggested to be a noninvasive diagnosis for monitoring the status of the disease and efficacy of therapy against AD (*11*). A clear understanding of the molecular mechanism by which tau is phosphorylated at Ser^{396} is an important step in understanding AD pathology and therefore is of considerable interest.

Ser^{396} of tau contains a consensus recognition sequence for proline-directed kinases (*12*). Current evidence indicates that GSK3β is the major kinase that phosphorylates tau at several proline-directed sites, including Ser^{396} in the brain (*13*−*20*). GSK3β is an ~45 kDa enzyme involved in the regulation of various physiological processes, including glycogen metabolism, signal transduction, cell fate specification, gene expression, and apoptosis, and may have a role in AD pathogenesis (*21*). A unique feature of GSK3β is that it phosphorylates some substrates only when the substrate has been previously phosphorylated (primed) by another kinase. It also phosphorylates some substrates which have not been previously primed (*22*). In the priming mechanism, phosphorylation of the substrate by the priming kinase at position $n + 4$ (n being the GSK3β target site) generates the S/TXXXS(p) sequence motif, which is recognized by GSK3β (*23*). The mechanism by which GSK3β phosphorylates tau is not fully understood. However, tau purified from bovine brain extract that is endogenously phosphorylated is efficiently phosphorylated by GSK3β (*15*). Upon dephosphorylation, the brain tau becomes a poor substrate for GSK3β (*15*). Similarly, in vitro tau phosphorylated by Cdk5, cAMP-dependent protein kinase, PKC, or MAP-kinase is phosphorylated by GSK3β more efficiently than its nonphosphorylated counterpart (*24*−*26*). These data indicate that GSK3β phosphorylates tau via a priming mechanism. However, nonphosphorylated recombinant tau is also phosphorylated by GSK3β in vitro (*27*). Since GSK3β phosphorylates tau at several sites (*15*, *27*), these observations suggest that tau is phosphorylated by GSK3β at some sites by priming and some sites without priming. The mechanisms behind the phosphorylation of tau sites in vivo are not fully understood.

When transfected into mammalian cells, tau becomes phosphorylated at various sites by endogenous kinases of the cells (*14*, *20*, *28*, *29*). When GSK3β is cotransfected with tau in these cells, GSK3β phosphorylates tau (*14*, *20*, *28*, *29*). Assuming that in mammalian cells GSK3β phosphorylates al least some tau sites by a mechanism requiring priming, these observations suggest that endogenous kinases of mammalian cells phosphorylate and prime tau for GSK3β. Henceforth, to elucidate the biochemical mechanism of tau phosphorylation, we have cotransfected GSK3β individually with various site-specific tau mutants containing alanine in place of possible priming phosphorylation sites and evaluated tau phosphorylation. We show that in HEK-293 cells, GSK3β phosphorylates tau at S^{202}, T^{231}, S^{396}, and S^{400}. Among these sites, GSK3β phosphorylates S^{202} without priming and T^{231}, S^{396}, and S^{400} via priming mechanisms. Our data suggest that upon phosphorylation of tau at S^{404} by endogenous priming kinase, GSK3β phosphorylates tau first at S^{400} and then at S^{396} sequentially.

MATERIALS AND METHODS

Proteins and Antibodies. Anti-tau and anti-GSK3β polyclonal antibodies to generate Figure 1 have been described previously (*19*). Monoclonal anti-Flag and anti-HA have also been described (*28*). Tau phosphorylation sensitive monoclonal antibodies TG3, MC6, and PHF1 were generous gifts from P. Davies (Albert Einstein College of Medicine, Bronx, NY). AT180 was from Zymed Laboratories Inc. Other tau phosphorylation-specific antibodies, 12E8 and AT8, have been described previously (*28*). Tau phosphorylation sensitive polyclonal antibodies pS396, pS400, and pS404 were obtained from Biosource Inc.

cDNA Cloning. Tau site-specific mutants S202A, T231A, S235A, S396A, S400A, and S404A containing Ala in place

of the indicated Ser or Thr were generated by PCR (polymerase chain reaction) using the longest human tau isoform as the template and a site-directed mutagenesis kit (Clontech) following the manufacturer's instructions. The following primers were used: 5′-CC GGC GCC CCA GGC ACT CCC-3′ and 5′-GCC TGG GGC GCC GGG GCT GC-3′ for S202A, 5′-TG GTC CGT GCT CCA CCC AAG TCG C-3′ and 5′-GT GGA GCA CGG ACC ACT GCC ACC T-3′ for T231A, 5′-CCC AAG GCG CCG TCT TCC GCC AAG A-3′ and 5′-AGA CGG CGC CTT GGG TGG AGT ACG-3′ for S235A, 5′-TG TAC AAG GCG CCA GTG GTG TC-3′ and 5′-CA CTG GCG CCT TGT ACA CGA TC-3′ for S396A, 5′-GTG GTG GCT GGG GAC ACG TCT C-3′ and 5′-C CCC AGC CAC CAC TGG CGA CTT G-3′ for S400A, and 5′-GAC ACG GCT CCA CGG CAT CT-3′ and 5′-CC G TG G AG CCG TGT CCC CAG AC-3′ for S404A. All mutations were confirmed by DNA sequencing, and each mutant was cloned into mammalian expression plasmid Flag-pcDNA3.1/Zeo.

Cell Culture, Transfection, Immunoprecipitation, and Western Blot Analysis. HEK-293 cells were maintained in Dulbecco's modified Eagle's (high-glucose) medium supplemented with 10% fetal bovine serum (Invitrogen). Cells were plated in 100 mm culture dishes and were transfected with the indicated plasmid(s) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The cells were harvested 48 h after transfection. Cells in each culture dish were homogenized in lysis buffer [50 mM Tris-HCl (pH 7.4) 150 mM NaCl, 100 mM β-glycerophosphate, 10 mM EDTA, 10 mM EGTA, 10 mM NaF, 10 mM $MgCl_2$, 0.2% Nonidet P-40, 400 nM okadaic acid (Sigma-Aldrich), 2 nM cypermethrin (Sigma-Aldrich), and aprotinin, pepstatin, and leupeptin (0.2 μg/mL each)]. The homogenate was incubated on ice for 30 min and then centrifuged for 20 min. The supernatant was designated as cell lysate and used for various experiments. To immunoprecipitate Flag-tau, cell lysate ($\sim$500 μL) was precleared with $\sim$50 μL of mouse IgG agarose (Sigma-Aldrich). Precleared cell lysate ($\sim$200 μL) was mixed with $\sim$50 μL of anti-Flag agarose (Sigma-Aldrich) or mouse IgG agarose (control). Each mixture was incubated at 4 °C for 6 h with end-to-end shaking. After incubation, each sample was centrifuged and the recovered beads were washed three times (30 min each). The washed beads were dissolved in 50 μL of SDS−PAGE sample buffer, boiled, and centrifuged, and 20 μL of supernatant was immunoblotted using the indicated antibody. Cells were treated with LiCl as follows. After transfection, cells were replaced with fresh medium containing 20 mM LiCl and incubated in the incubator for 1 h before being harvested. Control cells for LiCl were treated with equal amounts of KCl. Immunoprecipitations and immunoblottings were performed as described previously (*28*). GSK3β and tau were immunoprecipitated from brain bovine extract essentially as described previously (*19*).

RESULTS

GSK3β Interacts with Tau in Brain Extract and Transfected HEK-293 Cells. When tau was immunoprecipitated from a fresh bovine brain extract and analyzed by immunoblot analysis, GSK3β specifically co-immunoprecipitated with tau (Figure 1A, lane 3). Likewise, when GSK3β was immunoprecipitated from the brain extract, tau co-immunoprecipitated with GSK3β (Figure 1B, lane 3). These data are consistent with the previous report (*19*) and indicate that GSK3β and tau interact in the brain.

Table 1: Tau Phosphorylation Sensitive Antibodies Used in This Study

antibody	type	specificity	ref
AT8	mouse monoclonal	S^{202}/T^{205}	Pierce Biotechnology Inc.
12E8	mouse monoclonal	S^{262}/S^{356}	*30*
PHF1	mouse monoclonal	S^{396}/S^{404}	*7*
pS396	rabbit polyclonal	S^{396}	Biosource International Inc.
AT180	mouse monoclonal	T^{231}	Zymed Laboratories
TG3	mouse monoclonal	T^{231}	*40*
MC6	mouse monoclonal	S^{235}	*40*
pS400	rabbit polyclonal	S^{400}	Biosource International Inc.
pS404	rabbit polyclonal	S^{404}	Biosource International Inc.

To examine if tau and GSK3β interact in transfected mammalian cells, we cotransfected Flag-tagged tau (Flag-tau) and HA-tagged GSK3β (HA-GSK3β) in HEK-293 cells. Transfected cells were lysed and analyzed by co-immunoprecipitation. GSK3β specifically co-immunoprecipitated with tau (Figure 1C, lane 2). Thus, GSK3β and tau also associate when cotransfected in HEK-293 cells. This observation suggests that interaction of GSK3β with tau can be studied in this cell model.

Identification of Tau Sites Phosphorylated in HEK-293 Cells by Endogenous Kinases and Specificities of Various Tau Phosphorylation Sensitive Antibodies. We observed that tau is phosphorylated when cotransfected with GSK3β in HEK-293 cells. If GSK3β phosphorylates tau via a priming mechanism, priming phosphorylation of tau by endogenous kinase(s) must have occurred. Therefore, to elucidate the mechanism of tau phosphorylation in HEK-293 cells, it became important to identify tau sites that are phosphorylated by endogenous kinases of the cells. To do this, we transfected Flag-tau in HEK-293 cells and analyzed tau phosphorylation by immunoblot analysis using a series of antibodies directed to tau phosphorylated at various sites (Table 1). Surprisingly, transfected Flag-tau exhibited reactivity against all these antibodies (data not shown but see below). These data indicated that either tau is phosphorylated at all the sites described above by endogenous kinases or our antibodies bind with tau nonspecifically.

To discriminate between these two possibilities, we generated a series of site-specific mutants of Flag-tau. The wild type (WT) and various mutants were individually transfected into HEK-293 cells. Transfected cells were lysed and analyzed by immunoblot analysis for binding to various tau phosphorylation sensitive antibodies.

As shown in Figure 2A, WT tau is recognized by AT8 antibody (lane 1). Tau mutants T231A, S235A, S396A, and S404A are also immunoreactive with respect to AT8 in a manner similar to that of WT (lanes 2−4 and 6). However, AT8 completely failed to react with S202A (lane 5). Since an anti-Flag immunoblot confirmed equal tau loading in different lanes (Figure 2A, top blot), these data indicate that AT8 specifically binds with tau phosphorylated at S^{202}. To further confirm these data, we treated the blot with alkaline phosphatase and then incubated the blot with the antibody. Tau in the blot exhibited reactivity with the anti-Flag antibody but not with AT8 (data not shown). On the basis of these results, we concluded that AT8 recognizes tau phosphorylated at S^{202}.

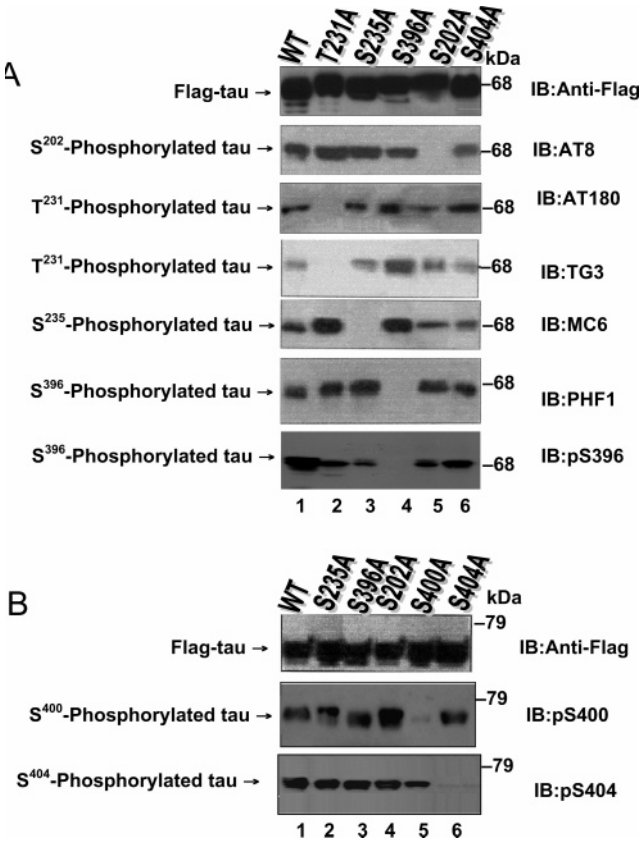

FIGURE 2: Specificities of tau phosphorylation sensitive antibodies. The indicated WT and site-specific mutants of Flag-tau were individually transfected in HEK-293 cells. Lysates of transfected cells were analyzed by immunoblot analysis using the indicated antibodies. The specificity of each antibody against the phosphorylated tau residue is given. Anti-Flag immunoblots demonstrate expression of Flag-tau in transfected cells.

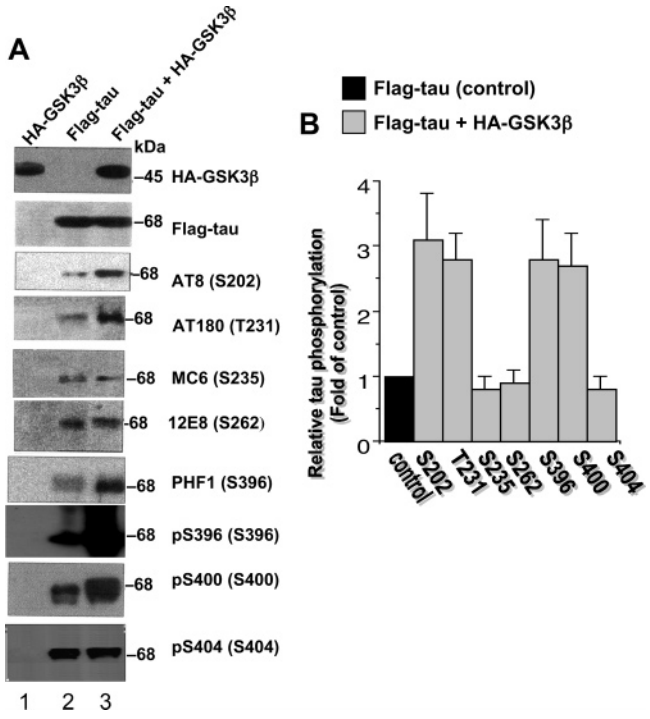

FIGURE 3: Tau phosphorylation by GSK3β. HEK-293 cells transfected with the genes of the indicated proteins were analyzed by immunoblot analysis to evaluate tau phosphorylation and expression of various transfected genes: (A) immunoblots and (B) relative tau phosphorylation. Blots representing tau in panel A were scanned, and band intensity values for each tau band were obtained. The tau band intensity value for each lane of each blot representing phosphorylated tau was divided by the respective total tau band intensity in the blot representing Flag-tau. The resulting value for each lane in each blot was then divided by the value of the control lane (lane 2) of that blot. The values are an average of three independent determinations. The relative level of phosphorylation of S^{396} was calculated on the basis of band intensities of the blot representing PHF1 antibody.

Both AT180 and TG3 recognize WT, S235A, S396A, S202A, and S404A but not T231A (Figure 2A). MC6 recognizes WT, T231A, S396A, S202A, and S404A but not S235A. PHF1 and pS396 react with WT, T231A, S235A, S202A, and S404A but not S396A, whereas pS400 reacts with WT, S235A, S396A, S202A, and S404A but not S400A (Figure 2B). pS404 reacts with WT, S235A, S396A, S202A, and S400A but not S4004A. When blots were treated with alkaline phosphatase and then exposed to the antibodies listed above, tau in the blot displayed almost no reactivity (data not shown). These observations indicate that AT180 and TG3 specifically recognize tau phosphorylated at T^{231}, whereas PHF1 and pS396 react with tau phosphorylated at S^{396}. Similarly, MC6, pS400, and pS404 specifically bind with tau phosphorylated at S^{235}, S^{400}, and S^{404} respectively. In addition to these antibodies, we also used monoclonal antibody 12E8. 12E8 recognizes tau phosphorylated at S^{262} and/or S^{356} in vitro (*30*). However, in vivo, tau is not phosphorylated at S^{356} (*12*). On the basis of these results, we concluded that our antibodies are specific, and in HEK-293 cells, tau is phosphorylated at a basal level at S^{202}, T^{231}, S^{235}, S^{262}, S^{396}, S^{400}, and S^{404} by endogenous kinase(s).

Identification of Tau Sites Phosphorylated in HEK-293 Cells by GSK3β. Tau in the lysates of cells transfected with both Flag-tau and HA-GSK3β exhibited ~3.1-fold enhanced phosphorylation at S^{202} compared to those cells transfected with Flag-tau and mock (Figure 3A,B). Similarly, tau became ~2.8-, ~2.8-, and ~2.7-fold more phosphorylated at T^{231}, S^{396}, and S^{400}, respectively, in cells cotransfected with HA-GSK3β than in those cotransfected with mock (compare lanes 2 and 3 of corresponding blots in Figure 3A, and see Figure 3B). When compared to Flag-tau, Flag-tau cotransfected with HA-GSK3β showed almost no change in phosphorylation at S^{235}, S^{262}, and S^{404} (compare lanes 2 and 3 of corresponding blots in Figure 3A, and see Figure 3B). The above enhanced phosphorylation of S^{202}, T^{231}, S^{396}, and S^{400} in cells transfected with Flag-tau and HA-GSK3β was not apparent when cells were treated with the GSK3β-specific inhibitor LiCl (data not shown). These data indicate that in vivo, GSK3β phosphorylates tau at S^{202}, T^{231}, S^{396}, and S^{400} and does not significantly phosphorylate S^{235}, S^{262}, and S^{404}.

Tau Phosphorylation of S^{202} by GSK3β. GSK3β phosphorylates some of its substrates only after they have been previously phosphorylated (primed) by another kinase (*22*). In this mechanism, phosphorylation of the target protein on a specific site by priming kinase generates a recognition sequence for GSK3β (*23*). However, GSK3β also phosphorylates some sites that do not require priming (*22*). As shown in Figure 3, Flag-tau is phosphorylated when cotransfected with HA-GSK3β on S^{202}. However, in HEK-293 cells, tau is already phosphorylated to a basal level at T^{231}, S^{235}, S^{396}, S^{400}, and S^{404} (Figure 2) and perhaps at other sites, too, which we were not able to determine because of the unavailability of specific antibodies. It is possible that basal phosphorylation

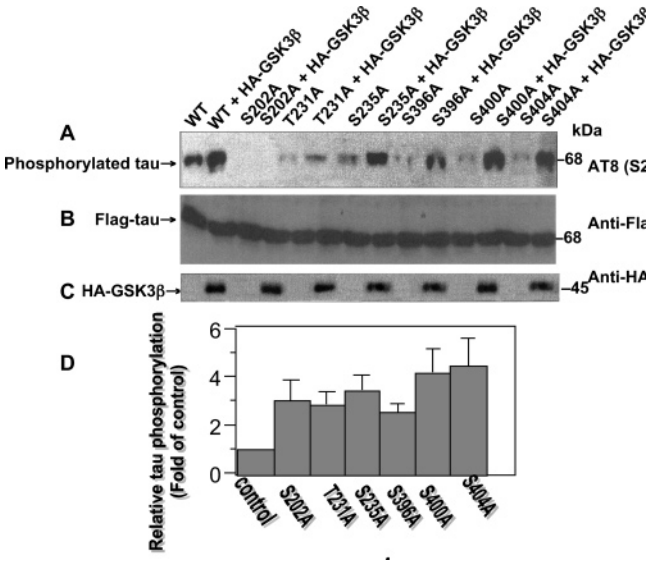

FIGURE 4: Phosphorylation of tau (WT) and site-specific tau mutants at S^{202} by GSK3β. Flag-tau (WT) or indicated site-specific Flag-tau mutants were cotransfected with HA-GSK3β in HEK-293 cells. Transfected cells were then analyzed by immunoblot analysis for phosphorylation of tau at S^{202} as well as for the expression of the respective genes. The blot representing AT8 monitors phosphorylation of tau at S^{202}, whereas those representing anti-Flag and anti-HA show expression levels of the respective genes: (A−C) immunoblots and (D) relative levels of tau phosphorylation. Blots representing panels A and B were scanned. The band intensity value of each phosphorylated tau species in panel A was then divided by the band intensity value of the respective Flag-tau in panel B. The resulting value for each tau species in each lane was then divided by the value of that tau species in the control lane (tau species transfected alone). The values are an average of three independent determinations. The control indicates each tau species transfected alone.

at one of the phosphorylation sites mentioned above may have primed tau for GSK3β. As a result, GSK3β could phosphorylate tau at S^{202}. If this is true, blocking basal phosphorylation on corresponding priming site should prevent GSK3β from phosphorylating tau at S^{202}. This will also allow us to identify the priming site whose phosphorylation facilitates phosphorylation of tau at S^{202} by GSK3β.

To test this idea, we cotransfected GSK3β with WT or various tau mutants in HEK-293 cells. Cells were lysed and analyzed for phosphorylation of tau at S^{202} by GSK3β. WT tau was ~2.9-fold more phosphorylated when cotransfected with HA-GSK3β than with mock (compare lanes 1 and 2 of Figure 4A, and see Figure 4D). Similarly, tau mutants T231A, S235A, S396A, S400A, and S404A were ~2.8-, ~3.3-, ~2.3-, ~4.0-, and 4.5-fold more phosphorylated, respectively, when cotransfected with HA-GSK3β than when with mock (Figure 4A,D). These observations suggest that GSK3β phosphorylates S^{202} of WT, T231A, S235A, S396A, S400A, and S404A. To substantiate this suggestion, we performed a similar experiment but treated transfected cells with LiCl. LiCl suppressed phosphorylation of each tau species that was cotransfected with HA-GSK3β to a basal level (data not shown). On the basis of these results, we concluded that GSK3β phosphorylates S^{202} of WT, T231A, S235A, S396A, S400A, and S404A, and blocking possible priming phosphorylation of tau at T^{231}, S^{235}, S^{396}, S^{400}, or S^{404} sites does not prevent tau phosphorylation by GSK3β at S^{202}. Thus, previous tau phosphorylation at T^{231}, S^{235}, S^{396}, S^{400}, or S^{404} is not required for phosphorylation of tau at S^{202} by GSK3β.

Phosphorylation of Tau at T^{231} by GSK3β. To examine tau phosphorylation of T^{231}, we cotransfected GSK3β with WT or various tau mutants in HEK-293 cells. Cells were lysed and analyzed by immunoblot analysis using the TG3 antibody, specific for tau phosphorylated at T^{231} (Figure 5A,D). WT, S202A, S396A, and S404A were ~4-, ~6.4-, 5.1-, and ~4.4-fold more phosphorylated, respectively when cotransfected with HA-GSK3β compared to the mock control cotransfection (Figure 5D). When LiCl was included, it suppressed phosphorylation of each tau species in cells cotransfected with HA-GSK3β to a basal level observed in cells in which the corresponding tau species was cotransfected with mock (data not shown). Importantly, S235A was not phosphorylated above the basal level in cells in which it was cotransfected with HA-GSK3β (in Figure 5A, compare lanes 7 and 8). These data indicate that GSK3β phosphorylates T^{231} of WT, S202A, S396A, and S404A but not that of S235A. Thus, mutations of tau residues S^{202}, S^{396}, and S^{404} to A have no effect on the phosphorylation of tau at T^{231} by GSK3β. However, mutation of S^{235} to A almost completely prevents phosphorylation of tau at T^{231} by GSK3β. These observations are consistent with a previous report (*13*) and indicate that previous phosphorylation of tau at S^{235} is required for GSK3β to phosphorylate tau at T^{231}.

Phosphorylation of Tau at S^{396} by GSK3β. To evaluate tau phosphorylation by GSK3β at S^{396}, we cotransfected HA-GSK3β with WT or various tau mutants in HEK-293 cells. Cell lysates were analyzed by immunoblot analysis using the PHF1 and pS396 antibodies, specific for tau phosphorylated at S^{396}. WT, S202A, T231A, and S235A were ~3.4-, 4.0-, ~5.0-, and ~6.2-fold more phosphorylated, respectively, when cotransfected with HA-GSK3β than the control cotransfection with mock (compare corresponding lanes in Figure 6A, and see Figure 6H). When a similar experiment was performed in which transfected cells were treated with LiCl, phosphorylation of each of the above-mentioned tau species cotransfected with HA-GSK3β was inhibited to a basal level (data not shown). These data demonstrate that GSK3β phosphorylates S^{396} of WT, S202A, T231A, and S235A.

However, as shown in panels A and B of Figure 6, S400A was phosphorylated at S^{396} only at a basal level in cells in which it was cotransfected with HA-GSK3β (compare lanes 11 and 12). These data indicate that GSK3β does not phosphorylate S400A at S^{396} and blocking S^{400} phosphorylation prevents S^{396} phosphorylation by GSK3β. This means previous phosphorylation of tau at S^{400} is required for phosphorylation of tau at S^{396} by GSK3β. Surprisingly, S404A was also not phosphorylated at S^{396} significantly when cotransfected with HA-GSK3β (compare lanes 13 and 14 in panels A and B of Figure 6). This result indicates that blocking S^{404} phosphorylation also prevents GSK3β from phosphorylating tau at S^{396}, and therefore, previous phosphorylation of S^{404} is also required for GSK3β to phosphorylate tau at S^{396}.

Phosphorylation of Tau at S^{400} by GSK3β. To examine phosphorylation of tau at S^{400}, we analyzed tau phosphorylation in the lysates of transfected HEK-293 cells mentioned above by immunoblot analysis using a pS400 antibody specific for tau phosphorylated at S^{400} (Figure 6C). WT,

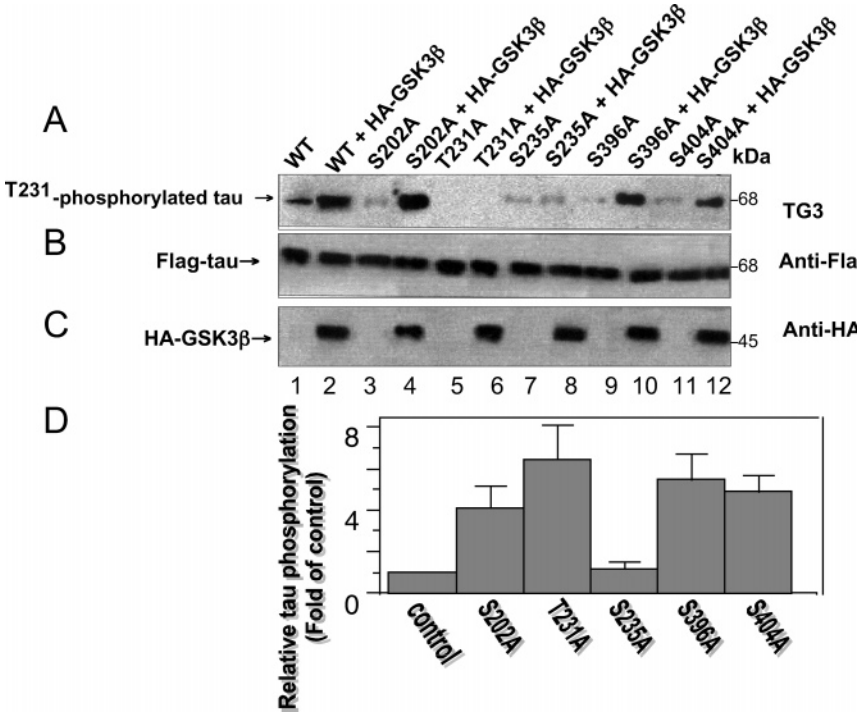

FIGURE 5: Phosphorylation of tau (WT) and various site-specific tau mutants at T^{231} by GSK3β. HEK-293 cells transfected with HA-GSK3β and Flag-tau (WT) or the indicated tau mutants were immunoblotted against the TG3 antibody to monitor tau phosphorylated at T^{231}m and against anti-Flag and anti-HA antibodies to show the expression of Flag-tau and HA-GSK3β, respectively: (A−C) immunoblots and (D) relative levels of tau phosphorylation. To determine the relative level of tau phosphorylation for each tau species, blots representing panels A and B were scanned. Blot band intensities of various tau species were then used to determine the relative levels of tau phosphorylation as in Figure 4. The values are an average of three independent determinations. The control indicates the tau species transfected alone.

S202A, T231A, S235A, and S396A were ~3.9-, ~3.4-, ~3.6-, ~2.4-, and ~2.8-fold more phosphorylated, respectively, when cotransfected with HA-GSK3β than when cotransfected with mock (Figure 6C,H). When LiCl was included, it inhibited phosphorylation of all the tau species mentioned above to an almost basal level in cells in which they were cotransfected with HA-GSK3β (data not shown). More importantly, S404A was phosphorylated at S^{400} only at a basal level when cotransfected with HA-GSK3β (compare lanes 13 and 14 of Figure 6C). This result indicates that GSK3β efficiently phosphorylates S^{400} of WT, S202A, T231A, S235A, and S396A but not that of S404A. Thus, previous phosphorylation of tau at S^{404} is required for phosphorylation of tau at S^{400} by GSK3β.

Sequential Phosphorylation of Tau at S^{404}, S^{400}, and S^{396} by GSK3β. GSK3β phosphorylates tau at S^{396} and S^{400} but not S^{404} (Figure 3). Previous phosphorylation of tau at S^{404} controls subsequent phosphorylation of both S^{400} and S^{396} by GSK3β (Figure 6A,C). These data appear to suggest that phosphorylation of S^{404} primes tau for phosphorylation of both S^{400} and S^{396} by GSK3β. However, previous phosphorylation of tau at S^{400} is also required for GSK3β to phosphorylate tau at S^{396} (Figure 6A). It is therefore possible that GSK3β phosphorylates S^{396} by a two-step mechanism. In the step 1, following the phosphorylation of S^{404} by a priming kinase, GSK3β phosphorylates S^{400}. Phosphorylation of S^{400} now primes tau for step 2 phosphorylation by GSK3β of S^{396}. Subsequently, GSK3β phosphorylates S^{400}-phosphorylated tau at S^{396}. For this mechanism to be true, previous phosphorylation of tau at S^{400} and not at S^{404} is required for GSK3β to phosphorylate tau at S^{396}.

To test the suggestion given above, we analyzed HEK-293 cells cotransfected with HA-GSK3β and WT or various tau mutants for tau phosphorylation at S^{404}. As shown in Figure 6D, S400A, which cannot be phosphorylated at S^{400}, was phosphorylated at S^{404} in a manner similar to that of WT, S202A, T231A, and S235A by GSK3β (compare lane 12 with lanes 2, 4, 6, 8, and 10). However, unlike the above-mentioned tau species, S400A was phosphorylated at S^{396} at a basal levels when cotransfected with HA-GSK3β or mock (compare lanes 11 and 12 in panels A and B of Figure 6). These data demonstrate that GSK3β does not phosphorylate S^{396} if phosphorylation of S^{400} is blocked despite S^{404} being previously phosphorylated. Thus, previous phosphorylation of S^{400} and not S^{404} is required for GSK3β to phosphorylate tau at S^{396}. Previous phosphorylation of tau at S^{404}, on the other hand, is essential for phosphorylation of tau at S^{400} by GSK3β.

DISCUSSION

PHF-tau is phosphorylated at 21 sites (*9*). These sites include all the sites that are phosphorylated in normal adult brain (normal sites) as well as those which are phosphorylated in PHF-tau but not in normal adult brain (abnormal sites) (*12*). Currently, the individual contributions of all the phosphorylation sites of PHF-tau in NFT pathology are not fully known. Tau in the normal adult brain is phosphorylated yet does not form PHFs (*12*). This observation suggests that tau sites that are phosphorylated in both normal adult brain and PHF-tau may not be involved in NFT pathology and implies that the abnormal tau phosphorylation sites are responsible for PHF formation. However, some of the abnormal sites are only partially phosphorylated in PHFs (*9*). These sites therefore are unlikely to be involved in tau dysfunction associated with AD. These observations suggest that phosphorylation of some of the abnormal sites may

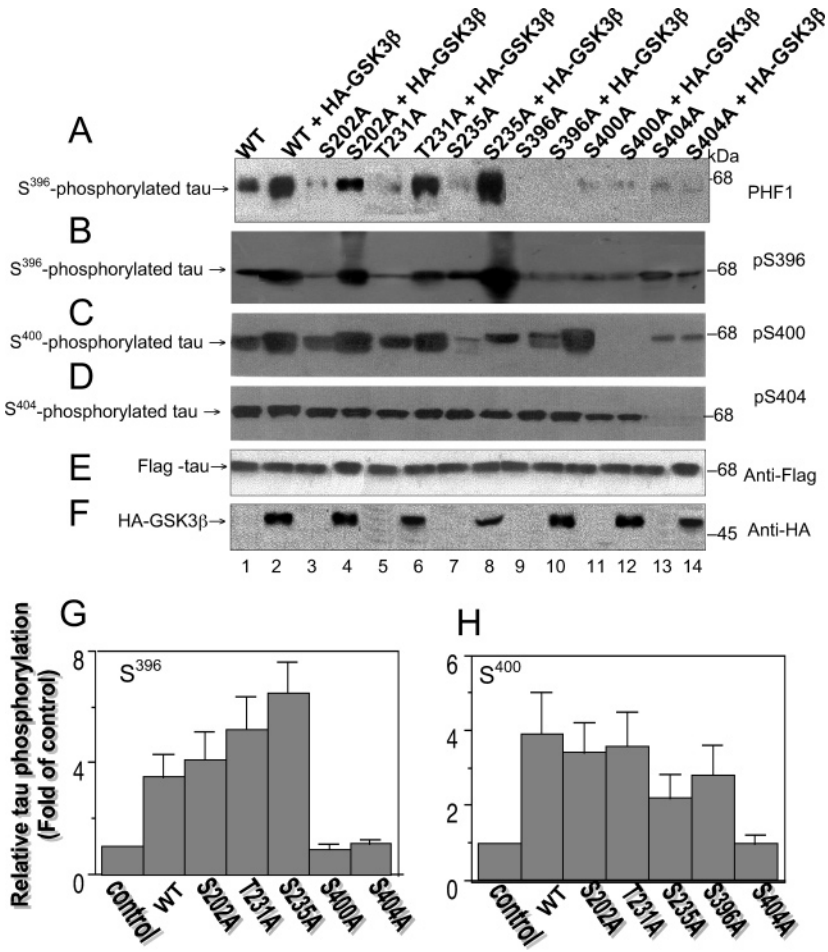

FIGURE 6: Sequential tau phosphorylation of S^{400} and S^{396} by GSK3β. Flag-tau (WT) or indicated tau mutants were cotransfected with HA-GSK3β or mock in HEK-293 cells. Lysates of transfected cells were analyzed for phosphorylation of S^{396}, S^{400}, and S^{404} and the expression of transfected genes by immunoblot analysis using the respective antibodies. Blots A−E were scanned, and blot band intensities were used to determine relative levels of tau phosphorylation of the indicated sites: (A−F) immunoblots and (G and H) relative levels of tau phosphorylation. The relative level of tau phosphorylation of indicated tau species was determined as in Figure 4. To generate panel G, blot A representing PHF1 antibody was used. Similar results were obtained from at least three independent experiments. The control indicates tau species transfected alone.

trigger PHF formation in AD brain. Among all the abnormal phosphorylation sites of PHF-tau, Ser^{396} phosphorylation is thought to play a major role in NFT pathology (*7*, *10*, *11*). Since tau is not phosphorylated at Ser^{396} in the normal adult brain (*7*, *12*), preventing or reducing the level of tau phosphorylation at this site alone offers a promising therapy for AD without affecting the normal function of tau.

S^{396} is a potential target for Cdk5 and GSK3β in vivo. Although few studies have reported that Cdk5 does not phosphorylate tau in vivo (*31*, *32*), several other studies have demonstrated Cdk5-dependent phosphorylation of tau at S^{396} and/or S^{404} in the brains of transgenic mice overexpressing Cdk5 (*33*−*35*). However, these studies used an antibody that recognized tau phosphorylated at both S^{396} and S^{404} and, therefore, did not determine whether tau is phosphorylated by Cdk5 at S^{396}. Two studies analyzed tau phosphorylation in brains of transgenic mice overexpressing Cdk5 activity and in COS-7 cells transfected with Cdk5 and tau using antibodies specific for tau phosphorylated at S^{396} or S^{404} (*29*, *36*). Both studies found that tau is phosphorylated by Cdk5 at S^{404} but not at S^{396} in vivo. Taken together, these studies indicate that tau is not phosphorylated by Cdk5 at S^{396}.

GSK3β robustly phosphorylates tau at S^{396} when cotransfected with tau in various mammalian cells (*16*, *20*, *28*, *29*). In transgenic mice overexpressing GSK3β in the brain, tau becomes phosphorylated at S^{396} (*17*, *18*). More importantly, Michel et al. (*29*) used antibodies that specifically recognized tau phosphorylated at S^{396} or S^{404}. They demonstrated that when GSK3β was cotransfected with tau in mammalian cells, it phosphorylates S^{396} and not S^{404}. Our data are consistent with these reports and demonstrate that in HEK-293 cells transfected with tau and GSK3β, GSK3β phosphorylates tau at S^{396} (Figure 3). Current evidence indicates that GSK3β is the major kinase that phosphorylates tau at S^{396} in the brain.

Previous studies indicated that GSK3β phosphorylates tau directly and by a mechanism that requires priming (*13*, *37*). We observed that blocking phosphorylation at T^{231}, S^{235}, S^{396}, or S^{404} does not prevent S^{202} phosphorylation by GSK3β (Figure 4). Moreover, GSK3β recognizes the S/TXXXS(P) sequence motif (*22*, *23*). For S^{202} to be phosphorylated by a priming mechanism, tau must be phosphorylated by a priming kinase at P^{206} (*9*), which is a nonphosphorylatable amino acid residue. These observations argue that GSK3β phosphorylates tau at S^{202} by a mechanism that does not require tau priming. However, as shown in Figure 4A, GSK3β phosphorylates WT, S235A, S396A, S400A, and S404A with almost equal efficiencies. T231A, on the other hand, is phosphorylated by GSK3β with an efficiency

significantly lower than that of WT (compare lanes 2 and 6 in Figure 4A). These observations suggest that blocking T^{231} phosphorylation inhibits S^{202} phosphorylation by GSK3β. This means that previous phosphorylation of T^{231} facilitates subsequent S^{202} phosphorylation by GSK3β. It should be noted that tau is also phosphorylated endogenously in HEK-293 cells on various sites. Interestingly, S231A and S396A are phosphorylated more efficiently at S^{235} than WT (Figure 2A). Similarly, S202A is phosphorylated more efficiently than WT at S^{400} (Figure 2B). Taken together, these observations suggest that previous phosphorylation of tau at some sites influences the subsequent phosphorylation of other specific sites.

One of the GSK3β phosphorylation sites, T^{231}, is located four residues upstream of S^{235}, which is phosphorylated in vivo (*9*). Phosphorylation of S^{235} generates a $T^{231}XXXSp^{235}$ sequence motif recognized by GSK3β (*22*, *23*). Indeed, in our transfected cells, GSK3β completely failed to phosphorylate T^{231} of tau mutant S235A (Figure 5). These data are consistent with a previous report (*13*) and indicate that previous phosphorylation of S^{235} primes tau for phosphorylation by GSK3β at T^{231}.

GSK3β phosphorylated S396A at S^{400} to an extent similar to that of WT in three experiments (compare lanes 2 and 10 of Figure 6C) but slightly less than that of WT in one experiment (data not shown). Despite this inconsistency, in all of the experiments described above, blocking of S^{396} phosphorylation by mutating S^{396} to A did not prevent GSK3β from phosphorylating S^{400}. These observations indicate that the previous phosphorylation of S^{396} is not required for the subsequent S^{400} phosphorylation by GSK3β. However, GSK3β phosphorylates tau at S^{400} only when tau has been previously phosphorylated at S^{404} (Figure 6). Likewise, once tau is phosphorylated at S^{400}, GSK3β phosphorylates tau at S^{396} (Figure 6). Since GSK3β does not phosphorylate tau at S^{404} (Figure 3 and ref *29*), this site has to be phosphorylated by a priming kinase other than GSK3β. This means that once tau is phosphorylated at S^{404} by the priming kinase, GSK3β sequentially phosphorylates tau at S^{400} and then at S^{396} in the carboxyl to amino-terminal direction with each phosphorylation generating an SXXXSp motif. It should be noted that GSK3β phosphorylates glycogen synthase also by a sequential mechanism that requires priming phosphorylation of S^{565} by casein kinase 2. Once S^{565} is phosphorylated, GSK3β sequentially phosphorylates glycogen synthase at S^{652}, S^{648}, S^{644}, and S^{640} in the carboxyl to amino-terminal direction with each phosphorylation depending on the prior phosphorylation of position $n + 4$ (*23*). Likewise, once β-catenine is phosphorylated at S^{45} by the priming kinase, GSK3β phosphorylates the substrate sequentially from the carboxyl to amino terminus sequentially at S^{41}, S^{37}, S^{33}, and S^{29} (*38*, *39*).

ACKNOWLEDGMENT

We thank Dr. Peter Davies of the Albert Einstein College of Medicine for providing PHF-1, TG3, and MC6 antibodies.

REFERENCES

1. Lee, V. M.-Y., Goedert, M., and Trojanowski, J. Q. (2001) Neurodegenerative tauopathies, *Annu. Rev. Neurosci. 24*, 1121−1159.
2. Lee, V. M.-Y., Balin, B. J., Otvos, L., Jr., and Trojanowski, J. Q. (1991) A68: A major subunit of paired helical filaments and derivatized forms of normal tau, *Science 251*, 675−678.
3. Grundke-Iqbal, I., Iqbal, K., Quinalan, M., Tung, Y. C., Zaidi, M. S., and Wisniewski, H. M (1986) Microtubule-associated protein tau. A component of Alzheimer paired helical filaments, *J. Biol. Chem. 261*, 6084−6089.
4. Grundke-Iqbal, I., Iqbal, K., Tung, Y. C., Quinalan, M., Wisniewski, H. M., and Binder, L. I. (1986) Abnormal phosphorylation of the microtubule-associated protein τ (tau) in Alzheimer cytoskeletal pathology, *Proc. Natl. Acad. Sci. U.S.A. 83*, 4913−4917.
5. Wang, J. Z., Gong, C.-X., Zaidi, T., Grundke-Iqbal, I., and Iqbal, K. (1995) Dephosphorylation of Alzheimer paired helical filaments by protein phosphatase 2A and 2B, *J. Biol. Chem. 270*, 4854−4860.
6. Wang, J. Z., Grundke-Iqbal, I., and Iqbal, K. (1996) Restoration of biological activity of Alzheimer abnormally phosphorylated tau by depohosphorylation with protein phosphatase 2A, 2B and 1, *Mol. Brain Res. 38*, 200−208.
7. Bramblett, G. T., Goedert, M., Jakes, R., Merrick, S. E., Trojanowski, J. Q., and Lee, V. M.-Y. (1993) Abnormal tau phosphorylation at Ser396 in Alzheimer's disease recapitulates development and contributes to reduced microtubule binding, *Neuron 10*, 1089−1099.
8. Alonso, A. d. C., Grundke-Iqbal, I., and Iqbal, K. (1996) Alzheimer's disease hyperphosphorylated tau sequesters normal tau into tangles of filaments and disassembles microtubules, *Nat. Med. 2*, 783−787.
9. Morishima-Kawashima, M., Hasegawa, M., Takio, K., Suzuki, M., Yoshida, H., Titani, K., and Ihara, Y. (1995) Proline-directed and non-proline directed phosphorylation of PHF-tau, *J. Biol. Chem. 270*, 823−829.
10. Alonso, A. d. C., Maderlyova, A., Novak, M., Grundke-Iqbal, I., and Iqbal, K. (2004) Promotion of hyperphosphorylation of frontotemporal demetia tau mutations, *J. Biol. Chem. 279*, 34873−34881.
11. Hu, Y., He, S. S., Wang, X., Duan, Q. H., Grundke-Iqbal, I., Iqbal, K., and Wang, J. (2002) Levels of nonphosphorylated and phosphorylated tau in cerebrospinal fluid of Alzheimer's disease patient. An ultrasensitive enzyme−substrate-recycle enzyme-linked immunosorbent assay, *Am. J. Pathol. 160*, 1269−1278.
12. Watanabe, A., Hasegawa, M., Suzuki, M., Takio, K., Morishima-Kawashima, M., Titani, K., Arai, T., Kosik, K. S., and Ihara, Y. (1993) In vivo phosphorylation sites in fetal and adult rat tau, *J. Biol. Chem. 268*, 25712−25717.
13. Cho, J. H., and Johnson, G. V. (2004) Primed phosphorylation of tau at Thr231 by glycogen synthase kinase 3β plays a critical role in regulating tau's ability to bind and stabilize microtubules, *J. Neurochem. 88*, 349−358.
14. Hong, M., Chen, D. C., Klein, P. S., and Lee, V. M.-Y. (1997) Lithium reduces tau phosphorylation by inhibition of glycogen synthase kinase-3, *J. Biol. Chem. 272*, 25326−25332.
15. Ishiguro, K., Omori, A., Takamatsu, M., Sato, K., Arioka, M., Ucida, T., and Imahori, K. (1992) Phosphorylation sites on tau by tau protein kinase I, a bovine derived kinase generating an epitope of paired helical filaments, *Neurosci. Lett. 148*, 202−206.
16. Lee, C. W.-C., Lau, K.-F., Miller, C. C. J., and Shaw, P.-C. (2003) Glycogen synthase kinase 3β mediated tau phosphorylation in cultured cell lines, *Mol. Neurosci. 14*, 257−260.
17. Lucas, J. J., Hernandez, F., Gomez-Ramos, P., Moran, M. A., Hen, R., and Avila, J. (2001) Decreased nuclear β-catenin, tau hyperphosphorylation and neurodegeneration in GSK3β conditional transgenic mice, *EMBO J. 20*, 27−39.
18. Spittaels, K., Van den Haute, C., Van Dorpe, J., Geerts, H., Mercken, M., Bruynseels, K., Lasrado, R., Vandezaride, K., Laenen, I., Boon, T., Van Lint, J., Vandenheede, J., Moechars, D., Loos, R., and Van Leuven, F. (2000) Glycogen synthase kinase-3β phosphorylates protein tau and rescues the axonopathy in the central nervous system of human four-repeat tau transgenic mice, *J. Biol. Chem. 275*, 41340−41349.
19. Sun, W., Qureshi, H. Y., Cafferty, P. W., Sobue, K., Agarwal-Mawal, A., Neufield, K. D., and Paudel, H. K. (2002) Glycogen synthase kinase-3β is complexed with tau protein in brain microtubules, *J. Biol. Chem. 277*, 11933−11940.
20. Wagner, U., Utton, M., Gallo, J. M., and Miller, C. C. J. (1996) Cellular phosphorylation of tau by GSK3β influences tau binding to microtubules and microtubule organization, *J. Cell Sci. 109*, 1537−1543.

21. Kim, L., and Kimmel, A. R. (2000) GSK3, a master switch regulating cell-fate specification and tumorigenesis, *Curr. Opin. Genet. Dev. 10*, 508−514.
22. Dajani, R., Fraser, E., Roe, S. M., Young, N., Good, V., Dale, T. C., and Pearl, L. H. (2001) Crystal structure of glycogen synthase kinase 3β: Structural basis for phosphate primed substrate specificity and autoinhibition, *Cell 105*, 721−732.
23. Fiol, J. F., Mahrenholz, A. M., Wang, Y., Roeske, R. W., and Roach, P. J. (1987) Formation of protein kinase recognition sites by covalent modification of the substrate. Molecular mechanism for the synergestic action of casein kinase 2 and glycogen synthase kinase 3, *J. Biol. Chem. 262*, 14042−14048.
24. Liu, S. J., Zhang, J. Y., Li, H. L., Fang, Y. F., Wang, Q., Deng, H. M., Gong, C. X., Grundke-Iqbal, I., Iqbal, K., and Wang, Z. (2004) Tau becomes more favorable substrate of GSK3 when it is prephosphorylated by PKA in rat brain, *J. Biol. Chem. 279*, 50078−50088.
25. Sengupta, A., Wu, Q., Grundke-Iqbal, I., Iqbal, K., and Singh, T. J. (1997) Potentiation of GSK-3-catalyzed Alzheimer-like phosphorylation of human tau by cdk5, *Mol. Cell. Biochem. 167*, 99−105.
26. Hanger, D. P., Hughes, K., Woodgett, J. R., Brion, J.-P., and Anderton, B. H. (1992) Glycogen synthase kinase-3 induces Alzheimer's disease-like phosphorylation of tau: Generation of paired helical filament epitopes and neuronal localization of the kinase, *Neurosci. Lett. 147*, 58−62.
27. Wang, J.-z., Wu, Q., Smith, A., Grundke-Iqbal, I., and Iqbal, K. (1998) τ is phosphorylated by GSK3 at several sites found in Alzheimer disease and its biological activity markedly inhibited only after it is pre-phosphorylated by A-kinase, *FEBS Lett. 436*, 28−34.
28. Agarwal-Mawal, A., Qureshi, H. Y., Cafferty, P. W., Yuan, Z., Han, D., Lin, R., and Paudel, H. K. (2003) 14-3-3 connects glycogen synthase kinase-3β to tau within a brain microtubule-associated tau phosphorylation complex, *J. Biol. Chem. 279*, 12723−12728.
29. Michel, G., Murayama, M. M., Noguchi, K., Ishiguro, K., Imahori, K., and Takashima, A. (1998) Characterization of tau phosphorylation in glycogen synthase kinase-3β and cyclin dependent kinase-5 activator (p23) transfected cells, *Biochim. Biophys. Acta 1380*, 177−182.
30. Seubert, P., Mawal-Dewan, M., Barbour, R., Jakes, R., Goedert, M., Johnson, G. V. W., Litersky, J. M., Schenk, D., Lieberburg, I., Trojanowski, J. Q., and Lee, V. M.-Y. (1995) Detection of phosphorylated Ser^{262} in fetal tau, adult tau, and paired helical filament tau, *J. Biol. Chem. 270*, 18917−18922.
31. Hallo, J. L., Chen, K., DePinho, R. A., and Vincent, I. (2003) Decreased cyclin-dependent kinase 5 (cdk5) activity is accompanied by redistribution of cdk5 and cytoskeletal proteins and increased cytoskeletal protein phosphorylation in p35 null mice, *J. Neurosci. 23*, 10644−10633.
32. Van den Haute, C., Spittaels, K., Van Dorpe, J., Lasrado, R., Vandezande, K., Laenen, I., Geert, H., and Van Leuven, F. (2001) Coexpression of human cdk5 and its activator p35 with human protein tau in neurons in brain of triple transgenic mice, *Neurobiol. Dis. 8*, 32−44.
33. Cruz, J. C., Tseng, H. C., Goldman, J. A., Shih, H., and Tsai, L. H. (2003) Aberrant Cdk5 activation by p25 triggers pathological events leading to neurodegeneration and neurofibrillary tangles, *Neuron 40*, 471−483.
34. Noble, W., Olm, V., Takata, K., Casey, E., Mary, O., Meyerson, J., Gaynor, K., LaFrancois, J., Wang, L., Kondo, T., Davies, P., Burns, M., Veeranna, Nixon, R., Dickson, D., Matsuoka, Y., Ahlijanian, M., Lau, L. F., and Duff, K. (2003) Cdk5 is a key factor in tau aggregation and tangle formation in vivo, *Neuron 38*, 555−565.
35. Ahlijanian, M. K., Barrezueta, N. X., Williams, R. D., Jakowski, A., Kowsz, K. P., McCarthy, S., Coskran, T., Carlo, A., Seymour, P. A., Burkhardt, J. E., Nelson, R. B., and McNeish, J. D. (2000) Hyperphosphorylated tau and neurofilament and cytoskeletal disruptions in mice overexpressing human p25, an activator of cdk5, *Proc. Natl. Acad. Sci. U.S.A. 97*, 2910−2915.
36. Takashima, A., Murayama, M., Yasutake, K., Takashi, H., Yokoyama, M., and Ishiguro, K. (2001) Involvement of cyclin-dependent kinase 5 activator p25 on tau phosphorylation in mouse brain, *Neurosci. Lett. 306*, 37−40.
37. Cho, J. H., and Johnson, G. V. (2003) Glycogen synthase kinase 3β phosphorylates tau at both primed and unprimed sites. Differential impact on microtubule binding, *J. Biol. Chem. 278*, 187−193.
38. Kang, D. E., Soriano, S., Xia, X., Eberhart, E. G., Strooper, B. D., Zheng, H., and Koo, E. H. (2000) Presenilin couples the paired phosphorylation of β-catenin independent of axin: Implications of β-catenin activation in tumorigenesis, *Cell 110*, 751−762.
39. Polakis, P. (1999) The oncogenic activation of β-catenin, *Curr. Opin. Genet. Dev. 9*, 15−21.
40. Vincent, I., Rosado, M., and Davies, P. (1996) Mitotic mechanisms in Alzheimer's disease? *J. Cell Biol. 132*, 413−425.

BI051634R