Cyclin-Dependent Protein Kinase 5 Primes Microtubule-Associated Protein Tau Site-Specifically for Glycogen Synthase Kinase 3β[†]

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ABSTRACT: In the preceding paper, we showed that GSK3β phosphorylates tau at S^{202}, T^{231}, S^{396}, and S^{400} in vivo. Phosphorylation of S^{202} occurs without priming. Phosphorylation of T^{231}, on the other hand, requires priming phosphorylation of S^{235}. Similarly, priming phosphorylation of S^{404} is essential for the sequential phosphorylation of S^{400} and S^{396} by GSK3β. The priming kinase that phosphorylates tau at S^{235} and S^{404} in the brain is not known. In this study, we find that in HEK-293 cells cotransfected with tau, GSK3β, and Cdk5, Cdk5 phosphorylates tau at S^{202}, S^{235}, and S^{404}. S^{235} phosphorylation enhances GSK3β-catalyzed T^{231} phosphorylation. Similarly, Cdk5 by phosphorylating S^{404} stimulates phosphorylation of S^{400} and S^{396} by GSK3β. These data indicate that Cdk5 primes tau for GSK3β in intact cells. To evaluate if Cdk5 primes tau for GSK3β in mammalian brain, we examined localizations of Cdk5, tau, and GSK3β in rat brain. We also analyzed the interaction of Cdk5 with tau and GSK3β in brain microtubules. We found that Cdk5, GSK3β, and tau are virtually colocalized in rat brain cortex. When bovine brain microtubules are analyzed by FPLC gel filtration, Cdk5, GSK3β, and tau coelute within an ~450 kDa complex. From the fractions containing the ~450 kDa complex, tau, Cdk5, and GSK3β coimmunoprecipitate with each other. In HEK-293 cells transfected with tau, Cdk5, and GSK3β in different combinations, tau binds to Cdk5 in a manner independent of GSK3β and to GSK3β in a manner independent of Cdk5. However, Cdk5 and GSK3β bind to each other only in the presence of tau, suggesting that tau connects Cdk5 and GSK3β. Our results suggest that in the brain, tau, Cdk5, and GSK3β are components of an ~450 kDa complex. Within the complex, Cdk5 phosphorylates tau at S^{235} and primes it for phosphorylation of T^{231} by GSK3β. Similarly, Cdk5 by phosphorylating tau at S^{404} primes tau for a sequential phosphorylation of S^{400} and S^{396} by GSK3β.

Paired helical filaments (PHFs),[1] the major structural component of neurofibrillary tangles (NFTs) associated with Alzheimer's disease (AD), are mainly composed of abnormally hyperphosphorylated tau protein (*1*−*3*). Inactive PHF-tau (tau isolated from PHFs) regains its biological activity to bind and promote microtubule assembly upon dephosphorylation (*4*, *5*). Abnormal tau phosphorylation has been suggested as being the early step leading to neurofibrillary pathology (for a review, see ref *2*). Therefore, a clear understanding of how tau is phosphorylated in the brain is essential for understanding AD pathology and designing therapeutic interventions. In AD brain, tau is phosphorylated mostly on sites which contain a motif recognized by proline-directed kinase(s) (*3*, *6*). Among proline-directed kinases, glycogen synthase kinase 3β (GSK3β) is strongly expressed in the brain and phosphorylates tau in vitro and in vivo (*7*−*19*). GSK3β is present in brain microtubules and interacts with tau within microtubules (*8*, *19*). Importantly, active GSK3β colocalizes with NFTs in AD brain (*20*). Current evidence indicates that GSK3β is one of the major kinases that phosphorylate tau in AD brain.

GSK3β displays a unique substrate specificity and often requires a priming phosphate at the $n + 4$ position, where n is the phosphorylation site (*21*, *22*). For example, GSK3β phosphorylates tau at T^{231} only when tau has been previously phosphorylated at S^{235} (*23*, *24*). Following the priming phosphorylation of S^{404}, GSK3β sequentially phosphorylates tau at S^{400} and then S^{396} (*24*). The physiological significance of this substrate specificity of GSK3β is not very well understood. However, this uniqueness allows GSK3β to sequentially phosphorylate and make tau hyperphosphorylated. More importantly, this mechanism provides a priming kinase that phosphorylates S^{235} control over T^{231} phosphorylation by GSK3β. Similarly, the priming kinase that phosphorylates S^{404} can regulate sequential phosphorylation

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

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of S^{400} and S^{396} by GSK3β. Studies have suggested that phosphorylation of tau at T^{231} and S^{396} plays a major role in regulating tau function and may have roles in the development of neurofibrillary pathology of AD (*4*, *5*, *24*−*26*). Though the priming kinase(s) that phosphorylates tau at S^{235} and S^{404} thereby controlling GSK3β-catalyzed phosphorylation of T^{231}, S^{396}, and S^{400} is not known, both phosphorylation sites, S^{235} and S^{404}, are proline-directed (*3*). This observation suggests that the priming kinase may be a proline-directed kinase.

Brain proline-directed kinase Cdk5 (also known as neuronal Cdc2-like kinase or tau kinase II) is a member of the cyclin-dependent kinase family with a predominant neural expression. It is a heterodimer consisting of a catalytic Cdk5 subunit and an ~25 kDa regulatory subunit (*27*). The p25 subunit is a proteolytic fragment of an ~35 kDa protein and is an activator of Cdk5 kinase activity. Initially, Cdk5 was purified from bovine brain microtubules on the basis of its ability to phosphorylate tau (*7*). Subsequent studies have shown that Cdk5 phosphorylates tau in vitro and in vivo (*28*−*33*) and has been suggested to phosphorylate tau in AD brain (*2*). More importantly, in transgenic mice overexpressing Cdk5 activity in the brain and in mammalian cells transfected with tau and Cdk5, Cdk5 phosphorylates tau at S^{235} and S^{404} (*14*, *33*, *34*). These observations suggest that Cdk5 may prime tau for GSK3β in the brain. To test this possibility, we have evaluated tau phosphorylation by Cdk5 and GSK3β individually and together. We have also investigated the interaction of these three proteins in rat brain and purified microtubules. Herein, we show that Cdk5 phosphorylates tau at S^{235}, thereby facilitating the phosphorylation of T^{231} by GSK3β. Similarly, Cdk5 phosphorylates tau at S^{404} and enhances GSK3β-catalyzed tau phosphorylation of S^{400} and S^{396}. We show that Cdk5 is colocated with tau and GSK3β in rat brain cortex and is part of a large macromolecular complex consisting of tau and GSK3β. Our results suggest that Cdk5 primes tau for GSK3β in the brain.

MATERIALS AND METHODS

Proteins, Antibodies, and Enzyme Assays. Anti-mouse GSK3β was purchased from BD Biosciences (Mississauga, ON). Anti-rabbit Cdk5, anti-rabbit p35 (that cross-reacts with both p35 and p25), and anti-goat tau were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-tau polyclonal, anti-Cdk5 monoclonal, and monoclonal antibody that recognizes GSK3α and GSK3β have been described previously (*19*). Monoclonal anti-Flag, anti-Xpress, anti-HA, and anti-Myc have also been described (*10*, *11*). 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, AT8, pS400, and pS404, have been described previously (*19*, *24*). Texas Red (TR)-conjugated and fluorescein isothiocyanate-conjugated (FITC) secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). Polyclonal anti-MAP kinase and anti-Cdc2 kinase antibodies were purchased from Upstate Biotechnology.

Fluorescence Immunohistochemisty. Four adult male Sprague-Dawley rats (HSLAS, University of Alberta) weighing 250−300 g each were deeply anesthetized with 4% chloral hydrate (VWR Canlab, Montreal, PQ) before perfusion with 0.01 M phosphate-buffered saline (PBS, pH 7.2) followed by 4% paraformaldehyde. Brains were removed, postfixed overnight, and stored at 4 °C in a 30% PBS/sucrose solution before being coronally sectioned (20 μm) on a cryostat and processed for double-immunofluorescence labeling as described previously (*35*). In brief, free-floating sections were sequentially incubated overnight at room temperature with either an anti-Cdk5 (1:50)/anti-GSK3β (1:50), an anti-Cdk5 (1:50)/anti-tau (1:50), or an anti-GSK3β (1:50)/anti-tau (1:100) mixture. After being washed with PBS, the sections were exposed to combinations of TR-conjugated anti-rabbit (1:100)/FITC-conjugated anti-mouse (1:100) or FITC-conjugated anti-rabbit (1:100)/TR-conjugated anti-goat (1:100) antibodies for 2 h. Sections were washed thoroughly with PBS, cover-slipped with Vectashield mounting medium (Vector Laboratories), and then visualized under a Zeiss fluorescence microscope. The captured images from two channels were then processed using Adobe Photoshop 6.0.

cDNA Cloning, Cell Culture, and Transfection. The human Cdk5 and bovine p25 cDNAs, each in the pGEX-2T vector, were amplified by polymerase chain reaction (PCR) using *pfu* DNA polymerase and the following primers: Cdk5 forward primer (5′-CGC GGA TCC TGC AGA AAT ACG AGA AAC TGG AA-3′) containing a *Bam*HI site, Cdk5 reverse primer (5′-AAA GGG CCC CTA GGG CGG ACA GAA GTC GGA GAA-3′) containing an *Apa*I site, p25 forward primer (5′-AA AAG AAT TCA GCC AAC CTG TCC ACG TTC-3′) containing an *Eco*RI site, and p25 reverse primer (5′-A ACG GGA TCC TTA GCT CTC GTT CTT CAG GTC-3′) containing a *Bam*HI site. After amplification, the Cdk5 cDNA was subcloned into a pcDNA 3.1/HisA vector (Invitrogen) at *Bam*HI and *Apa*I sites and p25 cDNA was subcloned into the Flag-pcDNA3.1 vector at *Eco*RI and *Bam*HI sites. Subcloning of p35 was performed as described above using a forward primer (ATT TGA ATT CAG GCA CGG TGC TGT TCC C), a reverse primer (ACG CGG ATC CTC ACC GGT CCA GCC CGA), and the pBluescript KS+ plasmid containing bovine p35. The subcloned DNA was ligated into *Eco*RI and *Bam*HI sites of 5′-Myc pcDNA3.1/Zeo, which expressed Myc-tagged p35. Other plasmids, pcDNA3.1 containing C-terminally HA-tagged GSK3β and Flag-pcDNA3.1/Zeo containing the longest human tau isoform, were described previously (*10*). Generation of tau site-specific mutants (S202A, T231A, S235A, S396A, S400A, and S404A) containing Ala in place of the indicated Ser or Thr has been described previously (*24*).

HEK-293 cells were maintained in 10 cm dishes and transfected with the indicated plasmid(s) using Lipofectamine 2000 reagent (Invitrogen) and harvested as described previously (*24*). The amount of DNA used for various transfections was 6 μg of Flag-tau, 8 μg of HA-GSK3β, 6 μg of Xpress-Cdk5, 4 μg of Flag-p25, or 4 μg of Myc-p35. Because transient transfection can stress cells and change activities of intracellular kinases and phosphatases, the total amount of DNA in each transfection was maintained at 24 μg. In some transfections in which the amount of DNA was less than 24 μg, an appropriate amount of mock DNA (His-tag pcDNA 3.1) was added to bring the total amount of DNA to 24 μg. Cells were treated with olomoucine or LiCl as

follows. After transfection, cells were replaced with fresh medium containing either 100 μM olomoucine or 20 mM LiCl and incubated for 1 h before being harvested. Control cells for olomoucine and LiCl were treated with equal amounts of DMSO and KCl, respectively. Other methods have been described previously (*24*).

Microtubule Purification and FPLC Gel Filtration. Unless otherwise stated, all procedures were performed at 4 °C. Microtubules were purified from a fresh bovine brain extract by temperature-induced microtubule assembly and disassembly (*19*). Purified microtubules were disassembled by being incubated at 0 °C for 30 min and then centrifuged at 27000*g* for 30 min. The supernatant was loaded onto a phosphocellulose column pre-equilibrated in PEM buffer [100 mM PIPES (pH 6.8), 1 mM EGTA, 1 mM $MgSO_4$, and 1 mM β-mercaptoethanol] containing 0.1 mM GTP. The column was washed, and column-bound tau, Cdk5, and GSK3β were eluted with 0.8 M NaCl in PEM buffer. Effluent fractions containing tau, Cdk5, and GSK3β were combined and dialyzed against Mops buffer [25 mM Mops (pH 7), 50 mM β-glycerol phosphate, 0.2 M NaCl, 10 mM NaF, 15 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT]. The dialyzed sample (~25 mL) was concentrated to ~2 mL by dialysis against aquacide III (Calbiochem). The concentrated sample was again dialyzed against Mops buffer and centrifuged and a portion of the supernatant (~1 mL) loaded onto a Pharmacia Superose 12 HR 10/30 analytical column (1 cm × 30 cm) pre-equilibrated in Mops buffer. Fractions (0.25 mL each) were collected.

Anti-GSK3β Immunoaffinity Chromatography. An anti-GSK3β immunoaffinity column was prepared using polyclonal anti-GSK3β antiserum and Affi-Gel HZ immunoaffinity beads (Bio-Rad) following the procedure described in the manufacturer's instruction manual. The column (~1 mL) was washed and then equilibrated with ~20 mL of 0.1 M Tris-HCl (pH 7.5), 1 M NaCl, 0.1 mM EDTA, and 0.1 mM DTT. The sample (~1.5 mL) was diluted with an equal volume of the equilibration buffer and then loaded onto the column. The column was washed with ~50 mL of the equilibration buffer and then eluted with 0.2 M glycine (pH 2.2). Fractions (0.25 mL each) were collected.

Kinase Activity Assay. A synthetic peptide derived from the cAMP response element-binding protein (KRREILSRRPSYR) which becomes a specific substrate of GSK3 upon phosphorylation by PKA was prepared and used as a substrate to assay GSK3 activity as described previously (*19*). The final concentrations of the various assay components were 25 mM Mops (pH 7.2), 50 mM β-glycerol phosphate, 0.2 M NaCl, 10 mM NaF, 15 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.5 mM [γ-^{32}P]ATP, and 50 μM PKA-phosphorylated peptide substrate. The reaction was initiated by adding 10 μL of the sample to 20 μL of the reaction mixture containing the rest of the assay components. After 30 min at 30 °C, 10 μL of 50% trichloroacetic acid was added to the assay mixture to stop the reaction. The mixture was incubated at 4 °C for 10 min and then centrifuged using a benchtop centrifuge, and 20 μL of supernatant was withdrawn and analyzed via a phosphocellulose filter paper assay to determine the amount of radioactivity incorporated into the peptide substrate (*19*). Cdk5 activity was assayed essentially as described above except the KTPKKAKKPKTPKKAKKL substrate peptide, derived from histone H1 (*30*), was used. To assay tau phosphorylation in the brain extract, 20 μL of the indicated column fraction was mixed with 5 μL of [γ-^{32}P]ATP. The final concentrations of various assay components were 20 mM Mops (pH 7.2), 40 mM β-glycerol phosphate, 0.16 M NaCl, 8 mM NaF, 12 mM $MgCl_2$, 0.8 mM EDTA, 0.8 mM DTT, and 0.5 mM [γ-^{32}P]-ATP. After the mixture had been incubated for 10 min at 30 °C, 20 μL was withdrawn and immunoblotted using the anti-tau antibody. The blot was then autoradiographed to evaluate tau phosphorylation.

RESULTS

Tau Site Phosphorylated by Cdk5 and GSK3β Individually and Together. To determine if Cdk5 primes tau for GSK3β, we examined tau phosphorylation by Cdk5 and GSK3β individually and together under identical conditions at T^{231}, S^{235}, S^{396}, S^{400}, and S^{404}. Since GSK3β does not phosphorylate S^{262} and phosphorylates S^{202} without requiring priming (*24*), we analyzed phosphorylation of S^{202} and S^{262} as controls. We transfected Flag-tau, HA-GSK3β, and Xpress-Cdk5/Flag-p25 in HEK-293 cells and evaluated tau phosphorylation using various tau phosphorylation sensitive antibodies. We used AT8, TG3, MC6, 12E8, PHF1, pS400, and pS404 that cross-react with tau phosphorylated at S^{202}, T^{231}, S^{235}, S^{262}, S^{396}, S^{400}, and S^{404}, respectively (*24*).

Cells transfected with Flag-tau and HA-GSK3β displayed no enhancement of phosphorylation at S^{235}, S^{262}, and S^{404} when compared to cells transfected with Flag-tau alone (compare lanes 1 and 2 of corresponding blots in Figure 1A, and see Figure 1B). However, tau became ~2.6-, ~3.3-, ~3.5-, and ~2.5-fold more phosphorylated at S^{202}, T^{231}, S^{396}, and S^{400}, respectively, when cotransfected with HA-GSK3β than when transfected alone (compare lanes 1 and 2 of corresponding blots in Figure 1A, and see Figure 1B). These data are consistent with a previous report (*24*) and indicate that in vivo GSK3β phosphorylated tau at S^{202}, T^{231}, S^{396}, and S^{400} and does not phosphorylate S^{235}, S^{262}, and S^{404}.

Tau was ~3.8-fold more phosphorylated at S^{202} in cells transfected with Flag-tau and Xpress-Cdk5/Flag-p25 (Xpress-Cdk5 and Flag-p25) than in those transfected with Flag-tau alone (compare lanes 1 and 3 in Figure 1A, and see Figure 1B). The extent of phosphorylation of S^{235} and S^{404} was ~4.9- and ~4.6-fold greater, respectively, in cells transfected with Flag-tau and Xpress-Cdk5/Flag-p25 compared to those with Flag-tau alone (compare lanes 1 and 3 of corresponding blots in Figure 1A, and see Figure 1B). However, the extent of phosphorylation of T^{231} and S^{396} was ~1.4- and ~1.3-fold greater, respectively, in cells transfected with Flag-tau and Xpress-Cdk5/Flag-p25 than in those transfected with Flag-tau alone (Figure 1A,B). At S^{262} and S^{400}, however, tau phosphorylation did not change significantly in cells transfected with Flag-tau and Xpress-Cdk5/Flag-p25 when compared with those transfected with Flag-tau alone (Figure 1A,B). When a similar experiment was performed and transfected cells were treated with Cdk5 inhibitor olomoucine (*36*), tau phosphorylation at S^{202}, S^{235}, and S^{404} was inhibited to a basal level (data not shown). These data demonstrate that Cdk5 phosphorylates tau at S^{202}, S^{235}, and S^{404} and does not phosphorylate significantly T^{231}, S^{262}, S^{396}, or S^{400} in vivo.

To prime tau for GSK3β, Cdk5 must phosphorylate S^{235} and S^{404} in the presence of GSK3β. Likewise, GSK3β must

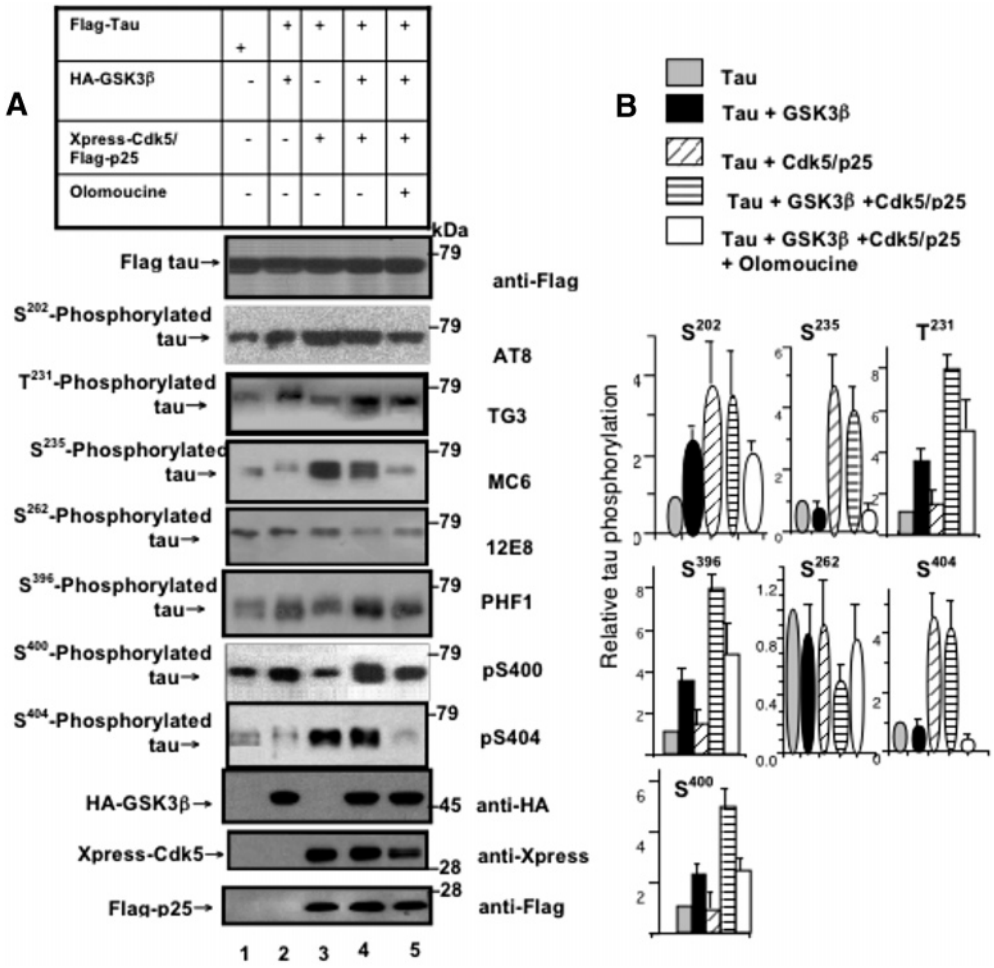

FIGURE 1: Tau phosphorylation by Cdk5 and GSK3β. HEK-293 cells transfected with the indicated genes were analyzed by immunoblot analysis to evaluate tau phosphorylation and expression of various transfected genes. Immunoblots representing antibodies AT8, TG3, MC6, 12E8, PHF1, pS400, and pS404 show tau phosphorylation on the indicated sites, whereas those representing anti-Flag, anti-HA, and anti-Xpress demonstrate expression of the respective genes. Note that both tau and p25 contain a Flag tag but migrate with ~68 and ~25 kDa sizes on SDS gels: (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 representing Flag-tau (lane 1) of that blot. The values are an average of three independent determinations.

phosphorylate T^{231} and S^{396} in the presence of Cdk5. To examine how Cdk5 and GSK3β each phosphorylate tau in the presence of the other, we analyzed cells transfected with Flag-tau, HA-GSK3β, and Xpress-Cdk5/Flag-p25. Phosphorylation of tau at S^{262} remained at a basal level in cells transfected with Flag-tau, HA-GSK3β, and Xpress-Cdk5/Flag-p25 (compare lanes 1 and 4 in Figure 1A, and see Figure 1B). These data indicate that neither Cdk5 nor GSK3β phosphorylates tau at S^{262} in these cells. However, tau became highly phosphorylated at S^{202}, T^{231}, S^{235}, S^{396}, S^{400}, and S^{404} in cells transfected with Flag-tau, HA-GSK3β, and Xpress-Cdk5/Flag-p25 when compared to control cells transfected with Flag-tau alone (Figure 1A, lane 4, and Figure 1B). These data show that in cells containing Flag-tau, HA-GSK3β, and Xpress-Cdk5/Flag-p25, tau is phosphorylated at S^{202}, T^{231}, S^{235}, S^{396}, S^{400}, and S^{404}. To identify the kinase(s) responsible for this, we treated cells transfected with Flag-tau, HA-GSK3β, and Xpress-Cdk5/Flag-p25 with the Cdk5-specific inhibitor olomoucine. Olomoucine almost completely suppressed phosphorylation of tau at S^{235} and S^{404} (Figures 1A, lane 5 of corresponding blots, and Figure 1B). When a similar experiment was performed using the GSK3β inhibitor LiCl, in place of olomoucine, phosphorylation of tau at T^{231}, S^{396}, and S^{400} was suppressed to a basal level without any effect on phosphorylation of S^{235} and S^{404} (data not shown but see Figure 2). Phosphorylation of S^{202}, on the other hand, in the transfected cells described above was not significantly influenced by olomoucine or LiCl but was suppressed to a basal level when cells were treated with both reagents together (data not shown). This observation is as expected since both Cdk5 and GSK3β phosphorylate tau at S^{202}. On the basis of these results, we concluded that in the cells containing tau, Cdk5, and GSK3β, S^{235} and S^{404} are phosphorylated by Cdk5 whereas T^{231}, S^{396}, and S^{400} are phosphorylated by GSK3β. S^{202} on the other hand is phosphorylated by both Cdk5 and GSK3β.

Cdk5 Primes Tau for GSK3β. Tau is phosphorylated at T^{231}, S^{396}, and S^{400} by GSK3β (Figure 1 and ref *24*). However, previous phosphorylation of S^{235} is required for GSK3β to phosphorylate T^{231} (*24*). Similarly, priming phosphorylation of S^{404} is essential for GSK3β to phosphorylate tau at S^{400} followed by that at S^{396} (*24*). As shown in Figure 1B, Cdk5 phosphorylates tau at S^{235} and S^{404} to a level over the basal level in cells transfected with Flag-tau

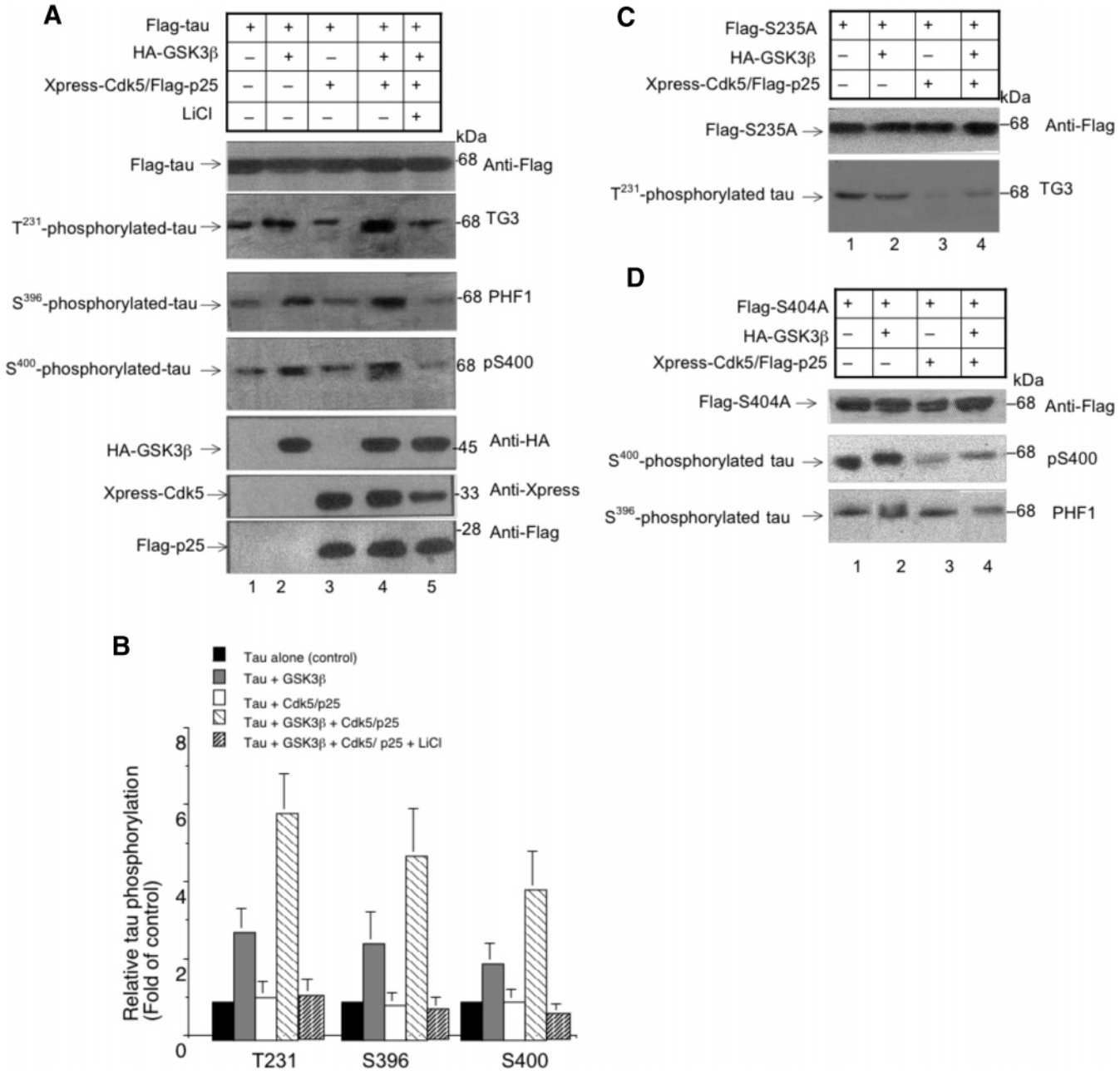

FIGURE 2: Effect of Cdk5 on phosphorylation by GSK3β of tau (WT) and tau site-specific mutants S235A and S404A. (A and B) Phosphorylation of tau (WT). HEK-293 cells transfected with the indicated genes were analyzed by immunoblot analysis to evaluate tau phosphorylation and expression of various transfected genes. (B) Relative tau phosphorylation. Blots representing tau in panel A were scanned, and band intensity values of each tau band were obtained and used to determine the relative level of tau phosphorylation as in Figure 1 using lane 1 representing Flag-tau as the control. The values are an average of three independent determinations. (C and D) Phosphorylation of tau site-specific mutants S235A and S404A. The Flag-tagged tau site-specific S235A or S404A mutant was cotransfected with the indicated genes in HEK-293 cells. Transfected cells were lysed and analyzed by immunoblot analysis to evaluate phosphorylation at indicated sites using the respective antibodies.

and Xpress-Cdk5/Flag-p25. Therefore, if Cdk5 primes tau for GSK3β, the level of phosphorylation of tau at T^{231}, S^{400}, and S^{396} by GSK3β should increase in cells transfected with tau, GSK3β, and Cdk5/p25 compared to that in cells transfected with Flag-tau and GSK3β.

To test this idea, we transfected HEK-293 cells with Flag-tau, HA-GSK3β, and Xpress-Cdk5/Flag-p25 in different combinations. Transfected cells were then analyzed for tau phosphorylation. As shown in panels A and B of Figure 2, T^{231}, S^{396}, and S^{400} of tau were ~2.8-, ~2.5-, and ~2-fold more phosphorylated, respectively, in cells transfected with Flag-tau and HA-GSK3β than in those transfected with Flag-tau. These data are consistent with results in Figure 1 and indicate that GSK3β phosphorylates tau at T^{231}, S^{400}, and S^{396}. More importantly, in cells transfected with Flag-tau, HA-GSK3β, and Xpress-Cdk5/Flag-p25, tau was ~5.9-, ~4.8-, and ~3.9-fold more phosphorylated at T^{231}, S^{396}, and S^{400}, respectively, than in those transfected with Flag-tau and ~2.1-, ~1.9-, and ~2-fold more phosphorylated, respectively, than in those transfected with Flag-tau and HA-GSK3β (Figure 2A,B). In cells transfected with Flag-tau, HA-GSK3β, and Xpress-Cdk5/Flag-p25 and treated with GSK3β inhibitor LiCl, phosphorylation of tau at T^{231}, S^{396}, and S^{400} was almost completely inhibited to a basal level

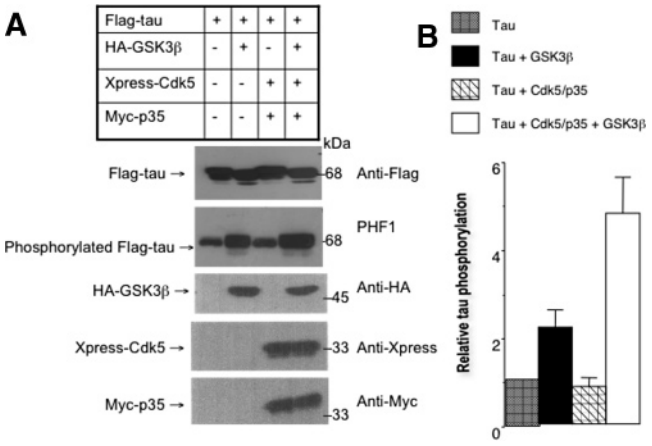

FIGURE 3: Effect of Cdk5/p35 on tau phosphorylation by GSK3β. HEK-293 cells transfected with the indicated genes were analyzed by immunoblot analysis using the indicated antibodies: (A) immunoblots and (B) relative tau phosphorylation. Blots representing phosphorylated tau and Flag-tau were scanned, and blot band intensities were used to calculate the relative level of tau phosphorylation as in Figure 1. The values are the average of three determinations.

(Figure 2A, lane 5, and Figure 2B). These data indicate that phosphorylation of tau at T^{231}, S^{235}, and S^{400} in these cells was catalyzed by GSK3β. Thus, in HEK-293 cells, Cdk5 stimulated GSK3β-catalyzed phosphorylation of T^{231}, S^{396}, and S^{400}. When a similar experiment was performed by replacing wild-type (WT) tau with a tau S235A mutant, Cdk5 completely failed to facilitate T^{231} phosphorylation by GSK3β (compare lanes 2 and 4 of Figure 2C, bottom panel). Similarly, Cdk5 could not stimulate GSK3β-mediated phosphorylation of S^{400} or S^{396} of tau mutant S404A (compare lanes 2 and 4 of Figure 2D, middle and bottom panels). On the basis of these data, we concluded that in HEK-293 cells transfected with Flag-tau, HA-GSK3β, and Xpress-Cdk5/Flag-p25, Cdk5 phosphorylates tau at S^{235} and stimulates GSK3β-catalyzed phosphorylation of T^{231}. Similarly, Cdk5 phosphorylates tau at S^{404} and enhances phosphorylation of S^{400} and S^{396} by GSK3β.

p25, the activating subunit of Cdk5, is the proteolytic fragment of a 35 kDa protein, p35. Both p35 and p25 can activate Cdk5 in vivo (*27*−*34*). To determine if Cdk5/p35 can prime tau for GSK3β, we cotransfected Flag-tau with HA-GSK3β, Xpress-Cdk5, and Myc-p35 in different combinations and analyzed tau phosphorylation. As shown in Figure 3A (second blot from the top), tau did not become significantly more phosphorylated in cells transfected with Flag-tau and Xpress-Cdk5/Myc-p35 when compared with those transfected with Flag-tau alone (compare lanes 1 and 3). This indicates that Cdk5/p35 does not phosphorylate S^{396} of tau. HA-GSK3β, on the other hand, phosphorylated S^{396} in the absence (lane 2) and presence of Xpress-Cdk5/Myc-p35 (lane 4). More importantly, HA-GSK3β phosphorylated S^{396} ~2.4-fold more strongly in the presence of Xpress-Cdk5/Flag-p35 than in its absence (compare lanes 2 and 4, and see Figure 3B). This result demonstrates that Cdk5/p35, like Cdk5/p25, stimulates phosphorylation of S^{396} by GSK3β. This observation is consistent with the idea that Cdk5/p35 also primes tau for GSK3β in vivo.

Tau, GSK3β, and Cdk5 Are Colocalized in Rat Brain Cortex. To prime tau for GSK3β in vivo, Cdk5 is expected to be present in the regions of the brain where GSK3β phosphorylates tau. Cdk5 is also expected to interact with tau and GSK3β. To locate regions of the brain where GSK3β phosphorylates tau, we determined and compared immunohistochemical localizations of tau and GSK3β in the adult rat brain cortex. GSK3β immunoreactive neurons were detected in most layers of the cortex with varying intensities (Figure 4A,G, green). Moderate immunoreactivity was noted in the neurons of layers I−IV, while neuronal perikarya in deeper cortical layers V and VI demonstrated strong labeling. GSK3β immunoreactivity was evident in cell bodies and many associated axonal processes. Tau, on the other hand, was labeled intensely in axonal processes and to some extent in the cell cytoplasm of neurons throughout the cortex (Figure 4B,E, red). These observations are consistent with previous reports (*37*−*39*). Importantly, immunoreactive tau was colocalized with GSK3β in the perinuclear domain of the cortical neurons and in the proximal component of the axons (Figure 4C, yellow). These data indicate that tau and GSK3β colocalize in the cortex, and hence, GSK3β likely phosphorylates tau in this area of the brain.

As observed previously (*40*), Cdk5 immunoreactive neurons were present throughout the cortex with a characteristic pattern of distribution. Neurons of the superficial layers were labeled moderately, whereas neurons in layers V and VI were labeled intensely (Figure 4D, green; Figure 4H, red). Within the neurons, Cdk5 immunoreactivity was evident in the cell body as well as in a number of axonal processes. Importantly, Cdk5-positive neurons also showed tau-positive fibers in all cortical layers (Figure 4F, yellow). Thus, Cdk5 colocalized with tau within all cortical layers and, hence, very likely phosphorylates tau in these areas of the brain.

Our double labeling results revealed that the majority of GSK3β-positive neurons in the cortex (Figure 4G, green) express Cdk5 (Figure 4H, red) and that GSK3β and Cdk5 within these neurons colocalize (Figure 4I, yellow). Furthermore, this colocalization was observed within areas of the cortical neurons where these two kinases colocalize with tau (Figure 4C,F). These observations suggest that GSK3β and Cdk5 phosphorylate tau within the same brain regions, and hence, Cdk5 can prime tau for GSK3β.

Interaction of Tau, Cdk5, and GSK3β in the Brain. Tau is a microtubule-associated protein. When microtubules are purified from brain extract, tau which is bound to microtubules is copurified with microtubules (*19*). GSK3β and Cdk5 also are copurified with microtubules from brain extract in a manner similar to that of tau (*7*, *8*, *19*, *30*). Moreover, both GSK3β and Cdk5 interact with tau within brain microtubules (*19*, *30*). Therefore, to examine if Cdk5 interacts with tau and GSK3β in the brain, we purified microtubules from bovine brain extract and subjected them to phosphocellulose chromatography. Tau, Cdk5, and GSK3β were bound to the column, whereas tubulins were recovered in the flow-through fractions (data not shown). The column was eluted, and fractions containing tau, Cdk5, and GSK3β were combined, concentrated, and subjected to FPLC gel filtration chromatography using a highly resolving Superose-12 column.

GSK3 activity eluted as several overlapping peaks within fractions 34−72 (Figure 5A). Previous studies have demonstrated that ~51 kDa GSK3α also is copurified with microtubules in a manner similar to that of GSK3β (*8*, *19*). Since the peptide substrate used in this study is phosphorylated by GSK3α and GSK3β with equal efficiencies, we

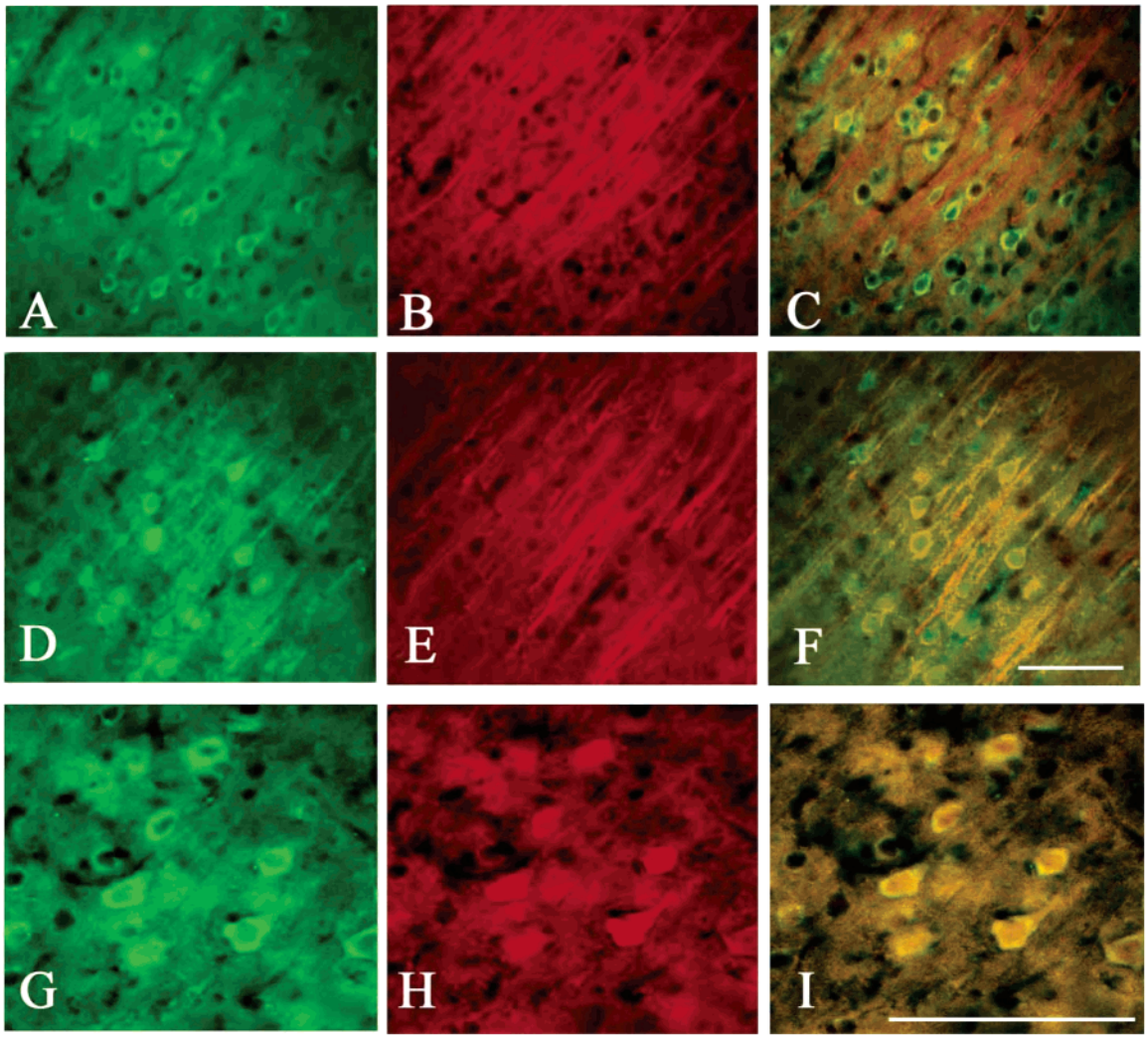

FIGURE 4: Immunofluorescence photomicrographs of the normal adult rat brain cortex transverse sections showing the distributions of GSK3β, tau, and Cdk5: (A and G, green) distribution of GSK3β, (B and E, red) distribution of tau, (D and H, red) distribution of Cdk5, and (C, F, and I, yellow) colocalization of tau and GSK3β, tau and Cdk5, and GSK3β and Cdk5, respectively. The scale bar is 10 μm.

became curious about which isoforms of GSK3 contribute to the different activity peaks. We therefore analyzed various effluent fractions by immunoblot analysis using an antibody that recognized both isoforms of GSK3. GSK3α was present within fractions 34−72 (Figure 5B). GSK3β on the other hand eluted within three overlapping peaks: first within fractions 34−42 with peak fraction 38 and a size of ~600 kDa, second within fractions 44−54 with peak fraction 48 and a size of ~450 kDa, and third within fractions 56−72 with peak fraction 66 and a size of ~50 kDa (Figure 5A).

GSK3β is a monomeric ~47 kDa protein (*40*). In vivo, it may exist as a monomer or may form a complex with variety of proteins to execute its action (*42*). GSK3β eluting within fractions 56−72 with a size of ~50 kDa, therefore, must be the low-molecular mass monomeric kinase. That within fractions 34−42 with a size of ~600 kDa and that within fractions 44−52 with a size of 450 kDa are likely to be GSK3β complexed with other proteins.

In a previous study, we determined that GSK3β eluting within fractions 44−52 is complexed with tau (*19*). Indeed, we detected tau within fractions 44−54 containing the ~450 kDa GSK3β (Figure 5C). However, the sum of the sizes of tau and GSK3β is ~97 kDa compared to the ~450 kDa size of the complex. Furthermore, when an aliquot from fraction 48 containing the ~450 kDa complex was incubated with [γ-^{32}P]ATP/Mg^{2+} and the product analyzed by immunoblot analysis using anti-tau antibody followed by autoradiography of the blot, tau was highly phosphorylated (data not shown). This observation suggested that GSK3β phosphorylates tau within the complex. Since GSK3β phosphorylates primed tau (*24*), this observation also suggested that the kinase that primes tau for GSK3β also may be present within the 450 kDa complex. To test this possibility, we analyzed various fractions by immunoblot analysis using antibodies directed to various kinases that are known to associate with microtubules and phosphorylate tau in vitro. MAP kinases ($p42^{erk1}$ and $p44^{ERk2}$) were present within fractions 62−68, whereas cdc2 kinase was undetectable (data not shown). Interestingly, Cdk5 activity eluted as a large peak within fractions 32−54 (Figure 5A). Activity rapidly peaked in fraction 40 but then eluted slowly, forming a shoulder. Immunoblot analysis indicated that Cdk5 and its p25 subunit were present within active fractions 34−50 (Figure 5D,E).

To examine if Cdk5 is a component of the ~450 kDa complex, we combined gel filtration fractions 46−52. From a portion of the combined column fraction, we performed a series of co-immunoprecipitation experiments. Tau co-immunoprecipitated with GSK3β (Figure 6A, lane 3 of the

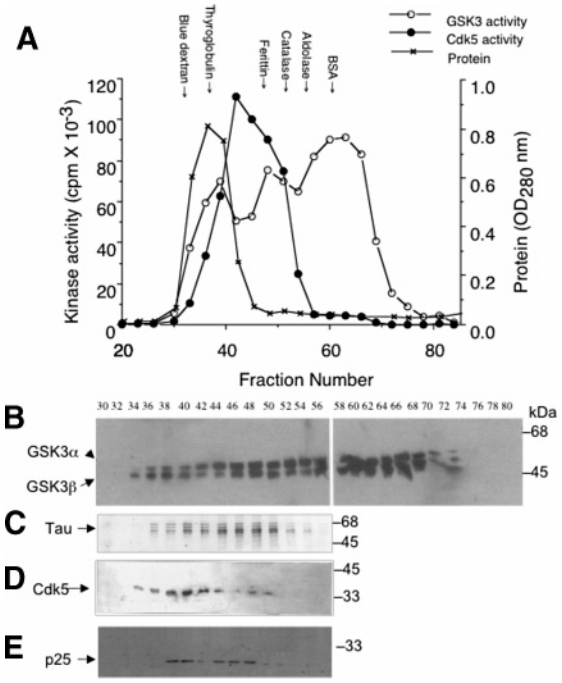

FIGURE 5: FPLC gel filtration of the microtubule fraction. Microtubules purified from bovine brain extract were chromatographed with a phosphocellulose column. The effluent fractions containing tau, Cdk5, and GSK3β were combined and then subjected to FPLC Superose 12 gel filtration. Chromatography was carried out using a Pharamacia FPLC Superose 12 HR 10/30 column (1 cm $\times$ 30 cm) with a flow rate of 0.25 mL/min. Fractions (0.25 mL each) were collected**,** and indicated fractions were analyzed by kinase activity assay and immunoblot analysis: (A) gel filtration profile showing elutions of GSK3 and Cdk5 activities and (B−E) immunoblots of the indicated fractions.

top panel) and Cdk5 (Figure 6B, lane 3 of the top panel). Similarly, Cdk5 co-immunoprecipitated with tau (Figure 6C, lane 3 of the top panel) and GSK3β (Figure 6A, lane 3 of the bottom panel). Interestingly, despite the presence of GSK3α and GSK3β in the combined column fraction (Figure 6B, lane 1 of the bottom panel, and Figure 6C, lane 1 of the bottom panel), only GSK3β co-immunoprecipitated with Cdk5 (Figure 6B, lane 3 of the bottom panel) and tau (Figure 6C, lane 3 of the bottom panel). These observations determined that Cdk5 interacts with both tau and GSK3β within the 450 kDa complex.

To further analyze the ~450 kDa complex, we examined the remainder of above pooled fractions 46−52 by anti-GSK3β immunoaffinity chromatography. GSK3β remained bound to the affinity beads and did not elute from the column (Figure 7A, lane 2). The peak effluent fraction exhibited faint bands with sizes of 25−35 and 50−65 kDa on a silver-stained gel (data not shown). By immunoblot analysis, we identified the 50−65 kDa bands as various tau isoforms and the ~25 and ~33 kDa bands as Cdk5 and p25, respectively (data not shown). Immunoblot analysis of various effluent fractions showed that Cdk5, p25, and tau had coeluted from the column (Figure 7B). Thus, both Cdk5 and tau bind to the anti-GSK3β immunoaffinity column. These observations indicate that GSK3β also interacts with tau and Cdk5 within the 450 kDa complex.

As shown in Figure 5, GSK3β also elutes within fractions 34−42 with a size of ~600 kDa. Interestingly, fractions containing the ~600 kDa complex also contain tau and Cdk5 (Figure 5C,D). To examine the ~600 kDa complex, we pooled fractions 34−42, concentrated them, and subjected them to gel filtration chromatography using the same column and conditions used to generate Figure 5A. Various effluent fractions were analyzed by immunoblot analysis using antibodies directed to GSK3β, Cdk5, and tau. Most of GSK3β, tau, and Cdk5 again eluted within fractions 34−42 (data not shown). Thus, the ~600 kDa GSK3β may represent a larger complex containing GSK3β, tau, and Cdk5 and possibly other biological component(s).

Tau, Cdk5, and GSK3β Interact in Transfected HEK-293 Cells. Within the 450 kDa complex, Cdk5 may interact with tau in a manner independent of GSK3β. Similarly, interaction of Cdk5 with GSK3β may not require tau. Alternatively, Cdk5, tau, and GSK3β may be the components of the same complex. To determine if Cdk5 interacts with tau and GSK3β directly or through any other biological molecule(s) in the brain, we immunoprecipitated either HA-GSK3β or Xpress-Cdk5 from lysates of HEK-293 cells transfected with different combinations of Flag-tau, HA-GSK3β, and Xpress-Cdk5. Xpress-Cdk5 co-immunoprecipitated with Flag-tau from lysates of cells transfected with Flag-tau and Xpress-Cdk5 (Figure 8A, lane 2 of the top panel). Similarly, HA-GSK3β co-immunoprecipitated with Flag-tau from lysates of cells transfected with Flag-tau and HA-GSK3β (Figure 8B, lane 2 of the top panel). Although these data indicated that tau interacts with GSK3β in a manner independent of Cdk5, and with Cdk5 in a manner independent of GSK3β, we could not rule out a possibility that tau may bind to GSK3β or Cdk5 through a molecule which is expressed in brain as well as in HEK-293 cells. Therefore, we performed a GST pull-down assay. Glutathione−agarose beads coated with GST−GSK3β, GST−Cdk5, or GST were incubated with recombinant tau. Incubated beads were washed and immunoblotted with anti-tau antibody. Tau bound to GST−GSK3β and GST−Cdk5 but not GST (Figure 8C). These results demonstrate that tau directly binds to Cdk5 in a manner independent of GSK3β and to GSK3β in a manner independent of Cdk5.

Cdk5 and GSK3β Interact through Tau. From lysates of cells transfected with HA-GSK3β and Xpress-Cdk5, Xpress-Cdk5 did not co-immunoprecipitate with HA-GSK3β (Figure 9A, lane 3) and HA-GSK3β did not co-immunoprecipitate with Xpress-Cdk5 (Figure 9B, lane 3). These data indicated that Cdk5 does not bind to GSK3β. However, Cdk5 and GSK3β co-immunoprecipitate with each other from microtubule fractions prepared from brain extract (Figure 6A, lane 3 of the bottom panel, and Figure 6B, lane 3 of the bottom panel). Therefore, these observations suggest that the interaction of Cdk5 and GSK3β is brain-specific and may require a biological molecule(s) that is not expressed in HEK-293 cells.

Tau is a neuronal protein (*2*). It is colocalized in the brain with Cdk5 and GSK3β (Figure 4). It is copurified from brain microtubules with GSK3β and Cdk5 (Figures 5 and 7). In vitro, it binds to Cdk5 as well as GSK3β (Figure 8C). Therefore, tau may bind to both Cdk5 and GSK3β simultaneously and form a ternary complex. Since HEK-293 cells do not express tau, this may be the reason Cdk5 and GSK3β do not interact when cotransfected in this cell line. If this is true, Cdk5 and GSK3β should bind to each other in HEK-293 cells expressing tau. To test this possibility, we immu-

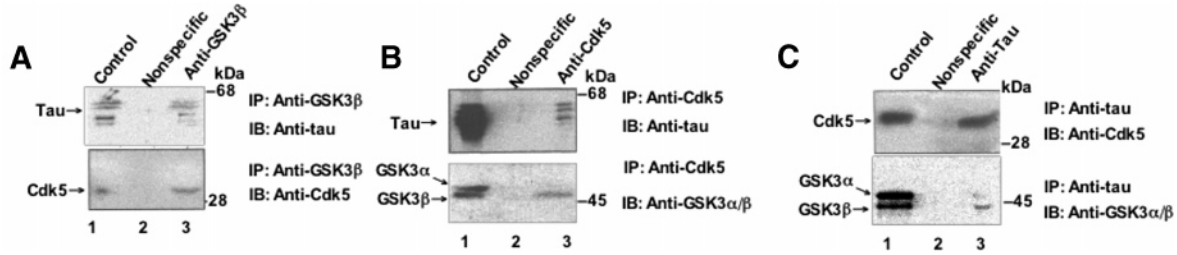

FIGURE 6: Co-immunoprecipitation. Fractions 46−52 from Figure 5A were combined and subjected to immunoprecipitation using the indicated antibodies. Each resulting immune complex was then immunoblotted as indicated. IP and IB indicate immunoprecipitation and immunoblot, respectively. Control (lane 1) represents the combined column fraction used for immunoprecipitation.

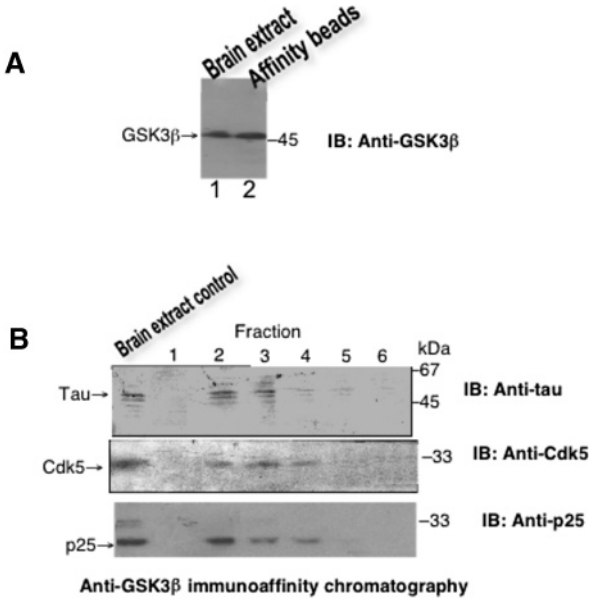

FIGURE 7: Anti-GSK3b immunoaffinity chromatography. A portion of the combined fractions 46−52 form Figure 6 was chromatographed with an anti-GSK3b immunoaffinity column. The indicated fractions were analyzed by immunoblot analysis using the indicated antibodies.

noprecipitated Cdk5 or GSK3β from lysates of cells transfected with Flag-tau, HA-GSK3β, and Xpress-Cdk5. Indeed, Xpress-Cdk5 co-immunoprecipitated with HA-GSK3β (Figure 9A, lane 2) and HA-GSK3β co-immunoprecipitated with Xpress-Cdk5 (Figure 9B, lane 2). These observations indicate that Cdk5 and GSK3β interact with each other through tau.

If tau simultaneously binds to Cdk5 and GSK3β, a complex containing tau, Cdk5, and GSK3β should be formed. Indeed, from the lysates of HEK-293 cells transfected with Flag-tau, Xpress-Cdk5, and HA-GSK3β, HA-GSK3β co-immunoprecipitated with Flag-tau (Figure 9F, lane 2) and Xpress-Cdk5 (Figure 9F, lane 3). Similarly, Xpress-Cdk5 co-immunoprecipitated with Flag-tau (Figure 9G, lane 2) and HA-GSK3β (Figure 9G, lane 5), and Flag-tau co-immunoprecipitated with Xpress-Cdk5 (Figure 9H, lane 3) and HA-GSK3β (Figure 9H, lane 5). Thus, Flag-tau, Xpress-Cdk5, and HA-GSK3β in the cell lysates could not be separated from each other. These data demonstrate the formation of a ternary complex consisting of Flag-tau, HA-GSK3β, and Xpress-Cdk5.

DISCUSSION

The ability of GSK3β to phosphorylate tau at T^{231} and S^{396} suggests that this kinase plays important roles in the regulation of tau function in the normal brain and in the development of NFT pathology in AD brain. Ironically, GSK3β cannot phosphorylate tau at T^{231} if phosphorylation of S^{235} is blocked (*23*, *24*). Similarly, GSK3β does not phosphorylate tau at S^{400} and S^{396}, if S^{404} is not phosphorylated (*24*). These observations indicate that in the brain, GSK3β works as a team with a kinase which primes tau by phosphorylating S^{235} and S^{404}.

Several lines of evidence indicate that Cdk5 primes tau for GSK3β in the brain. In rat brain cortex, Cdk5, GSK3β, and tau are virtually colocalized (Figure 4). From bovine brain microtubules, Cdk5, GSK3β, and tau co-immunoprecipitate with each other (Figure 6) and are copurified via anti-GSK3β immunoaffinity chromatography (Figure 7). In transfected HEK-293 cells, Cdk5 phosphorylates tau at S^{202}, S^{235}, and S^{404} (Figure 1). By phosphorylating S^{235}, Cdk5 enhances phosphorylation of T^{231} by GSK3β. Similarly, By phosphorylating S^{404}, Cdk5 stimulates sequential phosphorylation of S^{400} and S^{396} by GSK3β (Figure 2). Taken together, these observations suggest that Cdk5 by phosphorylating tau at S^{235} and S^{404} signals GSK3β to action. GSK3β subsequently phosphorylates tau at T^{231}, S^{400}, and S^{396}, leading to hyperphosphorylation of tau and perhaps a physiological response.

Cdk5 and GSK3β are widely expressed in various tissues and perform diverse cellular functions (*27*, *42*). Despite being components of different signaling pathways and having distinct cellular targets, both phosphorylate tau, associate with microtubules, and regulate microtubule dynamics in a very similar manner in vivo (*7*, *19*, *30*). Moreover, transgenic animals overexpressing Cdk5 or GSK3β activities display similar phenotypes: hyperphosphorylated tau and nerve damage (*17*, *18*, *29*, *31*, *32*). Importantly, in the brains of transgenic mice overexpressing Cdk5 activity, GSK3β is activated and Cdk5 and GSK3β colocalize with hyperphosphorylated tau (*31*). These observations are consistent with the idea that in the brain Cdk5 and GSK3β are parts of the same regulatory pathway that controls tau phosphorylation in the brain.

Phosphorylation of S^{396} prevents tau from binding to microtubules and accelerates tau aggregation (*4*, *43*). Phosphorylation of T^{231}, on the other hand, allows prolyl isomerase, Pin1, to bind to tau (*44*). Tau-bound Pin1 subsequently restores the ability of phosphorylated tau to associate with microtubules, prevents tau from being phosphorylated further, and accelerates tau's dephosphorylation (*44*). As discussed above, GSK3β phosphorylates T^{231} only when S^{235} has been previously phosphorylated. Likewise, GSK3β catalyzes sequential phosphorylation of S^{400} and S^{396} when S^{404} has been previously phosphorylated. Since

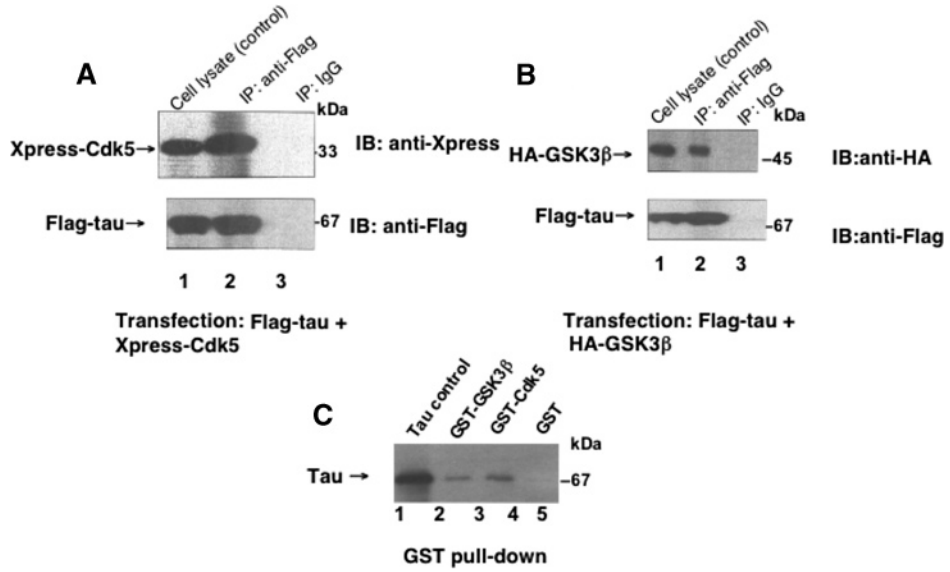

FIGURE 8: Binding of tau with GSK3β and Cdk5. (A and B) Binding in intact cells. Flag-tau was immunoprecipitated from HEK-293 cells transfected with Flag-tau and Xpress-Cdk5 (A) or Flag-tau and HA-GSK3β (B). Each resulting immune complex was immunoblotted against the indicated antibody. These experiments were repeated at least four times with similar results. (C) For in vitro binding, glutathione−agarose beads coated with GST−GSK3β, GST−Cdk5, or GST were mixed with recombinant tau, washed, and immunoblotted by using anti-tau antibody. GST−GSK3β, GST−Cdk5, GST, and the longest bacterially expressed human tau isoform were prepared as described previously (*9*, *19*).

phosphorylation of T^{231} and S^{396} imparts opposite effects on tau function, phosphorylation of T^{231} very likely occurs in a manner independent of S^{396} phosphorylation and vice versa. It is possible that Cdk5 under certain conditions phosphorylates tau at S^{235} and triggers phosphorylation of T^{231} by GSK3β. This is followed by binding of T^{231}-phosphorylated tau with Pin1 and subsequent restoration of damaged microtubule-related tau function. Under different conditions, however, Cdk5 may phosphorylate tau at S^{404} and initiate a phosphorylation cascade of S^{400} followed by S^{396}, making tau hyperphosphorylated and nonfunctional. It should be noted that although juvenile and PHF-tau are phosphorylated at both T^{231} and S^{396}, adult brain tau is phosphorylated at T^{231} but not at S^{396} (*3*, *6*).

In this study, we used HEK-293 cells to elucidate the interaction of tau with Cdk5 and GSK3β. Since tau is predominantly expressed in neurons where it is phosphorylated by Cdk5 and GSK3β, a question of whether the interaction observed in HEK-293 cells also occurs in neurons arises. However, as shown in Figure 4, tau, Cdk5, and GSK3β are colocalized in neurons of rat brain. From microtubules purified from the brain extract, Cdk5, tau, and GSK3β are copurified through an anti-GSK3β immunoaffinity column (Figure 7) and co-immunoprecipitate with each other (Figure 6). Together, these data strongly argue that tau, Cdk5, and GSK3β also interact in neurons. It should be noted that tau is also phosphorylated by the endogenous kinase(s) of HEK-293 cells (Figure 1A). In the previous study, we showed that these endogenous kinases phosphorylate tau and prime tau for GSK3β (*24*). We do not know the number and identity of the kinases of HEK-293 cells that phosphorylate tau. It is possible that some of these kinases may be expressed in neurons and may phosphorylate tau.

Both GSK3α and GSK3β phosphorylate tau in vitro (*45*). However, despite the presence of almost equal amounts, GSK3β but not GSK3α is purified as the tau kinase from bovine brain microtubules (*7*). This study and subsequent studies (*7*−*19*) indicate that GSK3β but not GSK3α is involved in tau phosphorylation in the brain.

In a previous study, we evaluated the interaction of tau with GSK3α and GSK3β (*19*). Consistent with a previous report (*45*), we observed that tau interacts with GSK3β with an affinity much higher than that for its interaction with GSK3α (*19*). In this study, we find that Cdk5 also preferentially interacts with GSK3β over GSK3α (Figure 6B, lane 3 of the bottom panel). However, Cdk5 and GSK3β, which do not co-immunoprecipitate from lysates of cells transfected with HA-GSK3β and Xpress-Cdk5 (Figure 8A, lane 3, and Figure 8B, lane 3), co-immunoprecipitate with each other from lysates of cells transfected with HA-GSK3β, Xpress-Cdk5, and Flag-tau (Figure 9A, lane 2, and Figure 7B, lane 2). Thus, tau simultaneously binds to Cdk5 and GSK3β. Taken together, these observations suggest that tau not only preferentially interacts with GSK3β over GSK3α but also mediates interaction between Cdk5 and GSK3β in the brain.

Microtubule-associated GSK3 activity elutes as a broad peak from a gel filtration column with a size ranging from ∼50 to ∼600 kDa (*19*). To gain insight into this behavior of microtubule-associated GSK3, we utilized a strongly resolving analytical FPLC Superose 12 gel filtration column in this study. We observed three GSK3β peaks (Figure 5B). The peak eluting within fractions 54−72 with a size of ∼50 kDa is very likely to be the monomeric kinase. The peak within fractions 34−42 with a size of ∼600 kDa is the macromolecular complex that contains tau, GSK3β, and 14-3-3 described by us previously (*10*, *11*). Interestingly, Cdk5 is also present in fractions 34−42 containing the ∼600 kDa complex (Figure 5D). It is possible that Cdk5 is also a component of the ∼600 kDa complex containing tau, GSK3β, and 14-3-3. The current ∼450 kDa complex may have been formed due to the dissociation of 14-3-3 from the parent complex.

It has been reported that previous phosphorylation of tau by PKA enhances subsequent phosphorylation of tau by

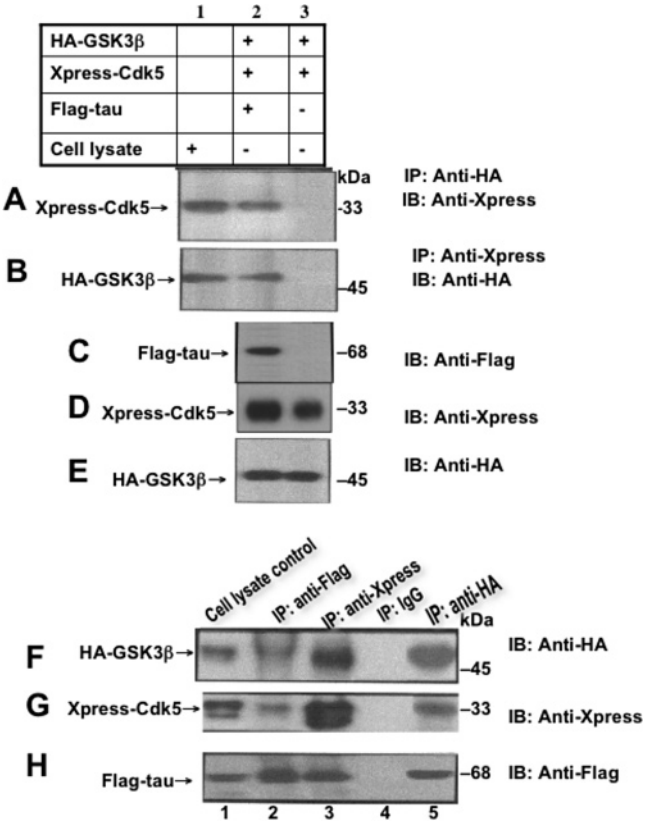

FIGURE 9: Interactions of tau, Cdk5, and GSK3β in HEK-293 cells. (A−E) Interaction of tau, Cdk5, and GSK3b. Lysates from HEK-293 cells transfected with the indicated constructs were immunoblotted to evaluate the expression of the transfected constructs and then subjected to co-immunoprecipitation to test binding. Immunoprecipitation was performed by using the indicated antibodies. Each immune complex was then immunoblotted using the indicated antibody. Panels A and B represent co-immunoprecipitation, whereas panels C−E show expression of the indicated genes in the indicated cells. Note that Cdk5 and GSK3β co-immunoprecipitate with each other in the presence of tau (lane 2) but not in its absence (lane 3). (F−H) Formation of the Cdk5·tau·GSK3β complex. Lysates from HEK-293 cells transfected with Flag-tau, HA-GSK3β, and Xpress-Cdk5 were analyzed by co-immunoprecipitation using the indicated antibodies. Similar observations were made in at least three experiments.

GSK3β (*46*). This study suggests that PKA also primes tau for GSK3β in the brain. PKA is a non-proline-directed kinase and does not phosphorylate tau at S^{235} and S^{404} (*47*). Hence, PKA is very likely not to prime tau for phosphorylation at T^{231} or S^{396} by GSK3β. Tau is phosphorylated on a number of proline-directed and non-proline-directed sites in the brain (*3*, *6*). PKA, therefore, may prime tau for phosphorylation by GSK3β on site(s) not examined in this study.

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