14-3-3 Connects Glycogen Synthase Kinase-3β to Tau within a Brain Microtubule-associated Tau Phosphorylation Complex*

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In a recent study, we reported that in bovine brain extract, glycogen synthase kinase-3β and tau are parts of an ~400–500 kDa microtubule-associated tau phosphorylation complex (Sun, W., Qureshi, H. Y., Cafferty, P. W., Sobue, K., Agarwal-Mawal, A., Neufield, K. D., and Paudel, H. K. (2002) J. Biol. Chem. 277, 11933–11940). In this study, we find that when purified microtubules are subjected to Superose 12 gel filtration column chromatography, the dimeric scaffold protein 14-3-3 co-elutes with the tau phosphorylation complex components tau and GSK3β. From gel filtration fractions containing the tau phosphorylation complex, 14-3-3, GSK3β, and tau co-immunoprecipitate with each other. From extracts of bovine brain, COS-7 cells, and HEK-293 cells transfected with GSK3β, 14-3-3 co-precipitates with GSK3β, indicating that GSK3β binds to 14-3-3. From HEK-293 cells transfected with tau, GSK3β, and 14-3-3 in different combinations, tau co-immunoprecipitates with GSK3β only in the presence of 14-3-3. In vitro, ~10-fold more tau binds to GSK3β in the presence of than in the absence of 14-3-3. In transfected HEK-293 cells, 14-3-3 stimulates GSK3β-catalyzed tau phosphorylation in a dose-dependent manner. These data indicate that in brain, the 14-3-3 dimer simultaneously binds and bridges tau and GSK3β and stimulates GSK3β-catalyzed tau phosphorylation.

Microtubules, the major cytoskeletal structures of eukaryotic cells, are dynamic structures, and their assembly and disassembly is regulated by microtubule-associated proteins (1). In neurons, tau is one of the major microtubule-associated proteins and is mainly found in the axonal compartment (for reviews, see Refs. 1–3). Tau binds to microtubules and stabilizes microtubule structure. Studies suggest that tau regulates microtubule dynamics, axonal transport, and neuronal morphology by binding and stabilizing the microtubule structure (1–3). There are six tau isoforms, which migrate with sizes 45–65 kDa on an SDS-polyacrylamide gel. These isoforms are phosphorylated on multiple sites in the brain and display a characteristic retarded mobility on an SDS gel upon phosphorylation (2, 3). Tau phosphorylation reduces the affinity of tau for microtubules and is one of the mechanisms that control microtubule structure and dynamics in vivo (1–3).

In Alzheimer’s disease (AD)1 brain, abnormally hyperphosphorylated tau accumulates and forms paired helical filaments (4, 5). Since abnormally phosphorylated tau does not bind to microtubules, abnormal tau phosphorylation in AD brain is thought to cause a loss of tau function, microtubule dysfunction, and neurodegeneration (2, 3). It is not understood how abnormally phosphorylated tau accumulates in AD brain, but a defect in the regulatory mechanism that controls tau phosphorylation/dephosphorylation is very likely to be involved. The elucidation of the regulatory mechanism that controls tau phosphorylation in normal brain and the determination of how this regulation fails in AD brain are essential steps in understanding disease ontogeny and developing therapeutic interventions.

Glycogen synthase kinase-3 (GSK3) is an important regulatory enzyme that phosphorylates numerous substrates and regulates diverse physiological processes such as glycogen metabolism, gene expression, apoptosis, signal transduction, and cell fate specification (6–8). There are two isoforms of GSK3 that are highly expressed in the brain: ~51-kDa GSK3α and ~47-kDa GSK3β (9). In transfected cells and transgenic mice, enhanced expression of GSK3β leads to tau phosphorylation and microtubule instability (10–15). In AD brain, GSK3β is activated in pretangle neurons and accumulates in paired helical filaments (16, 17). These observations suggest that GSK3β phosphorylates tau in both normal and AD brain. Previous studies have shown that a large amount of GSK3β in brain is associated with microtubules (18–20), and microtubule-associated GSK3β is part of an ~400–500-kDa multiprotein complex containing tau and GSK3β (20). These data indicate that GSK3β phosphorylates tau within a microtubule-associated multiprotein complex (hereon designated as tau phosphorylation complex). The enormity of the tau phosphorylation complex suggests that within the complex, there may be proteins other than tau and GSK3β (20). The identification of all the complex components and the determination of their functions within the complex are essential to understanding the mechanism by which GSK3β phosphorylates tau in the brain.

14-3-3 is a family of conserved acidic proteins that are widely expressed in all eukaryotic tissues (21, 22). There are seven 14-3-3 isoforms, which are products of distinct genes. 14-3-3 is

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‡ The abbreviations used are: AD, Alzheimer’s disease; FPLC, fast protein liquid chromatography; GSK3, glycogen synthase kinase-3; GST, glutathione S-transferase; MOPS, 4-morpholinepropanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; DTT, dithiothreitol; HA, hemagglutinin; P, pellet; S, supernatant.
a naturally dimeric scaffold protein with the size of the monomer being ~30 kDa (21). Within the 14-3-3 dimer, the ligand binding grooves of each monomer run in opposite directions, and hence a 14-3-3 dimer can interconnect and bring two different proteins together. 14-3-3 binds to diverse cellular proteins, and more than 100 14-3-3 binding proteins have been identified (21). 14-3-3 is a cofactor of bacterial toxin *Pseudomonas* (20). 14-3-3 binds to Raf kinase and regulates the mitogen-activated protein (MAP) kinase signaling pathway (24, 25). It also binds cdc25, polyoma virus middle tumor antigen, p53, protein kinase C, Bcr, PI3 kinase, insulin-like growth factor, BAD, and p53 (26–37). By binding to its targets, 14-3-3 regulates cellular activity, stabilizes enzymes, constitutions control subcellular localization of proteins, and mediates protein–protein interaction (21, 22). 14-3-3 regulates diverse cellular processes including cell growth, cell differentiation, cell division, apoptosis, and neuronal function (21, 22).

In the brain, ~1% of soluble protein is 14-3-3 and has been suggested to be critical for brain function (21). From bovine brain extract, 14-3-3ζ co-immunoprecipitates with tau (36). In *vitro*, 14-3-3ζ binds and changes the tau conformation, thus making tau susceptible for kinase phosphorylation (36). More importantly, a substantial amount of 14-3-3ζ co-purifies with microtubules from the brain extract (36). These observations suggest that 14-3-3ζ is an integral part of brain microtubules and is involved in the regulation of tau phosphorylation and microtubule dynamics. However, very little information is available about microtubule-associated 14-3-3ζ. In this study, we have further analyzed microtubule-associated 14-3-3ζ. Herein we report that brain microtubule-associated 14-3-3ζ is part of the tau phosphorylation complex containing GSK3β and tau. Our data indicate that 14-3-3ζ mediates GSK3β–tau interaction and facilitates tau phosphorylation by GSK3β within the complex.

**MATERIALS AND METHODS**

**cDNA Cloning and Plasmids**—The longest human tau isoform in a pET-3a vector (38) was amplified by *Fus* DNA polymerase-catalyzed PCR using the forward primer (5′-AAAA GAA TAC AGC TGG GCC CGC C-3′) containing EcoRI (italicized) and reverse primer (5′-AA AAG GGA TCC CTA CAA ACC CGT CTT GGA-3′) containing BamHI (italicized) sites. Adenine overhangs were added to the PCR product by TaqDNA polymerase, which was then ligated into a pGEX-T Easy vector (Promega) for amplification (20). After amplification, the insert Taq DNA polymerase, which was then ligated into a pGEX-T Easy vector (Promega) for amplification (20). After amplification, the insert Taq DNA polymerase, which was then ligated into a pGEX-T Easy vector (Promega) for amplification (20). After amplification, the insert was released and ligated into the EcoRI/BamHI cloning site of FLAG-pcDNA3.1 Zeo vector (Invitrogen, Madison, WI). Human 14-3-3ζ cDNA was subcloned into the BamHI/EcoRI site of Xpress-pcDNA3.1 (Invitrogen) as described above using 14-3-3ζ-pGEX-6p (36) as the template and forward primer (5′-GGG TAC TTC GAT GGC TAA AAG T-3′) containing the EcoRI (italicized) and reverse primer (5′-CGG GGA TCC TTA ATT TTC CCC TCC TCT-3′) containing BamHI sites. All cloning procedures were confirmed by DNA sequencing. pcDNA3.1 containing 14-3-3ζ transfection (20, 38). 

**Cell Culture and Transfection**—CF-7 and HEK-293 cells were maintained in Dulbecco’s modified Eagle’s medium (high glucose) medium (Invitrogen) supplemented with 10% fetal bovine serum. Cells were plated in 100-mm culture dishes, grown to ~80% confluency, and transfected by standard calcium phosphate method with various amounts of the appropriate plasmids. For each 100-mm dish, ~5–10 µg of DNA was mixed with 50 µl of CaCl₂ (2.5 M) to give a final volume of 500 µl with distilled water. The mixture of DNA and CaCl₂ was added to 500 µl of 2× HEPEPS-buffered saline (1.63% NaCl, 1.18% Hepes, 0.02% Na₃HPO₄, pH 7.2), and the mixture was allowed to settle at 22 °C for 30 min. DNA mixture was added to the cells dropwise, and cells were allowed to grow for 12–18 h. The medium was then changed, and cells were incubated for 48–72 h.

**Proteins and GSK3β Activity Assay**—Recombinant tau was purified from bacterial extract overexpressing the longest human tau isoform (29). GST-14-3-3ζ and GST-GSK3β were purified from the respective bacterial lysates overexpressing the respective proteins by gelanger agarose chromatography, and the GST tag was removed as described previously (20, 40). Polyclonal antibodies against tau, GSK3β, and 14-3-3ζ have been described (20, 36). Monoclonal antibodies against tau and GSK3β were obtained from NeoMarker (Fremont, CA), and Transduction Laboratories (Lexington, KY), respectively. Monoclonal anti-HA and anti-FLAG antibodies were from Sigma. Anti-Xpress monoclonal antibody was purchased from Invitrogen. Tau phosphorylation-sensitive monoclonal antibodies, AT8, PHF-1, and 12E8, are described previously (20, 36). GSK3β activity assay was performed essentially as described (20).

**Microtubule Assembly/Disassembly and Partial Purification of 14-3-3ζ**—Microtubule Fractionation—Fractionation of microtubules from a fresh bovine brain extract by the temperature-induced microtubule assembly/disassembly has been described previously (38). Microtubule pelleted obtained by centrifugation after first, second, third, and fourth cycles of assembly/disassembly were designated as P1, P2, P3, and P4, and the supernatants were designated as S1, S2, S3, and S4, respectively.

For a partial purification of 14-3-3ζ, all procedures were carried out at 4 °C. Microtubule pellet P3 (~4 mg) was homogenized in ~10 ml of PEM buffer (0.1 M PIPES, 1 mM EGTA, 1 mM MgSO₄, and 1 mM DTT) containing 0.1 mM GTP using a glass homogenizer and then incubated in ice for 30 min. After incubation, the sample was centrifuged at 25,000 g for 20 min, and the supernatant (~12 ml) was loaded onto a phosphatecellulose (Whatman) column (25 × 5 cm) equilibrated in PEM buffer. The column was washed extensively, and the column-bound 14-3-3ζ was eluted with 200 ml of NaCl gradient (0–1 M in PEM buffer). Effluent fractions were immunoblotted using anti-14-3-3ζ antibody, and those containing 14-3-3ζ were combined and dialyzed against Mops buffer (25 mM MOPS (pH 7.4), 50 mM β-glycerol phosphate, 0.1 mM EDTA, 1 mM DTT, 0.2 mM NaCl, 10 mM NaF, and 15 mM MgCl₂) for 4 h. Dialyzed sample was concentrated by Aquacide III (Calbiochem) and centrifuged at 27,000 g for 30 min. The supernatant (~8 ml) was loaded onto an FPLC Superose 12 (Amersham Biosciences) gel filtration column (2.6 × 50 cm), equilibrated, and eluted with Mops buffer. Effluent fractions (1 ml each) were collected, and protein bands of 14-3-3ζ were pooled and dialyzed against 15 mM MOPS (pH 7.4), 1 mM EDTA, 20 mM NaCl, and 1 mM DTT. The dialyzed sample was loaded onto an FPLC Mono S column (Amersham Biosciences) equilibrated in 25 mM MOPS (pH 7.4), 0.1 mM EDTA, and 0.1 mM DTT. The column was washed with the equilibration buffer and then eluted with an NaCl gradient (0–0.5 M) in the equilibration buffer. Fractions (200 µl each) were collected, and those containing 14-3-3ζ were combined (~1.5 ml) and chromatographed through a Sepharose 4B (Sigma) gel filtration column (2.5 × 60 cm) equilibrated and eluted with Mops buffer. Fractions (0.5 ml each) were collected.

**Immunoprecipitation and GST Pull-down Assay**—Cells in each culture were suspended in 1 ml of lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 25 mM β-glycerol phosphate, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 10 mM MgCl₂, 1% Nonidet P-40, 100 mM sodium deoxycholate (Sigma), 50 µM cypemethane (Calbiochem), 1 mg/ml phenylmethylsulfonyl fluoride, and 1 µg/ml of each of pepstatin, leupeptin, aprotinin). The cell suspension was incubated in ice for 1 h and then centrifuged at 4 °C for 15 min. The supernatant was either used for immunoprecipitation or used for GST pull-down assay.

For immunoprecipitation, the supernatant (~200 µl) was precleared with ~50 µl of protein G-agarose beads (Sigma) equilibrated in lysis buffer. The precleared sample was mixed with 10 µg of indicated antibody, and the mixture was shaken end-over-end for 6 h at 4 °C. After shaking, ~50 µl of protein G-agarose beads was added to the mixture, and the shaking was continued for another 5 h. The beads were then collected by centrifugation and 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 analyzed by immunoblot analysis using the indicated antibody. The immunoprecipitation procedure for generating Fig. 4 is essentially the same.

To perform GST pull-down assay, ~50 µl of glutathione-agarose beads (Sigma) coated with the indicated protein was incubated with 200 µl of the cell or brain extract with end-over-end shaking for 14 h at 4 °C. After shaking, beads were washed three times with 50 µl Tris-HCl (pH 7.5), 0.5% Nonidet P-40, 1 mM EDTA, and 1 mM DTT. The washed beads were dissolved in 50 µl of SDS-PAGE sample buffer, boiled, and centrifuged, and 20 µl of supernatant was analyzed by immunoblot analysis using the indicated antibody. To generate Fig. 7, the GST pull-down assay was carried out as described above, except the brain or cell extract was replaced by the tau sample (50 µl Tris-HCl (pH 7.5), 0.5% Nonidet P-40, 1 mM EDTA, 1 mM DTT).
RESULTS

Microtubule-associated 14-3-3ζ—To examine microtubule-associated 14-3-3ζ, we purified microtubules from a fresh bovine brain extract using repeated cycles of temperature-induced microtubule assembly and disassembly. SDS-PAGE and an immunoblot analysis showed that microtubules were enriched during each cycle of assembly and disassembly (Fig. 1, A and B). An immunoblot analysis using an anti-14-3-3ζ antibody indicated that 14-3-3ζ was present in all the fractions in a manner similar to tubulin (Fig. 1C). By quantitating the intensities of various bands in Fig. 1, B and C, we determined that ~6.6, ~2.8, ~0.96, and ~0.3% of total 14-3-3ζ in brain extract remained associated with first (P1), second (P2), third (P3), and fourth (P4) microtubule pellets, respectively. The amount of tubulin was ~29.5, ~12.4, ~7.2, and ~3.0% of the total in P1, P2, P3, and P4, respectively (data not shown). More importantly, the ratio of the amount of 14-3-3ζ to the amount of tubulin in P1, P2, P3, and P4 was ~0.23, ~0.30, ~0.20, and ~0.16, respectively (Fig. 1D). Thus, a fraction of 14-3-3ζ remained stably associated with microtubules during purification in a manner similar to tubulin. These observations indicated that a significant amount of 14-3-3ζ is stably bound to microtubules in the brain.

Microtubule-associated 14-3-3ζ Is Part of a Large Molecular Complex—To further characterize microtubule-associated 14-3-3ζ, we depolymerized P3 microtubules by cold incubation and then subjected them to a phosphocellulose chromatography. 14-3-3ζ was not recovered within the flow-through fractions and eluted from the column with an NaCl gradient along with the other microtubule-associated proteins (data not shown, but see “Materials and Methods”). We then combined the column fractions containing 14-3-3ζ and chromatographed through an FPLC Superose 12 gel filtration column. Most of 14-3-3ζ eluted within fractions 40–46 with a size of ~500-kDa (Fig. 2B). Since the size of dimeric 14-3-3ζ is ~60-kDa (21, 22), these data indicated that 14-3-3ζ is bound to another biological molecule within the brain microtubules.

Identification of Molecules Bound to 14-3-3ζ within Brain Microtubules—A silver-stained SDS gel of various column fractions from Fig. 2A showed numerous protein bands of various sizes within fractions 40–46 (data not shown) and did not give us any indication as to the identification of the 14-3-3ζ-bound protein(s). In a previous study, we found that 14-3-3ζ is associated with tau in bovine brain extract and binds to tau in vitro (36). In a recent study, we showed that within brain microtubules, GSK3β and tau are parts of a multiprotein complex that elutes from an FPLC gel filtration column used in this study to generate Fig. 2A with an ~400–500-kDa size (20). We noted a very similar gel filtration behavior between the high molecular size 14-3-3ζ present within fractions 40–46 (Fig. 2A) and the tau phosphorylation complex described by us in a previous study (20). We therefore analyzed various Fig. 2A column fractions for the presence of tau and GSK3β. As shown in Fig. 2, C and D, tau and GSK3β were indeed present within fractions 40–46 (Fig. 2C, D) and the 14-3-3ζ fraction was immunoblotted using the indicated antibody. A, gel filtration profile. BSA indicates bovine serum albumin. C and D, immunoblot.
40–46, indicating that 14-3-3ζ has co-eluted with the tau phosphorylation complex from the gel filtration column. We pooled fractions 40–46 containing 14-3-3ζ and a portion of the pooled fraction chromatographed through an FPLC Mono S column. SDS-PAGE and immunoblot analyses of various effluent fractions indicated that 14-3-3ζ, tau, and GSK3β had co-eluted from the column (data not shown). We then pooled column fractions containing 14-3-3ζ and chromatographed through a Sepharose 4B gel filtration column. Tau, GSK3β, and 14-3-3ζ again co-eluted (data not shown).

An SDS-polyacrylamide gel of the peak Sepharose 4B column fraction containing tau, GSK3β, and 14-3-3ζ showed at least 11 prominent protein bands that migrated with various sizes on the gel (Fig. 3A). To find out which of these bands may represent protein(s) bound to 14-3-3ζ in brain microtubules, we determined the intensity value of each prominent band on the gel and then calculated the molar ratio value (band intensity divided by molecular size) of each band (Fig. 3B). The ratios for the 25–35, 35–50, 50–100, 100–150, 150–200, and 200–250 kDa bands were 16, 14, 13, 8, 9, and 4, respectively. The ratios for the 30–40, 40–50, 50–60, 60–70, 70–80, 80–90, and 90–100 kDa bands were 28, 29, 28, 28, and 28, respectively. Our immunoblot analysis indicated that the 30-kDa band corresponds to 14-3-3ζ, the 47-kDa band corresponds to GSK3β, and the 55–65-kDa bands correspond to various Tau isoforms. Thus, in the column fraction containing partially purified 14-3-3ζ, the molar ratios of 14-3-3ζ, GSK3β, and tau were similar to and higher than those of 25–35, 35–50, 50–100, 100–150, 150–200, and 220–250 kDa bands. These observations suggested that 14-3-3ζ may be bound to GSK3β and/or tau within brain microtubules and may be a component of tau phosphorylation complex.

To determine whether 14-3-3ζ is part of the tau phosphorylation complex, we immunoprecipitated 14-3-3ζ, tau, or GSK3β from the rest of the above combined column fractions from Fig. 2A. Each resulting immune complex was then immunoblotted with anti-tau, anti-GSK3β, or anti-14-3-3ζ antibody. Tau and GSK3β co-immunoprecipitated with 14-3-3ζ (Fig. 4A). Similarly, GSK3β and 14-3-3ζ co-immunoprecipitated with tau (Fig. 4B), and tau and 14-3-3ζ co-immunoprecipitated with GSK3β (Fig. 4C). Thus, 14-3-3ζ, tau, and GSK3β within Fig. 2A fractions 40–46 could not be separated from each other. Based on these data and our previous study (20), we concluded that 14-3-3ζ is very likely to be one of the components of tau phosphorylation complex.

To gain more evidence in support of the above idea and to study the interactions of 14-3-3ζ, tau, and GSK3β within the tau phosphorylation complex, we first asked whether or not 14-3-3ζ could bind to GSK3β directly. When glutathione-agarose beads coated with GST-14-3-3ζ were incubated with a brain extract, GSK3β specifically precipitated with the GST-14-3-3ζ beads (Fig. 5A). Although this observation indicated that 14-3-3ζ associates with GSK3β in the brain extract, we could not rule out the possibility that tau, which can bind to both GSK3β and 14-3-3ζ, may have influenced observed 14-3-3ζ and GSK3β association (Fig. 5A). Therefore, we performed a similar GST pull-down assay as described above by using COS-7 cells that express GSK3β but not tau. As shown in Fig. 5B, GSK3β again came down with GST-14-3-3ζ from the cell extract. To confirm that it was GSK3β that came down with GST-14-3-3ζ and not any other protein of similar size that may be immunoreactive to our anti-GSK3β antibody used to generate Fig. 5, A and B, we transfected HEK-293 cells with HA-GSK3β. Transfected cells were lysed, and glutathione-agarose beads coated with GST-14-3-3ζ were incubated with the cell lysates. Incubated beads were washed and immunoblotted by using an anti-HA antibody to test 14-3-3ζ-GSK3β binding. As
expected, HA-GSK3β bound to GST-14-3-3ζ (Fig. 5C). Based on these data, we concluded that 14-3-3ζ directly binds to GSK3β.

There are three possible mechanisms by which tau, GSK3β, and 14-3-3ζ can interact within the tau phosphorylation complex. First, because in vitro tau binds to GSK3β (20) as well as 14-3-3ζ (36) and the respective binding sites do not overlap, tau may bridge GSK3β and 14-3-3ζ within the complex. Second, 14-3-3ζ is a scaffold protein that can bind two ligands at a same time (21, 22), and it binds tau (36) and GSK3β (Fig. 5). Therefore, 14-3-3ζ may anchor GSK3β to tau within the phosphorylation complex. Third, GSK3β can bind tau in vitro (20) and can bind 14-3-3ζ in vitro (Fig. 5). Thus, GSK3β may be the central molecule that may hold 14-3-3ζ and tau simultaneously within the complex.

To discriminate between the above possibilities, we transfected HEK-293 cells with FLAG-tau, Xpress-14-3-3ζ, and HA-GSK3β constructs in various combinations. Transfected cells were lysed, and GSK3β was immunoprecipitated from each lysate using an anti-HA antibody. Each immune complex was then immunoblotted with anti-FLAG antibody to detect tau. FLAG-tau did not co-immunoprecipitate from cells overexpressing HA-GSK3β and FLAG-tau (Fig. 6A, lane 6), indicating that GSK3β does not bind to tau directly in vivo. This means that neither can tau bridge GSK3β to 14-3-3ζ nor can GSK3β simultaneously bind to tau and 14-3-3ζ within the tau phosphorylation complex. Therefore, 14-3-3ζ must be the central molecule that holds tau and GSK3β within the complex. Indeed, FLAG-tau co-immunoprecipitated with HA-GSK3β from cells overexpressing FLAG-tau and HA-GSK3β only when these cells also overexpressed Xpress-14-3-3ζ (Fig. 6A, lanes 8 and 9), indicating that GSK3β associates with tau only in the presence of 14-3-3ζ. As discussed above, 14-3-3ζ binds to tau (36) and GSK3β (Fig. 5) directly. Taken together, these observations indicated that 14-3-3ζ connects GSK3β to tau in vivo.

To further confirm the above finding, we performed an in vitro GST pull-down assay. Glutathione-agarose beads coated with GST-GSK3β were mixed with bacterially expressed recombinant tau in the presence of a series of 14-3-3ζ concentrations. Beads were washed, and bead-bound tau was detected by immunoblot analysis using an anti-tau antibody. Comparatively very little tau bound to beads when GST-GSK3β was incubated with tau alone (Fig. 7, lane 3). However, when an increasing amount of 14-3-3ζ was included in the assay mixture, the amount of tau binding to GST-GSK3β increased progressively (Fig. 7, lanes 4–8). When the amount of 14-3-3ζ was 100 μg/ml in the assay mixture, ~10-fold more tau bound to GST-GSK3β than in the absence of 14-3-3ζ (compare lanes 3 and 7). Based on these data, we concluded that 14-3-3ζ promotes in vitro GSK3β-tau binding and is required for a stable association of tau and GSK3β in vivo.

14-3-3ζ Stimulates GSK3β-catalyzed Tau Phosphorylation—GSK3β is one of the kinases implicated to phosphorylate tau in vivo (10–20). Since we find that 14-3-3ζ is required for a stable association between GSK3β and tau, we examined the influence of 14-3-3ζ on GSK3β–catalyzed tau phosphorylation in vivo. We transfected HEK-293 cells in various combinations with FLAG-tau, Xpress-14-3-3ζ, and HA-GSK3β constructs. Transfected cells were lysed, and the cell lysates were analyzed for tau phosphorylation using various tau phosphorylation-sensitive antibodies: AT8, PHF1, and 12E8, which recognize tau phosphorylated on Ser199/Ser202, Ser396/Ser404, and Ser262, respectively (20, 36). As shown in Fig. 8, A–C, tau was slightly phosphorylated in cells transfected with FLAG-tau alone (lane 3). This phosphorylation increased in cells co-transfected with FLAG-tau and HA-GSK3β as expected (lane 5). In cells that were co-transfected with fixed amounts of FLAG-tau and HA-GSK3β but different amounts of Xpress-14-3-3ζ, FLAG-tau phosphorylation increased progressively with the increase in the amount of Xpress-14-3-3ζ (lanes 7–9). This increase was evident not only by an increased immunoreactivity against all tau phosphorylation-sensitive antibodies tested but also by a retarded mobility of FLAG-tau on the SDS gel, a characteristic feature of hyperphosphorylated tau (2, 8). Thus, 14-3-3ζ profoundly stimulated GSK3β-catalyzed tau phosphorylation in vivo.
 connects and mediates the association of GSK3β with tau within the brain. However, as discussed above, tau does not associate with GSK3β in HEK-293 cells transfected with only tau and GSK3β, although 14-3-3 is known to be widely expressed in various cell lines including HEK-293 cells (21). It is possible that within HEK-293 cells, the endogenous 14-3-3ζ either is not sufficient or is not available to mediate the interaction of transected GSK3β and tau.

In a previous study, we reported that in vitro GSK3β binds to the N-terminal region of tau (20). Consistent with that report, we find that tau comes down with GST-GSK3β in a GST pull-down assay (Fig. 7, lane 3). However, tau does not co-immunoprecipitate with GSK3β from lysates of HEK-293 cells co-transfected with GSK3β and tau (Fig. 6A, lane 6). These observations suggest that in the absence of 14-3-3ζ, GSK3β binds to tau with a low affinity. It thus appears that in the brain, GSK3β interacts with tau in two different ways: one with low affinity that does not require 14-3-3ζ and the other with high affinity that requires 14-3-3ζ.

The substrate recognition by GSK3 is regulated by two mechanisms. The first mechanism requires a priming phosphorylation of the substrate (41, 42). For example, casein kinase 2 phosphorylates glycogen synthase first and generates a recognition motif for GSK3. GSK3 then phosphorylates casein kinase 2-phosphorylated glycogen synthase (42). The second mechanism does not require priming phosphorylation. Instead, a scaffold protein bridges GSK3β to its substrate within a multiprotein complex (42). In the Wnt signaling pathway, GSK3β phosphorylates β-catenin within a β-catenin destruction complex. β-catenin alone is not a good substrate of GSK3β. The scaffold protein axin connects GSK3β to β-catenin and facilitates β-catenin phosphorylation by GSK3β within the complex (6, 42–44).

Biochemical analyses and studies involving transgenic mice and cultured mammalian cells have established that GSK3β phosphorylates tau in the brain (10–20). The mechanism by which GSK3β phosphorylates tau is not clear. Our recent study (20) and the results presented in this study indicate that GSK3β, tau, and 14-3-3ζ are parts of a microtubule-associated tau phosphorylation complex. Within the complex, 14-3-3ζ binds to tau and GSK3β simultaneously and assembles the complex. Thus, the role of 14-3-3ζ within the tau phosphorylation complex appears to be similar to that of axin within the β-catenin destruction complex. Furthermore, 14-3-3ζ binds to tau and changes the tau conformation, making tau susceptible for hyperphosphorylation in vitro (36) and perhaps in vivo (Fig. 8). Since 14-3-3ζ stimulates tau phosphorylation on Ser199, Ser189, Ser202, Ser204, Ser396, and Ser404 (Fig. 8), it appears that 14-3-3ζ-induced conformational change occurs within a large part of the C-terminal tau region, which is the main area of in vivo phosphorylation (45). These observations suggest that 14-3-3ζ not only enhances association of tau and GSK3β within the complex but also prepares tau for GSK3β action.

We have found a unique multiprotein complex containing tau, GSK3β, and 14-3-3ζ within brain microtubules. Thus, a pool of GSK3β in the brain is targeted to microtubules through a stable association with tau and 14-3-3ζ. Because the function of this complex is to regulate tau phosphorylation and microtubule dynamics, we named this complex the tau phosphorylation complex. It should be noted that the size of the phosphorylation complex is 400–500-kDa, whereas the sum of the sizes of tau, GSK3β, and 14-3-3ζ dimer is ~167-kDa. Therefore, it is possible that there may be proteins other than, tau, GSK3β, and 14-3-3ζ within the tau phosphorylation complex. These proteins may play important roles in regulating tau phosphorylation and interactions between various phosphorylation.
complex components. Studies are ongoing in our laboratory to identify all of the phosphorylation complex components.

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