GSK-3β Directly Phosphorylates and Activates MARK2/PAR-1*

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In Alzheimer disease (AD), the microtubule-associated protein tau is found hyperphosphorylated in paired helical filaments. Among many phosphorylated sites in tau, Ser-262 is the major site for abnormal phosphorylation of tau in AD brain. The kinase known to phosphorylate this particular site is MARK2, whose activation mechanism is yet to be studied. Our first finding that treatment of cells with LiCl, a selective inhibitor of another major tau kinase, glycogen synthase kinase-3 (GSK-3β), inhibits phosphorylation of Ser-262 of tau led us to investigate the possible involvement of GSK-3β in MARK2 activation. In vitro kinase reaction revealed that recombinant GSK-3β indeed phosphorylates MARK2, whereas it failed to phosphorylate Ser-262 of tau. Our further findings led us to conclude that GSK-3β phosphorylates MARK2 on Ser-212, one of the two reported phosphorylation sites (Thr-208 and Ser-212) found in the activation loop of MARK2. Down-regulation of either GSK-3β or MARK2 by small interfering RNAs suppressed the level of phosphorylation on Ser-262. These results, respectively, indicated that GSK-3β is responsible for phosphorylating Ser-262 of tau through phosphorylation and activation of MARK2 and that the phosphorylation of tau at this particular site is predominantly mediated by a GSK-3β-MARK2 pathway. These findings are of interest in the context of the pathogenesis of AD.

Neurofibrillary tangles (NFTs) are found in the brain in numerous neurodegenerative diseases, such as Alzheimer disease (AD), frontotemporal dementia, and parkinsonism linked to chromosome 17 (FTDP-17), and progressive supranuclear palsy (1–4). The major component of NFTs is the microtubule-associated protein tau. It has been reported that β-amyloid, a peptide observed as an aggregated form in senile plaques of AD (5), cannot exhibit neurotoxicity and induce neurodegeneration without the existence of tau (6, 7). As demonstrated in these studies, tau is considered to play a crucial role in inducing neurodegeneration. Tau is functionally modulated by phosphorylation (8–10), and it is hyperphosphorylated in NFTs (1, 11), indicating that phosphorylation might play a part in tau-induced neurotoxicity. Tau can be phosphorylated at specific Ser and Thr residues by two types of protein kinase: Ser/Thr-Pro motif kinase and KGS motif kinase (12). Ser/Thr-Pro motif kinases, including GSK-3β, Cdk5, and c-Jun amino-terminal kinase, phosphorylate tau in domains containing Ser-Pro or Thr-Pro motifs. Among Ser/Thr-Pro motif tau kinases, GSK-3β, ubiquitously expressed in mammalian tissues, is thought important for tau toxicity as it phosphorylates several disease-associated phosphoepitopes such as Ser-199, Thr-212, Thr-214, and Ser-404 (13, 14). The role of GSK-3β in the pathogenesis of AD has been suggested by other findings as well. Expression of familial AD mutation-bearing amyloid precursor protein (APP) or Presenilin 1 (PS1) results in elevated GSK-3β activity (15, 16). Takashima et al. (17–19) have demonstrated that β-amyloid-induced neurotoxicity is mediated by GSK-3β. Furthermore, overexpression of Drosophila GSK-3 can promote the pathology of NFTs in fly models of tauopathy (20). Although these findings taken together hint at a crucial role for GSK-3β in AD pathogenesis, this role is still a matter of controversy.

The latter type of tau kinase, KGS motif kinase, phosphorylates Ser-262 and Ser-356 in KGS motifs within the microtubule binding region of tau. Phosphorylation at Ser-262, and to a lesser extent Ser-356, dramatically reduces the microtubule binding capacity of tau (21). Microtubules are known to be essential in synaptic vesicle transport during neurotransmission and in the formation and maintenance of synaptic structures. Recent study suggests that synaptic dysfunction may be one of the earliest events in the pathogenesis of AD (22). Therefore, it is likely that phosphorylation of tau at Ser-262 leading to the detachment of tau from microtubules affects microtubule dynamics and disrupts synaptic function. Indeed, the level of phosphorylation of Ser-262 was reported to be elevated in tau protein isolated from brains of AD patients (11). One KGS motif kinase hypothesized to be important for tau-induced toxicity is the mitogen-activated protein/microtubules affinity-regulating kinase (MARK). MARK was originally purified from porcine brain as a KGS motif tau kinase and has been suggested to be the most effective kinase of Ser-262 (23, 24). Overexpression of MARK in Chinese hamster ovary cells results in increased phosphorylation of microtubule-associated proteins in the KGS motif and disruption of the microtubule array. Moreover, Nishimura et al. (25) reported that overexpression of Drosophila PAR-1, a fly orthologue of mammalian MARK, led to elevated tau phosphorylation and enhanced toxicity in a transgenic fly model, indicating that MARK/PAR-1 plays a central role in conferring tau-induced toxicity in vivo. However, little is known about the upstream events that act through MARK to regulate tau phosphorylation in disease progression.

On the basis that the phosphorylation of tau is an essential event in the pathogenesis of AD, the explicit role of GSK-3β in the pathogenesis and the activation mechanism of MARK are two enticing areas of study. In this study, we initially found that GSK-3β inhibition resulted in suppression of Ser-262 phosphorylation of tau in neurohybridoma F11 cell line, indicating that GSK-3β is involved in the phosphorylation of tau at Ser-262. Because GSK-3β cannot phosphorylate this site directly, our finding suggested that GSK-3β and MARK cooperated in inducing the phosphorylation of tau at Ser-262. Here, we have shown that GSK-3β acts as a physiologically phosphorylating and activating factor of MARK2 and that the phosphorylation of tau at Ser-262 in cultured cells is predominantly mediated by the GSK-3β-MARK2 pathway.
GSK-3β Phosphorylates and Activates MARK2

MATERIALS AND METHODS

Cell Culture and Reagents—Neurohybrid F11 cells (26–28) and HEK293T cells were maintained in Ham’s F-12 medium supplemented with 10% fetal bovine serum and Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, respectively. Lithium chloride was purchased from Wako Pure Chemical (Osaka, Japan).

Plasmid Construction and Transfection—Rat MARK2 cDNA was amplified from rat brain 5′-terminal cDNA by PCR using the synthetic primers 5′-ATACCAAGCGCATGGCCAGC-3′ and 5′-CCTCATGACTTGGCCTTGAATAA-3′. The amplified PCR product was inserted into the vector pcDNA3 (Invitrogen) to construct pcDNA3-MARK2. For construction of an N-terminal Myc-tagged MARK2, a fragment from pcDNA3-MARK2 was ligated into pcDNA3-Myc, pGEX2T, and pRK-5-GST (Invitrogen) to construct pcDNA3.1-tau. For construction of an N-terminal GST-fused MARK2, a fragment from pcDNA3-MARK2 was ligated into pGEX2T (Amersham Biosciences) to construct pGEX2T-MARK2. Point mutants of pcDNA3-Myc-MARK2 and pGEX2T-MARK2 were generated by PCR using the synthetic primers 5′-GGGAAAGAGGGTGATGCTGTCAGGATATTTCACTTGCAGGAAACAG-3′ and 5′-CTTCTTCTGTTAGTCTCCTTGCAGGAGCTACCTCTTTCCCC-3′ (K82R), 5′-GGGAAAGAGGGTGATGCTGTCAGGATATTTCACTTGCAGGAAACAG-3′ and 5′-GGGAAAGAGGGTGATGCTGTCAGGAGCTACCTCTTTCCCC-3′ (S212A). Mouse GSK-3β cDNA was kindly provided by Dr. Ishiguro (Mitsubishi Kasei Institute of Life Sciences, Tokyo, Japan). GSK-3β cDNA was amplified by the synthetic primers 5′-GGCTTTCAATGCAAG-3′ and 5′-GCATAGGAAGGATGTTGTCAGGAC-3′ using the provided plasmid as a template. The amplified PCR product was inserted into the vector pcDNA3-Myc, pGEX2T, and pRK-5-FLAG to construct an N-terminal Myc-tagged GSK-3β, an N-terminal GST-fused GSK-3β, and an N-terminal FLAG-tagged GSK-3β, respectively. Point mutant K85R of GSK-3β was generated by PCR using the synthetic primers 5′-CAGGAGAATCTGGGTGCACTCAGGCAAGGAGCTACCTCTTTCCCC-3′ and 5′-GCGATTTATCCCGGGTCAGGTAGAGTTGGAGGACCGAGAACTGGTGGCCATCAGGAAAGT-3′. Mouse GSK-3β cDNA was kindly provided by Dr. Yanagawa (Keio University, Yokohama, Japan). Tau cDNA was ligated into the vector pcDNA3.1 (Invitrogen) to construct pcDNA3.1-tau.

For transient transfection, F11 cells or HEK293T cells were seeded at 104 cells/well and 2.5 or 5×105 cells/well in a six-well plate, respectively. The next day, F11 cells or HEK293T cells were transfected with 2.5 or 5 μg of plasmid using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions.

Purification of Recombinant Proteins and in Vitro Kinase Assay—For purification of GST-fused MARK2 or GSK-3β, 0.1 mM isopropyl-1-thio-β-D-galactopyranoside-stimulated Escherichia coli was extracted in IP buffer (50 mM HEPES (pH 7.6), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM dithiothreitol, 1 mM NaF, 10% glycerol, 0.1% Tween 20, 1% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Roche Diagnostics)) by sonication. After centrifugation, the E. coli extracts were input into glutathione-agarose (Amersham Biosciences), and GST-MARK2 or GST-3β were eluted with elution buffer (phosphate-buffered saline containing 20 mM GSH). For purification of tau, 0.1 mM isopropyl-1-thio-β-D-galactopyranoside-stimulated E. coli was extracted in MT-phosphate-buffered saline buffer (150 mM NaCl, 16 mM Na2HPO4, and 4 mM NaH2PO4) by sonication. After centrifugation, the E. coli extracts were heat treated at 100 °C for 5 min and centrifuged again to remove the denatured protein debris.

For the kinase assays, the purified proteins were mixed in a reaction buffer (20 mM HEPES (pH 7.4), 10 mM MgCl2, 10 mM MnCl2, 1 mM dithiothreitol, and 0.2 mM EGTA) with 1.6 mM ATP or 1.6 mM [γ-32P]ATP (1 μCi). After incubating the reaction mixtures at 30 °C for 30 min, the reaction was terminated by adding 3× SDS sample buffer (84 mM Tris (pH 6.8), 20% glycerol, 4.6% SDS, 10% 2-mercaptoethanol, and 0.004% bromphenol blue) and boiled for 3 min.

Western Blotting—Western blotting was performed as described (29). Primary antibodies used were: anti-tau (TAU5), anti-pS199-tau, anti-pS262-tau, and anti-pS404-tau antibodies from BioSource (Sunnyvale, CA); anti-Myc (9E10), anti-GST (B14), and anti-GSK-3α/β (0011-A) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); anti-FLAG (M2) antibody from Sigma; and anti-MARK2 antibody generated by Sigma using a synthetic peptide with the sequence CNKRPREEETGRK as an antigen.

RNA Interference—The small interfering RNA duplex (siRNA) for GSK-3α/β was purchased from Cell Signaling (Beverly, MA). MARK2 siRNA was synthesized by Dharmacon (Chicago). The target sequence of MARK2 siRNA was 5′-AACGGAGAAUAACGGGCCC-3′ (nucleotide 1282–1302 of MARK2). The nonspecific siRNA was purchased from Dharmacon. GSK-3β or MARK2 siRNA was transfected with the plasmid pcDNA3.1-tau into HEK293T and F11 cells, respectively, by using Lipofectamine 2000. Cells were collected 48 h after transfection.

RESULTS

Inhibition of GSK-3β Abrogates Phosphorylation of Tau at Ser-262—To investigate the mechanism regulating the phosphorylation of tau, F11 cells were transiently expressed in F11 cells, a neurohybrid cell line (26–28), and its state was examined. As shown in Fig. 1A, many Ser residues in the Ser/Thr-Pro (S/T-P) motif, such as Ser-199, -212, -214, and -404, Ser-262 in the KXGS motif, were found to be phosphorylated, indicating that the tau-phosphorylating kinases to be investigated are present in F11 cells. When the F11 cells expressing tau were treated with LiCl, a selective GSK-3β inhibitor (30, 31), phosphorylation of several GSK-3β-targeted S/T-P residues in tau, such as Ser-199 and Ser-404, was inhibited in a concentration-dependent manner. Interestingly, when GSK-3β activity was inhibited by LiCl treatment, we found that phosphorylation of Ser-262 in the KXGS motif also decreased without a change in the expression level of either GSK-3β or total tau (Fig. 1A). Similar results were obtained when HEK293T cells were used (data not shown). The decrease in phosphorylation of Ser-262 on inhibition of GSK-3β suggested the involvement of GSK-3β in the phosphorylation of tau at Ser-262. We then examined whether overexpression of GSK-3β could increase the phosphorylation of tau in F11 cells. N-terminal Myc-tagged GSK-3β-3β was co-expressed with tau in F11 cells, and the phosphorylation level of tau-Ser-262 was determined by Western blotting using appropriate antibodies. As shown in Fig. 1B, not only the phosphorylation of GSK-3β-targeted Ser-199 but also that of non-GSK-3β-targeted Ser-262 increased in proportion to the amount of Myc-GSK-3β. In contrast, the kinase-negative Myc-GSK-3β (K85R) mutant, which was reported unable to associate with ATP (32), could not induce phosphorylation at either Ser-199 or Ser-262 in tau. Similar results were obtained when HEK293T cells were used (data not shown). Because GSK-3β was reported to be unable to phosphorylate Ser-262 of tau directly (33), our finding raised the possibility that GSK-3β phosphorylates and activates a KXGS motif kinase, leading to phosphorylation of tau at Ser-262. To confirm this possibility, we first conducted an in vitro kinase assay to examine whether GSK-3β actually fails to directly phosphorylate the Ser-262 of recombinant tau. Recombinant
GST-GSK-3β was incubated with recombinant tau in the presence of 1.6 mM ATP at 30 °C for 30 min, and the kinase reaction products were analyzed by Western blotting using appropriate antibodies. As shown in Fig. 1C, although both the anti-phospho-Ser-199-tau and anti-phospho-Ser-404-tau antibody-reactive bands intensified in the presence of GST-GSK-3β, the anti-phospho-Ser-262-tau antibody-reactive band was not affected. These results confirmed that GSK-3β is unable to directly phosphorylate Ser-262 of tau as reported; rather, it might induce it through activation of a KXGS motif kinase.

GSK-3β Directly Phosphorylates MARK2 in Vitro—The major KXGS motif kinase is MARK, which was originally purified from brain. In humans, four MARK isoforms (MARK1, 2, 3, and 4) have been identified (12). Among them, MARK2 is the best characterized and expressed throughout various tissues. Phosphorylation of MARK2 is reported to be required for its activation (24). However, the factor phosphorylating and activating MARK2 is not fully understood. Therefore, we hypothesized that GSK-3β phosphorylates and simultaneously activates MARK2, thereby inducing the phosphorylation of tau at Ser-262 in F11 cells. We initiated our investigation by conducting an in vitro kinase assay using [γ-32P]ATP to examine whether GSK-3β could phosphorylate MARK2 directly. Myc-MARK2 was immunoprecipitated with anti-Myc antibody from Myc-MARK2-expressing HEK293T cell extracts and was incubated with recombinant GST-GSK-3β. As shown in Fig. 2A, slight autophosphorylation was observed when Myc-MARK2 alone was incubated, whereas the incorporation of phosphate into Myc-MARK2 was dramatically increased in the presence of GST-GSK-3β. Similar results were obtained when bacterially expressing GST-MARK2 was used as the substrate of GST-GSK-3β (Fig. 2B). In contrast, GST-GSK-3β (K85R) mutant did not induce phosphorylation of GST-MARK2 (Fig. 2B). These results strongly implied the direct phosphorylation of MARK2 by GSK-3β in vitro.

Ser-212 in the Activation Loop of MARK2 Is Phosphorylated by GSK-3β—Next, we investigated which amino acid residue of MARK2 was phosphorylated by GSK-3β. Because it was reported that Thr-208 and Ser-212 in the activation loop of MARK2 are phosphorylated residues important for the activation of the kinase (24), we designed partially unphosphorylatable mutants by substituting each phosphorylation site in MARK2 with Ala and examined whether they could still
phosphorylated by GSK-3β. As shown in Fig. 3A, the in vitro kinase assay revealed that both GST-MARK2 (wild type) and GST-MARK2 (T208A) were phosphorylated in proportion to the amount of GST-GSK-3β, whereas GST-MARK2 (S212A) was not capable of being phosphorylated by GST-GSK-3β. Similar results were obtained when kinase-negative MARK2 (K82R) (24), or its derived double mutated MARK2 (K82R/T208) or MARK2 (K82R/S212), was used as the substrate (Fig. 3B). These results verified that GSK-3β phosphorylates MARK2 at Ser-212 in vitro, and the phosphorylation is not affected by Ala at Thr-208.

Requirement of Phosphorylation at Ser-212 of MARK2 for Kinase Activity—Our finding that MARK2 was phosphorylated at Ser-212 in its activation loop by GSK-3β in vitro led us to examine whether phosphorylation at Ser-212 is essential for the activation of MARK2 in cultured cells. When Myc-MARK2 was expressed in tau-expressing F11 cells, the phosphorylation of tau at Ser-262 was substantially increased (Fig. 4A). On the other hand, phosphorylation of Ser-199 remained unchanged, suggesting that Myc-MARK2 and the phosphorylation of tau at Ser-199 are irrelevant. Expression of Myc-MARK2 (T208A) resulted in the phosphorylation of tau at Ser-262 almost to the level induced by wild type Myc-MARK2, which implies that mutating Thr-208 of MARK2 does not affect the activation. However, Myc-MARK2 (S212A) could not induce the phosphorylation of tau at Ser-262 (Fig. 4B). These results strongly imply that the phosphorylation of MARK2 at Ser-212 is necessary for its kinase activity leading to the phosphorylation of tau-Ser-262.

To examine whether the phosphorylation of tau at Ser-262 seen in F11 cells is actually regulated by MARK2, the expression of endogenous MARK2 was suppressed by siRNA and the level of phosphorylation of tau-Ser-262 was assessed. As shown in Fig. 4C, knockdown of endogenous MARK2 by its siRNA induced a significant decrease in the level of phosphorylation of tau-Ser-262 without affecting the overall expression of tau protein, indicating that phosphorylation of tau at Ser-262 is predominantly regulated by MARK2 in F11 cells.

GSK-3β Phosphorylates MARK2 in Cultured Cells—We have shown so far that MARK2 is responsible for the phosphorylation of tau-Ser-262 in F11 cells, and it is phosphorylated and activated by GSK-3β in vitro. To further confirm that MARK2 is activated by GSK-3β, we next examined whether GSK-3β could phosphorylate MARK2 in cultured cells. HEK293T cells were transiently co-transfected with Myc-MARK2 and FLAG-GSK-3β, which would later be metabolically labeled with [32P]phosphate 3 h before harvesting. Myc-MARK2 was immunoprecipitated from cell extracts by using anti-Myc antibody, and then the precipitate was separated by SDS-PAGE and analyzed by autoradiography. As shown in Fig. 5A, the [32P]-labeled phosphate was incorporated into Myc-MARK2 in a manner dependent on the amount of FLAG-GSK-3β. The result suggested that increased expression of GSK-3β caused enhancement of the phosphorylation of MARK2 in HEK293T cells.

Suppression of Endogenous MARK2 Inhibits GSK-3β-induced Phosphorylation of Tau at Ser-262—To exclude the possibility that MARK2 is phosphorylated and activated by kinases other than GSK-3β, HEK293T cells were transiently co-transfected with GSK-3β siRNA and tau plasmid, and phosphorylation levels of tau-Ser-262 were examined. As demonstrated in Fig. 5B, the endogenous expression of GSK-3β was successfully suppressed by GSK-3β siRNA in a dose-dependent manner. The intensity of the phospho-Ser-199-tau and phospho-Ser-262-tau antibody-immunoreactive band abated in correlation to the GSK-3β expression level without affecting the expression levels of total tau protein. These results indicated that GSK-3β acts as a physiological...
Phosphorylation and activating factor of MARK2 in cultured cells. Furthermore, when MARK2 siRNA was co-transfected with GSK-3β plasmid together with tau plasmid, the level of GSK-3β-induced phosphorylation of tau at Ser-262 decreased in a dose-dependent manner (Fig. 5C). Collectively our results strongly indicate a novel pathway toward the phosphorylation of tau at Ser-262, where GSK-3β phosphorylates Ser-212 of MARK2 in its activation loop and activated MARK2 phosphorolates tau at Ser-262.

**DISCUSSION**

In AD it is generally argued that aberrant phosphorylation of tau reflects an imbalance of cellular signal transduction and is a prelude to neuronal degeneration. Although at least 24 amino acid residues are known as phosphorylation sites of tau in NFTs, the role of phosphorylation at each site in the toxicity of tau and the signal transduction mechanism are not fully understood. In the present study, we have focused on the mechanism regulating the phosphorylation of tau at Ser-262 for tau-Ser-262 phosphorylation, exclusively found in paired helical filament-tau, has a severe effect on its ability to bind to microtubules, which presumably results in neurodegeneration through microtubule disruption and/or tau aggregation. From our observations that the treatment of cells with a GSK-3β inhibitor caused a decrease in the phosphorylation level of tau-Ser-262, and alternatively overexpression of GSK-3β caused an increase in the phosphorylation level of tau-Ser-262 (Fig. 1, A and B), we have demonstrated the involvement of GSK-3β in the phosphorylation of tau at Ser-262 in tau-expressing neurohybrid cells. However, GSK-3β is known as an S/T-P motif kinase, and we confirmed that GSK-3β itself fails to phosphorylate Ser-262 within the KXS motif of tau directly (Ref. 33 and Fig. 1C). Therefore, we proposed that GSK-3β induces phosphorylation at the site through activation of a KXS motif kinase. One candidate for such a kinase is MARK, which was originally purified from brain. Indeed, GSK-3β directly phosphorylates MARK2 in vitro (Fig. 2, A and B), and GSK-3β-dependent phosphorylation of MARK2 was also observed in cultured cells (Fig. 5A). Active MARK2 is a phosphoprotein and its kinase activity is lost after treatment with phosphatase 2A, indicating that phosphorylation of MARK2 is required for its kinase activity (24). Peptide sequencing data showed that MARK2 contained two phosphorylated Ser/Thr residues (Thr-208 and Ser-212) in the activation loop (24). Our site-directed mutational analysis revealed that MARK2 bearing the mutation T208A could still be phosphorylated by GSK-3β, whereas MARK2 bearing S212A could not, indicating that GSK-3β phosphorylates Ser-212 of MARK2 (Fig. 3, A and B).
This finding is likely because Ser-212 of MARK2 is followed by a Pro (LDTFCFSPPYAA), which comprises a GSK-3β/H9252 consensus sequence of the S/T-P motif. This amino acid sequence (LDTFCFSPPYAA) is also observed in other MARK isoforms, MARK1, MARK3, and MARK4 in their activation loops; therefore, it is likely that GSK-3β might phosphorylate MARKs 1, 3, and 4 at Ser residues corresponding to Ser-212 of MARK2. Previously, LKB1 and TAO-1/Ste20-like kinase (MARKK) have been reported as protein kinases that phosphorylate the activating loop of MARK2 (34, 35). However, both kinases selectively phosphorylate Thr-208 of MARK2. Therefore, this is the first report to identify the kinase that phosphorylates Ser-212 of MARK2. However, this is not direct evidence that phosphorylation of Ser-212 by GSK-3β is required for the activation of MARK2, because Timm et al. (35) demonstrated that phosphorylation of Ser-212 is actually inhibitory, although Ser-212 must be present for basal activity. Nevertheless, the importance of the phosphorylation of Ser-212 by GSK-3β for the activation of MARK2 is logically suggested by our findings. 1) Overexpression of the wild type or T208A mutant of MARK2 in cells increased the phosphorylation level of tau-Ser-262, but overexpression of the S212A mutant of MARK2 did not have any effect (Fig. 4, A and B). 2) MARK2 acted as a master protein kinase responsible for the phosphorylation of tau at Ser-262 in the cells, because knockdown of endogenous MARK2 significantly reduced the phosphorylation levels of tau-Ser-262 (Fig. 4C). 3) Knockdown of GSK-3β completely diminished the MARK2-induced phosphorylation of tau-Ser-262 (Fig. 5, B and C), indicating that the activation of MARK2 is regulated by GSK-3β. 4) Kinase-negative GSK-3β (K85R) did not induce the phosphorylation of tau at Ser-262, indicating that the kinase activity of GSK-3β is required for the activation of MARK2 (Fig. 1B). Thus, taken together, we propose that GSK-3β phosphorylates Ser-212 of MARK2 and that this phosphorylation causes the activation of MARK2 leading to tau-Ser-262 phosphorylation. Furthermore, the findings obtained from our knockdown experiments indicated that GSK-3β and MARK2 are prominent physiological protein kinases that phosphorylate Ser-212 of MARK2 and Ser-262 of tau.
tau, respectively. Therefore, although we cannot exclude the possibility that other tau-Ser-262 kinases such as SAD kinases (36) might be involved in the phosphorylation mechanism of Ser-262 of tau, the GSK-3β-MARK2 pathway mainly operates the mechanism of phosphorylation at Ser-262 of tau under physiological conditions. Recently, Nishimura et al. (25) demonstrated that the phosphorylation of tau at Ser-262 (and Ser-356) by MARK/PAR-1 is a prerequisite for subsequent phosphorylation by the downstream kinase GSK-3β to generate toxic tau species in vivo. Considering our findings, GSK-3β may act both upstream and downstream of MARK2, and this positive feedback loop could regulate tau functions such as microtubule dynamics and neuronal toxicity.

MARK2 has been reported to be activated through phosphorylation at Thr-208 by LKB-1 or TAO-1/Ste20-like kinase (MARKK) in vitro (34, 35). These reports are inconsistent with our finding that the mutation of Thr-208 to Ala in MARK2 did not reduce tau-Ser-262 phosphorylation in F11 or in HEK293T cells (Fig. 4B), indicating that phosphorylation of Thr-208 did not significantly activate MARK2. At present, we are still in search of the exact reason for this discrepancy. However, Lizcano et al. (34) demonstrated that the ability of LKB-1 to activate MARK2 is much greater than that of TAO-1, and LKB-1, a tumor suppressor kinase (37), has been reported to play important roles not only in microtubule polarity, neuronal differentiation, and Wnt signaling (43–46). Thereafter, other tau-Ser-262 kinases such as SAD kinases (36) might be involved in this phosphorylation.

LKB-1 was measured employing AMARA peptide (AMA-XX/H9252), indicating that phosphorylation of Thr-208 to Ala in MARK2 did not reduce tau-Ser-262 phosphorylation but that other tau-Ser-262 kinases such as SAD kinases (36) might be involved in this phosphorylation.

In summary, we have shown that GSK-3β phosphatases Ser-212 in the activation loop of MARK2, thereby inducing the activation of MARK2 and resulting in the phosphorylation of tau at Ser-262. Ser-262 is known as a major site for abnormal phosphorylation of PHF-tau in AD brain (47). Therefore, our finding would provide a new insight into the functional significance of the GSK-3β-MARK2 pathway in the pathogenesis of AD.

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19. Indeed, the kinase activity of MARK2 phosphorylated at Thr-208 by LKB-1 was measured employing AMARA peptide (AMA-RAASAAALARRR) as a substrate, which does not contain a KXSβ motif (34). Therefore, another possible explanation is that Thr-208-phosphorylated MARK2 and Ser-212-phosphorylated MARK2 differ in substrate specificity, with Thr-208-phosphorylated MARK2 phosphorylating a Ser/Thr residue within the ΦβXXS/XXΦ motif, but not a Ser/Thr residue within the KXSβ motif, and vice versa. If that were the case, the target protein of Ser-212-phosphorylated MARK2 would be different from that of Thr-208-phosphorylated MARK2. MARK/PAR-1 has been reported to play important roles not only in microtubule dynamics and tau-dependent neuronal toxicity but also in epithelial cell polarity, neuronal differentiation, and Wnt signaling (43–46). Therefore, it is likely that each of these cellular events is controlled by the state of MARK2, whether Ser-212 is phosphorylated, Thr-208 is phosphorylated, or both are phosphorylated.
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GSK-3β Phosphorylates and Activates MARK2