

Regulatory B Subunits of Protein Phosphatase 2A Are Involved in Site-specific Regulation of Tau Protein Phosphorylation

Un Young Yu¹, Byong Chul Yoo², and Jung-Hyuck Ahn¹

¹Department of Biochemistry, Ewha Womans University College of Medicine, Seoul 158-710, ²Colorectal Cancer Branch, Research Institute, National Cancer Center, Goyang 410-768, Korea

Overexpression of amyloid precursor protein with the Swedish mutation causes abnormal hyperphosphorylation of the microtubule-associated protein tau. Hyperphosphorylated isoforms of tau are major components of neurofibrillary tangles, which are histopathological hallmarks of Alzheimer's disease. Protein phosphatase 2A (PP2A), a major tau protein phosphatase, consists of a structural A subunit, catalytic C subunit, and a variety of regulatory B subunits. The B subunits have been reported to modulate function of the PP2A holoenzyme by regulating substrate binding, enzyme activity, and subcellular localization. In the current study, we characterized regulatory B subunit-specific regulation of tau protein phosphorylation. We showed that the PP2A B subunit PPP2R2A mediated dephosphorylation of tau protein at Ser-199, Ser-202/Thr-205, Thr-231, Ser-262, and Ser-422. Down-regulation of PPP2R5D expression decreased tau phosphorylation at Ser-202/Thr-205, Thr-231, and Ser-422, which indicates activation of the tau kinase glycogen synthase kinase 3 beta (GSK3 β) by PP2A with PPP2R5D subunit. The level of activating phosphorylation of the GSK3 β kinase Akt at Thr-308 and Ser-473 were both increased by PPP2R5D knockdown. We also characterized B subunit-specific phosphorylation sites in tau using mass spectrometric analysis. Liquid chromatography-mass spectrometry revealed that the phosphorylation status of the tau protein may be affected by PP2A, depending on the specific B subunits. These studies further our understanding of the function of various B subunits in mediating site-specific regulation of tau protein phosphorylation.

Key Words: Alzheimer's disease, Phosphorylation, Protein phosphatase 2A, Tau protein

INTRODUCTION

Alzheimer's disease (AD) is an age-dependent neurodegenerative disorder characterized by progressive memory decline and deterioration of cognitive function. Hyperphosphorylated tau protein is the major component of paired helical filaments and neurofibrillary tangles, which are diagnostic hallmarks of AD. Abnormally hyperphosphorylated tau causes misfolding and disruption of microtubules [1,2]. The most common tau protein kinases are cyclin-dependent kinase 5 (CDK5) and glycogen synthase kinase 3 beta (GSK3 β), and both have multiple phosphorylation sites in tau protein [3]. The serine/threonine kinase Akt is a known regulator of GSK3 β activity via phosphorylation [4]. Phosphorylation by phosphoinositide 3-kinase at Thr-308

and Ser-473 activates Akt, which in turn, phosphorylates GSK3 β at inhibitory Ser-9, resulting in hypophosphorylated tau protein [5,6]. The role of PP2A in the regulation of Akt activity has been shown in the pathways controlling cell growth and survival [7]. Akt also forms a signaling protein complex with protein phosphatase 2A (PP2A), which is a known negative regulator of Akt through Thr-308 and/or Ser-473 dephosphorylation. PP2A regulates tau phosphorylation via direct or indirect mechanisms [8,9].

PP2A is composed of a 36-kDa catalytic subunit, a 64-kDa structural subunit, and a variety of B subunits that regulate subcellular localization and enzyme specificity. PPP2R3A subunit contains two calcium ion-binding motif (EF-hand), and upon Ca²⁺ binding, the activity of PP2A with PPP2R3A regulatory subunit is increased up to three times [10]. Another B subunit PPP2R5D involved in dopamine signaling pathway [11] or cell cycle regulation with the phosphorylation on its Ser residues [12]. Four protein families of the B subunit exist, and the expression of several B subunits is abnormally altered in an AD model cell line [13]. The activity of PP2A is regulated by protein kinases such as protein kinase A (PKA), protein kinase C (PKC), and also regulate the activity of several important kinases di-

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Corresponding to: Jung-Hyuck Ahn, Department of Biochemistry, Ewha Womans University School of Medicine, 911-1 Mok-6-dong, Yangcheon-gu, Seoul 158-710, Korea. (Tel) 82-2-2650-5712, (Fax) 82-2-2652-7846, (E-mail) ahnj@ewha.ac.kr



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ABBREVIATIONS: PP2A, protein phosphatase-2A; GSK, glycogen synthase kinase; CDK, cyclin-dependent kinase; DMSO, dimethyl sulfoxide.

rectly or indirectly [14,15]. However, little is known about the physiological function of the PP2A heterotrimeric holoenzyme in regulating tau hyperphosphorylation in AD pathogenesis such as tauopathy.

To characterize the nature of PP2A-mediated tau protein phosphorylation, we identified PP2A B subunits which regulate specific phosphorylation sites of tau protein and revealed the molecular mechanism of tau-phosphorylating Akt-GSK3 β signaling pathway by PP2A holoenzyme. We also used a proteomics approach to discover yet unknown novel PP2A-regulated phosphorylation sites in tau protein.

METHODS

Cell culture

Human neuroglioma H4 cells [16] were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Gaithersburg, MD, USA), supplemented with 10% fetal bovine serum (Gibco-BRL, Gaithersburg, MD, USA), 100 Units/mL penicillin and 100 μ g/mL streptomycin (Gibco-BRL, Gaithersburg, MD, USA). H4 swedish mutation (H4-swe) cells [16] and H4 cells stably transfected with swedish double mutation K595N/M596L of human APP695, were cultured in H4 growth media contained 0.5 mg/mL geneticin (Gibco-BRL, Gaithersburg, MD, USA). Cells were grown at 37°C and 5% CO₂.

Plasmid and siRNA Transfection

H4 and H4-swe cells were plated to be at 60~70% confluence overnight and transiently transfected with plasmid or plasmid and siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After incubation at 37°C for 6 h, the medium was replaced and the cells were harvested at 48 h after transfection. The final concentrations of plasmid and siRNA

were 1 μ g/mL and 25 nM respectively. For the experiment, pCMV6 plasmid vector containing cDNA encoding the longest human tau (tau 441) gene purchased Origene (Origene Technologies, Inc., Rockville, MD, USA) and On-Target plus smart pool siRNAs targeting human PPP2R2A, PPP2R3A and PPP2R5D and On-Target plus non-targeting siRNAs were purchased Dharmacon (Dharmacon, Thermo Fisher Scientific, Inc., Chicago, IL, USA). Expression and knock-down of proteins were confirmed by western blot analysis.

Okadaic acid treatment

To study the effect of PP2A on tau phosphorylation, tau 441 transiently transfected H4 cells were treated with 10 nM okadaic acid (Calbiochem, Nottingham, UK) for 12 h. DMSO was used as a vehicle control.

Protein extraction and Western blot analysis

Cells were washed twice with ice cold phosphate buffered saline (PBS, pH7.4) and lysed in RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and 2 mM EDTA) plus protease inhibitors and phosphatase inhibitors (Roche Diagnostics GmbH, Mannheim, Germany) then incubate on ice for 30 min. After centrifugation at 13,000 rpm for 30 min at 4°C, the supernatant was collected. Concentration of protein was determined using BCA Protein Assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA).

Cell lysates added 5X SDS-PAGE sample buffer (250 mM Tris-HCl, pH 6.8, 0.5 M DTT, 10% SDS, 0.5% Bromophenol blue and 50% Glycerol) were heated at 100°C for 5 min and resolved in 8~10% SDS-polyacrylamide gels. Separated proteins were transferred to PVDF membrane (Millipore, Bedford, Massachusetts, USA). The blots were incubated with a super blocking buffer (Pierce Biotechnology, Inc., Rockford, IL, USA) for 2 h and then the membranes were incubated with the primary antibodies overnight at 4°C.

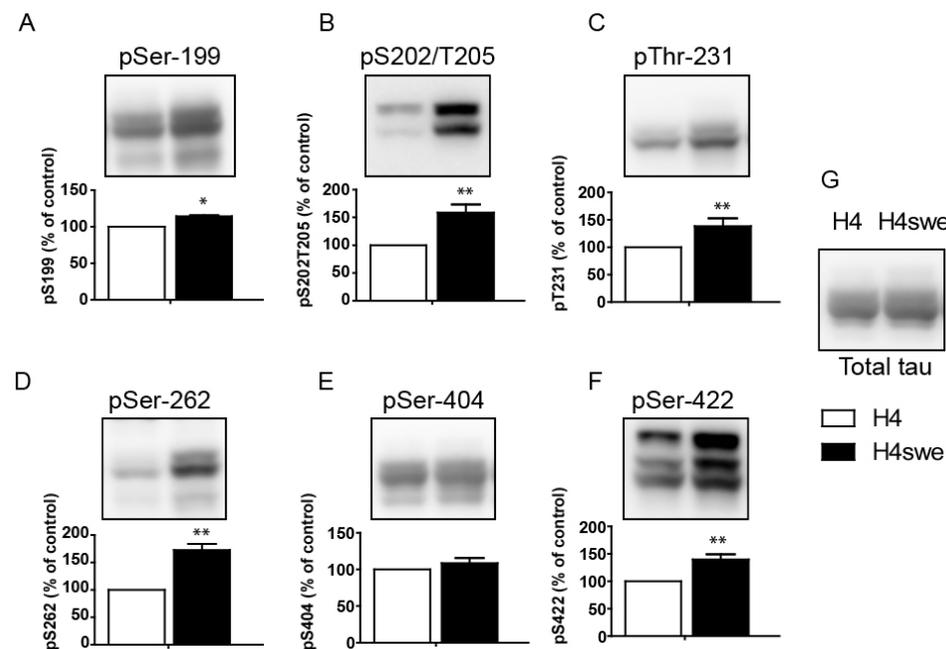


Fig. 1. Hyperphosphorylation of tau protein in the AD model cell line. A FLAG-tagged tau 441 expression construct was transfected into H4 wild-type cells (H4) or H4-swe cells, which express the Swedish mutant form of APP. The phosphorylation status was examined on (A) Ser-199, (B) Ser-202/Thr-205, (C) Thr-231, (D) Ser-262, (E) Ser-404, and (F) Ser-422 with the respective phospho-specific antibodies. Total tau protein was visualized with anti-FLAG antibody. Bar graphs show the phosphorylation levels of each phosphorylation site. Signals from H4-swe cells were normalized to values obtained for H4 wild-type cells. Statistical analyses were performed using a *t*-test (**p* < 0.001, ***p* < 0.05). Data are the means \pm standard error of the mean (SEM, *n* = 3).

After washing TBST, the membranes incubated with an anti-mouse or -rabbit HRP-conjugated secondary antibody for 2 h. The antibodies used in this study were against FLAG (1 : 1,000) from Sigma (Sigma Aldrich, St.Louis, MO, USA), Tau (1 : 1,000) and phospho Tau (Ser262) (1 : 1,000) from Invitrogen (Invitrogen, Carlsbad, CA, USA), phospho Tau (Ser202/Thr205, Thr231) (1 : 500) from Pierce (Pierce Biotechnology, Inc., Rockford, IL, USA), phospho Tau (Ser199, Ser404, Ser422) (1 : 1,000) from Abcam (Abcam, Cambridge, UK), PP2R2A (1 : 1,000) from cell signalling (Cell Signalling Technology, Danvers, MA, USA), and beta-actin (1 : 1,000) and secondary antibody (1 : 2,000) from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were developed by enhanced chemiluminescence detection (GE Healthcare, Little Chalfont, Buckinghamshire, UK) at LAS 3000 (Fugifilm UK Ltd., Bedfordshire, UK). The signals were quantified using image J program (<http://rsb.info.nih.gov/ij>).

Statistical analysis

Statistical analysis and graphing were performed using Graphpad Prism 5 (Graphpad, San Diego, CA, USA). Significance was determined using a two-tailed Student's *t*-test and *p* values <0.05 were considered significant.

RESULTS

Differential phosphorylation status of tau protein in the AD model cell line expressing the Swedish mutant form of amyloid precursor protein (APP-swe)

Our previous studies of differential gene expression of PP2A B subunits in the AD model cell line showed increased phosphorylation of tau protein at Ser-202/Thr-205, Thr-231, and Ser-422 with a variable amount of expression

of certain PP2A subunits and other tau kinases such as CDK5 and GSK3 β [16]. We further analyzed additional tau protein phosphorylation sites in this AD model cell line (Fig. 1). Human tau proteins constitute six isoforms with the range from 352 amino acids (tau 352) to 441 amino acids (tau 441) in brain. These isoforms detected as multiple bands in western blot analysis probed with anti-tau antibody, making analysis inconclusive. We transfected H4wt or H4-swe cells with FLAG-tagged 441-amino acid tau protein expression plasmid (tau 441) to enhance the signal in western blot analysis for measuring the status of tau protein phosphorylation. We performed total tau 441 measurement with anti-FLAG antibody to eliminate signals from short forms of tau protein which lacks important phosphorylation sites at C-terminus such as Ser-404 and Ser-422. Tau protein was significantly hyperphosphorylated at Ser-199, Ser-202/Thr-205, Thr-231, Ser-262, and Ser-422 in H4-swe cells compared to H4wt cells, implying that the activity of tau phosphatases or tau kinases is altered during AD pathogenesis.

PP2A activity is required for the regulation of tau protein phosphorylation

We next investigated the effects of PP2A on the regulation of tau protein phosphorylation using a selective PP2A inhibitor. H4wt cells were transiently transfected with tau 441 and treated with 10 nM okadaic acid, which specifically inhibits PP2A at a low dose [17]. Okadaic acid-treated H4wt cells showed similar phosphorylation of tau protein as in the AD model cell line H4-swe, indicating that PP2A activity is crucial in AD pathogenesis (Fig. 2). Phosphorylation at Ser-199, Ser-202/Thr-205, Thr-231, Ser-262, and Ser-422 was decreased 1.5 to 2-fold in okadaic acid-treated H4-swe cells compared to DMSO vehicle-treated cells.

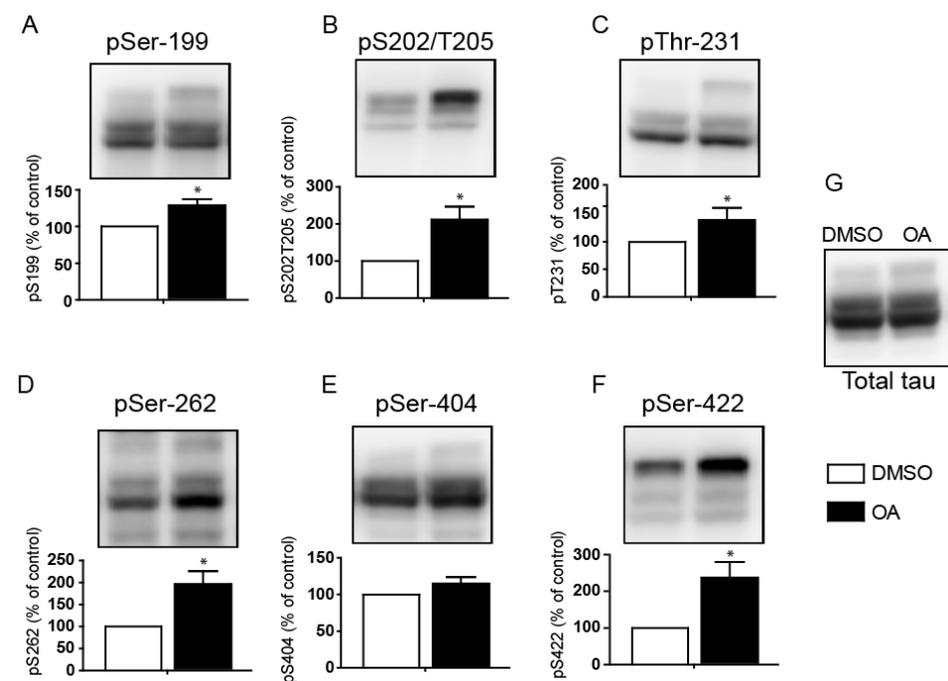


Fig. 2. PP2A-mediated regulation of the tau protein phosphorylation level. FLAG-tagged tau 441-transfected H4-swe cells were treated with 10 nM okadaic acid for 12 hours. The phosphorylation status was examined on (A) Ser-199, (B) Ser-202/Thr-205, (C) Thr-231, (D) Ser-262, (E) Ser-404, and (F) Ser-422 with a phospho-specific antibody corresponding to each site. Total tau protein was visualized with anti-FLAG antibody. Bar graphs show the phosphorylation levels of each phosphorylation site. Signals from H4-swe cells were normalized to values obtained for DMSO vehicle-treated cells. Statistical analyses were performed using a *t*-test (**p*<0.001, ***p*<0.05). Data are the means \pm standard error of the mean (SEM, *n*=3).

Regulatory B subunits are involved in site-specific regulation of tau phosphorylation

In the AD model cell line, H4-swe, the expression of various PP2A regulatory B subunits is largely altered, whereas the total amount of structural A subunit and catalytic C

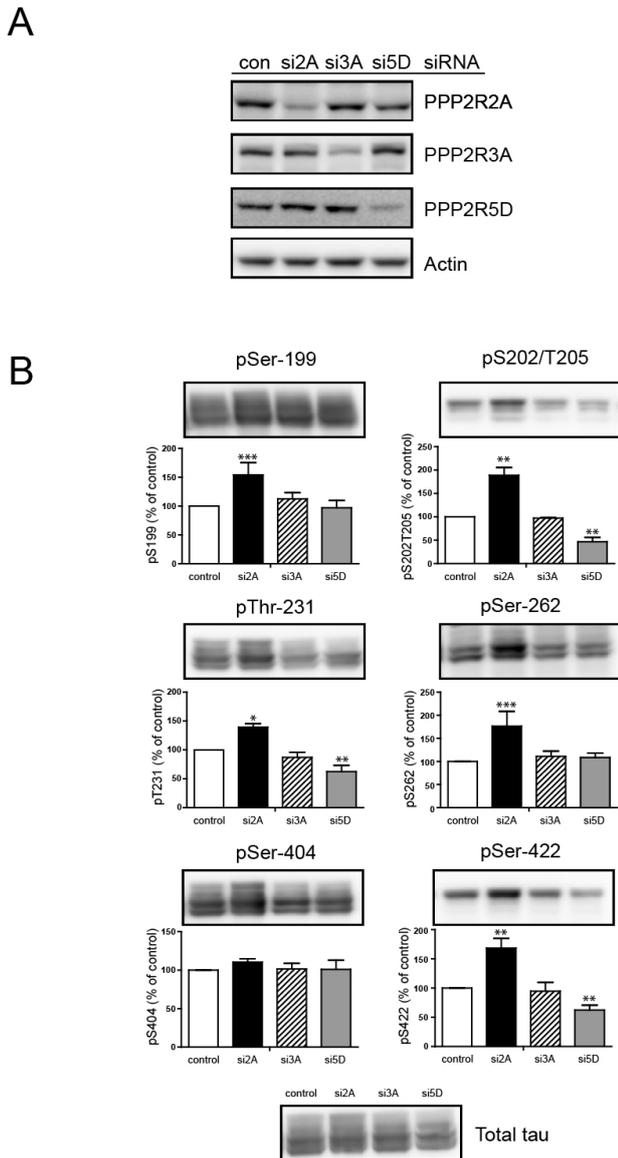


Fig. 3. Regulatory B subunits are involved in site-specific regulation of tau protein phosphorylation. B regulatory subunits were knocked down with siRNA specific to each B subunit; PPP2R2A siRNA (si2A), PPP2R3A siRNA (si3A), or PPP2R5D siRNA (si5D) was transfected into H4-swe cells. (A) The expression level of each B subunit was measured with antibodies corresponding to each B subunit. (B) Phosphorylation status analysis was performed with immunoblotting for each site as indicated. Bar graphs show the phosphorylation level for each site normalized to control siRNA-treated H4 cells. Statistical analyses were performed using a *t*-test (* $p < 0.001$, ** $p < 0.01$, *** $p < 0.05$). Data are the means \pm standard error of the mean (SEM, $n=3$).

subunit remains unchanged [16]. To address the effect of changes in B subunit expression, we used B subunit-specific small interfering RNA (siRNA) to knock down the expression of the B subunit in H4 cells. We chose PPP2R2A, PPP2R3A, and PPP2R5D, which represents the B, B', and B'' subunit families, respectively. The PPP2R2A subunit is known to be included in the tau-specific PP2A protein complex, but detailed information of the dephosphorylation sites in tau protein is limited. PPP2R3A and PPP2R5D subunits are expressed in brain and dephosphorylate CDK5 substrate proteins, implying that these subunits play a role in the tau dephosphorylation signaling process. We co-transfected H4wt cells with tau 441 and the B subunit siRNA and examined the expression level of each B subunit (Fig. 3A). Each siRNA down-regulated the expression of the target B subunit, and we measured the phosphorylation status of the tau protein (Fig. 3B). Down-regulation of the PPP2R2A subunit significantly increased phosphorylation at Ser-199, Thr-231, Ser202/Thr-205, Ser-262, and Ser-422, indicating that PP2A with the PPP2R2A B subunit is a major tau-specific phosphatase. Treatment with the siRNA for the PPP2R3A subunit did not change the tau phosphorylation status, and interestingly, PPP2R5D siRNA treatment reduced the phosphorylation level at Thr-231, Ser-202/Thr-205, and Ser-422, implying that PP2A with the PPP2R5D subunit activates certain tau-specific kinases via dephosphorylation.

The PPP2R5D subunit regulates tau phosphorylation via Akt-mediated GSK3 β activation

Down-regulation of PPP2R5D with siRNA reduced phosphorylation at Ser-202/Thr-205, Thr-231, and Ser-422, implying that PP2A with the PPP2R5D subunit activates a specific tau kinase through dephosphorylation. GSK3 β has

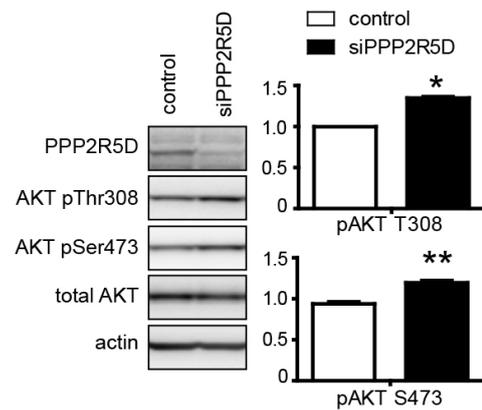


Fig. 4. PP2A with the PPP2R5D subunit regulates the AKT-GSK3 β signaling pathway. H4 cells were transfected with control siRNA or PPP2R5D siRNA (left panel), or a control vector or a PPP2R5D expression construct (right panel) as indicated. The status of inhibitory phosphorylation at Ser-9 of GSK3 β was visualized with a phospho-specific antibody. H4 cells were transfected with siRNA specific for the PPP2R5D subunit, and then the status of activating phosphorylation at Thr-308 and Ser-473 was visualized with a phospho-specific antibody corresponding to each site. The bar graph shows the relative signal intensity of siRNA-transfected samples compared to the control siRNA-transfected sample. Statistical analyses were performed using a *t*-test (* $p < 0.001$, ** $p < 0.01$). Data are the means \pm standard error of the mean (SEM, $n=3$).

Table 1. B subunit-dependent phosphorylation sites in tau protein from LC-MS analysis

Phospho-peptide	Peptide sequence	Frequency of phospho-peptide			
		Control	si2A	si3A	si5D
pT30	DQGGYtMHQDQEGDTDAGLK	0	2	1	0
pT175	IPAKtPPAPKTPPSSGEPK	9	2	3	0
pT181	TPPAPKtPPSSGEPKSGDR	77	69	50	35
pS185	TPPAPKTPP _s SGEPPK	1	2	1	0
pS199	SGYS _s PGSPGTPGSR	1	7	2	3
pS202	SGYSSPG _s PGTPGSR	126	160	72	57
pT205	SGYSSPGSPGtPGSR	0	4	0	0
pT212	SRtPSLPTPPTREPK	2	0	1	0
pS214	TP _s LPTPPTREPK	1	1	1	0
pT217	TPSLP _t PPTREPK	2	5	4	0
pT231	VAVVRtPPKSPSSAK	3	9	7	1
pS235	VAVVRTPPK _s PSSAK	0	1	3	0
pS237	VAVVRTPPKSP _s AK	0	3	2	0
pY394	VyKSPVVGDTSPR	0	2	2	0
pS396	VYK _s PVVSGDTSPR	5	7	5	1
pT403	TDHGAEIVYKSPVVGDTSPR	7	8	8	12
pS404	SPVVSGDT _s PR	167	133	84	57
pS409	HLsNVSS _t GSID _m VDSPQLATLADEV _S ASLAK	2	0	0	0
pT414	HLSNVSS _t GSID _m VDSPQLATLADEV _S ASLAK	0	1	1	0
pS416	HLSNVSS _t G _s ID _m VDSPQLATLADEV _S ASLAK	0	9	2	3

FLAG-tagged tau 441 was transfected into H4 cells with control siRNA, PPP2R2A siRNA (si2A), PPP2R3A siRNA (si3A), or PPP2R5D siRNA (si5D). Tau proteins were immunoprecipitated with anti-FLAG antibody 48 hours after transfection. Tau protein was in-gel digested with trypsin for LC-MS analysis. LC-MS analysis was repeated for four times using immunoprecipitated tau obtained from four independent experiments. The number indicates the frequency of each phospho-peptide from four independent experiments.

been reported to phosphorylate tau at these three sites, and Akt kinase regulates the activity of GSK3 β via inhibitory phosphorylation at Ser-9 of GSK3 β [18]. We assessed the phosphorylation status of Akt and GSK3 β in PPP2R5D knockdown or overexpressing cell lines (Fig. 4). Inhibitory phosphorylation at Ser-9 of GSK3 β was increased in the cell line treated with siRNA for PPP2R5D. Moreover, Ser-9 phosphorylation was decreased by PPP2R5D overexpression (Fig. 4). Next, we investigated the phosphorylation status of Akt kinase, which regulates the activity of GSK3 β by inhibitory phosphorylation at Ser-9. Akt-activating phosphorylation at Thr-308 and Ser-473 was significantly increased in PPP2R5D siRNA-treated H4 cells, indicating that PP2A with PPP2R5D is an Akt-specific phosphatase at these sites. Taken together, PP2A with the PPP2R5D subunit dephosphorylates Akt at Thr-308 and Ser-473 (Fig. 4), which inhibits overall Akt activity and results in activation of GSK3 β by decreasing the inhibitory phosphorylation at Ser-9.

Identification of regulatory B subunit-specific novel phosphorylation sites in tau protein

Although PP2A is known as a major tau phosphatase, little is known about the composition of the tau-specific PP2A heterotrimeric enzyme. We used a proteomics approach to identify regulatory B subunit-specific phosphorylation sites in tau protein. Tau 441 proteins were immunoprecipitated from B subunit siRNA-transfected cells, and then tau phosphorylation was analyzed with liquid chromatography-mass spectrometry (LC-MS). Twenty-three phosphorylation sites were identified in tau peptides, and

the frequency of phosphorylation was calculated using the total number of phospho-tau peptides identified in LC-MS analysis that was repeated four times (Table 1). Results from both western blotting with a phospho-specific antibody and LC-MS were similar, and additional PP2A-regulated tau phosphorylation sites were revealed.

DISCUSSION

In this study, we examined the molecular composition and substrate specificity of PP2A towards tau protein. Moreover, PPP2R5D was required for inhibition of Akt activity by dephosphorylating the activating phosphorylation sites at Thr-308 and Ser-473. Akt was previously reported to be associated with PP2A with PPP2R2A, and was also reported to be negatively regulated by PP2A through Thr-308 dephosphorylation [7,19]. Interestingly, PPP2R5D knockdown affects both phospho-Thr-308 and phospho-Ser-473, suggesting that PP2A with the PPP2R5D subunit may be a more effective regulator of Akt activity.

We selected a major regulatory B subunit from the three different currently identified subfamilies of the B subunit, which are PPP2R2A (B family), PPP2R5D (B' family), and PPP2R3A (B'' family) [3,20,21]. PPP2R2A, also known as B-alpha, is reported to be a component of tau phosphatase. PPP2R5D is phosphorylated by protein kinase A (PKA) or protein kinase C, and phosphorylation at Ser-566 of PPP2R5D enhances the overall activity of PP2A [11,22]. The PPP2R3A subunit has two calcium ion binding sites (EF-hand), and interaction with calcium ions enables PPP2R3A to bind the PP2A A/C core enzyme and also activates PP2A [10,23]. The

PPP2R2A subunit seems to be involved in reducing the phosphorylation level of tau at Ser-199, Ser-202/Thr-205, Thr-231, Ser-262, and Ser-422. Among them, phosphorylation at Ser-202/Thr-205, Thr-231, and Ser-422 was decreased by PPP2R5D knockdown, indicating that PPP2R2A and PPP2R5D work in opposite directions in regulating tau phosphorylation status.

The recent studies regarding the crystal structure of a PP2A A/C/B56gamma heterodimer has provided insight into holoenzyme assembly and the role of regulatory B subunit in substrate recognition [24]. The HEAT domains in B56 subunit make multiple contacts with the C subunit, implying that the activity of catalytic subunit is modulated through these contacts. It is interesting that non-enzymatic B subunit could affect the activity of PP2A holoenzyme towards tau protein phosphorylation status at more than twenty different phosphorylation sites. Another regulatory mechanism of PP2A, inhibitory Tyr-307 phosphorylation on catalytic C subunit was reported as a regulating factor of tau phosphorylation in Parkinsonism-dementia [25,26], however, phosphorylation of this phosphorylation site was unchanged in our H4-swe cells compared to wild-type H4 cells (data not shown).

Mass spectrometric analysis using LC-MS showed that more than 18 phosphorylation sites in tau were regulated by PP2A. LC-MS analysis showed that target phosphorylation sites for the PPP2R2A subunit include previously reported PPP2R2A-specific sites [27,28]. Another regulatory B subunit, PPP2R5D, is phosphorylated by PKA with forskolin treatment, and PP2A with a phosphorylated PPP2R5D subunit is activated [11]. Tau protein phosphorylation at Ser-214 and Ser-396/Ser-404 is regulated by protein kinase A (PKA) and increased with forskolin treatment. These sites were shown to be regulated by the PPP2R5D subunit with LC-MS analysis (Table 1) indicating that mass spectrometric analysis may be a useful technique for characterizing novel phosphorylation sites in tau protein.

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