Glycogen Synthase Kinase-3 β 2 Has Lower Phosphorylation Activity to Tau than Glycogen Synthase Kinase-3 β 1

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Glycogen synthase kinase-3 β (GSK-3 β) is a serine/threonine kinase that phosphorylate protein substrates involved in Alzheimer's disease (AD), such as microtubule-associated protein tau and amyloid precursor protein (APP). GSK-3 β consists of two splice variants; the major short form (GSK-3 β 1) distributes in many organs and the minor long form (GSK-3 β 2), whose structural difference is the insert of only 13 amino acid residues to the C-terminal side of the catalytic site of GSK-3 β 1, is present in central nervous system. However, the physiological significances of the two variants are unclear. Here we examined whether the phosphorylation activities of two variants to tau and APP are different in cells. We found that GSK-3 β 2 has lower phosphorylation activity to tau at AD-relevant epitope (Ser396) than GSK-3 β 1 in cells, whereas the two variants exhibit equivalent levels of phosphorylation activities to APP. Recombinant GSK-3 β 2 has also lower phosphorylation activity to tau than GSK-3 β 1 *in vitro*, although the phosphorylation activities of the two variants to a synthetic peptide substrate pGS-2 are comparable. Furthermore, the deletion of the C-terminal tail (CT) of GSK-3 β 2 resulted in considerable reduction of tau phosphorylation activity as compared with GSK-3 β 1, suggesting that the lower phosphorylation activity of GSK-3 β 2 to tau is attributed to weak interaction with tau through its unique higher-order structure of CT constructed by the 13 amino acids insertion. Such information may provide a clue for understanding of the physiological significance of the two splice variants of GSK-3 β and a new insight into the regulation of tau phosphorylation in central nervous system.

Key words glycogen synthase kinase-3 β ; splice variant; tau; amyloid precursor protein; Alzheimer's disease

Glycogen synthase kinase-3 β (GSK-3 β) is a serine/threonine kinase that is involved in the regulation of many signaling pathways in vertebrates.^{1–3} GSK-3 β is expressed in two splice variants. GSK-3 β 1 (the short form of GSK-3 β) ubiquitously distributes in organs. In contrast, GSK-3 β 2 (the long form of GSK-3 β), whose structural difference is the only 13 amino acids insert at the C-terminal side of the catalytic site, is present in central nervous system.^{4,5} Although, so far, many researches on GSK-3 β are concerned with GSK-3 β 1 because of its high level of expression in various tissues, there is little research attention to the minor form, GSK-3 β 2. At present, the substrate preferences and physiological significances of the two variants remains unclear.

GSK-3 β is known to phosphorylate the microtubule-associated protein tau at Alzheimer's disease (AD)-relevant epitopes *in vivo* and *in vitro*.^{6,7} The hyperphosphorylated tau is known to the major component of neurofibrillary tangles observed in AD and over-activation of GSK-3 β has been implicated in the aberrant phosphorylation of tau in AD.^{8,9} In addition, GSK-3 β phosphorylates the intracellular domain of amyloid precursor protein (APP), which is closely implicated in AD.^{10–12} However, since the above reports deal with GSK-3 β 1, the physiological significance of the neuron specific isoform GSK-3 β 2 yet remains to be dissolved.

In this study, we investigated whether the phosphorylation activities of GSK-3 β 1 and -3 β 2 to tau and APP are different in cells. We found that the phosphorylation level of tau at AD-relevant epitope (Ser396) by GSK-3 β 2 is considerably lower than that by GSK-3 β 1, while the phosphorylation levels of APP by the two variants are almost equivalent. Furthermore, *in vitro* analyses revealed that recombinant GSK-3 β 2 has lower phosphorylation activity to tau than GSK-3 β 1, although the phosphorylation activities of the two variants to

a synthetic peptide substrate, pGS-2, are almost equivalent. These results indicate that tau is an unfavorable substrate for GSK-3 β 2. Moreover, we found that, by the deletion analyses of C-terminal tail (CT), the phosphorylation activity of GSK-3 β 2 Δ CT to tau is significantly reduced as compared with that of GSK-3 β 1 Δ CT. These results suggest that the lower phosphorylation activity of GSK-3 β 2 to tau is attributed to its unique higher-order structure of CT constructed by the 13 amino acids insertion. Furthermore, these observations may imply that changes in the balance of GSK-3 β 2/-3 β 1 in neurons underlie tau hyperphosphorylation in AD. Our data provide new insights into the physiological significances of the two variants in the regulation of tau phosphorylation in central nervous system and into the mechanism of the onset of AD via dysregulation of the GSK-3 β 2/-3 β 1 balance.

MATERIALS AND METHODS

Materials The mouse anti-total-tau (TAU-5), and rabbit anti-phospho-tau (Ser396) antibodies were purchased from Biosource International (Camarillo, CA, U.S.A.). The rabbit anti-GSK-3 β (27C10) was from Cell Signaling Technology (Beverly, MA, U.S.A.). The rat anti-Hemagglutinin (HA) epitope tag (3F10) antibody was from Roche (Nutley, NJ, U.S.A.). The rabbit anti-phospho-APP (Thr668) and anti-rabbit total-APP antibodies were from IBL (Gunma, Japan). The anti-rabbit, anti-mouse and anti-rat immunoglobulin G (IgG) horseradish peroxidase (HRP)-coupled secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.). pGS-2 synthesized substrate peptide was from Millipore (Billerica, U.S.A.).

Cell Culture HEK293T cells were grown in Dulbecco's modified Eagle's medium/Ham's F-12 supplemented with

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10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 μ g/ml penicillin, and 100 μ g/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C.

Western Blot Analysis Harvested cell samples were lysed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (50 mM Tris(hydroxymethyl)aminomethane (Tris)-HCl, 2% SDS, 5% glycerol, 1% 2-mercaptoethanol, 0.1% bromophenol blue, pH 6.8). Cell lysates were separated by SDS-PAGE, and then transferred onto nitrocellulose membranes. The membranes were blocked with 2.5% skim milk and 0.25% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween 20 (TTBS) for 1 h at room temperature, and then probed with appropriate primary antibodies for overnight at 4 °C. After the membranes were washed with TTBS, incubated with the appropriate secondary antibody for 1 h at room temperature. After washing the membrane with TTBS, the blotted proteins were visualized with Light Capture System (ATTO) by using Supersignal West Femto Maximum Sensitivity Substrate (PIERCE).

In Vitro Measurement of Recombinant GSK-3 β 1 and -3 β 2 Activities The kinase activities of recombinant purified GSK-3 β 1 and -3 β 2 were determined by a luminescent kinase assay kit (Kinase-Glo; Promega, Madison, WI, U.S.A.) that quantitates decrements of ATP by kinase utilization. Briefly, recombinant GSK-3 β 1 or -3 β 2 was incubated with 20 μ l of reaction buffer (10 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 0.3 mM ethylenediaminetetraacetic acid (EDTA), 0.001% (v/v) Brij-35, 0.5% (v/v) glycerol, 0.01% (v/v) 2-mercaptoethanol, 0.1 mg/ml BSA, pH 7.2), 20 μ M pGS-2 synthesized substrate peptide, and 10 μ M ATP for 30 min at 30 °C. The reaction was stopped by adding 20 μ l of Kinase-Glo reagent and incubated an additional 10 min at room temperature. Luminescence was measured with a Wallac 1420 ARVO MX Multilabel Counter (Perkin Elmer, Waltham, MA, U.S.A.).

In Vitro Phosphorylation of Tau Recombinant GSK-3 β 1 or -3 β 2 was incubated with 10 μ l of reaction buffer (10 mM MOPS pH 7.2, 0.3 mM EDTA, 0.001% (v/v) Brij-35, 0.5% (v/v) glycerol, 0.01% (v/v) 2-mercaptoethanol, and 0.1 mg/ml BSA), 0.1 μ g/ μ l recombinant tau, 250 μ M ATP, and 0.1 μ Ci/ μ l of [γ -³²P]ATP (Perkin Elmer, Waltham, MA, U.S.A.) at 30 °C for 30 min. The reaction was stopped by adding 10 μ l of 2 $\times$ SDS-PAGE sample buffer to each sample. Samples were placed in a boiling water bath for 5 min, and proteins were separated on 10% SDS-PAGE gels. The gels were vacuum-dried and autoradiographed (BAS-1500; FUJIFILM).

RESULTS

The Phosphorylation Activities of GSK-3 β 1 and -3 β 2 to Tau and APP in Cells GSK-3 β has been shown to phosphorylate tau at AD-relevant epitope, Ser396.^{6,7} We compared kinase activities of GSK-3 β 1 and -3 β 2 to tau at Ser396 in HEK293T cells co-expressing tau and the two variants. The phosphorylation level of tau at Ser396 was determined by Western blot analysis using a specific anti-phospho-tau antibody that recognizes tau phosphorylated at Ser396. As shown in Fig. 1, the phosphorylation level of tau at Ser396 was considerably lower in cells expressing GSK-

Fig. 1. Comparison of Phosphorylation Activities of GSK-3 β 1 and -3 β 2 to Tau

HA-tagged GSK-3 β splice variants were co-expressed with tau in HEK293T cells. Whole cell lysates were analyzed by Western blotting using antibodies that recognize tau specifically phosphorylated at Ser396 (α tau (Ser396), upper panel), total tau (α tau, middle panel), and both GSK-3 β splice variants (α GSK-3 β , lower panel). The results shown are representative of three independent experiments. *: Endogenous GSK-3 β .

Fig. 2. Comparison of Phosphorylation Activities of GSK-3 β 1 and -3 β 2 to APP

HA-tagged GSK-3 β splice variants were co-expressed with APP in HEK293T cells. Whole cell lysates were analyzed by Western blotting using antibodies that recognize APP specifically phosphorylated at Thr668 (α pAPP (Thr668), upper panel), total APP (α APP, middle panel), and both GSK-3 β splice variants (α GSK-3 β , lower panel). The results shown are representative of three independent experiments. *: Endogenous GSK-3 β .

3 β 2 than GSK-3 β 1.

It has been reported that the intracellular domain (Thr668) of APP is phosphorylated by GSK-3 β ,¹⁰ and that the phosphorylation regulates APP function and metabolism.^{11,12} To examine the phosphorylation activities of GSK-3 β 1 and -3 β 2 to APP, the two variants were co-expressed with APP in HEK293T cells. Western blot analysis using a specific anti-phospho-APP antibody that recognizes APP phosphorylated at Thr668 showed that both GSK-3 β 1 and -3 β 2 phosphorylated APP at Thr668, and that the phosphorylation levels were similar each other (Fig. 2). These results reveal that tau is an unfavorable substrate of GSK-3 β 2.

GSK-3 β 2 Has Lower Phosphorylation Activity to Tau than GSK-3 β 1 in Vitro To investigate why GSK-3 β 2 has the lower phosphorylation activity to tau than GSK-3 β 1 in cells, we compared the phosphorylation activities of recombinants GSK-3 β 1 and -3 β 2 *in vitro*. To test the kinase activities of the purified recombinant GSK-3 β 1 and -3 β 2, the *in vitro* phosphorylation assay using pGS-2 synthesized peptide as the substrate was performed. As summarized in Table 1, the two variants displayed almost equivalent phosphorylation activities.

Next, we assessed the phosphorylation activities of the two recombinant GSK-3 β variants to recombinant tau. As shown in Fig. 3, the recombinant GSK-3 β 1 was able to phosphorylate tau at lower concentrations than GSK-3 β 2. These results reveal that GSK-3 β 2 has the lower phosphorylation activity to tau than GSK-3 β 1 *in vitro* as well as in cells.

Table 1. Evaluation of Kinase Activities of GSK-3 β 1 and -3 β 2 Using a Synthetic Peptide Substrate, pGS-2

	Kinase activity (nmol/min/mg)
GSK-3 β 1	234.5 $\pm$ 7.8
GSK-3 β 2	246.5 $\pm$ 2.4

Kinase activities of recombinant GSK-3 β 1 and -3 β 2 were measured by analyzing phosphorylation of pGS-2 synthesized substrate peptide as described under Materials and Methods. The results are shown by means $\pm$ S.E. of three independent experiments.

Fig. 3. Comparisons of Phosphorylation Activities of GSK-3 β 1 and -3 β 2 to Tau *in Vitro*

Recombinant tau was incubated with indicated concentrations of recombinant GSK-3 β 1 or -3 β 2 for 30 min at 30°C in the presence of [γ - ^{32} P] ATP, and was analyzed by SDS-PAGE and autoradiography (upper panel). Concentrations of recombinant GSK-3 β 1 and -3 β 2 were confirmed by Western blotting using an antibody that recognizes GSK-3 β (lower panel). The results shown are representative of three independent experiments.

The Lower Tau Phosphorylation Activity of GSK-3 β 2 Is Due to Its Weak Interaction of C-Terminal Tail with Tau Since GSK-3 β 2 has lower tau phosphorylation activity than GSK-3 β 1 even though the phosphorylation activities of the two variants to APP in cells and pGS-2 *in vitro* are equivalent, it is possible that higher-order structural differences outside the same catalytic domains of GSK-3 β 1 and -3 β 2 contribute to the differential phosphorylation activities to tau. Thus, we hypothesized that the lower phosphorylation activity of GSK-3 β 2 to tau is due to its unique higher-order structure in C-terminal region constructed by the insertion of 13 amino acid residues because of the location of the insert at the C-terminal side of catalytic domain (Fig. 4). At the C-terminal region, we focused on the C-terminal tail (CT) consisting of 40 amino acid residues, whose structure in GSK-3 β 1 has been invisible in all crystal structures because of its flexibility.^{13–24}

To test our hypothesis, deletion mutants of the two variants lacking CT were generated (Fig. 4). We examined the abilities of those mutants to phosphorylate co-expressed tau in HEK293T cells (Fig. 5). The phosphorylation of tau at Ser396 was determined by Western blot analysis using a specific anti-phospho-tau antibody that recognizes tau phosphorylated at Ser396. Importantly, GSK-3 β 2 Δ CT almost lost the phosphorylation activity to tau. In contrast, the deletion of CT from GSK-3 β 1 slightly reduced the tau phosphorylation activity. Thus, CT is critical to the tau phosphorylation activity of GSK-3 β 2. These results reveal that the interaction of CT in GSK-3 β 2 with tau is weak and critical, and support our hypothesis that the lower phosphorylation activity of GSK-3 β 2 to tau is attributed to its unique higher-order structure in the C-terminal region constructed by the insertion of 13 amino acid residues.

DISCUSSION

In this study, we compared the phosphorylation activities

Fig. 4. Schematic Structures of GSK-3 β 1 and -3 β 2

The 13 amino acids insert in the C-terminus of GSK-3 β 2 is indicated in black. The sequence of CT is identical between GSK-3 β 1 and GSK-3 β 2.

Fig. 5. Effects of Deletions of CT on the Phosphorylation Activities of GSK-3 β 1 and -3 β 2 to Tau

The C-terminal deletion mutants of GSK-3 β 1 (left panel) and -3 β 2 (right panel) were co-expressed with tau in HEK293T cells. Whole cell lysates were analyzed by Western blotting using an antibody that recognizes tau specifically phosphorylated at Ser396 (α tau (Ser396), upper panel), and both GSK-3 β splice variants (α GSK-3 β , lower panel). The results shown are representative of three independent experiments. Δ , Deleted; *: Endogenous GSK-3 β .

of GSK-3 β 1 and -3 β 2 to protein substrates closely implicated in AD, tau and APP. We found that GSK-3 β 2 has lower phosphorylation activity to tau at AD-relevant epitope (Ser396) as compared with GSK-3 β 1 in cells (Fig. 1), whereas the two variants exhibit almost equivalent levels of phosphorylation activities to APP at Thr668 (Fig. 2). We further showed that the phosphorylation activity of GSK-3 β 2 to tau is significantly lower than that of GSK-3 β 1 *in vitro* (Fig. 3), although the phosphorylation activities of the two variants to the synthesized peptide substrate pGS-2 are almost equivalent (Table 1). These results suggest that tau is an unfavorable substrate for GSK-3 β 2.

To investigate the molecular mechanism by which the phosphorylation activity of GSK-3 β 2 to tau is lower than GSK-3 β 1, we compared the phosphorylation activities of deletion mutants of the two variants lacking the common C-terminal tail (CT) consisting of 40 amino acid residues (Fig. 4). Surprisingly, the deletion of CT in GSK-3 β 2 resulted in the considerable reduction of tau phosphorylation activity as compared with the GSK-3 β 1 deletion mutant (Fig. 5). These results suggest that CT is indispensable for the tau phosphorylation activity of GSK-3 β 2, although CT is not essential for that of GSK-3 β 1. Probably, GSK-3 β 1 could utilize not only CT but also the adjacent region of CT for interaction with tau, while GSK-3 β 2 could utilize only CT uniquely constructed by the 13 amino acids insert. To elucidate the precise molecular mechanism of the interaction of CT in GSK-3 β 2 with tau, the structural analysis of GSK-3 β 2, whose structure has not yet been dissolved, is needed.

Our findings of differential substrate preferences between GSK-3 β 2 and -3 β 1 may provide important clues for understanding the physiological significances of the two variants in the regulation of tau phosphorylation in central nervous system. The hyperphosphorylated tau is known to be the major component of neurofibrillary tangles observed in AD and over-activation of GSK-3 β has been implicated in the aberrant phosphorylation of tau in the onset of AD.^{8,9} In this con-

text, we propose that changes in the balance of GSK-3 β 2/-3 β 1 in neurons underlie tau hyperphosphorylation associated with neuronal cell death in AD. We are now under investigation of changes in the balance of GSK-3 β 2/-3 β 1 in the brains of AD model animals and AD patients.

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