Involvement of aberrant glycosylation in phosphorylation of tau by cdk5 and GSK-3β

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Abstract **Microtubule-associated protein tau is abnormally hyperphosphorylated, glycosylated, and aggregated in affected neurons in the brains of individuals with Alzheimer's disease (AD). We recently found that the glycosylation might precede hyperphosphorylation of tau in AD. In this study, we investigated the effect of glycosylation on phosphorylation of tau catalyzed by cyclin-dependent kinase 5 (cdk5) and glycogen synthase kinase-3β (GSK-3β). The phosphorylation of the longest isoform of recombinant human brain tau, tau_{441}, at various sites was detected by Western blots and by radioimmuno-dot-blot assay with phosphorylation-dependent and site-specific tau antibodies. We found that cdk5 phosphorylated tau_{441} at Thr-181, Ser-199, Ser-202, Thr-205, Thr-212, Ser-214, Thr-217, Thr-231, Ser-235, Ser-396, and Ser-404, but not at Ser-262, Ser-400, Thr-403, Ser-409, Ser-413, or Ser-422. GSK-3β phosphorylated all the cdk5-catalyzed sites above except Ser-235. Deglycosylation by glycosidases depressed the subsequent phosphorylation of AD-tau (i) with cdk5 at Thr-181, Ser-199, Ser-202, Thr-205, and Ser-404, but not at Thr-212; and (ii) with GSK-3β at Thr-181, Ser-202, Thr-205, Ser-217, and Ser-404, but not at Ser-199, Thr-212, Thr-231, or Ser-396. These data suggest that aberrant glycosylation of tau in AD might be involved in neurofibrillary degeneration by promoting abnormal hyperphosphorylation by cdk5 and GSK-3β.**

Key words: Tau; Glycosylation; Phosphorylation; Cyclin-dependent kinase 5; Glycogen synthase kinase-3β; Alzheimer's disease

1. Introduction

The formation of neurofibrillary tangles (NFTs) in the brain is one of the neuropathological hallmarks of Alzheimer's disease (AD) and the number of NFTs correlates with the degree of dementia. Ultrastructurally, NFTs are composed of bundles of paired helical filaments (PHFs), the major protein subunit of which is microtubule-associated protein tau in an abnormally hyperphosphorylated form [1–4]. Hyperphosphorylated tau lacks normal tau's activity to bind to microtubules and to stimulate their assembly [5–8]. Instead, it sequesters normal tau and other microtubule-associated proteins and disassembles microtubules [6,9–11]. The abnormal hyperphosphorylation of tau also promotes its self-assembly into PHFs [11]. Thus, elucidating the molecular mechanism by which tau becomes abnormally hyperphosphorylated is critical to understanding the pathogenesis of AD.

Tau can be phosphorylated in vitro by several protein kinases. Among them, cyclin-dependent kinase 5 (cdk5) and glycogen synthase kinase-3β (GSK-3β) are believed to be the most important kinase candidates that regulate tau phosphorylation in the brain (for review, see [12–14]). Two kinases initially purified from microtubule preparation and identified as tau protein kinase I and II are actually GSK-3β and cdk5, respectively [15,16]. These two kinases phosphorylate tau at many phosphorylation sites seen in PHFs [17–29]. Phosphorylation of tau with these kinases inhibits its ability to bind to microtubules and to promote their assembly, and facilitates its polymerization into PHFs [29–34]. However, biochemical studies demonstrated no up-regulation of these kinases in AD brain [35–38]. Thus, one of the possibilities is that abnormal hyperphosphorylation might be the result of some modification of tau that converts it into a better substrate for phosphorylation.

In addition to being abnormally hyperphosphorylated, tau in AD brain is also aberrantly glycosylated with oligosaccharides [39]. This discovery was further supported and confirmed by strong staining of PHF tangles with lectins specific to glycoproteins on tissue sections of AD brain [40] and by analyses of the structure and sugar composition of the oligosaccharides hydrolyzed from the abnormally hyperphosphorylated tau purified from AD brains [41,42]. Our recent studies suggested that the aberrant glycosylation might precede the abnormal hyperphosphorylation and facilitate the phosphorylation of tau catalyzed by cyclic AMP-dependent protein kinase [42,43]. These findings urged us to investigate whether the aberrant glycosylation affects the subsequent phosphorylation of tau by cdk5 and GSK-3β, which are among the kinases most strongly implicated in the abnormal hyperphosphorylation of tau in AD brain.

In this study, we employed phosphorylation-dependent and site-specific tau antibodies to detect the phosphorylation of human tau at each specific site. We found that cdk5 phosphorylated the largest isoform of recombinant human tau, tau_{441}, at Thr-181, Ser-199, Ser-202, Thr-205, Thr-212, Ser-214, Thr-217, Thr-231, Ser-235, Ser-396, and Thr-404. GSK-3β phosphorylated all the cdk5-catalyzed sites above except Ser-235. The aberrant glycosylation facilitated the subsequent

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Abbreviations: AD, Alzheimer's disease; AD-tau, AD non-hyperphosphorylated tau; cdk5, cyclin-dependent protein kinase 5; GSK-3β, glycogen synthase kinase-3β; NFT, neurofibrillary tangle; PHF, paired helical filament

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phosphorylation catalyzed by either cdk5 or GSK-3β at several abnormal hyperphosphorylation sites in a site-specific manner.

2. Materials and methods

2.1. Materials

The tau polyclonal antibodies 92e, R111e, R134d, and R145 were raised in rabbits, as reported previously [44–46]. Phosphorylation-dependent and site-specific tau antibodies pT^{181}, pS^{199}, pS^{202}, pT^{205}, pT^{212}, pS^{214}, pT^{217}, pT^{231}, pS^{235}, pS^{262}, pS^{396}, pS^{400}, pT^{403}, pS^{404}, pS^{409}, and pS^{413} were purchased from Biosource International (Camarillo, CA, USA). ^{125}I-labeled anti-mouse and anti-rabbit whole antibodies were from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Alkaline phosphatase-conjugated goat anti-mouse IgG and anti-rabbit IgG were from Sigma (St. Louis, MO, USA). The total glycan detection kit, peptide-N-glycosidase F (PNGase F), O-glycosidase, and sialidase were purchased from Boehringer Mannheim (Indianapolis, IN, USA). GSK-3β was from Calbiochem (San Diego, CA, USA). Recombinant cdk5 and p25 (an activator of cdk5) were expressed, purified, and reconstituted into an active holoenzyme, as described previously [47]. [γ-^{32}P]adenosine triphosphate (ATP) was bought from ICN Biomedicals (Costa Mesa, CA, USA).

The autopsied human brain tissue used for this study was obtained within 6 h postmortem and was stored at −75°C until used. Two AD brains were included in this study. They were both histopathologically confirmed and were supplied by the Brain Tissue Resource Center of McLean Hospital (Belmont, MA, USA).

2.2. Preparation of tau proteins

The largest isoform of recombinant human tau, tau_{441}, was expressed and purified from *Escherichia coli* as described previously [11]. The cytosolic non-hyperphosphorylated tau (AD-tau) was purified from AD brains as described previously [48]. Briefly, 5% homogenate was prepared from the cerebral cortex of AD cases and centrifuged at 27 000×*g* for 30 min to remove cellular debris and NFTs. The supernatant was centrifuged again at 200 000×*g* for 45 min, and the second pellet was extracted with 8 M urea at room temperature (22°C) for 60 min. The extract was centrifuged at 334 000×*g* for 45 min at 22°C, and the resultant supernatant was dialyzed and subjected to phosphocellulose (PII, Whatman) cation-exchange chromatography. AD-tau was eluted by 0.31–0.8 M NaCl. The concentration of tau in the preparations was determined by radioimmuno-dot-blot assay [42,43,49] using a mixture of three polyclonal tau antibodies (92e, R111e and R134d) as a primary antibody, and recombinant tau_{441} as a standard.

2.3. Enzymatic deglycosylation of tau

AD-tau (400 μg) was diluted to a total volume of 4.0 ml with a deglycosylation buffer containing 20 mM sodium phosphate, pH 7.2, 20 mM ethylenediamine tetraacetic acid (EDTA), and 10 mM β-mercaptoethanol, and was heated in a 95°C water bath for 20 min. After being sonicated in a bath sonicator for 20 min, the sample was divided into two equal parts of 2.0 ml each. Into one part, PNGase F (6 U/ml), O-glycosidase (7.5 mU/ml), sialidase (50 mU/ml), and a cocktail of protease inhibitors were added. Into the other half, deionized water was added instead. The two tubes were then incubated at 37°C overnight, followed by heating at 95°C and bath sonication for 10 min each. The samples were then centrifuged at 14 000×*g* for 10 min to remove the denatured enzymes. The heat-stable tau in the supernatant was concentrated from 2.0 ml to approximately 400 μl by a speed vacuum concentrator, followed by dialysis against 40 mM HEPES, pH 7.5, 10 mM β-mercaptoethanol, and 10 mM $MgCl_2$. The deglycosylated and control-treated AD-tau were stored at −20°C until used. The tau concentration of the samples was determined by a radioimmuno-dot-blot assay [49,43], and the success of the deglycosylation was examined by using the total glycan detection kit (Roche Molecular Biochemicals).

2.4. Phosphorylation of tau

The in vitro phosphorylation was carried out by incubating tau_{441} (0.2 mg/ml) at 30°C in a phosphorylation reaction mixture. For cdk5-catalyzed phosphorylation, the reaction mixture contained 40 mM HEPES (pH 7.5), 10 mM β-mercaptoethanol, 10 mM $MgCl_2$, 0.2 mM [γ-^{32}P]ATP, 6.4 μg/ml cdk5, and protease inhibitors (2 μg/ml aprotinin, 2 μg/ml pepstatin, 5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)). For GSK-3β-catalyzed phosphorylation, the mixture contained 40 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 2 mM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.2 mM [γ-^{32}P]ATP, 200 U/ml GSK-3β, and protease inhibitors. After incubation for various periods of time, the reaction was stopped, the ^{32}P-labeled tau was separated from free [γ-^{32}P]ATP by paper chromatography, and the radioactivity of tau was determined by Cerenkov counting, as described previously [50]. For detecting site-specific phosphorylation, the phosphorylation reaction was carried out with non-radioactive ATP, and the reaction was stopped by adding 1/3 volume of four-fold concentrated sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer (240 mM Tris–HCl, pH 6.8, 8% SDS, 20% β-mercaptoethanol, 40% glycerol, and 0.08% bromophenol blue), followed by heating in boiling water for 5 min. The phosphorylation of tau at each specific site was detected by Western blots with various phosphorylation-dependent and site-specific tau antibodies (at a dilution of 1:1000), as described previously [51]. A phosphorylation-independent tau antibody, R134d (1:5000), was also used to detect total tau.

The phosphorylation of AD-tau with and without prior deglycosylation was carried out using the same conditions as described above for tau_{441}, except that 0.4 mg/ml of AD-tau was used. The reaction was terminated by heating in a boiling water bath for 5 min. After the mixture was cooled down, the phosphorylation of AD-tau at each specific site was measured by using a radioimmuno-dot-blot assay, as described [43].

3. Results and discussion

Several studies have reported in vitro phosphorylation of tau by cdk5 and GSK-3β at various sites as detected with different methods [17–29]. To study the effect of glycosylation on site-specific phosphorylation of tau catalyzed by cdk5 and GSK-3β, we first needed to ensure that the phosphorylation at each of these sites could be detected and quantitatively measured under the conditions used. For this purpose, we phosphorylated tau_{441} in vitro with these two kinases. We found that under the phosphorylation conditions used, cdk5 and GSK-3β each phosphorylated tau to a stoichiometry of approximately 1.5 mol Pi/mol tau during 3 h (Fig. 1A, B). These results are consistent with those reported previously [26,27,52]. Since tau is phosphorylated at multiple sites by either of these kinases, the stoichiometric phosphorylation of tau shown in Fig. 1A, B should be a sum of sub-stoichiometric phosphorylation of tau at these sites. We further examined the phosphorylation of tau at each specific site by Western blots developed with various phosphorylation-dependent and site-specific antibodies. None of these antibodies stained tau_{441} prior to the in vitro phosphorylation (Fig. 1C, D, left lanes), although the presence of tau was confirmed with the phosphorylation-independent antibody R134d. After incubation with cdk5 or GSK-3β for 3 h (right lanes), tau was recognized by several of these antibodies, indicating phosphorylation at the sites recognized by these antibodies. On the basis of immunostaining of tau with these antibodies after incubation with cdk5 or GSK-3β, we summarized the phosphorylation sites of tau with these kinases and compared our results with those reported in the literature (Table 1). By means of amino acid sequencing, mass spectrometry, and Western blots developed with phosphorylation-dependent antibodies, up to 29 phosphorylation sites of PHF-tau have been identified to date (see Table 1). Many of these tau sites can be phosphorylated in vitro with cdk5 or GSK-3β. Regarding the site-specific phosphorylation with cdk5 and GSK-3β,

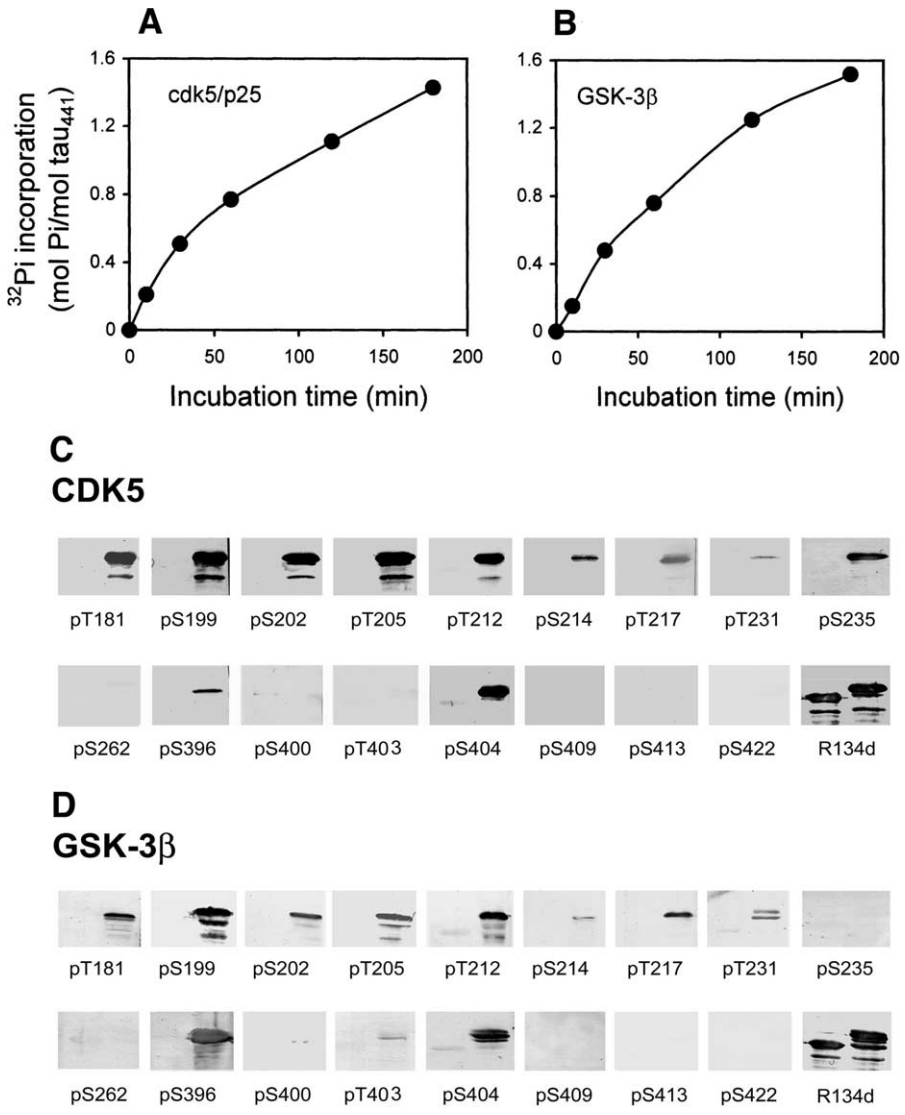

Fig. 1. Cdk5 and GSK-3β phosphorylate tau at multiple sites. The largest isoform of recombinant human brain tau, tau$_{441}$, was incubated at 30°C in a phosphorylation reaction mixture containing [γ-^{32}P]ATP and either cdk5/p25 (A) or GSK-3β (B). After various periods of incubation, the reaction was stopped, the ^{32}P incorporated into tau was separated from the free [γ-^{32}P]ATP by paper chromatography, and the radioactivity was determined by Cerenkov counting. C and D: tau$_{441}$ phosphorylated with cdk5/p25 (C) or GSK-3β (D) for 3 h, as described above, except that the [γ-^{32}P]ATP was replaced with non-radioactive ATP. The phosphorylation of tau at each specific site after incubation with (right lanes) or without (left lanes) the kinase was detected by Western blots (375 ng tau/lane) developed with phosphorylation-dependent and site-specific tau antibodies. These antibodies include pT^{181}, pS^{199}, pS^{202}, pT^{205}, pT^{212}, pS^{214}, pT^{217}, pT^{231}, pS^{235}, pS^{262}, pS^{396}, pS^{400}, pT^{403}, pS^{404}, pS^{409}, pS^{413}, and R145d for pS^{422}. A phosphorylation-independent antibody, R134d, was employed for detecting both phosphorylated and unphosphorylated tau. Cdk5 phosphorylated tau$_{441}$ at Thr-181, Ser-199, Ser-202, Thr-205, Thr-212, Ser-214, Thr-217, Thr-231, Ser-235, Ser-396, and Ser-404, but not at Ser-262, Ser-400, Thr-403, Ser-409, Ser-413, or Ser-422; and GSK-3β phosphorylated all the above cdk5-catalyzed sites except Ser-235. The phosphorylation by either kinase also produced an up-shift of gel mobility of tau$_{441}$. Not shown here are positive stainings of PHF-tau with antibodies pS^{262}, pS^{400}, pT^{403}, pS^{409}, pS^{413}, and R145.

our results were consistent with those reported previously, except that Ser-235, Ser-262, and Ser-400 were not phosphorylated by GSK-3β under our conditions. The in vitro phosphorylation of tau at these sites by GSK-3β can only be obtained in the presence of heparin or after prephosphorylation of tau by cyclic AMP-dependent protein kinase [21,23,26,27]. We did not prephosphorylate tau with cyclic AMP-dependent protein kinase or include heparin in the phosphorylation reaction mixture.

Fig. 1C indicates very strong immunoreactivity of tau phosphorylated by cdk5 with antibodies against phosphorylated Thr-181, Ser-199, Ser-202, Thr-205, Thr-212, and Ser-404. Hence, we developed a radioimmuno-dot-blot assay with these six antibodies to measure the cdk5-catalyzed phosphorylation of tau at each of the six phosphorylation sites. This assay was used to study the effect of aberrant glycosylation on the cdk5-catalyzed phosphorylation at each of these sites. For this purpose, we used AD-tau purified from AD brain as a substrate because it is aberrantly glycosylated but not hyperphosphorylated [42]. We first removed glycans from AD-tau by treatment with glycosidases. The deglycosylated and control-treated AD-tau were then used as substrates in parallel for in vitro phosphorylation with cdk5. We found that the deglycosylated AD-tau was phosphorylated by cdk5 at Thr-

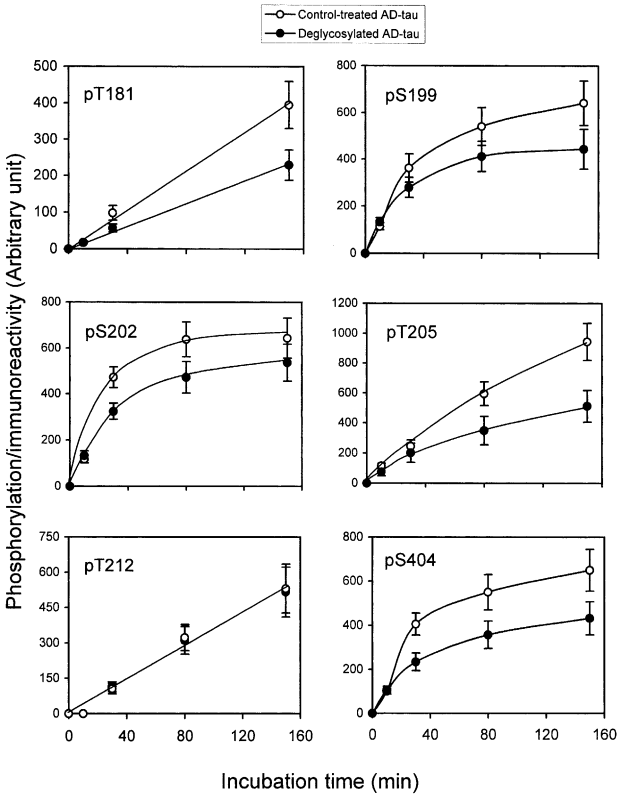

Fig. 2. Effect of deglycosylation on phosphorylation of tau with cdk5. AD-tau was deglycosylated by PNGase F, O-glycosidase, and sialidase overnight at 37°C. The deglycosylated (●) and control-treated AD-tau (○) was incubated with recombinant cdk5/p25 at 30°C for various periods. The phosphorylation of tau at each site was detected by a radioimmuno-dot-blot assay using pT^{181}, pS^{199}, pS^{202}, pT^{205}, pT^{212}, and pS^{404} as primary antibodies, and ^{125}I-labeled anti-rabbit whole antibody as secondary antibody. The error bar represents the S.D. of the three individual experiments. Compared with the control-treated AD-tau, the deglycosylated AD-tau was phosphorylated by cdk5 at a slower rate and to a smaller extent at Thr-181, Ser-199, Ser-202, Thr-205, and Ser-404, but not at Thr-212.

181, Ser-199, Ser-202, Thr-205 and Ser-404 at a slower rate and to a smaller extent than the control-treated AD-tau (Fig. 2). However, the deglycosylation did not affect the cdk5-catalyzed phosphorylation of tau at Thr-212. These results suggest that the aberrant glycosylation may facilitate phosphorylation of tau by cdk5 at selective sites.

The effect of glycosylation on the site-specific phosphorylation catalyzed by GSK-3β was similarly studied. We found that the deglycosylated AD-tau was phosphorylated by GSK-3β at Thr-181, Ser-202, Thr-205, Thr-217, and Ser-404 at a slower rate and to a smaller extent than the control-treated AD-tau (Fig. 3), whereas the phosphorylation of AD-tau at Ser-199, Thr-212, Thr-231, or Ser-396 by GSK-3β was not significantly affected by deglycosylation. These data suggest that the aberrant glycosylation differentially modulates GSK-3β-catalyzed phosphorylation in a site-specific manner, facilitating phosphorylation of tau at some of the sites.

Tau in AD brain is mainly N-glycosylated via N-linkage of the oligosaccharides at asparagine residues [39,41,42]. Unlike O-linked glycosylation that occupies the hydroxyl groups of serine/threonine residues and makes them unavailable for subsequent phosphorylation, the aberrant N-glycosylation of tau

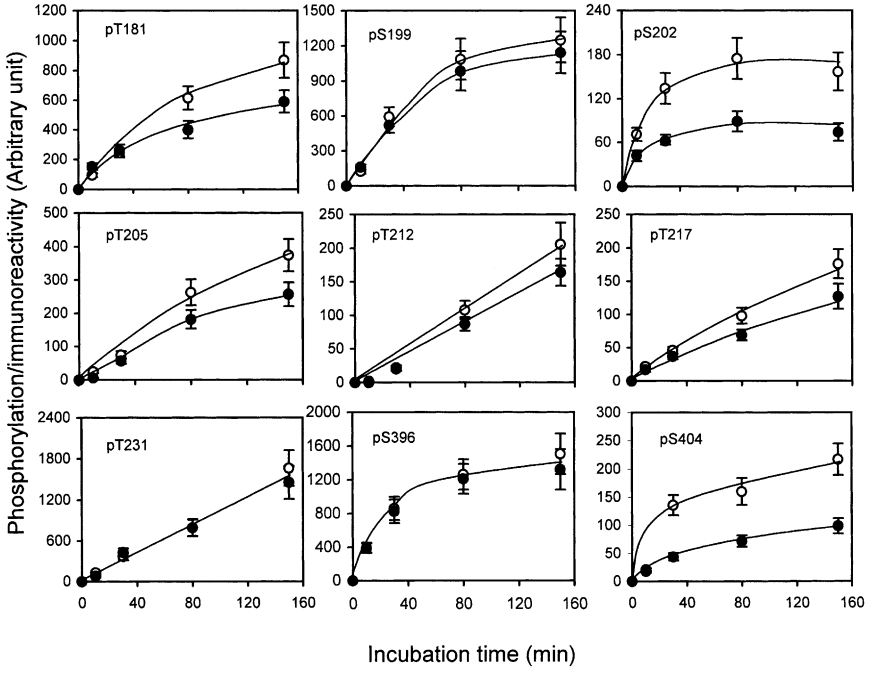

Fig. 3. Effect of deglycosylation on phosphorylation of tau with GSK-3β. The deglycosylated (●) and control-treated AD-tau (○) was incubated with GSK-3β at 30°C for various periods. Then, the phosphorylation of tau at specific sites was determined by radioimmuno-dot-blot assay, as in Fig. 2. The error bar represents the S.D. of the three individual experiments. The deglycosylation depressed phosphorylation of AD-tau with GSK-3β at Thr-181, Ser-202, Thr-205, Thr-217, and Ser-404, but had no significant effect on the phosphorylation at Ser-199, Thr-212, Thr-231, and Ser-396.

Table 1
Sites phosphorylated in PHF-tau and in normal tau in vitro phosphorylated by cdk5 or GSK-3β

Site	PHF-tau	Recombinant tau_{441}			
		Cdk5		GSK-3β	
		Reported	Present study	Reported	Present study
Ser-46	*		nd	*	nd
Thr-50			nd	*	nd
Thr-123	*		nd		nd
Ser-137	*		nd		nd
Thr-175	*		nd		nd
Thr-181	*	*	+	*	+
Ser-184			nd	*	nd
Ser-195		*	nd	*	nd
Ser-198	*		nd	*	nd
Ser-199	*	*	+	*	+
Ser-202	*	*	+	*	+
Thr-205	*	*	+	*	+
Ser-208	*		nd		nd
Ser-210	*		nd		nd
Thr-212	*	*	+	*	+
Ser-214	*	*	+		−/+
Thr-217	*	*	+	*	+
Thr-231	*	*	+	*	+
Ser-235	*	*	+	*[a]	−
Ser-237	*		nd		nd
Ser-238	*		nd		nd
Ser-262	*		−	*[a]	−
Ser-356	*		nd	*	nd
Thr-373		*	nd		nd
Ser-396	*	*	+	*	+
Ser-400	*		−	*[a]	−
Thr-403	*		−	*	−/+
Thr-404	*	*	+	*	+
Ser-409	*		−		−
Ser-412	*		nd		nd
Ser-413	*		−		−
Ser-422	*		−		−
References	[12,13,53,54]	[17,19, 20,25,29]		[12,18,21,22,26–28]	

*, identified in previous studies; +, phosphorylated; −, not phosphorylated; −/+, minimally phosphorylated; nd, not determined.
[a]Phosphorylation was obtained only in the presence of heparin or after prephosphorylation with cyclic AMP-dependent protein kinase.

in AD appears to make it a more favorable substrate for phosphorylation by cdk5 and GSK-3β at several but not all sites. Interestingly, the aberrant glycosylation did not inhibit the phosphorylation of tau by cdk5 or GSK-3β at any of the sites examined in the present study.

Because abnormal hyperphosphorylation of tau is pivotal to neurofibrillary degeneration in AD and other tauopathies, elucidating factors that modulate tau phosphorylation is significant. Our finding that aberrant glycosylation positively modulates phosphorylation of tau by cdk5, GSK-3β (this study) and cyclic AMP-dependent protein kinase [43] explains the possible role of aberrant glycosylation in neurofibrillary degeneration. The inhibition of aberrant glycosylation of tau might be a promising target to inhibit the abnormal hyperphosphorylation of tau and, consequently, the neurofibrillary degeneration.

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