Role of glycosylation in hyperphosphorylation of tau in Alzheimer's disease

Fei Liu[a], Tanweer Zaidi[a], Khalid Iqbal[a], Inge Grundke-Iqbal[a], Roberta K. Merkle[b], Cheng-Xin Gong[a,*]

[a] *Department of Neurochemistry, New York State Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, NY 10314, USA*

[b] *Complex Carbohydrate Research Center, the University of Georgia, 220 Riverbend Road, Athens, GA 30602, USA*

Received 3 December 2001; revised 18 December 2001; accepted 19 December 2001

First published online 11 January 2002

Edited by Jesus Avila

Abstract In Alzheimer's disease (AD) brain, microtubule-associated protein tau is abnormally modified by hyperphosphorylation and glycosylation, and is aggregated as neurofibrillary tangles of paired helical filaments. To investigate the role of tau glycosylation in neurofibrillary pathology, we isolated various pools of tau protein from AD brain which represent different stages of tau pathology. We found that the non-hyperphosphorylated tau from AD brain but not normal brain tau was glycosylated. Monosaccharide composition analyses and specific lectin blots suggested that the tau in AD brain was glycosylated mainly through *N*-linkage. In vitro phosphorylation indicated that the glycosylated tau was a better substrate for cAMP-dependent protein kinase than the deglycosylated tau. These results suggest that the glycosylation of tau is an early abnormality that can facilitate the subsequent abnormal hyperphosphorylation of tau in AD brain.

Key words: Tau; Glycosylation; Phosphorylation; Alzheimer's disease; Neurofibrillary degeneration; Lectin

1. Introduction

Tau protein is one of the major microtubule-associated proteins in neurons. Its normal function is to promote microtubule assembly and stabilize microtubules. In Alzheimer's disease (AD) brain, tau is abnormally hyperphosphorylated (AD P-tau) and aggregates into paired helical filaments (PHF) in affected neurons [1–4]. Accumulation of PHF in the neuronal cell bodies as neurofibrillary tangles (NFT) is one of the histopathological hallmarks of AD. The number of NFT in the brain correlates directly with the degree of dementia in AD patients [5–7]. In addition to PHF, there is a pool of AD P-tau in AD brain that is not aggregated into PHF [8]. Unlike normal tau (N-tau) isolated from normal human brain tissue, both PHF-tau and AD P-tau are unable to bind to microtubules and promote their assembly. This ability is, however, restored after the proteins are dephosphorylated with phosphatase [9–12]. In addition, AD brain also contains normal level of non-hyperphosphorylated tau (AD-tau) [13]. Unlike AD P-tau or PHF-tau, AD-tau has normal activity to promote microtubule assembly in vitro [9].

We have shown that unlike N-tau, AD P-tau and PHF-tau are glycosylated besides abnormal hyperphosphorylation [12]. The glycosylation of PHF in AD brain was also confirmed immunohistochemically by lectin staining [14]. The carbohydrate structures of isolated AD P-tau and PHF-tau have recently been identified [15]. This aberrant glycosylation of AD P-tau and PHF-tau is different from modification with *O*-linked *N*-acetyl-D-glucosamine (*O*-GlcNAcylation), a novel type of glycosylation recently found in bovine tau (B-tau) [16].

Post-translational modification by either glycosylation or phosphorylation usually alters the protein confirmation, and may consequently change its susceptibility to another modification and its biological activity. In the present study, we have investigated the nature and the temporal sequence of the abnormal glycosylation and the abnormal hyperphosphorylation of tau in AD. We found that the tau glycosylation is an early abnormality of neurofibrillary degeneration in AD, and that the glycosylation of tau might facilitate its abnormal hyperphosphorylation in AD brain.

2. Materials and methods

2.1. Materials

The polyclonal antibodies 92e to bovine brain tau and R134d to recombinant human brain tau were raised as previously reported [17]. The monoclonal antibodies Tau-1 and PHF-1 were gifts from Dr. Lester Binder of Northwestern University (Chicago, IL, USA) and Dr. Peter Davies of Albert Einstein College of Medicine (Bronx, NY, USA), respectively. Polyclonal antibodies anti-tau[pS^{199}], anti-tau[pS^{214}], anti-tau[pS^{262}], anti-tau[pS^{396}] and anti-tau[pS^{409}] were from Biosource International (Camarillo, CA, USA). Rabbit antiserum R145d was raised against a synthetic peptide corresponding to residues 417–426 of tau with phosphorylated Ser422 [18]. Goat anti-mouse IgG or anti-rabbit IgG conjugated with alkaline phosphatase, biotin-conjugated APA, horseradish peroxidase–RCA and avidin-conjugated horseradish peroxidase were from Sigma (St. Louis, MO, USA). Total Glycan Detection kit, Glycan Differentiation kit which contains digoxigenin-coupled GNA, MAA, SNA, PNA and DSA (see Table 1), peptide-*N*-glycosidase F (PNGase F), *O*-glycosidase and sialidase were purchased from Roche Diagnostic Corp. (Indianapolis, IN, USA). The catalytic subunit of cAMP-dependent protein kinase (PKA) was obtained from Sigma (St. Louis, MO, USA). DSA-

*Corresponding author. Fax: (1)-718-494 1080.
E-mail address: cgong@ultinet.net (C.-X. Gong).

Abbreviations: AD, Alzheimer's disease; AD-tau, AD non-hyperphosphorylated tau; AD P-tau, AD abnormally phosphorylated tau; B-tau, bovine tau; GalNAc, *N*-acetyl-D-galactosamine; GlcNAc, *N*-acetyl-D-glucosamine; N-tau, normal human tau; PHF, paired helical filaments; PKA, cAMP-dependent protein kinase; PNGase F, peptide-*N*-glycosidase F

0014-5793/02/$22.00
PII: S0014-5793(02)02228-7

coupled agarose was bought from EY laboratory (San Mateo, CA, USA). Seize™ X Protein G Immunoprecipitation kit was from Pierce (Rockford, IL, USA).

Autopsied human brain tissue used for this study was obtained within 6 h post-mortem and stored in liquid nitrogen or a −80°C freezer until used. Both of the AD and age-matched non-neuropathological control brains were histopathologically confirmed and supplied by brain banks.

2.2. Preparation of tau proteins

B-tau, N-tau and AD-tau were isolated as described previously from 100 000×*g* brain extracts by 2.5% perchloric acid treatment and carboxymethyl chromatography using Millipore MemSep CM 10/10 disk [9]. AD P-tau and some preparations of AD-tau were prepared from the 27 000×*g* to 200 000×*g* fraction of AD brain homogenate with 8 M urea treatment and phosphocellulose chromatography [8]. In some experiments, AD-tau and AD P-tau were purified with Seize™ X Immunoprecipitation kit (Pierce, IL, USA) according to the manufacturer's instruction, in which Tau-1 or a mixture of anti-tau[pS^{199}] and anti-tau[pS^{396}] was chemically cross-linked with protein G conjugated to agarose beads was used as immunoadsorbent.

PHF-tau was isolated from cerebral cortex of AD brain by Iqbal's long procedure [19]. The protein was quantitated by modified Lowry assay [20]. Immunoaffinity-purified PHF-tau was prepared by using monoclonal antibody MC-1 as described previously [21].

2.3. Lectin and Western blot analysis

Tau samples were resolved by 10% SDS–polyacrylamide gel electrophoresis (PAGE) as described originally by Laemmli [22], followed by electroblotting to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) for lectin blots and Western blots.

The detection of total glycans of tau proteins on PVDF membrane was carried out according to the manufacturer's instructions (Roche Diagnostic Corp., Indianapolis, IN, USA). The nature of sugar moieties attached to the tau proteins was identified by incubation of the PVDF blots with various lectins (Table 1) which specifically bind to different sugar moieties. These lectins were pre-conjugated with digoxigenin, horseradish peroxidase, or biotin. For some experiments, we included specific sugar in the lectin solution in order to specifically block the binding of the lectin to glycoproteins by competitive inhibition. For this purpose, 0.3 M lactose was added to the APA and MAA/SNA solution, 0.5 M α-methyl mannose to the GNA solution and 0.5 M galactose to the PNA solution.

The quantity and phosphorylation state of tau on the blots were confirmed by developing the blots with various phosphorylation-independent (92e and R134d) and -dependent (Tau-1, 12E8, PHF-1 and R145) tau antibodies as described by us previously [23].

2.4. Monosaccharide composition analysis

The immunoaffinity-purified PHF-tau was hydrolyzed with 2 N trifluoroacetic acid at 100°C for 4 h for the release of neutral and amino sugars. Sialic acids were released by mild acid hydrolysis using 2 M acetic acid at 80°C for 3 h. After hydrolysis, the samples were dried using a centrifugal vacuum evaporator and were resuspended with water. The samples were then subjected to Dionex high-pH anion-exchange chromatography (HPAEC). The calibration standards (0.5, 1.0 and 2.0 nmol each of fucose, *N*-acetyl-D-galactosamine (GalNAc), GlcNAc, galactose, glucose and mannose for neutral and amino sugars; 0.25, 0.5 and 1.0 nmol of *N*-acetyl neuraminic acid for sialic acid) were hydrolyzed and analyzed in parallel with PHF-tau samples.

2.5. In vitro deglycosylation and phosphorylation of AD-tau

When preparing the substrate for PKA, AD-tau (400 μg) was first diluted to a total volume of 4.0 ml with a deglycosylation buffer containing 20 mM sodium phosphate, pH 7.2, 20 mM EDTA and 10 mM β-mercaptoethanol. The solution was heated in a 95°C water bath and then sonicated in a bath sonicator for 20 min each. The sample was then divided into two parts, 2.0 ml each. Into one part, PNGase F (6 U/ml), *O*-glycosidase (7.5 mU/ml) and sialidase (50 mU/ml) were added. Into the other half, deionized water equal to the total volume of the added enzymes was added. The two tubes were then incubated at 37°C overnight, followed by boiling and bath sonication for 10 min each. The samples were centrifuged at 14 000×*g* for 10 min to remove any insoluble materials including the denatured enzymes. The heat-stable tau was recovered from the supernatant. The latter was concentrated from 2.0 ml to approximately 400 μl by a speed vacuum concentrator, followed by dialysis against 40 mM HEPES, pH 7.2, 10 mM β-mercaptoethanol and 10 mM $MgCl_2$. The deglycosylated and control-treated AD-tau were stored at −20°C until used. The protein concentration of the samples was determined by the Modified Lowry Method [20] and the success of the deglycosylation was examined by Total Glycan Detection kit.

Two types of assay were employed for measuring the phosphorylation of AD P-tau. One was the conventional radioactive assay. AD-tau (0.4 mg/ml) with and without pre-deglycosylation by glycosidases was incubated at 30°C in a phosphorylation reaction mixture containing 40 mM HEPES (pH 6.8), 10 mM β-mercaptoethanol, 1.0 mM EGTA, 10 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP and 20 μg/ml PKA. After various periods of incubation, an aliquot of the reaction mixture was transferred onto a strip of Whatman 32 ET chromatography paper, where the same volume of 20% trichloric acid and 5 mM ATP was pre-spotted to stop the phosphorylation reaction. The incorporated ^{32}Pi was then separated from the free [γ-^{32}P]ATP by paper chromatography [24], and the radioactivities were determined by Cerenkov counting. For the second method to measure the phosphorylation of AD-tau, the phosphorylation reaction was carried out as described above except that [γ-^{32}P]ATP was replaced by non-radioactive ATP. The reaction was terminated by transferring the reaction mixture into a tube containing hot (∼90°C) Tris-buffered saline, followed by heating in a boiling water bath for 5 min. Then, the reaction product, i.e. the phosphorylated tau, was quantitated by a radioimmuno-dot-blot assay using a mixture of polyclonal antibodies anti-tau[pS^{214}], anti-tau[pS^{262}] and anti-tau[pS^{409}] (1:1000 dilution each) as primary antibody and ^{125}I-labeled anti-rabbit IgG (0.1 μg/ml) as secondary anti-

Table 1
Specificity and concentrations of lectins employed

Lectins (abbreviation)	Binding specificity	Conjugated with	Concentration used (μg/ml)
Agaricus bisporus agglutinin (ABA)	β-Gal	biotin	0.5
Abrus precatorius agglutinin (APA)	β-Gal	biotin	2.0
Concanavalin A (ConA)	α-Man, α-Glc	peroxidase	4.0
Datura stramonium agglutinin (DSA)	Galβ1,4GlcNAc and GlcNAcβ1,4GlcNAc	digoxigenin	1.0
Erythrina corallodendron agglutinin (ECA)	Galβ1,4GalNAc	biotin	2.0
Galanthus nivalis agglutinin (GNA)	α-Man	peroxidase	4.0
Lens culinaris agglutinin (LCA)	α-Man	biotin	2.0
Maackia amurensis agglutinin (MAA)	SAα2,3Gal	digoxigenin	5.0
Peanut agglutinin (PNA)	Galβ1,3GalNAc	digoxigenin	10.0
Ricinus communis agglutinin (RCA)	βGal (terminal) and Galβ1,4GlcNAc	peroxidase	5.0
Sambucus nigra agglutinin (SNA)	SAα2,6Gal	digoxigenin	1.0
Tetragonolobus purpureaqs agglutinin (TGP)	α-L-Fuc	peroxidase	20.0
Ulex europaeus agglutinin (UEA I)	α-L-Fuc	peroxidase	40.0

Fuc: fucose; Gal: galactose; Glc: glucose; Man: mannose; SA: sialic acid.

body. The radioimmunoreactivity of the blots was visualized and quantitated by using a PhosphorImager (Fujifilm BAS-1500) and TINA 2.0 software (raytest, Isotopenmeβ geräte GmbH).

3. Results

3.1. Tau glycosylation is an early abnormality of neurofibrillary degeneration in AD

Based on phosphorylation state, biological activity and solubility, tau in AD brain can be divided into three states, AD-tau, AD P-tau and PHF-tau. These three states of tau appear to represent various stages of tau pathology of AD. We, therefore, first examined the glycosylation of these three states of tau isolated from AD brain as well as tau protein isolated from normal control human brain and, as a reference, bovine brain. The quality of the tau preparations used was examined by Western blots developed with various tau antibodies (Fig. 1A–E). Antibody 92e stained all tau preparations that displayed multiple tau bands due to multiple isoforms and various post-translational modifications (Fig. 1A). As compared with N-tau (lane 2) and AD-tau (lane 3), AD P-tau (lane 4) had a slower mobility in SDS–PAGE due to the abnormal hyperphosphorylation as reported previously [8]. PHF-tau (lane 5) is well known to display as a smear due to aggregation. Antibody Tau-1, which recognizes N-tau but not hyperphosphorylated tau, only strongly stained B-tau, N-tau and AD-tau (Fig. 1B). In contrast, 12E8, PHF-1 and R145, which recognize tau only when it is phosphorylated at Ser262/Ser356, Ser396/Ser404 and Ser422, respectively [23,25,26], did not or only faintly stained AD-tau, but strongly stained AD P-tau and PHF-tau (Fig. 1C–E).

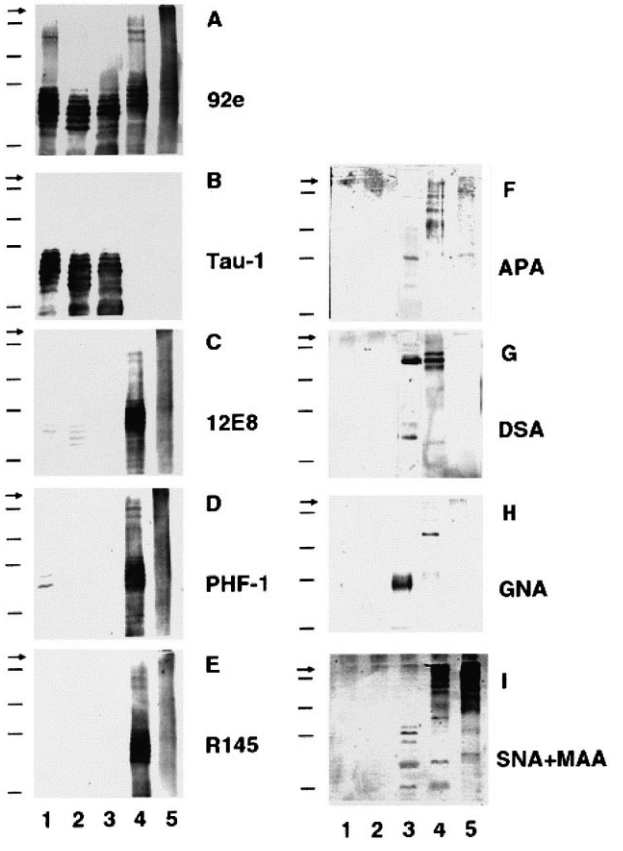

Fig. 1. Western and lectin blot analyses of various pools of tau. Isolated tau preparations (3.0 μg/lane) were separated by 10% SDS–PAGE and the polypeptides were then transferred onto PVDF membranes. The blots were developed either with tau antibodies or with lectins. Lanes 1–5 are B-tau, N-tau, AD-tau, AD P-tau and PHF-tau, respectively. Antibody 92e (A) was used to detect the total amount of tau loaded in each lane. Several phosphorylation-dependent tau antibodies (B–E) were used to verify the phosphorylation state of tau. Various lectins (F–I) were employed to identify glycosylation and the specific sugar moieties. At the left side of each blot, the arrow indicates the top of the separating gel, and the short bars indicate the position of molecular mass markers (200, 98, 67 and 43 kDa, respectively).

To identify the glycosylation state of these tau preparations, we employed various lectins to label tau transferred to PVDF membrane. These lectins specifically bind to different sugar moieties of glycoproteins (Table 1) and, therefore, are commonly used for glycoprotein detection. When incubated with the battery of lectins listed in Table 1, B-tau and N-tau were not stained (Fig. 1F–I, lanes 1–2). In contrast, AD-tau, AD P-tau and PHF-tau were all positively stained with lectins (Fig. 1F–I, lanes 3–5). Each of the tau preparations displayed different staining patterns with the lectins employed, suggesting that these different forms of tau were differentially glycosylated. Based on the known specificities of the lectins (Table 1), these data (Fig. 1F–I) suggested that all of the three pools of tau isolated from AD brain contained mannose (detected by GNA, Fig. 1H), galactose (detected by APA, Fig. 1F) and sialic acid (detected by SNA + MAA, Fig. 1I). In addition, AD-tau and AD P-tau also contained galactose-β1,3/1,4-*N*-acetylglucosamine (detected by DSA, Fig. 1G). As compared with AD P-tau, PHF-tau had much weaker staining with lectins DSA and GNA (Fig. 1G,H). To rule out the possibility of non-specific cross-reaction with these lectins, we examined these tau preparations with several other lectins that recognize the same sugar moieties as those shown in Fig. 1. These lectins include ABA, ConA, RCA and LCA (Table 1). Similar results were obtained with different lectins that recognize the same sugar moieties (data not shown). None of these five tau preparations were stained with the lectins UEA I, TGP or ECA (data not shown), suggesting that these proteins did not contain any fucose or galactose-β1,4-*N*-acetylgalactosamine.

The major lectin-positive bands as seen in Fig. 1 had higher apparent molecular weights than the most dominant tau isoforms seen on blots detected with antibody 92e to total tau. This might be because the aberrant glycosylation mainly occurs in an aggregated form of tau in AD brain or the lectin staining was due to glycoproteins other than tau present as contaminants in these tau preparations. To confirm the aberrant glycosylation of AD-tau and AD P-tau, we purified non-aggregated AD-tau and AD P-tau from three individual AD brains using Seize™ X Immunoprecipitation kit. This technique enabled purification of tau free from contaminating proteins as well as the antibody that is also a glycoprotein. The contamination of AD P-tau in these AD-tau preparations was excluded by Western blot analyses using anti-tau[pS^{199}] and anti-tau[pS^{396}], which react only with AD P-tau (Fig. 2Aa,b). Lectin staining indicated positive for AD-tau (Fig. 2Ac–f, lanes 1–3) and AD P-tau (lanes 4–6), although the staining for AD-tau was weaker than that of AD P-tau. One of the three AD-tau preparations did not display positive staining with APA, RCA or SNA/MAA (Fig. 2A, lane 1), indicating significant variations of the tau glycosylation between individual samples. These variations were most likely due to the heterogeneity of tau glycosylation among these

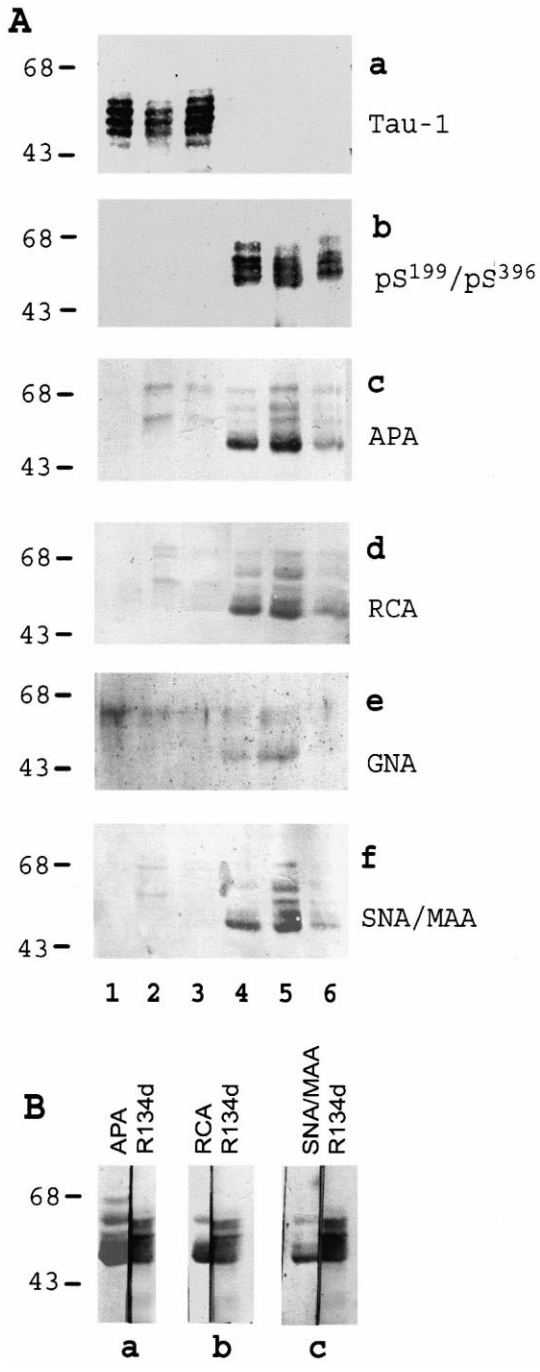

Fig. 2. Detection of glycosylation of immunoaffinity-purified AD-tau and AD P-tau. A: Immunoaffinity-purified AD-tau (lanes 1–3) and AD P-tau (lanes 4–6) from three AD brains (tau in lanes 1 and 4, 2 and 5, and 3 and 6 were from the same brain, respectively) were subjected to Western and lectin blotting. The phosphorylation-dependent antibodies Tau-1 (a) and a mixture of anti-tau[pS^{199}] and anti-tau[pS^{396}] (b) were used for detecting AD-tau and AD P-tau, respectively. The same samples were also subjected to detection for specific sugar moieties recognized by various lectins as indicated at right side of each blot (c–f). At the left of each blot, the bars indicate the positions of molecular mass markers in kDa. B: The affinity-purified AD P-tau was resolved by 10% SDS–PAGE and blotted onto PVDF membrane. Each lane of the AD P-tau was cut at the center into two strips. Half of each lane (one strip) was stained with R134d to total tau. The other half was developed with lectin APA (a), RCA (b), or a mixture of SNA and MAA (c). The molecular mass markers in kDa are indicated at the left of the blots.

individuals, which is a well-known characteristic of protein glycosylation [27,28].

We compared the lectin-positive bands with the tau antibody R134d-positive bands of the same lane and found that the lectin-positive bands matched the majority of the R134d-positive bands (Fig. 2B). When the tau blots were incubated with lectins in the presence of the specific competitive mono- or disaccharides, the lectin staining was abolished (data not shown). These results suggested that the lectin staining was the result of the specific binding of the lectins to the specific sugar moieties of tau, rather than non-specific binding of the lectins to the protein. To further confirm the glycosylation of AD-tau, we incubated AD-tau with DSA- and RCA-conjugated agarose beads, followed by Western blotting of the lectin-affinity-precipitated proteins. We found that AD-tau was affinity-precipitated by these lectins as determined with tau antibody R134d (data not shown). These data indicated that though it is not abnormally hyperphosphorylated, AD-tau is aberrantly glycosylated in AD brain.

3.2. *Tau is mainly modified by galactose, glucose, mannose, GlcNAc and sialic acid with N-linked glycosylation in AD brain*

The monosaccharide composition of the immunoaffinity-purified PHF-tau and AD P-tau was analyzed by Dionex HPAEC. We found that the PHF-tau contained galactose, glucose, mannose, GlcNAc, sialic acid and GalNAc, but not fucose moieties. Some of these monosaccharides were below the expected stoichiometry level (Table 2), indicating that only some of the PHF-tau molecules were glycosylated. This is in agreement with the lectin staining that only some of the tau polypeptides contained glycans. It is noted that the PHF-tau contained minimal GalNAc, a component of the core disaccharide of most *O*-linked oligosaccharides [29], suggesting that the oligosaccharides might be linked to tau mainly through *N*-linkage, but not *O*-linkage [30]. The lack of staining of the immunoaffinity-purified AD-tau, AD P-tau and PHF-tau by PNA (data not shown) also indicated that tau in AD brain does not contain Galβ1,3GalNAc, the core disaccharide of most *O*-linked oligosaccharides [29]. Furthermore, AD-tau could not be affinity-precipitated with PNA-conjugated agarose (data not shown). These findings together suggested that tau isolated from AD brain might mainly contain *N*-linked oligosaccharides.

3.3. *Tau phosphorylation modulates its subsequent phosphorylation*

We have investigated the pathophysiological significance of the tau glycosylation by examining its effect on tau phosphorylation. The deglycosylated AD-tau and the control-treated AD-tau were incubated in parallel with PKA in the presence

Table 2
Monosaccharide composition of PHF-tau

Monosaccharide	Stoichiometry (mol/mol of tau)
Galactose	2.9
Glucose	1.6
Mannose	0.6
GlcNAc	0.4
Sialic acid	0.2
GalNAc	0.1
Fucose	0.0

of [^{32}P]ATP and the tau phosphorylation was monitored by measuring ^{32}P incorporation to the protein. We found that the deglycosylated AD-tau was phosphorylated at a lower rate and to a smaller extent as compared with the control-treated AD-tau (Fig. 3A). During 170 min incubation, 0.9 and 1.7 mol Pi/mol of tau were incorporated in the deglycosylated and the control-treated AD-tau, respectively. Under the same conditions, recombinant human tau$_{441}$ and bovine brain tau were phosphorylated to a stoichiometry of approximately 1.2 mol Pi/mol of tau (data not shown).

We also used site-specific and phosphorylation-dependent antibodies to examine the phosphorylation of AD-tau at Ser214, Ser262/Ser356 and Ser409, which are among the six phosphorylation sites of tau catalyzed by PKA [31,32]. For this purpose, the deglycosylated and control-treated AD-tau were incubated with PKA in the presence of non-radioactive ATP and the tau phosphorylation was detected by a radioimmuno-dot-blot assay that selectively measures the phosphorylation of tau at Ser214, Ser262 and Ser409. Similar to the data observed with ^{32}P incorporation, the deglycosylated AD-tau was phosphorylated at a lower rate and to a smaller extent than the control-treated AD-tau (Fig. 3B). These results suggested that the tau glycosylation might facilitate tau phosphorylation by PKA.

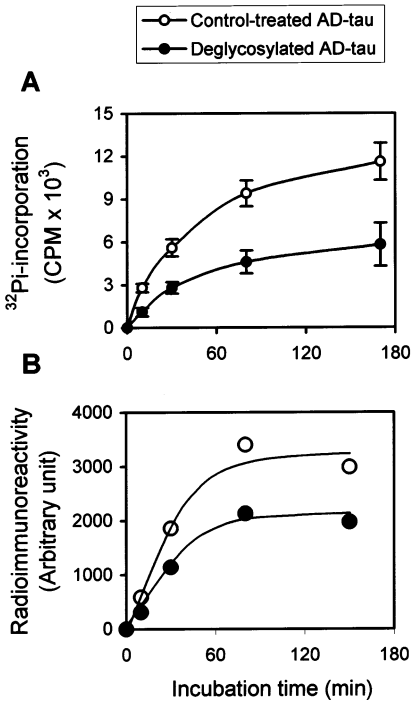

Fig. 3. Effect of deglycosylation on phosphorylation of tau by PKA. A: The deglycosylated (●) and control-treated (○) AD-taus as substrates were incubated with [γ-^{32}P]ATP and PKA in a phosphorylation buffer at 30°C for various periods. The ^{32}P incorporation was determined by counting the radioactivity of the AD-tau after removing the free [^{32}P]ATP by paper chromatography. B: The deglycosylated AD-tau (●) and the control-treated AD-tau (○) were incubated with PKA as in (A) except non-radioactive ATP was used instead of [^{32}P]ATP. The phosphorylation of tau in the reaction mixture was detected by a radioimmuno-dot-blot assay using a mixture of three phosphorylation-dependent antibodies, anti-tau[pS^{214}], anti-tau[pS^{262}] and anti-tau[pS^{409}], which detect the phosphorylation of tau at the corresponding sites. A representative result of three experiments is shown.

4. Discussion

In the present study, we have found that unlike N-tau, AD-tau, which is known to be normal with respect to its phosphorylation state and biological activity, is glycosylated. Monosaccharide composition analyses and specific lectin staining suggested that tau in AD brain is glycosylated mainly through *N*-linkage. This is in agreement with a recent report in which several *N*-linked oligosaccharides was identified in AD P-tau and PHF-tau [15]. Our data suggest that the aberrant modification of tau by glycosylation probably occurs at an early stage, i.e. before the abnormal hyperphosphorylation and loss of its biological activity. Thus, the tau pathology might begin at a much earlier stage than previously thought in AD. This finding may open a new avenue to investigate the pathogenesis of tau pathology in AD. Since AD-tau is active in binding to microtubules and stimulating microtubule assembly [9], the glycosylation of tau does not appear to directly affect tau's biological activity. However, our results suggest that the tau glycosylation facilitates PKA-catalyzed tau phosphorylation. PKA is one of the tau kinases [31,33,34] and the phosphorylation of tau by PKA accelerates subsequent Alzheimer-like hyperphosphorylation of tau catalyzed by glycogen synthase kinase-3β [35,36]. According to the present findings, we hypothesize that tau glycosylation is an early abnormality that facilitates its abnormal hyperphosphorylation in AD. It has been reported that the activity of the major tau phosphatase, protein phosphatase 2A [23,37,38], is decreased in AD brain as compared with age-matched controls [39,40]. Hence, the abnormal hyperphosphorylation of tau in AD brain might be the consequence of multiple metabolic abnormalities including the imbalance of the protein kinase and phosphatase activities, and the aberrant tau glycosylation. In addition, the aberrant glycosylation may coordinate with hyperphosphorylation to facilitate the aggregation of tau into PHF or stabilize the filamentous structure of PHF. Enzymatic deglycosylation of PHF was shown to untwist PHF into straight filaments 2.5 ± 0.5 nm in diameter [12].

We have noticed that AD P-tau appears to be glycosylated to a higher extent than AD-tau (Figs. 1 and 2). The more glycans in AD P-tau might partially contribute to the hyperphosphorylation of these tau molecules. The glycosylated tau is more difficult to be dephosphorylated in vitro by protein phosphatase 2A (unpublished observation). We also noticed the aberrant glycosylation on only some of the tau molecules and the individual variation. Thus, glycosylation might promote but might not be required for the abnormal hyperphosphorylation of tau. A recent study has shown that in vitro abnormal hyperphosphorylation of recombinant tau is sufficient to cause its self-assembly into PHF [41]. The various extents and sugar compositions of the aberrant glycosylation might affect the subsequent phosphorylation of tau differently. A detailed kinetic study remains to be carried out to elucidate the site- and sugar-specific correlation between glycosylation and phosphorylation of tau.

Previously, it was shown that B-tau is modified by a monosaccharide, GlcNAc, via GlcNAcylation [16]. Whether human tau is also GlcNAcylated is not yet known. GlcNAcylation is a special type of dynamic glycosylation that is different from classical protein glycosylation. The latter normally occurs in the rough endoplasmic reticulum and Golgi apparatus, and is restricted to membrane-bound and secreted proteins. Tau is a

cytosolic protein and does not contain the signal peptides required for entering the endoplasmic reticulum. It is currently not known how tau is glycosylated. A possibility is that glycosyltransferases that transfer sugar moieties to proteins leak from the endoplasmic reticulum and Golgi apparatus into cytoplasmic compartment of the affected neurons in AD brain. Abnormalities of these subcellular organelles of neurons have been reported in AD [42]. Alternatively, glycosylation of tau in AD brain might be a consequence of the association of tau protein with rough endoplasmic reticulum and plasma membrane [43,44], and abnormalities of membrane phospholipids [45] and membrane fluidity [46] have been described in AD brain. Due to one or more of these anomalies, tau might become accessible to specific glycosyltransferases and be glycosylated. Aberrations in the molecular topology of neuronal membranes might be involved in the pathogenesis of AD [47].

Acknowledgements: We express our sincere thanks to Dr. Peter Davies (Albert Einstein College of Medicine, Bronx, NY, USA) for providing immunoaffinity-purified PHF-tau and monoclonal antibody PHF-1, and to Dr. Lester Binder (Northwestern University, Chicago, IL, USA) for providing monoclonal antibody Tau-1. Autopsied brain specimens were provided by the Brain Tissue Resources Center (PHS Grant MH/NS 31862), McLean Hospital, Belmont, MA, USA, and by our Institute Brain Bank (Dr. Peter Koslowski). This work was supported in part by the New York State Office of Mental Retardation and Developmental Disabilities, NIH Grants AG16760 (to C.-X.G.), AG05892 (to K.I.), AG08076 (to K.I.), NS18105 (to I.G.-I.) and 2-P41-RR05351-06 (to the Complex Carbohydrate Research Center, University of Georgia).

References

[1] Grundke-Iqbal, I., Iqbal, K., Quinlan, M., Tung, Y.C., Zaidi, T. and Wisniewski, H.M. (1986) J. Biol. Chem. 261, 6084–6089.
[2] Grundke-Iqbal, I., Iqbal, K., Tung, Y.C., Quinlan, M., Wisniewski, H.M. and Binder, L.I. (1986) Proc. Natl. Acad. Sci. USA 83, 4913–4917.
[3] Iqbal, K., Smith, A.J., Zaidi, T. and Grundke-Iqbal, I. (1989) FEBS Lett. 248, 87–91.
[4] Lee, V.M.Y., Balin, B.J., Otvos, L. and Trojanowski, J.Q. (1991) Science 251, 675–678.
[5] Tomlinson, B.E., Blessed, G. and Roth, M. (1970) J. Neurol. Sci. 11, 205–242.
[6] Alafuzoff, I., Iqbal, K., Friden, H., Adolfsson, R. and Winblad, B. (1987) Acta Neuropathol. (Berl.) 74, 209–225.
[7] Dickson, D.W., Farlo, J., Davies, P., Crystal, H., Fuld, P. and Yen, S.H. (1988) Am. J. Pathol. 132, 86–101.
[8] Köpke, E., Tung, Y.C., Shaikh, S., del Alonso, A.C., Iqbal, K. and Grundke-Iqbal, I. (1993) J. Biol. Chem. 268, 24374–24384.
[9] del Alonso, A.C., Zaidi, T., Grundke-Iqbal, I. and Iqbal, K. (1994) Proc. Natl. Acad. Sci. USA 91, 5562–5566.
[10] Iqbal, K., Zaidi, T., Bancher, C. and Grundke-Iqbal, I. (1994) FEBS Lett. 349, 104–108.
[11] Wang, J.Z., Gong, C.-X., Zaidi, T., Grundke-Iqbal, I. and Iqbal, K. (1995) J. Biol. Chem. 270, 4854–4860.
[12] Wang, J.Z., Grundke-Iqbal, I. and Iqbal, K. (1996) Nat. Med. 2, 871–875.
[13] Khatoon, S., Grundke-Iqbal, I. and Iqbal, K. (1992) J. Neurochem. 59, 750–753.
[14] Takahashi, M., Tsujioka, Y., Yamada, T., Tsuboi, Y., Okada, H., Yamamoto, T. and Liposits, Z. (1999) Acta Neuropathol. 97, 635–641.
[15] Sato, Y., Naito, Y., Grundke-Iqbal, I., Iqbal, K. and Endo, T. (2001) FEBS Lett. 496, 152–160.
[16] Arnold, C.S., Johnson, G.V.W., Cole, R.N., Dong, D.L.-Y., Lee, M. and Hart, G.W. (1996) J. Biol. Chem. 271, 28741–28744.
[17] Grundke-Iqbal, I., Vorbrodt, A.W., Iqbal, K., Tung, Y.C., Wang, G.P. and Wisniewski, H.M. (1988) Mol. Brain. Res. 4, 43–52.
[18] Tanaka, T., Zhong, J., Iqbal, K., Trenker, E. and Grundke-Iqabal, I. (1998) FEBS Lett. 426, 248–254.
[19] Iqbal, K., Zaidi, T., Thompson, C.H., Merz, P.A. and Wisniewski, H.M. (1984) Acta Neuropathol. (Berl.) 62, 167–177.
[20] Bensadoun, A. and Weinstein, D. (1976) Anal. Biochem. 70, 241–250.
[21] Jicha, G.A., O'Donnell, A., Weaver, C., Angeletti, R. and Davies, P. (1999) J. Neurochem. 72, 214–224.
[22] Laemmli, U.K. (1970) Nature 227, 680–685.
[23] Gong, C.-X., Lidsky, T., Wegiel, J., Zuck, L., Grundke-Iqbal, I. and Iqbal, K. (2000) J. Biol. Chem. 275, 5535–5544.
[24] Huang, K.P. and Robinson, J.C. (1976) Anal. Biochem. 72, 593–599.
[25] Seubert, P., Mawal-Dewan, M., Barbour, R., Jakes, R., Goedert, M., Johnson, G.V., Litersky, J.M., Schenk, D., Leiberburg, I., Trojanowski, J.Q. and Lee, V.M. (1995) J. Biol. Chem. 270, 18917–18922.
[26] Otvos Jr., L., Feiner, L., Lang, E., Szendrei, G.I., Goedert, M. and Lee, V.M. (1994) J. Neurosci. Res. 39, 669–673.
[27] Rademacher, T.W., Parekh, R.B. and Dwek, R.A. (1988) Annu. Rev. Biochem. 57, 785–838.
[28] Dwek, R.A. (1995) Biochem. Soc. Trans. 23, 1–25.
[29] Kobata, A. (1992) Eur. J. Biochem. 209, 483–501.
[30] Cummings, R.D. (1992) in: Glycoconjugates: Composition, Structure and Function (Allen, H.J. and Kisailus, E.C., Eds.), pp. 333–360, Marcel Dekker Inc., New York.
[31] Scott, C.W., Spreen, R.C., Herman, J.L., Chow, F.P., Davison, M.D., Young, J. and Caputo, C.B. (1993) J. Biol. Chem. 268, 1166–1173.
[32] Litersky, J.M., Johnson, G.V., Jakes, R., Goedert, M., Lee, M. and Seubert, P. (1996) Biochem. J. 316, 655–660.
[33] Johnson, G.V.W. (1992) J. Neurochem. 59, 2056–2062.
[34] Jicha, G.A., Weaver, C., Lane, E., Vianna, C., Kress, Y., Rockwood, J. and Davies, P. (1999) J. Neurosci. 19, 7486–7494.
[35] Singh, T.J., Haque, N., Grundke-Iqbal, I. and Iqbal, K. (1995) FEBS Lett. 358, 267–272.
[36] Wang, J.-Z., Wu, Q., Smith, A., Grundke-Iqbal, I. and Iqbal, K. (1998) FEBS Lett. 426, 28–34.
[37] Goedert, M., Cohen, E.S., Jakes, R. and Cohen, P. (1992) FEBS Lett. 312, 95–99.
[38] Gong, C.-X., Grundke-Iqbal, I. and Iqbal, K. (1994) Neuroscience 61, 765–772.
[39] Gong, C.-X., Singh, T.J., Grundke-Iqbal, I. and Iqbal, K. (1993) J. Neurochem. 61, 921–927.
[40] Gong, C.-X., Shaikh, S., Wang, J.-Z., Zaidi, T., Grundke-Iqbal, I. and Iqbal, K. (1995) J. Neurochem. 65, 732–738.
[41] del Alonso, A.C., Zaidi, T., Novak, M., Grundke-Iqbal, I. and Iqbal, K. (2001) Proc. Natl. Acad. Sci. USA 98, 6923–6928.
[42] Gray, E.G., Paula-Barbosa, M. and Roher, A. (1987) Neuropathol. Appl. Neurobiol. 13, 91–110.
[43] Papasozomenos, S.C. (1989) Lab. Invest. 60, 375–389.
[44] Brandt, R., Léger, J. and Lee, G. (1995) J. Cell. Biol. 131, 1327–1340.
[45] Mason, R.P., Trumbore, M.W. and Pettegrew, J.W. (1995) Neurobiol. Aging 16, 531–539.
[46] Zubenko, G.S., Wusylko, M., Cohen, B.M., Boller, F. and Teply, I. (1987) Science 238, 539–542.
[47] Iqbal, K., Grundke-Iqbal, I. and Iqbal, K. (2000) Neurosci. News 3, 14–20.