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Glycogen synthase kinase 3β is identical to tau protein kinase I generating several epitopes of paired helical filaments

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We previously reported that tau protein kinase I (TPKI) induced normal tau protein into a state of paired helical filaments (PHF); this is further confirmed here by immunoblot analysis using several antibodies. We also present the amino acid sequence of TPKI, which is identical to glycogen synthase kinase 3β (GSK3β). Moreover, we found that TPKI activity was inseparable from GSK3 activity throughout the purification procedure. These results indicate that TPKI is identical to GSK3β.

Tau protein; Protein kinase; Phosphorylation; Paired helical filament; Alzheimer's disease; Glycogen synthase kinase 3β

1. INTRODUCTION

Paired helical filaments (PHF) are one of the hallmarks in the brain of Alzheimer's disease patients. The major component of PHF is abnormally phosphorylated tau. Identification of the kinase responsible for this abnormal phosphorylation is important for an understanding of the pathological change. We were the first to find a protein kinase activity generating a PHF epitope on tau from microtubule proteins of bovine brain [1]. The epitope was detected with anti-ptau 1 [2]. The protein kinase was independent of well-known second messengers, but tubulin stimulated the phosphorylation of tau by the kinase. The kinase activity was associated with tau, and the best substrates in brain extract were tau and MAP2. According to these properties relating to tau, this enzyme was named tau protein kinase (TPK) (EC 2.7.1.135) [3]. Further purification revealed that the TPK fraction contained two kinases, TPKI and TPKII, and that TPKI generated the PHF epitope [4]. Phosphorylation of tau by TPKI was accelerated by prior phosphorylation by TPKII, suggesting that TPKII indirectly regulates the epitope's formation [5]. We determined the phosphorylation sites on tau by both kinases, and found that these sites are in good agreement with those of PHF-tau [6]. Our immunochemical analyses suggested that TPKII works in normal brain [5]. On the other hand, the PHF epitope recognized by anti-ptau 1 and generated by TPKI was never detected in normal tau, suggesting that TPKI is a candidate for the kinase leading to the conversion of tau to a PHF-like state. To confirm this working hypothesis, we have to prove that TPKI also induces some other immunoreactivities characteristic of PHF. We present here a result strengthening this hypothesis.

Other groups have reported that mitogen-activated protein (MAP) kinase [7,8], cdc2 kinase [8,9] and glycogen synthase kinase 3 (GSK3) [10,11] are also candidates for the kinase inducing tau into the PHF-like state. Particularly, GSK3 exhibits a similar hierarchal phosphorylation process to TPKI [12,13]. In order to answer the question of whether or not TPKI is identical to these kinases, determination of the sequence of TPKI is required. Here, we report that the TPKI sequence is identical to that of GSK3β, which is derived from one of two classes of rat brain cDNA for GSK3, but not MAP kinase or cdc2 kinase. Furthermore, TPKI activity is inseparable from GSK3 activity throughout purification procedues. We conclude that TPKI is identical to GSK3β.

2. MATERIALS AND METHODS

2.1. *Materials*

TPKI, TPKII and tau were purified by the method described previously [4]. GSK3β was purified by the method of Woodgett [14], followed by gel filtration with G3000 SW (Tosoh). Casein kinase II was purified according to the method described by Cohen et al. [15]. *Xenopus* MAP kinase [16] was a kind gift from Dr. Nishida (University of Tokyo). The kinase has 95% sequence homology to mammalian MAP kinase, ERK2 [17]. Glycogen synthase was purchased from Sigma.

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Peptides named GS-1 and K2 were synthesized by the solid phase method, using a Biosearch model 9500 peptide synthesizer. The sequence of GS-1 was described in [18]. The sequence of K1, K2 and K3 corresponded to amino acid numbers 226–240, 189–224 and 396–439 of the longest human tau [19], respectively.

Anti-tau was purchased from Sigma. Monoclonal antibody, tau-1 was from Boehringer-Mannheim. Three monoclonal antibodies against neurofilament SMI31, SMI33 and SMI34 [20], were from Sternberger monoclonals Inc. A polyclonal antibody against PHF, anti-ptau 1 [2], was a kind gift from Dr. Ihara (University of Tokyo).

2.2. *Phosphorylation of peptides*

For the preparation of a substrate for TPKI assay, K2 was fully phosphorylated by TPKII and unlabelled ATP in a reaction medium described previously [4]. Then the solution was heated at 95°C for 5 min to inactivate TPKII. For TPKI assay, the phosphorylated K2 (p-K2) (0.12 mg/ml) was phosphorylated by TPKI and [γ-^{32}P]ATP (0.4 mM, 15 mBq/ml) The assay solution was soaked onto P81 paper, and the paper was washed four times in 1% phosphoric acid and then once in acetone, and was dried. The remaining radioactivity was counted by liquid scintillation counter. For GSK3 assay, we used GS-1 phosphorylated by casein kinase II as a substrate [18].

2.3. *Sequencing*

TPKI was digested by endoproteinase Lys-C (Boehringer-Mannheim) at a protein ratio of 20:1. The peptides were separated by reverse-phase column (C8) chromatography. The amino acid sequences were determined by a pulse-liquid-phase amino acid sequencer (Applied Biosystems 477A protein sequencer).

Bovine cerebrum cDNA was synthesized from poly(A)$^+$ RNA prepared from bovine cerebrum with a cDNA synthesis kit (Pharmacia) PCR amplification of an oligonucleotide primer corresponding to peptide D was carried out by GeneAmp DNA amplification reagent kit (Perkin-Elmer Cetus) using the bovine cerebrum cDNA as a template, and cDNA of rat TPKI was cloned from a λgt11 cDNA library (Clontech) using the PCR product as a probe. DNA was sequenced by dideoxy-mediated sequencing [21].

3. RESULTS

Previously, we reported that tau phosphorylated by TPKI had at least one PHF epitope [4]. It was reported that there are some other epitope changes characteristic of PHF. We investigated the other epitope changes accompanying the phosphorylation (Fig. 1). We also observed epitope changes induced by TPKII (a cdc2-like kinase; see section 4) and MAP kinase, since cdc2 kinase and MAP kinase have previously been suggested as other candidates for PHF formation [7–9]. Neurofilament antibodies SMI33, SMI34 and SMI31 are known to discriminate between tau and PHF. After phosphorylation of tau by TPKI, epitope SMI33 disappeared, epitope SMI34 increased, and epitope SMI31 appeared. These changes are expected for conversion of tau into PHF [20]. We have already reported that TPKI phosphorylated Ser-199, Thr-231, Ser-396 and Ser-413 [22] in the numbering of the longest human tau [19], whereas TPKII phosphorylated Ser-202, Thr-205, Ser-235 and Ser-404 [23]. Since the SMI33 epitope was reported to contain Ser-235 [20] which was phosphorylated by TPKII, TPKII made this epitope disappear. Considering that the same effect was shown

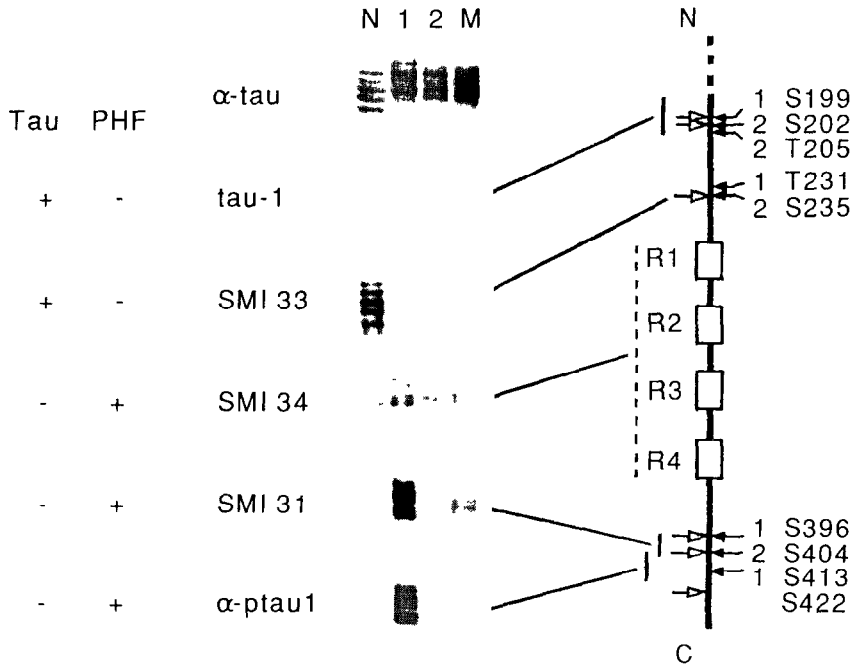

Fig. 1 Immunoblot analyses of tau. Normal bovine tau was phosphorylated by TPKI, TPKII or *Xenopus* MAP kinase. These proteins were immunoblotted with anti-tau (α-tau) (1:500), tau-1 (1:10,000), SMI33 (1:1,000), SMI34 (1:1,000), SMI31 (1:1,000) and anti-ptau 1 (α-ptau1) (1:50) The right panel indicates the recognition sites of tau-1 [24], SMI31, SMI33, SMI34 [20] and anti-ptau 1 [2] R1–R4 are repeats in the tubulin-binding region [19]. Open arrows indicate phosphorylation sites reported by Mandelkow et al. [7,20,35]. Closed arrows indicate phosphorylation sites reported by us [22]. Numbers 1 and 2 adjacent to these arrows indicate sites phosphorylated by TPKI and TPKII, respectively. Amino acid numbering is that found in the longest human tau [19]. The left table indicates known epitope differences between normal tau and PHF-tau. In this table, plus or minus indicates whether or not a reaction occurs with the antibodies, respectively

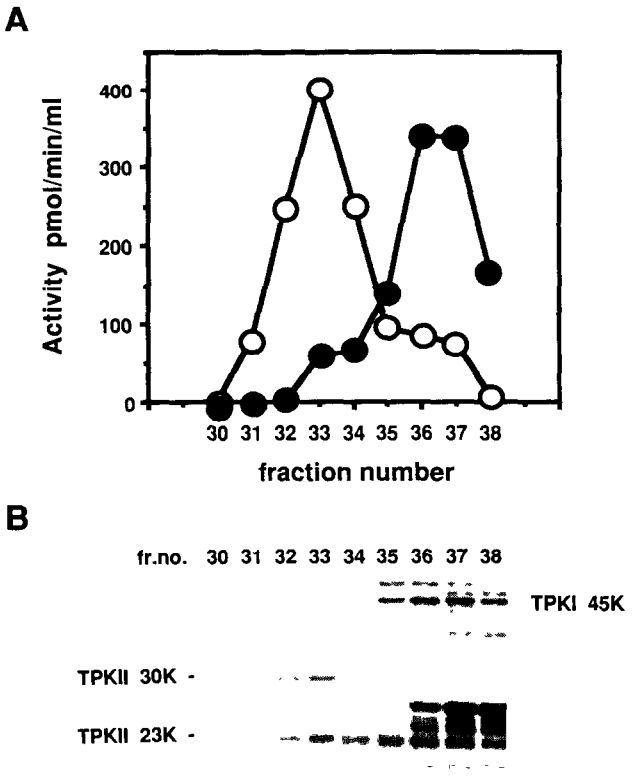

Fig. 2. Elution profile of TPKI and TPKII at S-Sepharose column chromatography (step 6 of our purification method [4]). (A) Activities of TPKI (●) and TPKII (○). TPKII activity was measured by phosphorylation of K2. TPKI activity was detected by phosphorylation of K2 previously phosphorylated by TPKII. (B) Protein profile of the fractions on SDS-PAGE. TPKI (45 kDa) and two polypeptides (30 kDa and 23 kDa) of TPKII are indicated.

by TPKI which phosphorylated Thr-231 near Ser-235, Thr-231 may also be essential for the SMI33 epitope. The SMI31 epitope was reported to be generated after phosphorylation of Ser-396 and Ser-404 [20]. Since normal tau was partially phosphorylated at Ser-404 [5], and TPKI phosphorylated Ser-396, it is reasonable to suggest that TPKI could generate the SMI31 epitope on normal tau. SMI34 was reported to recognize a conformational change of a repeating region. We show here that the change was induced by TPKI, although the epitope was already found weakly in normal tau. The tau-1 epitope disappeared on tau showing large mobility shift on SDS-PAGE after the phosphorylation by TPKI, indicating that the epitope contains Ser-199 [24]. TPKII and *Xenopus* MAP kinase also caused some, but not all, of the changes. In particular, MAP kinase could not generate the immunoreactivity recognized by anti-ptau 1, which was used as an antibody to detect conversion of tau into the PHF-like state for purification of TPKI [4]. The result strenghthened our working hypothesis that TPKI converts tau into the PHF state.

To characterize TPKI, we next developed a specific method of assaying for TPKI with a peptide substrate, based on the fact that phosphorylation of tau by TPKI was accelerated by its prior phosphorylation by TPKII [5]. Using three synthetic peptides each having a partial sequence of tau (K1, K2 and K3 [23]) we found that phosphorylation of Thr-231 in K1 and Ser-199 in K2 was enhanced by prior phosphorylation of Ser-235 and Ser-202, respectively. The enhancement was larger in the case of K2 than that in the case of K1. Therefore, we used K2 as a specific substrate for TPKI assay. Using the K2 phosphorylated by TPKII (p-K2) as a substrate, the activity of TPKI was measured at step 6 in our purification procedure [4], where TPKI was separated from TPKII. The phosphopeptide p-K2 was phosphorylated preferentially compared to K2, whereas TPKII phosphorylated only K2 (Fig. 2), indicating that this is a very effective and specific assay for TPKI.

To clarify the identity of TPKI, we next studied its amino acid sequence. Seven peptides (peptides A–G) were obtained from endopeptidase digests of TPKI, and subjected to peptide sequence analysis. About 90 amino acid residues in total were determined. A homology search of the amino acid sequences against the SWISS-PROT database showed that all of the sequence was present in GSK3β [14] (Fig. 3). PCR amplification of an oligonucleotide primer corresponding to peptide D was carried out using bovine cerebrum cDNA as a template, and synthetic sense- and antisense-guessmers corresponding to the N- and C-terminal of peptide D, respectively. A PCR product was obtained that contained the nucleotide sequence consisting of the middle portion of the peptide D. Using this PCR fragment as a hybridization probe, cDNA of TPKI was cloned from a rat brain cDNA library, and its sequence was determined. The sequence of TPKI is presented in Fig. 3. The amino acid sequence deduced from the nucleotide sequence is completely identical to that of GSK3β. In the nucleotide sequence, however, there are some differences; C(−103) in the sequence of GSK3β is deleted in that of TPKI, (−31)CC is TT, (718)ATG is GTA, (1,114)CCC is CCT, (1,168)CCT is CCG, (1,356)AAAAAA is AGAGGA (numbering in parentheses indicates nucleotide number of GSK3β [14]).

To confirm the identity, we purified GSK3β from bovine brain by Woodgett's method [14]. Detecting TPKI activity by the method mentioned above and GSK activity phosphorylating glycogen synthase, we confirmed that TPKI activity was inseparable from GSK activity throughout all steps of the purification. Additionally, phosphorylation of tau by the purified GSK3 was also stimulated by tubulin. This result supports the identity.

Furthermore, we proved that TPKI possessed GSK3 activity by using a specific phosphopeptide substrate for GSK3. Phosphorylation of glycogen synthase by GSK3 occurs at an amino acid very near the site phosphorylated by casein kinase II. GSK3 recognizes

the casein kinase II-modified serine residue and can then phosphorylate the second serine residue. This process was studied in detail using a peptide substrate, GS-1, that comprised a glycogen synthase sequence containing these phosphorylation sites [18]. Therefore, we measured GSK3 activity using the most highly purified TPKI using GS-1 peptide phosphorylated by casein kinase II as a substrate [18]. The phospho-GS-1, but not GS-1, was phosphorylated by the kinase activity in the purified TPKI (Fig. 4), indicating that the kinase is GSK3, i.e. TPKI.

4. DISCUSSION

Previously, we reported that TPKI generated one PHF epitope detected by anti-ptau 1 and diminished tau-1 epitope, as observed in PHF [1,4,22]. Here we showed that TPKI also induces some other immunoreactivities characteristic of PHF, supporting our previous hypothesis that TPKI is the most likely of the leading candidates for the role of the PHF-related kinase.

We also showed here that TPKI is identical to

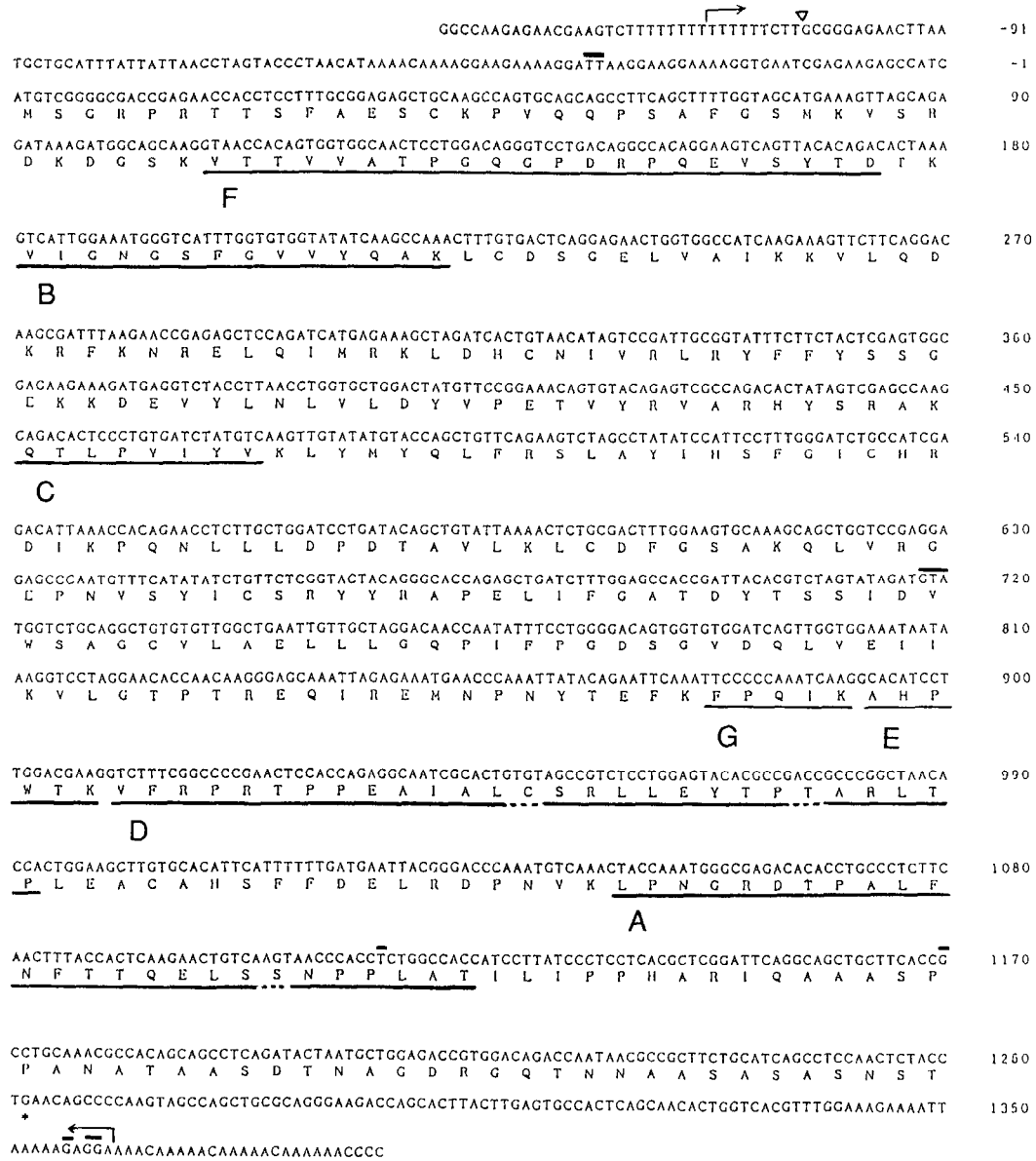

Fig. 3. Sequence of rat TPKI. A nucleotide sequence between two arrows is that of GSK3β already reported [14]. Lines above the sequence show nucleotide differences from those in GSK3β. ▽, indicates the deletion of a single nucleotide. Underlines indicate seven peptides, A–G determined from the TPKI digest with endoproteinase Lys-C (see text). Broken underlines show amino acids not determined.

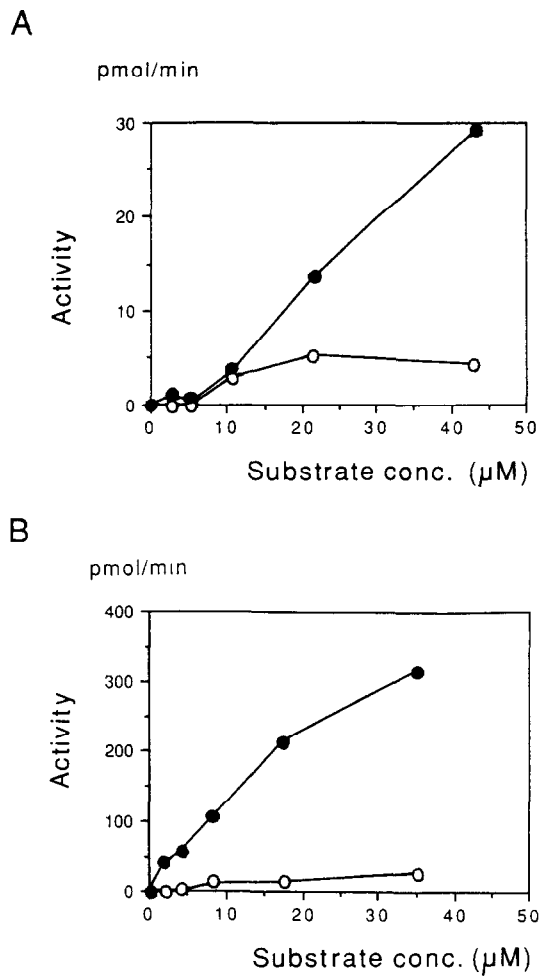

Fig. 4. TPKI activity (A) and GSK3 activity (B) of the mostly highly purified TPKI. The activities were measured with the following peptides as substrates; K2 (○) and K2 phosphorylated by TPKII (●) in A, and GS-1 (○) and GS-1 phosphorylated by casein kinase II (●) in B.

GSK3β. GSK3 was originally found to be a kinase phosphorylating glycogen synthase in muscle [15]. cDNA cloning showed the existence of another class of rat brain cDNA for GSK3 [14]. Thus the original GSK3 was called GSK3α, and the protein kinase found later was named GSK3β. Recently, both GSK3α and GSK3β have independently been reported to be the candidates for the abnormal phosphorylation of tau inducing PHF formation [10,11]. We detected the same epitope changes as those reported by Mandelkow et al. [11], although there were discrepancies between their and our estimation of the phosphorylation sites.

The identity is supported by other properties of TPKI. TPKI was purified from microtubules and induced a large mobility shift of tau on SDS-PAGE [1,4]. F_A/GSK3 was reported to be associated with microtubule and to induce large mobility shift of tau [25]. Furthermore, phosphorylation of tau by TPKI was accelarated by prior phosphorylation of tau by TPKII [5], a property which was useful for developing a specific assay for TPKI, as mentioned above. Considering that the recognition motif for GSK3 contains phosphoserine [12,13], it is reasonable to suggest that TPKI exhibits a similar hierarchal phosphorylation process.

GSK3 is known to be a multifunctional kinase. We found here that the phosphorylation of tau is also one of its functions. According to enzyme nomenclature, the kinase has been given various names based on the substrate; GSK3 [15], ATP-citrate lyase kinase [26], c-jun kinase [27], TPKI [4], and so on. Moreover, the kinase is also called factor A (F_A), because the kinase is an activating factor of phosphoprotein phosphatase 1 [28,29]. Properly, a multifunctional kinase should be given a name that represents its generalized function, and not a name dependent on the substrate in a single reaction. Further investigation will be necessary for providing an understanding of the features common among the various substrates. Until that time, we will call this kinase TPKI/GSK3β.

At present, it is impossible to determine which kinase actually works in Alzheimer's disease brain. One of the candidates is cdc2 kinase [8,9], which resembles TPKII based on partial sequencing (unpublished data) and substrate specificity [4,23], however, TPKII works in normal brain [5]. On the other hand, we have no evidence that phosphorylation sites by TPKI/GSK3β are phosphorylated in normal brain. These sites are reported to be phosphorylated in PHF [6]. It is reasonable to think that TPKI/GSK3β, rather than TPKII (cdc2-like kinase), induces tau to the PHF-like state, however, the possibility remains that TPKII aids the phosphorylation by TPKI/GSK3β with prior phosphorylation of tau in Alzheimer's disease brain [5].

Another candidate for PHF-related kinase is MAP kinase [7,8]. Phosphorylation of MAP kinase activates its activity [17]. Since we purified TPKI/GSK3β as the kinase inducing PHF epitopes without the activation step of MAP kinase [1,4], we may not have detected the MAP kinase for the PHF-related kinase. Our results indicate that MAP kinase generates epitope changes to a lesser extent than does TPKI/GSK3β. Therefore, we have no evidence that MAP kinase precedes TPKI/GSK3β in PHF formation.

Mammalian TPKI/GSK3β is 77% homologous to the *Drosophila* gene, shaggy [30] or zesta-white 3 [31]. Mutations in this gene cause abnormal differentiation of the nervous system [32]. In transgenic flies, rat GSK3β, but not GSK3α, can be substituted for shaggy [33]. Actually, our histochemical analysis with anti-TPKI/GSK3β antibodies suggested that the kinase plays an important role in the growth of axons during development of the brain (M. Takahashi et al., submitted elsewhere). Considering that degeneration of neurons is characteristic of Alzheimer's disease, TPKI/GSK3β may be implicated also in the degeneration process.

Further considering that GSK3β is also identical to

c-jun kinase [27], and that a programmed cell death is mediated by c-jun [34], TPKI/GSK3β may regulate the cell death through gene expression. Breakdown of the regulation may lead to the pathological state. We found that TPKI/GSK3β was involved in amyloid β protein-mediated neuronal cell death (A. Takashima et al., in press in Proc. Natl. Acad. Sci. USA). Tau phosphorylation may be one step in the process.

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