Calcium/calmodulin-dependent protein kinase II phosphorylates tau at Ser-262 but only partially inhibits its binding to microtubules

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Abstract PHF-tau, which is phosphorylated at 10 Ser/Thr-Pro and 11 non-Ser/Thr-Pro sites, is unable to promote microtubule assembly. Phosphorylation of the non-Ser/Thr-Pro site, Ser-262, is reported to be primarily responsible for this. The identities of kinase(s) responsible for Ser-262 phosphorylation are still to be clarified. In this study we have used the monoclonal antibody 12E8, which recognizes P-Ser-262 and P-Ser-356 on tau, to survey different kinases for their abilities to phosphorylate Ser-262 on human tau 3L (tau_{410}). In decreasing order of effectiveness we found that Ser-262 and Ser-356 phosphorylation can be catalyzed by CaM kinase II ≫ C-kinase ≫ GSK-3 ≅ A-kinase ≫ CK-1. CaM kinase II and C-kinase were shown to phosphorylate both Ser-262 and Ser-356. The binding of tau to taxol-stabilized microtubules was decreased by 35 and 42% after phosphorylation by CaM kinase II and C-kinase, respectively. Of the fraction of tau that bound to microtubules, about 50% was phosphorylated at Ser-262 and Ser-356. These results suggest that Ser-262 and Ser-356 are very good substrates for CaM kinase II but their phosphorylations are not sufficient to achieve maximal inhibition of tau binding to microtubules.

Key words: Tau protein; Microtubule; Protein kinase; Alzheimer's disease; Protein phosphorylation

1 Introduction

The paired helical filaments (PHF) found in Alzheimer disease (AD) brain are composed primarily of the microtubule-associated protein tau [1]. PHF-tau is in a highly phosphorylated state [2], and 21 phosphorylation sites have been identified. 10 of these sites are on Ser/Thr-Pro, the remaining 11 on non-Ser/Thr-Pro motifs [3–5]. These results suggest that both proline-dependent protein kinases (PDPKs) and non-PDPKs are involved in hyperphosphorylation of PHF-tau.

Phosphorylation of tau modifies its function. PHF-tau binds to microtubules and promotes microtubule assembly only after it is dephosphorylated [6]. Inhibition of microtubule assembly is also observed after tau is phosphorylated by A kinase [7] and cdc2 kinase [8]. Phosphorylation of tau by a 110 kDa kinase has been reported to strongly reduce the binding of tau to microtubules [9].

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Abbreviations: PHF, paired helical filaments; A-kinase, cyclic AMP-dependent protein kinase; CaM kinase II, calcium/calmodulin-dependent protein kinase II; C-kinase, calcium/phospholipid-dependent protein kinase; CK-1, casein kinase-1; GSK-3, glycogen synthase kinase-3; MAP kinase, mitogen-activated protein kinase; PDPK, proline-dependent protein kinase

The roles of Ser/Thr-Pro and non-Ser/Thr-Pro phosphorylation in regulating tau function are still unclear. It was initially reported that even after most of the Ser/Thr-Pro motifs on tau were phosphorylated by the PDPK, MAP kinase, the binding of tau was only moderately depressed [10]. Hence, the non-Ser/Thr-Pro phosphorylation sites in tau were implicated in regulating the binding of tau to microtubules The latter conclusion was further supported by a comparison of fetal tau and PHF-tau. Fetal tau [11], but not PHF-tau [6,11], can promote microtubule assembly. Fetal tau and PHF-tau are phosphorylated at 11 common sites. Of the additional 10 sites found only in PHF-tau, seven are on non-Ser/Thr-Pro motifs [3,12]. One of these sites is Ser-262. It was reported that phosphorylation of this site is primarily responsible for decreasing the affinity of tau for microtubules [9,10].

In this study we have evaluated the abilities of four non-PDPKs (A-kinase, C-kinase, CK-1, CaM kinase II) and a PDPK (GSK-3) to phosphorylate Ser-262 and modify binding of tau to microtubules. Our results indicate that CaM kinase II and C-kinase are the most effective kinases in both of these categories.

2. Materials and methods

2.1. Materials

The human tau clone 39 (kindly provided by M. Goedert) which encodes for tau isoform 3L, tau_{410} [13] was subcloned in *E. coli* and purified from cell extracts as described by us previously [14]. The purification of CK-1, GSK-3, CaM kinase II, C-kinase, and A-kinase has been described by us previously [14,15]. The monoclonal antibody 12E8 which recognizes tau phosphorylated at Ser-262 and/or Ser-356 [16] was a generous gift from Dale Schenk of Athena Neurosciences (San Francisco, CA). The polyclonal tau antibody 92e was raised in rabbits as previously reported [17]. Tubulin was isolated from rat brain through two temperature-dependent cycles of microtubule polymerization-depolymerization [18] followed by chromatography on phosphocellulose [19]. Taxol-stabilized microtubules were prepared by incubating tubulin (4 mg/ml) with taxol (20 μM) in the presence of MES (0.1 M, pH 6.8), EGTA (1 mM), and PMSF (1 mM) at 37°C for 30 min. The microtubules were collected by centrifugation over a cushion of 0.125 M sucrose at 50000×*g* for 30 min at 32°C.

2.2. Methods

To generate the tau fragments, tau 244–441 and tau 267–441, total RNA and mRNA were isolated by RNA purification kit (Pharmacia-LKB Biotechnology) from the temporal lobe of a patient (6 h post-mortem) who was histologically confirmed to have AD. Tau fragment cDNAs were prepared from AD mRNA using specific primers by PCR and reagents and conditions as described in Gene Amp RNA PCR kit (Perkin Elmer Cetus). The amplification products were cloned into p Bluescript II KS± (Stratagene) and sequenced. Recombinant plasmids (PRK 172) were transformed in *E. coli* B121 cells, expressed, and purified as described previously [20]. Tau 3L or tau fragments 244–441 and 267–441 were phosphorylated at 30°C by the

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different kinases in reaction mixtures normally containing 0.12 mg/ml tau or tau fragments, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.25 mM ATP, and 40 mM HEPES (pH 6.8 or 7.5) as described by us previously [14,15]. Reactions were initiated by addition of kinases and, after suitable time of incubation, terminated by heating (95°C/5 min). To assess the binding of tau to microtubules, 20 μg/ml tau (non-phosphorylated or phosphorylated by different kinases) was incubated with 0.4 mg/ml taxol-stabilized microtubules for 30 min at 37°C in buffer A (0.1 M HEPES (pH 6.8), 2 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 20 μM taxol, and 1 mM GTP). Tau bound to microtubules was separated from unbound tau by centrifugation at 50000×*g* for 30 min at 32°C. The pellet was washed once with buffer A. The combined supernatants and pellets were separately evaporated to dryness in a Speed Vac. All samples were run on either 10 or 12% (Fig. 3) SDS-polyacrylamide gels, transferred to nitrocellulose, and immunoblots probed by either 12E8 (1 μg/ml) or 92e (1/5000). Blots were then further probed with secondary antibodies which were either conjugated to alkaline phosphatase (Figs. 1–3) or labelled with ^{125}I (Fig. 4) [4]. The immunoreactive bands were visualized either by color development (Figs. 1–3) or by autoradiography (Fig. 4). Data were quantitated with the aid of either a Shimadzu CS-9000 Densitometer (Figs. 1–3) or a computerized Fuji 1500 Video Imaging System (Fig. 4).

3. Results

3.1. Phosphorylation of Ser-262/Ser-356 by different kinases

The monoclonal antibody 12E8 was previously shown to bind to tau which has been phosphorylated at Ser-262 and/or Ser-356 [16]. Using this antibody we have surveyed five kinases (A-kinase, C-kinase, CaM kinase II, CK-1, GSK-3) for their abilities to act as Ser-262 and/or Ser-356 kinases. In preliminary experiments, after phosphorylation of tau by the five kinases, acting either singly or in combination, binding of 12E8 to tau was variously increased (data not shown). To evaluate which kinase is kinetically most likely to phosphorylate Ser-262 and/or Ser-356 in situ we have analyzed the time course of phosphorylation of these sites by the different kinases (Figs. 1 and 2). Blots with 12E8 are shown for τ3L that was phosphorylated by A-kinase (Fig. 1A), C-kinase (Fig. 1B), and CaM kinase II (Fig. 1C). Tau 3L that was not phosphorylated bound only feebly to 12E8 (Fig. 1A, lane 1). To better appreciate the kinetics of phosphorylation of Ser-262 and/or Ser-356 by the above three kinases (as well as CK-1

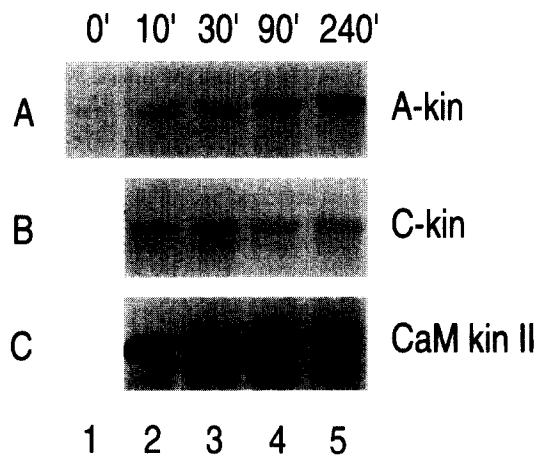

Fig. 1. Increased 12E8 immunoreactivity of tau after its phosphorylation by several kinases. Tau was incubated at 30°C in the presence of A-kinase (A) C-kinase (B), CaM kinase II (C), CK-1 (not shown), and GSK-3 (not shown). Samples were withdrawn at 0 min (lane 1), 10 min (lane 2), 30 min (lane 3), 90 min (lane 4), and 240 min (lane 5) and immunoblotted with 12E8.

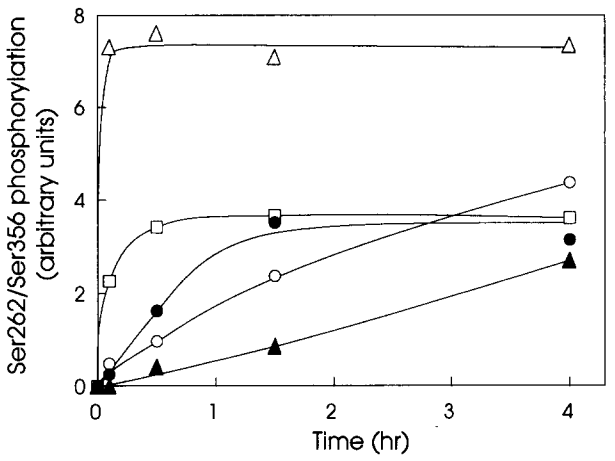

Fig. 2. Kinetics of phosphorylation of Ser-262 and/or Ser-356 by the different kinases. Immunoblots for tau phosphorylated by CK-1 (▲), A-kinase (○), GSK-3 (●), C-kinase (□), and CaM kinase II (△) were quantified by densitometry. Binding of 12E8 to tau observed at 0 min (Fig. 1A, lane 1) was subtracted from all values.

and GSK-3) blots were scanned by densitometry and the data presented in Fig. 2. It can be seen that the rate of phosphorylation (in decreasing order) of these sites is CaM kinase II ≫ C-kinase ≫ GSK-3 ≅ A-kinase ≫ CK-1. Further, CaM kinase II phosphorylated Ser-262 and/or Ser-356 to a 2-fold higher level (after 4 h) compared to the other kinases. It is obvious from these data that of the five kinases surveyed CaM kinase II is kinetically the best Ser-262 and/or Ser-356 kinase.

3.2. CaM kinase II and C-kinase phosphorylates both Ser-262 and Ser-356

We have addressed the question of whether CaM kinase II phosphorylates Ser-262, Ser-356, or both. To do this we used two tau constructs: one containing both Ser-262 and Ser-356 in the peptide Gln-244–Leu-441, the other containing only Ser-356 in the peptide Lys-267–Leu-441. These peptides were then used as substrates for CaM kinase II. The results with the Ser-356 peptide (Lys-267–Leu-441) are shown in Fig. 3. When this peptide was incubated in the absence of CaM kinase II (lane 1) the subsequent binding of 12E8 was only minimal. After incubation in the presence of CaM kinase II (lane 2) the binding of 12E8 was enhanced 7-fold. In the presence of C-kinase a 2.5-fold increase was detected (not shown). The extent of phosphorylation of the peptide containing both Ser-262 and Ser-356 (Gln-244–Leu-441) was 2-fold greater than that of the peptide containing only Ser-356 (Lys-267–Leu-441) (data not shown). These results suggest that CaM kinase II phosphorylates both Ser-262 and Ser-356 in Gln-244–Leu-441 and in full length (intact) tau.

3.3. Binding of CaM kinase II-phosphorylated tau to microtubules

We have evaluated what effect phosphorylation of Ser-262 and Ser-356 may have on the ability of tau to bind to microtubules (Fig. 4). When tau was not phosphorylated we found that 94% of it bound to microtubules. Of the five kinases (A-kinase, C-kinase, CK-1, CaM kinase II, GSK-3) tried, C-kinase and CaM kinase showed the largest effect. After phosphorylation of tau by these kinases the fraction of it that bound to microtubules was reduced to 52 and 59%, respec-

tively. Under the conditions of this experiment (1 h phosphorylation) A-kinase and CK-1 did not significantly affect binding of tau to microtubules. With GSK-3 13% inhibition of binding was observed. We also found that with C-kinase- and CaM kinase-phosphorylated tau, 54 and 46%, respectively, of the total 12E8 immunoreactivity was found in tau that bound to microtubules. These results suggest that the phosphorylation of Ser-262 and Ser-356 are not enough for abolishing the binding of tau to microtubules.

4. Discussion

In this study we have identified kinases that can phosphorylate the non-Ser/Pro sites, Ser-262 and Ser-356, to various extents. These kinases (in decreasing order of effectiveness) are CaM kinase II ≫ C-kinase ≫ GSK-3 ≅ A-kinase ≫ CK-1. Phosphorylation of Ser-262 and/or Ser-356 by CK-1 was very slow and became significant only after many hours of incubation at 30°C. CaM kinase II phosphorylated Ser-262 rapidly and to a greater than 2-fold higher extent compared to the other kinases. This is the first study to identify CaM kinase II and C-kinase as Ser-262 and Ser-356 kinases. Ser-262 and Ser-356 were previously shown to be phosphorylated by A-kinase [9] and GSK-3α [21], and Ser-262 by GSK-3β [22]. Unlike our results (Fig. 2), Ser-262 was phosphorylated by GSK-3α and GSK-3β only in the presence [21,22], and not in the absence [23], of heparin. One possible reason why we found phosphorylation of Ser-262 and Ser-356 in the absence of heparin is that we have used a single human tau isoform (τ3L) in our study compared to the use of bovine tau (a mixture of six tau isoforms) in the previous studies [21–23]. We have recently shown that tau isoforms are phosphorylated at different rates and to different extents by various kinases, including GSK-3 [24].

Our data support the conclusion that phosphorylation of Ser-262 and Ser-356 is not enough to prevent the binding of tau to microtubules. In recent studies [9] it was shown that phosphorylation of Ser-262 by a 110 kDa kinase was primarily responsible for reducing the affinity of tau for binding to microtubules. Our results do not support this conclusion. Although Ser-262 and Ser-356 are maximally phosphorylated by CaM kinase II, 59% of the tau remain bound to microtubules. This result suggests that the phosphorylation of Ser-262 and Ser-356 is not enough for eliminating the interaction of tau with microtubules. The phosphorylation of other sites is obviously required. A similar conclusion to ours was also reached using phosphorylated tau from human biopsy-brain samples [16].

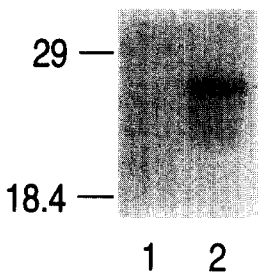

Fig. 3. CaM kinase II phosphorylates Ser-356 in addition to Ser-262. The tau construct Lys-267–Leu-441 lacking Ser-262 but containing Ser-356 was incubated for 1 h at 30°C in either the absence (lane 1) or presence (lane 2) of CaM kinase II. Samples were then immunoblotted with 12E8. The positions of marker proteins (in kDa) are shown on the left.

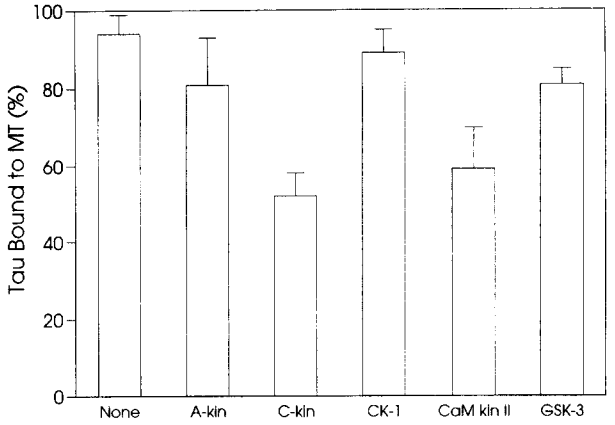

Fig. 4. Effect of phosphorylation of tau by different kinases on its ability to bind to microtubules. Tau was separately incubated for 1 h at 30°C in either the absence (none) or presence of A-kinase, C-kinase, CK-1, CaM kinase II, and GSK-3. In parallel incubations $^{32}P_i$ incorporation into tau by these kinases after 1 h was determined to be 0.90, 0.95, 3.10, 1.40, and 0.98 mol/mol tau, respectively. The reactions were stopped by heating at 95°C for 5 min. Binding of the different species of heat-stable phosphorylated tau to taxol-stabilized microtubules was then quantified by using the polyclonal phosphorylation-independent tau antibody 92e. Data (mean ± SD) were from three separate experiments.

The identities of the additional sites (besides Ser-262 and Ser-356) whose phosphorylation may be required to completely abolish binding of tau to microtubules are presently unclear. It is surprising that no inhibition of binding was detected with CK-1-phosphorylated tau. Of several non-PDPKs we have compared in our studies, CK-1 is among the best tau kinases. Under the conditions used for this study τ3L was phosphorylated by CK-1 to the extent of ~3 mol P_i/mol tau (see legend to Fig. 4 and [14,24]). Although we have not yet identified the sites phosphorylated by CK-1 it is possible that this kinase does not effectively phosphorylate sites in the microtubule-binding domains of tau. Ser-262 and Ser-356 are both located in these domains and are rapidly phosphorylated by C-kinase and CaM kinase II thereby causing inhibition of binding of tau to microtubules. In addition, C-kinase has been reported to also phosphorylate Ser-305 [9] and Ser-324 [25], both also located in the microtubule binding domains. Similarly, A-kinase has been reported to phosphorylate several sites in tau microtubule-binding domains: Ser-262, Ser-293, Ser-305, Ser-324, Ser-356 [7,9]. Yet we did not observe any significant inhibition of tau binding to microtubules by A-kinase. As we observed for Ser-262 and Ser-356 (Fig. 2) it is possible that the rates of phosphorylation of these sites by A-kinase are slow and they are incompletely phosphorylated after 1 h (Fig. 4). Although Ser-262 and Ser-356 are phosphorylated by GSK-3 at 75% the level achieved with C-kinase (after 1 h) the binding of tau to microtubules was reduced by only 13% compared to a 42% reduction with C-kinase (Fig. 4). These results further reinforce the idea that additional sites need to be phosphorylated for effective inhibition of tau binding to microtubules. It is not known whether GSK-3 can phosphorylate other sites (besides Ser-262 and Ser-356) in the microtubule binding domains in the absence of heparin. In the presence of heparin Ser-324, is also phosphorylated [21,22].

It is possible that phosphorylation of both Ser/Thr-Pro and non-Ser/Thr-Pro sites on tau may be required for a maximal effect on microtubule function to be observed. This seems to be true for PHF-tau. PHF-tau in its fully phosphorylated state (10 Ser/Thr-Pro sites, 11 non-Ser/Thr-Pro sites) fails to promote microtubule assembly, but does so after dephosphorylation [6,11]. We have recently shown that after an initial prephosphorylation of tau by non-PDPKs (A-kinase, C-kinase, CaM kinase II) several sites on tau that were previously inaccessible became readily accessible to a PDPK such as GSK-3 [14,24]. Hence potent regulation of tau function may require the concerted actions of both PDPK and non-PDPKs.

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