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Non-proline-dependent protein kinases phosphorylate several sites found in tau from Alzheimer disease brain

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Abstract

Of 21 phosphorylation sites identified in PHF-tau 11 are on ser/thr-X motifs and are probably phosphorylated by non-proline-dependent protein kinases (non-PDPKs). The identities of the non-PDPKs and how they interact to hyperphosphorylate PHF-tau are still unclear. In a previous study we have shown that the rate of phosphorylation of human tau 39 by a PDPK (GSK-3) was increased several fold if tau were first prephosphorylated by non-PDPKs (Singh *et al.*, FEBS Lett 358: 267–272, 1995). In this study we have examined how the specificity of a non-PDPK for different sites on human tau 39 is modulated when tau is prephosphorylated by other non-PDPKs (A-kinase, C-kinase, CK-1, CaM kinase II) as well as a PDPK (GSK-3). We found that the rate of phosphorylation of tau 39 by a non-PDPK can be stimulated if tau were first prephosphorylated by other non-PDPKs. Of the four non-PDPKs only CK-1 can phosphorylate sites (thr 231, ser 396, ser 404) known to be present in PHF-tau. Further, these sites were phosphorylated more rapidly and to a greater extent by CK-1 if tau 39 were first prephosphorylated by A-kinase, CaM kinase II or GSK-3. These results suggest that the site specificities of the non-PDPKs that participate in PHF-tau hyperphosphorylation can be modulated at the substrate level by the phosphorylation state of tau. (Mol Cell Biochem **154**: 143–151, 1996)

Key words: GSK-3, tau protein, protein kinases, Alzheimer disease, paired helical filaments, microtubules

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; CK-2 – casein kinase-2; GSK-3 – glycogen synthase kinase-3; MAP kinase – mitogen-activated protein kinase; PDPK – proline-dependent protein kinase

Introduction

In brain the primary function of tau is to promote the assembly of tubulin into microtubules. Tau is a phosphoprotein, and in its phosphorylated state it inhibits microtubule assembly from tubulin [1, 2]. Tau is a primary component of the paired helical filaments (PHFs) [3] found in the brain of patients with Alzheimer's disease (AD). Unlike normal tau, PHF-tau is in a hyperphosphorylated state [4]. Twenty-one phosphorylation sites in PHF-tau have recently been identified. Ten of these sites are on ser/thr-pro motifs, 11 on ser/thr-X motifs (X = ser, arg, thr, gly, asn) [5–7]. The ser/thr-pro and ser/thr-X sites are probably phosphorylated by proline-dependent protein kinases (PDPKs) and non-PDPKs, respectively.

The phosphorylation of purified tau *in vitro* by both PDPKs and non-PDPKs has been studied previously. The PDPKs include MAP kinase [8–10], cdc2 kinase [11], cdk2, cdk5 [12, 13], and GSK-3 [14–18]. The non-PDPKs include cyclic AMP-dependent protein kinase (A-kinase) [17–21], protein kinase C [17, 18, 21–23], calcium/calmodulin-dependent protein kinase II (CaM kinase II) [17, 18, 21, 24], calcium/calmodulin-dependent kinase Gr [18, 21, 25], casein kinase-1 (CK-1) [18, 21, 25], and casein kinase-2 (CK-2) [18, 21, 26].

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Several phosphorylation-dependent antibodies have been used to compare the sites phosphorylated on tau *in vitro* by purified kinases with those found in PHF-tau. The epitopes of several such antibodies have been mapped on tau. For instance, the antibodies PHF-1 [27], T3P [28] and 8D8 [29] have been shown to bind to epitopes that require ser 396 in the phosphorylated state. Similarly, SMI31 was shown to bind to P-ser 396 and P-ser 404 [30], M4 to P-thr 231 [31], and AT8 to P-ser 199 and P-ser 202 [32]. SMI34 binds to phosphorylated epitopes (undefined) on either side of the microtubule-binding domains of tau [30]. On the other hand the antibodies 102c (ser 46) [5], TP30 (thr 123) [6], SMI33 (ser 235), [30], and Tau-l (ser 199, ser 202) [32] have all been shown to bind to the dephosphorylated form of their epitopes.

The sites phosphorylated on tau *in vitro* by several protein kinases have also been identified directly by sequencing of phosphopeptides. Among the several sites phosphorylated by various protein kinases a few have been shown to be identical to those found in PHF-tau. A-kinase phosphorylated two such sites (ser 214, ser 409) [20], cdc2 kinase four sites (ser 202, thr 231, ser 235, ser 404) [11], GSK-3 two sites (ser 235, ser 404) [16], and cdk5 five sites (ser 202, thr 231, ser 235, ser 396, ser 404) [13].

It is presently unclear how PDPKs and non-PDPKs interact to bring about the final hyperphosphorylated state of PHF-tau. Further, the identities of the kinases that participate in the hyperphosphorylation are also unclear. To examine this aspect, we have recently shown that a prephosphorylation of human tau by non-PDPKs can stimulate a subsequent phosphorylation catalyzed by a PDPK such as GSK-3. Further, such prephosphorylation of tau by non-PDPKs permitted a rapid induction of several Alzheimer-like epitopes by GSK-3 [17, 18].

In the present study we have further characterized the potential interactions among different kinases in bringing about tau hyperphosphorylation. In particular, we investigated how the specificity of a non-PDPK for different sites on tau is affected when tau is first prephosphorylated by other kinases. We found that a prephosphorylation of tau by one non-PDPK can stimulate the level of phosphorylation achieved by other non-PDPKs. Most importantly, we found that CK-1 is the only non-PDPK (among five tested) that can phosphorylate some of the sites found in PHF-tau.

Materials

The human tau clone, tau 39 (kindly provided by M. Goedert), encodes for tau isoform 3L that has three tandem C-terminal repeats plus a 58-amino acid insert near the N-terminal end of the polypeptide [33]. Tau 39 was subcloned in *E. coli* and purified from cell extracts as described by us previously [17].

CK-1 [21] and GSK-3 were purified from bovine brain. GSK-3 was purified as described in [35] with some modifications. Instead of CM-Sepharose we used SP-Sepharose (after first Heparin-agarose step). This column effectively separated GSK-3 from cdk5 which was recovered in the flow-through. GSK-3 was eluted with 0.3 M NaCl, concentrated, and applied to Sephacryl S-300. The GSK-3 preparation contained both the α and β isoforms in ratio 1:3. CaM kinase II [36] and C-kinase [37] were purified from rat brain. CaM kinase II was a gift from Brad McDonald, Burroughs-Wellcome Laboratories (Research Triangle Park, NC, USA); C-kinase was generously supplied by V. Chauhan of this Institute. The catalytic subunit of A-kinase and calmodulin were purchased from Sigma (St. Louis, MO, USA). Monoclonal antibodies were obtained as follows: SMI31 and SMI34 (Sternberger Monoclonals, Inc., Baltimore, MD, USA), PHF-1 (gift from S. Greenberg, Cornell Medical College, White Plains, NY, and P. Davies, Albert Einstein College of Medicine, Bronx, NY, USA) [27], and M4 (gift from Y. Ihara, Institute for Brain Research, University of Tokyo, Tokyo, Japan) [31]. [γ-^{32}P] ATP was purchased from ICN Biomedicals (Costa Mesa, CA, USA).

Methods

Human tau 39 was phosphorylated by GSK-3 in a reaction mixture normally containing 0.15 mg/ml tau, 6 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.25 mM [γ-^{32}P] ATP, 40 mM Hepes (pH 7.5), and GSK-3. Phosphorylation of tau by the other kinases was recently described by us [25]. Reactions were normally initiated at 30°C by the addition of kinases. For determination of ^{32}P incorporation into protein, aliquots of the reaction mixtures were removed at different times, spotted on filter paper, and processed as described previously [38]. When prephosphorylation of tau was required [γ-^{32}P] ATP was replaced with unlabelled ATP. After incubation for 2 h at 30°C, the reactions were stopped by heating at 95°C for 5 min and denatured kinases removed by centrifugation (10,000 × g for 10 min). The prephosphorylated tau was then used as substrates for other kinases. Unless otherwise stated, further phosphorylation of prephosphorylated tau by a second kinase was for 2 h at 30°C.

Immunoblotting of tau by the different antibodies was carried out as described previously [5]. The following dilutions of the antibodies were used: SMI31 (1/75), SMI34 (1/75), PHF-1 (1/500), and M4 (1/1000). For quantitation, a Shimadzu CS-9000 Densitometer was used to scan immunoblots.

Results

Phosphorylation of tau by the combined actions of several non-PDPKs

We have recently shown that a prior phosphorylation of tau by several non-PDPKs stimulated both the rate and extent of a subsequent phosphorylation of tau catalyzed by a PDPK (GSK-3) [17, 18]. To further understand the roles of non-PDPKs in tau phosphorylation we have analyzed how the specificity of one non-PDPK is affected when the protein substrate (tau) is prephosphorylated by other non-PDPKs, as well as PDPKs. For these studies we have used four non-PDPKs (A-kinase, C-kinase, CK-1, CaM kinase II) and a PDPK (GSK-3). These kinases were then used to prepare five different species of phosphorylated tau 39. The phosphorylation of these different phosphorylated tau species by each kinase was then investigated and compared with nonphosphorylated tau. The results from such experiments are shown in Figs 1 and 2. For comparison, the level of ^{32}P incorporation achieved by each kinase after 3 h when a specific prephosphorylated form of tau is used as substrate will be considered. Such results are summarized in Table 1. After an initial prephosphorylation of tau by CK-1, CaM kinase II, or A-kinase, ^{32}P incorporation by the other three kinases is slightly elevated. The exception is tau that was prephosphorylated by A-kinase then further phosphorylated by CaM kinase II. Alternatively, after an initial phosphorylation of tau by C-kinase or GSK-3, ^{32}P incorporation by the other three kinases was slightly decreased. The exception is tau that was prephosphorylated by C-kinase then further phosphorylated by CK-1. We have also evaluated the five different species of prephosphorylated tau, as well as nonphosphorylated tau, as potential substrates for CK-2. In all cases ^{32}P incorporation into tau was less than 0.14 mol ^{32}P/mol tau after 3 h at 30°C (data not shown). Collectively, the data from Figs 1 and 2 suggest that the initial phosphorylation state of tau is important for determining the substrate specificities of various non-PDPKs.

Binding of tau antibodies to different species of phosphorylated tau

In our recent study [17] we have shown that a prior phosphorylation of tau by non-PDPKs changed the conformation of the protein in such a way that a subsequent phosphorylation by a PDPK (GSK-3) rapidly induced the binding of several phosphorylation-dependent tau antibodies. In the present study we have evaluated whether a prior phosphorylation of tau by the different kinases (see Figs 1 and 2) permitted non-PDPKs to similarly induce the epitopes to the tau antibodies. For these studies tau was separately prephosphorylated by each non-PDPK (A-kinase, C-kinase, CK-1, CaM kinase II) and GSK-3 for 2 h. Each prephosphorylated tau species was then used as a substrate for each of the other four kinases and

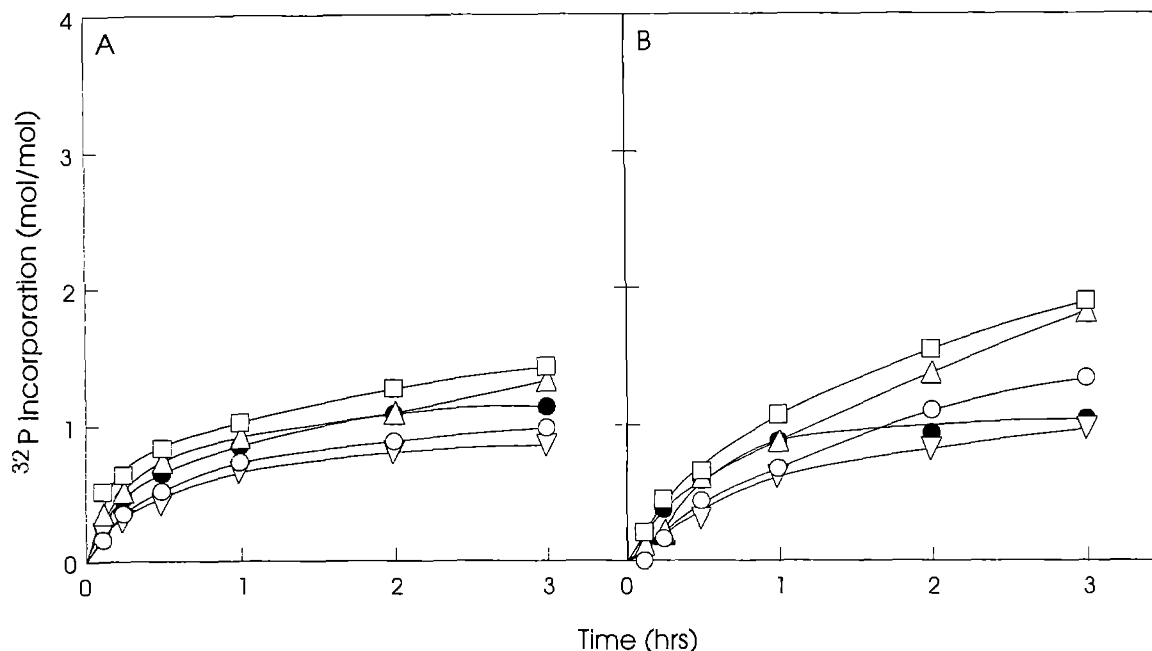

Fig. 1. Phosphorylation of different prephosphorylated tau species by A-kinase and C-kinase. Tau that was either prephosphorylated or not prephosphorylated (●) was used as a substrate for A-kinase (A) or C-kinase (B). Prephosphorylation of tau was for 2 h at 30°C and was catalyzed by GSK-3 (▽), CaM kinase II (Δ), CK-1 (□), C-kinase (O, A), and A-kinase (O, B).

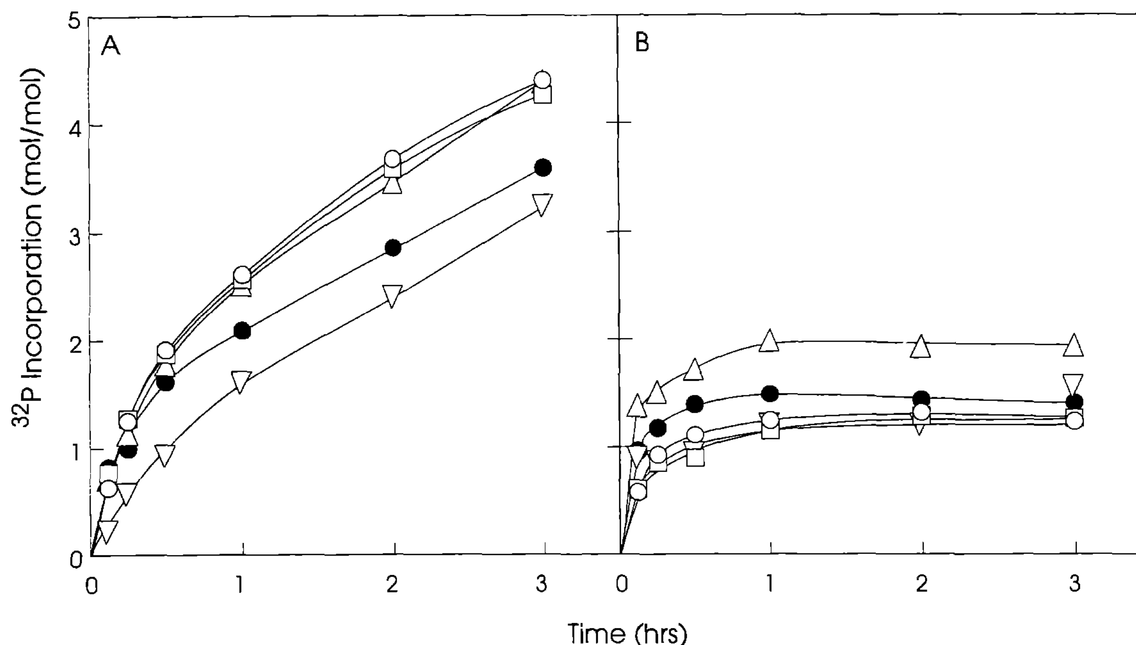

Fig. 2. Phosphorylation of different prephosphorylated tau species by CK-1 and CaM kinase II. Tau that was either prephosphorylated or not prephosphorylated (●) was used as a substrate for CK-1 (A) or CaM kinase II (B). Prephosphorylation of tau was for 2 h at 30°C and was catalyzed by GSK-3 (▽), C-kinase (O), A-kinase (□), CaM kinase II (Δ, A), and CK-1 (Δ, B). Results shown in Figs 1 and 2 have been confirmed in three separate experiments.

Table 1. Effect of prephosphorylation of tau on ^{32}P incorporation

Prephosphorylation	Phosphorylation[a]	^{32}P Incorp. (%)[b]
CK-1	A-kinase	127
	C-kinase	177
	CaM kinase II	135
CaM kinase II	A-kinase	127
	C-kinase	176
	CK-1	122
A-kinase	C-kinase	129
	CK-1	119
	CaM kinase II	90
C-kinase	A-kinase	87
	CK-1	122
	CaM kinase II	88
GSK-3	A-kinase	75
	C-kinase	93
	CK-1	90
	CaM kinase II	84

[a]Data taken from Figs 1 and 2 (3 h points). [b] ^{32}P incorporation observed with each kinase using a particular species of prephosphorylated tau is compared with ^{32}P incorporation observed by that same kinase when tau that is not prephosphorylated is used as a substrate. The latter is taken as 100% in each case.

phosphorylation continued for an additional 2 h. The binding of several tau antibodies to these different phosphorylated tau species was then analyzed by immunoblotting (Figs 3 and 4). The monoclonal antibodies SMI31, SMI34, and PHF-1 recognize only phosphorylated epitopes on tau [27, 30]. The binding of SMI31 and SMI34 to tau phosphorylated by a combination of different non-PDPKs is shown in Fig. 3. Significant binding of both antibodies is observed only with tau that was phosphorylated either by CK-1 alone (lane 9) or CK-1 in combination with A-kinase (lanes 3, 10), C-kinase (lanes 7, 11) and CaM kinase II (lanes 12, 16). SMI31 binding observed with tau phosphorylated either by CK-1 alone or CK-1 in combination with the other kinases is approximately the same (in Fig. 3A compare lane 9 with lanes 3, 7, 10, 11, 12, and 16). With SMI34, however, binding to the different phosphorylated tau species is variable. Compared to tau phosphorylated by CK-1 alone (lane 9, 100%), binding of SMI34 to tau phosphorylated by A-kinase + CK-1 was 148% (lane 3; see Fig. 5F also), C-kinase + CK-1 was 71% (lane 7), CK-1 + A-kinase was 88% (lane 10), CK-1 + C-kinase was 62% (lane 11), CK-1 + CAM kinase II was 57% (lane 12), and CAM kinase II + CK-1 was 70% (lane 16). By contrast to CK-1, the other three non-PDPKs (A-kinase, C-kinase, CaM kinase II), either by themselves, or in combination with each other, did not significantly induce binding of SMI31 and SMI34 to tau (Fig. 3). Unlike these two antibodies, PHF-1 showed weak binding to only one phosphorylated species of tau – that prephosphorylated by CaM kinase II followed by a further phosphorylation by CK-1 (data not shown).

The binding of SMI31 and SMI34 to tau phosphorylated

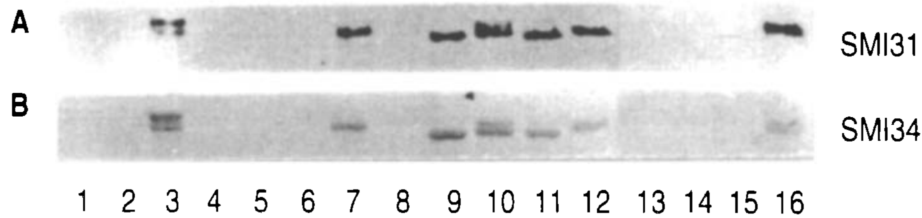

Fig. 3. Pattern of binding of tau antibodies to different species of phosphorylated tau. Tau was prephosphorylated by A-kinase (lanes 1–4), C-kinase (lanes 5–8), CK-1 (lanes 9–12), and CaM kinase II (lanes 13–16) for 2 h. The reactions were terminated by boiling for 5 min and removal of denatured kinases by centrifugation. The prephosphorylated tau was then incubated for a further 2 h at 30°C either in the absence of kinases (lanes 1, 5, 9, 13) or the presence of A-kinase (lanes 6, 10, 14), C-kinase (lanes 2, 11, 15), CK-1 (lanes 3, 7, 16), and CaM kinase II (lanes 4, 8, 12). The different phosphorylated tau species were immunoblotted with SMI31 (A), SMI34 (B) and PHF-1 (not shown). Immunoblots shown in Figs 3–6 have been analyzed by densitometry. In the text antibody binding to tau is expressed either as a percent (Fig. 3) or as fold increase compared to controls (Figs 4–6).

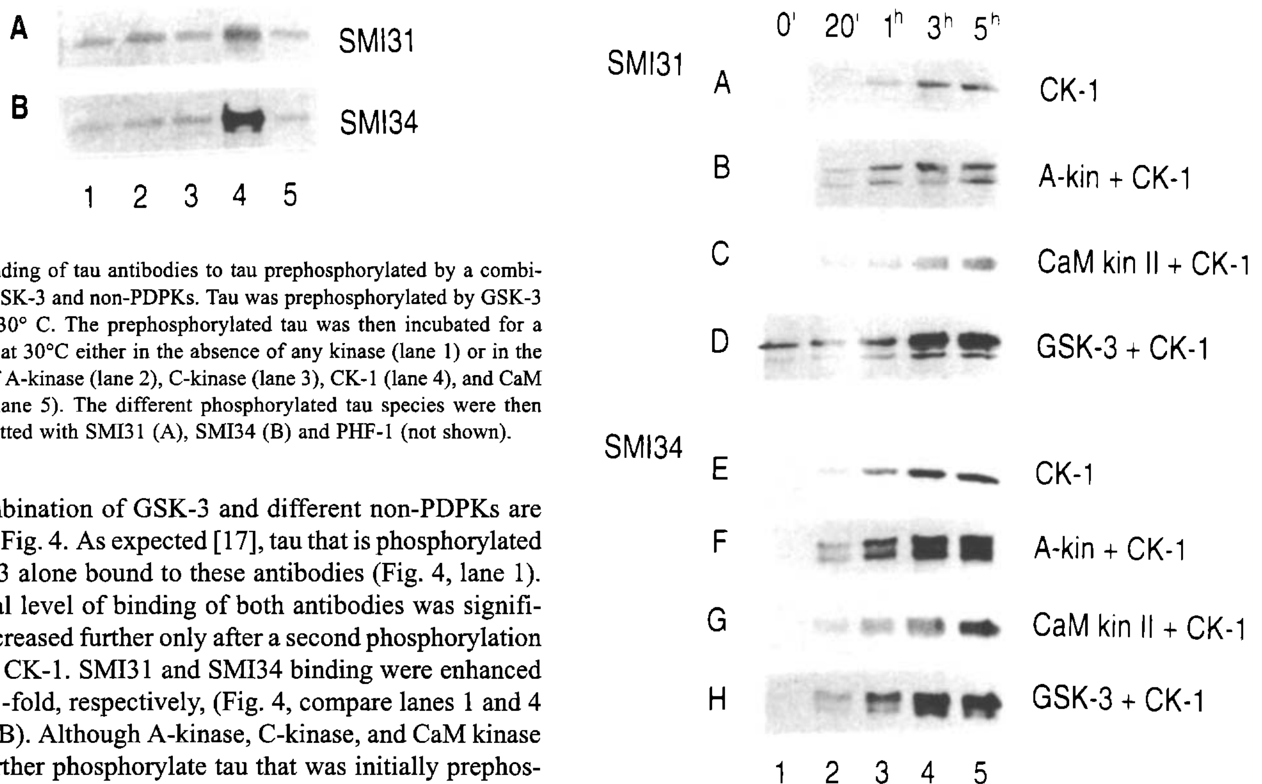

Fig. 4. Binding of tau antibodies to tau prephosphorylated by a combination of GSK-3 and non-PDPKs. Tau was prephosphorylated by GSK-3 for 2 h at 30° C. The prephosphorylated tau was then incubated for a further 2 h at 30°C either in the absence of any kinase (lane 1) or in the presence of A-kinase (lane 2), C-kinase (lane 3), CK-1 (lane 4), and CaM kinase II (lane 5). The different phosphorylated tau species were then immunoblotted with SMI31 (A), SMI34 (B) and PHF-1 (not shown).

Fig. 5. Time course of induction of SMI31 and SMI34 binding by CK-1 in the absence and presence of other kinases. Tau that was not prephosphorylated (A, E) or was prephosphorylated by A-kinase (B, F), CaM kinase II (C, G), and GSK-3 (D, H) for 2 h was then further phosphorylated by CK-1 for different times. Aliquots of the reaction mixtures were removed at 0 min (lane 1), 20 min (lane 2), 1 h (lane 3), 3 h (lane 4), and 5 h (lane 5) and immunoblotted with SMI31 (A-D), SMI34 (E-H), and PHF-1 (not shown).

by a combination of GSK-3 and different non-PDPKs are shown in Fig. 4. As expected [17], tau that is phosphorylated by GSK-3 alone bound to these antibodies (Fig. 4, lane 1). This basal level of binding of both antibodies was significantly increased further only after a second phosphorylation of tau by CK-1. SMI31 and SMI34 binding were enhanced 2- and 23-fold, respectively, (Fig. 4, compare lanes 1 and 4 in A and B). Although A-kinase, C-kinase, and CaM kinase II can further phosphorylate tau that was initially prephosphorylated by GSK-3 (Figs 1 and 2), such phosphorylation did not significantly increase the binding of SMI31 and SMI34 (Fig. 4). Unlike these two antibodies no significant increase in the binding of PHF-1 was observed when tau was phosphorylated by a combination of GSK-3 and the other non-PDPKs (including CK-1) (data not shown). These results suggest that the epitopes for SMI31 and SMI34 (but not PHF-1) are good substrates for CK-1 especially after tau is first prephosphorylated by another non-PDPK or GSK-3.

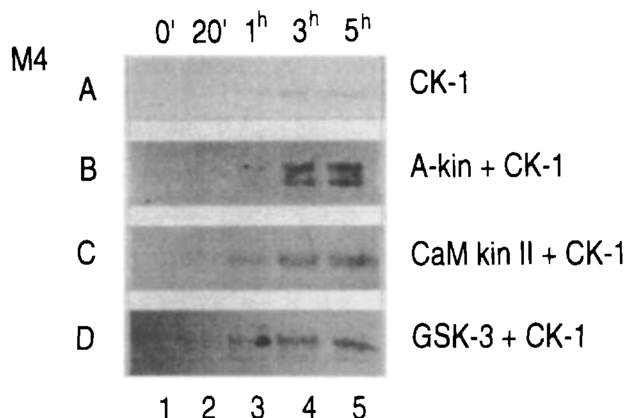

Fig. 6. Time course of induction of M4 binding by CK-1 in the absence and presence of other kinases. Tau that was not prephosphorylated (A) or was prephosphorylated by A-kinase (B), CaM kinase II (C), and GSK-3 (D) was then further phosphorylated by CK-1. Aliquots of the reaction mixtures were removed at the different times and immunoblotted with M4. Lane numbers and phosphorylation times are the same as in Fig. 5. The results presented in Figs 3, 4, 5, 6 have been repeated two, four, three, and two times, respectively.

Time course of induction of antibody epitopes by CK-1

From the results of Figs 3 and 4 it is apparent that CK-1 can promote greater binding of SMI31 and SMI34 to tau if the latter were first prephosphorylated by A-kinase, CaM kinase II, or GSK-3. We have further explored the interactions between the different kinases in tau phosphorylation by analyzing the time course of induction of the binding of three tau antibodies – SMI31, SMI34, and M4 (the latter recognizes phosphorylated thr 231). Figure 5 shows the results achieved with SMI31 (panels A–D) and SMI34 (panels E–H). Phosphorylation of tau by CK-1 alone (panels A and E) promotes binding of these antibodies. Enhanced binding of both antibodies is detected after only 20 min phosphorylation with CK-1. A prephosphorylation of tau by A-kinase (panels B and F), CaM kinase II (panels C and G), or GSK-3 (panels D and H) promoted an increase in both the rate and extent of binding of SMI31 and SMI34. In all cases maximal binding of the antibodies required about 5 h. Tau that was prephosphorylated with A-kinase, CAM kinase II or GSK-3 showed 2-fold, 1.5-fold, and 6-fold increase in the binding of SMI31, respectively, over that achieved with tau that was not prephosphorylated (compare lane 5 in A with B, C, D, Fig. 5). Similarly, the binding of SMI34 was increased 2.8-, 1.5-, and 2.2-fold after prephosphorylation of tau by A-kinase, CAM kinase II, and GSK-3, respectively (compare lane 5 in E with F, G, H, Fig. 5).

Figure 6 shows the time course of induction of the M4 epitope. Unlike SMI31 and SMI34 (Fig. 5) only minimal binding of M4 is observed after phosphorylation of tau by CK-1 (Fig. 6A). Prephosphorylation of tau by A-kinase (Fig. 6B), CAM kinase II (Fig. 6C) or GSK-3 (Fig. 6D) greatly stimulated both the rate and extent of M4 binding. After 5 h M4 binding was increased 20-fold, 9-fold, and 7-fold for tau prephosphorylated by A-kinase, CAM kinase II, and GSK-3, respectively (compare lane 5 in A with B, C, D, Fig. 5). The results of Figs 5 and 6 indicate that CK-1 can promote increased binding of several antibodies (SMI31, SMI34, M4) to tau if the latter is first prephosphorylated (by A-kinase, CaM kinase n, GSK-3) compared to no prephosphorylation.

Discussion

The results presented in this study are part of our continuing efforts to decipher the roles of non-PDPKs in tau hyperphosphorylation. Since 11 (of 21) phosphorylation sites identified in PHF-tau are not followed by pro [5–7] one possible role of non-PDPKs is a direct phosphorylation of these sites. A second possible role of non-PDPKs in PHF-tau hyperphosphorylation is the modulation of the site specificity of a PDPK. We have recently shown that when tau is prephosphorylated by several non-PDPKs, the rate and extent of a subsequent phosphorylation catalyzed by GSK-3 (a PDPK) is greatly increased. Additionally, the rate of induction of several 'Alzheimer-like' epitopes on tau were also greatly increased [17]. Still a third possible role of non-PDPKs is to modulate the specificities of other non-PDPKs for different phosphorylation sites on tau. The latter role of non-PDPKs was investigated in the present study.

We have explored the interactions between a PDPK (GSK-3) and several non-PDPKs in tau phosphorylation. Both types of kinases are apparently involved in the hyperphosphorylation of PHF-tau [7]. It is presently unclear how they interact to bring about the hyperphosphorylated state of PHF-tau. Two types of kinase interactions in tau phosphorylation were investigated in this study: non-PDPK-non-PDPK and PDPK-non-PDPK interactions. Four non-PDPKs (A-kinase, C-kinase, CaM kinase II, CK-1) were used. Each non-PDPK was used to prephosphorylate tau and the latter in turn used as a substrate for the other three non-PDPKs. Overall our results suggest that prephosphorylation of tau by one non-PDPK can positively modulate the activity of other non-PDPKs in the phosphorylation of tau. Previously [17, 18] we have shown that prior phosphorylation of tau by non-PDPKs serve to stimulate a subsequent phosphorylation catalyzed by a PDPK (GSK-3). In the present study we have asked whether the opposite situation is also true. That is, can prephosphorylation of tau by PDPK (GSK-3) serve to stimulate the level of phosphorylation achieved by non-PDPKs. Surprisingly, we found that for all four non-PDPKs (A-kinase, C-kinase, CK-1, CaM kinase II) tau prephosphorylated by GSK-3 was a

slightly worse substrate compared to tau that was not prephosphorylated. These observations are still to be further tested using other PDPKs and non-PDPKs that are tau kinases. Nevertheless, our results suggest that prior phosphorylation of tau by non-PDPKs may serve to stimulate the activities of both PDPKs and non-PDPKs. Alternatively, prephosphorylation of tau by a PDPK (such as GSK-3) may serve to inhibit the activities of non-PDPKs.

The phosphorylation of human tau versus bovine tau by different kinases deserves further comment. In our initial studies [18, 21, 25] we used bovine brain tau as a substrate for different kinases. This tau preparation is a mixture of six different isoforms [44]. Though the isoforms are highly homologous proteins, it has been reported that some tau isoforms may be better substrates for kinases compared to others [26]. Hence, we have recently started studying the phosphorylation of individual human tau isoforms by different kinases ([17]; this study). Both bovine and human (tau 39 isoform) taus were phosphorylated by all the individual kinases, with some differences in the level of phosphorylation observed. Major differences, however, were detected in the ability of GSK-3 to phosphorylate tau that was prephosphorylated by different non-PDPKs. For instance, prephosphorylation of human tau 39 by CaM kinase II stimulated a subsequent phosphorylation by GSK-3 several-fold compared to tau that was not prephosphorylated [17]. A similar stimulation was not observed with bovine tau prephosphorylated by CaM kinase II [18]. Further, prephosphorylation of human tau 39 by A-kinase and CaM kinase II dramatically increased the subsequent rate of induction of the SMI31, PHF-1, and M4 antibody epitopes by GSK-3. Prephosphorylation of bovine tau by these two non-PDPKs did not increase the rate at which these antibody epitopes were induced [17, 18]. It is possible that protein-protein interactions among the bovine tau isoforms may inhibit the increased rate of induction of the SMI31, PHF-1 and M4 epitopes observed with human tau 39 after prephosphorylation by the non-PDPKs.

Until quite recently a direct conversion of tau to an Alzheimer-like state by a non-PDPK was not demonstrated. In a separate study [21] we have shown that after phosphorylation of bovine tau by CK-1, increased binding primarily of SMI34 (and to lesser extents, SMI31 and PHF-1) was induced. These antibodies have all been demonstrated to bind to phosphorylated epitopes on PHF-tau [27, 30]. Hence, their binding suggest that CK-1 has phosphorylated some of the same sites found in PHF-tau which are necessary for binding of these antibodies. In the present study (using human tau 39) we have tested whether various combinations of non-PDPKs can phosphorylate some of the sites found in PHF-tau. Our results indicate that only CK-1, either singly or in combination, can phosphorylate such sites (as evidenced by binding of SMI31, SMI34 and M4). This fact, as well as the finding that tau can be rapidly phosphorylated to high stoichiometries by CK-1 ([17, 18, 25]; this study), suggests a potential important role of this non-PDPK in the conversion of normal tau to PHF-tau. The observation that a CK-1-like kinase is associated with PHF lends further support to this hypothesis [39].

The induction of Alzheimer-like epitopes on human tau by a PDPK (GSK-3) and a non-PDPK (CK-1) can be compared. First, when acting alone these kinases induce Alzheimer-like epitopes (SMI31, PHF-1, SMI34, M4) only slowly. Second, prephosphorylation of human tau 39 by non-PDPKs (A-kinase, CaM kinase II) can increase the subsequent rate of induction of the SMI31, PHF-1, and M4 epitopes by GSK-3. A similar prephosphorylation also increased the rate of induction of the SMI31 and M4, but not the PHF-1, epitope by CK-1 ([17]; this study). Third, prephosphorylation of tau 39 by CK-1 increased the subsequent rate at which GSK-3 induced the SMI31 epitope. Reciprocally, prephosphorylation of tau 39 by GSK-3 increased the subsequent rate of induction of the SMI31 (and SMI34, M4) epitope by CK-1 ([17]; this study). These results can possibly be explained by considering the kinetics of phosphorylation of the SMI31 epitope either by GSK-3 or CK-1. When acting alone GSK-3 requires greater than 16 h, whereas about 5 h is needed by CK-1, to maximally induce this epitope. After prephosphorylation of tau 39 by CK-1 only 1 h is required for maximal induction of the SMI31 epitope by GSK-3 ([17]; this study). In the present study (see Figs 4–6 for details) tau 39 was prephosphorylated with GSK-3 for 2 h. Under these conditions the SMI31 epitope will be induced only partially [17]. However, after this prephosphorylation by GSK-3 the conformation of tau will be so changed that the SMI31 epitope now becomes a good substrate for CK-1 and is thus further phosphorylated.

The finding that tau can be converted to an Alzheimer-like state after phosphorylation by CK-1 has important implications. First, the epitopes for SMI31 (P-ser 396, P-ser 404), SMI34 (phosphorylated residues on either side of the microtubule binding domains) [30] and M4 (P-thr 231) [31] are known to be followed by pro. The specificity determinants for CK-1 have been reported to be acidic residues primarily on the N-terminal side of the ser or thr to be phosphorylated [40]. To date pro has not been reported as a specificity determinant of CK-1. Hence our results suggest that the epitopes for SMI31, SMI34, and M4 may be directly phosphorylated by CK-1. If this is the case, then CK-1 would have to be reclassified as both a PDPK as well as a non-PDPK. Such a classification is true of GSK-3 which phosphorylates both proline-dependent and proline-independent sites in various protein substrates [41]. Second, it is possible that CK-1 may not directly phosphorylate the proline-dependent sites (thr 231, ser 396, ser 404) in the antibody epitopes. Instead, this kinase may phosphorylate other proline-independent sites that can possibly modify binding of SMI31, SMI34 and M4.

Binding of Tau-l to tau has been reported to be affected by the phosphorylation state of ser 199 and ser 202 [32]. However, it was recently demonstrated using synthetic phosphopeptides that the phosphorylation of other sites upstream and downstream of these two residues can also affect Tau-1 binding rather dramatically [42, 43].

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