Modulation of GSK-3-catalyzed phosphorylation of microtubule-associated protein tau by non-proline-dependent protein kinases

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Abstract The phosphorylation of bovine tau, either by GSK-3 alone or by a combination of GSK-3 and several non-proline-dependent protein kinases (non-PDPKs), was studied. GSK-3 alone catalyzed the incorporation of ~3 mol ^{32}P/mol tau at a relatively slow rate. Prephosphorylation of tau by A-kinase, C-kinase, or CK-2 (but not by CK-1, CaM kinase II or Gr kinase) increased both the rate and extent of a subsequent phosphorylation catalyzed by GSK-3 by several-fold. These results suggest that the phosphorylation of tau by PDPKs such as GSK-3 (and possibly MAP kinase, cdk5) may be positively modulated at the substrate level by non-PDPK-catalyzed phosphorylations.

Key words: GSK-3; Tau protein; Protein kinase; Alzheimer's disease; Paired helical filament

1. Introduction

The microtubule-associated protein tau is the main component found in the paired helical filaments (PHF) of Alzheimer disease (AD) brain. Compared to tau from normal brain, PHF-tau is hyperphosphorylated. Twenty phosphorylation sites have been identified in PHF-tau. Eleven of these sites are on Ser/Thr-Pro motifs [1]. These sites are probably phosphorylated by proline-dependent protein kinases (PDPKs). Members of this class include MAP kinase, GSK-3, and cdk5. The identities of the kinases responsible for the phosphorylation of the other nine sites in PHF-tau are not known.

Tau has been shown to be a substrate in vitro for several protein kinases. Stoichiometric phosphorylation was achieved with cyclic AMP-dependent protein kinase (A-kinase) [2,3] calcium/calmodulin-dependent protein kinase II (CaM kinase II) [3,4], protein kinase C [3,5], MAP kinase [6,7], GSK-3 [8–10], cdc2 kinase [11], cdk5 [12], casein kinase-1 (CK-1), and a calcium/calmodulin-dependent protein kinase from rat cerebellum (Gr kinase) [3].

The phosphorylation of tau in vivo may involve multiple kinases, both PDPKs and non-PDPKs. It is not known how such kinases may interact to bring about the final phosphorylation state of tau. One possibility is that the phosphorylation state of tau itself may serve as a determinant to dictate substrate specificity of different kinases. Such a mode of regulation for GSK-3 is well known using other protein substrates. The phosphorylation of glycogen synthase [13], the R11 subunit of A-kinase [14], phosphatase inhibitor-2 [15], and protein phosphatase-1 G-subunit [16] by GSK-3 are enhanced when these proteins are first phosphorylated by a different kinase. An initial phosphorylation of tau by a PDPK (TPKII/cdk5) was reported to stimulate a subsequent phosphorylation catalyzed by TPK I/GSK-3 [17]. Alternatively, prior phosphorylation of tau by one kinase may serve to inhibit the action of a second kinase.

In this study we have investigated the interactions among PDPKs and non-PDPKs in the phosphorylation of tau. We have analyzed how the specificity of GSK-3 (a PDPK) is affected when the initial phosphorylation state of tau is altered by various non-PDPKs.

2. Experimental

2.1. Materials

Bovine brain tau was purified from microtubules obtained by three cycles of assembly/disassembly as described [18]. CK-1 and CK-2 [19] were purified from bovine brain whereas GSK-3 [20] was from rat brain. The GSK-3 preparation contained both the α and β isoforms in the ratio 3:2. CaM kinase II, Gr kinase [21], and C-kinase [22] were purified from rat brain. CaM kinase II and Gr kinase were gifts from Brad McDonald, Burroughs-Wellcome Laboratories; C-kinase was generously supplied by V. Chauhan of this Institute. The catalytic subunit of A-kinase and calmodulin were purchased from Sigma Chemical Co. whereas calf alkaline phosphatase was from Boehringer-Mannheim. [γ-^{32}P]ATP was purchased from ICN Biomedicals.

2.2. Methods

Tau was phosphorylated by GSK-3 in a reaction mixture normally containing 0.15 mg/ml tau, 6 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.1 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 [3]. Reactions were initiated at 30°C by addition of [γ-^{32}P]ATP and, after the desired incubation time, stopped by addition of SDS-stop solution and processed as described [3]. GSK-3 activity was measured using myelin basic protein as a substrate. One unit of GSK-3 activity is defined as the amount of the kinase that will catalyze the incorporation of 1 nmol ^{32}P/min into myelin basic protein (1 mg/ml) at 30°C.

Tau was dephosphorylated with calf intestine alkaline phosphatase as previously described [17].

3. Results

3.1. Phosphorylation of tau by GSK-3

It was previously shown that maximal phosphorylation of tau by GSK-3 requires up to 20 h incubation at 37°C [8]. Since

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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; CK-2, casein kinase-2; Gr kinase, calcium/calmodulin-dependent protein kinase from rat cerebellum; GSK-3, glycogen synthase kinase-3; MAP kinase, mitogen-activated protein kinase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PDPK, proline-dependent protein kinase.

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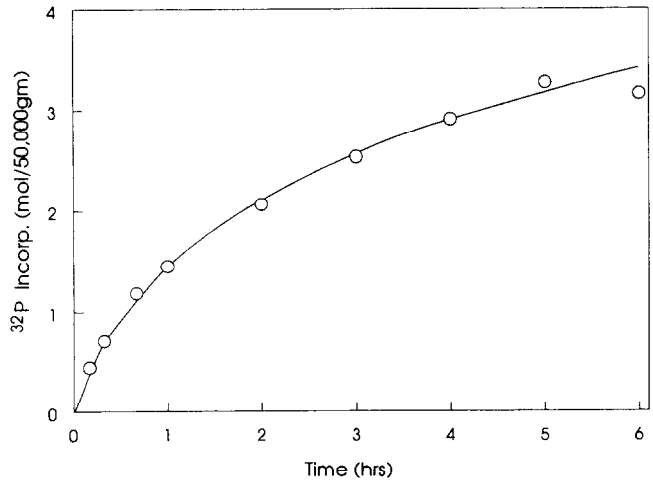

Fig. 1. Time-course of phosphorylation of tau by GSK-3. Tau was phosphorylated by GSK-3, aliquots of the phosphorylation mixture were removed at different times and the reaction stopped by addition of SDS-stop solution. ^{32}P-labelled tau was resolved from other proteins by SDS-PAGE, excised from the gels and ^{32}P incorporation determined by Cerenkov counting.

phosphorylation events occur relatively rapidly in cells, we have further examined tau phosphorylation by GSK-3. We first optimized the reaction conditions. We established that maximal ^{32}P incorporation into tau by GSK-3 occurred at 3–5 mM $MgCl_2$ and at 0.1 mM ATP at pH 7.5. $MnCl_2$ could not substitute for $MgCl_2$ in reaction mixtures: in the presence of 3 mM $MnCl_2$, GSK-3 activity was less than 2% of that found with 3 mM Mg^{2+} (data not shown). Under these optimal conditions GSK-3 catalyzed the incorporation of ~3 mol ^{32}P into tau in 4 h/30°C (Fig. 1). The first ~1.5 mol ^{32}P is incorporated in 1 h, additional phosphorylation occurring only slowly (~1.5 mol ^{32}P/3 h).

3.2. *Phosphorylation of tau by a combination of GSK-3 and other kinases*

One possible reason to explain the slow rate at which tau is phosphorylated by GSK-3 (Fig. 1 and [8]) is that the phosphorylation of other sites on tau by a second kinase may be required. Such modulation of GSK-3 activity at the level of the protein substrate has previously been shown [13–16]. We therefore separately phosphorylated tau with six different kinases (CK-1, CK-2, A-kinase, C-kinase, CaM kinase II, Gr kinase) and used these different phosphorylated tau species as substrates for GSK-3 (Figs. 2 and 3). The phosphorylation of tau by the above six kinases has been studied in detail elsewhere [3], and will be dealt with here only briefly. After 60 min, ^{32}P incorporation catalyzed into tau by A-kinase, C-kinase, CK-2, CK-1, CaM kinase II, and Gr kinase was approximately 2.0 mol, 1.6 mol, 0.6 mol, 3.5 mol, 3.0 mol, and 1.4 mol, respectively (Figs. 2 and 3). When these different phosphorylated species of tau were used as substrates for GSK-3, the latter kinase further phosphorylated tau. After 60 min incubation, the net ^{32}P incorporation catalyzed by GSK-3 was 2.8 mol, 3.3 mol, 4.2 mol, 1.1 mol, 1.0 mol, and 2.2 mol for tau that was prephosphorylated with A-kinase, C-kinase, CK-2, CK-1, CaM kinase II, and Gr kinase, respectively.

From the results of Figs. 2 and 3 it is clear that GSK-3 in combination with another kinase catalyzed a higher ^{32}P incorporation into tau than achieved by that kinase alone. From the data of Figs. 2 and 3, the net ^{32}P incorporation into tau catalyzed by GSK-3 alone was calculated. This was then compared with ^{32}P incorporation achieved by GSK-3 using tau that was not prephosphorylated. The results are shown in Fig. 4. It can be seen that when tau was prephosphorylated by A-kinase, C-kinase, and CK-2, both the rate and extent of ^{32}P incorporation was increased compared to tau that was not prephosphorylated. The rate of ^{32}P incorporation was increased 3.4-, 2.5-, and 2.4-fold and its extent (after 60 min) 1.9-, 2.3-, and 2.9-fold, respectively (Fig. 4A). After prephosphorylation of tau by CK-1 or CaM kinase II, both the rate and extent of

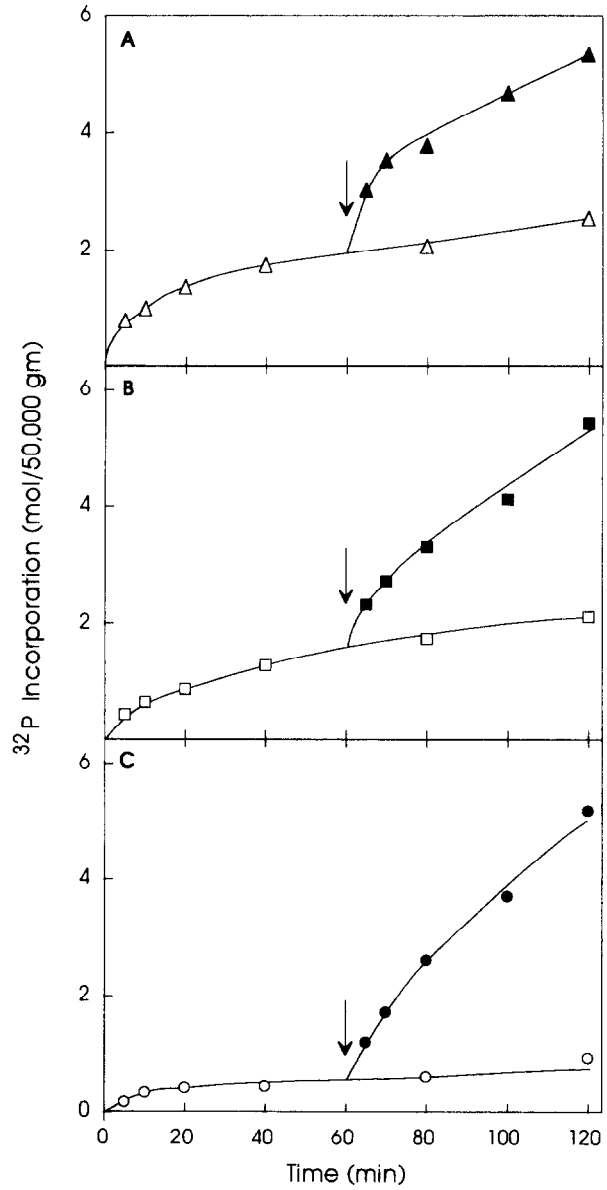

Fig. 2. Phosphorylation of tau by a combination of A-kinase, C-kinase, CK-2, and GSK-3. Tau was separately phosphorylated by A-kinase (△,A), C-kinase (□,B), and CK-2 (○,C), and aliquots removed at different times for determination of ^{32}P incorporation. After 60 min (↓), aliquots of the reaction mixtures were separately removed and supplemented with GSK-3 (▲,■,●). The incubations were continued and aliquots removed at different times to determine ^{32}P incorporation into tau.

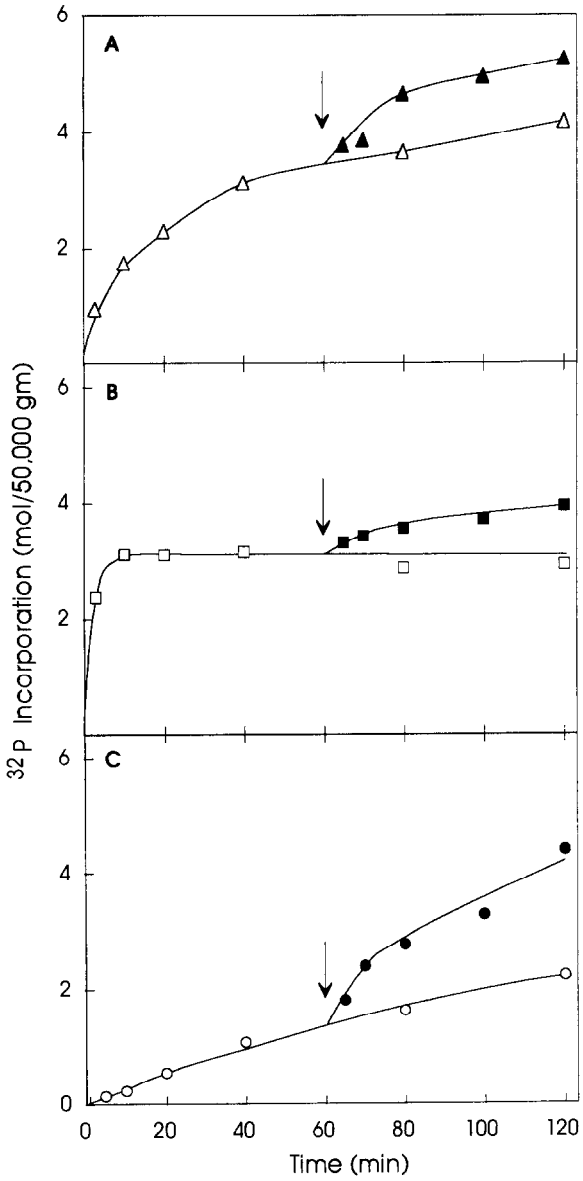

Fig. 3. Phosphorylation of tau by a combination of CK-1, CaM kinase II, Gr kinase, and GSK-3. Tau was separately phosphorylated by CK-1 (△,A), CaM kinase II (□,B) and Gr kinase (○,C). After 60 min (↓), aliquots of the reaction mixtures were separately removed and supplemented with GSK-3 (▲,■,●) and the incubations continued.

^{32}P incorporation by GSK-3 was decreased. Although the rate is also decreased after prephosphorylation with Gr kinase, the extent of ^{32}P incorporation is slightly elevated (1.5-fold) (Fig. 4B). These results suggest that GSK-3-catalyzed phosphorylation of tau may be positively modulated by A-kinase, C-kinase, and CK-2 but not by CK-1, CaM kinase II or Gr kinase.

3.3. Role of endogenous tau phosphorylation

It was previously shown that purified bovine tau is already phosphorylated at Ser-144 and Ser-315. Treatment of such tau with calf alkaline phosphatase can completely remove the phosphates from these sites [17]. We investigated what role, if any, these endogenous phosphates play in modulating the specificity of the seven kinases for different phosphorylation sites on tau. For these studies tau was dephosphorylated with calf alkaline phosphatase as described [17]. The rate and extent of phosphorylation of the dephospho-tau and non-dephospho-tau by GSK-3 and the six other kinases were then compared. A typical result is shown in Fig. 5. It can be seen that the same rate and extent of ^{32}P incorporation is catalyzed by A-kinase whether tau was initially dephosphorylated (Fig. 5B) or not (Fig. 5A). Similarly, no differences in the phosphorylation of these two species of tau was observed using C-kinase, CK-2, CK-1, CaM kinase II, Gr kinase, or GSK-3 (data not shown). Further, a repeat of the experiments outlined in Fig. 2 using dephosphorylated tau and a combination of GSK-3 and A-kinase, C-kinase, or CK-2, did not change the previous results. Fig. 5 also shows such an experiment using a combination of A-kinase and GSK-3. These results suggest that phosphorylation of Ser-144 and Ser-315 do not play a major role in modulating the specificity of GSK-3 for different sites on tau.

4. Discussion

In an earlier study [3] we have shown that tau can be stoichiometrically phosphorylated by five non-PDPKs: A-kinase, C-kinase, CK-1, CaM kinase II, and Gr kinase. Substoichiometric phosphorylation was obtained with a sixth non-PDPK–CK-2. CK-1 and CaM kinase II catalyzed the highest levels of ^{32}P incorporation into tau. When these two kinases were used in combination, an even higher level of ^{32}P incorporation into tau was achieved [3].

In the present study, the phosphorylation of tau by GSK-3 was studied. This kinase catalyzed the incorporation of ~2 mol ^{32}P/mol tau in 2 h. Additional phosphorylation of tau occurred only slowly. It has been established previously that the rate and extent of GSK-3-catalyzed phosphorylation can be modulated by a prior phosphorylation of the particular protein substrate [13–16]. Hence, we have explored the potential interaction between GSK-3 and six non-PDPKs (A-kinase, C-kinase, CK-1, CK-2, CaM kinase II, Gr kinase). We reasoned that prephosphorylation of tau by one or more of these kinases may make tau a better substrate for GSK-3. Our results indicate that prephosphorylation of tau by three of the kinases (A-kinase, C-kinase, CK-2) increased both the rate and level of ^{32}P incorporation catalyzed by GSK-3 several-fold. Although both A-kinase and CK-2 have been shown to modulate GSK-3 activity by prephosphorylation of other protein substrates [13 16], this is the first report of such modulation by C-kinase. After prephosphorylation by CK-2, for example, GSK-3 catalyzed an incorporation of greater than 4 mol ^{32}P/60 min. By comparison, a 20 h incubation with GSK-3 (no prephosphorylation of tau) was required to achieve this level of phosphorylation [8]. In a separate study [9], 4 mol ^{32}P/mol tau were incorporated after 3 h incubation with GSK-3. Even though the time is shorter compared to the previous study (3 h vs. 20 h), it is still inferior compared to that observed when tau is prephosphorylated with CK-2 (1 h vs. 3 h). In addition, the concentration of GSK-3 (25 μg/ml) used in the latter study [9] was relatively high. This can be compared to 2 μg/ml GSK-3 used in our study. Unlike A-kinase, C-kinase, and CK-2, prephosphorylation of tau by CK-1, CaM kinase II or Gr kinase did not enhance the subsequent GSK-3-catalyzed phosphorylation. On the contrary, GSK-3-catalyzed ^{32}P incorporation decreased after prephosphorylation of tau by CK-1 and CaM kinase II. These

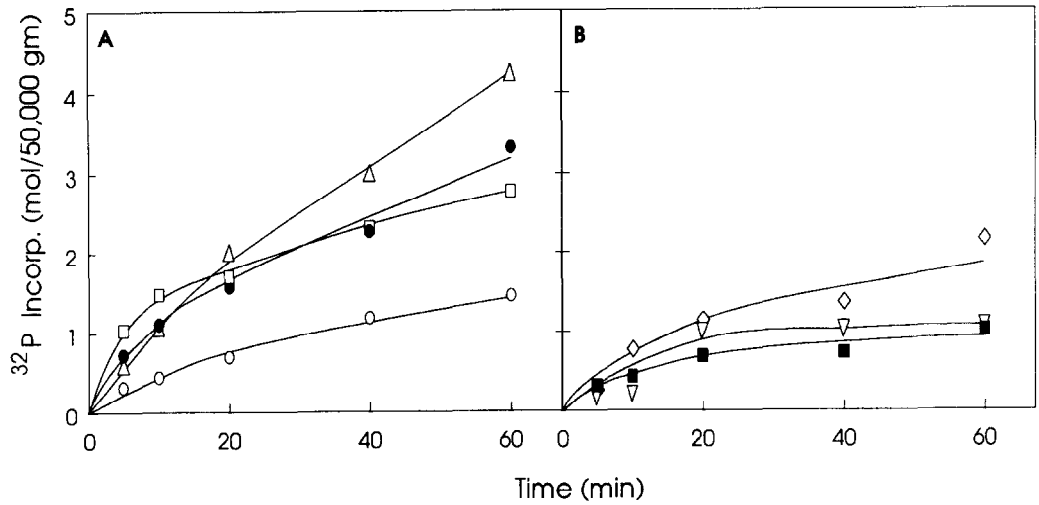

Fig. 4. Comparison of the GSK-3-catalyzed phosphorylations of tau which was prephosphorylated by various non-PDPKs. Tau was separately prephosphorylated for 60 min by CK-2 (△), C-kinase (●), A-kinase (□), Gr kinase (◇), CK-1 (▽), and CaM kinase II (■) and then used as a substrate for GSK-3. The net ^{32}P incorporation catalyzed by GSK-3 alone is shown. For comparison, GSK-3 catalyzed phosphorylation of tau that was not prephosphorylated by any kinase (○) is shown.

results suggest that after an initial phosphorylation of tau by the latter two kinases, it assumes conformations that are unfavorable for subsequent GSK-3-catalyzed phosphorylations.

We have also evaluated how the specificities of the different kinases are affected by endogenous phosphorylation of tau. It was previously shown that Ser-144 and Ser-315 are already in a phosphorylated state in purified bovine tau. These phosphates could be removed by alkaline phosphatase [17]. We have compared the rates of phosphorylation of dephospho-tau and non-dephospho-tau by GSK-3, A-kinase, C-kinase, and CK-2, either singly or in combination. Our results suggest that the phosphorylation states of Ser-144 and/or Ser-315 do not modulate the site specificities of GSK-3 or the other kinases. CK-2 and C-kinase have been shown to phosphorylated Thr-39 [23] and Ser-324 (equivalent to Ser-313 in bovine tau) [5], respectively, in human tau 40 (441 amino acids). A-kinase also phosphorylated Ser-324, in addition to Ser-214, Ser-356, Ser-409, and Ser-416 [24]. From our results it can be inferred that prephosphorylation of Thr-39 and Ser-324 changes tau conformation in such a way that additional sites not normally accessible to GSK-3 can now be readily phosphorylated by this kinase. Previous studies have shown that GSK-3 can phosphorylate Ser-199, Thr-231, Ser-235, Ser-396, Ser-404, and Ser-413 in human tau 40 [9,25]. It is still to be clarified whether the higher levels of phosphorylation of tau by GSK-3 (see Fig. 2) lead to increased inhibition of tau-stimulated microtubule assembly. Phosphorylation of tau by either A-kinase or C-kinase was shown to inhibit such assembly [24,26].

Our results help to emphasize the importance of the phosphorylation state of tau in dictating kinase specificity. Since PHF-tau is phosphorylated at both pro- and non-pro-directed sites [1], both PDPKs and non-PDPKs may participate in bringing about its hyperphosphorylated state. In this study we have demonstrated that the specificity of a PDPK (GSK-3) can be modulated by several non-PDPKs. A similar type of modulation of the specificities of other PDPKs (MAP kinase, cdk5)

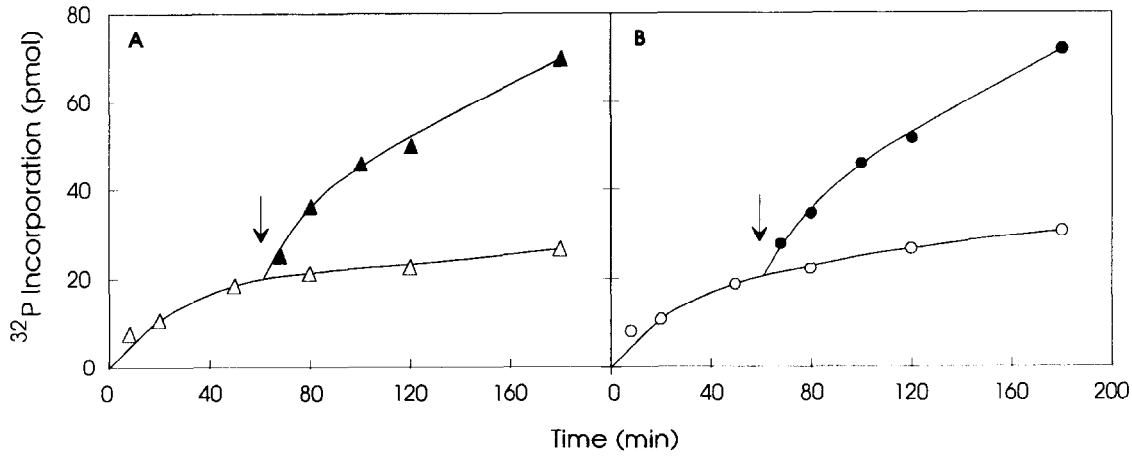

Fig. 5. Comparison of the phosphorylations of dephosphorylated tau with tau not previously dephosphorylated. Tau that was either dephosphorylated (B) with alkaline phosphatase or was not dephosphorylated (A) was used as a substrate for A-kinase (△,○). After 60 min (↓), aliquots of the reaction mixtures were separately removed and supplemented with GSK-3 (▲,●) as described for Fig. 2.

by the same or other non-PDPKs is also possible. Other types of modulation such as PDPK-PDPK and non-PDPK-non-PDPK are also possible in tau phosphorylation. It has been reported that a prior phosphorylation of tau by TPK II/cdk5 (a PDPK) can modulate the site specificity of TPK I/GKS-3 (another PDPK) [17].

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