Biochemical and Biophysical Research Communications **273,** 209–212 (2000)
doi:10.1006/bbrc.2000.2926, available online at http://www.idealibrary.com on IDEAL®

Dual Effects of PKNα and Protein Kinase C on Phosphorylation of Tau Protein by Glycogen Synthase Kinase-3β

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Received May 19, 2000

We analyzed the effects of PKNα and protein kinase C (PKC) on phosphorylation of tau protein by glycogen synthase kinase (GSK)-3β using monoclonal antibodies (AT8, AT180, and AT270). These antibodies are highly specific for phosphorylated tau in Alzheimer paired helical filaments, and recognize phosphorylated Ser202/Thr205, Thr231, and Thr181 of tau protein, respectively. Immunoblot analysis demonstrated that PKNα and PKC did not directly phosphorylate their sites, whereas GSK-3β efficiently did so. Incubating GSK-3β with PKNα or PKC subtypes inhibited subsequent GSK-3β-induced AT8 and AT270 immunoreactivity. However, the constitutive active form of the GSK-3β(S9A) mutant was almost totally inert to each enzyme. Incubating tau with PKNα increased the GSK-3β-induced AT180 immunoreactivity, which was further enhanced when the S9A mutant was used instead of the wild type GSK-3β. These results suggest that PKNα and PKC directly inhibit GSK-3β activity at least in part by phosphorylating Ser9 of GSK-3β, and that they indirectly suppress GSK-3β-stimulated phosphorylation of tau at amino acids Ser202/Thr205 and Thr181, but enhanced phosphorylation at Thr231 through phosphorylation at other sites of tau.

Key Words: **PKNα; PKC; tau protein; Alzheimer.**

Paired helical filaments (PHFs) that accumulate in affected neurons associated with Alzheimer's disease (AD) consist of hyper phosphorylated tau that exhibits electrophoretic and antigenic properties distinct from that of tau in the normal adult central nervous system (1, 2). Whether or not accumulation of hyperphosphorylated tau and/or actual PHF formation are causative of AD neuropathology or instead represent end-stage "byproducts" remain controversial. Nevertheless, their presence is indicator of AD. Accordingly, one way to understand the onset and progression of AD is to determine the kinase(s) responsible for phosphorylating tau. Several protein kinases, such as Ca^{2+}-calmodulin kinase, cyclin-dependent kinases, mitogen-activated (MAP) kinase and glycogen synthase kinase (GSK), phosphorylate tau in a manner that alters the antigenicity and electrophoretic migration characteristic of tau from AD brains. PHFs are induced by not one, but several kinases.

The serine/threonine kinase, PKNα, is activated by fatty acids and small GTPase Rho, and it has a catalytic domain that is highly homologous to that of protein kinase C (PKC) in the C-terminal region (3–6). This kinase phosphorylates tau *in vitro* and *in vivo* and is enriched in neurons, where it is concentrated in a subset of endoplasmic reticulum (ER), ER-derived vesicles, as well as in nuclei within the central nervous system (CNS) (7). In AD-affected neurons, PKNα is closely associated with neurofibrillary tangles (NFTs) and their major constituent, abnormally modified tau (7). This raises the possibility that PKNα is involved in the production of PHFs. The PKC family consists of at least 11 structurally related phospholipid-dependent serine/threonine protein kinases that are directly involved in the transmission of a wide variety of extracellular signals (8, 9). PKC also phosphorylates tau. It is postulated that PKN and PKC can mediate upstream signals to the same target in the CNS (10). The present study examines the effects of PKNα and PKC subtypes on the GSK-3β-mediated phosphorylation of tau.

Abbreviations used: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; GSK, glycogen synthase kinase.

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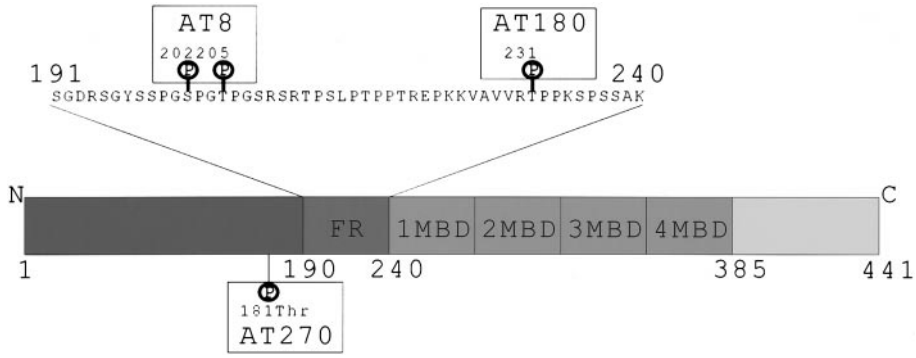

FIG. 1. Epitopes of antibodies on tau protein. The bar shows human tau (the longest isoform). Phosphorylated residues recognized by each monoclonal antibody are shown above and below the bar.

MATERIALS AND METHODS

Preparation of recombinant proteins. A human tau cDNA clone was provided by Dr. H. Mori. This cDNA encodes 383 amino acids of tau isoform. Tau was expressed in *Escherichia coli* using variants of the pTric-His vector (Invitrogen), and the proteins were purified by nickel column chromatography according to the manufacturer's instructions (Qiagen). The numbering of amino acid residues refers to the longest isoform of human tau (441 residues, Fig. 1). Full length PKNα fused to GST (GST/PKNα), the catalytic domain (GST/PKNα/AF3), and the kinase negative form of the catalytic domain (GST/PKNα/AF3 (K644E)) of human PKNα cDNA were expressed in Sf9 cells as described (11). The full length cDNA of each rat PKC subtype (δ (12), and ζ (13)) and human GSK-3β (provided by Dr. J. R. Woodegett) were inserted into the baculovirus transfer vector, pBlueBacHis/GST (11) to express each protein as a GST fused protein in Sf9 cells. A GSK-3β(S9A) mutant of the Serine 9 residue of wild type human GSK-3β to Alanine, was constructed by site directed mutagenesis using the QuickChange kit (Stratagene).

Phosphorylation of tau by each protein kinases. Phosphorylation proceeded at 30°C for 5 min in a reaction mixture containing 20 mM Tris/HCl at pH 7.5, 4 mM $MgCl_2$, 40 μM ATP, 616 kBq of [γ-^{32}P] ATP, 40 μM arachidonic acid, 3 μg recombinant tau and 200 ng of recombinant enzyme. Reactions were terminated by adding an equal volume of Laemmli's sample buffer. The mixtures were resolved by SDS–PAGE, and the gel was dried under vacuum. Phosphorylation was visualized using the Imaging analyzer, PACKARD INSTANT IMAGER.

Effects of PKNα and PKC subtypes on the phosphorylation of tau by GSK-3β. Tau protein was phosphorylated at 30°C for 4 h in a mixture containing 20 mM Tris/HCl at pH 7.5, 4 mM $MgCl_2$, 1 mM ATP, 40 μM arachidonic acid, 3 μg recombinant tau, 200 ng recombinant enzyme and 200 ng recombinant GSK-3β. To incubate GSK-3β with PKNα or PKC subtypes, GSK-3β was phosphorylated at 30°C for 30 min by individual PKC subtypes or PKNα in the absence of recombinant tau. Thereafter, tau was added and the samples were incubated at 30°C for 4 h. To incubate tau with PKNα, tau was phosphorylated at 30°C for 30 min by PKNα in the absence of GSK-3β. Thereafter, GSK-3β(S9A) was added and the samples were incubated at 30°C for 4 h. Reactions were terminated by adding an equal volume of Laemmli's sample buffer. The samples were resolved by SDS–PAGE, and phosphorylation was analyzed by immunoblotting.

Immunoblotting. Immunoblot analysis was performed as described (14). The monoclonal antibodies, AT8, AT180, and AT270 were purchased from Innogenetics. Blots were visualized using enhanced chemiluminescence.

RESULTS AND DISCUSSION

We reported that PKNα is closely associated with NFTs at an early stage of their development and that tau protein is efficiently phosphorylated *in vitro* by PKNα (7). We therefore surmised that PKNα is involved in the "AD-like" phosphorylation of tau protein. Monoclonal antibodies, AT8, AT180, and AT270 recognize phosphorylated Ser202/Thr205, Thr231, and Thr181 of tau protein, respectively (Fig. 1). These residues are fully phosphorylated in Alzheimer's and in fetal tau. Therefore, we first analyzed the phosphorylation of these residues of tau by PKNα and PKC subtypes using monoclonal antibodies. Recombinant enzymes fused to GST were affinity-purified from Sf9 cells (Fig. 2A). Figure 2B shows that tau was phosphorylated by each of the enzymes. However, immunoreactivity was not detected with any antibodies when tau protein was incubated with PKNα or PKC subtypes (data not shown), indicating that PKNα and PKC subtypes did not directly phosphorylate Thr181, Ser202/Thr205, and Thr231 of tau protein.

It is known that these sites were phosphorylated by GSK-3β. Because PKC stimulates c-Jun DNA binding by inhibiting the phosphorylation of c-Jun by GSK-3β (16) and since PKCβII stimulates the Wnt/adenomatous polyposis coli (APC)/β-catenin proliferative signaling pathway *in vivo* by inhibiting GSK-3β (17), we examined the effect of PKNα and each PKC subtype on phosphorylation at Thr181, Ser202/Thr205, and Thr231 induced by GSK-3β (18). Incubating GSK-3β with PKNα significantly suppressed subsequent AT8 and AT270 immunoreactivity (Fig. 3, top and bottom, lane 2). The result was similar when GSK-3β was initially incubated with the active form of PKNα or each PKC subtype (Fig. 3, top and bottom, lanes 3, 5 and 6), whereas the kinase negative form of PKNα did not suppress the immunoreactivity (Fig. 3, lane 4). The activity of GSK-3β is inhibited by the phosphorylation of Ser9 of GSK-3β (19) by different

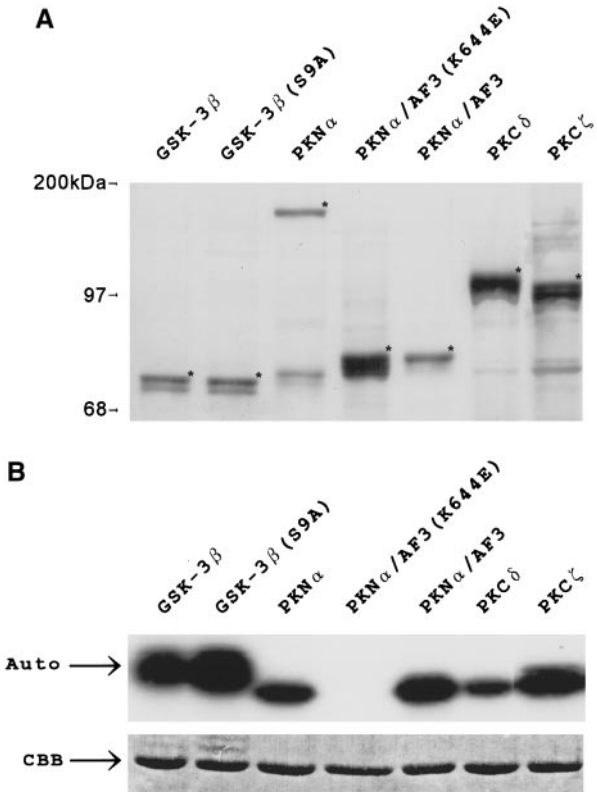

FIG. 2. Phosphorylation of tau protein. (A) Silver staining for recombinant kinases. Asterisk indicates each kinase. (B) Phosphorylation of tau protein by each protein kinase. Top: autoradiography (auto) of tau phosphorylation. Bottom: Coomassie Brilliant Blue (CBB) staining of tau in each reaction.

upstream kinases (p90rsk-1, and PKB/Akt), and GSK-3β becomes resistant to this inhibition by replacing Ser9 with Alanine. Thus, we performed the same

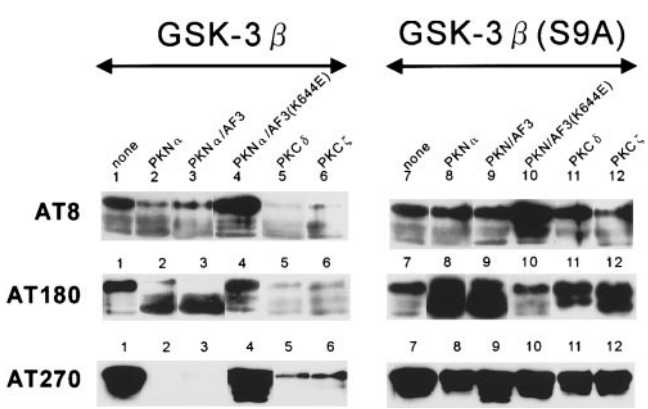

FIG. 3. Effect of GSK-3β incubated with PKNα or PKC subtypes on immunoreactivity of AT8, AT180, and AT270 monoclonal antibodies. Before phosphorylation of tau by GSK-3β or GSK-3β(S9A), GSK-3β or GSK-3β(S9A) were incubated with indicated protein kinases. Thereafter, tau protein was added as described under Materials and Methods.

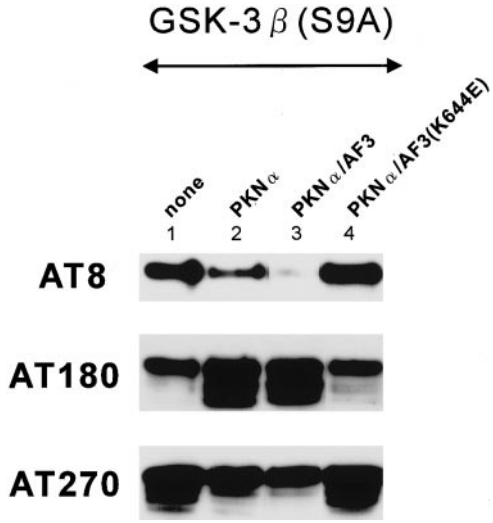

FIG. 4. Effect of tau protein incubated with PKNα or PKC subtypes on immunoreactivity of AT8, AT180, and AT270 monoclonal antibodies. Before phosphorylation of tau by GSK-3β(S9A), tau protein was incubated with indicated protein kinases. Thereafter, GSK-3β(S9A) was added as described under Materials and Methods.

experiment using GSK-3β(S9A) to verify whether these results were derived by phosphorylation of Ser9 by PKNα or by PKC. Incubating GSK-3β(S9A) with PKNα or each PKC subtype did not suppress AT8 and AT270 immunoreactivity (Fig. 3, top and bottom, lanes 8–12). These results suggest that PKNα and PKC inhibit GSK-3β activity at least in part by phosphorylating the Ser9 of GSK-3β. On the other hand, wild type GSK-3β incubated with PKNα did not suppress the total immunoreactivity against AT180, although more immunoreactive bands migrated faster in the gels (Fig. 3, middle, lanes 2 and 3). When the S9A mutant of GSK-3β was added instead of wild type GSK-3β, AT180 immunoreactivity was significantly increased, (Fig. 3, middle, lane 8 and 9).

Next, we examined the effect of incubating tau with PKNα on the phosphorylation of these sites by GSK-3β. To avoid the effect of inhibitory phosphorylation on Ser9 of GSK-3β by PKNα, we used the GSK-3β(S9A) mutant. Figure 4 shows that incubating of tau with PKNα suppressed immunoreactivity against AT8 and AT270. On the other hand, AT180 immunoreactivity was significantly enhanced. These results suggest that PKNα changes the conformation of tau, making it difficult for GSK-3β to access Thr181 and Ser202/205, but facilitating access Thr231. Reports indicate that GSK-3β is a kinase that changes normal tau to "AD-like" tau by phosphorylation *in vitro* (20). Almost all GSK-3β targeted residues were phosphorylated in "AD-like" tau, and one of them is regulated by sequential phosphorylation. The epitope was Thr212, and the complete phosphorylation is supposed to require the phosphorylation of Ser214 by PKA (21).

Our study suggests that PKNα and PKC induce a conformational change of phospho-epitopes. Phosphorylation was enhanced more by the GSK-3β(S9A) mutant than by wild type GSK-3β. These results suggest that PKNα and PKC have dual regulation functions such as a conformational change of tau and the inhibition of GSK-3β, both caused by phosphorylation. Immunoreactivity against AT180 was enhanced in the presence of PKNα and PKC *in vitro*. A similar effect of MAP and other kinases has been reported (22, 23). These results are supported by the similar biphasic generation of immunoreactivity towards the conformation dependent ALZ-50 epitope (24). These results suggest that the phosphorylation of tau by PKNα may play crucial roles in AD pathology by regulating the biological functions of tau. The present study did not find a significant difference in the effects of PKNα and PKC subtypes on phosphorylation of tau protein by GSK-3β. However, we found that PKNα phosphorylates different sites of tau protein from those phosphorylated by the PKC subtypes (submitted). Therefore, each PKC related protein kinase might have a unique function in tau phosphorylation. Signal transduction kinases such as PKC, PKA, MAP kinase cross-talk in neuronal homeostasis (15, 25–29). The findings of the present study indicate that such cross talk influences the net phosphorylation state of tau not only by cross talk among kinase pathways, but also by the sequential phosphorylation of tau protein.

ACKNOWLEDGMENTS

We thank Dr. Y. Nishizuka for encouragement. This work was supported in part by research grants from the Ministry of Education, Science, Sports, and Culture of Japan and the Research for Future Program of the Japan Society for the Promotion of Science.

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