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A potential role for tau as a signal transducing molecule.

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A POTENTIAL ROLE FOR TAU AS A SIGNAL TRANSDUCING MOLECULE

by

SCOTT M. JENKINS

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham, in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT OF DISSERTATION
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Title A Potential Role for Tau as a Signal Transducing Molecule

Tau was originally identified as a protein factor that copurifies with microtubules through repeated cycles of polymerization and induces microtubule stability in a phosphorylation-dependent manner. Because increasing tau phosphorylation decreases its microtubule-binding capacity and because a hyperphosphorylated form of tau comprises the paired helical filaments and neurofibrillary tangles of the Alzheimer's disease brain, the enzymes that modulate tau phosphorylation have been intensely investigated. The present studies examined the ability of specific protein kinases and signaling pathways to modulate tau phosphorylation in intact cells.

The first study demonstrated that, in response to osmotic stress, tau phosphorylation was increased at both Ser/Thr-Pro and non-Ser/Thr-Pro sites. Although multiple members of the stress-activated protein kinase family were activated by osmotic stress, only a single member, SAPK3, was able to phosphorylate tau in situ in response to activation. Osmotic stress-induced tau phosphorylation at non-Ser/Thr-Pro sites within tau's microtubule-binding domain was mediated by a staurosporine-sensitive protein kinase and was partially responsible for an osmotic stress-induced decrease in tau-cytoskeleton interactions.

The second study demonstrated that the thiol-reactive agent phenylarsine oxide (PAO) increased tau phosphorylation within its microtubule-binding domain. Similar to

osmotic stress, this PAO-stimulated increase in tau phosphorylation decreased tau-cytoskeleton interactions and was mediated by a staurosporine-sensitive protein kinase activity. In an effort to identify the protein kinase/s responsible for tau phosphorylation in response to PAO, the third study examined a 100-kD protein kinase activity that was activated by PAO and phosphorylated tau within its microtubule-binding domain. Because this protein kinase activity was also inhibited by staurosporine, it was hypothesized to mediate the PAO-induced tau phosphorylation response in situ. This protein kinase activity was identified as the protein kinase, MARK, shown previously to regulate microtubule/microtubule-associated protein interactions.

The final study provided evidence that a 100-kD MARK-like protein kinase activity was enriched in embryonic rat brain relative to adult brain. This embryo-enriched protein kinase activity copurified with microtubules and decreased tau's microtubule-binding capacity in vitro. These studies provide novel insights into the signaling pathways and specific protein kinases that regulate tau phosphorylation, and they have implications for tau functioning under both normal and pathological conditions.

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INTRODUCTION

In 1975, a heat-stable protein that copurified with tubulin through repeated cycles of polymerization and facilitated the formation of microtubules was described (Weingarten et al., 1975). This protein was called tau due to its ability to induce tubule formation (Weingarten et al., 1975). Subsequently, tau was purified and found to migrate as several closely spaced bands on a sodium dodecyl sulfate (SDS)-polyacrylamide gel with apparent molecular masses between 55 and 62 kD (Cleveland et al., 1977a, 1977b). Further characterization revealed that tau is a family of neuronal proteins residing predominantly in the axon that are derived from alternative splicing of a single gene (Binder et al., 1985; Goedert et al., 1989; Lee et al., 1988; Papasozomenos and Binder, 1987).

In human brain, alternative splicing of the tau RNA gives rise to the expression of six distinct isoforms containing 3 or 4 imperfect sequence repeats of 31 or 32 amino acids in the carboxy terminal region of the molecule (Goedert et al., 1989). These repeats, in conjunction with the flanking regions, mediate the binding of tau to microtubules (Butner and Kirschner, 1991; Gustke et al., 1994). Additionally, tau isoforms can contain a 29 amino acid insert, a 58 amino acid insert, or no insert in the amino terminal region of the molecule (Goedert et al., 1989). Although no definitive function for the amino terminal region of tau has been established, some data suggest

that this region mediates an interaction between tau and the neural plasma membrane that may be important for neurite outgrowth (see below).

Modulation of tau's microtubule-binding capacity has focused largely on the effects of phosphorylation. Initial data suggested that increasing the phosphorylation state of microtubule-associated proteins in general (Jameson et al., 1980) and of tau in particular (Lindwall and Cole, 1984) decreased their ability to bind microtubules. Because phosphorylation modulates tau's microtubule-binding capacity, much interest has focused on characterizing the specific kinases and/or phosphatases that modulate tau's phosphorylation state. Numerous protein kinases have been shown to phosphorylate tau in vitro, including calcium-calmodulin-dependent protein kinase II (CaMKII) (Steiner et al., 1990), casein kinase II (Greenwood et al., 1994), cAMP-dependent protein kinase (PKA) (Litersky and Johnson, 1992), mitogen-activated protein kinase (MAPK) (Drewes et al., 1992), a neuronal cdc2-like kinase (cdk5/p35) (Paudel et al., 1993), glycogen synthase kinase 3 (GSK-3) (Hanger et al., 1992; Mandelkow et al., 1992), the stress-activated protein kinases (SAPK) (Goedert et al., 1997; Reynolds et al., 1997a, 1997b), and microtubule affinity regulating kinase (MARK) (Drewes et al., 1995).

More detailed examination of the effects of phosphorylation on tau's microtubule-binding capacity has revealed that phosphorylation of specific sites, rather than the overall extent of phosphorylation, is the important determinant in tau's ability to bind microtubules. For example, phosphorylation of tau in vitro by PKA decreases tau-tubulin binding, whereas phosphorylation by CaMKII does not decrease tau's microtubule-binding capacity (Johnson, 1992). Thus, sites phosphorylated by PKA are

more important in regulating tau-microtubule interactions than sites phosphorylated by CaMKII. Assigning a functional significance, in terms of modulating tau's microtubule-binding capacity, to specific phosphorylation sites in vivo has proved difficult. However, several sites within the microtubule-binding domain of tau appear to be especially important in modulating tau's ability to bind microtubules in vitro. Specifically, Ser^{262}, within the first microtubule-binding repeat, and Ser^{356}, in the fourth repeat, appear to be important in regulating tau's microtubule-binding capacity (Biernat et al., 1993; Drewes et al., 1995). These sites are non-Ser-Pro, suggesting that they are phosphorylated in the cell by non-Pro-directed protein kinases. Although phosphorylation of non-Ser-Pro sites within tau's microtubule-binding region is particularly important for regulation of tau-microtubule interactions, phosphorylation of Ser/Thr-Pro sites that lie in regions flanking the microtubule-binding repeats has a measurable, although less pronounced, effect (Trinczek et al., 1995).

Although the in vitro phosphorylation of tau has been studied extensively, phosphorylation of tau in situ is less well characterized. Recent studies have begun to address the physiological regulation of tau's phosphorylation state. For example, casein kinase II phosphorylates tau on Thr^{39} in vitro, a site that is phosphorylated in LAN-5 human neuroblastoma cells (Greenwood et al., 1994). Although these data support the conclusion that tau is a substrate for casein kinase II in these cells, further studies are required to provide definitive evidence. Similarly, when rat brain cortical slices are stimulated with forskolin, an activator of adenylyl cyclase, and rolipram, a cAMP phosphodiesterase inhibitor, the in situ phosphorylation state of tau increases significantly. The resulting phosphopeptide map of tau phosphorylated in situ in

response to this treatment and the map of tau phosphorylated by PKA in vitro are virtually identical (Fleming and Johnson. 1995). These data suggest that PKA is involved in the regulation of tau's phosphorylation state in brain.

Transfection studies also provide clues concerning the identity of the protein kinases that modify tau in situ. Cotransfection of tau and GSK3 into COS cells increased reactivity with a panel of monoclonal antibodies against phosphoepitopes on tau (Lovestone et al., 1994). More recent work has demonstrated that treatment of either NT2N cells (Hong et al., 1997) or SH-SY5Y human neuroblastoma cells (Lesort et al., 1999) with insulin decreased tau phosphorylation by inhibiting the activity of GSK-3β. These results suggest that GSK-3β phosphorylates tau in the intact cell. In contrast, transient cotransfection of MAPK and tau into COS cells did not result in any apparent increase in the phosphorylation state of tau (Anderton et al., 1995; Lovestone et al., 1994), and tau transfected into mouse Swiss 3T3 cells was not phosphorylated to a significant extent by the constitutive activation of MAPK (Latimer et al., 1995). Therefore, although MAPK readily phosphorylates tau in vitro, it does not do so in intact cells. These results emphasize the potential pitfalls of interpreting in vitro data as representative of the in vivo situation.

Transfection studies also implicate the non-Pro-directed protein kinase MARK in regulating tau phosphorylation and function in the cell (Drewes et al., 1995, 1997). MARK preferentially phosphorylates KXGS motifs in the microtubule-binding domains of tau, MAP-2, and MAP-4 (Drewes et al., 1995). In tau, these residues correspond to Ser^{262} in the first microtubule-binding repeat and to Ser^{293}, Ser^{324}, and Ser^{356} in the second, third, and fourth repeats, respectively. In vitro phosphorylation of tau by

MARK decreased tau's microtubule-binding capacity and decreased microtubule stability (Drewes et al., 1995). Similarly, overexpression of MARK in Chinese hamster ovary cells resulted in disruption of the microtubule network which was reversible by coexpression of a nonphosphorylatable MAP2c (Drewes et al., 1997). These results suggest that MARK can regulate MAP functioning in situ, and that this kinase may play a major role in regulating microtubule dynamics in vivo.

While the importance of protein kinase activity in modulating the phosphorylation state of tau has been appreciated for some time, only recently has the role of protein phosphatases been recognized as being important, as well. Treatment of brain slices (Harris et al., 1993) or cultured cells (Arias et al., 1993; Vincent et al., 1994) with the protein phosphatase inhibitor okadaic acid resulted in a hyperphosphorylation of tau. Because okadaic acid inhibits phosphatase 1 (PP1) and phosphatase 2A (PP2A) but not phosphatase 2B (PP2B) at the submicromolar concentrations used in these studies (Bialojan and Takai, 1988), it is likely that PP1 and/or PP2A modulate the phosphorylation state of tau in situ. However, since micromolar concentrations of okadaic acid were required to induce maximal phosphorylation of tau (Harris et al., 1993), PP2B may be involved, as well. While further studies are required to characterize fully the involvement of specific protein phosphatases, these data suggest that phosphatases do play an important role in the physiological regulation of tau's phosphorylation state.

Although most studies of tau function have focused on tau-microtubule interactions and microtubule stabilization, more recent research indicates that tau may have novel functions not involving the modulation of microtubule dynamics.

Phosphorylation is likely to modulate these novel functions, as well. For example, using monoclonal antibodies, tau has been detected in the nucleus (specifically in the nucleolus) of two human neuroblastoma cell lines (Loomis et al., 1990), as well as in fresh human frontal cortex (Brady et al., 1995). Because tau was localized to the fibrillar centers of the nucleolus (the sites of rRNA transcription) in interphase cells and to the nucleolar organizer region (NOR) (the location of rRNA genes) in dividing cells, nuclear tau has been proposed to play a role in the formation and/or the function of ribosomes (Loomis et al., 1990). Support for this hypothesis comes from the earlier observation that tau staining was observed on the surface of the rough endoplasmic reticulum and on free cytoplasmic granular aggregates believed to be ribosomes (polysomes) (Papasozomenos and Binder, 1987).

Tau has also been proposed to mediate an interaction between microtubules and the neural plasma membrane (Brandt et al., 1995). Overexpression of either full length tau or tau's amino terminal region in PC-12 cells resulted in tau localizing to the plasma membrane, suggesting that tau interacts with the membrane via its amino terminal region. Overexpression of the amino terminal fragment of tau but not full length tau suppressed nerve growth factor (NGF)-induced neurite outgrowth in PC-12 cells. Thus, it was suggested that the interaction of tau's amino terminus with the plasma membrane is important for neurite outgrowth and that the full length tau molecule may play a role in mediating microtubule-plasma membrane interactions necessary for neurite formation (Brandt et al., 1995).

Tau is also enriched in the growth cones of sympathetic neurons, and this enrichment does not correspond to a greater extent of microtubule polymerization or to

increased resistance of assembled microtubules to depolymerizing agents (Black et al., 1996). Thus, the tau localized to growth cones may be participating in processes other than promoting the assembly and stability of microtubules. Tau found in growing axons exhibits a spatial gradient of phosphorylation at the tau-1 epitope, with 80% of tau phosphorylated at this site in the soma and 20% in the axonal growth cone (Mandell and Banker, 1996). This gradient is hypothesized to be involved in the establishment of neuronal cell polarity (Mandell and Banker, 1996).

More recently, tau has been shown to affect the activity of microtubule motor proteins. Specifically, overexpression of tau in both Chinese hamster ovary cells and differentiated neuroblastoma cells impaired plus-end-directed transport mediated by kinesin-like motors (Ebneth et al., 1998). This tau-mediated inhibition of microtubule motor activity resulted in altered cellular distribution of specific organelles, including mitochondria and endoplasmic reticulum. These data suggest that tau's role in the cell is more complex than the regulation of microtubule dynamics.

Tau received a great deal of attention in 1986 when a hyperphosphorylated and aggregated form was found to make up the paired helical filaments (PHFs) and neurofibrillary tangles (NFTs) in the Alzheimer's disease (AD) brain (for review, see Johnson and Jenkins, 1996). Because tau from AD brains is hyperphosphorylated and because increasing tau phosphorylation decreases its microtubule-binding capacity, neurodegeneration in AD brains has been hypothesized to result, at least in part, from decreased microtubule stability in AD brains. This microtubule destabilization is hypothesized to interfere with intracellular protein trafficking, resulting ultimately in cell death (for review, see Johnson and Jenkins, 1996).

The relationship between tau hyperphosphorylation and the formation of PHFs is unclear at present. However, there are data suggesting that pools of non-PHF tau in AD brain are abnormally phosphorylated. For example, a non-PHF pool of tau from AD brains was found to be 3 to 4 times more phosphorylated than either normal tau from AD brain or tau from control brain (Kopke et al., 1993). The phosphate content of this non-PHF tau was similar to that found in PHF preparations (Ksiezak-Reding et al., 1992). Additionally, immunological methods using a phosphorylation-dependent antibody to tau have demonstrated a pool of soluble tau from AD brains that is phosphorylated at $Ser^{199/202}$ and/or Thr^{205}, Ser^{396}, and Ser^{404} (Ledesma et al., 1995). The presence of these specific pools of abnormally phosphorylated, non-PHF tau in the AD brain suggests that abnormal tau phosphorylation may precede and even be necessary for aggregation into PHFs.

However, in vitro research has provided contradictory findings concerning the effect of phosphorylation on tau aggregation. Depending on the specific protein kinase used, the sites phosphorylated, and the method of aggregation, phosphorylation can either increase or decrease tau aggregation. For example, phosphorylation of tau increases its susceptibility to aluminum-induced aggregation (Li et al., 1998), and phosphorylation of tau in vitro with cdk5 increases its tendency to dimerize (Paudel, 1997). These results suggest that the abnormal increase in tau phosphorylation in AD may contribute to tau aggregation and formation of PHFs. However, phosphorylation of tau by CaMKII actually decreases tau dimerization (Guttmann et al., 1997), and phosphorylation at specific sites that control tau-microtubule interactions (specifically Ser^{262} and Ser^{214}) decrease polyanion-induced tau aggregation (Schneider et al., 1999).

Therefore, the effect of tau phosphorylation on its aggregation into PHFs may depend on the specific sites phosphorylated or, in the case of in vitro assays, on the method used to induce aggregation.

The specific protein kinases involved in hyperphosphorylating tau in AD brain have not yet been identified. Several protein kinases that have been shown to phosphorylate tau in intact cells have been hypothesized to contribute to the pathological regulation of tau phosphorylation. One possibility is the lipid-activated, protein kinase C (PKC)-related kinase, protein kinase N (PKN) (Mukai and Ono, 1994). PKN is activated by rho in vitro (Amano et al., 1996; Watanabe et al., 1996) and is able to interact with components of the cytoskeleton, including intermediate filament proteins (Mukai et al., 1996; Matsuzawa et al., 1997) and α-actinin (Mukai et al., 1997). These findings suggest that PKN may regulate cytoskeletal functioning. In normal neurons, PKN was associated with microtubules; however, in AD, PKN was found to be closely associated with NFTs (Kawamata et al., 1998). Additionally, tau was phosphorylated by PKN in vitro and in situ in human neuroblastoma cells expressing active PKN (Kawamata et al., 1998). These results suggest that PKN could potentially be involved in regulation of tau phoshporylation in normal brain, as well as in the abnormal hyperphosphorylation seen in AD brain.

The presence in tau from AD brains of phosphoepitopes that are normally found only in mitotic cells has also led to the examination of mitotic kinases in tau hyperphosphorylation. The mitotic kinase complex cdc2/cyclin B1 was enriched in NFT-bearing neurons (Vincent et al., 1997). Additionally, immunologically detected active cdc2 was found in NFT-containing neurons but not in normal neurons, and

mitotic kinase purified from AD brain showed greater in vitro kinase activity than kinase from normal brain (Vincent et al., 1997). These findings suggest that pathological reexpression of mitotic kinases, such as cdc2, could contribute to tau pathology seen in AD brains.

Another family of protein kinases, the SAPKs, have also been hypothesized to contribute to tau hyperphosphorylation in AD. SAPKs can be activated by environmental stressors (osmotic stress, UV irradiation, and protein synthesis inhibitors), as well as by proinflammatory cytokines (for review, see Cohen, 1997). It has been hypothesized that the AD brain is characterized by increased release of certain proinflammatory cytokines as part of a feed forward, autotoxic loop (McGeer and McGeer, 1995). This inflammation response, which may be secondary to the primary lesions of AD (McGeer and McGeer, 1995), could then activate members of the SAPK family (Meier et al., 1996). In support of this hypothesis, tau is readily phosphorylated in vitro by multiple members of the SAPK family (Goedert et al., 1997; Reynolds et al., 1997a, 1997b). Additionally, staining for activated p38, a specific SAPK, revealed increased p38 activation in AD brains relative to age matched control brains. This activated p38 was found to be associated with NFT-bearing neurons (Hensley et al., 1999). Based on this evidence, members of the SAPK family have been hypothesized to contribute to tau hyperphosphorylation in AD.

In order to understand fully the regulation of tau functioning in the cell, the stimuli and pathways that modulate tau phosphorylation must be understood. While much effort has gone into studying the signaling pathways and enzymes that regulate tau phosphorylation and function in vivo, only recently have their identities begun to be

elucidated. Continued study of the regulation of tau phosphorylation and function in situ will help us to understand tau's role under both normal and pathological conditions in vivo.

The studies presented herein examine the signaling pathways and the specific enzymes that regulate tau phosphorylation and function in situ. The first study examined changes in tau phosphorylation in response to induction of osmotic stress and to treatment with the protein synthesis inhibitor anisomycin, both of which have been used as SAPK activators in multiple cell types. Osmotic stress and anisomycin treatment both activated the SAPKs. However, osmotic stress increased tau phosphorylation at Ser/Thr-Pro sites, while anisomycin did not. Although osmotic stress activated multiple SAPKs, only SAPK3 was able to phosphorylate tau in situ in response to activation. Tau phosphorylation in response to osmotic stress was augmented in cells overexpressing SAPK3 at the same sites phosphorylated by SAPK3 in vitro. These results suggest that SAPK3 may be involved in the normal and/or the pathological regulation of tau phosphorylation in vivo.

Osmotic stress also increased tau phosphorylation at $Ser^{262/356}$, within tau's microtubule-binding domain. Because these sites are hypothesized to be important in regulating tau-microtubule interactions, changes in tau localization in response to osmotic stress were examined. Osmotic stress-induced increase in tau phosphorylation correlated with a decrease in the amount of tau associated with the cytoskeleton and an increase in the amount of soluble tau. This change in tau localization was partially but not completely due to tau phosphorylation at $Ser^{262/356}$ within the microtubule-binding region. Osmotic stress-induced tau phosphorylation within the microtubule-binding

domain was potently inhibited by the protein kinase inhibitor staurosporine. suggesting that tau phosphorylation at $Ser^{262/356}$ in response to osmotic stress occurs through a staurosporine-sensitive protein kinase.

The second study examined changes in tau phosphorylation in response to the thiol-reactive agent phenylarsine oxide (PAO). PAO stimulated tau phosphorylation within the microtubule-binding domain, specifically at $Ser^{262/356}$. PAO treatment also decreased the amount of tau associated with the cytoskeleton, increased the amount of soluble tau, and decreased microtubule stability. Similar to the osmotic stress-induced tau phosphorylation, this change in tau localization was only partially due to tau phosphorylation at $Ser^{262/356}$ within the microtubule-binding region. PAO-induced tau phosphorylation at $Ser^{262/356}$ was potently inhibited by staurosporine suggesting that the same protein kinase activity may phosphorylate tau at $Ser^{262/356}$ in response to both osmotic stress and PAO.

To identify the protein kinase/s responsible for phosphorylating tau within its microtubule-binding domain, the third study identified a 100-kDa protein kinase activity that phosphorylated tau at $Ser^{262/356}$, was activated by PAO, copurified with microtubules, and was inhibited by low concentrations of staurosporine. These findings suggest that this 100-kDa protein kinase activity is likely to mediate the tau phosphorylation response at $Ser^{262/356}$ to PAO. Based on relative molecular mass and substrate specificity, this protein kinase was hypothesized to be MARK. Mass spectrometric analysis of the protein bands at 100 kDa that contain tau-directed protein kinase activity revealed overlap between the peptide masses from these protein bands and the predicted peptide masses from MARK1 and MARK2. Immunoblotting with a

MARK antibody confirmed that the isolated protein kinase bands that phosphorylated tau at $Ser^{262/356}$ in vitro and that were activated by PAO correspond to MARK.

The final study examined the developmental regulation of a MARK-like protein kinase activity in rat brain. This 100-kDa protein kinase activity copurified with microtubules, phosphorylated tau on its microtubule-binding domain, and was enriched in embryonic compared to adult rat brain. Tau was phosphorylated at Ser^{262} by the microtubule-associated protein kinase activity in vitro, and this phosphorylation decreased tau's microtubule-binding capacity. These results suggest that a MARK-like protein kinase activity is enriched in embryonic rat brain and may contribute to the developmental regulation of tau phosphorylation within its microtubule-binding domain.

MODULATION OF TAU PHOSPHORYLATION AND INTRACELLULAR LOCALIZATION BY CELLULAR STRESS

by

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ABSTRACT

Tau is a microtubule-associated protein that is functionally modulated by phosphorylation and that is hyperphosphorylated in several neurodegenerative diseases. Because phosphorylation regulates both normal and pathological tau functioning, it is of great interest to identify the signaling pathways and enzymes capable of modulating tau phosphorylation in vivo. The present study examined changes in tau phosphorylation and localization in response to osmotic stress, which activates the SAPKs, a family of proline-directed protein kinases shown to phosphorylate tau in vitro and hypothesized to phosphorylate tau in Alzheimer's disease. Immunoblot analysis with phosphorylation-dependent antibodies revealed that osmotic stress increased tau phosphorylation at Ser/Thr-Pro sites outside tau's microtubule-binding domain, as well as at the non-Ser/Thr-Pro sites $Ser^{262/356}$ within the microtubule-binding domain. Although all SAPKs examined were activated by osmotic stress, only SAPK3 was able to phosphorylate tau in situ in response to activation by osmotic stress. Osmotic stress-induced tau phosphorylation correlated with a decrease in the amount of tau associated with the cytoskeleton and an increase in the amount of soluble tau. This stress-induced alteration in tau localization was partially but not totally due to phosphorylation at $Ser^{262/356}$ by a staurosporine-sensitive protein kinase. Taken together, these results suggest that osmotic stress activates at least two tau-directed protein kinases, one proline-directed and one non-proline-directed, SAPK3 can phosphorylate tau on Ser/Thr-Pro residues in situ, and $Ser^{262/356}$ phosphorylation only partially modulates tau localization in the cell.

INTRODUCTION

Tau is a family of neuronal phosphoproteins originally identified by its ability to copurify with microtubules through repeated cycles of polymerization (Weingarten et al., 1975). Although tau is traditionally described as a microtubule-stabilizing protein, it may also participate in other cellular processes such as linking signal transduction pathways to the cytoskeleton (Lee et al., 1998), activation of phospholipase C (Hwang et al., 1996), and regulation of microtubule motor activity (Ebneth et al., 1998). Widespread interest in tau was generated when a hyperphosphorylated and aggregated form was shown to comprise the PHFs which make up the NFTs in AD brain (see Johnson and Jenkins, 1996 for review). Further, it was demonstrated that the ability of tau to bind and stabilize microtubules correlates inversely with phosphorylation (Jameson et al., 1980) and that phosphorylation of a small number of specific sites within tau's microtubule-binding domain (e.g., Ser^{262} and Ser^{356}) dramatically decreases tau's microtubule-binding capacity (Drewes et al., 1995). These and other findings suggest that the abnormal phosphorylation of tau in AD may contribute to the neurodegenerative process.

Because phosphorylation is involved in regulating tau function under both normal and pathological conditions, many studies have focused on identifying the protein kinases that phosphorylate tau. While numerous protein kinases have been shown to phosphorylate tau and regulate its function in vitro (Johnson and Jenkins, 1996; Johnson and Hartigan, 1998), elucidation of the specific enzymes that regulate tau's phosphorylation state in vivo has proven more difficult. Recently, stress-responsive members of the MAPK family, known as the SAPKs, were shown to

phosphorylate tau in vitro (Goedert et al., 1997; Reynolds et al., 1997a, 1997b). Specifically, SAPK1 (c-jun N-terminal kinase/JNK), SAPK2a (p38), SAPK2b (p38β), SAPK3 (ERK 6), and SAPK 4 all phosphorylate tau in vitro on residues that are hyperphosphorylated in PHF-tau (Goedert et al., 1997). Activation of SAPKs occurs in response to multiple forms of cellular stress (e.g., osmotic shock and inhibition of protein synthesis) and by certain proinflammatory cytokines [interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α)] (for reviews, see Cohen, 1997; Woodgett et al., 1996). Downstream targets of SAPK activation include other protein kinases, such as MAPK-activated protein kinases 2 and 3 (MAPKAP-K2/3), and transcription factors such as c-jun and cyclic-AMP-response-element-binding protein (CREB) (Cohen, 1997; Woodgett et al., 1996).

SAPKs phosphorylate serine residues and/or threonine residues that are followed by a proline (Cohen, 1997; Woodgett et al., 1996). Because multiple Ser/Thr-Pro residues are phosphorylated in PHF-tau (Morishima-Kawashima et al., 1995), it has been proposed that the tau hyperphosphorylation in AD is due, at least in part, to aberrant activation of one or more proline-directed protein kinases. In order to assess the plausibility of the hypothesis that tau's phosphorylation state is modulated by specific SAPKs in an intact cellular environment, the present paper examined the ability of specific SAPKs to phosphorylate tau in situ in response to cellular stress.

Utilizing human neuroblastoma SH-SY5Y cells, the present study provides the first demonstration that tau phosphorylation increases at multiple epitopes in response to osmotic stress and that this increase in phosphorylation correlates with a decrease in the amount of tau associated with the cytoskeleton. Although all SAPKs examined were

activated by osmotic stress, only SAPK3 phosphorylated tau in situ providing the first demonstration that tau phosphorylation can be modulated by a specific SAPK in the intact cell. In addition to Ser/Thr-Pro sites, osmotic stress increased tau phosphorylation at $Ser^{262/356}$, non-Ser/Thr-Pro sites in tau's microtubule-binding domain, as indicated by immunostaining with the phosphorylation-specific antibody 12E8. Phosphorylation of $Ser^{262/356}$ was mediated by a staurosporine-sensitive protein kinase activity, and inhibition of $Ser^{262/356}$ phosphorylation partially but not completely inhibited the osmotic stress-induced decrease in cytoskeleton-associated tau.

MATERIALS AND METHODS

Cell culture

SH-SY5Y human neuroblastoma cells were grown on Corning 100-mm tissue culture dishes in RPMI 1640 (Cellgro) supplemented with 10% (vol/vol) heat-inactivated horse serum (GIBCO), 5.0% heat-inactivated Fetal Clone II (HyClone), 100 U/ml penicillin (GIBCO), 100 μg/ml streptomycin (GIBCO) and 2.0 m*M* glutamine (GIBCO). For some experiments, cells were differentiated in RPMI 1640 supplemented with 5.0% horse serum, 1.0% Fetal Clone II, penicillin, streptomycin, glutamine, and 20 μ*M* retinoic acid (Sigma) for 7-10 days before use.

Immunoblotting

SH-SY5Y cells were incubated at 37° C in media containing 0.5 *M* or 1.0 *M* sorbitol (Sigma) for 30 min unless otherwise indicated or containing 50 μg/ml anisomycin (Sigma) for 60 min, or with vehicle alone. Treated cells were washed once

with ice-cold phosphate buffered saline (PBS) and collected in a 2x stop buffer without dithiothreitol (DTT) or dye [500 m*M* Tris-Cl (pH 6.8), 10% SDS, 100 m*M* EGTA, 100 m*M* EDTA, and 10% glycerol]. Cell extracts were sonicated and protein concentrations were determined using the bicinchoninic acid (BCA) method (Pierce). The samples were diluted in 2x stop buffer containing 25 m*M* dithiothreitol (DTT) and bromophenol blue as the tracking dye, and the indicated amounts of cell extract were electrophoresed on 7.5% SDS-polyacrylamide gels. Proteins were then transferred to nitrocellulose for immunoblotting. Blots were probed with antibodies as indicated in the text and developed by enhanced chemiluminescence (ECL; Pierce). Antibodies to tau include the phosphorylation-independent antibodies tau-5 and 5A6 (Carmel et al., 1996; Kosik et al., 1988; Trojanowski et al., 1989) and the phosphorylation-dependent antibodies tau-1 (Binder et al., 1985; Szendrei et al., 1993), PHF-1 (Otvos et al., 1994; Greenberg and Davies, 1990), 12E8 (Seubert et al., 1995), and AT270 (Goedert et al., 1994).

Kinase activity measurements

Naive SH-SY5Y cells grown on 60-mm Corning dishes were treated with either sorbitol or anisomycin as described above. Cells were washed once in ice-cold PBS and scraped from the plates into 150 µl of SAPK immunoprecipitation buffer [20 m*M* Tris-Cl (pH 7.4), 1.0% NP-40, 500 m*M* NaCl, 1.0 m*M* EDTA, 1.0 m*M* EGTA, 1.0 m*M* Na_3VO_4, 1.0 µ*M* okadaic acid (Sigma), 1.0 m*M* phenylmethylsulfonyl fluoride (PMSF), and 10 µg/ml each of leupeptin, aprotinin, and pepstatin]. SAPK3, SAPK4, and SAPK5 immunoprecipitation buffer contained 0.25% NP-40 and 100 m*M* NaCl. Extracts were sonicated briefly and cleared by centrifugation at 16,000 *g* for 10 min. Protein

determinations were made and the indicated amount of extract was incubated overnight at 4° C in the presence of polyclonal antibodies to SAPK1 (JNK), SAPK 2a (p38), SAPK 2b (p38β), c-myc (Upstate Biotechnology Inc.) (for transfected SAPK3 and SAPK4), or flag epitope (Santa Cruz) (for transfected SAPK5). Protein A sepharose beads were added and the incubations were continued for another 4 h. SAPK1, SAPK2a, and SAPK2b immunoprecipitates were washed with immunoprecipitation buffer and resuspended in SAPK phosphorylation buffer {20 mM MOPS (pH 7.2), 25 m*M* β-glycerophosphate, 5.0 m*M* EGTA, 1.0 m*M* Na_3VO_4, 1.0 m*M* DTT, 25 m*M* $MgCl_2$, 1.0 μ*M* okadaic acid, 100 μ*M* ATP, 1.0 μCi/nmol [γ-^{32}P]ATP, 1.0 m*M* PMSF, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin} containing either c-jun-GST (Upstate Biotechnology Inc.) (3.0 μg/tube for JNK) or GST-MAPKAP-Kinase-2-myc (Upstate Biotechnology Inc.) (0.3 μg/tube for SAPK 2a and 2b). SAPK3, SAPK4, and SAPK5 immunoprecipitates were incubated in phosphorylation buffer containing 1.0 m*M* cold ATP, without [γ-^{32}P]ATP, and recombinant human tau (T4L) (prepared as described in Fleming et al., 1996) (0.1 μg/μl) as a substrate. Immunoprecipitates were incubated for 30 min at 30° C. The reactions were stopped by addition of 2x SDS stop with 25 m*M* DTT and bromophenol blue. Samples were electrophoresed on 8% SDS-polyacrylamide gels and either vacuum dried, exposed to a phosphor screen (Molecular Dynamics), and analyzed on a phosphorimager (SAPK1, 2a, and 2b) or transferred to nitrocellulose and immunoblotted with phosphorylation-dependent tau antibodies (SAPK3, 4, and 5).

Fractionation

SH-SY5Y cells were incubated in the presence or absence of sorbitol as described above. Cells were rinsed once with warm PBS and once with warm extraction buffer [80 m*M* piperazine-N,N-bis-{2-ethanesulfonic acid}(PIPES) (pH 6.8), 1.0 m*M* $MgCl_2$, 2.0 m*M* EGTA, 30% glycerol, 10 m*M* benzamidine, 50 μg/ml leupeptin, 1.0 m*M* PMSF, and 0.5 μ*M* okadaic acid]. Next, cells were incubated in 300 μl (for 60-mm plate) of extraction buffer containing 0.1% Triton X-100 for 10 min at 37° C. Cells were transferred into Eppendorf tubes and centrifuged for 2 min at 16,000 *g*. The supernatant was removed and incubated in a boiling water bath for 10 min, while the pellet was resuspended in 100 μl of 2x SDS stop buffer, without DTT or dye, and sonicated. Protein determinations were made and samples were diluted with 2x stop buffer, boiled, electrophoresed on SDS-polyacrylamide gels, and immunoblotted as described above.

Transfection

Naive SH-SY5Y cells were plated onto either 35- or 60-mm Corning dishes. Cells were incubated in the presence of 6.0 μl of FuGene 6 Transfection Reagent (Boehringer Mannheim) and 2.0 μg of the indicated cDNA according to the manufacturer's protocol. Myc-tagged SAPK3 in the pCMVN vector and myc-tagged SAPK4 in the pCDNA3.1 vector were the generous gifts of Dr. M. Goedert. Flag-tagged BMK1 (SAPK5) in pcDNA3, BMK1(AEF) (dominant negative) (SAPK5(AEF)) in pcDNA3, and MEK5(D) (constitutively active) in pCMV5 (Zhou et al., 1995; Abe et al., 1996; Kato et al., 1997; English et al., 1998) were the generous gifts of Dr. J.-D. Lee. Twenty-four to 48 h after transfection, cells were treated as described above.

RESULTS

Osmotic stress increases tau phosphorylation at multiple sites

To determine whether osmotic stress modulates tau phosphorylation. SH-SY5Y human neuroblastoma cells, naive or differentiated (Fig. 1A), were incubated in the presence or absence of sorbitol (0.5 M). Sorbitol-induced osmotic stress resulted in a decrease in the electrophoretic mobility of tau, as indicated by tau-5/5A6 immunoblots (Fig. 1A, top panel). Decreased tau mobility on a polyacrylamide gel is indicative of increased tau phosphorylation (Lindwall and Cole, 1984). Consistent with the observation of decreased electrophoretic mobility, sorbitol treatment resulted in an increase in tau phosphorylation at multiple epitopes as indicated by alterations in the immunoreactivity of phosphorylation-specific antibodies. Tau phosphorylation was increased at the Ser/Thr-Pro epitopes tau-1 (indicated by decreased tau-1 immunoreactivity), PHF-1, and AT270, as well as at the non-Ser/Thr-Pro epitope 12E8. The pattern of sorbitol-induced tau phosphorylation was the same for both naive and differentiated cells at all epitopes examined with the exception of AT270, which showed a greater increase in immunoreactivity in response to osmotic stress in naive cells than differentiated cells. Osmotic stress induced by incubation of cells in the presence of 1.0 *M* mannitol also resulted in increased tau phosphorylation at the tau-1 and the PHF-1 epitopes (data not shown). To characterize further the sorbitol-induced increases in tau phosphorylation, the time dependence, concentration dependence, and reversibility of the effect were examined (Fig. 1B, C, D). In Fig. 1B, SH-SY5Y cells were incubated in the presence of 0.5 *M* sorbitol for the times indicated. The sorbitol-induced increase in tau-1 phosphorylation, indicated by a decrease in tau-1 immunoreactivity, was evident

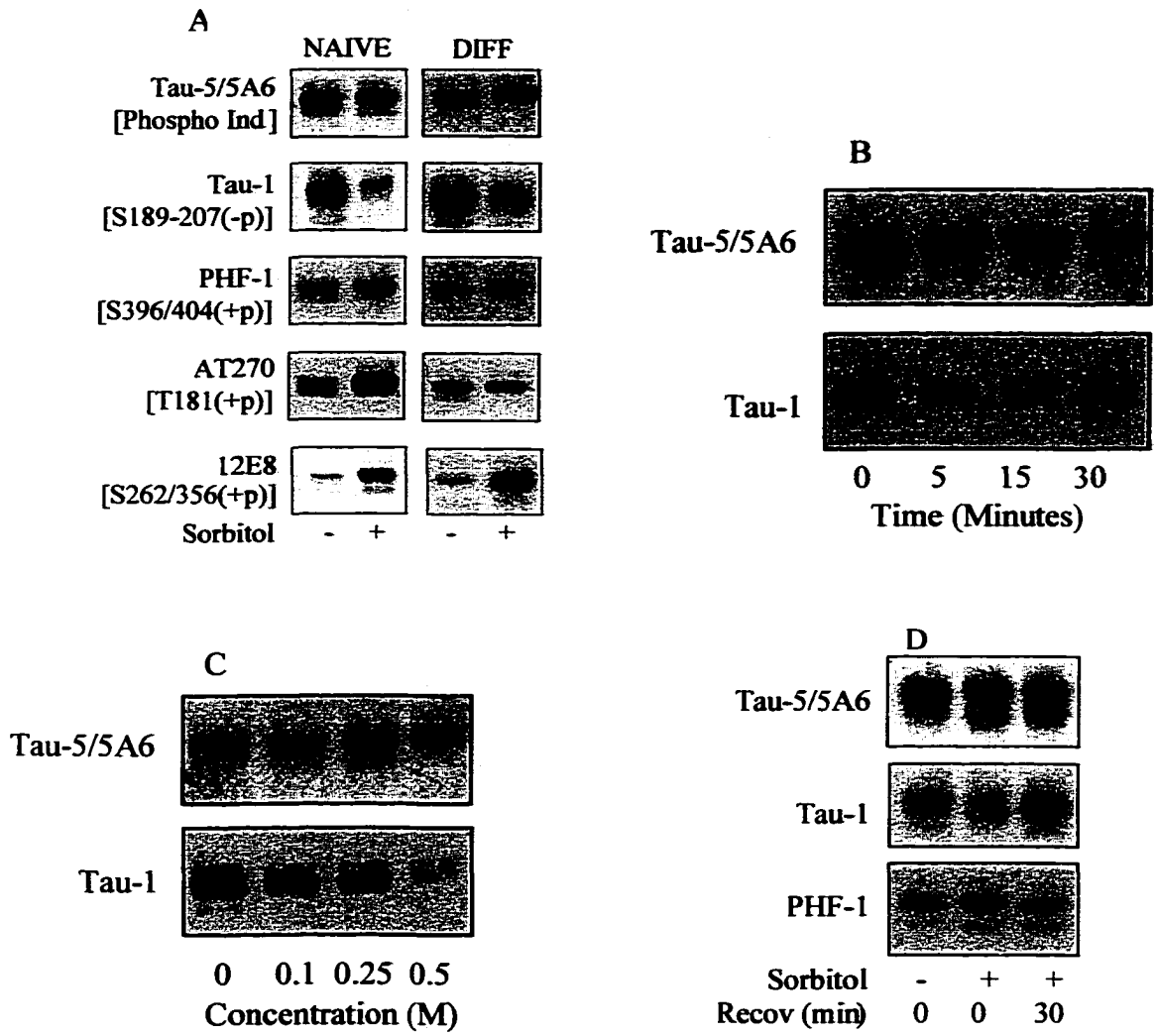

FIG. 1. Sorbitol-induced osmotic stress increases tau phosphorylation at multiple sites in a time-dependent, dose-dependent, and reversible manner. In (**A**), either undifferentiated (NAIVE) or differentiated (DIFF) SH-SY5Y cells were incubated for 30 min at 37° C in media +/- 0.5 *M* sorbitol. In (**B**), undifferentiated SH-SY5Y cells were incubated in the presence of 0.5 *M* sorbitol for the indicated time. In (**C**), SH-SY5Y cells were incubated for 30 min in the presence of the indicated concentration of sorbitol. In (**D**), SH-SY5Y cells were incubated in the presence (+) or the absence (-) of sorbitol. Sorbitol-treated cells were either harvested immediately following treatment (Recov = 0) or incubated an additional 30 min in media containing no sorbitol (Recov = 30). Extract (10 μg) was electrophoresed and immunoblotted with the indicated tau antibody. Epitopes recognized by each phosphorylation-dependent antibody are indicated in (A). Antibodies that recognize a phosphorylated epitope are designated (+p), while recognition of a dephosphorylated epitope is designated (-p). Immunoblots are representative of at least 3 separate experiments.

by 5 min and increased in a time-dependent manner. The decrease in tau-1 immunoreactivity was not due to changes in tau levels (Fig. 1B, top panel). In Fig. 1C, SH-SY5Y cells were incubated in the presence of increasing concentrations of sorbitol. At sorbitol concentrations of 0.1 and 0.25 *M*, decreases in tau-1 immunoreactivity, indicating an increase in phosphorylation at this epitope, were observed (Fig. 1C). However, a much larger increase in phosphorylation at the tau-1 epitope was observed at 0.5 *M* sorbitol (Fig. 1C). The apparent increase in immunoreactivity of the tau-5/5A6 band with 0.5 *M* sorbitol is most likely due to an increase in the phosphorylation state of the faster migrating forms of tau and a subsequent decrease in their mobility. This decreased mobility results in a compaction of the tau bands into a single slower migrating form. In Fig. 1D, the reversibility of sorbitol-induced tau phosphorylation was examined. SH-SY5Y cells were incubated in the presence of 0.5 *M* sorbitol for 30 min. Following this initial incubation, cells were either harvested as described above or were incubated an additional 30 min in the absence of sorbitol. Fig. 1D indicates that sorbitol-induced tau phosphorylation is completely reversed at both the tau-1 and PHF-1 epitopes following a 30-min incubation in the absence of sorbitol.

Anisomycin treatment decreases tau phosphorylation

To determine if the tau phosphorylation effect was specific for osmotic stress or was a general response to SAPK-activating cellular stressors, SH-SY5Y cells were incubated in the presence of the protein synthesis inhibitor anisomycin, which has been shown to activate specific members of the SAPK family (Cuenda et al., 1997). Anisomycin treatment resulted in an increase in tau's electrophoretic mobility, indicated

by the downward shift in the tau-5/5A6 immunoreactive bands (Fig. 2). This increase in tau electrophoretic mobility is indicative of a decrease in tau phosphorylation. Consistent with this finding, anisomycin treatment resulted in decreased tau phosphorylation at both the tau-1 and PHF-1 epitopes (Fig. 2).

Osmotic stress decreases the amount of tau associated with the cytoskeleton

Because sorbitol treatment increased tau phosphorylation, specifically at $Ser^{262/356}$ within the microtubule-binding domain (see Fig. 1A), the effect of sorbitol treatment on tau localization was examined. Differentiated SH-SY5Y cells were incubated in the presence or absence of sorbitol and were fractionated to separate the detergent-insoluble cytoskeleton from the detergent-soluble fraction. As indicated by the phosphorylation-independent tau antibodies, tau-5/5A6, incubation in the presence of sorbitol decreased the amount of tau associated with the cytoskeleton and increased the amount of tau in the soluble fraction (Fig. 3).

Activated SAPK1, SAPK2a, and SAPK2b do not phosphorylate tau in situ

To examine the involvement of specific SAPKs in osmotic stress-induced tau phosphorylation, SH-SY5Y cells were incubated in the presence of either sorbitol or anisomycin. SAPK1, SAPK2a, and SAPK2b were immunoprecipitated from either treated or untreated extracts, and the extent of activation by the stressors was measured using either c-jun (SAPK1) or MAPKAP-K2 (SAPK2a and 2b) as substrates.

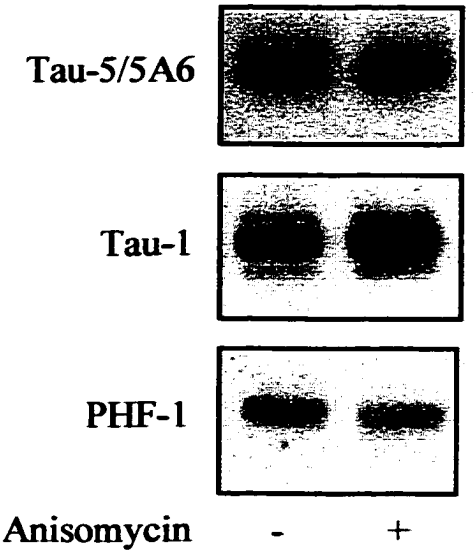

FIG. 2. Anisomycin-induced stress decreases tau phosphorylation. Undifferentiated SH-SY5Y cells were treated for 60 min in the presence (+) or absence (-) of 50 μg/ml anisomycin. Extract (10 μg) was electrophoresed and immunoblotted with the indicated tau antibodies. Immunoblots are representative of at least 3 separate experiments.

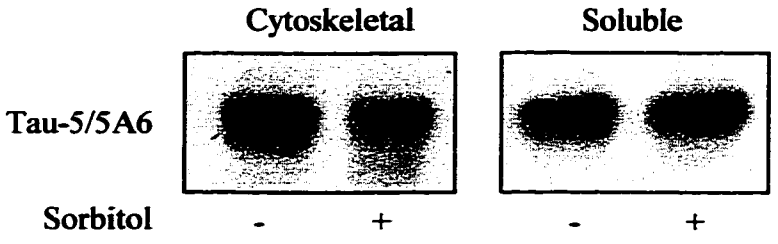

FIG. 3. Sorbitol-induced osmotic stress decreases the amount of tau associated with the cytoskeleton and increases the amount of soluble tau. Differentiated SH-SY5Y cells were treated for 30 min in the presence (+) or absence (-) of 0.5 *M* sorbitol. The detergent-insoluble cytoskeleton was then separated from the detergent-soluble fraction as described in the methods. Cytoskeletal and soluble fractions were electrophoresed and immunoblotted with the phosphorylation-independent tau-5 and 5A6 antibodies. During ECL development, soluble immunoblots were exposed longer relative to cytoskeletal immunoblots. Immunoblots are representative of at least 3 separate experiments.

Quantitation of substrate phosphorylation (Fig. 4A) demonstrates that SAPK1 was activated by both sorbitol and anisomycin. Similar results were found for SAPK2a and SAPK2b (data not shown). In Fig. 4B, the SAPK2a and SAPK2b inhibitor SB202190 (Calbiochem) (Cuenda et al., 1997) was used to examine further the involvement of these two protein kinases in the osmotic stress-induced tau phosphorylation response. Inhibition of SAPK2a and 2b resulted in a slight basal dephosphorylation of tau at the tau-1 epitope; however, the sorbitol-stimulated increase in tau phosphorylation was unaffected at either the tau-1 or PHF-1 epitopes (Fig. 4B).

Osmotic stress-activated SAPK3 phosphorylates tau in vitro and *in situ*

Because endogenous SAPK3 was virtually undetectable by antibody staining in untransfected SH-SY5Y cells (data not shown) and because immunoprecipitation of untransfected cell extract with an SAPK3 polyclonal antibody (a generous gift of Dr. M. Goedert) failed to precipitate any protein kinase activity (data not shown), it is unlikely that endogenous SAPK3 is involved in the osmotic stress-induced increase in tau phosphorylation. However, it was of interest to determine whether SAPK3 is able to phosphorylate tau in situ in response to osmotic stress. Therefore, alterations in tau phosphorylation in response to osmotic stress were examined in SAPK3 overexpressing cells. SH-SY5Y cells overexpressing myc-tagged SAPK3 were treated with either sorbitol or anisomycin, and cell extracts were immunoprecipitated with an antibody against c-myc. Immunoprecipitates were incubated in the presence of recombinant tau and ATP. Following incubation, the tau was electrophoresed and immunoblotted with the indicated antibodies. Fig. 5A (lane 2) demonstrates that SAPK3 from sorbitol-

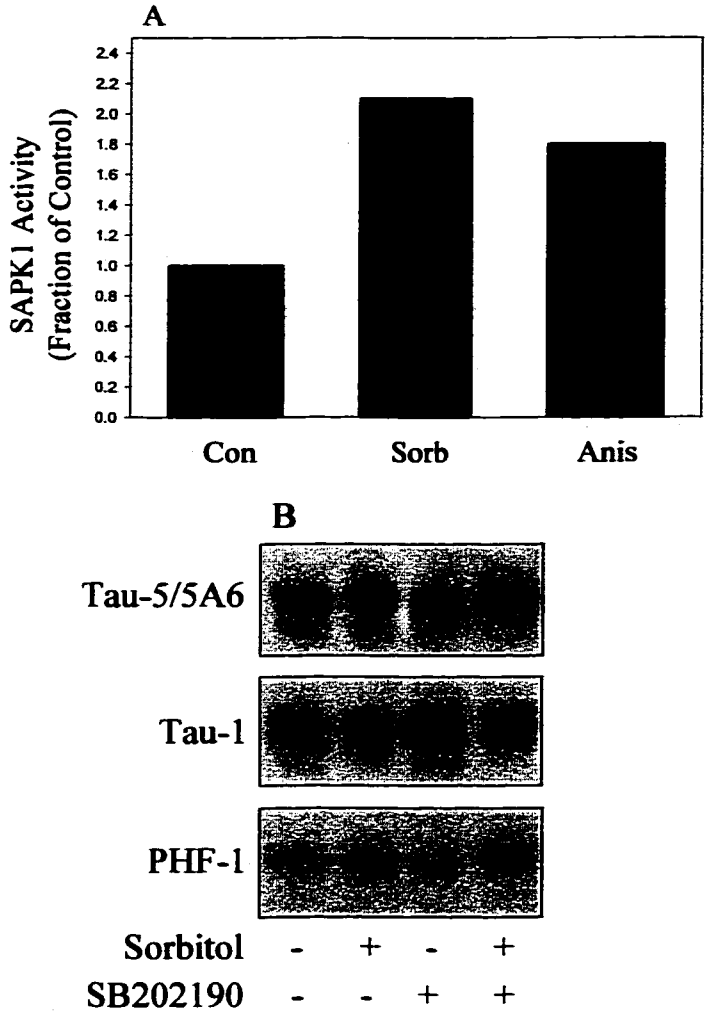

FIG. 4. SAPK1, SAPK2a, and SAPK2b are not involved in sorbitol-induced tau phosphorylation. Undifferentiated SH-SY5Y cells were not treated (con), were treated for 30 min with 0.5 *M* sorbitol (sorb), or were treated for 60 min with 50 μg/ml anisomycin (anis). Extracts were then incubated with a polyclonal antibody to SAPK1 (**A**). The immune complexes were assayed for protein kinase activity using c-jun-GST as a substrate. Phosphorylated c-jun was electrophoresed, exposed to a phosphor screen, and analyzed on a phosphorimager. The results were quantitated and expressed as the fraction of control. Similar results were obtained in 4 (Sorb) or 2 (Anis) separate experiments. In (**B**) SH-SY5Y cells were incubated in the presence of 15 μ*M* SB202190 or vehicle alone (10 min) and then in the presence (+) or absence (-) of 0.5 *M* sorbitol. Extracts (10 μg) were electrophoresed and immunoblotted with the indicated tau antibodies. Immunoblots are representative of at least 3 separate experiments.

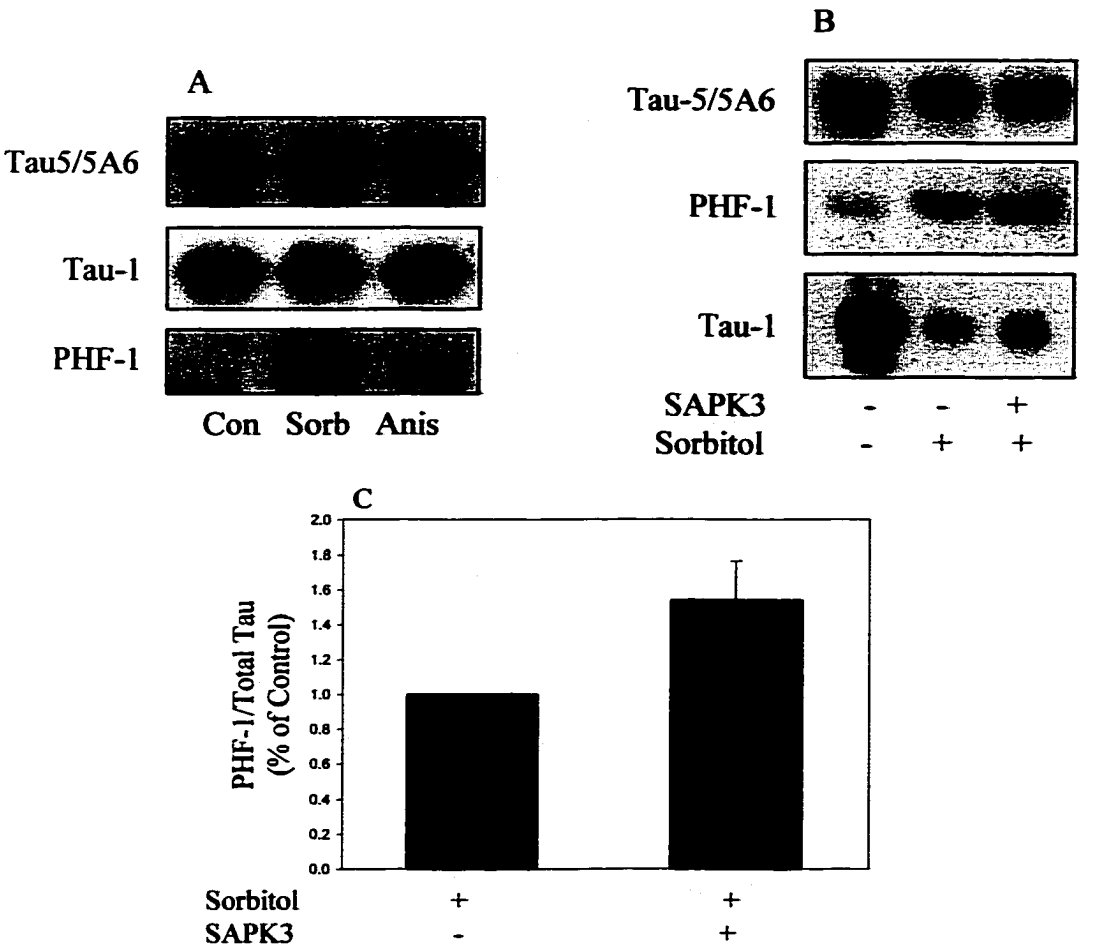

FIG. 5. Overexpressed SAPK3 phosphorylates tau at the PHF-1 epitope in vitro and in situ. **A:** Naive SH-SY5Y cells overexpressing myc-tagged SAPK3 were incubated in media in the absence of treatments (Con), in the presence of 1.0 *M* sorbitol for 30 min (Sorb), or in the presence of 50 μg/ml of anisomycin (Anis) for 60 min. Extracts were immunoprecipitated with a polyclonal antibody to c-myc. Immunoprecipitates were incubated with recombinant tau in the presence of ATP. The reaction was stopped after 60 min, tau (100 ng) was electrophoresed, and it was immunoblotted with the indicated antibodies. In **(B)**, SH-SY5Y cells were transiently transfected with either SAPK3 (+) or pCDNA3.1 vector alone (-). Cells were then incubated in media in the presence (+) or absence (-) of 1.0 *M* sorbitol for 30 min. Extracts were electrophoresed and immunoblotted with the indicated tau antibodies. In **(C)**, the sorbitol-stimulated increase in PHF-1 immunoreactivity was quantitated for SAPK3 overexpressing cells. The ratio of PHF-1 immunoreactivity to tau-5/5A6 (total tau) immunoreactivity from 7 separate experiments was determined and expressed as a fraction of control. SAPK3 overexpressing cells showed significantly ($p < 0.05$) higher phosphorylation at PHF-1 than did control transfected cells.

treated cells phosphorylates recombinant tau at the PHF-1 epitope, resulting in the appearance of a PHF-1 immunoreactive band. SAPK3 from anisomycin-treated cells also phosphorylated recombinant tau at the PHF-1 epitope, although to a much lower extent than SAPK3 from sorbitol-treated cells (Fig. 5A, lane 3). SAPK3 from either sorbitol- or anisomycin-treated cells did not phosphorylate tau at the tau-1 epitope.

To test the hypothesis that activated SAPK3 can increase tau phosphorylation in situ, SH-SY5Y cells overexpressing SAPK3 were incubated in 1.0 *M* sorbitol for 30 min, and extracts were immunoblotted with phosphorylation-dependent tau antibodies. SAPK3 overexpression had no effect on basal tau phosphorylation at either the tau-1 or the PHF-1 epitopes (data not shown). However, sorbitol-induced tau phosphorylation at the PHF-1 epitope was increased when SAPK3 was overexpressed (Fig. 5B, compare lanes 2 and 3). Sorbitol-stimulated tau phosphorylation at the tau-1 epitope was unaffected by SAPK3 overexpression (Fig. 5B, bottom panel). Quantitation of the effect of SAPK3 overexpression on phosphorylation of the PHF-1 epitope is shown in Fig. 5C. SAPK3 overexpression led to a significant ($p < 0.05$) increase in sorbitol-stimulated tau phosphorylation at the PHF-1 epitope.

Osmotic stress-activated SAPK4 phosphorylates tau in vitro but not in situ

SH-SY5Y cells transiently overexpressing myc-tagged SAPK4 were also incubated in the presence of either sorbitol or anisomycin. Cell extracts were immunoprecipitated with a polyclonal antibody against c-myc, and the protein kinase activity of the immunoprecipitate was evaluated using recombinant tau as a substrate as described for SAPK3. Fig. 6A demonstrates that SAPK4 was activated by both sorbitol

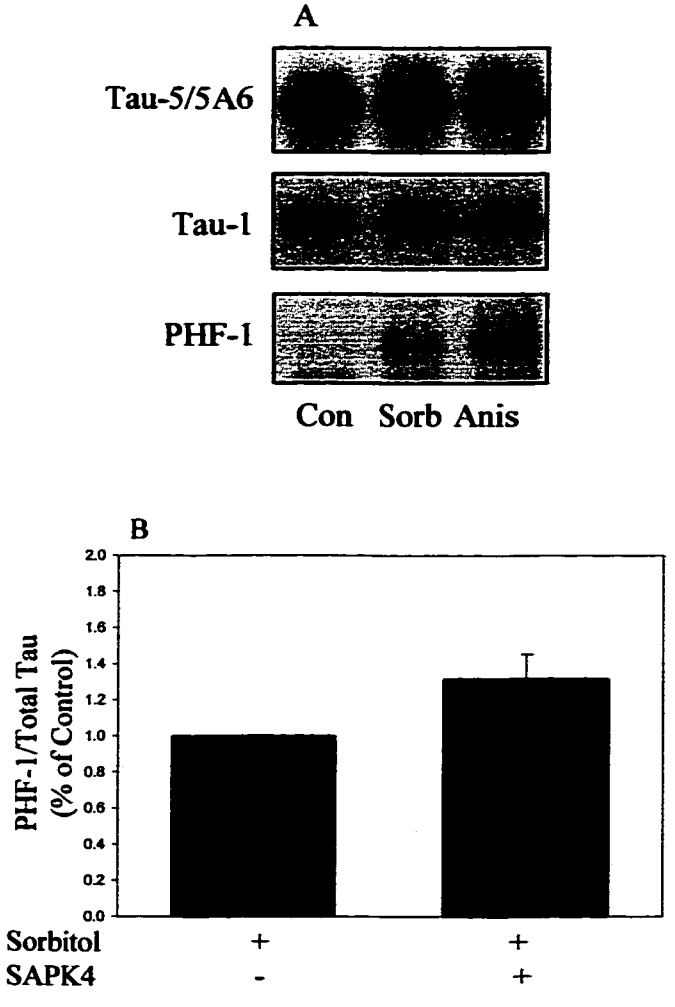

FIG. 6. Overexpressed SAPK4 from sorbitol- and anisomycin-treated cells phosphorylates tau in vitro but not in situ. **A:** Naive SH-SY5Y cells overexpressing myc-tagged SAPK4 were incubated in media in the absence of treatments (Con), in the presence of 1.0 *M* sorbitol for 30 min (Sorb), or in the presence of 50 μg/ml of anisomycin (Anis) for 60 min. Extracts were immunoprecipitated with a polyclonal antibody to c-myc. Immunoprecipitates were incubated with recombinant tau in the presence of ATP. The reaction was stopped after 60 min, tau (100 ng) was electrophoresed, and blots were immunoblotted with the indicated antibodies. In **(B)**, SH-SY5Y cells were transiently transfected with either SAPK4 (+) or pcDNA3.1 vector alone (-). Cells were then incubated in media in the presence (+) or absence (-) of 1.0 *M* sorbitol for 30 min. Extracts were electrophoresed and immunoblotted with the PHF-1 and the tau-5/5A6 antibodies. The ratio of PHF-1 immunoreactivity to tau-5/5A6 (total tau) immunoreactivity from 4 separate experiments was expressed as a fraction of control. Overexpression of SAPK4 did not significantly ($p > 0.05$) increase sorbitol-induced tau phosphorylation at the PHF-1 epitope.

and anisomycin treatment and phosphorylated tau at the PHF-1 epitope. Anisomycin treatment was more effective at activating SAPK4 than sorbitol treatment. The ability of SAPK4 to phosphorylate tau in situ was then assessed in cells transiently transfected with either vector alone or SAPK4. SH-SY5Y cells were incubated in the presence of sorbitol (1.0 *M*) and extracts were immunoblotted with phosphorylation-dependent tau antibodies. Quantitation revealed that SAPK4 overexpression did not significantly increase osmotic stress-mediated tau phosphorylation at the PHF-1 epitope (Fig. 6B). There was also no increase in phosphorylation of the tau-1 epitope (data not shown). Similar experiments with SAPK5 demonstrated that SAPK5 is activated by osmotic stress, as indicated by decreased electrophoretic mobility; however, SAPK5 failed to phosphorylate tau at the PHF-1 or tau-1 epitopes in vitro or in situ (data not shown).

Osmotic stress-induced tau phosphorylation at $Ser^{262/356}$ is inhibited by staurosporine

To examine the effect of protein kinase inhibition on osmotic stress-induced tau phosphorylation, SH-SY5Y cells were preincubated with the protein kinase inhibitor staurosporine followed by incubation in the presence of sorbitol for 30 min. Fig. 7A demonstrates that preincubation of SH-SY5Y cells with staurosporine almost completely prevented the osmotic stress-induced increase in tau phosphorylation at the non-Ser/Thr-Pro epitope $Ser^{262/356}$ but not at the Ser/Thr-Pro epitopes examined. The change in 12E8 immunoreactivity was not due to changes in tau levels as no change in immunoreactivity was seen with the phosphorylation-independent antibodies tau-5/5A6 (top panel). A similar staurosporine-sensitive increase in tau phosphorylation at $Ser^{262/356}$ has been sho-

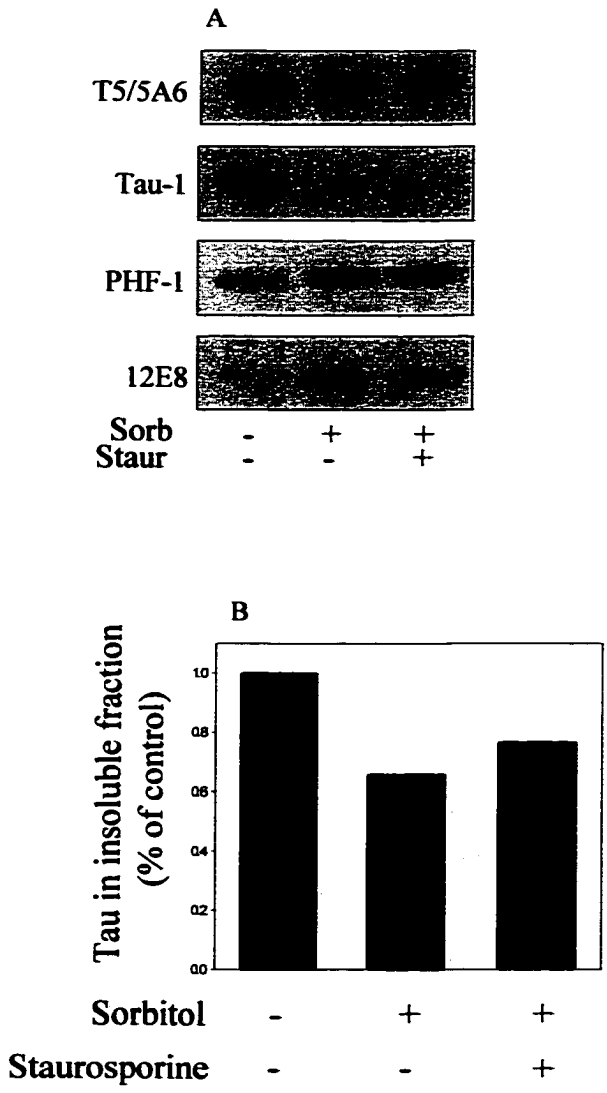

FIG. 7. Staurosporine prevents the osmotic stress-induced increase in tau phosphorylation at $Ser^{262/356}$ and partially reverses the change in tau localization. SH-SY5Y cells were preincubated in the presence (+) or absence (-) of 10 n*M* staurosporine and then in the presence (+) or absence (-) of 0.5 *M* sorbitol. In (**A**), extracts were electrophoresed and immunoblotted with the indicated antibody against tau. In (**B**), extracts were separated into the detergent-insoluble cytoskeleton and the detergent-soluble fraction. Cytoskeletal fractions were electrophoresed and immunoblotted for total tau. Tau levels were quantitated and are expressed as a percent of control values from 2 separate experiments.

wn recently in SH-SY5Y cells incubated in the presence of PAO (Jenkins and Johnson. 1999).

Because phosphorylation of residues within tau's microtubule-binding domain, specifically Ser^{262}, dramatically decreased tau-microtubule interactions in vitro. the effect of staurosporine on osmotic stress-induced change in tau localization was examined. SH-SY5Y cells were preincubated in the presence or absence of staurosporine and treated with sorbitol. Cytoskeletal fractions were separated from soluble fractions as described and the amount of tau in the cytoskeletal fraction was quantitated. Fig. 7B demonstrates that sorbitol treatment decreased the amount of tau associated with the detergent-insoluble cytoskeleton. This decrease was only minimally affected by preincubation with staurosporine.

DISCUSSION

Although many signaling pathways are likely to modulate tau phosphorylation in vivo, little is known about the specific enzymes involved. Identification of the protein kinases that phosphorylate tau is important for understanding both the normal and pathological regulation of tau function. In this study, several novel findings are reported about the regulation of tau phosphorylation in situ. Incubating human neuroblastoma cells under hyperosmotic conditions resulted in increased tau phosphorylation at multiple Ser/Thr-Pro sites, as well as the non-Ser/Thr-Pro sites $Ser^{262/356}$. This osmotic stress-induced tau phosphorylation at specific Ser/Thr-Pro residues was enhanced by overexpression of SAPK3, suggesting that SAPK3 phosphorylates tau in situ. Additionally, phosphorylation of tau at $Ser^{262/356}$ by a staurosporine-sensitive kinase only

partially contributed to the decrease in cytoskeletally associated tau following osmotic stress.

Because tau phosphorylation increased at multiple Ser/Thr-Pro sites, it is likely that one or more proline-directed protein kinases are activated by and phosphorylate tau in response to osmotic stress. Many proline-directed protein kinases can phosphorylate tau in vitro, including MAPK (ERK), cdk5/p35, GSK-3β (Johnson and Jenkins, 1996), and the SAPKs (Goedert et al., 1997; Reynolds et al., 1997a, 1997b). Although these protein kinases clearly phosphorylate tau in vitro, only GSK-3β has been convincingly shown to phosphorylate tau in intact cells (Hong et al., 1997). However, GSK-3β is not involved in the osmotic stress-induced tau phosphorylation response as pretreatment with LiCl, which potently inhibits GSK-3β activity, did not eliminate the sorbitol-induced increase in tau phosphorylation (data not shown).

Endogenous SAPK1 (JNK), SAPK2a (p38), and SAPK2b (p38β) were all shown to be activated by both sorbitol and anisomycin treatment in the present study. Because only sorbitol treatment increased tau phosphorylation in our paradigm, these specific SAPKs are most likely not involved in the osmotic stress-induced increase in tau phosphorylation. This finding was supported by the observation that an inhibitor of SAPK2a and 2b did not attenuate sorbitol-stimulated tau phosphorylation at the sites examined. Because SAPK1, SAPK2a, and SAPK2b did not phosphorylate tau in response to osmotic stress and because these SAPKs were previously shown to be less effective than SAPK3 and 4 at phosphorylating tau in vitro (Goedert et al., 1997), we next focused on SAPK3 and SAPK 4.

The lack of detectable SAPK3 (by immunoblotting) in untransfected SH-SY5Y cells makes it unlikely that this protein kinase is involved in the endogenous tau phosphorylation response. Therefore, SH-SY5Y cells overexpressing SAPK3 were used to investigate the ability of this protein kinase to phosphorylate tau in situ. Consistent with previous reports on the ability of SAPK3 to phosphorylate recombinant tau, transfected SAPK3 was activated by sorbitol treatment and phosphorylated tau in vitro at the PHF-1 epitope. The relevance of this in vitro phosphorylation for the in vivo situation was supported by the finding that when overexpressed in SH-SY5Y cells, SAPK3 enhanced sorbitol-induced tau phosphorylation at the PHF-1 epitope. Because SAPK3 is capable of phosphorylating this epitope in vitro, the effect of SAPK3 on PHF-1 phosphorylation in SH-SY5Y cells may be direct, although a role for an intermediate protein kinase cannot be ruled out. SAPK3 may be unique in its ability to phosphorylate tau in the intact cell as demonstrated by the finding that neither SAPK4 nor SAPK5, when transfected into cells, phosphorylated tau in situ, although both were activated by osmotic stress. Consistent with previous findings (Goedert et al., 1997; Reynolds et al., 1997a, 1997b), SAPK4 did phosphorylate tau in vitro, demonstrating the importance of caution when interpreting in vitro results as representative of the in vivo situation.

The finding that SAPK3 can phosphorylate tau in the intact cell is interesting in light of the hypothesis that abnormal activation of SAPKs in AD contributes to tau hyperphosphorylation. It has been hypothesized that the AD brain is characterized by increased release of certain proinflammatory cytokines as part of a feed forward, autotoxic loop (McGeer and McGeer, 1995). This inflammation response, which may be secondary to the primary lesions of AD (McGeer and McGeer, 1995), could then

activate members of the SAPK family (Meier et al., 1996). Activation of SAPKs, specifically SAPK3, could contribute to the tau hyperphosphorylation characteristic of AD brains. This hypothesis has gained some recent support by the finding that SAPK2b is abnormally activated in AD brains compared to control brains (Hensley et al., 1999), suggesting that there are signals present in brains of AD patients that favor SAPK activation. It is, therefore, possible that other SAPKs, including SAPK3, are also activated in the AD brain.

Because tau phosphorylation has traditionally been examined in terms of tau's microtubule-binding capacity, changes in tau localization following the induction of osmotic stress were also examined. Osmotic stress-induced increases in tau phosphorylation resulted in a decrease in the amount of tau associated with the cytoskeleton and an increase in the amount of soluble tau. This is in agreement with the general finding that increasing tau phosphorylation decreases tau's ability to bind microtubules (Jameson et al., 1980). Because the non-Ser/Thr-Pro epitope $Ser^{262/356}$ was phosphorylated in response to sorbitol treatment, the osmotic stress-induced change in tau localization was hypothesized to be due to this phosphorylation within tau's microtubule-binding domain. In fact, inhibition of osmotic stress-induced $Ser^{262/356}$ phosphorylation with staurosporine caused only a minor reversal in the osmotic stress-induced decrease in cytoskeletally associated tau. Therefore, while phosphorylation of $Ser^{262/356}$ does appear to contribute to the regulation of tau localization, other sites are at least as important. Recent work indicates that phosphorylation of Ser/Thr-Pro sites has a measurable effect on tau's microtubule-binding capacity (Trinczek et al., 1995) and that phosphorylation of Ser^{214} by PKA dramatically decreases interactions between tau and

the cytoskeleton in situ (Illenberger et al., 1998). Therefore, it is likely that the Ser/Thr-Pro sites phosphorylated in response to osmotic stress, as well as perhaps other sites such as Ser^{214}, also contribute to the osmotic stress-induced decrease in tau-cytoskeleton interactions.

The finding that osmotic stress-induced $Ser^{262/356}$ phosphorylation is eliminated by staurosporine is intriguing given the recent report of a staurosporine-sensitive protein kinase phosphorylating tau at $Ser^{262/356}$ in response to the vicinal thiol-reactive agent PAO (Jenkins and Johnson, 1999). Because in both cases the $Ser^{262/356}$-directed protein kinase activity is inhibited by very low concentrations of staurosporine (10 n*M*), a single protein kinase or group of kinases may be involved in both responses. Staurosporine was originally identified as a PKC inhibitor (for review see Gescher, 1998) but has more recently been shown to inhibit several protein kinases. Neither the specific PKC inhibitor GF109203X nor PKC down-regulation with phorbol myristate acetate (PMA) was found to inhibit sorbitol-induced tau phosphorylation at the 12E8 epitope (data not shown), suggesting that PKC is unlikely to be involved. Additionally, overexpression of the atypical PKC iota had no effect on PAO-induced tau phosphorylation at $Ser^{262/356}$ (unpublished data in collaboration with Dr. M. Wooten). However, the possibility that a GF109203X and PMA-insensitive PKC isoform, or a PKC-related protein kinase such as PKN, is involved cannot be ruled out. Additionally, because staurosporine is not specific for PKC (for reviews, see Reugg and Burgess, 1989; Gescher, 1998), its inhibitory effect could also be due to the inhibition of other protein kinases. For example, the recently identified MARK phosphorylates tau in vitro and in situ within its microtubule-binding domain (Drewes et al., 1997). The susceptibility of MARK to

staurosporine is unknown, and MARK involvement in the present response is difficult to investigate due to the lack of available inhibitors.

In conclusion, the present study provides the first demonstration that tau phosphorylation is increased in response to osmotic stress; that a specific SAPK, SAPK3, can phosphorylate tau in situ; that osmotic stress-induced tau phosphorylation altered tau localization within the cell; and that this change in localization is partially dependent on phosphorylation of $Ser^{262/356}$ by a staurosporine-sensitive protein kinase. These findings represent an important step in understanding how tau phosphorylation and function can be regulated by cellular stress, and the present findings have implications for understanding tau function under normal and, perhaps, under pathological conditions.

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MODULATION OF TAU PHOSPHORYLATION WITHIN ITS MICROTUBULE-BINDING DOMAIN BY CELLULAR THIOLS

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ABSTRACT

Tau is a microtubule-stabilizing protein that is functionally modulated by alterations in its phosphorylation state. Because phosphorylation regulates both normal and pathological tau functioning, it is of importance to identify the signaling pathways that regulate tau phosphorylation in vivo. The present study examined changes in tau phosphorylation and function in response to modulation of cellular thiol content. Treatment of cells with PAO, which reacts with vicinal thiols, selectively increased tau phosphorylation within its microtubule-binding domain, at the non-Ser/Thr-Pro sites $Ser^{262/356}$, while decreasing tau phosphorylation at Ser/Thr-Pro sites outside this region. This increase in tau phosphorylation correlated with a decrease in the amount of tau associated with the cytoskeleton and decreased microtubule stability. PAO-induced tau phosphorylation was inhibited by oxidants and by the protein kinase inhibitor staurosporine. Although staurosporine completely eliminated the increase in tau phosphorylation at $Ser^{262/356}$, as detected by immunostaining with 12E8, it had a comparatively minor effect on the changes in tau localization induced by PAO. The results suggest that regulation of cellular thiols is important for modulating tau phosphorylation and function in situ. Additionally, although phosphorylation of $Ser^{262/356}$ decreases tau's interaction with the cytoskeleton, phosphorylation of these residues alone is not sufficient for the PAO-induced changes in tau localization.

INTRODUCTION

Tau is a family of neuronal phosphoproteins originally identified by its ability to copurify with microtubules through repeated cycles of polymerization (Weingarten et

al., 1975). Although tau is traditionally described as a microtubule-stabilizing protein, it may also participate in other cellular processes, such as the linking of signal transduction pathways to the cytoskeleton (Lee et al., 1998), activation of phospholipase C (Hwang et al., 1996; Jenkins and Johnson, 1998), and regulation of microtubule motor activity (Ebneth et al., 1998). Widespread interest in tau was generated when a hyperphosphorylated and aggregated form was shown to comprise the PHFs which make up the NFTs in AD brain (for review, see Johnson and Jenkins, 1996). Further, it was demonstrated that the ability of tau to bind and stabilize microtubules correlates inversely with phosphorylation (Jameson et al., 1980; Lindwall and Cole, 1984), and that phosphorylation of a small number of specific sites dramatically decreases tau's microtubule-binding capacity (Drewes et al., 1995). For example, tau phosphorylation within the microtubule-binding region, specifically on Ser^{262} and Ser^{356}, dramatically reduces tau's microtubule binding capacity in vitro (Biernat et al., 1993; Drewes et al., 1995). Based on these data, it was hypothesized that Ser^{262} and Ser^{356} regulate tau-microtubule interactions in vivo (Biernat et al., 1993; Drewes et al., 1995). Given these findings, much effort has gone into identifying the enzymes and signaling pathways that modulate tau phosphorylation within the microtubule-binding region (for review, see Johnson and Jenkins, 1996). Although several protein kinases have been shown to phosphorylate these sites in vitro, the exact signaling cascades and protein kinases that regulate phosphorylation of these sites in situ have not been fully elucidated.

Regulation of protein function by modulation of cellular thiols has been documented for several enzymes, including the calcium-activated protease calpain (Guttmann et al., 1997; Guttmann and Johnson, 1998), glucosamine-6-phosphate

deaminase (Altamirano et al., 1992), and phosphoenolpyruvate carboxykinase (Lewis et al., 1993). Additionally, binding of vicinal thiol groups by arsenoxides, such as PAO, inhibits the activity of a certain class of protein tyrosine phosphatases (Zhang et al., 1992). Therefore, regulation of cellular thiols can affect signaling through multiple pathways, and stimuli that affect cellular thiols are likely to be important in regulating cell function in vivo.

The present study provides the first evidence that tau phosphorylation within the microtubule-binding region is modulated by cellular thiols in both SH-SY5Y cells and rat primary cortical cultures. Treatment of cells with the vicinal thiol-reactive agent PAO increased tau phosphorylation at $Ser^{262/356}$, which are non-Ser-Pro motifs (Goedert et al., 1989), as determined by increases in reactivity with the antibody 12E8 (Seubert et al., 1995). This increase in tau phosphorylation correlated with a decrease in cytoskeletally associated tau and a decrease in microtubule stability. In contrast, PAO did not increase tau phosphorylation at the Ser/Thr-Pro motifs comprising the tau-1 epitope (Binder et al., 1985; Szendrei et al., 1993). The PAO-induced tau phosphorylation was independent of tyrosine phosphorylation, was inhibited by oxidants, and was sensitive to staurosporine. Surprisingly, the PAO-induced changes in tau localization were only minimally dependent on phosphorylation of the 12E8 epitope within the microtubule-binding domain. These results suggest the presence of a vicinal thiol-dependent pathway that modulates tau phosphorylation and function in situ.

MATERIALS AND METHODS

SH-SY5Y cell cultures

SH-SY5Y human neuroblastoma cells were grown on 100-mm Corning tissue culture dishes in RPMI 1640 (Cellgro) supplemented with 10% (vol/vol) heat-inactivated horse serum (GIBCO), 5.0% heat-inactivated Fetal Clone II (HyClone), 100 U/ml penicillin (GIBCO), 100 μg/ml streptomycin (GIBCO) and 2.0 m*M* glutamine (GIBCO). For some experiments, cells were differentiated in RPMI 1640 supplemented with 5.0% horse serum, 1.0% Fetal Clone II, penicillin, streptomycin, glutamine, and 20 μ*M* retinoic acid (Sigma) for 7-10 days before use.

Primary cortical cultures

Embryonic cortices (embryonic days 15-17) from Sprague-Dawley rats were dissected, dissociated by trituration, and plated on 35-mm Falcon dishes coated with poly-L-lysine as described previously (Lesort et al., 1997). Cells were grown for 6-8 days in GIBCO modified Eagle's medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, 1.0% glutamine, 0.3% glucose, 5.0% fetal calf serum, and 0.01% BSA.

Immunoblotting

SH-SY5Y cells were incubated at 37° C in media containing 5.0 μ*M* PAO in dimethyl sulfoxide (DMSO) or DMSO alone for 45 min unless otherwise indicated. The final concentration of DMSO did not exceed 0.1%. Treated cells were washed once with ice-cold PBS and collected in 2x stop buffer [500 m*M* Tris-Cl (pH 6.8), 10% SDS, 100 m*M* EGTA, 100 m*M* EDTA, and 10% glycerol] without DTT or dye. Cell extracts

were sonicated, and protein concentrations were determined using the BCA method (Pierce). The samples were diluted in 2x stop buffer containing 25 m*M* DTT and bromophenol blue as the tracking dye, and the indicated amounts of cell extract were electrophoresed on 7.5% SDS-polyacrylamide gels. Proteins were then transferred to nitrocellulose for immunoblotting. Blots were probed with antibodies as indicated in the text and developed by ECL (Pierce). Antibodies to tau include the phosphorylation-independent antibodies tau-5 and 5A6 (Carmel et al., 1996; Johnson et al., 1997) and the phosphorylation-dependent antibodies tau-1 (Binder et al., 1985; Szendrei et al., 1993), PHF-1 (Greenberg and Davies, 1990; Otvos et al., 1994), and 12E8 (Seubert et al., 1995). Other antibodies used were against α-tubulin and acetylated tubulin (Sigma).

Fractionation

Fractionation was performed by modification of the procedure described by Esmaeli-Azad et al. (1994). Differentiated SH-SY5Y cells were incubated in the presence or absence of PAO as described above. Cells were rinsed once with warm PBS and once with warm fractionation buffer [80 m*M* PIPES (pH 6.8), 1.0 m*M* $MgCl_2$, 2.0 m*M* EGTA, 30% glycerol, 10 m*M* benzamidine, 50 μg/ml leupeptin, 1.0 m*M* PMSF, and 0.5 μ*M* okadaic acid], followed by incubation in 300 μl (for 60-mm plate) of fractionation buffer containing 0.1% Triton X-100 for 10 min at 37° C. Cells were transferred into Eppendorf tubes and centrifuged for 2 min at 16,000 *g*. The supernatant was removed and incubated in a boiling water bath for 10 min, and the pellet was resuspended in 100 μl of 2x SDS stop buffer, without DTT or dye, and sonicated. Protein determinations were made, and samples were diluted with 2x stop buffer

(containing dye and DTT), boiled, electrophoresed on SDS-polyacrylamide gels, and immunoblotted as described above. Where indicated, immunoblots were analyzed on a densitometer, and quantified data were analyzed for statistical significance using a Student's *t* test.

RESULTS

PAO treatment increases tau phosphorylation within its microtubule-binding domain

To determine whether modification of thiol groups could modulate tau phosphorylation, SH-SY5Y cells (Fig. 1A) or embryonic rat primary cortical cultures (Fig. 1B) were incubated in the presence of 5 μM PAO for 45 min. Routine morphological analysis indicated that some cell shrinkage occurred in the presence of PAO (data not shown). However, PAO treatment did not compromise cellular integrity as determined by measurement of lactate dehydrogenase release (data not shown). Extracts were electrophoresed and immunoblotted with the indicated phosphorylation-dependent and -independent tau antibodies. Incubation of cells in the presence of PAO increased tau phosphorylation within the microtubule-binding domain at the 12E8 epitope in both naive SH-SY5Y cells (Fig. 1A) and rat primary cortical cultures (Fig. 1B). A similar increase in $Ser^{262/356}$ phosphorylation was seen in SH-SY5Y cells that had been differentiated in retinoic acid (data not shown). Additionally, a dephosphorylation in the presence of PAO was observed at the tau-1 epitope, indicated by increased tau-1 immunoreactivity (tau-1 recognizes a dephosphorylated epitope containing Ser/Thr-Pro sites (Fig. 1, middle panels). PAO treatment of rat primary cortical cultures increased tau's electrophoretic mobility as indicated by a downward sh-

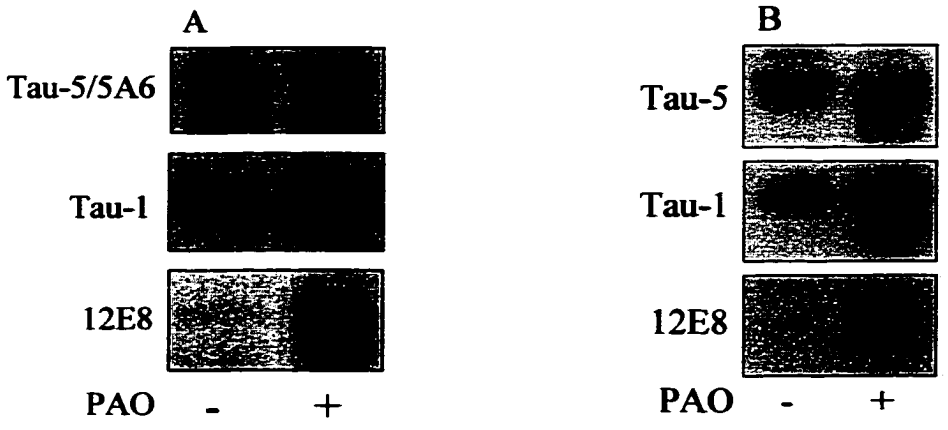

FIG. 1. PAO treatment of SH-SY5Y cells and rat primary cortical cells increases tau phosphorylation within the microtubule-binding region. Naive SH-SY5Y cells **(A)** or rat primary cortical cells **(B)** were incubated in the presence (+) or absence (-) of 5 μM PAO for 45 min at 37°C. Extracts (10 μg) were electrophoresed and immunoblotted with the indicated tau antibodies.

ift in the phosphorylation-independent tau-5 band (Fig. 1B, top panel). This increase in electrophoretic mobility is indicative of tau dephosphorylation at Ser/Thr-Pro epitopes (Lindwall and Cole, 1984). PAO treatment induced either no change or a slight decrease in tau phosphorylation at the PHF-1 epitope, which is also a Ser/Thr-Pro motif (data not shown). The changes in tau immunoreactivity with phosphorylation-specific antibodies were not due to changes in tau levels, as indicated by the fact that there was no change in reactivity with the phosphorylation-independent antibodies tau-5 and 5A6 (Fig. 1, top panels).

PAO treatment decreases the amount of tau associated with the cytoskeleton and decreases microtubule stability in SH-SY5Y cells

The effect of the PAO-induced increase in $Ser^{262/356}$ phosphorylation on tau localization within the cell was assessed. Differentiated SH-SY5Y cells incubated in the presence or absence of PAO were fractionated as described to separate the detergent-insoluble cytoskeleton from the detergent-soluble fraction. Fig. 2A demonstrates that incubation of SH-SY5Y cells in the presence of PAO decreased the amount of tau associated with the cytoskeleton (top left panel). There was a concomitant increase in the amount of soluble tau in PAO-treated cells (Fig. 2A, top right panel). It should be noted that the soluble fractions contained more protein and were overexposed relative to the cytoskeletal fractions to aid the visualization of changes. To determine the effect of these changes in tau localization on tubulin dynamics, the amount of α-tubulin in each fraction was determined. Fig. 2A (bottom panels) demonstrates that the amount of detergent-insoluble α-tubulin decreased in cells exposed to PAO. There was a simultan-

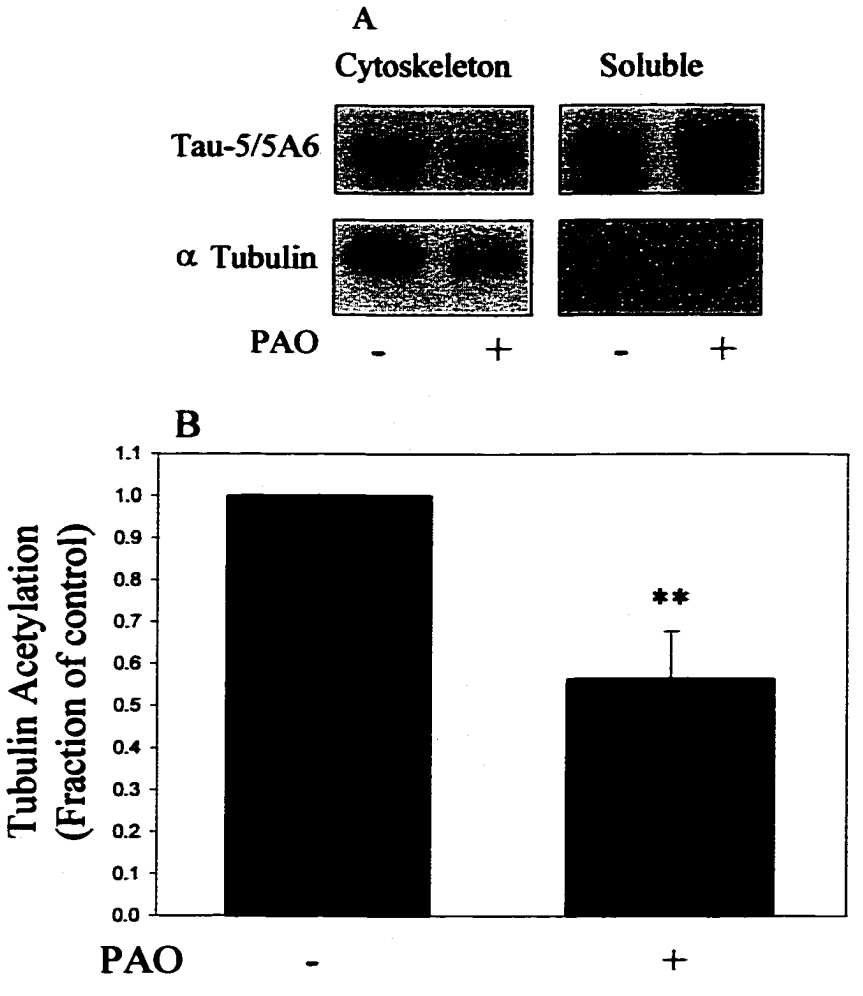

FIG. 2. PAO treatment of SH-SY5Y cells decreases the amount of tau associated with the cytoskeleton and alters microtubule dynamics. **A:** Differentiated SH-SY5Y cells were incubated in the presence (+) or absence (-) of PAO (5 μ*M*) for 45 min. Extracts were fractionated as described to separate the detergent-insoluble cytoskeleton from the detergent-soluble fraction. Each fraction was electrophoresed (2 μg of protein for cytoskeleton and 5 μg for soluble) and immunoblotted with the indicated antibody. **B:** Differentiated SH-SY5Y cells were incubated in the presence (+) or absence (-) of PAO (5 μ*M*) for 45 min. Extracts were fractionated as in A, and Triton X-insoluble fractions were electrophoresed and immunoblotted with antibodies to α-tubulin or acetylated tubulin. Immunoblots of the cytoskeletal samples were analyzed by densitometry and expressed as a fraction of control. Values for acetylated tubulin were normalized to levels of α–tubulin to provide a relative measure of tubulin acetylation. Data are means ± SEM (n = 4). ** $p < 0.01$.

eous increase in the amount of soluble α-tubulin in cells exposed to PAO, suggesting a destabilization of microtubules in PAO-treated cells. To examine changes in markers of tubulin stability, detergent-insoluble extracts were electrophoresed and immunoblotted for acetylated α-tubulin, an indicator of stable microtubules (for review, see MacRae, 1997). Levels of insoluble, cytoskeletal-associated acetylated tubulin were normalized for the amount of α-tubulin present in each fraction (Fig. 2B). Tubulin acetylation decreased significantly ($p < 0.01$, Student's t test) in cells incubated with PAO, indicating decreased microtubule stability.

PAO treatment decreases the amount of tau associated with the cytoskeleton and decreases microtubule stability in rat primary cortical cells

The effect of PAO treatment on tau localization and microtubule dynamics was next assessed in rat primary cortical cultures. Cells were incubated in the presence or absence of PAO and fractionated to separate the detergent-insoluble cytoskeleton from the detergent-soluble fraction. In Fig. 3A, levels of tau associated with the cytoskeleton were quantified and are expressed as a fraction of control. Cells incubated in the presence of PAO have significantly less ($p < 0.01$, Student's t test) cytoskeletally associated tau compared with untreated cells (Fig. 3A). Primary cortical cells treated with PAO also contained significantly lower ($p < 0.01$, Student's t test) levels of detergent-insoluble α-tubulin than cells incubated in the absence of PAO (Fig. 3B). Fig. 3C indicates that tubulin acetylation is also significantly ($p < 0.01$, Student's t test) lower in rat primary cortical cells incubated in the presence of PAO than in cells incubated in the absence of PAO.

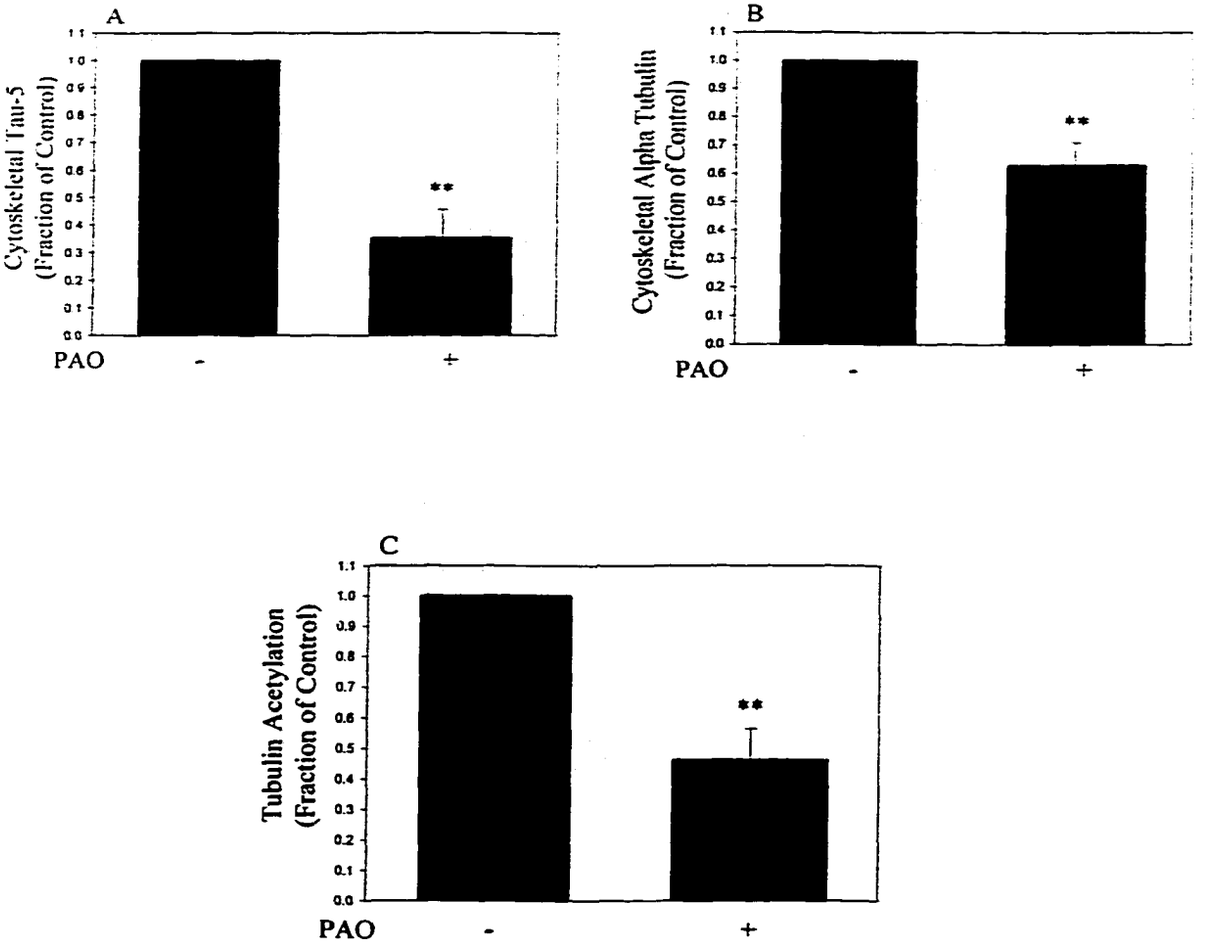

FIG. 3. PAO treatment of rat primary cortical cells decreases the amount of tau associated with the cytoskeleton and alters microtubule dynamics. **A:** Rat primary cortical cultures were incubated in the presence of 5 μM PAO for 45 min, and extracts were fractionated as described. Cytoskeletal fractions were electrophoresed, immunoblotted with the phosphate-independent tau-5 antibody, and analyzed by densitometry. Tau levels are expressed as a fraction of control. Data are means $\pm$ SEM (n = 4). **B:** Cytoskeletal fractions were immunoblotted with an antibody to α–tubulin, analyzed by densitometry, and expressed as a fraction of control. Data are means $\pm$ SEM (n = 4). **C:** Cytoskeletal fractions were immunoblotted with an antibody to acetylated tubulin, analyzed by densitometry, normalized for the amount of α–tubulin, and expressed as a fraction of control. Data are means $\pm$ SEM (n = 4). ** $p < 0.01$

PAO-induced tau phosphorylation is not due to stimulation of celluar tyrosine phosphorylation

Because PAO has been shown previously to inhibit certain protein tyrosine phosphatases (Zhang et al., 1992) and to increase tyrosine phosphorylation in cells (Oetken et al., 1992), the involvement of tyrosine phosphorylation in PAO-stimulated tau phosphorylation was examined. SH-SY5Y cells were preincubated in the absence or presence of the protein tyrosine kinase inhibitor genistein followed by PAO treatment. Genistein pretreatment had no effect on PAO-induced tau phosphorylation at the 12E8 epitope (Fig. 4). An inhibitor of src-like protein tyrosine kinase activity, PP1 (Biomol), also failed to block the PAO-induced tau phosphorylation (data not shown). Additionally, other tyrosine phosphatase inhibitors, including sodium orthovanadate and pervanadate, had no effect on tau phosphorylation at $Ser^{262/356}$ (data not shown).

PAO-induced tau phosphorylation at $Ser^{262/356}$ is inhibited by oxidants

Because PAO binds vicinal thiol groups (Zhang et al., 1992), the effect of cellular redox state on PAO-induced tau phosphorylation was examined. SH-SY5Y cells were depleted of glutathione (GSH) by chronic treatment with either 50 or 200 μM L-Buthionine-[*S,R*]-sulfoximine (BSO), an inhibitor of γ-glutamylsynthase (Wullner et al., 1999). The depletion of GSH was confirmed by measurement of GSH in cellular extracts (data not shown) (Makar et al., 1994). BSO treatment resulted in a dose-dependent decrease in PAO-stimulated tau phosphorylation at the 12E8 epitope (Fig. 5A). The ability of diamide, a thiol cross- linker that works through an oxidative mechanism (Becker et al., 1986), to modulate either basal or PAO-stimulated tau phosp-

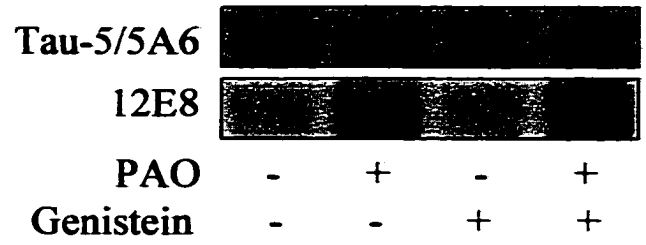

FIG. 4. Inhibition of tyrosine kinase activity does not inhibit PAO-induced $Ser^{262/356}$ phosphorylation. SH-SY5Y cells were incubated in the presence (+) or absence (-) of 200 μM genistein for 30 min followed by incubation in the presence (+) or absence (-) of 5 μM PAO for 45 min. Extracts were electrophoresed and immunoblotted with the indicated antibodies.

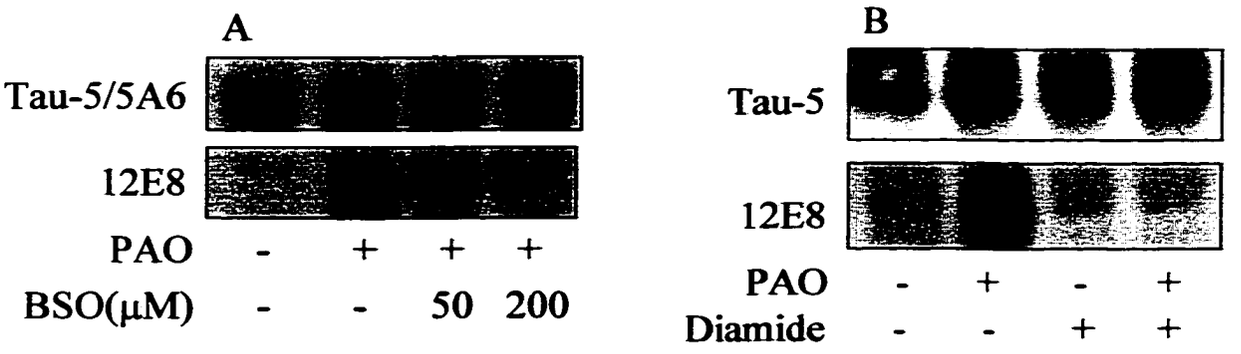

FIG. 5. PAO-induced tau phosphorylation occurs through an oxidant-sensitive pathway. **A:** SH-SY5Y cells were incubated in the presence (+) or absence (-) of the indicated concentration of BSO for 48 h and then incubated in the presence (+) or absence (-) of 5 μM PAO for 45 min. Extracts were electrophoresed and immunoblotted with the indicated antibodies. **B:** SH-SY5Y cells were preincubated for 30 min in the presence (+) or absence (-) of 500 μM diamide (in ethanol) followed by incubation in the presence (+) or absence (-) of 5 μM PAO. Extracts were electrophoresed and immunoblotted with the indicated antibodies.

horylation was examined next. Pretreatment with diamide eliminated PAO-induced $Ser^{262/356}$ phosphorylation (Fig. 5B). Diamide had no effect on basal tau phosphorylation. Additionally, pretreatment with H_2O_2 eliminated the PAO-induced increase in tau phosphorylation at $Ser^{262/356}$ (data not shown). These results show that the PAO-induced increase in tau phosphorylation at $Ser^{262/356}$ is inhibited by enhancement of the oxidative state of the cell and, therefore, likely requires a reducing environment.

PAO-induced tau phosphorylation at $Ser^{262/356}$ is inhibited by staurosporine

Because PAO appears to activate, either directly or indirectly, a tau-directed protein kinase, the effect of the protein kinase inhibitor staurosporine was examined. Pretreatment with 10 n*M* staurosporine virtually eliminated the PAO-induced increase in $Ser^{262/356}$ phosphorylation (Fig. 6A). Staurosporine did not decrease basal phosphorylation of the 12E8 epitope (data not shown). Other inhibitors, including 30 μ*M* H-89 (which inhibits PKA), 30 μ*M* KN-62 (which inhibits CaMKII), and 20 μ*M* GF109203X (which inhibits PKC), did not block PAO-induced $Ser^{262/356}$ phosphorylation (data not shown). Additionally, down-regulation of PKC levels by treating cells with 1 μ*M* PMA for 48 h did not inhibit the PAO-induced increase in $Ser^{262/356}$ phosphorylation (data not shown). The downregulation of PKC by PMA was confirmed by immunoblot analysis (data not shown).

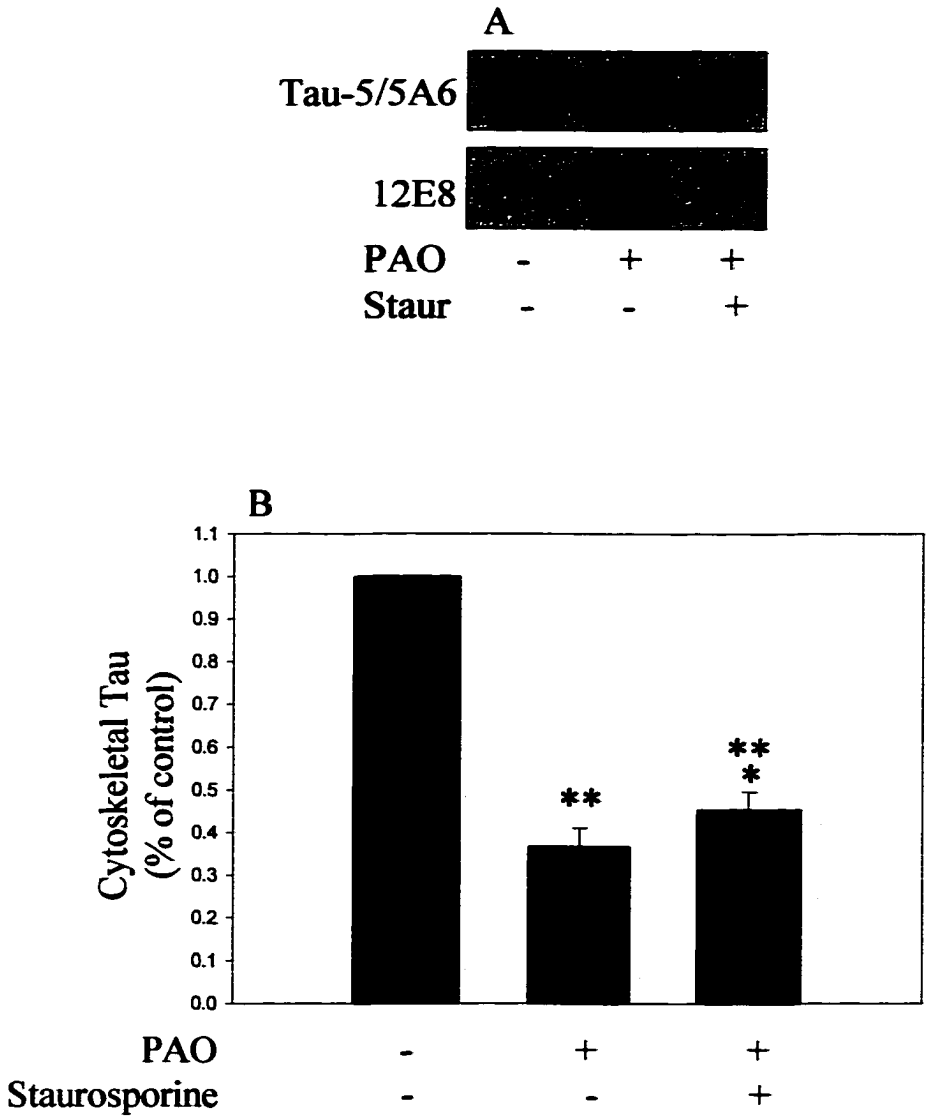

FIG. 6. Inhibition of PAO-induced 12E8 phosphorylation with staurosporine has only a minor effect on PAO-induced changes in tau localization. A: Differentiated SH-SY5Y cells were preincubated for 30 min in the presence (+) or absence (-) of 10 n*M* staurosporine (staur) followed by incubation in the presence (+) or absence (-) of 5 μ*M* PAO. Extracts were electrophoresed and immunoblotted with the indicated antibodies. B: Differentiated SH-SY5Y cells were treated as in A. Extracts were fractionated as described, and cytoskeletal fractions were electrophoresed and immunoblotted with the tau-5 antibody. Immunoblots were analyzed by densitometry and are expressed as a fraction of control. Data are means ± SEM (n = 3), ** $p < 0.01$ compared with control; * $p < 0.01$ compared with the presence of PAO and the absence of staurosporine.

Inhibition of PAO-induced tau phosphorylation at $Ser^{262/356}$ has a relatively small effect on the PAO-induced changes in tau localization

To examine the effect of inhibition of $Ser^{262/356}$ phosphorylation on the changes in tau localization, SH-SY5Y cells were pretreated with staurosporine, incubated in the presence or absence of PAO, and fractionated to separate the detergent-insoluble cytoskeleton from the detergent-soluble fraction. Fig. 6B demonstrates that PAO treatment decreased the amount of cytoskeletal tau by ~ 60%. Pretreatment with staurosporine, which prevented the PAO-induced increase in $Ser^{262/356}$ phosphorylation, resulted in a slight but statistically significant inhibition of the PAO-induced change in tau localization ($p < 0.01$, paired t test) (Fig. 6B).

The PAO-induced change in tau localization is not due to a direct effect of PAO on microtubule dynamics

To determine whether the PAO-induced increase in tau phosphorylation was a consequence, rather than a cause, of microtubule destabilization, SH-SY5Y cells were pretreated for 60 min with the microtubule-stabilizing agent taxol (20 μM) before incubation in the presence or absence of PAO. Extracts were electrophoresed and immunoblotted with 12E8 and tau-5 antibodies. Fig. 7 demonstrates that the PAO-induced increase in tau phosphorylation at $Ser^{262/356}$ was not inhibited by taxol pretreatment (Fig. 7, compare lanes 2 and 4). As has been shown before, taxol treatment decreased tau's basal phosphorylation state resulting in increased electrophoretic mobility (Xie et al., 1998). Taxol treatment, which prevented PAO- induced microtubule depolymerization as indicated by the amount of insoluble tubulin following detergent extraction (data not shown), did not inhibit the PAO-induced decrease in cyto-

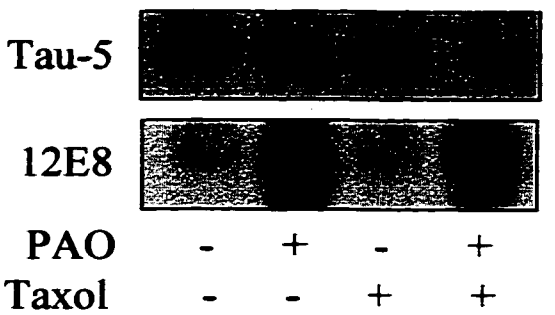

FIG. 7. The PAO-induced change in tau localization is not due to a direct effect of PAO on microtubule dynamics. SH-SY5Y cells were preincubated in the presence (+) or absence (-) of 20 μ*M* taxol for 60 min followed by incubation in the presence (+) or absence (-) of 5 μ*M* PAO. Extracts were electrophoresed and immunoblotted.

skeletally associated tau as measured by immunoblotting the cytoskeletal fraction (data not shown).

DISCUSSION

Little is known about the pathways that regulate tau phosphorylation and function in vivo. The present article contains the first evidence that tau phosphorylation within its microtubule-binding region is regulated by a thiol-reactive agent. PAO, a trivalent arsenical, reacts with neighboring thiol groups to form a stable dithioarsine ring (Gorin et al., 1997). This reaction with vicinal thiols likely mediates the varied effects of PAO on intracellular signaling cascades, including inhibition of the cytokine-stimulated DNA-binding activity of nuclear factor-κ B in rat hepatocytes (Taylor et al., 1999), activation of the receptor tyrosine kinase ErbB-4 in NIH-3T3 cells (Vecchi et al., 1998), and modulation of acetylcholine receptor clustering in *Xenopus* muscle cells (Dai and Peng, 1998). Although PAO has been shown to inhibit protein tyrosine phosphatases (Zhang et al., 1992), the inability of genistein and PP1, inhibitors of protein tyrosine phosphorylation, to inhibit PAO-mediated tau phosphorylation suggests that the observed tau phosphorylation response is independent of the ability of PAO to increase cellular tyrosine phosphorylation. In support of this hypothesis, other tyrosine phosphatase inhibitors, including sodium orthovanadate and pervanadate, failed to increase tau phosphorylation at the 12E8 epitope (data not shown). Therefore, the ability of PAO to increase tau phosphorylation may be due to the ability of PAO to bind vicinal thiol groups of proteins other than protein tyrosine phosphatases. In fact, the activity of many cellular proteins is modulated by thiol groups (see introductory section). Alternatively, the possibility cannot be excluded that PAO uniquely inhibits a

specific tyrosine phosphatase that regulates the phosphorylation of genistein- and PP1-insensitive sites.

Because PAO modifies vicinal thiol groups, it was hypothesized that cellular oxidants might have a similar effect on tau phosphorylation. However, increasing the oxidative state of the cell, either by depleting intracellular GSH with BSO (Wullner et al., 1999) or by direct addition of H_2O_2, actually inhibited PAO-induced tau phosphorylation at the 12E8 epitope. Therefore, it is unlikely that PAO is stimulating tau phosphorylation through a general oxidative mechanism. In support of this hypothesis, treatment with diamide, a thiol cross-linker thought to act through an oxidative mechanism (Becker et al., 1986), also inhibited PAO-induced tau phosphorylation. These data are consistent with previous reports indicating that tau phosphorylation is not increased in response to an acute oxidative insult. In fact, oxidative stress frequently results in tau dephosphorylation (Ko et al., 1997), suggesting that specific tau-directed protein kinases are inhibited by oxidation. The present results indicate that a reducing environment is necessary for the PAO-induced activation of the protein kinase that phosphorylates tau at $Ser^{262/356}$.

The mechanism by which PAO stimulates tau phosphorylation at $Ser^{262/356}$ is unknown. However, because PAO binds vicinal thiol groups, it is hypothesized that some component of the PAO-stimulated tau phosphorylation pathway contains thiol groups important for activity of the pathway. For example, PAO may inhibit the activity of an enzyme that constitutively inactivates the tau phosphorylation pathway in the absence of PAO. Relief of this inhibition by PAO treatment could activate some non-Pro-directed protein kinase that then phosphorylates tau within its microtubule-binding

domain. Multiple protein kinases, both Pro-directed and non-Pro-directed, have been hypothesized to phosphorylate tau in the cell. For example, PKA, CaMKII (for review, see Johnson and Jenkins, 1996), and PKC (Correas et al., 1992) phosphorylate tau in vitro within its microtubule-binding domain. However, the involvement of PKA and CaMKII were ruled out because their inhibitors, H-89 and KN-62, respectively, failed to block the PAO-induced increase in tau phosphorylation. Another protein kinase, MARK, has been shown to phosphorylate tau at Ser^{262} and Ser^{356} (Drewes et al., 1995). However, the lack of available inhibitors of MARK makes its involvement difficult to examine. Alternatively, we cannot rule out the possibility that PAO is inhibiting a constitutively active tau-directed protein phosphatase. Tau phosphorylation *in situ* has been hypothesized to be regulated by protein phosphatases 1, 2a and 2b (see Johnson and Jenkins, 1996 for review). However, these protein phosphatases dephosphorylate both Ser/Thr-Pro and non-Ser-Pro sites on tau. It is; therefore, unlikely that inhibition of one of these protein phosphatases would be responsible for the selective increase in phosphorylation of Ser 262/356 following PAO treatment.

Although no kinase-specific inhibitors were identified that eliminated PAO-induced tau phosphorylation, the general protein kinase inhibitor, staurosporine, did inhibit the response. Because staurosporine was identified originally as a potent inhibitor of PKC (for review see Gescher, 1998), and because staurosporine inhibited PAO-induced tau phosphorylation at a low concentration (10 n*M*), the involvement of PKC was examined. Neither the specific PKC inhibitor GF109203X nor PKC down-regulation with PMA was found to inhibit PAO-induced tau phosphorylation at the 12E8 epitope (data not shown), suggesting that PKC is unlikely to be involved. Additionally,

overexpression of the atypical PKC-iota had no effect on PAO-induced tau phosphorylation at $Ser^{262/356}$ (unpublished data in collaboration with Dr. M. Wooten). However, the possibility that a GF109203X- and PMA-insensitive PKC isoform, or a PKC-related protein kinase such as PKN, is involved cannot be ruled out. Additionally, because staurosporine is not specific for PKC (for reviews, see Ruegg and Burgess, 1989; Gescher, 1998), its inhibitory effect is likely due to the inhibition of other protein kinases.

Tau phosphorylation within the microtubule-binding region, specifically $Ser^{262/356}$, regulates tau-microtubule interactions in vitro (Drewes et al., 1995); therefore, the effect of PAO on tau localization was examined. Following PAO treatment, both the amount of tau associated with the cytoskeleton and the degree of microtubule polymerization were decreased, suggesting that PAO-induced tau phosphorylation caused tau to dissociate from microtubules resulting in microtubule destabilization. This is consistent with previous work suggesting that tau phosphorylation within the microtubule-binding domain is important in regulating tau-microtubule interactions and tubulin stability (Drewes et al., 1995). In addition to a decrease in microtubule polymerization, PAO treatment also decreased tubulin acetylation, which has been used as a marker for stable microtubules (Xie et al., 1998). These data are consistent with the hypothesis that PAO treatment increases tau phosphorylation within the microtubule-binding domain resulting in tau coming off the microtubules and a subsequent reduction in microtubule stability.

However, this idea was challenged by the finding that inhibition of PAO-induced tau phosphorylation at $Ser^{262/356}$ resulted in only a modest reversal of the PAO-induced

decrease in cytoskeletally associated tau. This finding suggests that residues other than $Ser^{262/356}$ are involved in regulating tau localization in situ. In fact, recent research suggests that residues outside tau's microtubule-binding domain are involved in regulating tau-microtubule interactions. Specifically, the importance of Ser^{214} in regulating tau's microtubule-binding capacity has been clearly demonstrated (Illenberger et al., 1998). It is, therefore, possible that other residues, perhaps phosphorylation of Ser^{214}, act either independently of, or in conjunction with, phosphorylation of $Ser^{262/356}$ to regulate tau localization in the cell.

An alternative interpretation is that PAO directly causes microtubule depolymerization resulting in tau delocalization and subsequent phosphorylation. However, this is unlikely, because taxol stabilization of microtubules had no effect on PAO-induced tau phosphorylation or the PAO-induced decrease in tau associated with the cytoskeleton. Therefore, tau phosphorylation likely occurs independently of, and results in, microtubule destabilization, suggesting that residues in addition to $Ser^{262/356}$ are involved in regulating tau localization.

The present results suggest that regulation of cellular thiols is important for modulating tau phosphorylation and function and that the role of $Ser^{262/356}$ phosphorylation in regulating tau localization in situ needs to be examined further. To understand fully the regulation of tau functioning in the cell, the stimuli and pathways that modulate tau phosphorylation must be understood. Furthering our understanding of the regulation of tau phosphorylation and function in situ will help us to understand tau's role under both normal and pathological conditions in vivo.

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MARK IS ACTIVATED BY PAO IN SITU AND PHOSPHORYLATES TAU WITHIN ITS MICROTUBULE-BINDING DOMAIN

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ABSTRACT

Tau is a microtubule-associated protein that is functionally modulated by phosphorylation and that is hyperphosphorylated in several neurodegenerative diseases. Because phosphorylation regulates both normal and pathological tau functioning, it is of interest to identify the signaling pathways and enzymes capable of modulating tau phosphorylation in vivo. Previous work demonstrated that in SH-SY5Y human neuroblastoma cells and rat primary cortical cultures tau is phosphorylated at $Ser^{262/356}$, within its microtubule-binding domain, by a staurosporine-sensitive protein kinase in response to the vicinal thiol-directed agent PAO. The present study demonstrates the presence of a 100-kDa protein kinase activity in SH-SY5Y cells that phosphorylates tau at $Ser^{262/356}$, is activated by PAO, and is inhibited by the protein kinase inhibitor staurosporine. Isolation of individual protein bands from a polyacrylamide gel revealed two closely spaced proteins containing $Ser^{262/356}$-directed protein kinase activity. Mass spectrometry analysis indicated that these protein bands correspond to the 100-kDa protein kinase MARK, which has previously been shown to phosphorylate tau within its microtubule-binding domain. Immunoblot analysis of the protein kinase bands confirmed that they were MARK, demonstrating that PAO activates MARK, which then phosphorylates tau within its microtubule-binding domain, resulting in a decrease in the association of tau with the cytoskeleton.

INTRODUCTION

The function of the microtubule-associated protein tau is largely regulated by site-specific phosphorylation (Drewes et al., 1995). Tau not only modulates microtubule

dynamics (Jameson et al., 1980; Lindwall and Cole, 1984) but also participates in other cellular processes such as linking signal transduction pathways to the cytoskeleton (Lee et al., 1998), activation of phospholipase C (Hwang et al., 1996; Jenkins and Johnson, 1998), and regulation of microtubule motor activity (Ebneth et al., 1998). Tau is also involved in the neuropathology of AD as a hyperphosphorylated and aggregated form was shown to comprise the PHFs which make up the NFTs in AD brain (for review, see Johnson and Jenkins, 1996). Although tau is phosphorylated at numerous sites, phosphorylation of residues within the microtubule-binding domain appears to play a dominant role in regulating the affinity of tau for microtubules (Biernat et al., 1993; Drewes et al., 1995). For example, phosphorylation of tau within the microtubule-binding region, specifically on Ser^{262} and^{356} [numbering based on the longest form of human brain tau (Goedert et al., 1989)], dramatically reduces tau's microtubule-binding capacity in vitro (Biernat et al., 1993; Drewes et al., 1995). Based on these data, it was hypothesized that Ser^{262} and, to a lesser extent, Ser^{356} play a significant role in regulating tau-microtubule interactions in vivo (Biernat et al., 1993; Drewes et al., 1995).

Much effort has gone into identifying the enzymes and signaling pathways that modulate tau phosphorylation within the microtubule-binding region (for reviews, see Johnson and Jenkins, 1996; Johnson and Hartigan, 1998). Although several protein kinases have been shown to phosphorylate these sites in vitro (Johnson and Jenkins, 1996; Johnson and Hartigan, 1998), the exact signaling cascades and protein kinases that regulate their phosphorylation in situ have not been fully elucidated. One protein kinase hypothesized to be important for the regulation of tau phosphorylation within its microtubule-binding domain in vivo is MARK (Drewes et al., 1997). Two MARK

isoforms, MARK1 and MARK2, have been identified in rat brain thus far (Drewes et al., 1997). MARK overexpression resulted in increased phosphorylation of MAPs on KXGS sites such as Ser^{262} and Ser^{356} in tau and in disruption of the microtubule array (Drewes et al., 1997). However, agents that activate MARK in situ have not been identified.

Recently, we showed that the thiol-reactive agent PAO (Jenkins and Johnson, 1999) dramatically increased tau phosphorylation at $Ser^{262/356}$ as measured by immunoreactivity with the 12E8 antibody (Seubert et al., 1995). PAO-induced tau phosphorylation correlated with a decrease in the amount of tau associated with the cytoskeleton. The increase in tau phosphorylation at $Ser^{262/356}$ was potently inhibited by low nanomolar concentrations of the protein kinase inhibitor staurosporine. Therefore, modification of cellular thiols by PAO was hypothesized to activate a staurosporine-sensitive protein kinase capable of phosphorylating the non-Ser/Thr-Pro sites within tau's microtubule-binding region.

The present study demonstrates that PAO activates a staurosporine-sensitive 100-kDa protein kinase activity. This protein kinase activity is actually composed of two separate protein bands isolated from a polyacrylamide gel. These protein bands were identified as MARK by both mass spectrometry and immunoblot analysis, providing the first demonstration of endogenous MARK activation and suggesting that MARK modulates MAP-cytoskeletal interactions in vivo.

MATERIALS AND METHODS

SH-SY5Y cell cultures

SH-SY5Y human neuroblastoma cells were grown on Corning 100-mm tissue culture dishes in RPMI 1640 (Cellgro) supplemented with 10% (vol/vol) heat-inactivated horse serum (GIBCO), 5.0% heat-inactivated Fetal Clone II (HyClone), 100 U/ml penicillin (GIBCO), 100 μg/ml streptomycin (GIBCO), and 2.0 m*M* glutamine (GIBCO). For some experiments, cells were differentiated in RPMI 1640 supplemented with 5.0% horse serum, 1.0% Fetal Clone II, penicillin, streptomycin, glutamine, and 20 μ*M* retinoic acid (Sigma) for 7-10 days prior to use.

Preparation of T4L

cDNA clone for the longest isoform of human tau (T4L) was the generous gift of Dr. M. Goedert. The cDNA clone in the expression plasmid pRK172 was expressed in the *Escherichia coli* strain BL21 (DE3) following induction with isopropyl-β-D-thiogalactoside (IPTG) at 0.4 mM. T4L was purified as previously described (Fleming et al., 1996).

Immunoblotting

SH-SY5Y cells were incubated at 37° C in media containing either 5.0 μ*M* PAO in DMSO or DMSO alone (45 min) unless otherwise indicated. The final concentration of DMSO did not exceed 0.1%. Treated cells were washed once with ice-cold PBS and collected in 2x stop buffer [500 m*M* Tris-Cl (pH 6.8), 10% SDS, 100 m*M* EGTA, 100 m*M* EDTA, and 10% glycerol] without DTT or dye. Cell extracts were sonicated and

protein concentrations were determined using the BCA method (Pierce). The samples were diluted in 2x stop buffer containing 25 m*M* DTT and bromophenol blue as the tracking dye, and the indicated amounts of cell extract were electrophoresed on 8% SDS-polyacrylamide gels. Proteins were then transferred to nitrocellulose for immunoblotting. Blots were probed with antibodies as indicated in the text and developed by ECL (Pierce). Antibodies to tau include the phosphorylation-independent antibodies tau-5 and 5A6 (Carmel et al., 1996; Johnson et al., 1997) and the phosphorylation-dependent antibody 12E8 (Seubert et al., 1995). A polyclonal antibody against MARK was also used (Drewes et al., 1997).

For immunoblotting of reelectrophoresed protein bands, taxol-stabilized microtuble/MAP-enriched fractions (see below) were electrophoresed on an 8% polyacrylamide gel. Gels were stained for total protein content with Gelcode Blue stain (Pierce), and the indicated protein bands were excised from the gel with a razor blade. Individual protein bands were soaked in 5x stop and then reelectrophoresed, transferred to nitrocellulose, and immunoblotted with the polyclonal antibody to MARK (Drewes et al., 1997).

Taxol-stabilized microtubule enrichment

Taxol stabilization of microtubules was performed by a modification of a previous procedure (Vallee, 1982). SH-SY5Y cells were homogenized in 100 m*M* PIPES (pH 6.9); 1 m*M* $MgSO_4$; 1 m*M* EGTA; 1 μ*M* okadaic acid; 1 m*M* Na_3VO_4; 1 m*M* PMSF; and 10 μg/ml each of leupeptin, aprotinin, and pepstatin. Cell homogenates were centrifuged for 15 min at 111,000 *g* at 4° C. The supernatant was incubated for 30 min

in a 37° C water bath in the presence of 20 μ*M* taxol and 2 m*M* GTP. Polymerized tubulin was pelleted by centrifugation at 57,000 *g* for 25 min at 37° C. The pellet, containing microtubules and MAPs, was resuspended in 2x Stop and stored at –80° C until use.

In gel protein kinase assay

The in gel kinase assay was performed essentially as described by Gonzalez-Nicolas et al. (1994). SH-SY5Y cells were incubated in the presence or absence of 5 μ*M* PAO. Extracts containing 10 μg of protein were electrophoresed on an 8% polyacrylamide gel containing 100 μg/ml of the longest human brain tau isoform, T4L, polymerized into the matrix. Following electrophoresis, protein within the gels was denatured and renatured by incubating consecutively in 50 m*M* Tris-Cl (pH 8.0) containing 20% 2-propanol (1 h), 50 m*M* Tris-Cl containing 5 m*M* 2-mercaptoethanol (buffer A) (1 h), 6 *M* guanidine HCl in buffer A (1 h), and 0.04% Tween 20 in buffer A (overnight at 4°C with 5 changes). The gels were then incubated in phosphorylation buffer (buffer A containing 0.04% tween 20 and 5 m*M* $MgSO_4$, pH 7.4) for 30 min at room temperature followed by addition of 300 μ*M* ATP and incubation for 5 min (or indicated time) at room temperature. Gels were then rinsed once with ddH_2O and washed 5 min in 10% SDS. Gels were transferred for 4 h at 100 volts to nitrocellulose and immunoblotted with the 12E8 antibody.

For measurement of protein kinase activity in reelectrophoresed protein bands, the taxol-stabilized microtubule/MAP-enriched fraction (see above) was electrophoresed and gels were stained with Gelcode Blue stain (Pierce) to visualize total protein content.

Gelcode Blue stain allowed protein staining in the absence of acetic acid, which could permanently impair protein kinase activity. Indicated protein bands were then excised from the gel and reelectrophoresed on an 8% polyacrylamide gel containing 100 μg/ml T4L.

Isolation of protein bands and mass spectrometry

Microtubule/MAP-enriched fractions were electrophoresed on a polyacrylamide gel. Gels were stained with Coomassie Brilliant Blue and destained with 20% methanol and 10% acetic acid. Indicated protein bands were excised from the gel using a razor blade and submitted to the UAB Comprehensive Cancer Center mass spectrometry shared facility. Mass spectrometric analysis was carried out by a previously established procedure (see *http://donatello.ucsf.edu/MSF/ingel.html* for method). Masses of resulting peptides were compared to predicted peptide masses for MARK, PKN, PKA, and CaMKII using the protein prospector program (*http://donatello.ucsf.edu*).

RESULTS

The ability of PAO to induce tau phosphorylation at $Ser^{262/356}$ was examined using the phosphorylation-specific antibody 12E8. Incubation of differentiated SH-SY5Y cells in the presence of PAO (5 μ*M*) increased tau phosphorylation at the 12E8 epitope (Fig. 1A, compare lanes 1 and 2). This PAO-induced tau phosphorylation at $Ser^{262/356}$ is completely eliminated by preincubation with the protein kinase inhibitor staurosporine (10 n*M*) (Fig. 1A, compare lanes 2 and 3). Changes in 12E8 immunoreactivity are not due to changes in tau levels as indicated by the

phosphorylation-independent antibodies tau-5 and 5A6 (Fig. 1A. top panel). Similar findings were obtained in undifferentiated SH-SY5Y cells (data not shown). Next. the ability of PAO to activate a $Ser^{262/356}$-directed protein kinase activity was investigated using the in gel protein kinase assay as described. Extracts from cells treated with 5.0 μM PAO (Fig. 1B) were electrophoresed on a polyacrylamide gel containing T4L polymerized into the matrix. Gels were washed and incubated in the presence of phosphorylation buffer and ATP to allow any renatured, active, and tau-directed protein kinases contained in the extract to phosphorylate the tau polymerized into the gel. Gels were then transferred to nitrocellulose and immunoblotted with the 12E8 antibody revealing only protein kinase activity that phosphorylates tau at $Ser^{262/356}$. Fig. 1B demonstrates that a 100-kDa protein kinase activity phosphorylates tau at $Ser^{262/356}$ under unstimulated conditions (lane 1). This 100-kDa, $Ser^{262/356}$-directed protein kinase activity was activated in extracts from cells incubated in the presence of PAO (Fig. 1B, compare lanes 1 and 2). Activation by PAO causes an apparent decrease in the electrophoretic mobility of the 100-kDa protein kinase activity. Fig. 1B also demonstrates that no other protein kinase activities in the cell extract phosphorylate tau at $Ser^{262/356}$ to a comparable extent as measured by the in gel kinase assay. No 12E8 immunoreactivity was seen when the in gel kinase assay was performed either in the absence of ATP in the phosphorylation buffer or in the absence of tau polymerized into the gel matrix (data not shown).

Because PAO-stimulated tau phosphorylation at $Ser^{262/356}$ was inhibited by pretreatment with staurosporine (Jenkins and Johnson, 1999), the effect of staurosporine on the 100-kDa protein kinase activity was investigated. In Fig. 2, extract from PAO-tr-

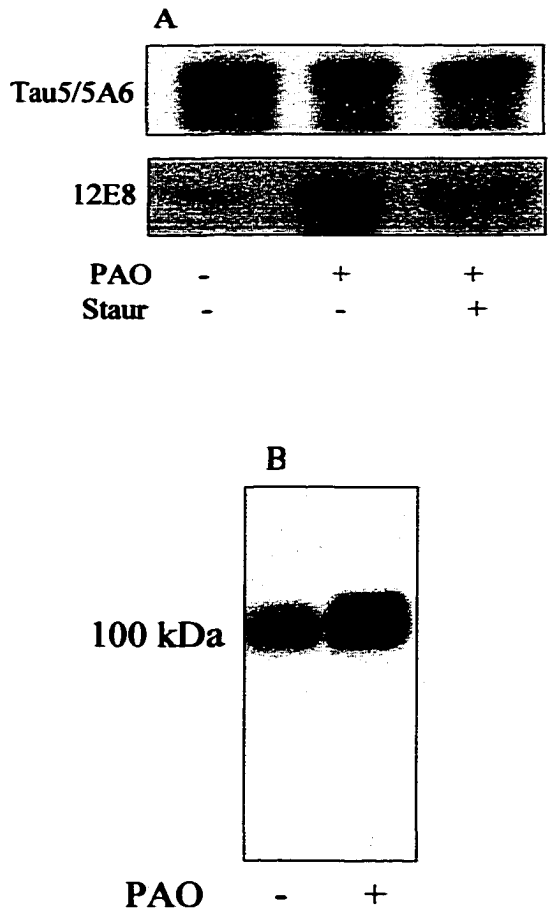

FIG. 1. PAO stimulates tau phosphorylation and activation of a 100-kDa protein kinase activity in situ. In **(A)**, differentiated SH-SY5Y cells were incubated in the presence (+) or absence (-) of 10 n*M* staurosporine for 30 min followed by incubation in the presence (+) or absence (-) of PAO (5 μ*M*) for 45 min. Extracts were electrophoresed and immunoblotted with the indicated antibodies. In **(B)**, SH-SY5Y cells were incubated in the absence (-) or presence (+) of 5.0 μ*M* PAO for 45 min. Extracts (10 μg) were electrophoresed on an 8% polyacrylamide gel containing 100 μg/ml of T4L polymerized into the matrix. Gels were washed as described and incubated in phosphorylation buffer containing 300 μ*M* ATP for 5 min. Gels were then transferred to nitrocellulose and immunoblotted with the 12E8 antibody.

eated SH-SY5Y cells was electrophoresed on a polyacrylamide gel containing tau polymerized into the matrix. Individual lanes were preincubated for 60 min with 0, 1, or 10 n*M* staurosporine prior to phosphorylation. At 1 n*M*, staurosporine dramatically reduced the amount of $Ser^{262/356}$-directed protein kinase activity migrating at 100-kDa (compare lanes 1 and 2 in Fig. 2). This protein kinase activity was virtually eliminated by pretreatment with 10 n*M* staurosporine (compare lanes 1 and 3 in Fig. 2). To determine whether the 100-kDa protein kinase activity associates with microtubules, a taxol-stabilized, microtubule/MAP-enriched fraction was prepared as described. This microtubule/MAP fraction was electrophoresed on a polyacrylamide gel containing tau polymerized into the matrix and was assayed for $Ser^{262/356}$-directed protein kinase activity. Fig. 3A demonstrates the presence of a 100-kDa protein kinase activity that associates with microtubules and that phosphorylates tau at $Ser^{262/356}$. In Fig. 3B, the microtubule/MAP fraction was electrophoresed on a polyacrylamide gel and stained for total protein content. Two bands, labeled 1 and 2 in Fig. 3B and migrating at the approximate molecular mass of the $Ser^{262/356}$-directed protein kinase activity, were cut from the gel. In Fig. 3C, these bands were reelectrophoresed on a polyacrylamide gel containing tau polymerized into the matrix for measurement of protein kinase activity. Both bands contained $Ser^{262/356}$-directed protein kinase activity, suggesting that the 100-kDa protein kinase activity seen in crude extracts and in microtubule/MAP preparations is actually composed of two protein bands. Protein kinase activity of reelectrophoresed bands appears circular on the in gel kinase blot because cut bands were loaded vertically into the gel, causing compression of the electrophoresed protein.

Based on molecular mass and substrate specificity, the 100-kDa protein kinase

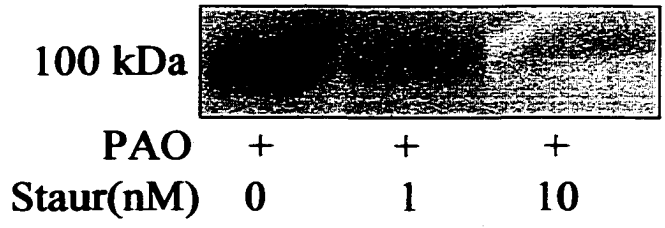

FIG. 2. The 100-kDa protein kinase activity is inhibited by staurosporine. SH-SY5Y cells were incubated in the presence of 5 μ*M* PAO for 45 min. Extract (10 μg) was electrophoresed on an 8% polyacrylamide gel containing 100 μg/ml of T4L polymerized into the matrix. Gels were washed as described and incubated in the presence of 0, 1, or 10 n*M* staurosporine (staur) as indicated. Gels were then incubated in phosphorylation buffer containing 300 μ*M* ATP for 5 min, transferred to nitrocellulose, and immunoblotted with the 12E8 antibody.

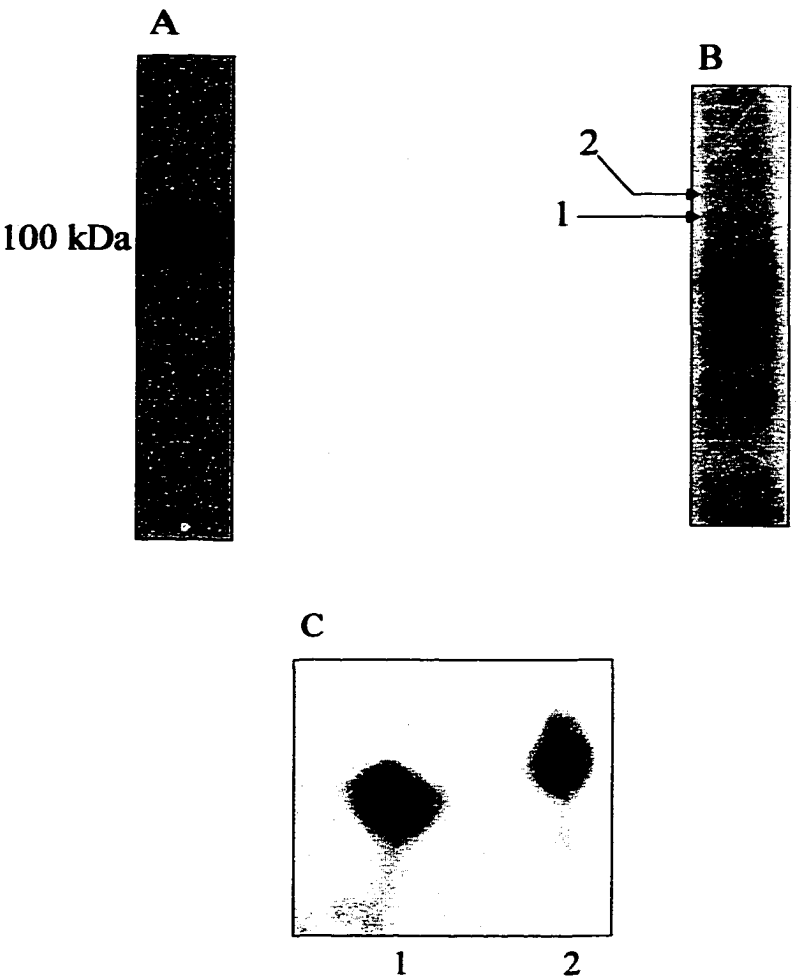

FIG. 3. Two protein bands from the microtubule/MAP fraction contain $Ser^{262/356}$-directed protein kinase activity. In **(A)**, the taxol-stabilized microtubule fraction (1 μg) from SH-SY5Y cells was electrophoresed on an 8% polyacrylamide gel containing 100 μg/ml of T4L polymerized into the matrix. The gel was washed as described, incubated in phosphorylation buffer containing 300 μ*M* ATP for 15 min, transferred to nitrocellulose, and immunoblotted with the 12E8 antibody. In **(B)**, the taxol-stabilized microtubule fraction from SH-SY5Y cells was electrophoresed on an 8% polyacrylamide gel and total protein was stained with Coomassie Brilliant Blue stain. In (C), the bands indicated in (B), stained with Gelcode Blue stain reagent (Pierce), were cut from the gel and reelectrophoresed on an 8% polyacrylamide gel containing 100 μg/ml of T4L polymerized into the matrix. The gel was washed as described, incubated in phosphorylation buffer containing 300 μ*M* ATP for 60 min, transferred to nitrocellulose, and immunoblotted with the 12E8 antibody. Lanes 1 and 2 correspond to bands 1 and 2, respectively, in (B).

activity was hypothesized to be the previously identified MARK (Drewes et al., 1997). To evaluate this hypothesis, each band was cut from a polyacrylamide gel, proteolyzed with trypsin, and subjected to mass spectrometry. The resulting peaks, representing peptide masses, were compared to predicted trypsin peptides for MARK. Predicted peptide masses were generated using the Protein Prospector program (*http://donatello.uscf.edu*). Of the peptides generated from protein band 1 (from Fig. 3B), 41% matched predicted peaks in MARK1/2 from rat brain (Drewes et al., 1997). It should be noted that SH-SY5Y cells are of human origin. To determine whether the peptide matches were specific for MARK, or whether the same degree of overlap would be found in unrelated proteins, three control protein kinases, PKN, PKA, and CaMKII, were chosen for comparison. The predicted peak masses for these three protein kinases exhibited minimal overlap, 8% for PKN, 8% for PKA, and 10% for CaMKII, with peptide masses from band 1. Similarly, 43% of the peptides generated from protein band 2 (from Fig. 3B) matched the masses of predicted peptides for MARK1/2, while minimal overlap was found for PKN (13%), PKA (10%), and CaMKII (11%). These results suggest that both protein bands correspond to MARK. To evaluate this hypothesis further the protein bands indicated in Fig. 3B were cut from a polyacrylamide gel, reelectrophoresed, transferred to nitrocellulose, and immunoblotted with an antibody against rat MARK. Fig. 4 demonstrates that both bands were immunoreactive with the MARK antibody. A control band of comparable intensity was not recognized by the MARK antibody (data not shown).

DISCUSSION

Although many signaling pathways are likely to modulate tau phosphorylation in vivo, little is known about the specific enzymes involved. Identification of the protein kinases that phosphorylate tau in intact cells is crucial for understanding both the normal and pathological regulation of tau function. The present study reports several novel findings and represents an important step toward understanding the regulation of tau function. The thiol-reactive agent, PAO, which stimulates tau phosphorylation within its microtubule-binding domain by a staurosporine-sensitive protein kinase (Jenkins and Johnson, 1999), was shown to activate a 100-kDa protein kinase activity. This 100-kDa protein kinase activity phosphorylates tau at $Ser^{262/356}$, is inhibited by staurosporine, and copurifies with microtubules. Two separate protein bands on a polyacrylamide gel constitute the kinase activity, and these bands correspond to the previously described protein kinase MARK. This is the first demonstration of activation of endogenous MARK, and the results suggest that MARK is a physiologically relevant regulator of tau phosphorylation at $Ser^{262/356}$.

Because mass spectrometric analysis of peptides generated by trypsin cleavage of the identified protein kinase bands revealed much greater overlap with predicted peptide masses from MARK than with predicted peptide masses from PKN, PKA, or CaMKII, the kinase bands were tentatively identified as MARK. The overlap between peptides from the protein bands and the predicted peptides for MARK was just over 40% despite the fact that the MARK sequence used for prediction of peptide masses was from rat (Drewes et al., 1997), while the peptides generated from the protein bands were of human origin. Slight sequence differences between species, even conservative amino

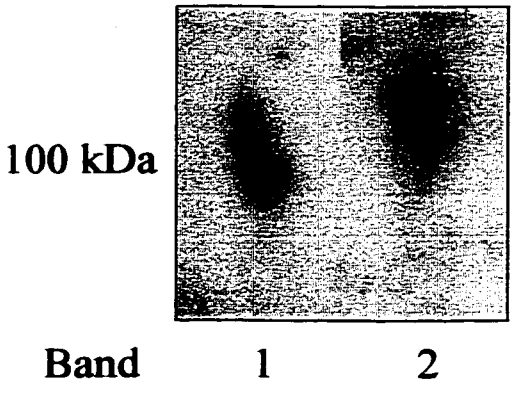

FIG. 4. Protein kinase bands from microtubule/MAP fraction correspond to MARK. The microtubule-enriched fraction was electrophoresed and stained for total protein, and the bands indicated in Fig. 3 were excised from the gel. Individual protein bands were electrophoresed on a polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with a polyclonal antibody against MARK (1:250). Bands 1 and 2 correspond to 1 and 2 in Fig. 3B.

acid substitutions, would be expected to change the trypsin cleavage profile of the protein resulting in different peptide masses. The three control protein kinases, PKN, PKA, and CaMKII (all from rat), were chosen because all three phosphorylate tau (Johnson and Jenkins, 1996; Kawamata et al., 1998); however, two of the three, PKA and CaMKII, cannot be involved in the 100-kDa protein kinase activity based on molecular mass of the protein. The finding that the predicted peptide masses for these three protein kinases exhibit little overlap with the peptides obtained from the microtubule/MAP preparation strengthens the argument that the more extensive matching seen with MARK represents a real overlap in sequences.

Confirmation of the hypothesis that the cut protein bands correspond to MARK was achieved by immunoblotting the individual protein bands with a MARK antibody. Given previous findings that a PAO-activated, staurosporine-sensitive protein kinase phosphorylates tau at $Ser^{262/356}$ (Jenkins and Johnson, 1999), the present results suggest that PAO-activated MARK phosphorylates tau at $Ser^{262/356}$ in situ. The mechanism by which PAO activates MARK is unknown, although, based on previous work (Jenkins and Johnson, 1999), it is unlikely to be due to inhibition of tyrosine phosphatase activity. MARK activation by PAO may involve stimulation of a signaling pathway that results in phosphorylation of MARK at specific Ser and Thr residues critical for kinase activity (Drewes et al., 1997). Phosphorylation of these residues decreases MARK's electrophoretic mobility, consistent with the present observation that PAO treatment decreases the electrophoretic mobility of the 100-kDa protein kinase activity. Because the effects of PAO on cell functioning are thought to be mediated by its ability to bind

vicinal thiols, the MARK activation pathway may contain a vicinal thiol-containing protein that is susceptible to PAO activation.

MARK is likely related to two recently identified 100-kDa protein kinase activities enriched in embryonic rat (Jenkins and Johnson, 1996) and chick (Lopez and Sheetz, 1995) brains. The embryonic rat brain protein kinase activity copurified with microtubules, phosphorylated tau at $Ser^{262/356}$, and decreased the ability of tau to bind microtubules in vitro (Jenkins and Johnson, 1996). In addition to tau, this embryonic rat brain protein kinase activity phosphorylated MAP-2. The embryonic chick brain protein kinase also phosphorylated MAP-2, causing release of MAP-2 from microtubules (Lopez and Sheetz, 1995). This is consistent with a previous report that, in addition to tau, MARK phosphorylates MAP-2 and MAP-4 on KXGS sites within the microtubule-binding domains of these MAPs (Illenberger et al., 1996). These KXGS sequences are homologous to sequences surrounding Ser^{262} and Ser^{356} within tau's microtubule-binding domain. Thus, MARK may be a developmentally regulated protein kinase, with expression of highest activity in the embryonic brain, that regulates the microtubule-binding activity of multiple MAPs. Enhancement of MARK activity in the embryonic brain could partially account for the higher levels of tau phosphorylation observed in embryonic compared to adult brain (for review, see Johnson and Jenkins, 1996).

Although the present results indicate that MARK contributes to PAO-induced tau phosphorylation, the involvement of other protein kinases cannot be excluded. Because the in gel protein kinase assay requires that a kinase renature inside the polyacrylamide gel following electrophoresis, some protein kinases are likely to become permanently inactivated by sample preparation. Additionally, any protein kinases requiring co-

factors (other than magnesium) or associated protein subunits for activity would not be visible on the in gel kinase assay because of the denaturing conditions present during sample preparation and electrophoresis. This leaves open the possibility that protein kinases, in addition to MARK, also contribute to PAO-induced tau phosphorylation.

The present finding that MARK is inhibited by low concentrations of staurosporine is interesting in light of previous work demonstrating that tau is phosphorylated at $Ser^{262/356}$ by a staurosporine-sensitive protein kinase in response to osmotic stress (Jenkins et al., 1999). This osmotic stress-induced tau phosphorylation at $Ser^{262/356}$ is eliminated by 10 n*M* staurosporine, the same concentration used to inhibit PAO-induced tau phosphorylation (Jenkins and Johnson, 1999), suggesting the protein kinases involved in osmotic stress-induced and PAO-induced tau phosphorylation are either closely related or the same protein kinase. There may be other staurosporine-sensitive protein kinases that contribute to PAO-induced tau phosphorylation at $Ser^{262/356}$. For example, staurosporine potently inhibits PKC. However, neither PKC down-regulation nor inhibition eliminated PAO-induced or osmotic stress-induced tau phosphorylation (Jenkins and Johnson, 1999; Jenkins et al., 1999). Thus, while other staurosporine-sensitive protein kinases may participate in PAO-stimulated tau phosphorylation, PKC is not likely to be involved.

In conclusion, the present study examined a 100-kDa protein kinase activity that phosphorylates tau within its microtubule-binding domain in response to treatment with PAO. This protein kinase activity was identified as MARK, which is hypothesized to be phosphorylate tau in situ in response to PAO treatment. These findings represent an important step in understanding the regulation of tau phosphorylation and function.

Future work will further our understanding of the specific pathways involved in tau phosphorylation by MARK and will have implications for understanding tau function under normal and, perhaps, under pathological conditions.

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PHOSPHORYLATION OF MICROTUBULE-ASSOCIATED PROTEIN TAU ON SER^{262} BY AN EMBRYONIC 100-KD PROTEIN KINASE

by

SCOTT M. JENKINS AND GAIL V. W. JOHNSON

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ABSTRACT

This study examined the phosphorylation of tau on Ser^{262}, within the first microtubule-binding domain, by a developmentally regulated 100-kD protein kinase exhibiting significantly greater activity in the embryonic rat brain than in the adult rat brain. This protein kinase copurified with microtubules and coimmunoprecipitated with both tau and MAP-2. In addition to phosphorylating tau, MAP-2, and a Ser^{262}-containing peptide, the present protein kinase activity was shown to autophosphorylate as determined by the in gel kinase assay in the absence of any protein or peptide polymerized into the matrix. Phosphorylation of tau with this protein kinase significantly reduced the tau-microtubule interaction, and the effect was significantly greater with MAP preparations from embryonic brain than with preparations from the adult. Ser^{262} is phosphorylated extensively in PHF tau from AD brain, to a lesser extent in fetal tau, and only to a very minor extent in biopsy-derived human tau. Because the 100-kD protein kinase activity phosphorylates Ser^{262} and is higher in the fetal brain than the adult brain, it is hypothesized that an inappropriate reexpression and/or reactivation of this or a similar developmentally regulated protein kinase could contribute to the phosphorylation of Ser^{262} in PHF-tau, and thus play a role in the pathogenesis of AD.

INTRODUCTION

Tau is a predominantly neuronal family of phosphoproteins enriched in axons that bind to and stabilize microtubules in vitro (Weingarten et al., 1975; Lindwall and Cole, 1984; Binder et al., 1985) and presumably perform a similar function in vivo

(Lovestone et al., 1996). The adult human brain contains six isoforms of tau resulting from alternative splicing of a single RNA transcript (Goedert et al., 1989). Tau isoforms differ by the presence of either three or four imperfect repeats of 31-32 amino acids in the carboxy-terminal region, and either no, one, or two amino-terminal inserts (Goedert et al., 1989). Early studies demonstrated that tau in a more extensively phosphorylated state had a decreased affinity for microtubules and was less able to promote microtubule assembly compared to dephosphorylated tau (Lindwall and Cole, 1984; Gustke et al., 1992). In addition, hyperphosphorylated tau, which is the major protein component of PHFs found in AD brains (Grundke-Iqbal et al., 1986; Wood et al., 1986; Kosik et al., 1986; Ihara et al., 1986), was found to be microtubule-binding incompetent (Bramblett et al., 1992, 1993). These findings have led to the hypothesis that abnormal phosphorylation of tau may be involved in the neurodegeneration seen in AD, possibly through a mechanism involving microtubule depolymerization (Trojanowski and Lee, 1995; Johnson and Jenkins, 1996). Considering this hypothesis, many studies have focused on identifying the protein kinases that phosphorylate tau and modulate its microtubule affinity.

Although PHF tau from AD brain is extensively hyperphosphorylated on multiple residues, hyperphosphorylation itself may be a result of, rather than an initiating event in, compromising tau's ability to bind microtubules. In fact, recent evidence indicates that site-specific phosphorylation, rather than an increase in the overall phosphorylation state, plays a more important role in decreasing tau's microtubule-binding capacity. Previously, it has been shown that phosphorylation of tau on a specific

serine residue within the first repeat of the microtubule-binding domain of tau [Ser^{262}: numbering based on longest human tau isoform (Goedert et al., 1989)] dramatically decreases tau's ability to bind and stabilize microtubules (Biernat et al., 1993). In fact, phosphorylation of tau at only two sites within the microtubule-binding region (Ser^{262} and Ser^{356}) decreases tau's microtubule-binding capacity to a greater extent than does phosphorylation of many Ser/Thr-Pro sites by MAP kinase (Biernat et al., 1993). In addition, Ser^{262} is phosphorylated in AD brain but not in autopsied normal adult brain (Hasegawa et al., 1992; Morishima-Kawashima et al., 1995), and only minimally in brain biopsy samples from controls (Seubert et al., 1995).

Based on mass spectrometric measurements and immunoblot analyses, tau from embryonic brain is phosphorylated to a greater extent than normal adult tau on multiple residues [including Ser^{262} (Seubert et al., 1995)] and promotes microtubule assembly less efficiently than does normal adult tau (Hasegawa et al., 1992; Seubert et al., 1995). However, fetal tau is phosphorylated to a lesser extent than tau in AD brain (Peng et al., 1986) and fetal tau remains microtubule-binding competent (Yoshida and Ihara, 1993). The similarity in phosphorylation states of fetal and PHF tau, as well as the possible increases in mitotic protein kinases in AD brain (Liu et al., 1995; Vincent et al., 1996), has led to the hypothesis that AD in part represents an inappropriate recapitulation of the developmental state (Bramblett et al., 1993; Goedert et al., 1993; Vincent et al., 1996). It is therefore important to examine the developmentally regulated expression of protein kinases which may play a role in modulating the phosphorylation state of tau.

Given the importance of the phosphorylation state of Ser^{262}, as well as homologous sites in the other 3 microtubule-binding repeats (KXGS motif, Ser^{293}, ^{324}, and ^{356}) in modulating the microtubule-binding competency of tau, identifying the protein kinases that phosphorylate tau at these sites is critical. Both PKA and CaMKII are able to phosphorylate tau in vitro on Ser^{262} and Ser^{356} within the microtubule-binding domain, although only to a relatively low stoichiometry (Litersky et al., 1996). However, a 110-kD protein kinase known as MARK recently purified from bovine brain phosphorylates tau predominantly on the KXGS motifs located in the microtubule-binding repeats (Drewes et al., 1995; Illenberger et al., 1996). Phosphorylation of tau by MARK dramatically decreases tau's ability to bind microtubules. This protein kinase was subsequently shown to phosphorylate both MAP-2 and MAP-4 on KXGS sites corresponding to those within the tau molecule (Illenberger et al., 1996). This has led to the hypothesis that the phosphorylation of tau by MARK may be inappropriately increased in AD and result in a decrease in tau's ability to bind to and stabilize microtubules. This loss of microtubule-binding capacity by tau due to increased MARK phosphorylation could disrupt axonal transport and compromise neuronal function (Trojanowski and Lee, 1995; Johnson and Jenkins, 1996). Further, the abnormal accumulation of tau in the neuronal perikarya could secondarily result in tau hyperphosphorylation due to its inappropriate localization (Johnson and Jenkins, 1996).

In the present study, a developmentally regulated 100-kD protein kinase from rat brain that copurifies with microtubules and coimmunoprecipitates with MAP-2 and tau was examined. This protein kinase readily phosphorylates tau on Ser^{262} and significantly

decreases tau's microtubule-binding capacity. High levels of the 100-kD protein kinase activity are expressed in the embryonic brain, while activity levels are significantly lower in the adult. It is likely that this 100-kD protein kinase from embryonic rat brain shares homology with bovine MARK, as well as with an independently identified 100kD protein kinase from chicken brain that was shown to phosphorylate MAP-2 and decrease its association with microtubules (Lopez and Sheetz, 1995).

MATERIALS AND METHODS

Tau isoform expression

cDNA clone for the longest isoform of human tau (T4L) was the generous gift of Dr. M. Goedert. The cDNA clone in the expression plasmid pRK172 was expressed in the *Escherichia coli* strain BL21 (DE3) following induction with IPTG at 0.4 m*M* (Goedert and Jakes, 1990). T4L was purified as previously described (Fleming et al., 1996).

MAP-2 purification

MAP-2 was purified from bovine brain as previously described (Johnson et al., 1991).

Peptide synthesis

Tau peptides were synthesized at the UAB Comprehensive Cancer Center core peptide synthesis and analysis facility. The sequence of the Ser^{262} peptide,

KSKIGSTENLKHQPGGG, corresponds to amino acid residues 257-273 of the first microtubule-binding repeat of the largest isoform of human tau (Goedert et al., 1989).

Taxol-stabilized microtubule preparation

Fresh bovine or porcine brains from a local slaughterhouse were cleaned of meninges and homogenized in 100 m*M* PIPES (pH 6.9), 1 m*M* $MgSO_4$, 1 m*M* EGTA, 1 m*M* PMSF, and 10 μg/ml each of leupeptin, aprotinin, and pepstatin. Twice-cycled microtubules were prepared from the homogenates as described previously (Shelanski et al., 1973). Tubulin was isolated from the twice-cycled microtubules using a phosphocellulose column (Johnson et al., 1991). The tubulin that eluted in the void volume was used immediately to prepare taxol-stabilized microtubules by the method of Vallee (1982). The resulting taxol-stabilized microtubules were stored in aliquots at –80°C until use.

MAP preparation

Pregnant Sprague-Dawley rats were sacrificed following CO_2-induced anesthesia. Embryos (E15-E21) were excised and the forebrains of both embryos and adults were removed. Embryonic and adult forebrains were homogenized (1:1.5 wt/vol) in 35 m*M* PIPES (pH 7.4), 5 m*M* $MgSO_4$, 1 m*M* EGTA, 0.5 m*M* EDTA, 1 m*M* PMSF, and 10 μg/ml each of leupeptin, aprotinin, and pepstatin. MAPs and associated proteins were prepared from each homogenate using the taxol and salt-dependent procedure of Vallee (1982). Following salt extraction, the MAP preparation was dialyzed overnight

against homogenization buffer and concentrated by dialysis against 50% glycerol in homogenization buffer. Samples were removed for protein determination by the BCA (Smith et al., 1985) and the remainder of the MAP preparation was stored in aliquots at –80°C until use.

Phosphorylation of Ser^{262} peptide by MAP preparation

Embryonic and adult MAP preparations were incubated at a final concentration of 0.4 μg/μl in a phosphorylation buffer containing 35 m*M* PIPES (pH 7.2), 0.1 m*M* DTT, 10 m*M* $MgSO_4$, 1 m*M* EGTA, 0.5 m*M* EDTA, 2 μ*M* protein kinase inhibitor (Sigma), 100 μ*M* ATP, 0.1 μ*M* okadaic acid, protease inhibitors (as indicated above), [γ-^{32}P]ATP (0.1 μCi/nmol ATP) and either 100 μ*M* Ser^{262} peptide (see above) or an equivalent amount of ddH_2O in a final volume of 30 μl. Protein kinase inhibitor is a selective inhibitor of PKA (Cheng et al., 1986). The reaction mixture was incubated at 37°C for 1 h and was stopped by spotting a 5-μl aliquot of the phosphorylation mixture onto 2 cm x 1 cm P81 filter paper (Whatman) and immersing the paper in 0.5% phosphoric acid. After extensive washing, the filter paper was rinsed in 95% ethanol and air dried. ^{32}P incorporation into the peptide was determined with a Beckman LS 6500 liquid scintillation counter by subtracting control values (without peptide) from the corresponding values with peptide present.

Coimmunoprecipitation of 100-kD protein kinase with tau and MAP-2

Adult and embryonic MAP preparations (100 μg) were incubated with either a monoclonal antibody to MAP-2 (AP-14) (Binder et al., 1984; Peng et al., 1986), a non-phosphate-dependent monoclonal antibody to tau (tau-5) (Carmel et al., 1996), non-immune rabbit IgG, or nonimmune mouse IgG (approximately 1 μg of antibody per 10 μg of protein) overnight at 4°C (rotating gently). Following incubation, washed protein A-linked Sepharose beads equilibrated with homogenization buffer (see above) were added (approximately 2 μl beads/μg antibody) and rotated gently at 4°C for 4 h. Immunoprecipitates were rinsed 5x with homogenization buffer, boiled for 8 min in 2x SDS stop solution (containing 10% SDS, 100 m*M* EGTA, 100 m*M* EDTA, 25 m*M* DTT, 10% glycerol, and 0.5 *M* Tris-HCl, pH 6.8), and centrifuged at 9,000 *g* for 45 seconds to pellet the Sepharose beads. Supernatants were removed and stored at -20°C until use in the in gel kinase assay (see below).

In gel kinase assays

The in gel kinase assay using [γ-^{32}P]ATP was performed according to the method of Kameshita and Fujisawa (1989). In brief, immunoprecipitated fractions or the MAP preparations (10 μg) were electrophoresed on 8% SDS-polyacrylamide gels with either 100 μg/ml of T4L, 100 μg/ml of bovine MAP-2, or 1 m*M* (unless specified otherwise) Ser^{262} peptide (see above) polymerized into the matrix. For autophosphorylation studies, no protein or peptide was polymerized into the gel. Following electrophoresis, protein within the gels was denatured and renatured by incubating consecutively in 50 m*M* Tris-

HCl (pH 8.0) containing 20% 2-propanol (1 h). 5 m*M* 2-mercaptoethanol in 50 m*M* Tris-HCl (buffer A) (1 h). 6 *M* guanidine HCl in buffer A (1 h). and 0.04% Tween 20 in buffer A (overnight at 4°C). The gels were then incubated in homogenization buffer (see above) for 30 min at room temperature followed by addition of [γ-^{32}P]ATP (10 μCi/ml) and 50 μM ATP, and incubation for 2 h at room temperature. Following the incubation. gels were washed extensively with 5% trichloroacetic acid containing 1% sodium pyrophosphate until background radioactivity was negligible. Gels were then vacuum dried and exposed to X-ray film for autoradiography.

The in gel kinase assay using nonlabeled ATP was performed as described by Gonzalez-Nicolas et al. (1994) with 100 μg/ml of T4L polymerized into the gel. Electrophoresed polyacrylamide gels were transferred to nitrocellulose and probed with a monoclonal antibody (12E8) which recognizes tau only when $Ser^{262/356}$ are phosphorylated (Seubert et al., 1995) at a concentration of 50 ng/ml. Blots were then probed with the appropriate secondary antibody and developed with ECL (Pierce).

Tau phosphorylation for microtubule-binding assay

Fifty micrograms of T4L was incubated with 1 μg of adult or embryonic MAP preparations in 25 m*M* Tris (pH 7.4), 10 m*M* $MgCl_2$, 10 μM protein kinase inhibitor, 1 m*M* EGTA, 1 μM okadaic acid, and 2 μM KN-62 (a selective CaMKII inhibitor) (Seikagaku Corp.) in the absence (control) or the presence (phosphorylated) of 1 m*M* ATP in a final volume of 50 μl for 4 h at 37°C. Samples were frozen at -20°C until use in microtubule-binding assay (see below).

Microtubule-binding assay

Phosphorylated or control T4L (see above) was incubated at a final concentration of 0.01 μg/μl with 1 μg/μl of taxol-stabilized bovine or porcine microtubules (see above) in 50 m*M* PIPES (pH 6.9), 0.5 m*M* $MgSO_4$, 1 m*M* EGTA, 20 μ*M* taxol, and 1 m*M* GTP (binding buffer) (prewarmed to 37°C) in a final volume of 50 μl for 30 min at 37°C. Samples were then underlaid with prewarmed binding buffer containing 10% sucrose before centrifugation in a Beckman Airfuge at 100,000 *g* for 30 min. The microtubule pellet was rinsed gently in prewarmed binding buffer, resuspended in 2x SDS stop solution, boiled, and frozen at -20°C until use. Samples and appropriate standards were electrophoresed on SDS-polyacrylamide gels, transferred to nitrocellulose, and blotted with the monoclonal antibody Tau-5. The amount of tau in each lane, corresponding to the amount of tau bound to microtubules, was quantitated using densitometry.

Time course of T4L phosphorylation

T4L at a final concentration of 0.2 μg/μl was incubated with the embryonic MAP preparation (0.3 μg/μl) in 25 m*M* Tris (pH 7.4), 10 m*M* $MgCl_2$, 1 m*M* ATP, 2 μ*M* protein kinase inhibitor, 1 m*M* EGTA, and 1 μ*M* okadaic acid at 37°C. Aliquots of 5 μl were removed at 0, 15, 30, 60, 120, and 240 min and diluted to 0.02 μg/μl T4L in 2x SDS stop solution. Fifteen nanograms of T4L from each sample were electrophoresed on 8% SDS-polyacrylamide gels followed by immunoblotting with the monoclonal antibody (12E8) at a concentration of 50 ng/ml.

RESULTS

100-kD protein kinase activity associated with microtubules phosphorylates tau and MAP-2

To identify protein kinases associated with microtubules that phosphorylate either MAP-2 or tau, MAP preparations from embryonic and adult rat brain were analyzed using an in gel kinase assay with either MAP-2 or T4L polymerized into the matrix. In this assay, electrophoresed protein kinases are renatured within the gel thereby regaining biological activity. After incubation of the gels in [γ-^{32}P]ATP, kinases capable of phosphorylating the protein polymerized into the matrix in a cofactor-independent manner can be visualized by autoradiography. Fig. 1 demonstrates the presence of a protein kinase activity associated with MAP preparations that migrates at 100-kD and phosphorylates both MAP-2 (Fig. 1A) and tau (Fig. 1B). This kinase activity is significantly greater in embryonic (lane 1) than in adult (lane 2) rat brain.

Phosphorylation of tau in its microtubule-binding region

To examine the time course of tau phosphorylation within its microtubule-binding region, MAP preparations from embryonic rat brains were incubated with T4L in the presence of ATP (see methods) for the indicated time. Protein kinase inhibitor and EGTA were included to inhibit PKA and CaMKII, respectively. Aliquots taken at each time point were electrophoresed and blotted with the monoclonal antibody 12E8 which recognizes tau when Ser^{262} and/or Ser^{356} are phosphorylated (Seubert et al., 1995) (both of these residues are within the microtubule-binding region). Fig. 2 demonstrates the ability of a protein kinase activity in the embryonic MAP preparation to phosphory-

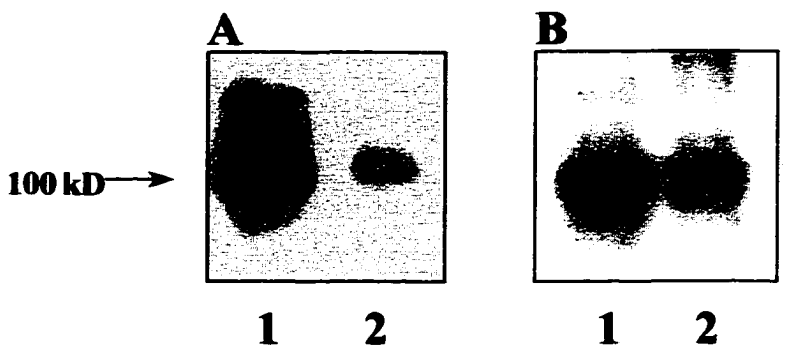

FIG. 1. Autoradiograph of an in gel kinase assay with embryonic (lane 1) or adult (lane 2) MAP preparations (10 μg) electrophoresed on 8% SDS-polyacrylamide gels and 100 μg/ml of either MAP-2 (**A**) or T4L (**B**) polymerized into the matrix.

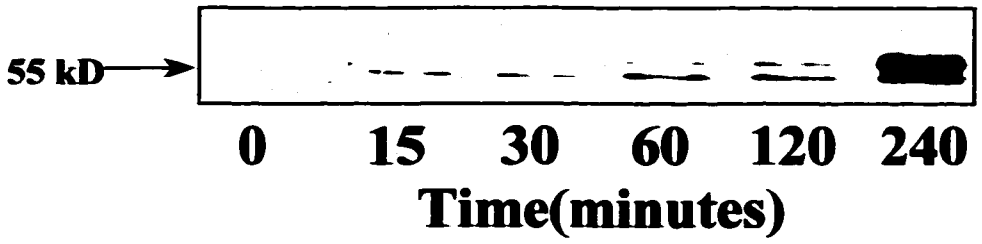

FIG. 2. A microtubule-associated protein kinase from embryonic brain phosphorylates tau at $Ser^{262/356}$. Immunoblots (12E8, 50 ng/ml) of T4L phosphorylated for the times indicated by protein kinases present in the embryonic MAP preparation. Ten nanograms of T4L was electrophoresed in each lane. These results demonstrate the ability of protein kinases in the embryonic MAP preparation to phosphorylate tau at $Ser^{262/356}$.

late tau within its microtubule-binding region in a time-dependent manner. Phosphorylation of $Ser^{262/356}$ is detectable by 15 min and continues to increase up to 4 h. A similar time course of phosphorylation was observed when tau was phosphorylated by protein kinases associated with the adult MAP preparation (data not shown).

The ability of the 100-kD protein kinase to specifically phosphorylate Ser^{262} was evaluated with the in gel kinase procedure described above with the exception that 1 m*M* Ser^{262} peptide (see methods) was polymerized into the gel (Fig. 3A) rather than full-length protein. The in gel kinase assay was also performed without any protein or peptide polymerized into the matrix to adjust for autophosphorylation of the kinase (Fig. 3B). The autoradiograph in Fig. 3A shows that a 100-kD protein kinase activity that is greater in the embryonic than the adult MAP preparation phosphorylates the Ser^{262} peptide. As Fig. 3B demonstrates, this 100-kD protein kinase also autophosphorylates. However, the amount of ^{32}P detected that migrated at an apparent molecular mass of 100-kD was significantly greater in the gel containing the Ser^{262} peptide compared to the levels in the absence of peptide (control), for both the embryonic (133% of control gel) and the adult (145% of control gel) MAP preparations. Thus, although the 100-kD protein kinase autophosphorylates, it also phosphorylates the Ser^{262} peptide.

To quantitatively determine the ability of protein kinases associated with the adult and embryonic MAP preparations to phosphorylate Ser^{262}, kinases present in the MAP preparations were used to phosphorylate the Ser^{262} peptide, and the amount of ^{32}P incorporated into the peptide was determined. Protein kinase inhibitor and EGTA were included in the incubation to inhibit PKA and CaMKII, respectively. All calculations

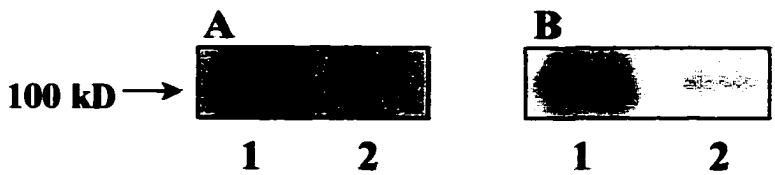

FIG. 3. Autoradiograph of an in gel kinase assay with embryonic (lane 1) and adult (lane 2) MAP preparations electrophoresed on 8% SDS-polyacrylamide gels and either 1 m*M* Ser^{262} peptide (**A**) or no peptide (**B**) polymerized into the matrix. Band intensity was evaluated by densitometry.

were made after subtracting the values obtained in the absence of peptide from those obtained in the presence of peptide (see methods). Fig. 4 shows that protein kinases present in the embryonic MAP preparation incorporate 2.0 ± 0.5 pmol $^{32}P/\mu g$ protein/h (n = 4) into the Ser^{262} peptide, while kinases present in the adult MAP preparation only incorporate 0.7 ± 0.2 pmol $^{32}P/\mu g$ protein/h (n = 4) into the peptide. Thus, the embryonic MAP preparation contains approximately three fold more protein kinase activity that is able to phosphorylate the Ser^{262} peptide compared to the protein kinases present in the adult MAP preparation.

Microtubule-binding

T4L was incubated in the presence (phosphorylated) or absence (control) of ATP and either embryonic or adult MAP preparations as described in the methods. Protein kinase inhibitor was included to inhibit PKA, and both KN-62 and EGTA were included to inhibit CaMKII activity. Control and phosphorylated tau was then incubated with taxol-stabilized microtubules, and the amount of tau associating with microtubules was determined. Fig. 5 shows that tau phosphorylated by the protein kinases associated with the embryonic MAP preparation exhibits a 59 ± 3% decrease in microtubule-binding capacity compared to control tau incubated with the embryonic MAP preparation in the absence of ATP. In contrast, tau phosphorylated by the protein kinases in the adult MAP preparation displayed a 41 ± 5% decrease in microtubule-binding capacity compared to control tau. Thus, protein kinases present in the embryonic MAP preparati-

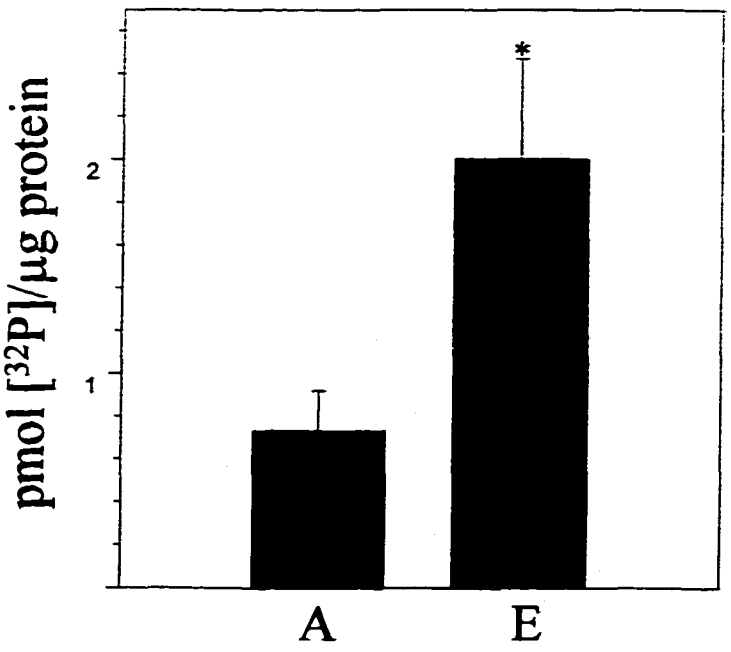

FIG. 4. Adult (A) and embryonic (E) MAP preparations were used to [^{32}P] phosphorylate the Ser^{262} peptide. Embryonic MAP preparations contained significantly more protein kinase activity towards Ser^{262} peptide than did the adult preparations. Mean $\pm$ SEM (n = 4), * $p < 0.05$.

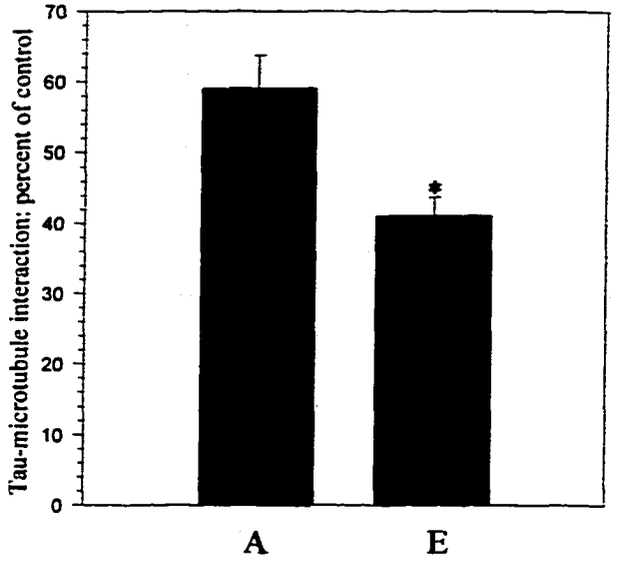

FIG. 5. Tau phosphorylated by protein kinases present in either the adult (A) or embryonic (E) MAP preparation was incubated with taxol-stabilized microtubules, and the amount of tau that bound the microtubules was determined. Results are expressed as the percentage of phosphorylated tau bound to microtubules compared to control tau (nonphosphorylated). Phosphorylation of tau with protein kinases associated with embryonic MAPs resulted in a significantly greater reduction in microtubule-binding capacity compared to protein kinases associated with the adult MAPs. Mean $\pm$ SEM (n = 3 separate experiments in duplicate), $^{*}p < 0.05$.

on phosphorylate tau and decrease microtubule-binding capacity to a significantly greater extent than the protein kinases present in the adult MAP preparation.

Immunoprecipitation of the 100-kD protein kinase

Adult and embryonic MAP preparations were immunoprecipitated with either a monoclonal antibody to MAP-2 (AP-14) (Binder et al., 1984; Peng et al., 1986), a phosphate-independent monoclonal antibody to tau (Tau-5) (Carmel et al., 1996), or non-immune rabbit IgG. The immunoprecipitates were analyzed using the in gel kinase assay with either T4L (Fig. 6A) or the Ser^{262} peptide (Fig. 6B) polymerized into the matrix. The autoradiograph in Fig. 6A demonstrates that a 100-kD protein kinase that selectively immunoprecipitates with both MAP-2 and tau phosphorylates full length tau. This 100-kD protein kinase activity is significantly higher in embryonic than in adult rat brain. In Fig. 6B, the 100-kD protein kinase that immunoprecipitates with MAP-2 and tau is shown to phosphorylate the Ser^{262} peptide. Again, kinase activity is significantly greater in embryonic brain than in adult brain. Thus, the 100-kD protein kinase that immunoprecipitates from rat brain with MAP-2 and tau phosphorylates the microtubule-binding region of tau, specifically on Ser^{262}.

Embryonic MAP preparations, adult MAP preparations, MAP-2 immunoprecipitates, or tau immunoprecipitates were electrophoresed on an SDS-polyacrylamide gel containing tau (T4L) polymerized into the matrix for the in gel kinase assay using nonlabeled ATP. The blots were transferred to nitrocellulose and probed with the monoclonal tau antibody 12E8 (see methods). Tau polymerized into the

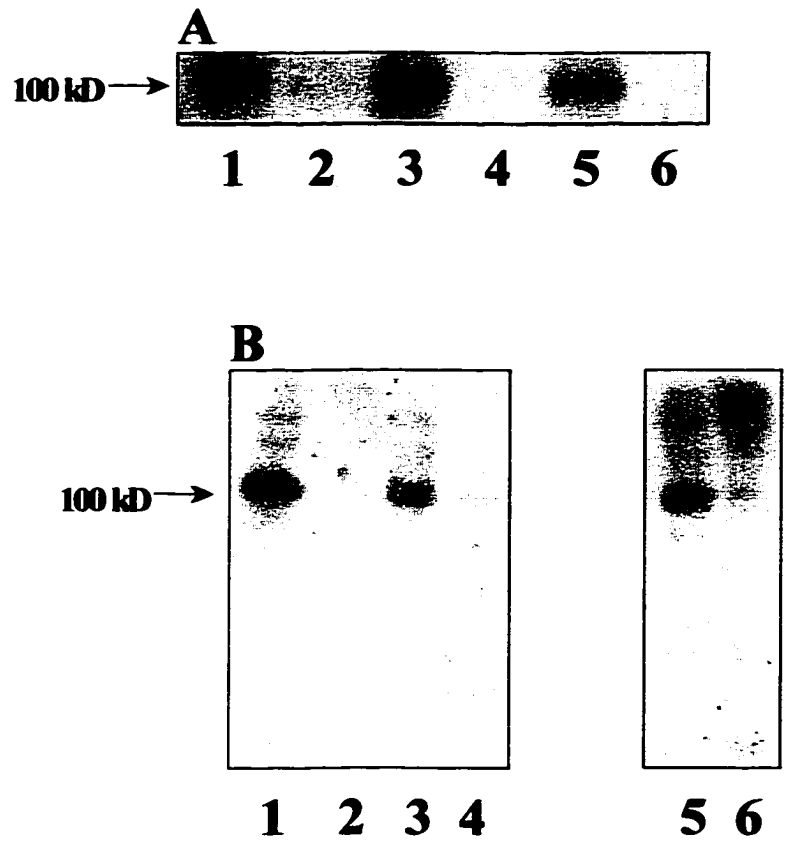

FIG. 6. Autoradiograph of an in gel kinase assay with either MAP preparations or immunoprecipitates electrophoresed on 8% polyacrylamide gels and either T4L (**A**) or Ser^{262} peptide (300 μM) (**B**) polymerized into the matrix. Lanes in (**A**) correspond to: embryonic (lane 1) and adult (lane 2) MAP-2 immunoprecipitates, embryonic (lane 3) and adult (lane 4) tau immunoprecipitates, and embryonic (lane 5) and adult (lane 6) control IgG immunoprecipitates. Lanes 1-4 in (**B**) are the same as lanes 1-4 in (**A**). Lanes 5 and 6 in (**B**) represent embryonic (lane 5) and adult (lane 6) MAP preparations.

matrix transfers to the nitrocellulose; however, only the tau that is phosphorylated on $Ser^{262/356}$ is recognized by the antibody. Fig. 7A shows a protein kinase activity migrating at 100-kD that phosphorylates tau on $Ser^{262/356}$, coprecipitates with both MAP-2 (lanes 1 and 2) and tau (lanes 3 and 4), and is significantly greater in embryonic brain (lanes 1 and 3) than in adult brain (lanes 2 and 4). In Fig. 7B, the exposure time of the film (using ECL) was significantly increased to demonstrate the presence of a protein kinase activity phosphorylating tau on $Ser^{262/356}$ that migrates at approximately 40 kD. This activity may represent the catalytic subunit of PKA based on its molecular mass and previous findings (Drewes et al., 1995). As indicated by the necessity to increase the exposure time in order to visualize this band, the 40-kD protein kinase activity phosphorylates tau at $Ser^{262/356}$ with lower efficiency than does the 100-kD protein kinase, or alternatively may be present at a significantly lower level. In contrast to the 100-kD protein kinase, the activity levels in the 40-kD protein kinase were not significantly different between the embryonic and the adult preparations.

DISCUSSION

Phosphorylation has been shown to affect tau's ability to bind to and stabilize microtubules both in vitro and in vivo (Lindwall and Cole, 1984; Preuss et al., 1995; Lovestone et al., 1996). Early work investigating the relationship between phosphorylation and microtubule-binding capacity focused on tau's numerous Ser/Thr-Pro sites which are hyperphosphorylated in PHFs found in AD brain (Gustke et al., 1992; Mandelkow et al., 1992). However, phosphorylation of tau on only one or two si-

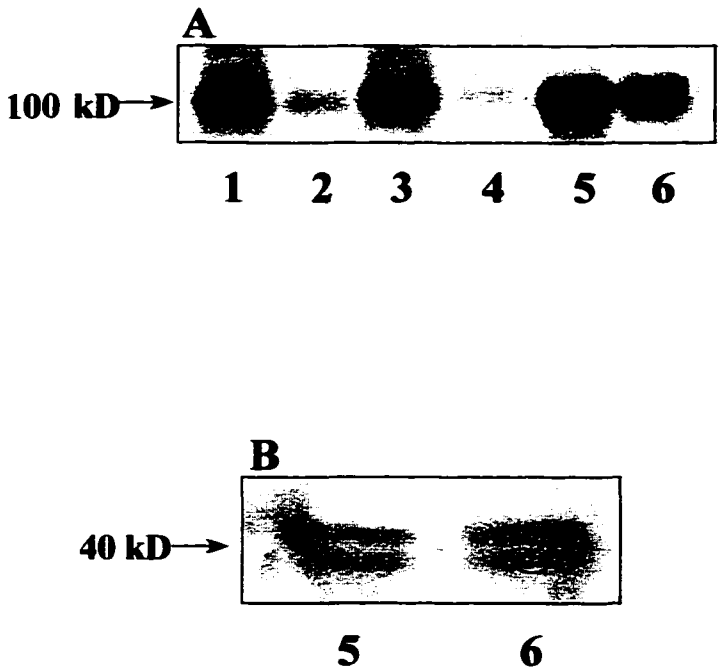

FIG. 7. Immunoblot of an in gel kinase assay using non-labeled ATP with embryo MAP preparation (10 μg), adult MAP preparation (10 μg), and tau and MAP-2 immunoprecipitates electrophoresed on an 8% polyacrylamide gel and T4L polymerized into the matrix. In (**A**) the ECL immunoblots were exposed briefly to X-ray film to detect the 100-kD protein kinase activity. In (**B**) the same blot was exposed for a greater period of time to demonstrate the presence of a 40-kD protein kinase which phosphorylates $Ser^{262/356}$. In (**B**) only the last 2 lanes of the blot in (**A**) are shown. Lanes correspond to embryonic (lane 1) and adult (lane 2) MAP-2 immunoprecipitates, embryonic (lane 3) and adult (lane 4) tau immunoprecipitates, and embryonic (lane 5) and adult (lane 6) MAP preparations.

tes within the microtubule-binding region (including Ser^{262}) decreases tau's ability to bind and stabilize microtubules to a much greater extent than phosphorylation on numerous Ser/Thr-Pro sites outside the microtubule-binding region (Biernat et al., 1993). These data, combined with the finding that Ser^{262} is phosphorylated in PHF-tau and not in tau derived from autopsied normal adult brain (Hasegawa et al., 1992; Morishima-Kawashima et al., 1995), has led to much interest in investigating protein kinases capable of phosphorylating tau within the microtubule-binding region, specifically on Ser^{262}.

In this study we have demonstrated the presence of a 100-kD protein kinase activity that is present at higher levels in embryonic than adult rat brain and phosphorylates both tau and MAP-2. This protein kinase activity phosphorylates tau within its microtubule-binding domain, on Ser^{262}, and coimmunoprecipitates from rat brain with both MAP-2 and tau. As determined previously by immunoblot analysis with 12E8, phosphorylation of tau at $Ser^{262/356}$ is highest in fetal brain and almost undetectable in the adult brain (Seubert et al., 1995). Interestingly, the 100-kD protein kinase activity described in the present study selectively associates with tau and is significantly enhanced in the embryonic brain. Considering these findings, it can be hypothesized that this protein kinase phosphorylates tau in vivo. Additionally, tau phosphorylated by kinases in the embryonic MAP preparation (and to a lesser extent by those in the adult MAP preparation) in the presence of inhibitors of CaMKII and PKA displayed a significant reduction in microtubule-binding capacity. Because the 100-kD protein kinase coprecipitates with tau, is enriched in embryonic brain, and phosphorylates Ser^{262},

it is likely that the effects of tau phosphorylation on microtubule-binding are due, at least in part, to the 100-kD protein kinase.

An embryonic protein kinase recently characterized in chicken brain with a molecular weight of 100-kD has been shown to phosphorylate MAP-2 and decrease its interaction with microtubules (Lopez and Sheetz, 1995). However, the sites phosphorylated by this protein kinase were not determined. Similarly, in vitro phosphorylation of MAP-2, MAP-4 (Illenberger et al., 1996), and tau (Drewes et al., 1995) by MARK results in dissociation from microtubules and their subsequent destabilization. The similarity in molecular mass between the 100-kD protein kinase described in this study, MARK (Drewes et al., 1995; Illenberger et al., 1996), and the chicken brain protein kinase (Lopez and Sheetz, 1995) as well as their similar ability to decrease the microtubule-binding capacity of microtubule-associated proteins, suggest that these protein kinases may share some homology. They may be involved in the general regulation of cytoskeletal assembly/disassembly by regulating MAP-microtubule interactions.

The precise relationship between tau phosphorylation and PHF pathology in AD has yet to be elucidated. Due to the presence of mitotic protein kinases in AD brain (Liu et al., 1995; Vincent et al., 1996) and of phosphorylated residues in fetal tau that are present in PHF-tau (Bramblett et al., 1993; Goedert et al., 1993; Seubert et al., 1995), it has been hypothesized that an inappropriate recapitulation of a developmental state may contribute to the pathology of AD (Bramblett et al., 1993; Goedert et al., 1993; Vincent et al., 1996). For example, Ser^{396} and Ser^{202} are phosphorylated extensively in embryonic

brain, but not to a significant extent in normal adult brain (Bramblett et al., 1993; Goedert et al., 1993; Seubert et al., 1995). However, these residues have been found to be phosphorylated in AD brain to a significantly greater extent than in either normal adult or fetal brain (Bramblett et al., 1993; Goedert et al., 1993; Vincent et al., 1996). Additionally, tau from fetal brain binds microtubules less efficiently than tau from normal adult brain (Hasegawa et al., 1992) similar to PHF-tau which is microtubule-binding incompetent (Bramblett et al., 1992, 1993). Based on this evidence, it has been suggested that inappropriate reexpression and/or re-activation of "fetal" protein kinases may contribute to the abnormal phosphorylation state of tau in AD brain (Bramblett et al., 1993; Goedert et al., 1993).

Phosphorylation of structural proteins is a key mechanism by which cytoskeletal dynamics and organization are regulated during development (Matus, 1988; Mandell and Banker, 1995). Developmental regulation of protein kinases is likely to be an important process by which specific, temporal phosphorylation events are controlled thereby maintaining appropriate levels of neuronal plasticity. The increased expression of certain protein kinases early in development, such as the 100-kD protein kinase described here, may contribute to the dynamic nature of microtubules during neurogenesis by phosphorylating certain MAPs and decreasing their ability to stabilize microtubules. Although the continued presence of these protein kinases is likely to be required in the adult brain, expression levels would be expected to be significantly lower than in the fetal brain in order to maintain the more stable neuronal architecture that exists in the adult brain (Richardson and Wuillemin, 1981; Bruno et al., 1984).

However, if an inappropriate increase in the expression or activation of "fetal" protein kinases, such as the 100-kD protein kinase, occurred in the adult brain, the result could be an abnormal increase in the phosphorylation of tau and other MAPs on sites that modulate microtubule-binding. Because Ser^{262}, as well as similar sites within the other microtubule-binding domains, is an important site in regulating MAP-microtubule interactions, increased phosphorylation of Ser^{262} could result in increased microtubule instability (Biernat et al., 1993; Drewes et al., 1995). Further, tau inappropriately phosphorylated at Ser^{262} may accumulate within neuronal perikarya and be an initiating factor in the abnormal aggregation and hyperphosphorylation of tau which occurs in AD brain (Johnson and Jenkins, 1996).

In conclusion, the results of the present study demonstrate the presence of a 100-kD protein kinase activity that phosphorylates tau on Ser^{262} and is significantly higher in embryonic rat brain compared to the adult. Further studies are required to elucidate the role of this protein kinase in development and its putative contribution to the pathology of AD (Weingarten et al., 1975).

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CONCLUSIONS

The ability of phosphorylation to modulate tau's microtubule-stabilizing capacity and the fact that hyperphosphorylated tau is aggregated in brains of AD patients suggest the importance of understanding the regulation of tau phosphorylation for understanding the normal and pathological functioning of the microtubule cytoskeleton. Most of the work examining the enzymes capable of regulating tau phosphorylation has been done in vitro and may or may not be relevant for tau phosphorylation in vivo. Examining tau phosphorylation and function in situ will help researchers to identify the physiologically relevant modulators of tau and will shed light on tau's role in both normal and diseased brain. The present set of studies identified signaling pathways, initiated by osmotic stress and thiol modification, that stimulate tau phosphorylation in situ. Examination of the enzymes involved in these responses has provided novel insights into the regulation of tau phosphorylation and function under normal and, potentially, under pathological conditions.

Because many sites on tau that are hyperphosphorylated in AD are Ser/Thr-Pro sites (Morishima-Kawashima et al., 1995), abnormal activation of one or more Pro-directed protein kinases in AD brain has been hypothesized to account, at least in part, for the pathogenesis of the disease. In fact, pools of tau have been isolated from AD brains that are hyperphosphorylated at Ser/Thr-Pro sites but are not aggregated into PHFs/NFTs (Ledesma et al., 1995), suggesting that Ser/Thr-Pro hyperphosphorylation in AD is an early event in the progression of the disease. Because the SAPKs

phosphorylate Ser/Thr-Pro sites on tau in vitro (Goedert et al., 1997; Reynolds et al., 1997a, 1997b) and because the AD brain has been hypothesized to contain SAPK-activating signals (McGeer and McGeer, 1995), these protein kinases have been hypothesized to contribute to tau hyperphosphorylation in AD. The plausibility of this hypothesis is supported by the present finding that overexpressed SAPK3 phosphorylated tau in situ following activation by osmotic stress. If SAPK3 is abnormally activated in AD, it could potentially contribute to tau hyperphosphorylation. The AD brain has been hypothesized to be subject to a chronic inflammation response characterized by increased release of certain proinflammatory cytokines as part of a feed forward, autotoxic loop (McGeer and McGeer, 1995). Because cytokines have been shown to activate members of the SAPK family, cytokine release in the AD brain could activate specific SAPKs (Meier et al., 1996), including SAPK3. This hypothesis has gained some recent support by the finding that SAPK2b is abnormally activated in AD brains compared to control brains (Hensley et al., 1999), suggesting that there are signals present in brains of AD patients that favor SAPK activation. It is, therefore, possible that other SAPKs, including SAPK3, are also activated in the AD brain.

Because SAPK1, 2a, 2b, 4, and 5 were also activated by osmotic stress but did not contribute to tau phosphorylation, SAPK3 may be unique in its ability to phosphorylate appropriately localized tau in the intact cell. However, this does not necessarily exclude the involvement of other SAPKs in tau hyperphosphorylation in AD. Abnormally activated SAPKs other than SAPK3 could potentially gain access to tau in the AD brain after it becomes delocalized from the cytoskeleton. Therefore, it is possible that abnormal activation of SAPK3 in the AD brain could contribute to the

early stages of tau hyperphosphorylation and contribute to tau delocalization from the cytoskeletal compartment (see Fig. 1). Once removed from the cytoskeleton, tau may become accessible to other abnormally activated SAPKs which could contribute to the later stages of tau hyperphosphorylation. Future work should examine the activation state of SAPK3 in the AD brain, as well as the ability of specific SAPKs to phosphorylate tau in cells with intact versus compromised microtubule cytoskeletons. Destabilizing microtubules should cause delocalization of microtubule-bound tau. If SAPKs other than SAPK3 can phosphorylate tau only after microtubule disruption, it would support the hypothesis that abnormal activation of these protein kinases in the AD brain could contribute to tau hyperphosphorylation after tau delocalization from microtubules.

A complete understanding of tau regulation by phosphorylation also requires examination of the non-Ser/Thr-Pro sites in tau's microtubule-binding region. Phosphorylation of these non-Ser/Thr-Pro sites dramatically decreases tau's in vitro microtubule-binding capacity (Biernat et al., 1993; Drewes et al., 1995), and they are likely to regulate tau's association with the cytoskeleton in vivo. Several protein kinases have been shown to phosphorylate tau at these sites in vitro, including PKA, CaMKII, and PKC (see introduction), although none of these protein kinases was involved in either the osmotic stress- or the PAO-induced tau phosphorylation at Ser $^{262/356}$ seen in the present studies. Another protein kinase that has been shown to phosphorylate tau at Ser $^{262/356}$ and has been proposed to be the main protein kinase in brain that phosphorylates tau at these residues is MARK (Drewes et al., 1995). Our finding that a MARK-like protein kinase activity, later confirmed as MARK, was activated by PAO

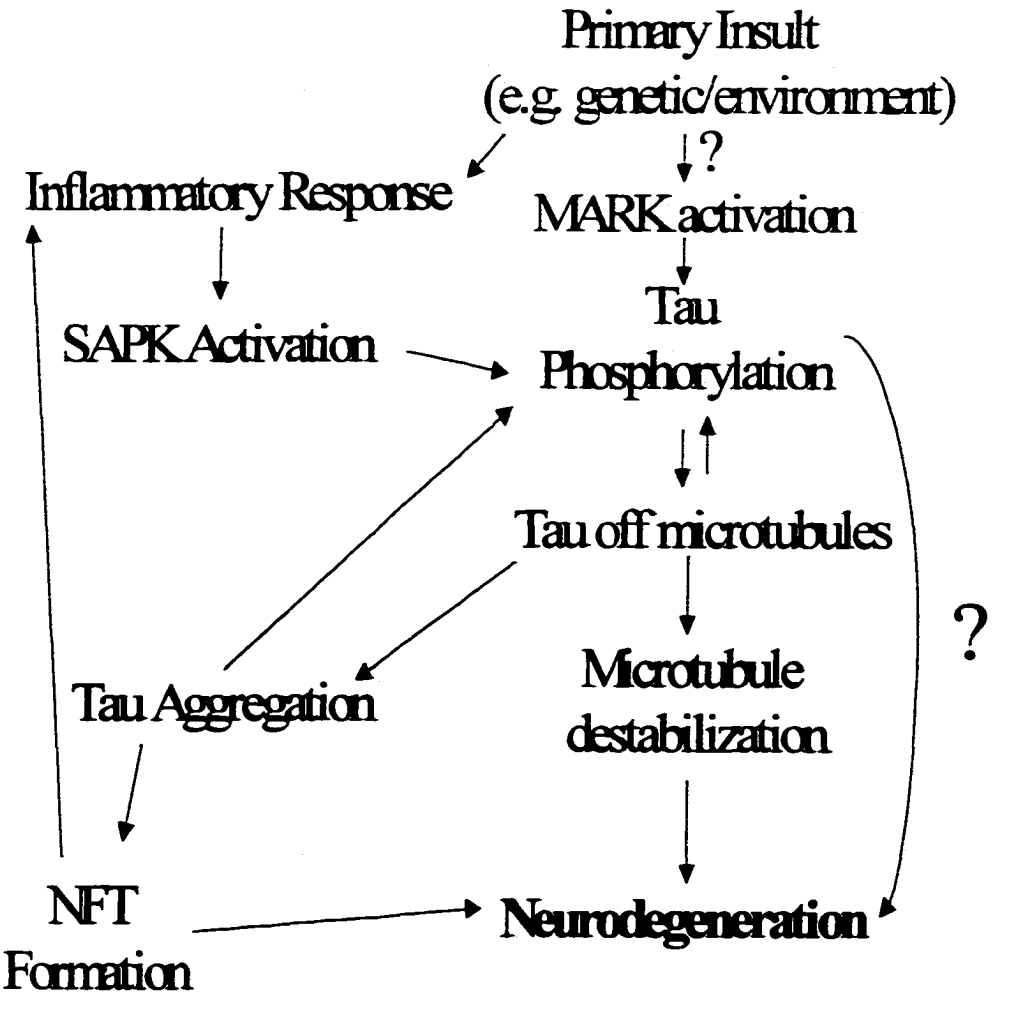

FIG. 1. Schematic representation of potential involvement of SAPK3 and MARK in tau hyperphosphorylation and neurodegeneration in AD brain. The primary insult initiating the disease could be genetic, environmental, or some combination of the two. The presence of a chronic inflammatory response has been hypothesized in AD brain and could result in cytokine release and subsequent SAPK3 activation. The primary insult could simultaneously, through an unknown mechanism, activate a non-Ser/Thr-Pro-directed protein kinase such as MARK which would phosphorylate tau at key residues involved in controlling tau-microtubule interactions. Tau hyperphosphorylation would be expected to decrease tau's microtubule-binding capacity, resulting in tau coming off the microtubules. Microtubule-binding incompetent tau could then be more susceptible to aggregation into NFTs which could be further phosphorylated as a result of abnormal cellular localization and exposure to novel protein kinases. Microtubule destabilization, cessation of protein transport, and cell death may result following tau dissociation from microtubules. Additionally, aggregated tau in the form of the NFT could contribute directly to cell death and could exacerbate the inflammatory response, resulting in a feed forward, self-propagating loop.

provides the first evidence of an activator of endogenous MARK. This MARK-like protein kinase activity was by far the most prominent Ser $^{262/356}$-directed protein kinase activity, as judged by the in gel kinase assay, supporting the hypothesis that MARK is the major protein kinase that phosphorylates tau at Ser $^{262/356}$. It is likely that MARK activation by PAO is mediated by phosphorylation as PAO treatment decreased MARK's apparent electrophoretic mobility. Previous studies have shown that phosphorylation of Ser and Thr residues and, to a lesser extent, Tyr is necessary for protein kinase activity and that this activating phosphorylation decreases MARK's electrophoretic mobility (Drewes et al., 1997). Because MARK is inhibited by low concentrations of staurosporine, it may be involved in osmotic stress-stimulated tau phosphorylation at Ser $^{262/356}$, as well as in PAO-stimulated tau phosphorylation.

How MARK activation might be regulated is unknown; however, the present finding that PAO, which binds vicinal thiols, increases MARK activity suggests that a thiol-dependent pathway might be involved. Regulation of protein function by modulation of cellular thiols has been documented for several enzymes, including the calcium-activated protease calpain (Guttmann et al., 1997; Guttmann and Johnson, 1998), glucosamine-6-phosphate deaminase (Altamirano et al., 1992), and phosphoenolpyruvate carboxykinase (Lewis et al., 1993). Therefore, regulation of cellular thiols can affect signaling through multiple pathways and could be involved in the ability of PAO to activate MARK.

Because MARK has been shown to phosphorylate MAPs other than tau, including MAP2 and MAP4 (Drewes et al., 1997), the effects of MARK activity in the cell can only be fully understood within the context of the multiple MAP environment.

MARK may be a general regulator of cytoskeletal dynamics and could be involved in many cellular processes that require changes to the cytoskeleton such as mitosis, apoptosis, and neurite outgrowth. Through its effect on the ability of structural MAPs to bind microtubules, MARK may also regulate the association of nonstructural MAPs, such as the microtubule-motor proteins kinesin and dynein, with microtubules. Recent research suggests that tau (Ebneth et al., 1998) and MAP-2 (Lopez and Sheetz, 1995) can decrease microtubule-motor activity, presumably by interfering with either the motor's ability to bind microtubules or the ability to translocate along microtubules. Localized activation of microtubule-associated MARK could decrease the ability of either tau or MAP-2 to bind microtubules removing the MAP-mediated inhibition of motor activity. This would be predicted to result in increased microtubule-motor activity. In support of this hypothesis, a 100-kD protein kinase activity from embryonic chick brain was shown to phosphorylate MAP-2, decreasing its association with microtubules and restoring MAP-2-inhibited motor activity (Lopez and Sheetz, 1995). Thus, it is possible that, in vivo, MARK regulates microtubule-motor activity indirectly through its effect on structural MAP-microtubule interactions.

In addition to the normal regulation of tau phosphorylation and localization, MARK may also be involved in tau hyperphosphorylation in AD brains. Abnormal activation of MARK could increase tau phosphorylation in its microtubule-binding region, resulting in tau coming off the microtubules and subsequent microtubule destabilization. Microtubule destabilization could then contribute to neurodegeneration by interrupting intracellular protein transport (see Fig. 1). In fact, microtubule stabilization with taxol has been shown to partially prevent apoptosis induced by protein

phosphatase inhibitors (Tanaka et al., 1998). These results suggest the possibility that abnormal MARK activation could contribute to microtubule destabilization and subsequent cell death. Future work on this topic should examine the effect of MARK activation on cell survival.

Findings that tau from embryonic brain is hyperphosphorylated compared to adult tau, although not as highly phosphorylated as tau from AD brains (Morishima-Kawashima et al., 1995), have led to the hypothesis that AD represents an unsuccessful attempt by brain cells to return to an embryonic state (for review, see Johnson and Jenkins, 1996). If correct, this hypothesis might predict that the protein kinases contributing to tau hyperphosphorylation in AD would be up-regulated in embryonic brain. This prediction is especially interesting in light of the present findings that a 100-kD MARK-like protein kinase activity is increased in embryonic rat brain compared to adult rat brain. A similar protein kinase activity, based on relative molecular mass and substrate specificity, was isolated from embryonic chick brains (see above) (Lopez and Sheetz, 1995), suggesting that MARK or MARK-like protein kinases may play an important developmental role in cytoskeletal dynamics. It is, therefore, possible that developmental regulation of MARK activity could account for the developmental regulation of tau phosphorylation. High MARK activity expressed in embryonic brain could account for the higher levels of tau phosphorylation, within its microtubule-binding region, observed in the embryo. Future work examining the mechanisms of MARK regulation could shed light on the normal developmental regulation of tau phosphorylation, as well as on the pathological tau phosphorylation seen in AD.

The regulation of tau phosphorylation under normal conditions and the aberrant regulation of tau phosphorylation in AD suggest that examining tau's phosphorylation state in situ could have important implications for both normal and pathological brain functioning. By developing an understanding of how tau's phosphorylation state is regulated in intact cells, the role of tau and how that role is modulated will be clarified. Identification of the protein kinases that act on tau in vivo is a necessary step toward this goal of clarification. Continued study will enhance our understanding of how tau fits into overall cell functioning, as well as increasing our knowledge of tau's role in cell dysfunction.

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I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that he may be recommended for the degree of Doctor of Philosophy.

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