Modulation of tau phosphorylation and intracellular localization by cellular stress

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Tau is a microtubule-associated protein that is functionally modulated by phosphorylation and hyperphosphorylated in several neurodegenerative diseases. Because phosphorylation regulates both normal and pathological tau functioning, it is of great interest to identify the signalling 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 stress-activated protein kinases (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 the non-Ser/Thr-Pro sites Ser-262/356, within the microtubule-binding domain, as well as Ser/Thr-Pro sites outside of tau's microtubule-binding domain. Although all SAPKs examined were activated by osmotic stress, none of the endogenous SAPKs mediated the

increase in tau phosphorylation. However, when transfected into SH-SY5Y cells, SAPK3, but not the other SAPKs examined, phosphorylated 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 only partially due to phosphorylation at Ser-262/356 by a staurosporine-sensitive, non-proline-directed, 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, that SAPK3 can phosphorylate tau on Ser/Thr-Pro residues *in situ*, and that Ser-262/356 phosphorylation only partially regulates tau localization in the cell.

Key words: Alzheimer's disease, cytoskeleton, microtubule-associated protein, osmotic stress, stress-activated protein kinase.

INTRODUCTION

Tau is a family of neuronal phosphoproteins originally identified by its ability to co-purify with microtubules through repeated cycles of polymerization [1]. 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 [2], activation of phospholipase C [3] and regulation of microtubule motor activity [4]. Widespread interest in tau was generated when a hyperphosphorylated and aggregated form was shown to comprise the paired helical filaments (PHFs) which make up the neurofibrillary tangles in Alzheimer's disease (AD) brain (see [5] for review). Further, it was demonstrated that the ability of tau to bind and stabilize microtubules correlates inversely with phosphorylation [6], 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 [7]. 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. Whereas numerous protein kinases have been shown to phosphorylate tau and regulate its function *in vitro* [5,8], elucidation of the specific enzymes that regulate tau's phosphorylation state *in vivo* has proven more difficult. Recently, stress-responsive members of the mitogen-activated protein

kinase family, known as the stress-activated protein kinases (SAPKs), were shown to phosphorylate tau *in vitro* [9–11]. Specifically, SAPK1 (c-Jun N-terminal kinase, JNK), SAPK2a (p38), SAPK2b (p38 β), SAPK3 (ERK 6) and SAPK 4 all phosphorylate tau *in vitro* on residues that are hyperphosphorylated in PHF tau [9–11]. Activation of SAPKs occurs in response to multiple forms of cellular stress (e.g. osmotic shock and inhibition of protein synthesis) and by certain pro-inflammatory cytokines (interleukin-1 and tumour necrosis factor- α ; for reviews see [12,13]). Downstream targets of SAPK activation include other protein kinases, such as mitogen-activated-protein-kinase-activated protein kinases 2 and 3 (MAPKAP-K2/3), and transcription factors such as c-Jun and cAMP-response-element-binding protein (CREB) [12,13].

SAPKs phosphorylate serine residues and/or threonine residues that are followed by a proline [12,13]. Because multiple Ser/Thr-Pro residues are phosphorylated in PHF tau [14], 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 this increase in phosphorylation correlates with a decrease in the amount of tau associated with the cytoskeleton. Although all

Abbreviations used: PHF, paired helical filament; AD, Alzheimer's disease; SAPK, stress-activated protein kinase; MAPKAP-K, mitogen-activated protein kinase-activated protein kinase; DTT, dithiothreitol; JNK, c-Jun N-terminal kinase; PKC, protein kinase C; GSK-3 β , glycogen synthase kinase 3 β ; MEK, mitogen-activated-protein-kinase kinase.

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SAPKs examined were activated by osmotic stress, only SAPK3, when transfected into the cells, phosphorylated tau *in situ*. These data provide 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 and Ser-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 only partially 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, Mediatech, Herndon, VA, U.S.A.) supplemented with 10% (v/v) heat-inactivated horse serum (Gibco), 5.0% heat-inactivated Fetal Clone II (HyClone, Logan, UT, U.S.A.), 100 units/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco) and 2.0 mM 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 µM retinoic acid (Sigma) for 7–10 days prior to use.

Transfection

Naive SH-SY5Y cells were plated on to 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 (MRC Centre, Cambridge, U.K.). FLAG-tagged BMK1 (big map kinase 1, SAPK5) in pcDNA3, BMK1(AEF) (dominant-negative) and SAPK5(AEF) in pcDNA3, and MEK5(D) (mitogen-activated-protein-kinase kinase; constitutively active) in pCMV5 [15–18] were the generous gifts of Dr. J.-D. Lee (Scripps Research Institute, La Jolla, CA, U.S.A.). Following transfection (24–48 h), cells were treated as described above.

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 50 µg/ml anisomycin (Sigma) for 60 min, or with vehicle alone. Treated cells were washed once with ice-cold PBS and collected in a 2 × stop buffer without dithiothreitol (DTT) or dye [500 mM Tris/HCl (pH 6.8), 10% SDS, 100 mM EGTA, 100 mM EDTA and 10% glycerol]. Cell extracts were sonicated and protein concentrations were determined using the bicinchoninic acid (BCA) method (Amersham). The samples were diluted in 2 × stop buffer containing 25 mM DTT and Bromophenol Blue as the tracking dye, and the indicated amounts of cell extract were electrophoresed on SDS/polyacrylamide (7.5%) gels. Proteins were then transferred on 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 [19–21] and the phosphorylation-dependent antibodies tau-1 [22,23], PHF-1 [24], 12E8 [25] and AT270 [26].

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 mM Tris/HCl (pH 7.4), 1.0% Nonidet P-40, 500 mM NaCl, 1.0 mM EDTA, 1.0 mM EGTA, 1.0 mM Na₃VO₄, 1.0 µM okadaic acid (Sigma), 1.0 mM PMSF and 10 µg/ml each of leupeptin, aprotinin and pepstatin]. SAPK3, SAPK4 and SAPK5 immunoprecipitation buffers contained 0.25% Nonidet P-40 and 100 mM NaCl. Extracts were sonicated briefly and cleared by centrifugation at 16000 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, Waltham, MA, U.S.A.; for transfected SAPK3 and SAPK4) or FLAG epitope (Santa Cruz, Santa Cruz, CA, U.S.A.; 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 mM β-glycerophosphate, 5.0 mM EGTA, 1.0 mM Na₃VO₄, 1.0 mM DTT, 25 mM MgCl₂, 1.0 µM okadaic acid, 100 µM ATP, 1.0 µCi/nmol [γ-³²P]ATP, 1.0 mM PMSF and 10 µg/ml each of aprotinin, leupeptin and pepstatin} containing either c-Jun-GST (glutathione S-transferase; 3.0 µg/tube for SAPK1; Upstate Biotechnology) or GST-MAPKAP-K2-Myc (0.3 µg/tube for SAPK 2a and 2b; Upstate Biotechnology). SAPK3, SAPK4, and SAPK5 immunoprecipitates were incubated in phosphorylation buffer containing 1.0 mM unlabelled ATP, without [γ-³²P]ATP, and recombinant human tau (T4L, 0.1 µg/µl; prepared as described in [27]) as a substrate. Immunoprecipitates were incubated for 30 min at 30 °C. The reactions were stopped by addition of 2 × SDS stop buffer with 25 mM DTT and Bromophenol Blue. Samples were electrophoresed on SDS/polyacrylamide (8%) gels and either vacuum dried, exposed to a phosphor screen (Molecular Dynamics) and analysed on a PhosphorImager (SAPK1, 2a and 2b) or transferred on 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 mM Pipes (pH 6.8), 1.0 mM MgCl₂, 2.0 mM EGTA, 30% glycerol, 10 mM benzamidine, 50 µg/ml leupeptin, 1.0 mM PMSF and 0.5 µM okadaic acid]. Next, cells were incubated in 300 µl (for a 60-mm plate) of extraction buffer containing 0.1% Triton X-100 for 10 min at 37 °C. Cells were transferred into microcentrifuge tubes and centrifuged for 2 min at 16000 g. The supernatant was removed and incubated in a boiling-water bath for 10 min, while the pellet was resuspended in 100 µl of 2 × SDS stop buffer, without DTT or dye, and sonicated. Protein determinations were made and samples were diluted with 2 × stop buffer, boiled, electrophoresed on SDS/polyacrylamide gels and immunoblotted 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

Figure 1 Sorbitol-induced osmotic stress increases tau phosphorylation at multiple sites in a time-dependent, dose-dependent and reversible manner

(A) Undifferentiated (NAIVE) or differentiated (DIFF) SH-SY5Y cells were incubated for 30 min at 37 °C in media $\pm$ 0.5 M sorbitol. (B) Undifferentiated SH-SY5Y cells were incubated in the presence of 0.5 M sorbitol for the indicated time. (C) SH-SY5Y cells were incubated for 30 min in the presence of the indicated concentration of sorbitol. (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 for an additional 30 min in media containing no sorbitol (Recov = 30). Extract (10 μ g) 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 whereas recognition of a dephosphorylated epitope is designated – p. In SH-SY5Y cells the shortest form of tau predominates [44]. However, tau is differentially phosphorylated and this results in the various immunoreactive bands that are present on the blots in (A). Because the blots of naïve and differentiated cells in (A) are from separate experiments and were run on different gels, direct comparisons of the migration of the different bands cannot be made. In all cases immunoblots are representative of at least 3 separate experiments.

or differentiated (Figure 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 (Figure 1A, top panel). Decreased tau mobility on a polyacrylamide gel is indicative of increased tau phosphorylation [28]. 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 similar for both naïve 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 naïve cells, and 12E8, which showed a slightly greater response in 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 PHF-1 epitopes (results not shown). To characterize further the sorbitol-induced increases in tau phosphorylation, the time-dependence, concentration-dependence and reversibility of the effect were examined (Figures 1B–1D). In Figure 1(B), 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 by 5 min and increased in a time-dependent manner. The decrease in tau-1 immunoreactivity was not due to changes in tau levels (Figure 1B, top panel). In Figure 1(C), SH-SY5Y cells were incubated in the presence of increasing concentrations of sorbitol. At sorbitol concentrations of 0.1 and 0.25 M,

Figure 2 Anisomycin-induced stress decreases tau phosphorylation

Undifferentiated SH-SY5Y cells were treated for 60 min in the presence (+) or absence (–) of 50 μ g/ml anisomycin. Extract (10 μ g) was electrophoresed and immunoblotted with the indicated tau antibodies. Immunoblots are representative of at least 3 separate experiments.

decreases in tau-1 immunoreactivity, indicating an increase in phosphorylation at this epitope, were observed (Figure 1C). However, a much larger increase in phosphorylation at the tau-1 epitope was observed at 0.5 M sorbitol (Figure 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 Figure 1(D), 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 incubated for an additional 30 min in the absence of sorbitol. Figure 1(D) 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 if it 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 [29,30]. Anisomycin treatment resulted in an increase in tau's electrophoretic mobility indicated by the downward shift in the tau-5/5A6 immunoreactive bands (Figure 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 (Figure 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 Figure 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 fractionated to separate the detergent-insoluble cytoskeleton from the

Figure 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 Materials and methods section. Cytoskeletal and soluble fractions were electrophoresed and immunoblotted with the phosphorylation-independent tau-5 and 5A6 antibodies. During ECL[®] development, immunoblots of the soluble fractions were exposed for longer relative to immunoblots of the cytoskeletal fractions. Immunoblots are representative of at least 3 separate experiments.

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 (Figure 3).

Sorbitol and anisomycin activate SAPK1, SAPK2a and SAPK2b *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. Quantification of substrate phosphorylation demonstrated that SAPK1 was activated by sorbitol 2.1-fold and by anisomycin 1.8-fold. Both sorbitol (10-fold) and anisomycin (8.5-fold) robustly activated SAPK2a. SAPK2b was also activated by both stressors to an extent similar to what was observed for SAPK1 (2.1-fold for sorbitol and 1.7-fold for anisomycin). The SAPK2a and SAPK2b inhibitor SB202190 (Calbiochem) [30] 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 (Figure 4).

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 (results not shown) and because immunoprecipitation of untransfected cell extract with a SAPK3 polyclonal antibody failed to precipitate any protein kinase activity (results not shown), it is unlikely that endogenous SAPK3 is involved in the osmotic-stress-induced increase in tau phosphorylation. However, because SAPK3 is present in brain [31] and efficiently phosphorylates tau *in vitro* [11] it was important to determine whether SAPK3 phosphorylates tau *in situ* in response to osmotic stress. SH-SY5Y cells transfected with Myc-tagged SAPK3 were treated with either sorbitol or anisomycin, and cell extracts were immunoprecipitated with an antibody against c-Myc. Immuno-

Figure 4 Inhibition of SAPK2a/b does not decrease the osmotic-stress-induced increase in tau phosphorylation

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 μ g) were electrophoresed and immunoblotted with the indicated tau antibodies. Immunoblots are representative of at least 3 separate experiments.

precipitates were incubated in the presence of recombinant tau and ATP. Following incubation, the tau was electrophoresed and immunoblotted with the indicated antibodies. Figure 5(A; 2nd lane) demonstrates that SAPK3 from sorbitol-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 lesser extent than SAPK3 from sorbitol-treated cells (Figure 5A, 3rd lane). SAPK3 immunoprecipitated from either sorbitol- or anisomycin-treated cells did not phosphorylate tau at the tau-1 epitope (Figure 5A).

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 PHF-1 epitopes (results not shown). However, sorbitol-induced tau phosphorylation at the PHF-1 epitope was increased when SAPK3 was overexpressed (Figure 5B, compare the 2nd and 3rd lanes). Sorbitol-stimulated tau phosphorylation at the tau-1 epitope was unaffected by SAPK3 overexpression (Figure 5B, bottom panel). SAPK3 overexpression led to a significant $156 \pm 22\%$ ($P < 0.05$) increase in sorbitol-stimulated tau phosphorylation at the PHF-1 epitope compared with mock-transfected (vector only) cells ($n = 7$ separate experiments).

Osmotic-stress-activated SAPK4 phosphorylates tau *in vitro*

Because SAPK4 phosphorylates tau *in vitro* [11], the ability of this protein kinase to be activated by cellular stress and to phosphorylate tau was examined. Endogenous levels of SAPK4 could not be determined because currently there is no antibody available that specifically recognizes SAPK4. Therefore SH-SY5Y cells were transfected with Myc-tagged SAPK4 and incubated subsequently in the absence or 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. Figure 6

Figure 5 Overexpressed SAPK3 phosphorylates tau at the PHF-1 epitope *in vitro* and *in situ*

(A) Naïve 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 μ g/ml 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 immunoblotted with the indicated antibodies. (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. The tau-1 immunoblot was overexposed to determine whether SAPK3 transfection affected sorbitol-stimulated tau phosphorylation at the tau-1 epitope.

demonstrates that SAPK4 was activated by both sorbitol and anisomycin treatment and phosphorylated tau at the PHF-1 epitope. Further, 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. Quantification revealed that SAPK4 overexpression increased osmotic-stress-mediated tau phosphorylation at the PHF-1 epitope $132 \pm 16\%$ compared with the mock-transfected cells ($n = 4$ separate experiments). However, this increase was not significant. There was also no increase in phosphorylation of the tau-1 epitope (results not shown).

Effect of SAPK5 on osmotic-stress-induced tau phosphorylation

SH-SY5Y cells overexpressing FLAG-tagged SAPK5 were incubated in the presence of either sorbitol or anisomycin. Extracts

Figure 6 Overexpressed SAPK4 from sorbitol- and anisomycin-treated cells phosphorylates tau *in vitro*

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 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, and tau (100 ng) was electrophoresed and immunoblotted with the indicated antibodies. Although both sorbitol and anisomycin treatment resulted in the activation of SAPK4, anisomycin was a more potent activator of the kinase.

were immunoprecipitated with a polyclonal antibody against the flag epitope and protein kinase activity in the immunoprecipitates was measured using tau as a substrate. Figure 7(A) demonstrates

that SAPK5 from sorbitol-treated cells exhibits an upward mobility shift, which is indicative of SAPK5 being phosphorylated and activated [17]. Anisomycin did not induce this mobility shift in SAPK5, suggesting that anisomycin did not activate SAPK5. In Figure 7(B), SAPK5 was immunoprecipitated from transfected cells that had been treated with either sorbitol or anisomycin, and the ability of the immunoprecipitated SAPK5 to phosphorylate tau was measured. SAPK5 from untreated, sorbitol-treated and anisomycin-treated cells did not phosphorylate tau at either the tau-1 or the PHF-1 epitopes.

To examine whether SAPK5 is an indirect mediator of tau phosphorylation *in situ*, SH-SY5Y cells were transfected with either vector alone, wild-type SAPK5, dominant-negative SAPK5 [SAPK5(AEF)] or constitutively active MEK5 [MEK5(D)], the upstream activator of SAPK5 [17]. Figure 7(C) demonstrates that none of these constructs significantly affected either basal or sorbitol-stimulated tau phosphorylation at either the tau-1 or PHF-1 epitopes. Additionally, co-transfection of MEK5(D) and SAPK5 had no effect on tau phosphorylation (results 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 pre-incubated with the protein kinase inhibitor staurosporine fol-

Figure 7 SAPK5 does not phosphorylate tau *in vitro* or *in situ*

Naive SH-SY5Y cells overexpressing FLAG-tagged SAPK5 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 anisomycin (Anis) for 60 min. Extracts were immunoprecipitated with a polyclonal antibody to the FLAG epitope. (A) Immunoprecipitates electrophoresed and immunoblotted with a polyclonal antibody against SAPK5. (B) Immunoprecipitates incubated with recombinant tau in the presence of ATP. The reaction was stopped after 60 min, and tau (100 ng) was electrophoresed, and immunoblotted with the indicated antibodies. (C) SH-SY5Y cells were transiently transfected with either pCDNA3.1 vector alone, wild-type SAPK5, dominant-negative SAPK5 [SAPK5(AEF)] or constitutively active MEK5 [MEK5(D)]. 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 antibodies.

Figure 8 Staurosporine blocks the osmotic-stress-induced increase in tau phosphorylation at Ser-262/356

SH-SY5Y cells were pre-incubated in the presence (+) or absence (–) of 10 nM staurosporine and then in the presence (+) or absence (–) of 0.5 M sorbitol. Extracts were electrophoresed and immunoblotted with the indicated antibodies against tau.

lowed by incubation in the presence of sorbitol for 30 min. Figure 8 demonstrates that pre-incubation 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 tau5/5A6 (Figure 8, top panel). Osmotic-stress-induced tau phosphorylation at the 12E8 epitope was not inhibited by the protein kinase A inhibitor H-89 [32], the specific protein kinase C (PKC) inhibitor GF109203X [33] or PKC down-regulation with PMA (results not shown).

Because phosphorylation of residues within tau's microtubule-binding domain, specifically Ser-262, dramatically decreases tau-microtubule interactions *in vitro* [7], the effect of staurosporine on osmotic-stress-induced change in tau localization was examined. SH-SY5Y cells were pre-incubated 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 quantified. Sorbitol treatment decreased the amount of tau associated with the detergent-insoluble cytoskeleton to 65 % of control values. In cells preincubated with staurosporine prior to sorbitol treatment, the amount of tau associated with the cytoskeleton was 77 % of control values ($n = 2$ separate experiments). These findings demonstrate that phosphorylation at Ser-262/356 alone only contributes minimally to the association of tau with the cytoskeleton.

DISCUSSION

Although many signalling 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 hyper-osmotic 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 glycogen synthase kinase β (GSK-3 β ; for a review see [5]) and the SAPKs [9–11]. Nonetheless, only GSK-3 β has been shown convincingly to phosphorylate tau in intact cells [34,35]. However, GSK-3 β is not involved in the osmotic-stress-induced tau phosphorylation response as pretreatment with LiCl, which potentially inhibits GSK-3 β activity [36], did not eliminate the sorbitol-induced increase in tau phosphorylation (results not shown).

Endogenous SAPK1 (JNK), SAPK2a (p38) and SAPK2b (p38 β) 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 probably 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.

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. However, SAPK3 is present in brain [31] and efficiently phosphorylates tau *in vitro* [11]. Therefore it was important to determine whether SAPK3 phosphorylates tau *in situ*. Consistent with previous reports on the ability of SAPK3 to phosphorylate recombinant tau [11], transfected SAPK3 was activated by sorbitol treatment and phosphorylated tau *in vitro* at the PHF-1 epitope. The relevance of this phosphorylation *in vitro* for the situation *in vivo* 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. SAPK4 also efficiently phosphorylates tau *in vitro* [11] and when transfected into cells it enhanced the sorbitol-induced increase in tau phosphorylation, although the enhancement was not significant. However, when transfected SAPK4 was immunoprecipitated from sorbitol or anisomycin-treated cells, anisomycin was found to more effectively activate SAPK4. Because only sorbitol treatment increased tau phosphorylation *in situ*, these findings decrease the likelihood that SAPK4 mediates tau phosphorylation in this paradigm. In contrast to the findings with SAPK3 and SAPK4, SAPK5 did not phosphorylate tau *in situ* or *in vitro*.

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 pro-inflammatory cytokines as part of a feed-forward, autotoxic loop [37]. This inflammation response, which may be secondary to the primary lesions of AD [37], could then activate members of the SAPK

family [38]. Activation of SAPKs, specifically SAPK3, could contribute to the tau hyperphosphorylation characteristic of AD brains. This hypothesis has gained some support by the finding that SAPK2b is abnormally activated in AD brains compared with control brains [39], suggesting that there are signals present in brains of AD patients that favour SAPK activation. Therefore, it is possible that other SAPKs, including SAPK3, are also activated in the AD brain.

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 [6]. 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. However, inhibition of osmotic-stress-induced Ser-262/356 phosphorylation with staurosporine caused only a minor reversal in the osmotic-stress-induced decrease in cytoskeletonally associated tau. Recent work indicates that phosphorylation of Ser/Thr-Pro sites has a measurable effect on tau's microtubule-binding capacity [40], and therefore it is likely that the Ser/Thr-Pro sites phosphorylated in response to osmotic stress 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. Staurosporine was originally identified as a PKC inhibitor (see [41] for review) but has more recently been shown to inhibit several protein kinases. Neither the specific PKC inhibitor GF109203X [33] nor PKC down-regulation with chronic PMA treatment was found to inhibit sorbitol-induced tau phosphorylation at the 12E8 epitope (results not shown), suggesting that PKC is unlikely to be involved. However, the possibility that a GF109203X- and PMA-insensitive PKC isoform, or a PKC-related protein kinase such as protein kinase N [42], is involved cannot be ruled out. Additionally, because staurosporine is not specific for PKC (for reviews see [41,43]), its inhibitory effect could also be due to the inhibition of other protein kinases. Further work is required to identify the kinase(s) responsible for the phosphorylation of Ser-262/356 in response to osmotic stress.

In conclusion, the findings presented in this study 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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