Evidence that phosphorylation of the microtubule-associated protein Tau by SAPK4/p38δ at Thr50 promotes microtubule assembly

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Summary

Phosphorylation regulates both normal and pathological Tau functioning. This microtubule-associated protein plays a role in the organization and integrity of the neuronal cytoskeleton under normal conditions and becomes hyperphosphorylated and aggregated in a number of neurodegenerative diseases referred to as tauopathies. In this study, we identify and compare the residues in human Tau phosphorylated in vitro by all four p38 MAPK isoforms, and study the regulation of the phosphorylation of Thr50, under conditions where p38 MAPKs are active in cells. Through biochemical analysis, loss of function studies and analysis of endogenous and overexpressed Tau proteins, we show that SAPK4/p38δ is the major kinase phosphorylating Thr50 in Tau, when cells are exposed to osmotic stress. We also show that mutation of Thr50 to glutamic acid, which mimics phosphorylation, increases the ability of Tau to promote tubulin polymerisation in vitro and in vivo. Moreover, we show that Thr50 is phosphorylated in filamentous Tau from Alzheimer's disease brain. These findings suggest a role for Tau in the adaptative response of neurons to stress and indicate that SAPK4/p38δ and/or SAPK3/p38γ may contribute to the hyperphosphorylation of Tau in the human tauopathies.

Key words: Tau protein, Phosphorylation, p38 MAPK, Osmotic shock, Tauopathy

Introduction

Tau protein belongs to the family of microtubule-associated proteins. It is expressed mainly in neurons, where it is believed to play major regulatory roles in the organization and integrity of the cytoskeletal network. In adult human brain, six Tau isoforms are produced from a single gene by alternative mRNA splicing. They differ by having three or four conserved repeats, located in tandem in the C-terminal region, and no, one or two insertions in the N-terminal portion (Goedert et al., 1989a; Goedert et al., 1989b). The repeats constitute the microtubule-binding domains of Tau (Lee et al., 1989). Tau binds to β-tubulin through these repeats and promotes microtubule assembly (Kar et al., 2003), but may also participate in other cellular processes, such as the linking of signal-transduction pathways to the cytoskeleton (Lee et al., 1998).

Tau is functionally modulated by phosphorylation and is highly phosphorylated in several neurodegenerative diseases (Goedert, 2001). It has been demonstrated that the ability of Tau to bind to and stabilise microtubules correlates inversely with its phosphorylation, which may in turn facilitate self-assembly (Bramblett et al., 1993; Yoshida and Ihara, 1993; Drewes et al., 1995). Hyperphosphorylated and aggregated Tau (PHF-Tau) is the major component of the paired helical filaments (PHFs) that make up the neurofibrillary tangles of Alzheimer's disease (Goedert, 2001). Protein chemical and immunochemical studies have identified 25 phosphorylation sites in PHF-Tau (Morishima-Kawashima et al., 1995; Hanger et al., 1998). Hyperphosphorylation of Tau could result from an increased activity of Tau kinases or a decreased activity of Tau phosphatases. Whereas numerous protein kinases have been shown to phosphorylate Tau and regulate its function in vitro, including glycogen synthase kinase 3β (GSK-3β) (Hanger et al., 1992; Mandelkow et al., 1992), cyclin-dependent kinase-5 (cdk5) (Paudel et al., 1993; Kobayashi et al., 1993), mitogen-activated protein kinase (MAPK) (Drewes et al., 1992; Ledesma et al., 1992; Goedert et al., 1992a), microtubule-affinity regulating kinase (MARK) (Drewes et al., 1997) and stress-activated protein kinases (SAPK; p38 and JNK) (Reynolds et al., 1997a; Reynolds et al., 1997b; Goedert et al., 1997; Yoshida et al., 2004), identification of the specific enzymes that regulate phosphorylation of Tau in vivo has proved difficult.

Given that more than half of the phosphorylation sites in PHF-Tau are serine and threonine residues followed by proline (Hanger et al., 1998), it is conceivable that members of the MAPK family play an important role in phosphorylating Tau. Since aberrantly activated JNK and p38s have been reported to be associated with cells that contain filamentous Tau in some neurodegenerative diseases (Hensley et al., 1999; Zhu et al., 2000; Atzori et al., 2001; Ferrer et al., 2001), these kinases may contribute to the hyperphosphorylation of Tau protein. Moreover, the p38 activator, MKK6, has been found to be

active in neurodegenerative diseases (Zhu et al., 2001). The p38 isoforms are MAPK family members that are activated by cellular stresses, bacterial lipopolysaccharide and cytokines. The group of p38s comprises p38α, p38β, SAPK3/p38γ and SAPK4/p38δ (Cohen, 1997), which phosphorylate Tau in vitro on residues that are phosphorylated in PHF-Tau (Reynolds et al., 1997a; Reynolds et al., 1997b; Reynolds et al., 2000; Goedert et al., 1997). It has previously been shown that Tau is a good in vitro substrate for SAPK3/p38γ and SAPK4/p38δ and that phosphorylation by these two enzymes reduces its ability to promote microtubule assembly (Goedert et al., 1997). Furthermore, p38 MAPKs can phosphorylate Tau in transfected neuroblastoma cells and COS cells in response to osmotic stress (Jenkins et al., 2000; Buée-Scherrer and Goedert, 2002). In neuroblastoma cells, overexpressed SAPK3/p38γ, but not other SAPKs, induces Tau phosphorylation and this correlates with a decrease in the amount of Tau associated with the cytoskeleton and an increase in the amount of soluble Tau (Jenkins et al., 2000).

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. Here, we have identified the residues phosphorylated in human Tau by all four p38MAPKs in vitro, and have studied how phosphorylation of one specific residue, Thr50, is regulated by cellular stress, and the effects of phosphorylation at this site on Tau function.

Materials and Methods

Antibodies and peptides

The peptide KESPLQphosTPTEDG (residues 44-55) from human Tau40 (longest isoform) was used to generate the Phos-Thr50 antibody. It was coupled to bovine serum albumin and keyhole limpet haemocyanin, and injected into sheep at Diagnostics Scotland (Pennicuik, UK). An antibody that recognises both phosphorylated and non-phosphorylated Tau was generated by injecting sheep with glutathione S-transferase (GST)-tagged Tau. Anti-SAPK3 and anti-SAPK4 antibodies were generated by injecting sheep with the GST-tagged kinases. The antibodies were affinity-purified as previously described (Sabio et al., 2004).

p38α antibody was obtained from Upstate Inc. (Dundee, UK). Antibodies that recognise p38α phosphorylated at Thr180 and Tyr182 (these antibodies also recognise phosphorylated p38β, SAPK3/p38γ and SAPK4/p38δ), phosphorylated MAPKAP-K2 at Thr334 and both phosphorylated and non-phosphorylated MAPKAP-K2 were purchased from New England Biolabs (Hitchin, UK). The phosphorylation-independent anti-Tau antibody BR134 has been described before (Goedert et al., 1989a). The AT270 antibody, which recognises Tau phosphorylated at Thr181, was obtained from Pierce (Perbio Science, UK). Anti-β-tubulin is a mouse monoclonal antibody purchased from Zymed (Cambridge Bioscience).

Phospho-peptides CRTPPKphosSPSSAKS, KTKIAphosTPRGA and SDAKSphosTPTA corresponding to residues 229-241, 148-157 and 64-72, respectively, were used in the characterization of the Phos-Thr50 antibody. The peptide KKKKESPLQTPTEDG corresponding to residues 44-55 of Tau plus four Lys at the N-terminus, and containing Thr50, was used for assaying SAPK/p38 activity in vitro (Cuenda et al., 1997). All peptides used in this study were synthesised by G. Bloomberg (University of Bristol, UK).

DNA constructs

For overexpression in mammalian cells, HA-tagged hTau40 was cloned into the vector pCMV5, whereas for expression in *E. coli* hTau40 was cloned into the vector pGEX-6P-1. HA-p38α and HA-p38β were cloned into pCMV5, and c-myc-SAPK4/p38δ and c-myc-SAPK3/p38γ was cloned into pcDNA3.1.

Thr50 in Tau was mutated to alanine or glutamic acid using the QuikChange kit (Stratagene). All DNA constructs and clones were verified by DNA sequencing, which was carried out by The Sequencing Service, School of Life Sciences, University of Dundee (www.dnaseq.co.uk).

Protein expression, purification and activity

Wild type and mutant GST-hTau40 were expressed in *E. coli* and purified as described (Knebel et al., 2001). All human GST-SAPK/p38s were prepared and activated by the Protein Production Team of the Division of Signal Transduction Therapy, University of Dundee. SAPK/p38 family members were assayed routinely using myelin basic protein (MBP) from Invitrogen (Paisley, UK) as substrate (Cuenda et al., 1997). Phosphorylation of Tau by active GST-SAPK/p38s was carried out as described (Cuenda et al., 1997; Goedert et al., 1997). PHF-Tau was prepared from frontal cortex of two Alzheimer's disease brains as described (Goedert et al., 1992b). Recombinant hTau46 (a 412 amino acid isoform of human brain Tau) was expressed and purified as previously described (Goedert and Jakes, 1990).

Identification of phosphorylation sites in GST-hTau40

Human GST-hTau40 (~0.5 μM) was incubated for 30 minutes at 30°C with activated (2 U/ml) human GST-p38α, GST-p38β, GST-SAPK3/p38γ or GST-SAPK4/p38δ, 10 mM magnesium acetate and 1 mM [γ-^{32}P]ATP (Amersham Bioscience) in 50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1 mM sodium orthovanadate and 0.1% (v/v) 2-mercaptoethanol. The procedure for mapping the phosphorylation sites is detailed elsewhere (Campbell and Morrice, 2002). The ^{32}P-labelled protein was reduced with DTT and incubated with 0.5% (v/v) 4-vinylpyridine to alkylate cysteine residues and subjected to SDS-PAGE. The band corresponding to ^{32}P-labelled GST-hTau40 was excised, digested with trypsin and chromatographed on a Vydac C_{18} column (2 mm i.d. × 15 cm) equilibrated in 0.1% (v/v) trifluoroacetic acid (TFA). The column was developed with a gradient of acetonitrile in 0.1% (v/v) TFA from 0-30% acetonitrile (0-90 minutes), 30-50% acetonitrile (90-110 minutes) and 50-100% acetonitrile (110-120 minutes). The flow rate was 0.2 ml/minute, fractions of 0.1 ml were collected and ^{32}P-radioactivity determined by Cerenkov counting. Briefly, sites of phosphorylation within the peptides were determined by solid-phase Edman sequencing of peptides coupled to a Sequelon-arylamine membrane. The release of ^{32}P-radioactivity after a cycle of Edman degradation was counted. Phosphoaminoacid analysis was performed as described (Cuenda et al., 1997).

Cell culture conditions, transfection and cell lysis

Human embryonic kidney (HEK)293 cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) and 2 mM L-glutamine supplemented with 10% (v/v) foetal calf serum (Biowhittaker, UK). SH-SY5Y human neuroblastoma cells were grown in DMEM-F12 supplemented with 15% (v/v) fetal bovine serum, 2 mM L-glutamine and 1% (v/v) nonessential amino acids. Transfection of HEK293 cells was carried out using the calcium phosphate method (Cuenda et al., 1997). Cells were exposed to 0.5 M sorbitol, 0.5 mM sodium arsenite or 200 J/m^2 UV-C radiation and lysed in buffer A (50 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 50 mM sodium β-glycerophosphate, 5 mM pyrophosphate, 0.27 M sucrose, 0.1 mM phenylmethylsulphonyl fluoride, 1% (v/v) Triton X-100, 1 μM microcystin) plus 0.1% (v/v) 2-mercaptoethanol and complete

Proteinase inhibitor cocktail (Roche, East Susset). Lysates were centrifuged at 13,000 ***g*** for 5 minutes at 4°C, the supernatants removed, quick frozen in liquid nitrogen and stored at –80°C until use. When required, cells were pre-incubated for 1 hour with 10 μM SB203580 (Calbiochem, Nottingham) or 10 μM PD184352, prior to stimulation with the above-mentioned agonists.

Immunoprecipitation of endogenous Tau and SAPK4/p38δ

Two micrograms anti-Tau antibody or 4 μg anti-SAPK4/p38δ antibody were coupled to protein G-Sepharose (Amersham Bioscience) and incubated with 1 mg or 10 mg protein extracts from SH-SY5Y cells, respectively. After 2 hours incubation at 4°C, the captured proteins were centrifuged at 13,000 ***g***, the supernatant discarded and the beads washed twice in buffer A containing 0.5 M NaCl, then twice in buffer A alone. Samples were denatured, electrophoresed in precast polyacrylamide gels (Invitrogen, Paisley) and immunoblotted with the appropriate antibodies.

siRNA construction and transfection

For the generation of siRNA against SAPK4/p38δ, we used the pSUPER (pS) technology (Brummelkamp et al., 2002). pSUPER vectors termed pS1, pS2 and pS3 were generated by cloning in the oligonucleotides:
(5′GATCCCCGGATTTCACTCAGCTGTTCTTCAAGAGAGAACAGCTGAGTGAAATCCTTTTTGGAAA3′), (5′GATCCCCACAAAGCGGCCAAATCCTATTCAAGAGATAGGATTTGGCCGCTTTGTTTTTTGGAAA3′) (5′GATCCCCAGCGGCCAAATCCTACATCTTCAAGAGAGATGTAGGATTTGGCCGCTTTTTTGGAAA3′).
They were directed against the human SAPK4/p38δ sequence and selected using the siDESIGN center programme at http://www.oligoengine.com/. SH-SY5Y cells were transfected with the empty pSUPER vector or pS1, pS2 or pS3, employing the standard calcium chloride procedure (Cuenda et al., 1997).

For the generation of siRNA against SAPK3/p38γ we used the pSUPER and the Silencer siRNA construction kit from Ambion. pSUPER vectors were generated by cloning in the oligonucleotides:
(5′GATCCCCGTTCCTCGTGTACCAGATGTTCAAGAGACATCTGGTACACGAGGAACTTTTTGGAAA3′),
(5′GATCCCCGAGCGATGAGGCCAAGAACTTCAAGAGAGTTCTTGGCCTCATCGCTCTTTTTGGAAA3′)
(5′GATCCCCCCGCACACTGGATGAATGGTTCAAGAGACCATTCATCCAGTGTGCGGTTTTTGGAAA3′).
They were directed against the human SAPK3/p38γ sequence and selected as described above. SH-SY5Y cells were transfected with the empty pSUPER vector or with a mixture of the three pSUPER vectors.

Other siRNAs for human SAPK3/p38γ were used:
(5′GACGACGTTGACCGCACACTGCCTGTCTC3′)
(5′GACGTTGACCGCACACTGGATCCTGTCTC3′)
(5′AACATGAGAAGCTAGGCGAGGCCTGTCTC3′)
(5′GACCAGCTGAAGGAGATCATGCCTGTCTC3′)
(5′AAGAACAACATGAAGGGCCTCCCTGTCTC3′).
They were prepared using the Silencer siRNA construction kit according to the manufacturer's instructions. SH-SY5Y cells were transfected with 100 nM of the siRNA mixture using Lipofectamine 2000 reagent (Invitrogen). The same amount of GAPDH siRNA sequence derived from the 5′ medial region of the GAPDH mRNA sequence and provided by Ambion was used as a control in these experiments.

Microtubule assembly

In vitro microtubule assembly was carried out as described (Goedert et al., 1997), using 2 μM wild-type (WT) or mutant (T50A and T50E) GST-tagged hTau40. Tubulin and GTP were obtained from Cambridge Bioscience. Microtubule assembly in vivo was performed as previously described (Pérez et al.2000). HEK293 cells were transfected with constructs encoding wild-type (WT) and mutant (T50A and T50E) Tau, and treated with 2.5 μg/ml nocodazole for 2 hours, before being released in fresh media for 20 minutes, and tubulin re-polymerization visualised by immunofluorescence.

Immunofluorescence

HEK293 cells were grown on 22 mm diameter coverslips coated with 10 μg/ml poly-L-Lysine. Transient transfections with the corresponding plasmids were performed using FuGene reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. Cells were fixed in a –20°C methanol-acetone solution at 4°C for 5 minutes, blocked in 1% donkey serum and 5% bovine serum albumin (BSA) in PBS for 1 hour at room temperature and then incubated with anti-β-Tubulin antibody (dilution 1/50) for 1 hour at room temperature. Secondary antibodies (Molecular Probes, Eugene) were applied at 1:250 in 5% BSA for 45 minutes in a dark humidified chamber. Epifluorescence microscopy was performed using a Leica DM-IRS or Zeiss axiovert 200M inverted microscope equipped with PlanApo x63 1.32NA oil immersion objectives. Images were captured using Improvision®Openlab 3.0.7 software and assembled using Adobe Photoshop® 4.0.

Statistical analysis

Kruskall-Wallis with Dunn's post test was performed using GraphPad Prism version 3.0a for Macintosh (GraphPad Software, San Diego, CA) $P \leq 0.05$ was considered significant.

Results

Analysis of the phosphorylation of Tau protein by all four p38 isoforms in vitro

We have shown previously, using phosphospecific antibodies, that Tau protein is an in vitro substrate for all SAPK/p38 isoforms (Goedert et al., 1997). Although identification of phosphorylation sites using antibodies has proved useful, it is not definitive; direct sequencing is required to identify unequivocally the residues phosphorylated and to identify sites that available antibodies do not recognise. Human Tau40 (the longest Tau isoform in human brain with 441 residues) was therefore phosphorylated in vitro. All p38 isoforms showed a similar K_m towards recombinant Tau protein (1.3-1.7 μM), although initial rates of Tau phosphorylation showed that, among all the SAPK/p38s, SAPK3/p38γ and SAPK4/p38δ were better Tau kinases than p38α and p38β (data not shown) (Goedert et al., 1997). Moreover, the phosphate incorporation per Tau molecule was ~14 mol/mol with SAPK3/p38γ, ~12 with SAPK4/p38δ, ~10 with p38α and ~5 with p38β.

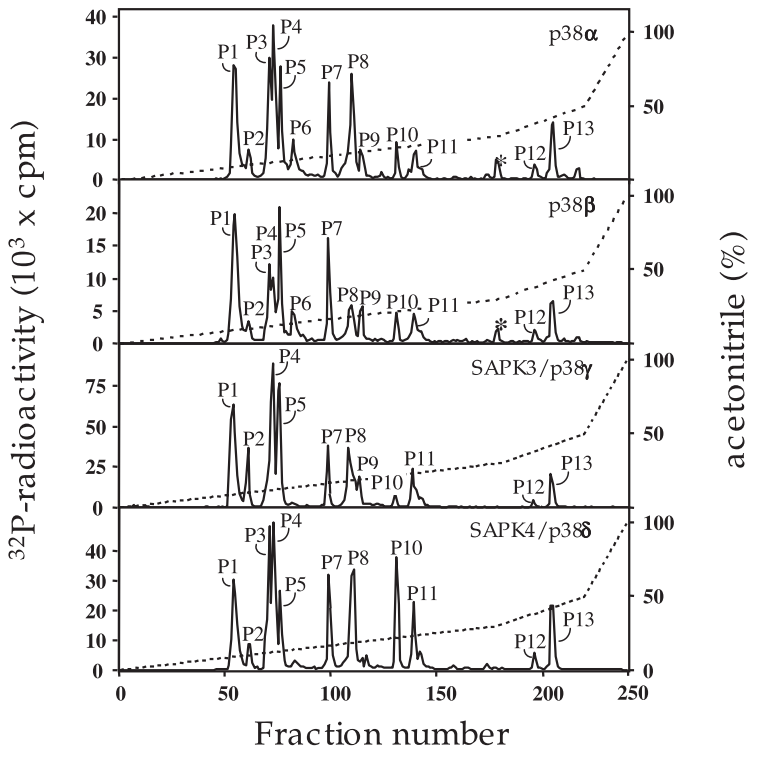
Phosphorylated Tau protein was digested with trypsin and the resulting peptides chromatographed on a Vydac C_{18} column. More than ten major peaks of ^{32}P-radioactivity, termed P1 to P13, were observed (Fig. 1A). p38α and p38β showed the same phosphorylation pattern, except that the proportion of peaks P4 and P8 compared to other peaks was higher in p38α than in p38β. P6 was not detectable after phosphorylation by SAPK3/p38γ or SAPK4/p38δ, whereas P3 and P9 were not found in Tau phosphorylated by SAPK3/p38γ or SAPK4/p38δ, respectively. The proportion of P10 was larger in Tau phosphorylated by SAPK4/p38δ compared with other SAPK/p38s (Fig. 1A). A peak (*) of radioactivity was also observed that eluted at 30% acetonitrile (Fig. 1A).

Radioactivity in this peak is due to phosphorylation of a Ser residue in the GST tag (data not shown). Tryptic peptide(s) contained in each peak were identified using the methodology described previously (Campbell and Morrice, 2002) and the results are listed in Fig. 1B.

The determination of phosphorylated sites was repeated at least twice for each SAPK/p38, with similar results. Results using GST-hTau40 or hTau40 protein without any tag were similar (data not shown), indicating that the N-terminal tag had no effect on Tau phosphorylation by SAPK/p38s in vitro.

The sites in Tau phosphorylated by the different SAPK/p38s are indicated (Fig. 1C). Eight sites were phosphorylated in vitro by all four SAPK/p38s: Thr50, Thr69, Thr153, Ser202, Thr205, Ser235, Ser404 and Ser422. Four of these (Ser202, Ser235, Ser404 and Ser422) are known to be phosphorylated in PHF-Tau (Morishima-Kawashima et al., 1995; Hanger et al., 1998), Thr153 has been found in the PHF smear (Morishima-Kawashima et al., 1995) and Thr205 and Thr50 have been reported to be phosphorylated in vitro by members of the MAPK family and by GSK3β (Goedert et al., 1997; Illenberger et al., 1998; Godemann et al., 1999; Reynolds et al., 2000), whereas Thr69 is a new phosphorylation site. Two sites (Thr181 and Thr217) were phosphorylated by p38α and p38β, but not detectably by SAPK3/p38γ or SAPK4/p38δ. Thr111 was only phosphorylated by p38β and SAPK3/p38γ. Residues Ser199 and Ser396 were not phosphorylated by SAPK4/p38δ, whereas Thr212 was not phosphorylated by p38α. Surprisingly, residues Ser195 or Ser220, which are not followed by proline, were phosphorylated by SAPK3/p38γ or SAPK4/p38δ and p38α, respectively, but not by p38β. MKK6, the enzyme used to activate each p38MAPK, did not phosphorylate Tau (data not shown).

A



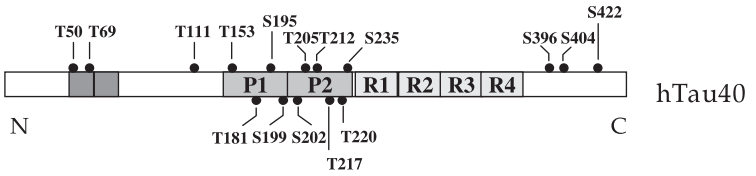
B

HPLC peak	Phospho-peptide sequence in the peak	Phosphorylated residue(s)
P1	TPPKSPSSAK (α, β, γ, δ)	S^{235}
P2	IATPR (α, β, γ, δ)	T^{153}
P3	SGYSSPGSPGTPGSR (β, δ) SGYSSPGSPGTPGSR (α)	S^{202} T^{205} S^{199} S^{202} T^{205}
P4	SPVVSGDTSPR (α, β, γ, δ)	S^{404}
P5	SGYSSPGSPGTPGSR (α, β) SGYSSPGSPGTPGSR (γ) SGYSSPGSPGTPGSR (δ)	S^{199} S^{202} T^{205} S^{195} S^{199} S^{202} T^{205} S^{202} T^{205}
P6	TPPAPKTPPSSGEPPK (α,β)	T^{181}
P7	ESPLQTPTEDGSEEPGSETSDAK (α, β, γ, δ)	T^{50}
P8	TPSLPTPPTR (α, β) SRTPSLPTPPTR (β, γ, δ) SRTPSLPTPPTR (α, δ)	T^{217} T^{212} T^{220}
P9	TDHGAEIVYKSPVVSGDTSPR (α, β, γ)	S^{396}
P10	STPTAEDVTAPLVDEGAPGK (α, β, γ, δ)	T^{69}
P11	QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQAR (β, γ)	T^{111}
P12	HLSNVSSTGSIDMVDSPQLATLADEVSASLAK (α, β, γ, δ)	S^{422}
P13	HLSNVSSTGSIDMVDSPQLATLADEVSASLAK (α, β, γ, δ)	S^{422}

Cellular stresses induce phosphorylation of endogenous Tau in SH-SY5Y human neuroblastoma cells

Among the residues in Tau phosphorylated in vitro by SAPK/p38s, Thr50 is the only one that is present in human Tau isoforms, but not in isoforms from some other species, such as rodents. Moreover, Thr50 is located in the N-terminal region of Tau, phosphorylation of which has not been extensively studied. Since the in vitro phosphorylation of Tau revealed that Thr50 is phosphorylated efficiently by all four SAPK/p38 isoforms, we decided to examine its phosphorylation in intact cells. First, we generated a phospho-specific antibody that recognises Tau phosphorylated at residue Thr50. Its specificity was established by the finding that it only recognised GST-Tau after phosphorylation in vitro by SAPK3/p38γ and did not recognise the non-phosphorylated form of Tau (Fig. 2A). Furthermore, the recognition of phosphorylated Tau was abolished when the antibody was incubated with the phosphopeptide used as the antigen, but not when the non-phosphorylated form of this peptide or

C



Residue	p38α	p38β	SAPK3/p38γ	SAPK4/p38δ
T^{50}	+	+	+	+
T^{69}	+	+	+	+
T^{111}		+	+	
T^{153}	+	+	+	+
T^{181}	+	+		
S^{195}			+	
S^{199}	+	+	+	
S^{202}	+	+	+	+
T^{205}	+	+	+	+
T^{212}		+	+	+
T^{217}	+	+		
T^{220}	+			+
S^{235}	+	+	+	+
S^{396}	+	+	+	
S^{404}	+	+	+	+
S^{422}	+	+	+	+

Fig. 1. Phosphorylation of GST-hTau40 by different SAPK/p38 isoforms. (A) Human GST-Tau40 (hTau40) was labelled with Mg[γ-^{32}P]ATP in the presence of p38α, p38β, SAPK3/p38γ or SAPK4/p38δ, and subjected to SDS-PAGE. Phosphorylated hTau40 was excised from the gel, digested with trypsin and the peptides separated by chromatography. The column was developed with an acetonitrile gradient (broken line) and ^{32}P-radioactivity is shown by the solid line. Phosphopeptides P1 to P13 are indicated; the asterisks indicate a phosphopeptide from the GST tag. (B) Identification of the sites in hTau40 phosphorylated by SAPK/p38s. The kinases used to phosphorylate Tau are indicated in brackets: α, p38α; β, p38β; γ, SAPK3/p38γ; δ, SAPK4/p38δ. P3 in Tau phosphorylated by p38α is a mixture of one to three di-phosphopeptides, whereas P5, for all p38 isoforms, is a mixture of two to four mono-phosphopeptides. P12 and P13 contained the same phosphopeptide, but the Met in P12 was oxidised. All residues were identified by a combination of techniques (MALDI-TOF, Q-TOF, MS/MS, solid phase sequencing and phospho-amino acid analysis) (Campbell and Morrice, 2002), with the exception of residue Thr69 for SAPK3/p38γ, which was identified only by HPLC. The phosphorylated residue(s) in each phosphopeptide is/are underlined and numbered according to the sequence of the longest Tau isoform in human brain. (C) The Tau isoform (hTau40) used in this study. The identified phosphorylation sites are shown. The C-terminal domain contains three or four repeats (R1-R4) that are flanked N-terminally by proline-rich regions (P1 and P2) which extend into the N-terminal or projection domain. The proline-rich regions contain most of the Ser/Thr-Pro motifs. The table indicates the in vitro phosphorylation sites on hTau40 phosphorylated by the different p38s.

phosphopeptides corresponding to other phosphorylation sites were used (Fig. 2B). One of these phosphopeptides was Phos-Thr69 peptide, which contains Thr69 and possesses a similar sequence to the peptide containing Thr50 (Fig. 2B). Moreover, the recognition of phosphorylated Tau by Phos-Thr50 antibody is not abolished when phosphorylated Tau is incubated with the phospho-Tau(T50A), the Tau mutant in which Thr50 was mutated to Ala and then phosphorylated at all possible sites with SAPK3/p38γ (Fig. 2B). However, phospho-Tau(T50A) was not recognised by Phos-Thr50 antibody but was recognised by Phos-Thr181 antibody (Fig. 2B).

To examine whether Tau also became phosphorylated at Thr50 in vivo under conditions where p38 MAPKs were activated, we first transfected HEK293 cells with either a vector expressing HA-Tau, or HA-Tau and a vector expressing HA-p38α, HA-p38β, myc-SAPK3/p38γ or myc-SAPK4/p38δ. Cells were exposed to osmotic stress, arsenite or UV-C radiation to trigger the activation of p38 MAPKs, and the phosphorylation of Tau analysed using the Phos-Thr50 antibody. In cells where Tau was expressed alone, significant phosphorylation at Thr50 was observed only after treatment with sorbitol (Fig. 2C). Under these conditions, endogenous p38α and SAPK3/p38γ were activated. SAPK4/p38δ was weakly activated by sorbitol and it was not by UV-C or arsenite (not shown). In cells co-transfected with Tau and either p38α or SAPK3/p38γ, phosphorylation at Thr50 was detected even in the absence of sorbitol treatment, suggesting that the basal activity of these kinases, when overexpressed, was sufficient to phosphorylate this site. An increase in the phosphorylation of Thr50 was observed only after cellular stress treatment when Tau was co-expressed with p38β or SAPK4/p38δ (Fig. 2C). Retardation in the electrophoretic mobility of Tau protein was observed when cells had been exposed to osmotic shock or arsenite, indicating phosphorylation of Tau at multiple sites (Fig. 2C).

Phosphorylation of Thr50 is not mediated by p38α or p38β

We next examined whether endogenous Tau became phosphorylated at Thr50 in SH-SY5Y neuroblastoma cells exposed to osmotic shock, sodium arsenite or UV-C (Fig. 3A). Tau became phosphorylated at Thr50 after sorbitol treatment, whereas arsenite and UV-C radiation did not cause detectable phosphorylation (Fig. 3A). The phosphorylation of Tau at Thr50 was further increased when cells were starved of serum and non essential amino acids for 6 hours before stress treatment (Fig. 3A). After sorbitol treatment a lower molecular weight band became apparent with both Phos-Thr50 and total Tau antibodies. This band did not appear when cells were pre-incubated with the phosphatase inhibitor okadaic acid before stimulation with sorbitol (C.F. and A.C., unpublished), suggesting that it may have resulted from the dephosphorylation of residues other than from Thr50.

In order to get information about which kinase(s) is/are

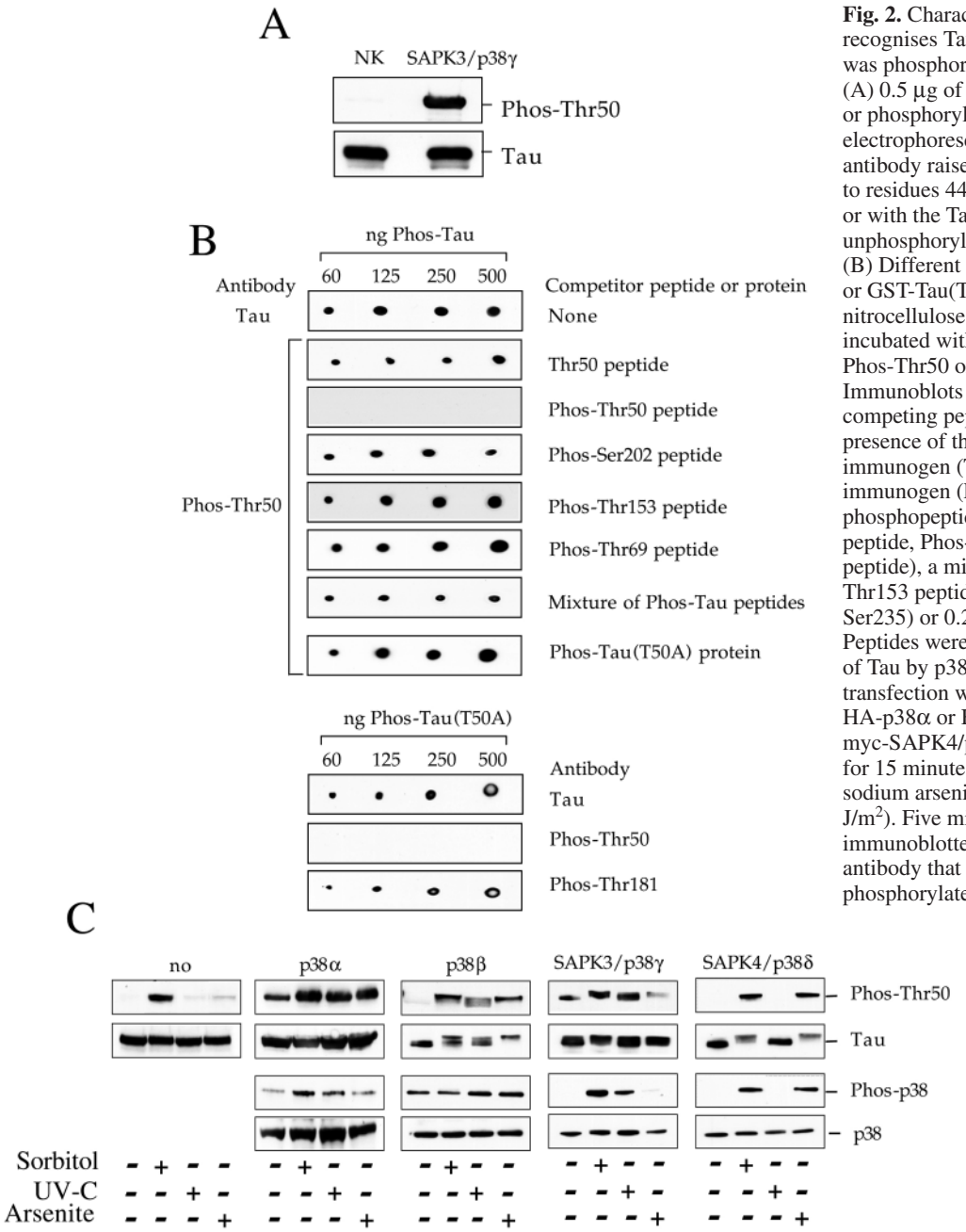


Fig. 2. Characterisation of the antibody that recognises Tau phosphorylated at Thr50. GST-Tau was phosphorylated by 2 U/ml of SAPK3/p38γ. (A) 0.5 μg of GST-Tau non-phosphorylated (NK) or phosphorylated (SAPK3/p38γ) was electrophoresed and blotted with the Phos-Thr50 antibody raised against the peptide corresponding to residues 44-55 of Tau phosphorylated at Thr50 or with the Tau antibody that recognises both the unphosphorylated and phosphorylated proteins. (B) Different amounts of phosphorylated GST-Tau or GST-Tau(T50A) were spotted onto a nitrocellulose membrane. Membranes were incubated with 1 μg/ml of Tau antibody, antibody Phos-Thr50 or antibody Phos-Thr181. Immunoblots were carried out in the absence of competing peptide or protein (None), or in the presence of the unphosphorylated peptide immunogen (Thr50 peptide), the phosphopeptide immunogen (Phos-Thr50 peptide), phosphopeptide for another site (Phos-Thr153 peptide, Phos-Ser202 peptide or Phos-Thr69 peptide), a mixture of phosphopeptides (Phos-Thr153 peptide, Phos-Ser202 peptide and Phos-Ser235) or 0.2 mg/ml Phos-Tau(T50A) protein. Peptides were at 100 μg/ml. (C) Phosphorylation of Tau by p38 MAPKs in HEK293 cells. After transfection with plasmids encoding HA-Tau and HA-p38α or HA-p38β, or myc-SAPK3/p38γ or myc-SAPK4/p38δ, HEK293 cells were exposed for 15 minutes to 0.5 M sorbitol or 0.5 mM sodium arsenite, or for 30 minutes to UV-C (200 J/m^2). Five micrograms of cell lysate was immunoblotted using antibody Phos-Thr50 and an antibody that recognises unphosphorylated and phosphorylated Tau equally. To examine activation of p38 MAPKs, 10 μg of cell lysate was used in the immunoblot. The p38α phospho-specific antibody also recognises phosphorylated p38β, SAPK3/p38γ and SAPK4/p38δ.

responsible for the phosphorylation of Tau at Thr50 in SH-SY5Y cells after sorbitol treatment, we incubated cells with SB203580 or PD184352, prior to osmotic shock. SB203580 is a relatively specific inhibitor of p38α and p38β activity, whereas PD184352 is a potent inhibitor of the ERK1/2 and ERK5 pathways (Davies et al., 2000; Mody et al., 2003). Neither compound had any significant effect on the phosphorylation of endogenous Tau at Thr50 (Fig. 3B). As expected, SB203580 prevented the phosphorylation of the p38α substrate MAPKAP-K2 (Fig. 3B).

Knock-down of SAPK4/p38δ and SAPK3/p38γ blocks Tau phosphorylation at Thr50

Our results suggested that phosphorylation of Tau at Thr50 in SH-SY5Y cells might be mediated by SAPK3/p38γ and/or SAPK4/p38δ, since sorbitol treatment activated both kinases and since their activity is not known to be inhibited by SB203580. Moreover, comparison of initial rates of phosphorylation by different SAPK/p38s of a synthetic peptide corresponding to residues 44 to 55 of hTau40, showed that Thr50 was phosphorylated more efficiently by SAPK4/p38δ and SAPK3/p38γ than by p38α or p38β (Fig. 4A).

To examine the role of SAPK3/p38γ and SAPK4/p38δ in phosphorylating endogenous Tau at Thr50, we employed a specific small interfering RNA (siRNA) approach, due to the impossibility of using cells from mice lacking these kinases, since Thr50 is not present in murine Tau. All three siRNA sequences chosen against SAPK4/p38δ (pS1, pS2 and pS3) reduced the levels of endogenous SAPK4/p38δ by more than

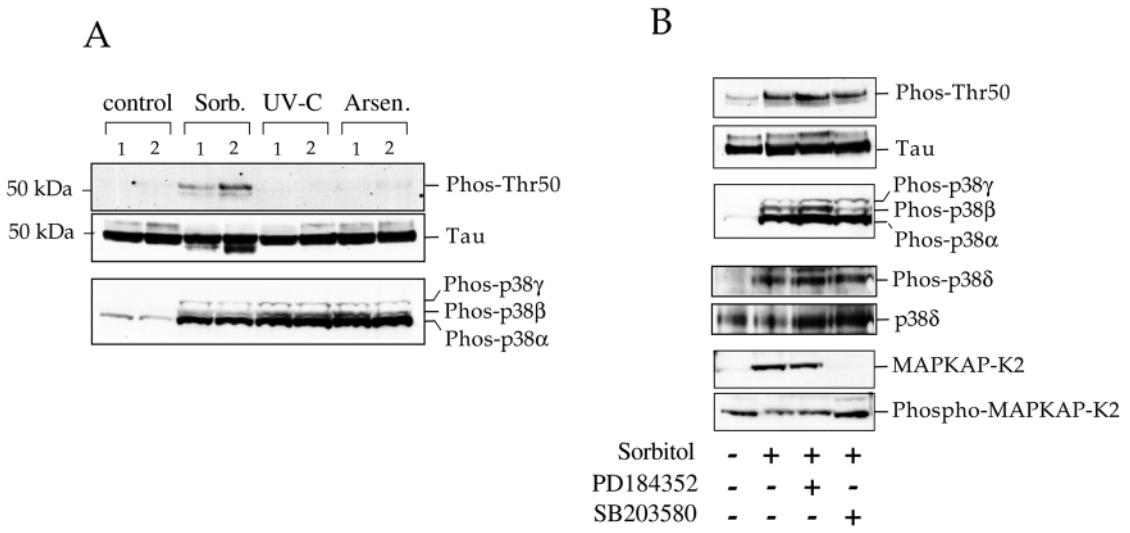


Fig. 3. Phosphorylation of endogenous Tau in SH-SY5Y human neuroblastoma cells. (A) SH-SY5Y cells were incubated for 6 hours in medium with (1) or without (2) serum and exposed for 15 minutes to 0.5 M sorbitol or 0.5 mM sodium arsenite, or for 30 minutes to UV-C (200 J/m^2). Endogenous Tau was immunoprecipitated with an anti-Tau antibody from 1.0 mg of cell lysate and immunoblotted using the Phos-Thr50 antibody or an antibody that recognises both unphosphorylated and phosphorylated Tau. To examine SAPK/p38 activation, 50 μg of cell lysate was used in the immunoblot with the p38α phospho-specific antibody, as shown in Fig. 2. (B) SH-SY5Y cells were preincubated for 1 hour with or without 10 μM SB203580 or 10 μM PD184352 prior to a 15 minutes exposure to 0.5 M sorbitol. Tau protein was immunoprecipitated and immunoblotted as indicated in A. Endogenous SAPK4/p38δ was immunoprecipitated from 10 mg cell lysate with anti-SAPK4/p38δ antibody, and immunoblotted using the p38α phospho-specific antibody, or with an antibody that recognises both unphosphorylated and phosphorylated SAPK4/p38δ. Activation of other p38s was examined as described in A. 60 μg of cell lysates were immunoblotted with an antibody that recognises MAPKAP-K2 phosphorylated at Thr334 or with an antibody that recognises both unphosphorylated and phosphorylated MAPKAP-K2.

90% in SH-SY5Ycells, without affecting the levels or activation of the other SAPK/p38 isoforms (Fig. 4B). To obtain knock-down in the expression of SAPK3/p38γ by more than 90%, a mixture of either five different siRNA sequences (S3mix) or a mixture of the three pSUPER vectors (pS3mix) was used (Fig. 4B). The levels of other p38MAPKs, namely p38α and SAPK4/p38δ, were also significantly reduced (Fig. 4B). This result indicated that the mixture of SAPK3/p38γ siRNAs needed to knock-down these kinases cross-reacted with similar sequences, such as other p38 MAPKs, causing a reduction in their levels of expression.

We next transfected cells with either siRNA control, the empty pSUPER vector or the different siRNA construct against SAPK3/p38γ or SAPK4/p38δ, and examined the phosphorylation of endogenous Tau at Thr50 in lysate from osmotically shocked cells. All siRNAs reduced the level of phosphorylation at Thr50 to a certain extent (Fig. 4C). The siRNA against SAPK4/p38δ reduced the phosphorylation by more than 80%, whereas the siRNA against SAPK3/p38γ reduced phosphorylation at Thr50 by more than 90%. The complete blockade of Thr50 phosphorylation by the SAPK3/p38γ siRNA was probably due to a reduction in the level of expression of both SAPK3/p38γ and SAPK4/p38δ. These results suggest that SAPK4/p38δ is the major kinase phosphorylating Thr50 in Tau after osmotic stress.

Mutation of Thr50 to Glu increased the ability of Tau to promote microtubule assembly

Tau binds to microtubules through the conserved repeats located in the C-terminal region. Modifications, such as phosphorylation, in sequences flanking the repeats, can affect the affinity of this region for microtubules. We therefore wanted to examine the effect of phosphorylation at Thr50 on the ability of Tau to promote microtubule assembly and its binding to tubulin. We generated Tau mutants in which residue Thr50 was mutated either to Glu(T50E), to mimic phosphorylation by introducing a negative charge, or to Ala(T50A) to prevent phosphorylation.

We first checked microtubule polymerization in vitro. The mutant Tau(T50E) promoted microtubule assembly approximately threefold more efficiently than the Tau(WT) or the mutant Tau(T50A), as judged by initial rates (Fig. 5A). Statistical analysis of these results showed that the efficiencies of Tau(WT) and Tau(T50A) at promoting microtubule polymerization in vitro were similar (data not shown). Moreover, we also examined the effect of phosphorylation of Tau at Thr50 on the reassembly of microtubules in living cells. For this, HEK293 cells transfected with cDNAs encoding either Tau(WT), Tau(T50E) or Tau(T50A) were treated with nocodazole to depolymerise microtubules. The drug was removed when the microtubule network had completely disappeared, and microtubule repolymerization was analysed after 20 minutes.

Expression of Tau(T50E) resulted in the appearance of a large number of cytoplasmic extensions bearing bundled microtubules which were not seen for expression of Tau(WT) and Tau(T50A) (Fig. 5B). Quantitation revealed that 30% of cells expressing Tau(T50E) had initiated re-polymerization of microtubules, whereas only ~15% of cells expressing either

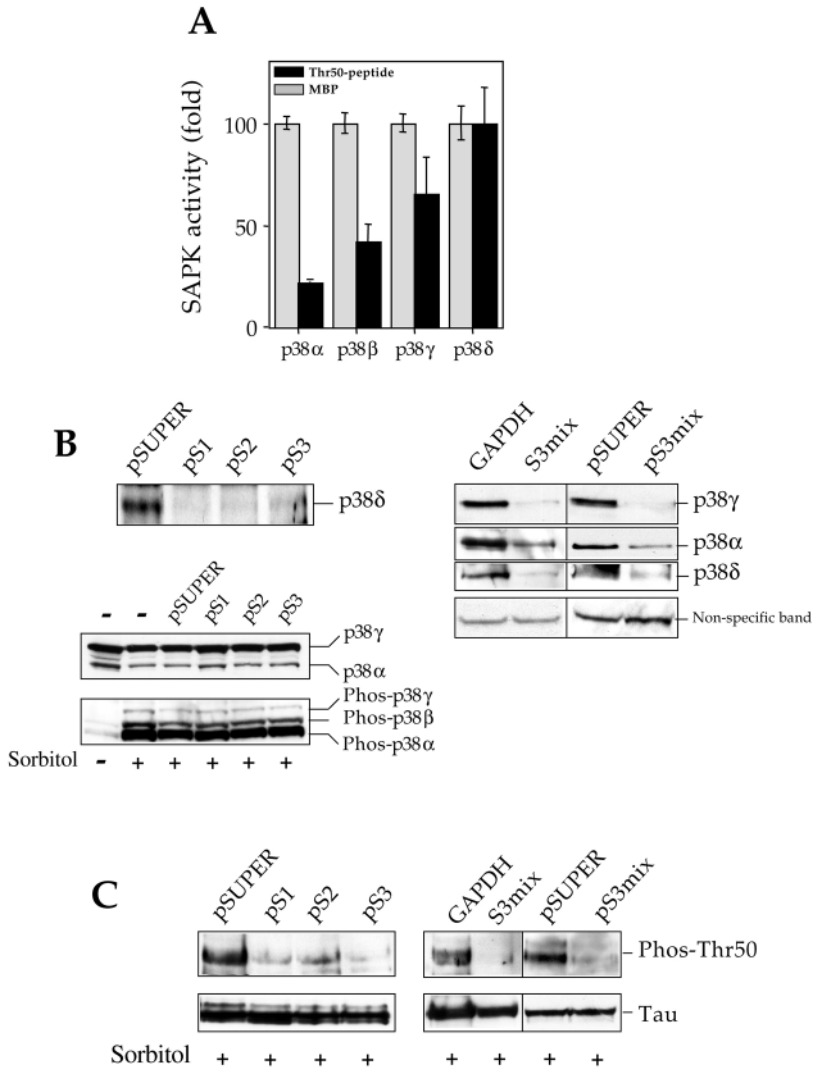


Fig. 4. Knock-down of SAPK3/p38γ and SAPK4/p38δ inhibits Tau phosphorylation at Thr50 after osmotic stress. (A) Comparison of substrate specificities of different SAPK/p38s. Each kinase (0.2 U/ml) was assayed under initial rate conditions, as described in Materials and Methods. The final concentrations of the Thr50-peptide (black bars) or MBP (grey bars) were 100 μM and 20 μM, respectively. Substrate phosphorylation is plotted as a percentage of the maximal phosphorylation. Results in A are shown as the mean±s.e.m. for triplicate determinations from a single experiment. (B) SH-SY5Y cells were transfected in the absence of siRNA (empty pSuper vector or control GAPDH siRNA), or in the presence of siRNA against SAPK4/p38δ (pS1, pS2 and pS3 constructs) or siRNA against SAPK3/p38γ (S3mix and pS3mix) as detailed in Materials and Methods. SAPK4/p38δ from 10 mg lysate or SAPK3/p38γ from 1 mg lysate was immunoprecipitated and immunoblotted as indicated above. (C) Endogenous Tau was immunoprecipitated from the cell lysates indicated in panel B, and immunoblotted as indicated in Fig. 3.

Tau(WT) or Tau(T50A) contained cytoplasmic extension bearing bundled microtubules (Fig. 5B). The levels of expression of wild-type and mutant Tau proteins were similar (Fig. 5C).

Since the ability of Tau to promote microtubule assembly is regulated by phosphorylation, we studied the possibility that Thr50 phosphorylation could alter the level of phosphorylation of other sites in the Tau molecule. Tau(WT), Tau(T50E) and Tau(T50A) were expressed in HEK293 cells and phosphorylation of residues in the Ser/Thr-Pro-rich region (Thr181, Ser199, Ser202 and Thr205) analysed in control cells and in cells exposed to osmotic shock. Only phosphorylation of Thr181 was affected by mutation of Thr50 to Glu (Fig. 5C). The phosphorylation of the other residues studied was unaffected (data not shown). The basal phosphorylation of Thr181 in Tau(WT) decreased after osmotic shock. In Tau(T50E), phosphorylation of Thr181 was less than in Tau(WT) and did not increase significantly after treatment with sorbitol. By contrast, in Tau(T50A) phosphorylation of Thr181 stayed high and did not change with osmotic shock treatment (Fig. 5C). These results indicate that phosphorylation at Thr50 can influence the phosphorylation of other sites, such as Thr181, and that this decrease in phosphorylation may contribute to the increased tubulin polymerization observed with the mutant Tau(T50E) in transfected cells.

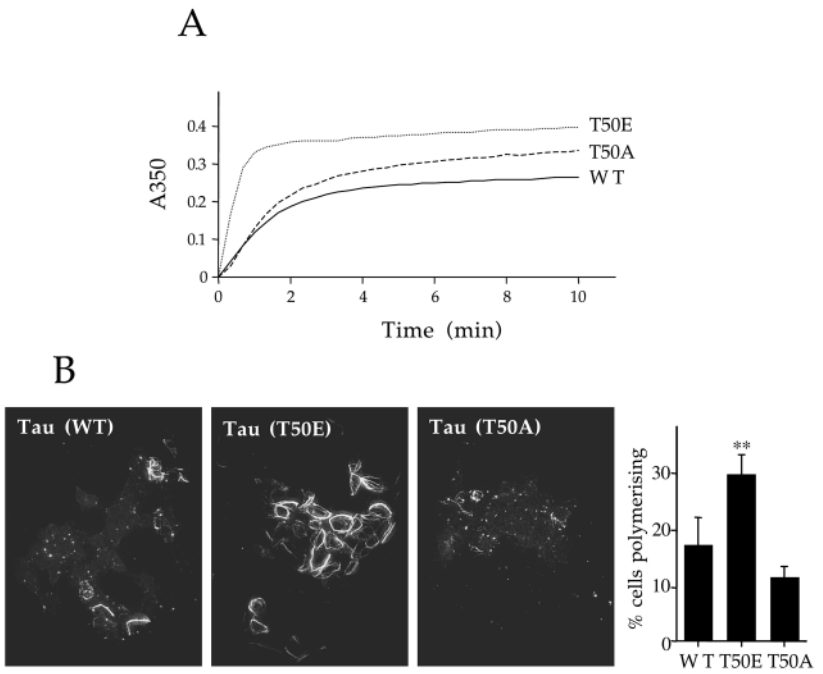
Thr50 is phosphorylated in PHF-Tau from Alzheimer's disease brain

Once the specificity of the Phos-Thr50 antibody was established, we examined whether Thr50 was phosphorylated in PHF-Tau from Alzheimer's disease brain. PHF-Tau was purified as described (Goedert et al., 1992b). Immunoblotting using anti-Tau serum showed the classical pattern of bands for PHF-Tau from Alzheimer's disease brain (Bueé et al., 2000). Phos-Thr50 antibody recognised the 64 and 68 kDa PHF-Tau bands but not the 55 kDa band (Fig. 6). This shows that Thr50 is phosphorylated in PHF-Tau from Alzheimer's disease brain.

Discussion

We have shown previously that p38 MAPK isoforms phosphorylate the microtubule-associated protein Tau at physiologically relevant sites (Thr181, Ser202, Thr205, Ser396 and Ser404) and at Ser422, which is phosphorylated in neurofibrillary tangles but not in normal adult brain (Hasegawa et al., 1996; Goedert et al., 1997). These phosphorylation sites were identified using phosphoantibodies (Goedert et al., 1997; Reynolds et al., 2000) and, although useful, this approach is not definitive. In the present study, we have identified unequivocally the residues in Tau phosphorylated by all four p38 MAPK isoforms through direct sequencing of ^{32}P-labelled recombinant hTau40 (Goedert et al., 1989a).

Out of the eight sites phosphorylated in vitro by all four SAPK/p38s (Thr50, Thr69, Thr153, Ser202, Thr205, Ser235, Ser404 and Ser422), four (Ser202, Ser235, Ser404 and Ser422) are phosphorylated in PHF-Tau, as shown by protein chemistry (Morishima-Kawashima et al., 1995; Hanger et al., 1998), whereas Thr153 has been found in the PHF smear (Morishima-



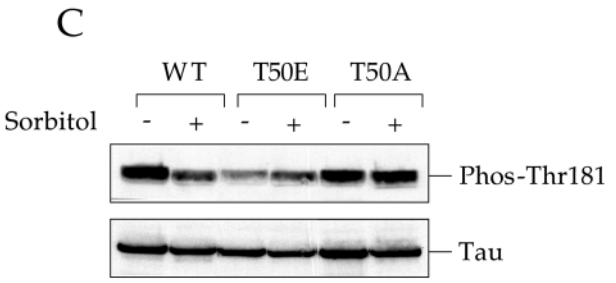


Fig. 5. Effects of Tau mutations T50E and T50A on tubulin polymerisation and reassembly of microtubules. (A) Polymerisation of tubulin promoted by GST-Tau(WT), GST-Tau(T50A) and GST-Tau(T50E) was monitored over time by measuring turbidity. A typical experiment is shown; similar results were obtained using at least three separate preparations of each protein. (B) HEK293 cells transfected with Tau(WT), Tau (T50E) or Tau(T50A) were treated with nocodazole for 2 hours. The microtubule network in transfected cells is shown 20 minutes after nocodazole had been washed out. Immunofluorescence staining was performed using a monoclonal antibody that recognises β-tubulin. The percentages of cells with re-polymerising microtubules were determined by counting five different optical fields containing at least 200 cells per field. ** indicates that the differences between the three groups were statistically significant (P<0.01). (C) HEK293 cells transfected with Tau(WT), Tau(T50A) or Tau(T50E) were exposed to 0.5 M sorbitol for 15 minutes. Five micrograms of cell lysate was immunoblotted using antibody AT270, which recognises phospho-Thr181 in Tau, and an antibody that recognises unphosphorylated and phosphorylated Tau.

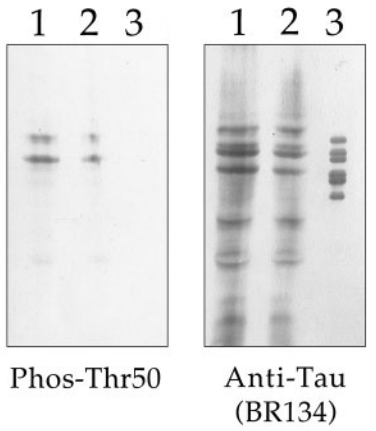


Fig. 6. Phosphorylation of Thr50 in PHF-Tau from Alzheimer's disease brains. Immunoblots of PHF-Tau with either Phos-Thr50 antibody (1 μg/ml) in the presence of 50 μg/ml of hTau46 (the 412 amino acid form), to further ensure the specificity of the antibody, or anti-Tau serum BR134 (1:1,000). Lanes 1 and 2, PHF-Tau prepared from two Alzheimer's disease brains; lane 3, mixture of recombinant human brain Tau isoforms.

Kawashima et al., 1995). Moreover, five residues (Thr181, Ser199, Thr212, Thr217 and Ser396) that are phosphorylated by one or more p38 MAPKs (see Fig. 1D) are phosphorylated in PHF-Tau (Morishima-Kawashima et al., 1995; Hanger et al., 1998), which suggests that cellular stresses that trigger activation of p38 MAPKs in the disease or misfunction of these kinases, and/or the phosphatase(s) that oppose them, might contribute to Tau hyperphosphorylation.

In addition, other residues phosphorylated by p38 MAPKs, such as Thr50 and Thr205, can be phosphorylated in vitro by GSK3β and/or MAPK (Goedert et al., 1997; Illenberger et al., 1998; Godemann et al., 1999; Reynolds et al., 2000), whereas Thr69 and Thr111 are new phosphorylation sites. Thr205 forms part of the epitope of antibody AT8, one of the most widely used markers for PHF-Tau (Goedert et al., 1995), implying phosphorylation of this residue in pathological Tau. Surprisingly, phosphorylation of residues Ser195 or Ser220, which are not followed by proline, was detected after phosphorylation of Tau by SAPK3/p38γ or SAPK4/p38δ and p38α, respectively. Previous work has shown that, on occasion, p38α, p38β and ERK5 phosphorylate serine and threonine residues that are not followed by proline (Cheung et al., 2003; Mody et al., 2003; Kuma et al., 2004). The results presented here also revealed some differences between the p38 MAPK isoforms (see Fig. 1), extending previous results that indicated different in vitro substrate specificities of individual p38 MAPKs (Cuenda et al., 1997; Kuma et al., 2004; Sabio et al., 2004).

We examined in more detail the regulation of the phosphorylation of Thr50 in Tau. This site is phosphorylated efficiently by all p38 MAPK isoforms in vitro and was chosen because it is present in Tau isoforms from species with spontaneous formation of Tau filaments, such as humans and non-human primates, as well as some ungulates, including goats and cows, but not in Tau isoforms from species where this pathology has not been found (Nelson et al., 1996; Holzer et al., 2004). Furthermore, using a phosphorylation-dependent antibody, we show here that Thr50 is phosphorylated in PHF-Tau from Alzheimer's disease brain.

Endogenous Tau was phosphorylated at Thr50 after osmotic shock of SH-SY5Y cells. Several lines of evidence point to SAPK4/p38δ and/or SAPK3/p38γ as Thr50 kinases. First, both kinases phosphorylated recombinant Tau and a synthetic peptide containing Thr50 at a faster initial rate than other p38s, and both were activated after exposure to osmotic stress. Moreover, phosphorylation of endogenous Tau at Thr50 after sorbitol treatment was not abolished by pre-incubation of neuroblastoma cells with SB203580 or PD184352, indicating that ERK1/2, ERK5, p38α and p38β were not rate-limiting for the phosphorylation of this site. Phosphorylation of Thr50 was not inhibited by pre-treatment of the cells with either kenpaullone and roscovitine, which inhibit GSK3β and/or CDKs (Bain et al., 2003) (data not shown). By contrast, siRNA against SAPK4/p38δ reduced phosphorylation of Thr50 by 80%, and siRNA against SAPK3/p38γ blocked it completely. One concern about the use of siRNAs to knock-down kinases is cross-hybridisation with transcripts of similar sequence, causing silencing of related proteins (Jackson et al., 2003). Our results show that the mixture of SAPK3/p38γ siRNA needed to knock-down this kinase cross-hybridised with other p38 MAPKs, causing a reduction in their levels. Thus, the complete blockade of Thr50 phosphorylation by the SAPK3/p38γ siRNA was due not only to the reduction in the level of SAPK3/p38γ, but also to a reduction in the level of expression of SAPK4/p38δ. These results suggest that SAPK4/p38δ is the major in vivo kinase that phosphorylates Thr50 in Tau following osmotic stress.

This study raises the question of the physiological role of Tau phosphorylation at Thr50. We have shown previously that phosphorylation by SAPK4/p38δ or SAPK3/p38γ decreases the ability of Tau to promote microtubule assembly (Goedert et al., 1997). Therefore, one possibility is that phosphorylation at Thr50 may also affect this function. The importance of individual sites in Tau in regulating microtubule binding has been studied for some time. For example, phosphorylation of residues located either within or adjacent to the microtubule-binding repeats reduces the association of Tau with microtubules (Buée et al., 2000; Lee et al., 2001). Thus, phosphorylation of Ser262 or Ser396 reduces the affinity of Tau for microtubules (Biernat et al., 1993; Bramblett et al., 1993). To examine the effect of phosphorylation at Thr50 on the ability of Tau to promote tubulin polymerization, this residue was mutated to Ala or Glu to prevent or mimic phosphorylation, respectively, and polymerization of tubulin was studied in vitro and in vivo. We found that mutation of Thr50 to Glu caused an increase in the polymerization and re-polymerization of tubulin. In addition, the mutation to Glu caused a decrease in the basal phosphorylation of other sites, such as Thr181 in the proline-rich region of Tau. Thr50 is located in exon 2 in the projection domain of Tau in a region with a high proportion of acidic residues. Different roles for this projection domain have been proposed, including determination of the spacing between axonal microtubules (Chen et al., 1992), modulation of the binding of Tau to proteins associated with the plasma membrane (Brandt et al., 1995) and interactions with other cytoskeletal proteins (Hirokawa et al., 1988). Moreover, a role for exons 2 and 3 in microtubule bundling has been suggested (Kanai et al., 1992). Taken together, these results suggest that phosphorylation of Thr50 could cause a conformational change in Tau that favours tubulin assembly. Such a change could also affect the phosphorylation of other sites, perhaps by modifying the affinity of a kinase and/or facilitating the action of a phosphatase. Previously, pseudophosphorylation of other sites has been shown to cause a reduced ability of Tau to promote microtubule assembly (Smith et al., 2000; Eidenmuller et al., 2001). However, it has also been reported that microtubule reduction in Alzheimer's disease is not dependent on PHF accumulation in neurons (Cash et al., 2003). Thr50 is the only known site whose pseudophosphorylation increases the ability of Tau to promote microtubule assembly. Although further experiments need to be performed to address this point, one possible explanation for the results shown in this study is that phosphorylation of Tau is a temporally ordered process that is responsible for generating toxic forms of Tau. Thus, phosphorylation of Tau at Thr50 may be an early event after SAPK4/p38δ activation. This early phosphorylation at Thr50 may cause an increase in the ability of Tau to promote microtubule assembly and help in the adaptive response of neurons to osmotic shock, which causes disruption of the cytoskeleton of the cells and cellular death (Morris et al., 2003) (data not shown). Subsequently, hyperphosphorylation of Tau by SAPK4/p38δ or/and by other protein kinase(s) in the neuron may instead induce the detachment of Tau from the microtubule and reduce the microtubule assembly promoted by Tau. Hyperphosphorylation in vitro of the Tau mutant T50E by either SAPK4/p38δ or SAPK3/p38γ decreased its ability to promote microtubule assembly (data not shown). Supporting this idea, it has been shown by genetic studies in *Drosophila*, that different kinases phosphorylate Tau in an ordered fashion, with PAR-1 phosphorylation of Tau facilitating subsequent phosphorylation by GSK3 and Cdk5 (Nishimura et al., 2004).

In conclusion, we show that Tau is phosphorylated by all four p38 MAPK isoforms and suggest a possible role for SAPK4/p38δ in the adaptation of neurons to changes in osmolarity. Our results also indicate that misfunction of SAPK4/p38δ and/or SAPK3/p38γ, or the phosphatases that oppose them, may contribute to the hyperphosphorylation of Tau that characterises Alzheimer's disease and other tauopathies.

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