

Phosphorylation of microtubule-associated protein tau by stress-activated protein kinases

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Abstract The paired helical filament, which comprises the major fibrous element of the neurofibrillary lesions of Alzheimer's disease, is composed of hyperphosphorylated microtubule-associated protein tau. Many of the hyperphosphorylated sites in tau are serine/threonine-prolines. Here we show that the stress-activated protein (SAP) kinases SAPK1 γ (also called JNK1), SAPK2a (also called p38, RK, CSBPs, Mpk2 and Mxi2), SAPK2b (also called p38 β), SAPK3 (also called ERK6 and p38 γ) and SAPK4 phosphorylate tau at many serine/threonine-prolines, as assessed by the generation of the epitopes of phosphorylation-dependent anti-tau antibodies. Based on initial rates of phosphorylation, tau was found to be a good substrate for SAPK4 and SAPK3, a reasonable substrate for SAPK2b and a relatively poor substrate for SAPK2a and SAPK1 γ . Phosphorylation of tau by SAPK3 and SAPK4 resulted in a marked reduction in its ability to promote microtubule assembly. These findings double the number of candidate protein kinases for the hyperphosphorylation of tau in Alzheimer's disease and other neurodegenerative disorders.

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Key words: Stress-activated protein kinase; Tau protein; Microtubule assembly; Alzheimer's disease

1. Introduction

Abundant neurofibrillary lesions made of paired helical filaments (PHFs) constitute one of the major neuropathological hallmarks of Alzheimer's disease and other neurodegenerative disorders. Hyperphosphorylated microtubule-associated protein tau (PHF-tau) is the major component of the PHF (reviewed in [1]). Extensive protein chemical and immunochemical studies have identified about 20 hyperphosphorylated amino acids in PHF-tau, almost all of which flank the microtubule-binding repeats [2–6]. Approximately half of these sites are serine/threonine-prolines [6]. Phosphorylation at many of these sites occurs in a significant fraction of fetal tau [5,7,8] and studies using biopsy-derived tau have shown that they are also phosphorylated in a small fraction of tau from adult human brain [9]. However, some sites, such as S422 [10], are only phosphorylated in PHF-tau.

Hyperphosphorylation of tau could result from an increased activity of tau kinases or the decreased activity of tau phosphatases. Mitogen-activated protein (MAP) kinase [11,12], neuronal cdc2-like kinase (NCLK) [13,14] and glycogen synthase kinase-3 (GSK3) [15–17] are candidate protein kinases for the hyperphosphorylation of tau and protein phos-

phatase 2A [12,18,19] is a candidate phosphatase. Of the above protein kinases, only MAP kinase phosphorylates recombinant tau at S422 [10]. Additional members of the MAP kinase family have been identified which are activated by cellular stresses (chemical, heat and osmotic shock, UV irradiation, inhibitors of protein synthesis), by bacterial lipopolysaccharide and by the cytokines interleukin-1 and tumour necrosis factor, and have, therefore, been called stress-activated protein kinases or SAPKs (reviewed in [20]). In view of their relatedness to MAP kinase, we have investigated the phosphorylation of tau protein by SAPKs.

In this paper we show that activated recombinant SAPK1 γ (also called JNK1), SAPK2a (also called p38, p40, RK, CSBPs and Mxi2), SAPK2b (also called p38 β), SAPK3 (also called ERK6 and p38 γ) and SAPK4 phosphorylate recombinant tau protein at multiple Ser/Thr-Pro sites that are hyperphosphorylated in PHF-tau, with SAPK4 and SAPK3 being the most effective. Phosphorylation of tau by SAPK3 and SAPK4 resulted in a marked reduction in the ability of tau to promote microtubule assembly.

2. Materials and methods

2.1. Protein kinases

The open reading frame of human SAPK1 γ (also called JNK1 [21]) was amplified by PCR, verified by DNA sequencing and subcloned into the (His)₆-tagged vector pRSET Xpress (Invitrogen). 6-His-SAPK1 γ was expressed in *E. coli* and purified by affinity chromatography using ProBond (Invitrogen). Murine GST-MKK4 (a gift from Dr. J.R. Woodgett, Ontario Cancer Institute, Toronto, Ont., Canada) was expressed in *E. coli* and purified by affinity chromatography on glutathione-agarose. The GST-MKK4 (1 μ M) was activated by incubation for 60 min at 30°C with 0.1 mg/ml active MalE-MEK kinase (a MalE fusion with the kinase domain of MEK kinase, a gift from Dr. A.R. Nebreda, EMBL, Heidelberg, Germany) in 50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.5 mM sodium orthovanadate, 0.03% (w/v) Brij 35, 0.1% (v/v) 2-mercaptoethanol, 5% (v/v) glycerol, 10 mM magnesium acetate and 0.1 mM ATP. Activated GST-MKK4 was used to activate 6-His-SAPK1 γ which was assayed routinely by the phosphorylation of GST-ATF2(19-96) (a gift from Dr. N. Jones, ICRF, London, UK). One unit of activity was that amount of enzyme which incorporated 1 nmol phosphate into GST-ATF2 in 1 min. MalE-Mpk2, the *Xenopus* homologue of SAPK2a (a gift from Dr. A.R. Nebreda [22]), human GST-SAPK2b [23,24], rat GST-SAPK3 [25] and human GST-SAPK4 [24] were expressed in *E. coli* and purified as described previously [24]. SKK3 (also called MKK6) was purified from rabbit skeletal muscle [26] and used to activate MalE-Mpk2, GST-SAPK2b, GST-SAPK3 and GST-SAPK4, as described previously [24]. SAPK2a, SAPK2b, SAPK3 and SAPK4 were assayed routinely by the phosphorylation of myelin basic protein (MBP). One unit of activity was that amount of enzyme which incorporated 1 nmol phosphate into MBP in 1 min.

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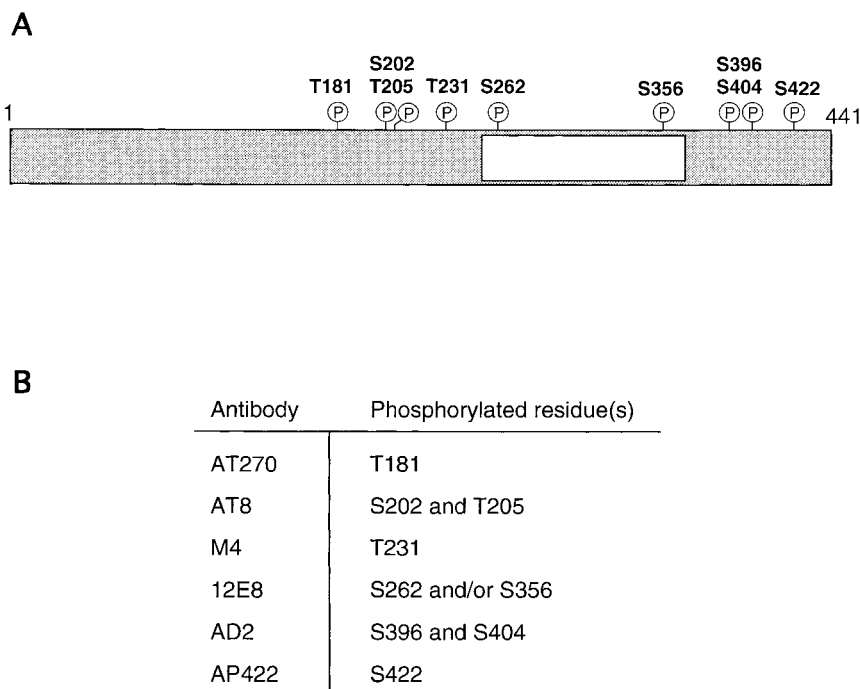


Fig. 1. Epitopes of phosphorylation-dependent anti-tau antibodies. A: Schematic drawing of the 441 amino acid isoform of human brain tau with phosphorylated amino acids recognised by phosphorylation-dependent anti-tau antibodies. The microtubule-binding repeat region of tau is shown in white. B: Phosphorylated residues in tau recognised by each phosphorylation-dependent monoclonal anti-tau antibody. With the exception of 12E8 all these antibodies recognise S/T-P sites in tau.

2.2. Phosphorylation assays

The 441 amino acid isoform of human brain tau was expressed in *E. coli* from cDNA clone htau40 [27] and purified as described [28]. Phosphorylation assays (0.025 ml) were carried out at 30°C and comprised 25 mM Tris-HCl, pH 7.4, 0.1 mM EGTA, 0.1 mM sodium orthovanadate, 2.5 μ M PKI (a specific inhibitor of cyclic AMP-dependent protein kinase), protease inhibitors (0.5 mM phenylmethylsulphonyl fluoride, 5 μ g/ml aprotinin, 5 μ g/ml leupeptin and 0.5 μ g/ml pepstatin), tau protein (2 μ M) or myelin basic protein (2 μ M), 10 mM magnesium acetate, 2 mM [γ - 32 P]ATP (approximately 10^6 cpm/nmol) and activated SAPK1 γ , SAPK2a, SAPK2b, SAPK3 or SAPK4 (0.2 U/ml or 1 U/ml). Reactions were initiated with ATP and aliquots were removed after various times ranging from 2 min to 18 h and used for SDS-PAGE and immunoblotting. Immunoblots were performed as described [12]. Alternatively, incorporation of 32 P-radioactivity was measured after adsorption to Whatman P81 paper, as described [12]. In some experiments 100 μ g/ml heparin or dextran sulphate were included in the assays.

2.3. Microtubule assembly

Non-phosphorylated htau40 (2 μ M) and htau40 phosphorylated with 1 U/ml SAP kinase-3 or SAP kinase-4 was incubated with tubulin (10 μ M, Cytoskeleton Inc.) in assembly buffer (80 mM PIPES, 1 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol, 1 mM GTP, pH 6.8) at 37°C. Polymerisation of microtubules was monitored by measuring the absorbance at 350 nm.

3. Results

Tau protein incubated with 1 U/ml of each SAP kinase for 18 h incorporated 2 mol phosphate/mol tau (SAPK1 γ), 4 mol phosphate/mol tau (SAPK2a), 8 mol phosphate/mol tau (SAPK2b), 11 mol phosphate/mol tau (SAPK3) and 14 mol phosphate/mol tau (SAPK4). Similar differences between the five SAP kinases (each used at 0.2 U/ml) were observed when initial rates of tau phosphorylation were measured relative to the standard substrates MBP and ATF2 (Table 1).

High stoichiometric ratios required long incubation periods and resulted in the generation of the epitopes of phosphorylation-dependent anti-tau antibodies (see Fig. 1 for antibody epitopes). Tau phosphorylated by individual SAP kinases showed a variable reduction in gel mobility, as seen by SDS-PAGE and immunoblotting with anti-tau serum 134 which recognises the carboxy-terminus of tau in a non-phosphorylation-dependent manner [29] (Fig. 2). Thus, whereas tau phosphorylated by SAPK1 γ showed almost no shift, tau phosphorylated by SAPK4 ran at 75 kDa apparent molecular mass compared to 67 kDa for non-phosphorylated htau40 (Fig. 2). Phosphorylation by SAPK2a and SAPK2b resulted in intermediate reductions in the gel mobility of tau (Fig. 2). Tau phosphorylated by each of the five SAP kinases was strongly immunoreactive with AT270, a phosphorylation-dependent monoclonal antibody which recognises tau phosphorylated at T181 [30] (Fig. 2). Phosphorylated tau was also immunoreactive with AP422, a monoclonal anti-tau anti-

Table 1
Rates of tau phosphorylation by SAP kinases relative to myelin basic protein and ATF2 (%)

	Myelin basic protein	ATF2	Tau
SAPK1 γ	n.d.	100	11 \pm 3
SAPK2a	100	40 \pm 2	14 \pm 4
SAPK2b	100	62 \pm 2	39 \pm 8
SAPK3	100	107 \pm 20	67 \pm 7
SAPK4	100	130 \pm 2	91 \pm 12

SAP kinases were activated in vitro and phosphorylation of myelin basic protein, ATF2 and tau (each at 2 μ M) studied at a SAPK concentration of 0.2 U/ml ($n=5$). The data for phosphorylation of ATF2 by SAPK2a, SAPK2b, SAPK3 and SAPK4 are taken from [24]. n.d., not determined.

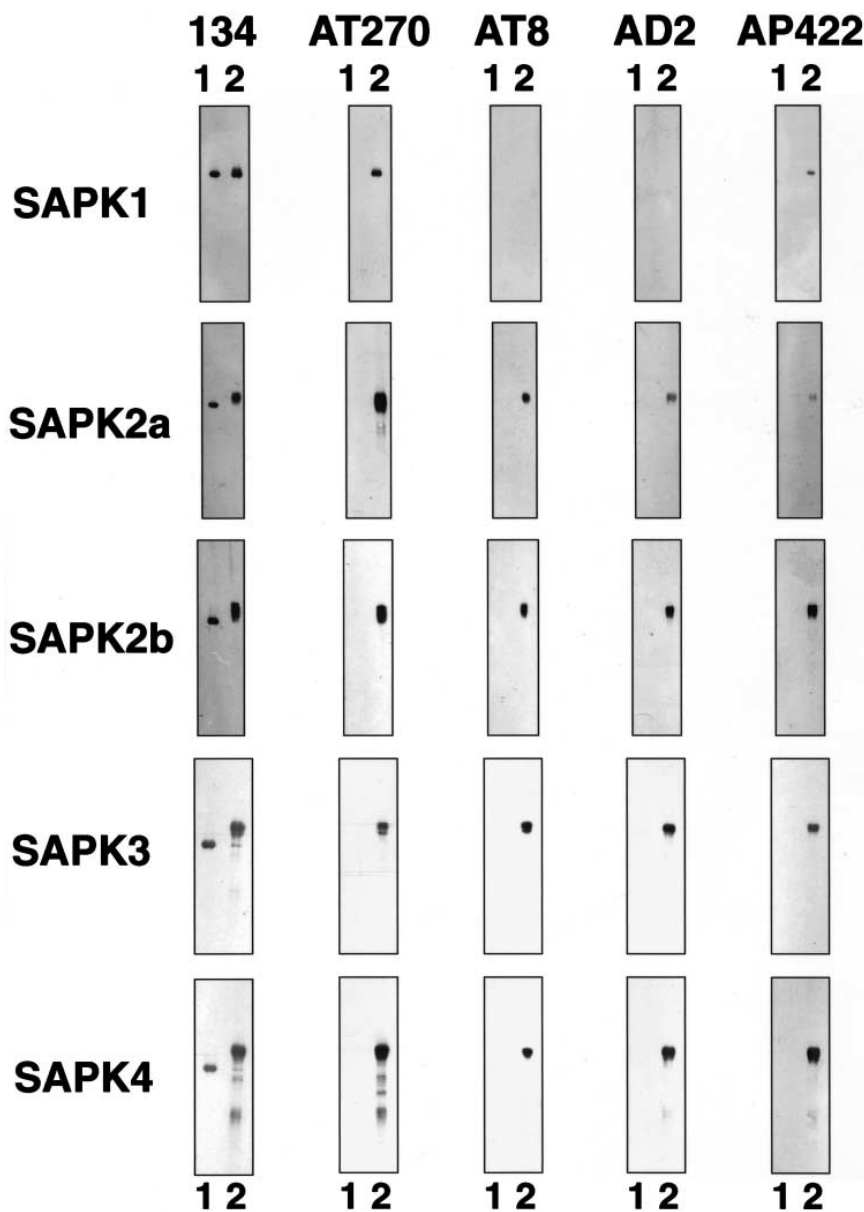


Fig. 2. Phosphorylation of recombinant tau protein (expressed from clone htau40) with 1 U/ml SAPK1 γ , SAPK2a, SAPK2b, SAPK3 and SAPK4. Immunoblots were incubated with anti-tau serum 134 and the phosphorylation-dependent monoclonal anti-tau antibodies AT270, AT8, AD2 and AP422. Lanes: 1, htau40; 2, htau40+SAPK.

body which recognises tau phosphorylated at S422 [10] (Fig. 2). However, whereas tau phosphorylated by SAPK4, SAPK3 and SAPK2b reacted strongly with AP422, tau phosphorylated with SAPK2a and SAPK1 γ reacted only weakly. Tau phosphorylated by SAPK4 and SAPK3 also reacted strongly with AT8, a monoclonal anti-tau antibody which recognises tau phosphorylated at S202 and T205 [31] (Fig. 2). Tau phosphorylated by SAPK2b and SAPK2a also reacted with AT8, whereas AT8 failed to recognise tau phosphorylated by SAPK1 γ . Similar results were obtained with AD2, a monoclonal anti-tau antibody that recognises tau phosphorylated at S396 and S404 [32] (Fig. 2). Tau phosphorylated by SAPK3 and SAPK4 reacted strongly with AD2, whereas tau phosphorylated by SAPK1 γ failed to react. We also used monoclonal antibody M4 which is specific for tau phosphorylated at T231 [10,33]. However, we failed to observe M4 immuno-

reactivity upon phosphorylation of tau by SAP kinases. Similarly, tau phosphorylated by SAP kinases failed to react with 12E8, a monoclonal antibody which recognises tau phosphorylated at S262 and/or S356 [34] (data not shown).

Sulphated glycosaminoglycans are known to stimulate tau phosphorylation by a number of protein kinases [10,35,36] and to induce the formation of tau filaments very similar to the PHFs from Alzheimer's disease brain [28]. We therefore examined the effects of heparin and dextran sulphate on tau phosphorylation by SAP kinases. Addition of 100 μ g/ml heparin (data not shown) or dextran sulphate to the phosphorylation reaction increased tau phosphorylation by SAPK1 γ , SAPK3 and SAPK4 (Fig. 3). Tau phosphorylated by SAPK1 γ additionally became immunoreactive with antibodies AT8 and AD2 (data not shown). The effect of heparin and dextran sulphate was particularly pronounced on tau phosphorylated

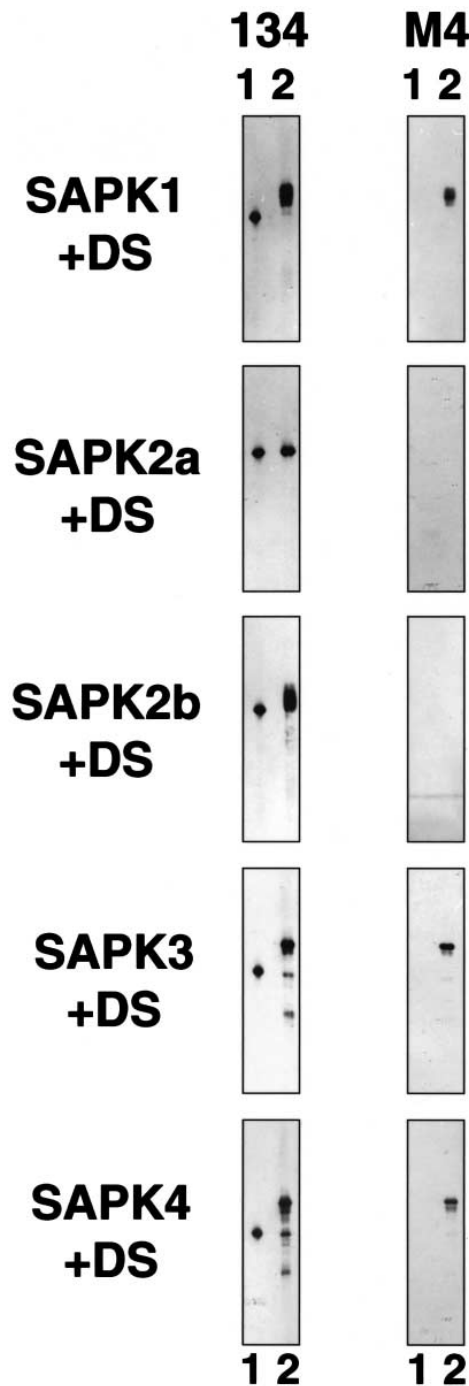


Fig. 3. Phosphorylation of recombinant tau protein (expressed from clone htau40) with 1 U/ml SAPK1 γ , SAPK2a, SAPK2b, SAPK3 and SAPK4, in presence of 100 μ g/ml dextran sulphate (DS). Immunoblots were incubated with anti-tau serum 134 and the phosphorylation-dependent monoclonal anti-tau antibody M4. Lanes: 1, htau40+DS; 2, htau40+DS+SAPK.

by SAPK4, with an eight-fold stimulation of the initial rate of tau phosphorylation and the incorporation of 16 mol phosphate/mol tau after 18 h. Sulphated glycosaminoglycans had no significant effect on phosphorylation of MBP by SAPK4. In contrast, phosphorylation of tau by SAPK2a and SAPK2b was reduced in the presence of heparin (data not shown) and dextran sulphate (Fig. 3). Phosphorylation of tau by SAPK1 γ , SAPK3 and SAPK4 in presence of dextran sulphate resulted

in the appearance of the epitope of antibody M4, indicative of phosphorylation at T231 (Fig. 3).

We tested whether phosphorylation of htau40 by SAPK3 and SAPK4 to high stoichiometries has an effect on the ability of tau to promote microtubule assembly. Tau phosphorylated by SAPK3 and SAPK4 promoted microtubule assembly approximately five-fold less efficiently than non-phosphorylated htau40, as judged by initial rates (Fig. 4). Phosphorylation of tau by SAPK4 was slightly more inhibitory than phosphorylation by SAPK3 (Fig. 4), in accordance with tau being a somewhat better substrate for SAPK4.

4. Discussion

SAP kinases are MAP kinase family members which are activated by a wide variety of cellular stresses, by bacterial lipopolysaccharide and by the pro-inflammatory cytokines interleukin-1 and tumour necrosis factor [20]. The activation of SAP kinases requires their phosphorylation on a threonine and a tyrosine residue, both phosphorylations being catalysed by SAP kinase kinases (SKKs) that are themselves dependent on serine/threonine phosphorylation for activity. SAPK1 is activated by SKK1 (also called MKK4), SAPK2a is activated by SKK2 (also called MKK3) and SKK3 (also called MKK6), whereas SAPK2b, SAPK3 and SAPK4 are activated by SKK3 [20]. Where examined, SAP kinase mRNAs are expressed in brain [21,23,24,37,38]. However, their normal role and the factors that stimulate their activity in this tissue are not well understood.

In this paper we show that the SAP kinases SAPK1 γ , SAPK2a, SAPK2b, SAPK3 and SAPK4 phosphorylate tau protein to various extents, as judged by relative initial rates of phosphorylation, incorporation of phosphate after extended periods of incubation, reduced gel mobility of phosphorylated tau and the production of epitopes of phosphorylation-dependent anti-tau antibodies. By these criteria, tau is a good substrate for SAPK4 and SAPK3, a reasonable substrate for SAPK2b and a relatively poor substrate for SAPK2a and SAPK1 γ . SAPK1 exists as multiple isoforms that are produced from three different genes by alternative mRNA splicing [39]. A very recent study has shown that tau is also a relatively poor substrate for non-activated recombinant rat SAPK1 β [40]. It remains to be seen whether tau is a better substrate for other isoforms of activated SAPK1. Initial rates of phosphorylation showed that tau is as good a substrate for SAPK4 as MBP which is used as the standard substrate for this protein kinase. This suggests that SAPK4 may play a physiological role in tau phosphorylation. Following an 18 h incubation with SAPK4, tau incorporated 14 mol phosphate/mol tau and showed a marked reduction in gel mobility. This resulted in the production of the epitopes of the phosphorylation-dependent anti-tau antibodies AT270, AT8, AD2 and AP422, indicating phosphorylation of T181, S202, T205, S396, S404 and S422 in tau. These antibodies recognise specific S/T-P sites in tau, in accordance with the known specificities of SAP kinases. Phosphorylation of tau by SAPK1 γ only produced the epitopes of AT270 and AP422, whereas phosphorylation by SAPK2a, SAPK2b and SAPK3 resulted in the appearance of the epitopes of all the above antibodies, albeit to variable intensities. Interestingly, all five SAP kinases phosphorylated S422 in tau, a site which is phosphorylated in PHF-tau, but not in biopsy-derived adult human brain tau

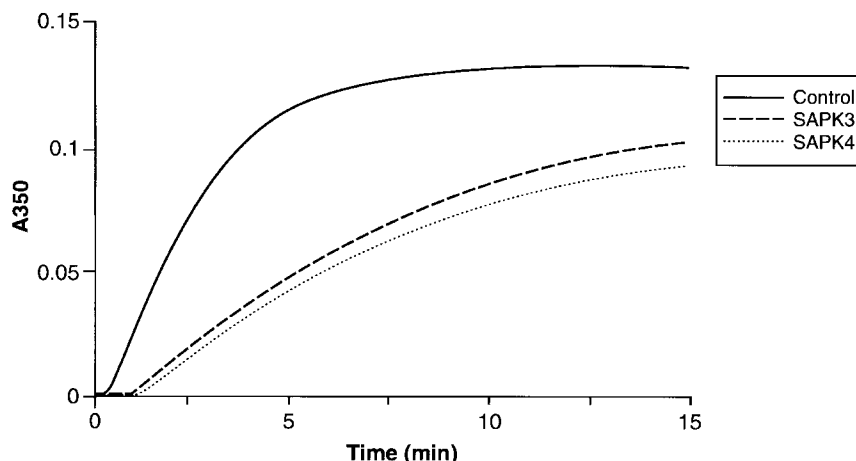


Fig. 4. Effects of phosphorylation of recombinant tau protein (expressed from clone htau40) with 1 U/ml SAPK3 and SAPK4 on promotion of microtubule assembly, as compared to non-phosphorylated htau40 (Control).

[10]. We have shown previously that MAP kinase also phosphorylates this site, whereas GSK3 β and NCLK fail to do so [10]. Of the available phosphorylation-dependent anti-tau antibodies of known epitope with a specificity for S/T-P sites, only antibody M4 failed to react with tau phosphorylated by SAP kinases. Similar results were obtained previously with tau phosphorylated by MAP kinase [30].

It is well established that phosphorylation of tau by a number of protein kinases is strongly stimulated in the presence of heparin [10,35,36] and we have shown recently that sulphated glycosaminoglycans also induce bulk assembly of tau into filaments with a morphology similar to the PHFs from Alzheimer's disease brain [28]. We therefore examined the effects of heparin and dextran sulphate on tau phosphorylation by SAP kinases. Phosphorylation by SAPK1 γ , SAPK3 and SAPK4 was strongly stimulated, resulting in an eight-fold stimulation of the initial rate of tau phosphorylation by SAPK4. In contrast, phosphorylation of tau by SAPK2a and SAPK2b was markedly inhibited by heparin and dextran sulphate. Addition of sulphated glycosaminoglycans resulted in the appearance of the epitope of antibody M4 when tau was phosphorylated with SAPK1 γ , SAPK3 and SAPK4, indicating phosphorylation of T231.

These findings extend previous results indicating different in vitro substrate specificities of individual SAP kinases. SAPK1 is the only member of this family to phosphorylate the activation domain of c-Jun [21]. The known substrate specificities of SAPK4 resemble those of SAPK3 in that, while both enzymes phosphorylate a number of proteins (including the activation domains of several transcription factors) at similar rates to SAPK2a and SAPK2b, they are far less effective in activating MAP kinase-activated protein kinase-2 (MAPKAP-K2) and MAPKAP-K3 than either SAPK2a or SAPK2b [24]. Moreover, the pyridinyl-imidazole compounds SB 203580 and SB 202190 [41] inhibit SAPK2a and SAPK2b, but not SAPK3 and SAPK4 [24]. Here we show that tau protein is a better substrate for SAPK4 and SAPK3 than for SAPK2b, SAPK2a and SAPK1 γ .

Depending on the isoform, the microtubule-binding region of tau consists of three or four repeats of 31 or 32 amino acids each [29]. The sequences flanking the repeats are known to enhance the affinity of the repeat region for microtubules. In particular, the proline-rich region upstream of the repeats,

which contains many of the S/T-P sites phosphorylated by SAP kinases, is essential for tubulin binding in vitro and in vivo [42–44]. We therefore examined the effects of phosphorylation by SAPK3 and SAPK4 on the ability of tau to promote microtubule assembly. Phosphorylation of tau by SAPK3 and SAPK4 markedly reduced the ability of tau to promote microtubule assembly, suggesting that SAP kinases could play a role in the regulation of microtubule dynamics in normal brain and in the inability of PHF-tau to bind to microtubules.

Taken together, the present in vitro findings which indicate that tau is phosphorylated by SAP kinases at many S/T-P sites, suggest a possible role for SAP kinases in the hyperphosphorylation of tau that characterises Alzheimer's disease and other neurodegenerative disorders.

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