

Phosphorylation of microtubule-associated protein tau by stress-activated protein kinases in intact cells

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Received 12 February 2002; accepted 14 February 2002

First published online 26 February 2002

Edited by Jesus Avila

Abstract Tau is a microtubule-associated protein that is abnormally hyperphosphorylated in the filamentous lesions that define a number of neurodegenerative diseases collectively referred to as tauopathies. We previously showed that stress-activated protein (SAP) kinases phosphorylate tau protein at many of the hyperphosphorylated sites in vitro. Here we have developed a system to study the effects of five SAP kinases (SAPK1c/JNK1, SAPK2a/p38 α , SAPK2b/p38 β , SAPK3/p38 γ and SAPK4/p38 δ) on tau phosphorylation in intact cells. All kinases phosphorylated tau, albeit at different efficiencies. Tau was a good substrate for SAPK3/p38 γ and SAPK4/p38 δ , a reasonable substrate for SAPK2b/p38 β and a relatively poor substrate for SAPK2a/p38 α and SAPK1c/JNK1. These findings indicate that the aberrant activation of SAP kinases, especially SAPK3/p38 γ and SAPK4/p38 δ , could play an important role in the abnormal hyperphosphorylation of tau that is an invariant feature of the tauopathies. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Abnormal hyperphosphorylation; Stress-activated protein kinase; Tau protein; Tauopathy

1. Introduction

Filamentous deposits made of the microtubule-associated protein tau constitute a defining neuropathological feature of Alzheimer's disease and other neurodegenerative diseases, such as progressive supranuclear palsy, corticobasal degeneration, Pick's disease and the inherited frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17) [1]. The discovery of mutations in the tau gene in FTDP-17 has established that dysfunction of tau protein is sufficient to cause neurodegeneration and dementia [2–4]. Human brain tau exists as six isoforms that are alternatively spliced from a single gene [5]. They differ by having three or four conserved repeats towards the carboxy-terminus and no, one or two insertions in the amino-terminal half. The repeats constitute the microtubule-binding domains of tau [6].

Abnormal hyperphosphorylation of tau protein is an invariant feature in diseases with filamentous tau deposits [1]. It is an early event that appears to precede assembly into filaments

[7,8]. Hyperphosphorylation renders tau unable to interact with microtubules which may facilitate its self-assembly [9,10]. Extensive protein chemical and immunochemical studies have identified over 20 hyperphosphorylated amino acids in filamentous tau from Alzheimer's disease brain, almost all of which flank the repeat region [11,12]. Approximately half of these sites are serine/threonine-prolines. Phosphorylation at many of these sites occurs in a significant fraction of tau from developing brain and a small fraction of tau from adult human brain [13–15]. However, some residues are only phosphorylated in filamentous tau [16,17].

Hyperphosphorylation of tau could result from an increased activity of tau kinases or the decreased activity of tau phosphatases. Based largely on in vitro experiments, mitogen-activated protein (MAP) kinase [18–20], stress-activated protein (SAP) kinases [21,22], neuronal cdc2-like kinase (NCLK), also known as cyclin-dependent kinase 5 [23,24], glycogen synthase kinase-3 (GSK3) [25,26] and dual-specificity tyrosine-phosphorylated and regulated kinase [27] are candidate protein kinases for the hyperphosphorylation of tau at serine/threonine-prolines and protein phosphatase 2A is a candidate phosphatase [20]. Since a number of protein kinases are capable of phosphorylating tau at many relevant sites in vitro, it is important to identify which of these kinases phosphorylate tau in vivo. Previous studies have demonstrated the phosphorylation of tau by GSK3 [28,29] and NCLK [30–32] in intact cells. By contrast, activated MAP kinase produced only a small increase in tau phosphorylation in intact cells [28,33]. Here, we have examined the ability of the SAP kinases SAPK1c/JNK1, SAPK2a/p38 α , SAPK2b/p38 β , SAPK3/p38 γ and SAPK4/p38 δ to phosphorylate human tau protein in transfected COS cells.

2. Materials and methods

2.1. Expression constructs

The previously described [34,35] cDNA clones encoding SAPK1c/JNK1, SAPK2a/p38 α , SAPK2b/p38 β , SAPK3/p38 γ and SAPK4/p38 δ , as well as clones encoding the upstream activators MAP kinase kinase-4 (MKK4) and MKK6 were subcloned into the eukaryotic expression vector pSG5 (Stratagene). A *c-myc* tag was added to the carboxy-termini of SAPK2b/p38 β , SAPK3 and SAPK4. The cDNA encoding htau40 (the 441 amino acid isoform of human brain tau) [5] was also subcloned into pSG5.

2.2. Cell culture and transfection

COS-7 cells were grown in 25 cm² flasks in Dulbecco's modified Eagle's medium containing glutamax (Life Technologies) supplemented with 10% foetal calf serum in a 95% O₂/5% CO₂ incubator at 37°C and transiently transfected with 10 µg/ml plasmid DNA using DEAE-dextran chloroquine. After 48 h, cells transfected with the tau

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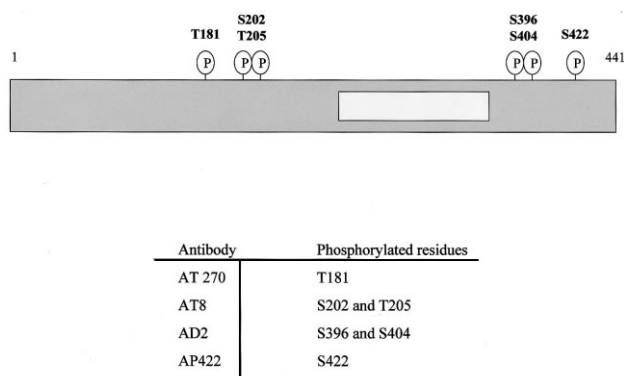


Fig. 1. Epitopes of phosphorylation-dependent anti-tau antibodies. A: Schematic diagram of the 441 amino acid isoform of human brain tau with phosphorylated amino acids recognised by phosphorylation-dependent anti-tau antibodies. The microtubule-binding repeat region of tau is shown in white. B: Phosphorylated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

construct alone, or with the tau, SAPK and SAPKK constructs were treated with 0.5 M sorbitol for 30 min in serum-free medium. Cells were harvested in phosphate-buffered saline, pH 7.4, at 4°C and centrifuged. The pellets were resuspended in 50 mM Tris–NaCl, boiled for 2 min and re-centrifuged. Protein concentrations were determined in the supernatants (Bio-Rad protein assay).

2.3. Immunoblots

Electrophoresis and immunoblotting were performed as described [36]. Briefly, an equal amount of protein from each sample was loaded onto 10% SDS–PAGE. Following transfer and blocking, membranes were incubated with the primary antibody for 90 min at room temperature. Horseradish peroxidase-conjugated secondary antibody (Sigma Fine Chemicals) was then used and the reaction product visualised using enhanced chemiluminescence (ECL, Amersham-Pharmacia). Expression of SAP kinases was determined by Western blotting using anti-myc antibody 9E10 (for SAPK2b/p38 β , SAPK3/p38 γ and SAPK4/p38 δ), as well as anti-SAPK1/JNK and anti-SAPK2a/p38 α antibodies (Santa Cruz Biotechnology). The phosphorylation state of tau was monitored using the phosphorylation-dependent anti-tau antibodies AT270, AT8, AD2 and AP422 (Fig. 1) [12,16,36,37]. Tau levels were determined using antiserum 134 [5], which recognises the carboxy-terminus of tau in a phosphorylation-independent manner. Band intensities were quantified using densitometry (Molecular Dynamics).

3. Results

COS cells were transfected with the tau construct alone or with the tau/SAP kinase/SAP kinase kinase constructs. After 2 days, the cells were exposed to osmotic stress using sorbitol, followed by analysis of the phosphorylation state of tau using anti-tau antibodies. Preliminary experiments had established that similar levels of each SAP kinase were expressed in COS cells 2 days after transfection (not shown). In cells transfected with the tau construct alone, a slightly reduced gel mobility of tau protein was observed following sorbitol treatment. However, this was not accompanied by a significant change in phosphorylation at the epitopes recognised by anti-tau antibodies AT8, AT270, AD2 and AP422 (Fig. 2). Following transfection of the tau/SAP kinase/SAP kinase kinase constructs and osmotic stress, tau protein showed a greatly reduced gel mobility and an increased phosphorylation at the sites recognised by AT8, AT270, AD2 and AP422 (Fig. 2). The labelling given by each phosphorylation-dependent anti-

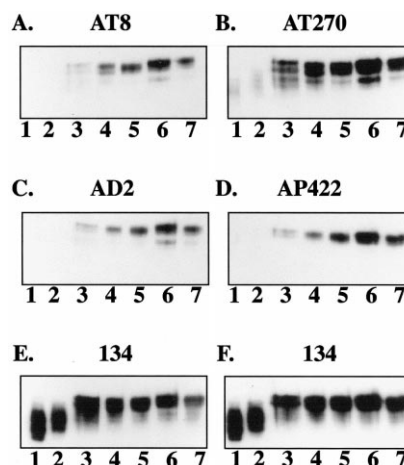


Fig. 2. Phosphorylation of tau protein by SAP kinases in transfected COS-7 cells. Lanes: 1, transfected tau; 2, transfected tau+osmotic stress; 3, transfected tau, SAPK1c/JNK1 and MKK4+osmotic stress; 4, transfected tau, SAPK2a/p38 α and MKK6+osmotic stress; 5, transfected tau, SAPK2b/p38 β and MKK6+osmotic stress; 6, transfected tau, SAPK3/p38 γ and MKK6+osmotic stress; 7, transfected tau, SAPK4/p38 δ and MKK6+osmotic stress. Tau phosphorylation was assessed using phosphorylation-dependent monoclonal anti-tau antibodies AT8 (A), AT270 (B), AD2 (C) and AP422 (D). Tau levels were determined using the phosphorylation-independent polyclonal anti-tau antibody BR134, which was used following each monoclonal antibody incubation (E, F after AD2 and AP422, respectively). A typical experiment is shown. Similar results were obtained in five separate transfection experiments.

tau antibody was quantified and normalised to that given by the phosphorylation-independent anti-tau serum 134, to correct for variations in tau levels. As shown in Fig. 3, of the five SAP kinases tested, SAPK3/p38 γ had the largest effect on the phosphorylation of tau, followed by SAPK4/p38 δ , SAPK2b/p38 β and SAPK2a/p38 α . The effect of SAPK1c/JNK1 was the smallest.

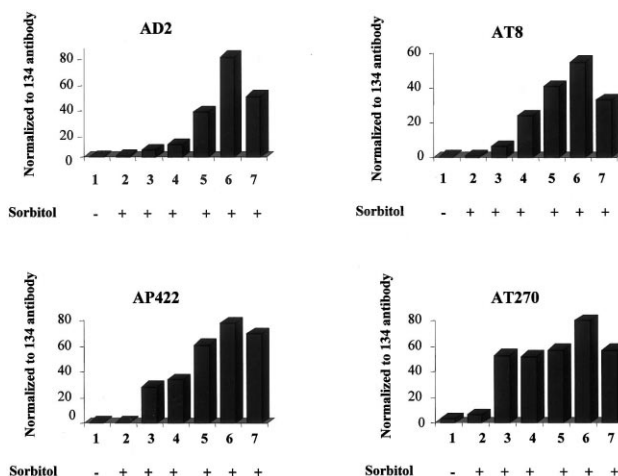


Fig. 3. Quantitative analysis of tau phosphorylation by SAP kinases in transfected COS-7 cells. For each phosphorylation-dependent anti-tau antibody, immunoreactivity was expressed as a percentage of that given by the phosphorylation-independent antibody BR134 (taken as 100%). Lanes: 1, 2, transfected tau; 3, transfected tau, SAPK1c/JNK1 and MKK4; 4, transfected tau, SAPK2a/p38 α and MKK6; 5, transfected tau, SAPK2b/p38 β and MKK6; 6, transfected tau, SAPK3/p38 γ and MKK6; 7, transfected tau, SAPK4/p38 δ and MKK6. Sorbitol-induced osmotic stress is indicated (+).

4. Discussion

SAP kinases are MAP kinase family members that are activated by a variety of cellular stresses, by bacterial lipopolysaccharide and by the pro-inflammatory cytokines interleukin-1 and tumour necrosis factor [38]. The activation of SAP kinases requires their phosphorylation on a threonine and a tyrosine residue. Both phosphorylations are catalysed by SAP kinase kinases that are themselves dependent on phosphorylation for activity. SAPK1c/JNK1 is activated by MKK4 and MKK7, SAPK2a/p38 α is activated by MKK3 and MKK6, whereas SAPK2b/p38 β , SAPK3/p38 γ and SAPK4/p38 δ are activated by MKK6. SAP kinase mRNAs are widely expressed, including in the brain.

In this study, we show that following transfection into COS cells and osmotic stress SAPK1c/JNK1, SAPK2a/p38 α , SAPK2b/p38 β , SAPK3/p38 γ and SAPK4/p38 δ phosphorylate tau protein to various extents, as judged by a reduced gel mobility of phosphorylated tau and the generation of the epitopes of phosphorylation-dependent anti-tau antibodies AT270, AT8, AD2 and AP422. All five SAP kinases generated the AT270 epitope, indicative of phosphorylation of T181 in tau. Quantitative analysis showed similar labelling with AT270 for all the SAP kinases. This was not the case of antibodies AT8, AD2 and AP422. For AT8, which recognises phosphorylated S202 and T205 in tau, SAPK3/p38 γ gave the strongest labelling, followed by SAPK2b/p38 β , SAPK4/p38 δ , SAPK2a/p38 α and SAPK1c/JNK1. The AD2 epitope, which corresponds to phosphorylated S396 and S404 in tau, was generated most effectively by SAPK3/p38 γ and SAPK4/p38 δ , followed by SAPK2b/p38 β , SAPK2a/p38 α and SAPK1c/JNK1. Finally, phosphorylation of S422 in tau, as recognised by antibody AP422, was generated most efficiently by SAPK3/p38 γ , SAPK4/p38 δ and SAPK2b/p38 β , and less so by SAPK2a/p38 α and SAPK1c/JNK1. S422 is an abnormal phosphorylation site, rather than a hyperphosphorylated site, since it is phosphorylated in filamentous tau, but not in tau from control human brain [16]. SAP kinases may therefore play a role in the abnormal phosphorylation of tau that is characteristic of the tauopathies.

From the above, it follows that in transfected COS cells tau is a good substrate for SAPK3/p38 γ and SAPK4/p38 δ , a reasonable substrate for SAPK2b/p38 β and a relatively poor substrate for SAPK2a/p38 α and SAPK1c/JNK1. These effects mirror our previous findings [22] on the *in vitro* phosphorylation of tau protein by SAP kinases. One study has described the effects of osmotic stress on tau phosphorylation in SH-SY5Y cells that had been transiently transfected with SAPK3/p38 γ or SAPK4/p38 δ [39]. Expression of SAPK3/p38 γ resulted in increased phosphorylation of tau at S396 and S404, in agreement with the present findings. However, in contrast to the present findings, SAPK4/p38 δ had no significant effect on the phosphorylation of tau. It appears likely that the co-transfection of SAP kinase and SAP kinase kinase provides a more sensitive measure for the study of tau phosphorylation than the single transfection of SAP kinase. Phosphorylation of tau negatively regulates its ability to interact with microtubules [40]. It therefore appears likely that phosphorylation by SAP kinases results in an increase in soluble, unbound tau and a reduced stability of the microtubule network. It remains to be determined whether SAP kinases, in particular SAPK3 and SAPK4, play a role in the

normal phosphorylation of tau in developing and adult brain.

SAP kinases may play a role in the abnormal hyperphosphorylation of tau in neurodegenerative diseases, since aberrantly activated SAPK1/JNK and SAPK2/p38 have been found to be associated with cells that contain filamentous tau in Alzheimer's disease, progressive supranuclear palsy, corticobasal degeneration, Pick's disease, FTDP-17 and Gerstmann-Sträussler-Scheinker disease with tangles [41–44]. Moreover, activated MKK6 has been described in cells with neurofibrillary lesions in Alzheimer's disease, progressive supranuclear palsy and Pick's disease [45]. It will be important to determine whether these are early changes in the pathway that leads from normal soluble to abnormal filamentous tau. If so, specific inhibitors of SAP kinases could well be of therapeutic benefit in the treatment of the tauopathies.

Acknowledgements: We are grateful to Professor Sir Philip Cohen for providing the MAP kinase kinase clones and for his helpful comments on the manuscript.

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