FEBS Letters 365 (1995) 42–46 FEBS 15506

Stimulation of MAP kinase by v-*raf* transformation of fibroblasts fails to induce hyperphosphorylation of transfected tau

Donna A. Latimer[a], Jean-Marc Gallo[b], Simon Lovestone[a], Christopher C.J. Miller[a], C. Hugh Reynolds[a], Betina Marquardt[c], Silvia Stabel[c], James R. Woodgett[d], Brian H. Anderton[a,*]

[a]*Department of Neuroscience, Institute of Psychiatry, De Crespigny Park, London SE5 8AF, UK*
[b]*Department of Neurology, Institute of Psychiatry, De Crespigny Park, London SE5 8AF, UK*
[c]*Max-Delbruck-Laboratorium in der Max-Planck-Gesselschaft, D-5000 Cologne 30, Germany*
[d]*Ontario Cancer Research Institute/Princess Margaret Hospital, 500 Sherbourne Street, Toronto, Ont. M4X 1K9, Canada*

Received 9 March 1995; revised version received 11 April 1995

Abstract A proportion of the microtubule-associated protein, tau, is in an elevated state of phosphorylation in foetal and adult brain whereas all of the tau in paired helical filaments, which are characteristic of Alzheimer's disease is hyperphosphorylated; it is important therefore to elucidate the mechanisms that regulate tau phosphorylation. Here we describe results that show that although MAP kinase can hyperphosphorylate tau in vitro, activation of MAP kinase in transformed fibroblasts does not result in hyperphosphorylation of transfected tau, whereas glycogen synthase kinase-3β (GSK-3β) when co-transfected with tau does result in tau hyperphosphorylation. The findings imply that GSK-3β may be a stronger candidate than MAP kinase for inducing tau hyperphosphorylation in vivo.

Key words: Alzheimer's disease; Development; Tau; PHF; Phosphorylation; MAP kinase; glycogen synthase kinase-3

1. Introduction

Tau is a microtubule-associated protein that is expressed principally in neurones. Adult brain tau comprises six isoforms generated by alternative splicing of a single gene transcript. The isoforms differ by either having three or four imperfect homologous repeat domains of 31 amino acids in the C-terminal half of the molecule, in addition both three and four repeat forms can each have zero, one or two non-homologous 29 amino acid inserts towards the N-terminus [1]. Only the smallest isoform (i.e. three repeats and zero inserts) is expressed in foetal brain [2].

Tau is most likely the principal structural component of the paired helical filaments (PHF) that are one of the characteristic neuropathological hallmarks of Alzheimer's disease [1]. The phosphorylation state of tau in PHF has been shown to be one of the properties that distinguish PHF-tau from normal tau since compared to tau isolated from normal adult brain, PHF-tau is hyperphosphorylated. However, if tau is isolated rapidly from adult brain, about 25% of the tau is in a similar hyperphosphorylated state to PHF-tau and a significant proportion of foetal tau is also hyperphosphorylated [2–8]. Some of the phosphorylated sites in PHF-tau and in the hyperphosphorylated fraction of adult and foetal tau have been identified by protein sequencing and by mapping of epitopes for a panel of monoclonal antibodies that discriminate PHF-tau from the non-hyperphosphorylated fraction of adult brain tau. These monoclonal antibodies differentiate tau phosphorylation states by virtue of their dependency for binding to tau upon the presence or absence of phosphate on specific serine residues [9–14]. Most of the sites identified to date are serine residues immediately followed in the sequence of tau by a proline and hence, are likely to be phosphorylated by proline directed kinases. Indeed, several members of the proline-directed kinase family of protein kinases have been found to phosphorylate recombinant tau at these sites in vitro and generate the corresponding PHF-tau epitopes. The candidate proline-directed kinases for generating hyperphosphorylated tau are certain members of the MAP kinase family (eg. $p44^{MAPK}$ and $p42^{MAPK}$), cdk5, and glycogen synthase kinases-3α and -3β (GSK-3α, GSK-3β) [11,15–18].

The functions of tau include stabilisation of microtubules since treatment of primary cultures of neurones with antisense oligonucleotides to tau results in neurite retraction [19,20]. Phosphorylation of tau may be a regulatory mechanism for this function since increased levels of phosphorylation reduce or abolish (eg. PHF-tau) the ability of tau to bind to microtubules [9,21–25]. It is therefore of some interest to elucidate the regulatory mechanisms for tau phosphorylation since they may be important in neurite outgrowth in the developing nervous system and may be aberrant in Alzheimer's disease. The next step, however, is to identify which of the candidate kinases are capable of phosphorylating tau in living cells.

We have previously reported that of the several MAP kinases and GSK-3α and GSK-3β that can generate hyperphosphorylated tau in vitro, only GSK-3α and GSK-3β but not $p44^{MAPK}$ nor $p42^{MAPK}$ phosphorylate tau in COS cells transiently transfected with tau and kinase cDNAs [13]. In this earlier study, the activation of MAP kinase was only transient following treatment of transfected cells with TPA, whereas it might be that more prolonged MAP kinase activation would result in tau hyperphosphorylation. We now report that in mouse Swiss 3T3 cells transformed with the oncogene v-*raf*, which results in constitutive activation of MAP kinase [26–28], the activated MAP kinase still did not result in the generation of PHF-tau-like hyperphosphorylation although when the MAP kinase activity was further elevated by co-transfection with a $p44^{MAPK}$ cDNA and treatment of the cells with phorbol ester, the phosphorylation state of transfected tau was different from tau in non-transformed 3T3 cells. However, co-transfection of the 3T3 cells with tau and GSK-3β did induce hyperphosphorylation of tau.

*Corresponding author. Fax: (44) (171) 708-0017.

SSDI 0014-5793(95)00434-3

2. Materials and methods

2.1. Cell culture and transfection

Swiss 3T3 cells and v-*raf*-transformed Swiss 3T3 cells were routinely cultured in DMEM containing 10% (v/v) foetal bovine serum supplemented with 2 mM glutamine and 100 U/ml penicillin and 100 μg/ml streptomycin. cDNAs encoding rat $p44^{MAPK}$, human GSK-3β and human tau with one N-terminal insert and four repeats (1N4R) were ligated into *Eco*RI/*Hin*dIII sites of the expression vector pGW1-CMV [29]. For transfection, cells were harvested by trypsinisation and resuspended in Optimem medium (Life Technologies, Strathclyde, UK). 15 μg of each plasmid DNA was introduced by electroporation using a BioRad electroporator operating at 0.22 kV, 960 μF in a 0.4 cm path length electroporation chamber containing 10^7 cells in 0.4 ml. Following electroporation, cells were immediately resuspended in DMEM containing 10% (v/v) foetal bovine serum and plated onto 160 mm diameter petri dishes. The cells were allowed to recover for 4 h and then the medium was routinely replaced with serum-free DMEM and the cells incubated for a further 20 h. For TPA treatment, TPA (phorbol 12-myristate 13-acetate; Sigma UK) dissolved in ethanol was added to the cells to a final concentration of 600 ng/ml and incubated for 10 min before harvesting.

For analysis of tau phosphorylation, cells were harvested by scraping into 1.5 ml of chilled phosphate-buffered saline, pelleted by centrifugation and then resuspended in 250 μl (a 50 μl aliquot was removed for protein analysis using Pierce BCA reagents) pelleted again and resuspended in 60 μl of MES-sodium chloride buffer comprising 100 mM MES, pH 6.5, 0.5 mM $MgCl_2$, 1 mM EGTA and 1 M NaCl supplemented with 2 mM DTT, 17.5 μg/ml PMSF, 20 μg/ml aprotinin, 50 mM sodium fluoride and 0.1 mM sodium vanadate. To isolate tau, the harvested cells were heated in a boiling water bath for 5 min and the insoluble fraction removed by centrifugation in a microcentrifuge for 15 min. The supernatant containing tau was prepared for SDS-PAGE by addition of SDS-PAGE sample buffer and analysed by Western blotting.

Analysis of tau phosphorylation by Western blotting was performed as previously described [13]. TP70 is a polyclonal antibody raised in rabbits to a tau synthetic peptide corresponding to the C-terminus of tau [30]. Monoclonal antibody (mAb) Tau.1 was obtained from Dr. L. Binder, SMI mAbs from Sternberger Monoclonals (Affiniti Research Products Ltd., UK) and mAb AT8 from Innogenetics, Ghent, Belgium. Monoclonal antibodies 121.5, BF10, RT97 and 8D8 have been described previously [31].

2.2. MAP kinase assays

MAP kinase was extracted and batch-purified by binding to phenyl-Sepharose (Pharmacia) and assayed by using bovine myelin basic protein (Sigma) as substrate, as previously described [13].

3. Results

3.1. Transfected tau is similarly phosphorylated in v-raf-transformed 3T3 cells as in untransformed cells

Swiss 3T3 cells were transfected with the cDNA encoding the human brain tau isoform with four repeat domains and one N-terminal insert, since this is an abundantly expressed isoform in brain [32]. Using the TP70 polyclonal antibody to tau that recognises normal tau as well as PHF-tau, Western blot analysis of the heat-stable fraction from these cells revealed two bands, the faster-migrating band co-migrated with the recombinant protein of this isoform (Fig. 1A, lanes 1 and 2). The slower migration of the upper band is probably due to phosphorylation since phosphorylation of tau results in changes in electrophoretic mobility, both in vitro with a number of kinases [11,33–36] and in transfected cells [3,13,37–39]. Nevertheless, neither tau band was hyperphosphorylated similar to PHF-tau nor to the hyperphosphorylated components of adult and foetal tau since they were not recognised by monoclonal antibodies AT8 or 8D8; these mAbs recognise phosphorylated serines-202

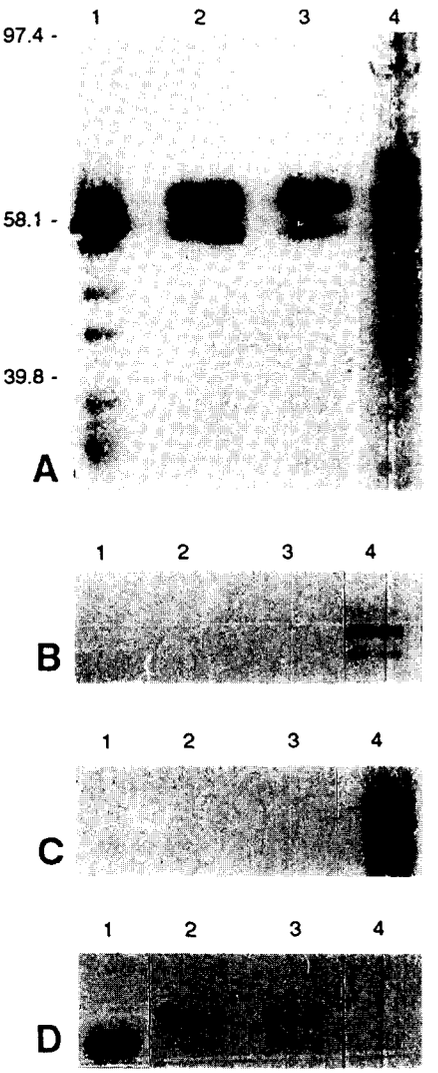

Fig. 1. Western blots with (A and E) polyclonal antibody, TP70, that recognises both normal tau and hyperphosphorylated PHF-tau; (B and F) mAb AT8 that recognises a tau epitope requiring phosphorylation of Ser_{202}; (C and G) mAb 8D8 that recognises a tau epitope requiring phosphorylation of Ser_{396}; (D and H) mAb Tau 1 that recognises a tau epitope requiring non-phosphorylation of Ser_{199} and Ser_{202}. For A–D: Lanes 1 = recombinant human brain tau; lanes 2 = heat-stable fraction from 3T3 cells transfected with tau; lanes 3 = heat-stable fraction from v-*raf*-transformed 3T3 cells transfected with tau; lanes 4 = PHF-tau.

and -396, respectively, in PHF-tau but do not recognise the non-hyperphosphorylated fractions of adult or foetal brain tau, nor do they recognise recombinant tau (Fig. 1B and C, lanes 1, 2 and 4). The mAbs RT97, 1215, SMI31 and SMI34 which all recognise phosphorylated sites in PHF-tau [13], also all failed to label the tau from the transfected 3T3 cells (data not shown). Both tau bands from the transfected cells were labelled by mAb Tau 1 which recognises unphosphorylated serine-202 (Fig. 1D, lane 2). Tau 1, which is specific for the non-hyperphosphorylated fractions of adult and foetal brain tau, also labelled non-phosphorylated recombinant tau (Fig. 1D, lane 1) but not PHF-tau (Fig. 1D, lane 4) and so again these results demonstrated a lack of hyperphosphorylation of the transfected tau. If the tau expressed in the 3T3 cells were hyper-

phosphorylated, we would predict from our in vitro phosphorylation studies that this isoform would co-migrate with the uppermost band of the three prominent PHF-tau bands, and transfected tau from the 3T3 cells clearly does not migrate in this position (Fig. 1A, lanes 2 and 4) [36].

The Western blot pattern of tau in heat-stable fractions from transfected v-*raf*-transformed 3T3 cells was identical to tau from transfected control 3T3 cells (Fig. 1A, lanes 2 and 3); no differences in electrophoretic mobility was observed, and neither of the two tau bands was labelled by mAbs AT8 or 8D8 (Fig. 1B and C, lanes 3), but both bands reacted with mAb Tau 1 (Fig. 1D, lane 3). The level of MAP kinase activity, measured using myelin basic protein as substrate, in extracts from transformed cells was at least 3-fold higher than that in extracts from non-transformed 3T3 cells which is comparable to that previously reported for these cell lines [28]. Thus, although transformation of 3T3 cells by v-*raf* resulted in constitutive elevation of MAP kinase activity, transfected tau was still not hyperphosphorylated under these conditions.

3.2. GSK-3β induced hyperphosphorylation of tau in 3T3 cells

When the 3T3 cells were co-transfected with tau and GSK-3β, hyperphosphorylation of tau was observed, although not all of the tau was hyperphosphorylated since two bands were labelled by the polyclonal antibody, TP70, and only the slower-migrating of which co-migrated with the expected component in PHF-tau (Fig. 2A, lanes 1 and 2). This slower-migrating band was also labelled by mAbs AT8 (Fig. 2B) and 8D8 (Fig. 2C) but not by Tau 1 (Fig. 2D), whereas Tau 1 but not AT8 or 8D8 labelled the faster-migrating band of the pair (Fig. 2D). Thus, as we previously demonstrated in transfected COS-7 cells [13], GSK-3β leads to hyperphosphorylation of at least a proportion of tau in 3T3 cells and this was further substantiated by the reactivity of the upper band with mAbs RT97, 1215, SMI31 and SMI34 (data not shown).

3.3. Co-transfection of tau and p44^{MAPK} into control and v-raf-transformed 3T3 cells results in elevated tau phosphorylation but not hyperphosphorylation

The GSK-3β stimulated hyperphosphorylation of tau clearly demonstrated that 3T3 cells have the capacity to support the formation of hyperphosphorylated tau and so a further attempt to elevate the activity of MAP kinase in the cells was made by transfection of a MAP kinase cDNA and treatment of the cells with TPA. A p44^{MAPK} cDNA was transiently co-transfected with tau into control and v-*raf*-transformed 3T3 cells and the cells treated with TPA; for comparison, control 3T3 cells transiently transfected only with tau cDNA were also treated with

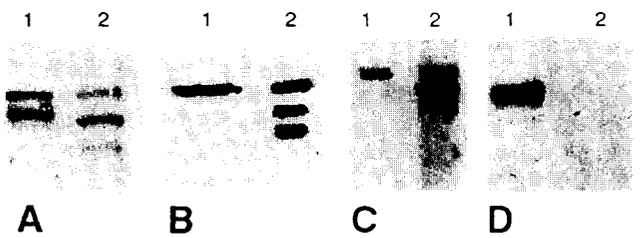

Fig. 2. Western blots with: (A) polyclonal antibody, TP70; (B) mAb AT8; (C) mAb 8D8; (D) mAb Tau 1. For A–D: Lanes 1 = heat-stable fraction from 3T3 cells co-transfected with tau and GSK-3β; lanes 2 = PHF-tau.

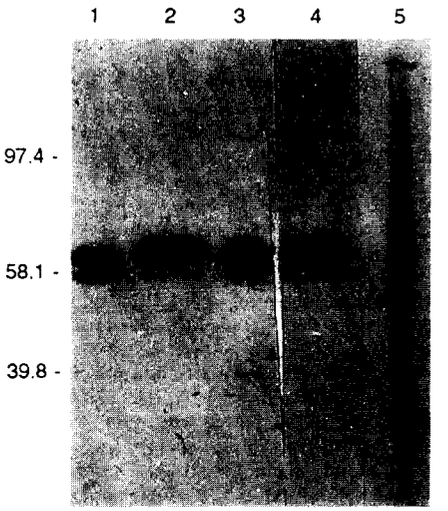

Fig. 3. Western blot with polyclonal tau antibody, TP70. Lanes 1–4 = heat-stable fraction from 3T3 cells (and all treated with TPA); lane 1 = transfected with tau; lane 2 = 3T3 cells co-transfected with p44^{MAPK} and tau; lane 3 = v-*raf*-transformed 3T3 cells transfected with tau; lane 4 = v-*raf*-transformed 3T3 cells co-transfected with p44^{MAPK} and tau; lane 5 = PHF-tau.

TPA. The tau in heat-stable fractions from all of these cells was analysed by Western blotting. The tau from both control and v-*raf*-transformed 3T3 cells co-transfected with tau and p44^{MAPK} cDNAs migrated mainly as a single band (Fig. 3, lanes 2 and 4, respectively) with the same mobility as the slower-migrating band of the tau doublet from either control or v-*raf*-transformed cells transfected only with tau cDNA (Fig. 3, lanes 1 and 3, respectively). Although this implies that elevation of MAP kinase activity through transfection with p44^{MAPK} cDNA and TPA treatment resulted in an increased level of tau phosphorylation, none of the bands migrated in the same position as the slowest of the three main PHF-tau bands, as would be expected if the tau was hyperphosphorylated similarly to PHF-tau (Fig. 3, lanes 2, 4 and 5) [36]. Similarly, none of the tau from these cells was labelled by mAbs specific for PHF-tau and hyperphosphorylated adult and foetal tau, ie, mAbs AT8, 8D8, RT97 1215, SMI31 and SMI34 (data not shown).

4. Discussion

The results of this study extend our previous findings that GSK-3α and GSK-3β hyperphosphorylate tau in co-transfected COS-7 cells but that neither p44^{MAPK} nor p42^{MAPK} led to a similar increase in tau phosphorylation in COS cells [13]. It is possible that the absence of a MAP kinase-induced hyperphosphorylation of tau that we observed previously was because the MAP kinase activity was only transiently activated with the peak occurring after 10 min treatment with TPA, and noticeably declining after 60 min. However, v-*raf*-transformation that leads to constitutive elevation of MAP kinase activity also failed to result in tau hyperphosphorylation. This is unlikely to be the result of some other difference between COS-7 and 3T3 cells because transfected GSK-3β did induce tau hyperphosphorylation in the 3T3 cells.

There are several possible explanations why tau is not hyperphosphorylated by activated MAP kinase in these two non-

neuronal cell types even though MAP kinase does utilise tau as a substrate in vitro. It is unlikely that the lack of phosphorylation is due to all activated MAP kinase being translocated to the nucleus because we demonstrated previously that a significant fraction remains cytoplasmic [13]. Furthermore in this study of 3T3 cells, a shift in mobility of tau was observed when it was analysed from cells in which MAP kinase was maximally elevated but without generation of PHF-tau or hyperphosphorylated adult and foetal tau epitopes, as monitored with available monoclonal antibodies, and the lack of a maximal decrease in mobility on SDS-PAGE. This implies that cytoplasmic MAP kinase is either capable of phosphorylating tau, albeit at non-PHF sites, or activates another kinase which utilises the cellular tau as substrate. Indeed, several kinases distinct from proline directed kinases can induce a shift in electrophoretic mobility of tau [33–35,40]. Interestingly, this change in tau mobility in $p44^{MAPK}$ transfected cells was not restricted to the v-*raf*-transformed cells which suggests that transfection with MAP kinase and treatment with TPA to stimulate the MAP kinase transiently, was sufficient to lead to a change in tau phosphorylation over and above that in TPA-treated control cells transfected only with tau.

It has been shown in several non-neuronal cells, including NIH3T3 cells, that activation of MAP kinase results in a decrease in activity of endogenous GSK-3. This is because MAP kinase stimulates MAP kinase-activated protein kinase-1β (MAPKAP), also known as p90 ribosomal protein S6 kinase-2 (Rsk2), which in turn phosphorylates and inactivates GSK-3 [41–44]. There are therefore interesting implications for treating Alzheimer's disease since nerve growth factor, which is considered as a potential therapeutic agent [45,46], activates MAP kinase in neuronal cells and so this neurotrophin might lead to a reduction in the amount of hyperphosphorylated neuronal tau in degenerating neurones. Indeed, we demonstrated previously that treatment of primary neurones in culture with TPA did activate MAP kinase and produced a modest reduction in the proportion of foetal tau that is hyperphosphorylated in these cells [13]. Clearly, it is now important to establish unequivocally the identity of the kinases responsible for the phosphorylation of neuronal tau in vivo and the mechanisms regulating these kinases.

Acknowledgements: This work was supported by the British Medical Research Council and The Wellcome Trust. We thank Innogenetics, Belgium, for the gift of mAb AT8 and Dr. L. Binder for Tau 1, and Dr. Fritz Propst for the gift of v-*raf*-transformed Swiss 3T3 cells. We are grateful for the human brain tissue for the preparation of PHF-tau provided by the MRC Neurodegenerative Diseases Brain and Tissue Bank, Department of Neuropathology, Institute of Psychiatry, London.

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