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p42 map kinase phosphorylation sites in microtubule-associated protein tau are dephosphorylated by protein phosphatase $2A_1$

Implications for Alzheimer's disease

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The paired helical filament (PHF), which comprises the major fibrous element of the neurofibrillary tangle of Alzheimer's disease, is composed of abnormally phosphorylated microtubule-associated protein tau. Here we show that p42 MAP kinase phosphorylates recombinant tau and converts it to a form which is similar to PHF tau. Of the major serine/threonine protein phosphatases found in mammalian tissues only protein phosphatase 2A (PP2A) could dephosphorylate tau phosphorylated in this manner, with $PP2A_1$ being the most effective form of the enzyme.

Alzheimer's disease; Microtubule-associated protein tau; Mitogen-activated protein kinase; Protein phosphatase 2A

1. INTRODUCTION

Abundant senile plaques and neurofibrillary tangles constitute the major pathological characteristics of Alzheimer's disease. Neurofibrillary tangles appear within the vast majority of nerve cells that degenerate during the course of the disease, where their presence is indicative of dementia (reviewed in [1]). Paired helical filaments (PHFs), which are composed of the microtubule-associated protein tau, form the principal fibrous component of the neurofibrillary tangle [2–4]. In human adult brain, tau comprises six isoforms of 48–67 kDa apparent molecular mass [5], while tau isolated from Alzheimer PHFs runs as three bands of 60, 64 and 68 kDa apparent molecular mass [2,6]. After dephosphorylation with alkaline phosphatase these bands align with the six tau isoforms expressed in *E. coli*, indicating that PHF tau consists of all six isoforms in an abnormally phosphorylated state [7]. Abnormal phosphorylation probably produces a change in tau that favours self-association over microtubule binding, resulting in the formation of PHFs.

Several sites that are abnormally phosphorylated in PHF tau have been identified through the use of phosphorylation-dependent antibodies which recognize PHF tau but do not recognize normal adult brain tau. Thus, antiserum T3P recognizes phosphoserine-396 [2] and antibody AT8 recognizes phosphoserine-199 and/or phosphoserine-202 [8] (using the numbering of the largest human brain tau isoform [5]). Each of these serines is followed by a proline, suggesting that protein kinases or protein phosphatases with specificity for phosphoseryl-proline may be involved. Recently, a protein kinase with the characteristics of a mitogen-activated protein (MAP) kinase was purified from pig brain and shown to phosphorylate recombinant tau to an Alzheimer-like state, as evidenced by a reduction in gel mobility and the phosphorylation of serine/threonine-proline sites [9]. Moreover, two brain protein kinases (PK36 and PK40) phosphorylate tau at serine-396 and other serine/threonine–proline sites [10] and a kinase called tau protein kinase I also phosphorylates tau at some serine/threonine–proline sites [11]. The relationships, if any, of these protein kinases to MAP kinase are not clear. Nothing is known about the protein phosphatases that can dephosphorylate tau phosphorylated at serine/threonine–proline sites.

In this paper we show that activated recombinant p42 MAP kinase (also known as ERK2) phosphorylates recombinant tau, converting it to a form which is similar to PHF tau, as judged by a reduction in gel mobility and the appearance of the T3P and AT8 epitopes. Tau phosphorylated in this manner could only be dephosphorylated by a type 2A protein phosphatase (PP2A). This suggests that an increase in the activity of a MAP kinase or a defect in a PP2A enzyme could underlie the abnormal state of tau in Alzheimer's disease.

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2. MATERIALS AND METHODS

2.1. *Materials*

The largest human brain tau isoform was expressed in *E. coli* from cDNA clone htau40 and purified as described [12]; the tau concentrations were determined by amino acid analysis. Protein phosphatases $2A_1$ and $2A_2$ partially purified from rat liver extracts by chromatography on Mono-Q ([13]; Mr. P. Ferrigno) and highly purified catalytic subunits of protein phosphatase 1 and protein phosphatase 2A from rabbit skeletal muscle ([14]; Dr. D. Schelling) were isolated at Dundee by the investigators indicated in parentheses. The specific 20 residue inhibitor of cyclic AMP-dependent protein kinase (PKI) was synthesized by Mr. F.B. Caudwell. Recombinant mouse p42 MAP kinase expressed in *E. coli* [15] was provided by Ms. S. Leevers and Dr. C. Marshall (Chester Beatty Laboratories, Institute for Cancer Research, London) and activated at Dundee by incubation with MAP kinase kinase ([16], Ms. S. Nakielny) and MgATP ([15]; Mr. D. Stokoe). Recombinant inhibitor-2 expressed in *E. coli* and okadaic acid were gifts from Dr. A. de Paoli-Roach (University of Indiana, Indianapolis, USA) and Dr. Y. Tsukitani (Fujisawa Pharmaceutical Co., Tokyo, Japan). Antiserum T3P [2] was a gift from Dr. V.M.-Y. Lee (University of Pennsylvania, Philadelphia, USA) and antibody AT8 [8] a gift from Dr. A. Van de Voorde (Innogenetics, Gent, Belgium).

2.2. *Assay of MAP kinase activity*

Incubations (0.05 ml) were carried out at 30°C and comprised 25 mM Tris-HCl, pH 7.0, 0.1 mM EGTA, 0.1 mM sodium orthovanadate, 2.5 μM PKI, 0.2 mM phenylmethylsulphonyl fluoride, tau protein (1 μM) or myelin basic protein (0.33 mg/ml), activated p42 MAP kinase (1 U/ml or 5 U/ml), 10 mM magnesium acetate and 2 mM [γ-^{32}P]ATP (approximately 10^6 cpm/nmol). Reactions were initiated with ATP, aliquots removed after various times ranging from 5 min to 24 h and used for SDS-PAGE. Alternatively, incorporation of ^{32}P-radioactivity was measured after adsorption to Whatman P81 paper, as described [17]. One unit of activity (U) was defined as the amount which catalysed the incorporation of 1 nmol of phosphate into myelin basic protein per min. Prior to the protein phosphatase assays tau phosphorylated with 1 U/ml MAP kinase for 24 h was run over a Sephadex G50 superfine column (20 × 1 cm) in 50 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 0.1% β-mercaptoethanol (v/v), 0.02% Brij 35 (w/v), in order to separate labelled tau from [γ-^{32}P]ATP. The tau fractions were concentrated to approximately 30,000 cpm/10 μl and dialyzed extensively against 50 mM Tris-HCl, pH 7.4, 0.1% β-mercaptoethanol (v/v), 0.02% Brij 35 (w/v). Precipitation with 20% TCA (w/v) indicated that all [γ-^{32}P]ATP had been removed.

2.3. *Assay of protein phosphatases*

^{32}P-Labelled phosphorylase *a* (containing 1.0 mol phosphate/mol subunit) was prepared as in [14] and ^{32}P-labelled tau (containing 7 mol phosphate/mol) as in section 2.2. Protein phosphatase assays were carried out as described previously [14,18,19]. One unit of activity (U) was that amount which catalysed the release of 1 μmol of phosphate from ^{32}P-labelled substrate in 1 min. To assess the relative contributions of PP1 and PP2A to the dephosphorylation of phosphorylase and tau in brain extracts, assays were carried out in the absence and presence of 2 nM okadaic acid or 200 nM inhibitor-2. PP2B was assayed in an identical manner, except that 0.1 mM EGTA was replaced by 0.1 mM $CaCl_2$, while PP2C was assayed in the presence of 10 mM $MgCl_2$.

3. RESULTS

3.1. *Phosphorylation of tau by p42 MAP kinase*

Tau protein incubated with p42 MAP kinase for 24 h incorporated 6–8 mol phosphate per mol protein at 1 U/ml MAP kinase and 10–12 mol phosphate per mol protein at 5 U/ml MAP kinase. A typical time-course of phosphorylation is shown in Fig. 1. High stoichiometric ratios required long incubation times, with only 1–2 mol phosphate per mol tau incorporated within 30 min. By SDS-PAGE phosphorylated tau showed a reduced gel mobility, running at 72 kDa apparent molecular mass after a 24 h incubation with MAP kinase instead of 67 kDa when non-phosphorylated (Fig. 2); the apparent molecular mass increased throughout the incubation period and was most pronounced at late time-points (Fig. 2a–c). Phosphorylated tau became immunoreactive with the anti-PHF tau-specific antibodies T3P and AT8, demonstrating phosphorylation of serine-396 and serines-199 and/or -202 (Fig. 2d,e). These residues only became phosphorylated following prolonged incubation of tau with MAP kinase (Fig. 2d,e). Phosphoamino acid analysis of ^{32}P-labelled tau containing 7 mol phosphate per mol protein revealed similar amounts of phosphoserine and phosphothreonine (Fig. 3). No phosphotyrosine was present.

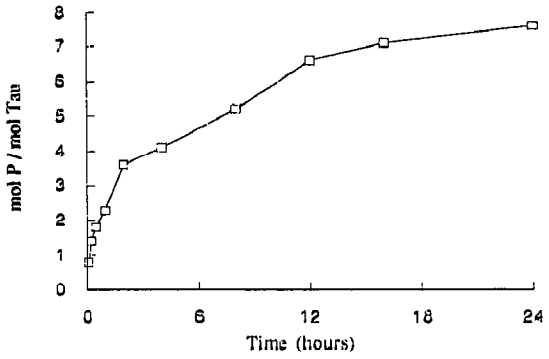

Fig. 1. Time-course of phosphorylation of recombinant human tau protein with p42 MAP kinase. One μM of the 441 amino acid brain isoform was phosphorylated with 1 U/ml p42 MAP kinase, as described in Materials and Methods. Similar results were obtained in four separate experiments.

3.2. *Identification of the major tau phosphatase in brain extracts*

Four major types of serine/threonine protein phosphatase catalytic subunit have been identified in mammalian tissues, termed protein phosphatases 1, 2A, 2B and 2C (PP1, PP2A, PP2B and PP2C). PP1 and PP2A are active in the absence of divalent cations, while PP2B and PP2C have absolute requirements for Ca^{2+} and Mg^{2+}, respectively (reviewed in [20]). In highly diluted extracts of brain and other tissues, it has been shown (using [^{32}P]-phosphorylase as a substrate) that PP2A is inhibited almost completely by 2 nM okadaic acid, whereas PP1 is unaffected at this concentration. Conversely, PP1 is inhibited almost completely by 200 nM inhibitor-2, whereas PP2A is unaffected [21]. Table I shows that 2 nM okadaic acid and inhibitor-2 inhibited the dephosphorylation of phosphorylase by 56% and

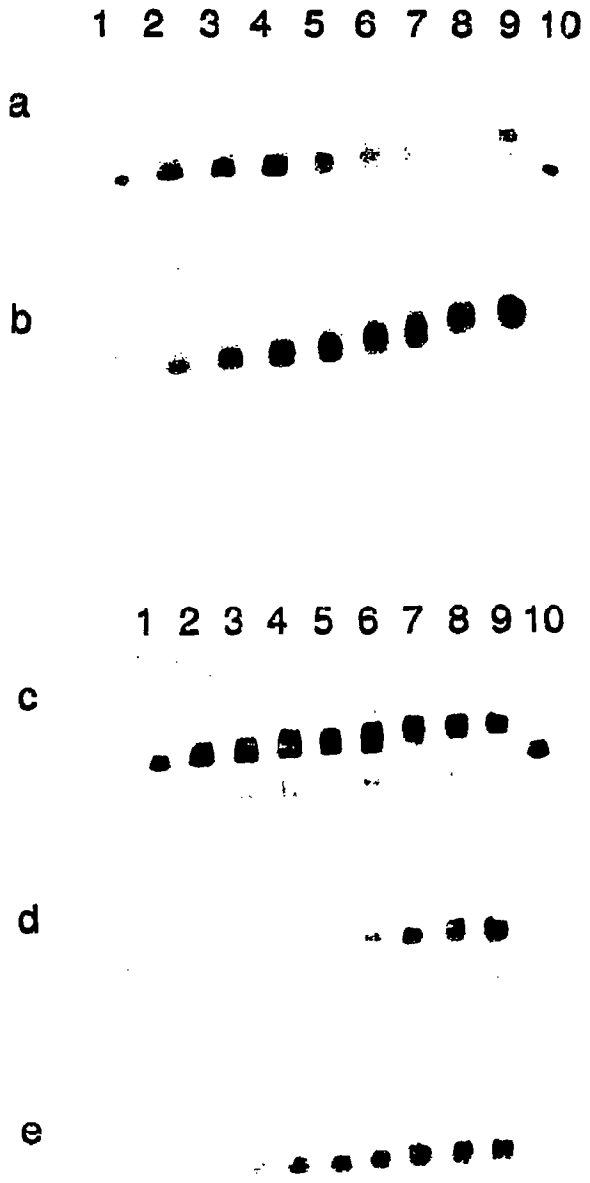

Fig. 2. SDS-PAGE of recombinant human tau protein phosphorylated with p42 MAP kinase for various times. (a) Coomassie-stained gel; (b) Autoradiogram of (a); (c) Immunoblot with the general anti-tau antiserum 133; (d) Immunoblot with the anti-PHF tau-specific antiserum. T3P; (e) Immunoblot with the anti-PHF tau-specific antibody AT8. Lanes: (1) no MAP kinase; (2) 0.5 h; (3) 1 h; (4) 2 h; (5) 4 h; (6) 8 h; (7) 12 h; (8) 16 h; (9) 24 h; (10) no MAP kinase. Note the increase in apparent molecular mass as a function of time and the appearance of the T3P and AT8 epitopes. Similar results were obtained in three separate experiments.

41%, respectively. Thus, PP1 contributed 43 ± 2% and PP2A 57 ± 2% of the phosphorylase phosphatase activity in brain extracts, a similar result to that reported in [21]. In contrast, when the same extracts were assayed with ^{32}P-labelled tau, 2 nM okadaic acid inhibited activity by 98% and 200 nM inhibitor-2 by only 4%. Thus,

Table 1

Effect of okadaic acid, inhibitor-2 and divalent cations on the dephosphorylation of glycogen phosphorylase and tau by rat brain extracts

Additions to assay	PhP activity (%)	TP activity (%)
None	100	100
Inhibitor-2 (0.2 μM)	59	96
Okadaic acid (2 nM)	44	2
Okadaic acid (10 nM)	ND	1
Okadaic acid (10 nM) + Mg^{2+} (10 mM)	ND	1
Okadaic acid (10 nM) + Ca^{2+} (0.1 mM)	ND	4

Rat brain was homogenised in 3 vols. of buffer/g wet weight as described [39], except for inclusion of a proteinase inhibitor cocktail [40], and the homogenates centrifuged for 15 min at 16,000 × g (prepared by Mr. P. Ferrigno). The supernatant was decanted and assayed at a 300-fold final dilution (5 mg/ml) using 10 μM [^{32}P]phosphorylase or 1.5 μM [^{32}P]tau as substrates. The 100% value corresponds to 12 mU/ml phosphorylase phosphatase (PhP) and 0.3 mU/ml tau phosphatase (TP) in the undiluted brain extract. Diluted extracts and inhibitor-2 were preincubated for 15 min prior to initiating the reaction with substrate, in order to achieve maximal inhibition of PP1. Similar results were obtained with two different brain extracts and two preparations of [^{32}P]tau substrate. ND = not determined.

in the absence of divalent cations, forms of PP2A accounted for virtually all the tau phosphatase activity, the contribution of PP1 being negligible.

PP2B is only affected by okadaic acid at concentrations above 500 nM, while PP2C is resistant [22]. The contributions of PP2B and PP2C to the tau phosphatase activity in brain extracts were therefore assessed in the presence of 10 nM okadaic acid (to inhibit PP2A) and either 0.1 mM Ca^{2+} or 10 mM Mg^{2+}. As shown in Table I, tau phosphatase activity was negligible in either the presence or absence of Ca^{2+} or Mg^{2+}, indicating that neither PP2B nor PP2C are significant tau phosphatases.

The PP2A catalytic subunit (termed $PP2A_C$) does not exist as such in vivo, but is complexed to a 60 kDa A-subunit, which in turn can be complexed to other subunits, termed B, B′ and B″ (reviewed in [20]). The heterodimeric species consisting of $PP2A_C$ complexed to the A-subunit is termed $PP2A_2$ and a major trimeric species comprising $PP2A_C$, the A-subunit and a 55 kDa B-subunit is termed $PP2A_1$. $PP2A_C$, the A-subunit and the α-isoform of the B-subunit are found in a variety of mammalian tissues, the highest levels of mRNA being found in brain, although an additional β-isoform of the B-subunit specific to the nervous system has also been identified [23–26]. The tau phosphatase:phosphorylase phosphatase activity ratios of different forms of PP2A are summarized in Table II. $PP2A_1$ has a 7–8-fold higher ratio than $PP2A_2$, and a 40-fold higher ratio than $PP2A_C$. The activity ratio in brain extracts is similar to that of $PP2A_1$. The free catalytic subunit of PP1 had

Table II

Different forms of protein phosphatase 2A have distinct tau phosphatase (TP):phosphorylase phosphatase (PhP) activity ratios

Form of PP2A	TP/PhP ratio
Brain extract	0.36
$PP2A_1$	0.48
$PP2A_2$	0.062
$PP3A_C$	0.011
$PP1_C$	0.002

Brain extracts were assayed at a 300-fold final dilution in the presence of 200 nM inhibitor-2 to inactivate PP1. Assays were carried out at 10 μM phosphorylase or 1.5 μM [^{32}P]tau. Similar results were obtained in separate experiments with two different preparations of tau and phosphorylase and two different brain extracts.

negligible tau phosphatase activity (Table II), in agreement with the results obtained in brain extracts (Table I).

Incubation of ^{32}P-labelled tau at 30°C with concentrations of $PP2A_1$ two-fold higher than those employed in the standard (10 min) assay released almost 40% of the [^{32}P]phosphate after 1 h and 45% after 2 h. The proportion of phosphoserine and phosphothreonine in tau that had been incubated for 1 h or 2 h with $PP2A_1$ was similar to that of tau which had not been treated with PP2A (Fig. 3), indicating that both threonine and serine residues in tau are dephosphorylated by $PP2A_1$ (Fig. 3).

4. DISCUSSION

MAP kinases are a group of serine/threonine kinases that are activated rapidly in response to many extracellular signals, that include hormones and growth factors whose receptors are protein tyrosine kinases. The activation of MAP kinases involves their phosphorylation on a threonine and a tyrosine residue (reviewed in [27]), both phosphorylations being catalysed by a single MAP kinase kinase, that is itself dependent on serine/threonine phosphorylation for activity [16]. MAP kinases are therefore turned on through the activation of a complex protein kinase cascade that has yet to be fully elucidated. MAP kinases transmit the actions of extracellular agonists by phosphorylating and activating target proteins, that may either be other protein kinases such as S6 kinase-II (reviewed in [27]) or MAPKAP kinase 2 [15], or transcription factors such as c-jun [28]. The preferred consensus sequence for phosphorylation by MAP kinase is Pro-Xaa-Ser/Thr-Pro [29,30], although the N-terminal proline is not obligatory.

The highest levels of MAP kinase mRNA and protein are found in brain [31]. Its normal role and the factors that stimulate its activity in this tissue are not well understood, although NMDA receptor activation and electroconvulsive treatment have been reported to induce tyrosine phosphorylation of a MAP kinase [32,33].

In this paper we demonstrate that the p42 isoform of MAP kinase phosphorylates tau protein in a similar manner to that observed in Alzheimer's disease, as judged by a large reduction in gel mobility, and the phosphorylation of serine-396 and serines-199 and/or -202, two of the sites that are abnormally phosphorylated in tau from PHFs [2,8]. These results are in agreement with a recent study showing that a MAP kinase from pig brain phosphorylates tau and converts it to an Alzheimer-like state [9].

Activation of MAP kinase in the absence of an appropriate stimulus is likely to be one of the mechanisms that causes cell transformation, since it occurs in response to tumour-promoting phorbol esters or when cells are transfected with oncogenes such as *ras, raf, gip2* and *src* (reviewed in [34]) which appear to lie upstream of MAP kinase kinase in the growth factor-stimulated MAP kinase cascade. Likewise, the neurofibrillary pathology of Alzheimer's disease could result from the uncoupling of a component of the MAP kinase activation cascade from regulation by an appropriate extracellular signal. Since brain cells cannot undergo cell division, the consequence of sustained MAP kinase activation would not be uncontrolled cell proliferation. Instead, normal brain function might be disrupted by the abnormal phosphorylation of proteins that are not normally phosphorylated to such an extent in vivo. Abnormal phosphorylation of proteins, such as tau (which is not phosphorylated at the T3P and AT8 epitopes in normal adult brain [2,8]), might trigger changes in their conformation that have a deleterious effect on normal function.

It is also possible that the abnormal phosphorylation

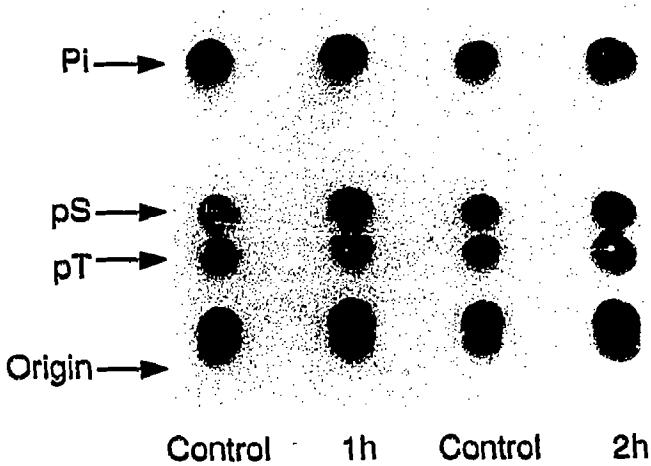

Fig. 3. Phosphoamino acid analysis of ^{32}P-labelled tau. ^{32}P-labelled tau was incubated in the absence (Control) or presence of $PP2A_1$ for 1 h or 2 h, which released 40% and 45% of the ^{32}P-radioactivity, respectively. The samples were hydrolysed and phosphorylated amino acids separated by thin layer electrophoresis, as described [17]. The positions of phosphothreonine (pT), phosphoserine (pS), inorganic phosphate (P_i) and the point of sample application (origin) are marked. The other ^{32}P-labelled spot represents phosphopeptides resulting from incomplete hydrolysis.

of tau in Alzheimer's disease does not arise from the activation of MAP kinase, but from a decrease in the activity of a tau phosphatase. We show here that PP2A is the only protein phosphatase with significant activity towards ^{32}P-labelled tau phosphorylated by MAP kinase, under our assay conditions. Furthermore, $PP2A_1$ is easily the most effective species of PP2A in dephosphorylating tau, the free catalytic subunit being almost inactive.

These findings are very similar to those obtained recently with several physiological substrates of p34cdc2 ([13,35]; P. Ferrigno, T. Langan and P. Cohen, in preparation). p34cdc2 resembles MAP kinase in phosphorylating serine and threonine residues that are followed by a proline residue. However, the preferred consensus sequence is (Lys)-Ser/Thr-Pro-Xaa-Lys [36], explaining why p34cdc2 is unable to phosphorylate myelin basic protein (one of the most effective in vitro substrates for MAP kinase) and why MAP kinase cannot phosphorylate histone H1 (the standard substrate for p34cdc2). A proline immediately C-terminal to the phosphorylated residue has been shown to prevent dephosphorylation of small synthetic peptides by all the major types of protein Ser/Thr phosphatase catalytic subunits [37,38], presumably explaining why the catalytic subunit of PP2A dephosphorylates proteins phosphorylated by p34cdc2 [35] or MAP kinase so poorly. The higher activity of $PP2A_2$ and even higher activity of $PP2A_1$, towards both p34cdc2 substrates and tau, strongly suggests that the A-subunit in $PP2A_2$ and the A and B subunits in $PP2A_1$ are able to counteract the negative effect of a C-terminal proline residue.

This raises the intriguing possibility that a defect in one of the B-subunits in brain might underlie the abnormal phosphorylation of tau which occurs in Alzheimer's disease, since this would be expected to lead to selective increases in the phosphorylation of MAP kinase substrates.

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