Epitope mapping of monoclonal antibodies to the paired helical filaments of Alzheimer's disease: identification of phosphorylation sites in tau protein

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Tau is a neuronal phosphoprotein the expression of which is developmentally regulated. A single tau isoform is expressed in fetal human brain but six isoforms are expressed in adult human brain, with the fetal isoform corresponding to the shortest adult isoform. Phosphorylation is also developmentally regulated, as fetal tau is phosphorylated at more sites than adult tau. In Alzheimer's disease, the six adult tau isoforms become hyperphosphorylated and form the paired helical filament (PHF), the major fibrous component of the neurofibrillary lesions. One way to identify phosphorylated sites in tau is to use antibodies that

recognize phosphorylated residues within a specific amino acid sequence. We here characterize the two novel phosphorylation-dependent anti-tau antibodies AT270 and AT180 and identify their epitopes as containing phosphorylated Thr-181 and Thr-231 respectively. With these antibodies we show that these two threonine residues are partially phosphorylated in fetal and adult tau and almost fully phosphorylated in PHF tau. This result contrasts with previous studies of Ser-202 and Ser-396 which are partially phosphorylated in fetal tau, unphosphorylated in adult tau but almost fully phosphorylated in PHF tau.

INTRODUCTION

The microtubule-associated protein tau promotes the assembly and stabilization of neuronal microtubules (for a review, see ref. [1]). Multiple tau isoforms are produced from a single gene by alternative mRNA splicing [2–8]. Each isoform contains in the C-terminal half three or four tandem repeats of 31 or 32 amino acids each, which constitute microtubule-binding units [9,10]. Additional isoforms contain 29 or 58 amino acid inserts in the N-terminal region in conjunction with three or four repeats, giving rise in adult human brain to a total of six isoforms [11].

Tau expression is developmentally regulated; in fetal human brain there is only one isoform, whereas in adult human brain all six isoforms are expressed, with the fetal isoform corresponding to the smallest of the adult isoforms [4,11]. Tau is a phosphoprotein (for a review, see ref. [1]) and developmental regulation extends to the level of phosphorylation, in that fetal tau is phosphorylated at more sites than adult tau.

In Alzheimer's disease, tau becomes hyperphosphorylated and self-associates through the microtubule-binding domain to form the paired helical filament (PHF) [12–14], the principal fibrous structure of the neurofibrillary lesions (neurofibrillary tangles, neuropil threads and senile plaque neurites). These appear within the vast majority of nerve cells that degenerate during the course of Alzheimer's disease, where their presence is indicative of dementia [15,16]. PHF tau is unable to bind to microtubules without prior dephosphorylation, indicating one consequence of abnormal phosphorylation [17,18]. Tau from Alzheimer PHFs runs as three bands of 60, 64 and 68 kDa apparent molecular mass [12,19]. Dephosphorylation generates six bands, indicating that PHF tau consists of all six brain tau isoforms in an abnormally phosphorylated state [20–22].

An understanding of the mechanisms that lead to PHF tau formation requires knowledge of the phosphorylated residues. M.s. and phosphopeptide sequencing were used to identify Thr-231, Ser-235 and Ser-262 as phosphorylation sites in PHF tau and Ser-404 as a phosphorylation site in normal adult tau (in the numbering of the largest adult human brain tau isoform) [23,24]. In addition, the phosphorylation-dependent anti-tau antibodies AT8 and T3P were used to identify Ser-202 and Ser-396 respectively as phosphorylation sites in PHF tau [12,17,25]. These two sites are not phosphorylated in normal adult tau; however, they are phosphorylated in immature brain tau [17,25]. With the exception of Ser-262, which is phosphorylated only in a fraction of PHF tau [23], all the above sites are located in serine/threonine-proline sequences. Accordingly, recombinant tau can be converted into a form that is similar to PHF tau by *in vitro* phosphorylation with mitogen-activated protein (MAP) kinase, glycogen synthase kinase-3 (GSK3) or proline-directed protein kinases [26–32]. The trimeric form of protein phosphatase 2A accounts for 96% of the phosphatase activity in rat brain extract towards tau phosphorylated by MAP kinase [28].

We report here the characterization of monoclonal antibodies AT270 and AT180, two novel phosphorylation-dependent anti-tau antibodies. On immunoblots they recognized PHF tau and a proportion of immature brain tau, but also a proportion of adult brain tau. Phosphorylated recombinant human tau mutated individually at each of its 14 serine/threonine-prolines was used to identify their epitopes. AT270 failed to recognize phosphorylated tau with a Thr-181 to Ala mutation. AT180 failed to recognize phosphorylated tau with a Thr-231 to Ala mutation and recognized tau with a Ser-235 to Ala mutation only weakly. AT270 recognized recombinant tau phosphorylated with p42 MAP kinase, but not tau phosphorylated with GSK3 β . AT180

Abbreviations used: PHF, paired helical filament; MAP kinase, mitogen-activated protein kinase; GSK3, glycogen synthase kinase-3; cdk5, cyclin-dependent kinase 5; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride.

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failed to recognize recombinant tau phosphorylated with either p42 MAP kinase or GSK3 β alone. However, it recognized tau phosphorylated by p42 MAP kinase and GSK3 β used in combination. We deduce that the epitopes of AT270 and AT180 require the phosphorylation of Thr-181 and Thr-231 respectively.

MATERIALS AND METHODS

Antibodies

PHF tau was used as the immunogen for the production of monoclonal antibodies AT270, AT180 and AT27. PHF tau preparation, immunizations, fusions and screening for anti-tau antibodies were performed as described previously [33]. The characteristics of anti-tau antibody 134 (directed against the C-terminal peptide) and of the phosphorylation-dependent anti-tau antibodies AT8 (requiring phosphorylation of Ser-202) and T3P (requiring phosphorylation of Ser-396) have been described [11,12,17,25,33].

Recombinant tau

A full-length cDNA clone (htau24) encoding a four-repeat isoform of human tau [4] and with an *Nde*I site in the context of the initiator codon was subcloned into the *Eco*RI site of M13mp18. This isoform contains 14 serine/threonine-proline sequences (residues T111, T153, T175, T181, S199, S202, T205, T212, T217, T231, S235, S396, S404 and S422, in the numbering of the largest human brain tau isoform). Site-directed mutagenesis was used to change each of the 14 codons individually from a serine or a threonine to an alanine. After cleavage with *Nde*I and *Eco*RI, the resulting fragments were subcloned downstream of the T7 RNA polymerase promoter in the expression plasmid pRK172 and the recombinant plasmids transformed into *Escherichia coli* BL21 (DE3) cells. The bacterial cultures were grown and induced as described [34]. Recombinant tau proteins were purified by an improvement [35] of the procedure described in [34].

Phosphorylation of recombinant tau by rat brain extract

An adult rat brain extract was prepared as described previously [25]. The supernatant was used for phosphorylation of recombinant tau. Incubations (0.05 ml) were carried out at 37 °C in 40 mM Hepes, pH 7.4, containing 2 mM ATP, 2 mM MgCl₂, 1 μ M tau protein, rat brain extract (1 μ l), 5 mM EGTA, 2 mM dithiothreitol (DTT), 1 μ M okadaic acid, 0.1 mM phenylmethanesulphonyl fluoride (PMSF), 1 μ M leupeptin, 1 μ M aprotinin and 1 μ M pepstatin. Reactions were initiated by the addition of brain extract and mixtures were incubated for 24 h. Samples were subjected to SDS/PAGE. Controls were incubated under the same conditions except that the brain extract was omitted.

Phosphorylation of recombinant tau by p42 MAP kinase and GSK3 β

Recombinant mouse p42 MAP kinase was expressed in *E. coli* as a glutathione transferase fusion protein and activated with purified MAP kinase kinase as described [36]. The β -isoform of GSK3 was purified from rabbit skeletal muscle as described [37].

Incubations were carried out at 30 °C and the mixtures comprised 25 mM Tris/HCl, pH 7.0, 0.1 mM EGTA, 0.1 mM sodium orthovanadate, 2.5 μ M PKI (an inhibitor of cyclic AMP-dependent protein kinase), 0.2 mM PMSF, tau protein (1 μ M),

activated p42 MAP kinase (1 unit/ml) and/or GSK3 β (1 unit/ml), 10 mM magnesium acetate and 2 mM [γ -³²P]ATP (approx. 10⁶ c.p.m./nmol). Reactions were initiated with ATP, and samples were removed after various times ranging from 20 min to 20 h and used for SDS/PAGE. Alternatively, incorporation of ³²P radioactivity was measured after adsorption to Whatman P81 paper [38]. One unit of MAP kinase activity was that amount that catalysed the incorporation of 1 nmol of phosphate into myelin basic protein/min [36]. One unit of GSK3 activity was that amount that catalysed the incorporation of 1 nmol of phosphate into the peptide substrate (YRRAAVPPSPSLSRHSSPHQSEDEEE) in 1 min [37].

Brain tau

Rats of various ages ranging from embryonic day 19 to 4 months were killed, their brains quickly dissected and frozen on solid CO₂. They were homogenized in 2.5% (v/v) perchloric acid (0.5 ml/g), allowed to stand on ice for 20 min and centrifuged for 10 min at 10000 g. The supernatant was dialysed overnight against 50 mM Tris/HCl, pH 7.4, containing 1 mM DTT and 0.1 mM PMSF and used for immunoblot analysis.

Human cerebral cortex was homogenized in 0.1 M Mes buffer containing 0.5 mM MgSO₄, 1 mM EGTA, 2 mM DTT and 0.75 M NaCl, boiled for 10 min, and centrifuged for 10 min at 10000 g. The heat-stable supernatant was treated with 2.5% perchloric acid, dialysed against distilled water, and concentrated by (NH₄)₂SO₄ precipitation before immunoblot analysis.

PHF tau proteins were extracted from the cerebral cortex of victims of Alzheimer's disease [20]. Tau (10 μ g/ml) was dephosphorylated by treatment with *E. coli* alkaline phosphatase (type III-N; Sigma) at 18 units/ml for 1 h at 67 °C.

Immunoblots

SDS/PAGE was carried out using 10% minigels. For immunoblot analysis, the proteins were transferred to a poly(vinylidene fluoride) membrane and residual protein-binding sites on the membrane were blocked with 1% gelatine in PBS. The blots were then incubated for 3 h at room temperature with anti-tau sera 134 (dilution 1:1000) or T3P (dilution 1:250) or with monoclonal antibodies AT270, AT180 or AT8 (each at a dilution of 1:500). Bound antibody was detected by the biotin/peroxidase system (Vectastain; Vector, Peterborough, U.K.).

Electron microscopy

Dispersed PHFs were extracted from an Alzheimer's diseased brain [20]. Methods for electron microscopy and immunolabelling were as described previously [20]. Monoclonal anti-tau antibodies AT270, AT180 and AT8 were used at a dilution of 1:100. Filaments were dephosphorylated by treatment with *E. coli* alkaline phosphatase (type III-N; Sigma) at 13.5 units/ml for 12 h at 67 °C. Micrographs were recorded on a Philips EM301, at an operating voltage of 80 kV and at a nominal magnification of $\times 25000$.

RESULTS

Monoclonal antibodies AT270 and AT180 were produced using PHF tau as the immunogen. They recognized PHF tau on immunoblots but failed to recognize the six non-phosphorylated recombinant human brain tau isoforms (Figure 1). However, they did stain a proportion of tau from immature and adult rat brain in a phosphorylation-dependent manner, as the staining

Figure 1 Immunoblots of fetal and adult brain tau and of PHF tau with anti-tau serum 134 and monoclonal antibodies AT8, AT270 and AT180

Lanes: 1, recombinant tau isoforms; 2, PHF tau; 3, tau from newborn rat brain; 4, tau from newborn rat brain, treated with alkaline phosphatase; 5, tau from adult rat brain; 6, tau from adult rat brain, treated with alkaline phosphatase.

Figure 2 Immunoblots of developing and adult rat brain tau with anti-tau serum 134 and monoclonal antibodies AT8, AT270 and AT180

Lanes: 1, 19 days of gestation; 2, 1 week old; 3, 2 weeks old; 4, 4 weeks old; 5, 8 weeks old; 6, 4 months old.

Figure 3 Immunoblots of fetal, postnatal and adult human brain with anti-tau serum 134 and monoclonal antibodies AT8, AT270 and AT180

Lanes: 1, 20 weeks of gestation; 2, 23 weeks of gestation; 3, 39 weeks of gestation; 4, postnatal day 5; 5, adult.

was completely abolished after treatment with alkaline phosphatase (Figure 1). In contrast, the previously characterized phosphorylation-dependent anti-tau antibody AT8 [25,33] reacted with PHF tau and immature rat brain tau, but not to any significant extent with adult rat brain tau.

This difference between AT270, AT180 and AT8 was confirmed when tau was extracted from the brains of rats of different ages (Figure 2). Antibody 134 showed the presence of two broad tau bands at embryonic day 19 and postnatal day 7, but thereafter it ran as a smear of increasing apparent molecular mass. AT8

Figure 4 Phosphorylation of wild-type and mutated recombinant tau (expressed from clone htau24) with the protein kinase activity from rat brain

Immunoblots were with anti-tau serum 134 and monoclonal antibodies AT8 and AT270. Lanes: 1, htau24; 2, htau24 plus brain extract; 3, T175A htau24; 4, T175A htau24 plus brain extract; 5, T181A htau24; 6, T181A htau24 plus brain extract.

Figure 5 Phosphorylation of wild-type and mutated recombinant tau (expressed from clone htau24) with a rat brain extract

Immunoblots were with anti-tau serum 134 and monoclonal antibodies AT8 and AT180. Lanes: 1, htau24; 2, htau24 plus brain extract; 3, T231A htau24; 4, T231A htau24 plus brain extract; 5, S235A htau24; 6, S235A htau24 plus brain extract.

strongly recognized the upper part of the tau band at embryonic day 19 and at postnatal day 7. At 2 weeks it still strongly recognized the same band, as well as a band of higher apparent molecular mass. At 4 weeks AT8 weakly labelled three bands, with no significant labelling at 8 weeks and 4 months (Figure 2).

AT270 gave only very weak labelling at embryonic day 19. At postnatal day 7 it strongly labelled one band. The labelling intensity peaked at 2 weeks; it then decreased somewhat in intensity, but was still clearly visible at 8 weeks and 4 months (Figure 2). AT180 recognized tau at embryonic day 19, where it labelled both bands. The same pattern was observed at 1 week, and it was stronger but more diffuse at 2 weeks. The staining

Figure 6 Phosphorylation of recombinant tau (expressed from clone htau24) with p42 MAP kinase, GSK3 β and p42 MAP kinase plus GSK3 β

Immunoblots were with anti-tau sera 134 and T3P and monoclonal antibodies AT270 (a) and AT180 (b). Lanes: 1, htau24; 2, htau24 plus p42 MAP kinase; 3, htau24 plus GSK3 β ; 4, htau24 plus p42 MAP kinase and GSK3 β ; 5, htau24.

persisted at 4 weeks, 8 weeks and 4 months, although the labelling intensity decreased somewhat with increasing age (Figure 2).

Similar results were obtained with developing human cerebral cortex (Figure 3). As described previously [25], AT8 recognized a proportion of tau extracted from cerebral cortex of fetuses at 20, 23 and 39 weeks of gestation, but failed to stain tau from postnatal day 5 or adult cortex. In contrast, AT270 and AT180 labelled some tau at 20, 23 and 39 weeks of gestation, as well as a proportion of tau from postnatal day 5 and adult cerebral cortex (Figure 3); strongest staining was around birth.

The epitopes of AT270 and AT180 were determined by phosphorylating wild-type tau and tau mutated individually at each of the 14 serine/threonine-proline sites using rat brain extract. The phosphorylation resulted in an increase in the apparent molecular mass of recombinant tau and in the generation of the AT8 epitope, as reported previously [25]; it also produced the AT270 (Figure 4) and AT180 (Figure 5) epitopes. Of the 14 mutant recombinant tau proteins, AT270 failed to recognize only recombinant tau with a Thr-181 to Ala mutation

Figure 7 Immunoelectron microscopy of dispersed PHF preparations

(a, c, e) Native material; (b, d, f) PHFs treated with alkaline phosphatase before immunodecoration. The monoclonal antibodies were AT270 (a and b), AT180 (c and d) or AT8 (e and f). Note immunogold decoration of filaments in (a), (c) and (e), but not in (b), (d) and (f). Scale bar, 200 nm.

(T181A), when phosphorylated with rat brain extract (Figure 4). AT27, another novel phosphorylation-dependent monoclonal anti-tau antibody, showed a specificity identical with that of AT270, indicating that the two antibodies recognize the same or a very similar epitope (results not shown). Of the 14 mutant recombinant tau proteins, AT180 failed to recognize recombinant tau with a Thr-231 to Ala mutation (T231A), when phosphorylated with rat brain extract (Figure 5). AT180 recognized tau with a Ser-235 to Ala mutation (S235A) only very weakly (Figure 5).

The effects of phosphorylation of recombinant tau by p42 MAP kinase and/or GSK3 β on the AT270 and AT180 epitopes were also investigated (Figure 6). A prolonged 20 h incubation with 1 unit/ml kinase resulted in the incorporation of 8 mol of phosphate/mol of tau with p42 MAP kinase and 4 mol of phosphate/mol of tau with GSK3 β . Tau phosphorylated with p42 MAP kinase showed a larger increase in apparent molecular mass than tau phosphorylated with GSK3 β (Figure 6). Recombinant tau phosphorylated with p42 MAP kinase was labelled by AT270 and T3P (Figure 6a), but not by AT180 (Figure 6b). Recombinant tau phosphorylated with GSK3 β was

labelled by T3P, but not by AT270 or AT180 (Figure 6). In contrast, recombinant tau phosphorylated using p42 MAP kinase and GSK3 β combined was immunoreactive with AT180 (Figure 6b).

By immunoelectron microscopy, both AT270 and AT8 decorated approx. 85 % of dispersed PHFs (Figures 7a and 7e). AT180 decorated approx. 70 % of dispersed PHFs, although more sparsely than AT270 or AT8 (Figure 7c). The labelling with all three antibodies was phosphorylation-dependent, as it was greatly reduced in PHFs treated with alkaline phosphatase (Figures 7b, 7d and 7f).

DISCUSSION

One way to identify amino acids that are phosphorylated in immature tau, adult tau or PHF tau is to use antibodies that recognize phosphorylated residues within a specific amino acid sequence [12,17,25,39–41]. We report here the characterization of AT270 and AT180, two novel monoclonal anti-tau antibodies that were produced using PHF tau as the immunogen. On immunoblots, AT270 and AT180 did not recognize recombinant

tau, but did so after phosphorylation with a rat brain extract. Recombinant human brain tau proteins mutated individually at each of the 14 serine/threonine-proline sites and phosphorylated with brain extract or purified kinases were then used to identify the AT270 and AT180 epitopes.

The AT270 epitope was produced by incubating recombinant tau with brain extract or with p42 MAP kinase but not with GSK3 β . The epitope was not produced in recombinant tau with a Thr-181 to Ala mutation, identifying phosphorylation of this residue as essential for the AT270 epitope. The situation with the AT180 epitope was more complicated. Incubation of recombinant tau with brain extract or with a mixture of p42 MAP kinase and GSK3 β but not with the purified kinases separately produced the AT180 epitope. AT180 failed to recognize phosphorylated recombinant tau with a Thr-231 to Ala mutation, while recognizing phosphorylated tau with a Ser-235 to Ala mutation only weakly.

The most likely explanation for these observations with AT180 is as follows. The preferred consensus sequence for phosphorylation by MAP kinase is PXS/TP [42,43], although the first proline in that sequence is not obligatory. GSK3 has an unusual specificity in that it phosphorylates serine and threonine residues positioned N-terminal to another phosphoserine [44,45], the preferred consensus sequence being S/TXXXX(P) [45]. The tau sequence in the region of the AT180 epitope corresponds to the consensus sequence of GSK3 (TPPKSP, residues 231–236). This suggests that p42 MAP kinase phosphorylates tau at Ser-235, but not at Thr-231. Once phosphorylated by MAP kinase, tau can then be phosphorylated by GSK3 β at Thr-231, resulting in the generation of the AT180 epitope. The fact that phosphorylated recombinant tau with the Ser-235 to Ala mutation is weakly recognized by AT180 probably indicates that Thr-231 can be phosphorylated to some extent by brain extract in the absence of prior phosphorylation of Ser-235. Although phosphorylation of Ser-235 may contribute, it is phosphorylation of Thr-231 that is decisive in producing the epitope for AT180. A similar dependence of phosphorylation of Thr-231 on prior phosphorylation of Ser-235 was observed when tau was phosphorylated with tau protein kinase I and tau protein kinase II [46]. It has recently been shown that tau protein kinase I is the same as GSK3 β , whereas tau protein kinase II is probably identical with cyclin-dependent kinase 5 (cdk5) [47,48]. This shows that *in vitro* GSK3 β phosphorylates Thr-231 after prior phosphorylation of Ser-235 by either MAP kinase or cdk5.

As shown by immunoelectron microscopy, AT270 decorated approximately 85 % and AT180 approximately 70 % of dispersed PHFs in a phosphorylation-dependent manner, indicating that the region in tau up to Thr-231 is exposed on the surface of the PHF. The microtubule-binding repeat region forms the core of the PHF, whereas the N- and C-terminal sequences with the serine/threonine-proline residues extend from its surface (for a review, see ref. [49]). The decoration with AT180 was sparser than that with AT270 or AT8, possibly indicating reduced accessibility in the PHF for this epitope. All three sites could be dephosphorylated without gross disruption of the structure of the assembled PHF.

On immunoblots, AT270 and AT180 labelled PHF tau, some tau from immature rat and human brain and some tau from adult rat and human brain. These results establish Thr-181 and -231 as normal phosphorylation sites in immature and adult brain tau; both sites are more heavily phosphorylated in PHF tau. Thus the present findings extend knowledge of tau phosphorylation in the physiological setting of the immature and adult brain. They also confirm and extend knowledge of tau phosphorylation in the pathological context of the PHF of Alzheimer's disease.

Thr-181 and Thr-231 and Ser-202 and Ser-396 are partially phosphorylated in tau from immature rat and human brain. Unlike Ser-202 and Ser-396 [12,17,25], Thr-181 and Thr-231 are still partially phosphorylated in adult brain tau, demonstrating that there is selective dephosphorylation of tau during brain development. It appears that phosphorylation of the different sites follows different developmental profiles, with phosphorylation of the AT8 epitope peaking somewhat earlier than that of the AT270 and AT180 epitopes. A very recent study on rat brain using mass spectrometry and phosphopeptide sequencing has also shown that Thr-181 and Thr-231 are phosphorylated more extensively in fetal tau than in adult tau [50].

Previously, serine residues 202, 235, 262 and 396 and threonine residue 231 were identified as phosphorylated sites in PHF tau [23]. We confirm Thr-231 as a phosphorylated site and show that Thr-181 is also phosphorylated in PHF tau. One difference between PHF tau and tau from immature and adult brain is that, for a given site, PHF tau appears to be fully phosphorylated, whereas only a proportion of tau is phosphorylated in normal brain. Future experiments will address this issue in a more quantitative manner and establish developmental profiles for the various sites.

The picture that emerges is that tau in normal adult brain is heterogeneously phosphorylated at a small number of serine/threonine-prolines, whereas in PHFs it is fully phosphorylated at a large number of such sites. In immature brain, as in PHFs, tau is phosphorylated at a large number of serine/threonine-prolines; however, as in adult brain, the phosphorylation is only partial. This quantitative difference between tau from immature brain and PHF tau may explain why PHFs do not form in developing brain. It may also explain why tau from immature brain can still bind to microtubules [18], whereas PHF tau has completely lost this ability [17,18].

In Alzheimer's disease tau is thus hyperphosphorylated, implying the deregulation of some phosphorylation/dephosphorylation mechanisms. MAP kinase, GSK3 and proline-directed protein kinases phosphorylate at least some of the abnormal phosphorylation sites in tau *in vitro* [26–32]. Moreover, three protein kinases that have been purified by virtue of their ability to phosphorylate tau *in vitro* to a form similar to PHF tau are closely related to or identical with the above [47,48,51]. However, it is unclear whether these kinases cause the abnormal phosphorylation of PHF tau in Alzheimer's disease. It is not known whether hyperphosphorylation results from an increased protein kinase or a decreased protein phosphatase activity or an inappropriate cellular localization of tau itself. Future experiments will address these issues.

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