

# Characterization of mAb AP422, a novel phosphorylation-dependent monoclonal antibody against tau protein

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Received 29 January 1996; revised version received 1 March 1996

**Abstract** A monoclonal antibody (AP422) specific for phosphoserine 422 in microtubule-associated protein tau has been produced. It strongly labels paired helical filament (PHF) tau from Alzheimer's disease brain in a phosphorylation-dependent manner. By contrast, AP422 only labels a small fraction of fetal tau and a very small fraction of tau from adult brain. The amount of tau phosphorylated at Ser-422 in normal brain is minor relative to that phosphorylated at sites recognized by other phosphorylation-dependent anti-tau antibodies of known epitope. It follows that AP422 is the most specific anti-tau antibody available for detecting the neurofibrillary lesions of Alzheimer's disease. We also show that Ser-422 in tau is a good *in vitro* substrate for mitogen-activated protein kinase, but not for glycogen synthase kinase-3 or neuronal cdc2-like kinase.

**Key words:** Tau protein; Phosphorylation; MAP kinase; 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 (AD). Hyperphosphorylated microtubule-associated protein tau (PHF-tau) is the major component of the PHF (for reviews, see Refs. [1–3]). 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 [4–8]. Approximately half of these sites are serine/threonine-prolines [8]. Phosphorylation at many of these sites is also present in a significant fraction of fetal tau [7,9,10] and studies using biopsy-derived tau have shown that they are also phosphorylated in a small fraction of tau from adult human brain [11]. The hyperphosphorylation of tau could result from an increased activity of a tau kinase or the decreased activity of a tau phosphatase. Based on *in vitro* experiments mitogen-activated protein (MAP) kinase [12], neuronal cdc2-like kinase (NCLK) [13,14] and glycogen synthase kinase-3 (GSK3) [15,16] are candidate protein kinases for the hyperphosphorylation of tau, whereas protein phosphatase 2A [17,18] is a candidate phosphatase. It has recently been shown that significant proportions of MAP kinase [19] and phosphatase 2A [20] are associated with microtubules in cultured cells. However, it is not clear which protein kinases and protein phosphatases act on tau in normal and AD brain.

In order to investigate the hyperphosphorylation of PHF-tau further, we have produced and characterized a monoclonal antibody (AP422) directed against phosphoserine 422 in

tau (using the nomenclature of the longest human brain tau isoform). We show here that AP422 strongly recognizes PHF-tau; it also recognizes a small fraction of fetal human tau and biopsy-derived adult human tau, as well as a small fraction of fetal and adult rat tau. However, the amount of tau phosphorylated at Ser-422 in normal brain is very small relative to that phosphorylated at sites recognized by other phosphorylation-dependent anti-tau antibodies. We also show that Ser-422 is a good substrate for MAP kinase, but not for NCLK and GSK3.

## 2. Materials and methods

### 2.1. Antibodies

Monoclonal antibody (mAb) AP422 was raised against a synthetic phosphopeptide, CGGIDMVD(S)PQLAT, where S(P) represents phosphoserine. 50 µg of peptide was injected into the footpads of BALB/c mice, which were boosted with another 50 µg of peptide on days 4, 7, and 12. On day 14 the regional lymph nodes were removed and the cells fused with myeloma cells (line PA1) using polyethylene glycol 4000. Screening of positive clones was done by enzyme-linked immunosorbent assay. Ascites was prepared by intraperitoneal injection of cloned hybridoma cells and used in all experiments. The production of an antiserum against phosphoserine 422 (AS AP422) has been described previously [8]. BR134 recognizes the carboxy-terminus of tau [21], whereas AT8, C5 and M4 are previously described phosphorylation-dependent anti-tau antibodies [7,22,23]. Antibodies were diluted as follows: 134 (1:2500), mAb AP422 (1:2000), M4 (1:3000), C5 (1:2000) for immunoblotting of site-directed mutants; 134 (1:3000), AT8 (1:2000), mAb AP422 (1:3000) for immunoblotting of Sarkosyl-PHF and dephosphorylated PHF-tau; 134 (1:5000), AT8 (1:2000), mAb AP422 (1:2000) for immunoblotting of human and rat tau; 134 (1:5000), AT8 (1:2000), mAb AP422 (1:5000) for immunoblotting of htau40 phosphorylated by MAP kinase, NCLK or GSK3.

### 2.2. Preparation of recombinant tau and phosphorylation

A full-length cDNA clone encoding the 383 amino acid isoform of human tau (htau24) [21] was used to mutate codons 202 (Ser to Ala; S202A), 231 (Thr to Ala; T231A), 235 (Ser to Ala; S235A), 396 (Ser to Ala; S396A), 404 (Ser to Ala; S404A) and 422 (Ser to Ala; S422A). Wild-type and mutated tau proteins were expressed and purified as described [24]. Phosphorylation of recombinant tau with activated recombinant p42 MAP kinase [24], reconstituted recombinant NCLK [25] and GSK3  $\alpha$  and  $\beta$  purified from rabbit skeletal muscle [26] were carried out as described, in the presence or absence of 100 µg/ml heparin [18,23]. Phosphorylation of recombinant tau by total rat brain protein kinase activity was carried out as described [24].

### 2.3. Extraction of tau protein and immunoblotting

Tau proteins were extracted from brains of 1-day-old and 10-week-old rats and immunoblotted, as described [24]. The brains were homogenized in 2.5% perchloric acid within 1 min after death, so as to prevent the action of phosphatases on tau. Tau from a fetal human brain (21 weeks of gestation) and biopsy-derived adult human brain tau were prepared as described [11]. PHF-tau was prepared from a Down's syndrome brain according to Greenberg and Davies [27], with minor modifications. The Sarkosyl-insoluble pellets were resuspended

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in 6 ml 50 mM Tris-HCl, pH 7.4, and re-centrifuged. The pellets were resuspended in 50 mM Tris-HCl (250  $\mu$ l per g starting material, Sarkosyl-PHF1), or in 50 mM Tris-HCl containing 7 M guanidine-HCl and 2%  $\beta$ -mercaptoethanol. Guanidine-HCl solubilized PHF-tau was dialyzed overnight at 4°C against 20 mM Tris-HCl, pH 7.4, and centrifuged for 10 min at 346 000 $\times$ g. The resulting supernatant was used (Sarkosyl-PHF2). Prior to alkaline phosphatase treatment Sarkosyl-insoluble pellets and guanidine-HCl solubilized PHF-tau were dialyzed against 50 mM Tris-HCl, pH 8.0, containing 1 mM PMSF and 0.1 mM EDTA. *E. coli* alkaline phosphatase (13.5 U/ml; type III-N, Sigma) and 1 mM MgCl<sub>2</sub> were added and incubations carried out at 67°C for 2 h.

#### 2.4. Immunohistochemistry and immunoelectron microscopy

Occipito-temporal cortex from an AD brain was fixed in phosphate-buffered 4% paraformaldehyde for 24 h at 4°C. Vibratome sections (50  $\mu$ m) were immunostained with mAb AP422 at a dilution of 1:1000 in PBS containing 10% fetal calf serum and developed using avidin-biotin (Vectastain). Immunolabelling of dispersed PHFs extracted from AD brain with mAb AP422 was carried out as described [7]. MAb AP422 was used at a dilution of 1:1000 in PBS containing 10% fetal calf serum. Filaments were dephosphorylated by treatment with *E. coli* alkaline phosphatase (27 U/ml; type III-N, Sigma) for various times at 67°C. Micrographs were recorded on a Philips EM301, at an operating voltage of 80 kV and at a nominal magnification of  $\times$ 25 000.

### 3. Results

#### 3.1. Antibody specificities

For epitope mapping of tau antibodies we used htau24 to prepare wild-type recombinant tau, S202A tau, T231A tau, S235A tau, S396A tau, S404A tau and S422A tau. Phosphorylation with brain extract resulted in a marked reduction in the gel mobility of recombinant tau. MAb AP422 failed to recognize wild-type or mutated recombinant tau prior to phosphorylation (Fig. 1). However, following an overnight

incubation with brain extract, mAb AP422 recognized wild-type tau and all mutated tau proteins, with the exception of phosphorylated S422A tau (Fig. 1). Immunoblotting with AS AP422 gave similar results; however, weak staining was detected with non-phosphorylated wild-type tau, when a large amount of tau was loaded, suggesting that this antiserum may not be entirely phosphorylation-dependent. By comparison, monoclonal antibodies M4 and C5, which were raised against PHF-tau, showed different patterns. M4 stained phosphorylated wild-type tau, S202A tau, S396A tau, S404A tau and S422A tau, but failed to stain T231A tau (Fig. 1). M4 also hardly recognized phosphorylated S235A tau, indicating that it is specific for phosphorylated Thr-231 and Ser-235. C5 stained phosphorylated wild-type tau and mutated tau proteins, with the exception of S396A tau (Fig. 1), indicating that it is specific for phosphorylated Ser-396.

#### 3.2. Immunoreactivity of PHF-tau, human brain tau and rat brain tau with mAb AP422

MAb AP422 strongly labelled PHF-tau in either the Sarkosyl-PHF1 or the cleaner Sarkosyl-PHF2 fraction (Fig. 2). The staining intensity was similar to that given by antibody 134 and the phosphorylation-dependent anti-tau antibody, AT8 (Figs. 2 and 3). Sarkosyl-insoluble PHFs and guanidine-HCl solubilized PHF-tau were dephosphorylated by alkaline phosphatase and immunoblotted with 134, AT8 and mAb AP422. Alkaline phosphatase treatment of intact PHFs resulted in marked changes in the electrophoretic mobility of PHF-tau and the loss of AT8 immunoreactivity (Fig. 2). By contrast, mAb AP422 immunoreactivity was only reduced. Similar results were obtained with PHF1 which recognizes phosphorylated serines 396 and 404 (data not shown). However, alkaline phosphatase treatment of guanidine-HCl solubilized PHF-tau

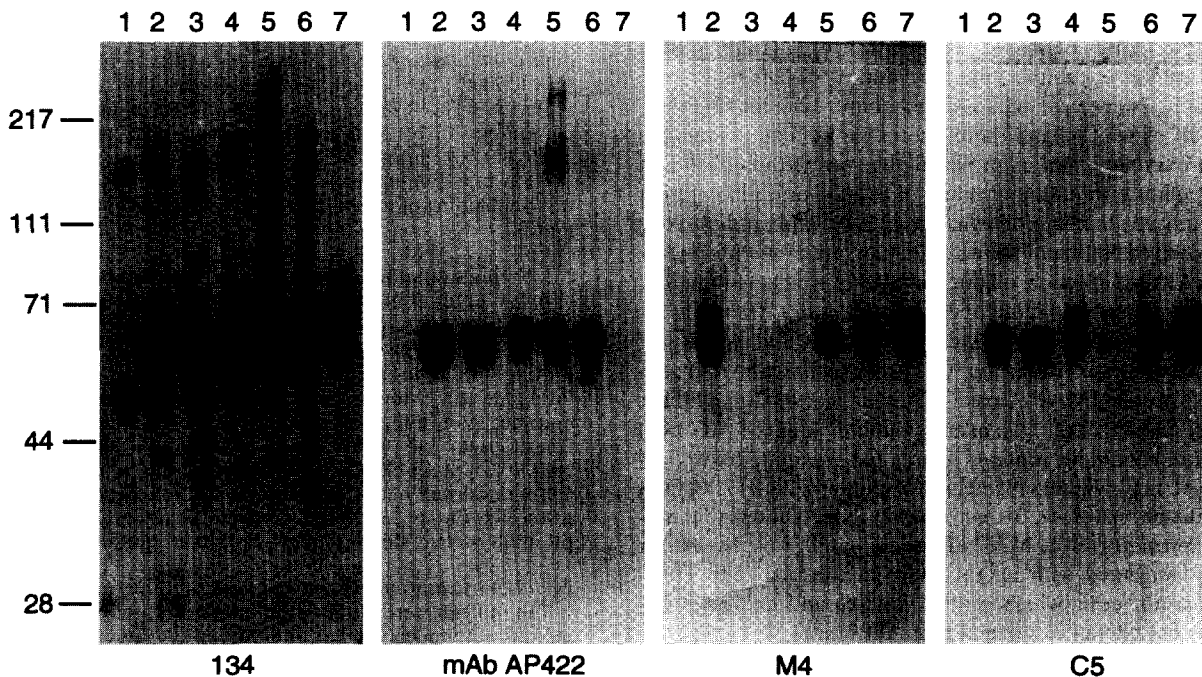


Fig. 1. Phosphorylation of wild-type and mutated recombinant tau proteins (htau24) with rat brain extract. Immunoblots were stained with anti-tau serum 134 and phosphorylation-dependent monoclonal anti-tau antibodies AP422, M4 and C5. Lanes: 1, htau24; 2, htau24+ brain extract; 3, T231A htau24+brain extract; 4, S235A htau24+ brain extract; 5, S396A htau24+brain extract; 6, S404A htau24+ brain extract; 7, S422A htau24+brain extract.

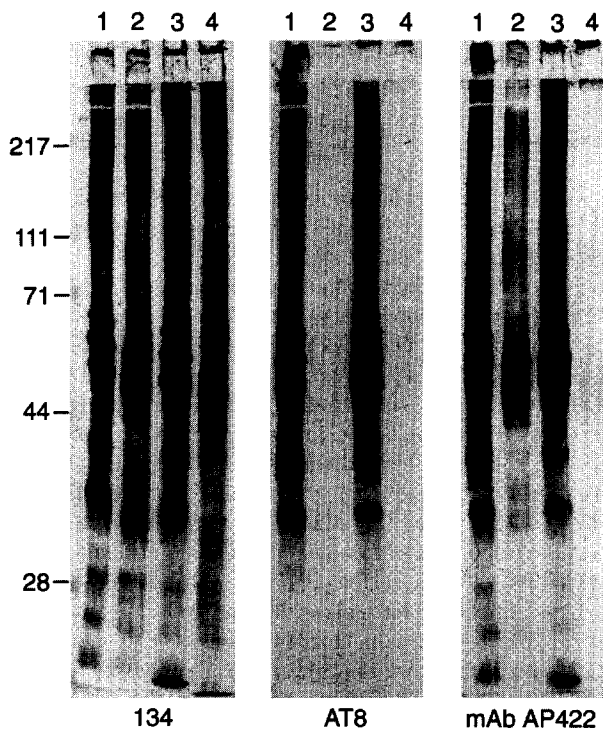


Fig. 2. Immunoblots of Sarkosyl-PHF-tau and dephosphorylated PHF-tau with anti-tau serum 134 and phosphorylation-dependent monoclonal anti-tau antibodies AT8 and AP422. Lanes: 1, Sarkosyl-PHF-tau; 2, Sarkosyl-PHF-tau incubated with alkaline phosphatase; 3, guanidine-treated PHF-tau; 4, guanidine-treated PHF-tau incubated with alkaline phosphatase.

resulted in the removal of mAb AP422 immunoreactivity (Fig. 2).

We next examined whether the phosphorylation of Ser-422 can be detected in fetal human tau and in biopsy-derived adult human tau. MAb AP422 only faintly stained human fetal tau which by comparison was strongly labelled by AT8 (Fig. 3). When the same amount of biopsy-derived adult hu-

man tau (2  $\mu$ g) as that of fetal tau was immunoblotted, no immunoreactivity could be detected with mAb AP422, while AT8 showed significant staining (data not shown). When the amount of biopsy-derived tau was increased 6-fold (12  $\mu$ g), very weak staining could be detected with mAb AP422 (Fig. 3). This faint staining cannot be explained by a low antibody titre, since PHF-tau was labelled equally strongly by mAb AP422 and AT8, while the converse was true of biopsy-derived adult human tau (Figs. 2 and 3). Similar to tau from human brain, a fraction of fetal rat tau was stained by mAb AP422, whereas tau from adult rat brain was only stained very weakly (Fig. 3). Tau was the only protein recognized by mAb AP422 in a crude extract from newborn rat brain.

### 3.3. Immunostaining with mAb AP422

Vibratome sections from AD cerebral cortex were stained with either mAb AP422 or AS AP422. Both antibodies strongly labelled intracellular neurofibrillary tangles, neuropil threads and plaque neurites (Fig. 4). By immunoelectron microscopy mAb AP422 strongly decorated a majority of dispersed PHFs (Fig. 5a), similar to the labelling with antibody 134. Alkaline phosphatase treatment of PHFs at 67°C for 2 h failed to remove the labelling with mAb AP422. However, when alkaline phosphatase was added twice over a 24 h period at 67°C, the labelling was abolished (Fig. 5b).

### 3.4. Immunoreactivity of tau following phosphorylation with MAP kinase, GSK3 and NCLK

The 441 amino acid isoform of human brain tau was phosphorylated with MAP kinase, NCLK or GSK3  $\alpha$  and  $\beta$ , in the presence or absence of 100  $\mu$ g/ml heparin, a known activator of tau phosphorylation with a number of protein kinases [28–30]. In the presence of heparin recombinant tau incorporated 8 mol phosphate/mol of protein; tau incubated with GSK3 $\beta$  and heparin incorporated 10 mol phosphate/mol protein, whereas tau incubated with NCLK and heparin incorporated 12 mol phos-

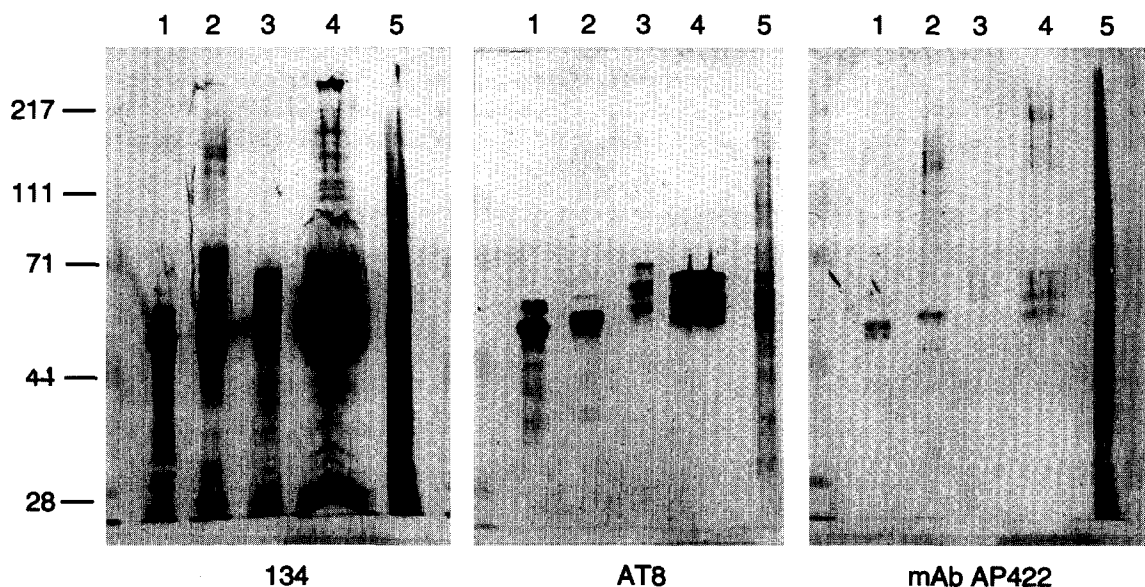


Fig. 3. Immunoblots of PHF-tau, fetal tau and biopsy-derived adult tau with anti-tau serum 134 and phosphorylation-dependent monoclonal anti-tau antibodies AT8 and AP422. Total protein loaded is indicated in brackets. Lanes: 1, fetal rat tau (2  $\mu$ g); 2, fetal human tau (2  $\mu$ g); 3, adult rat tau (2  $\mu$ g); 4, biopsy-derived adult human tau (12  $\mu$ g); 5, PHF-tau (Sarkosyl-PHF2) (1.5  $\mu$ g).



Fig. 4. AD cortex immunostained with mAb AP422. Note the staining of numerous neurofibrillary tangles, neuropil threads and plaque neurites. Magnification,  $\times 400$ .

phate/mol protein. Phosphorylation with NCLK or GSK3 in the presence of heparin resulted in a dramatic shift in the gel mobility of tau and generated the AT8 epitope, but not the epitope of mAb AP422 (Fig. 6). Phosphorylation by MAP kinase with or without heparin also resulted in a marked reduction in the gel mobility of tau and generated not only the AT8 epitope, but also the epitope of mAb AP422 (Fig. 6). Weak staining of tau phosphorylated by NCLK in the pres-

ence of heparin could be detected with mAb AP422 when a large amount of phosphorylated tau was immunoblotted (data not shown).

#### 4. Discussion

By using a combination of site-directed mutagenesis and *in vitro* phosphorylation we have shown that mAb AP422 is specific for tau phosphorylated at Ser-422. AP422 strongly recognized PHF-tau on immunoblots and decorated PHFs by electron microscopy, in agreement with previous mass spectrometric results demonstrating the presence of phosphoserine 422 in PHF-tau [8]. By contrast, AP422 recognized fetal human and rat tau only weakly. Similarly, biopsy-derived adult human tau and adult rat tau gave only a very weak signal when immunoblotted with AP422. These results show that mAb AP422 gives a staining pattern that is qualitatively similar to that given by other phosphorylation-dependent anti-tau antibodies of known epitope (antibodies AT8, AT180, M4, AT270, 12E8, PHF1 and C5)[7,11,22–24,31]. All these antibodies recognize PHF-tau more strongly than fetal tau which is in turn labelled more strongly than adult tau. However, there are important quantitative differences in that AP422 recognized a much smaller fraction of fetal and adult tau than the other antibodies. This was especially true of biopsy-derived adult human tau which was hardly recognized by mAb AP422. Thus, AP422 is almost entirely specific for PHF-tau and is presently the most specific such antibody of known epitope available. The low levels of phosphorylation probably explain previous failures to detect phosphorylation at Ser-422 in fetal and adult rat tau by mass spectrometry or by immunoblotting with AS AP422 [8]. It follows that in tau from normal brain Thr-181, Ser-202, Thr-205, Thr-231, Ser-235, Ser-262, Ser-396 and Ser-404 are phosphorylated to a greater extent than Ser-422, whereas similar proportions are phosphorylated at each site in PHF-tau. These results indicate that in tau from normal brain different sites are phosphorylated to different extents. Dephosphorylation experiments showed that mAb AP422 recognizes PHFs and PHF-tau in a phosphorylation-dependent manner. They also demonstrated that phosphorylated Ser-396, Ser-404 and Ser-422 in tau from PHFs are much more resistant to alkaline phosphatase than are phosphorylated Ser-202 and Thr-205.

Many of the phosphorylation sites in tau are serine/threo-



Fig. 5. Immunoelectron microscopy of dispersed PHFs with mAb AP422. (a) Native material; (b) PHFs treated with alkaline phosphatase before immuno-decoration. Note immuno-decoration of filaments in (a). Scale bar, 200 nm.

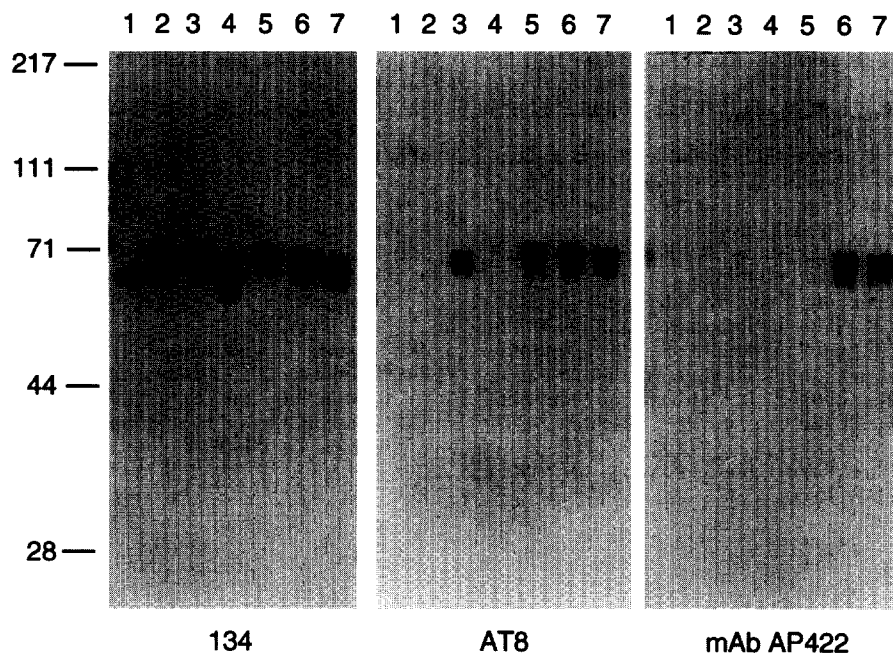


Fig. 6. Phosphorylation of the 441 amino acid isoform of human tau (htau40) with NCLK, GSK3 and MAP kinase in the presence or absence of heparin. Immunoblots were stained with anti-tau serum 134 and phosphorylation-dependent monoclonal anti-tau antibodies AT8 and AP422. Lanes: 1, htau40; 2, htau40+NCLK; 3, htau40+NCLK+heparin; 4, htau40+GSK3 $\beta$ ; 5, htau40+GSK3 $\beta$ +heparin; 6, htau40+MAP kinase; 7, htau40+MAP kinase+heparin.

nine-prolines which can be phosphorylated *in vitro* by proline-directed protein kinases, such as MAP kinase, NCLK and GSK3. Of these only MAP kinase phosphorylated recombinant tau at Ser-422, as demonstrated by the generation of the AP422 epitope. GSK3 failed to phosphorylate Ser-422, even in the presence of heparin, a known activator of tau phosphorylation by a number of protein kinases [28–30]. Phosphorylation of recombinant tau by NCLK resulted in very weak labelling with AP422 when a large amount of phosphorylated tau was loaded. These results indicate that MAP kinase or a MAP kinase-like enzyme may be involved in the phosphorylation of PHF-tau. It is unknown whether hyperphosphorylation of tau in the AD brain results from an increased protein kinase activity, a decreased protein phosphatase activity, or a combination of both. It is also not known whether hyperphosphorylation of tau precedes PHF formation nor whether it is either necessary or sufficient for PHF formation. However, it is clear that the inability of PHF-tau to bind to microtubules is the direct result of its hyperphosphorylation [32,33]. Moreover, immunohistochemical studies on transentorhinal cortex have indicated that hyperphosphorylation of tau may precede PHF formation [34]. Current evidence on phosphorylation of tau by known protein kinases indicates that multiple enzymes are likely to be involved in the hyperphosphorylation of tau. One challenge for the future is to evaluate the contributions made by phosphorylation at individual sites to the pathological behaviour of tau in the AD brain. MAb AP422 will be a valuable reagent in this undertaking.

**Acknowledgements:** We thank P. Cohen for MAP kinase and GSK3  $\alpha$  and  $\beta$ , J.H. Wang for NCLK and E. Vanmechelen for AT8. This work was supported by a grant from the Japan Foundation for Aging and Health (to M.H.).

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