Phosphorylation and accumulation of tau without any concomitant increase in tubulin levels in Chinese Hamster Ovary cells stably transfected with human tau_{441}

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Abstract **Eucaryotic expression vectors bearing a 1.4 kb cDNA encoding the 4 repeat isoform of human tau, tau_{441}, in either the sense or anti-sense orientation with respect to a cytomegalovirus (CMV) promoter were constructed. The resulting constructs were used to transiently express tau in Chinese Hamster Ovary cells and to generate non-neuronal stable cell lines. Immunocytochemical studies of these cells show that tau is expressed in the sense but not the anti-sense or vector containing lines. Some of the cells expressing tau showed fine elongated processes which were stained by tau antibodies. The general tau immunostaining pattern appeared diffuse and punctuate. The expressed tau was seen both unbound and bound to microtubules. In some cells labeling with antibodies that specifically recognize hyperphosphorylation of tau was observed. The size of this population increased with increasing numbers of cell passages. However, no increase in steady-state tubulin level was observed following tau_{441} expression. These studies show that tau can accumulate in the cells without a concomitant increase in tubulin.**

Key words: Alzheimer disease; Microtubule associated protein tau; Phosphorylation of tau; Tubulin; Neurofibrillary degeneration; Cell model

1. Introduction

Tau is one of the two major microtubule associated proteins (MAPs) found in neurons. In normal adult human brain there are six molecular species of tau. These isoforms are products of a single gene and are generated by alternate splicing [1]. The six adult human tau isoforms differ from one another by having three or four tubulin binding repeat domains of 31–32 amino acids in the carboxy terminal half of the protein [2] and by the presence or absence of one or two identical inserts of 29 amino acids, each near its amino terminal end.

Tau stimulates microtubule assembly by polymerizing with tubulin and maintains the microtubule structure [3,4]. In addition, tau is a phosphoprotein and its biological activity is regulated by its degree of phosphorylation [5,6]. In Alzheimer's disease (AD) tau is abnormally hyperphosphorylated. It is this form that accumulates in affected neurons [7–10]. The abnormally phosphorylated tau isolated from AD brain is biologically inactive, but on dephosphorylation its microtubule assembly promoting activity can be restored to near normal levels [6,11]. Under microtubule assembly conditions, abnormally phosphorylated tau inhibits the assembly of microtubules; this inhibition appears to be caused by the binding of the abnormal tau to normal tau, preventing the latter to interact with tubulin [6].

The lack of a cell or animal model system for Alzheimer disease has considerably hampered efforts to elucidate the pathophysiological mechanisms that lead to the deterioration and ultimately the death of neurons in AD brains. In this paper we show that, under the control of cytomegalovirus (CMV) promoter, a human tau_{441} cDNA can be stably expressed in Chinese Hamster Ovary (CHO) cells; these cells do not express endogenous tau. Some of the expressed tau is phosphorylated, is not associated with microtubules and accumulates in the transfected cells. The expression of tau in CHO cells does not result in any detectable increase in the levels of tubulin.

2. Materials and methods

2.1. Construction of expression plasmids

The prokaryotic expression vector pRK172, bearing the four repeat recombinant isoform of human tau_{441}, was kindly provided by Dr. Michel Goedert [1]. The entire 1.4 kb tau_{441} insert from this plasmid was amplified by PCR using a 5′ primer, 5′-ATGGCTGAGCCCCGCCAGGAGTT-3′, coding for the first 23 bases in the human tau_{441}, cDNA sequence and a 3′ primer, 5′-TAATGGACCGCCCAGGGGCCT-3′, complementary to the last 23 bases of the coding sequence and subsequently subcloned into pCR™II (Invitrogen Corp., San Diego, CA). Clones with tau in both the sense (TA-T10), and the anti-sense (TA-T8) orientation with respect to the pCR™ II Sp6 promoter were identified by restriction analysis using SacI. Restriction enzyme analysis with *Sac*I revealed a small fragment of 328 bp and a larger fragment of 1,137 bp for the sense and anti-sense clones respectively. Tau_{441} and anti-tau_{441} eucaryotic expression vectors (Fig. 1) were constructed by inserting the *Bam*HI/*Xho*I fragments from TA-T10 and TA-T8 into pCDNA3 (Invitrogen Corp). The resulting plasmids, pCDNA3-T10 and pCDNA3-T8 were characterized by restriction analysis and PCR (Fig. 1c,i–iii). Transient and stable transfection studies (see below) were performed with plasmid DNAs purified using the Wizard-Prep method (Promega Corp., Madison, WI) according to the manufacturer's protocol.

2.2. Cell culture and geneticin, G418 selection

CHO cells were maintained in RPMI 1640 (Gibco-BRL, Gaithersburg, MD), supplemented with 10% fetal calf serum, 100 μg/ml penicillin G, and 100 μg/ml streptomycin in a 37°C humidified CO_2 incubator. Approximatley 1×10^6 cells were plated on 35 mm culture dish two days prior to transfection. Ten micrograms of DNA-lipofectin (Gibco-BRL, Gaithersburg, MD) complex was prepared and overlaid on cells containing serum- and antibiotic-free media. After 20 hr the cells were fed with fresh media supplemented with 10% fetal calf serum. After two days the cells were either harvested for transient expression analysis or subjected to 400 μg/ml of geneticin G418 (Gibco-BRL, Gaithersburg, MD) to select for stable transfectants. After three weeks some neomycin resistant colonies were formed.

2.3. Immunocytochemistry

Transfected CHO cells were washed twice with PBS (phosphate-

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buffered saline: 200 mM NaCl, 10 mM Na_2HPO_4, pH 7.0) at 37°C and fixed for 5 min at 37°C with 4% paraformaldehyde and 0.1% Triton X-100 in 100 mM PIPES, 5 mM EGTA, 2 mM $MgCl_2$. In some cases cells were first extracted with 0.1% Triton X-100 for 1–2 min at 37°C after incubating in a microtubule-stabilizing buffer (100 mM PIPES, 5 mM EGTA, 2 mM $MgCl_2$, 2 M glycerol, pH 6.8) [12,13]. After fixation, the cells were overlaid with ~ −20°C cold methanol for five minutes. The cells were washed three times with PBS, blocked with 2% BSA (bovine serum albumin) in TBS (Tris-buffered saline: 200 mM NaCl, 50 mM Tris, pH 7.6) buffer and incubated with the primary antibodies overnight in a humid chamber. Unbound antibody was removed by three washes with PBS. Bound antibody was detected by incubation for one hour at 37°C with pre-absorbed fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit or anti-mouse IgG, respectively. Secondary antibodies were diluted 1:500 in TBS/BSA. Primary antibodies used were the mouse monoclonal tau antibodies Tau-1 (ascites, 1:10,000) [14], AT8 (1:100) (Immunogenetics, Ghent, Belgium) and PHF-1 (culture supernatant, 1:500), [15], rabbit polyclonal anti-tau antibody 92e (1:5000) [16] and mouse monoclonal antibody DMIA to α tubulin (1:500) (Sigma, St. Louis, MO). Coverslips were mounted in Vecta shield (Vector Laboratories, Inc., Burlingame, CA). The cells were examined and photographed with a Zeiss Axioskop microscope equipped for epifluorescence (Carl Zeiss, Inc., Thornwood, NY).

2.4. SDS-PAGE and immunoblotting

Control and transfected CHO cells were harvested, washed in PBS and centrifuged at 1000 × *g* for 5 min at 40°C. The pellet was suspended in 500 μl lysis buffer (2%, w/v, SDS, 2%, v/v, 2-mercaptoethanol), sonicated for 5 min and boiled for 10 min. Total protein was measured using the modified Lowry method [17]. Proteins were resolved by SDS-PAGE on slab gels (80 × 60 × 0.75 mm), using a linear polyacrylamide gradient (7.5–15%) and the Laemmli buffer system [18]. 100 μg of total protein was loaded in each well. After electrophoresis, protein and prestained high molecular weight protein standards (Gibco-BRL, Gaithersburg, MD) were transferred to Immobilon membranes (0.45 μm; Millipore, Bedford, MA) at 105 V for 1 h and immunostained as previously described [7]. In some cases, blots were dephosphorylated with 149 U/ml alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) [7], for 3 h at 37°C. Antibody binding was either detected with the peroxidase anti-peroxidase method [19] or with alkaline phosphatase conjugated secondary antibodies.

3. Results

As a first step towards developing a cell culture model in which the effect of various modulating factors on the largest isoform of human tau could be examined, tau_{441} cDNA was cloned into the eukaryotic expression vector pCDNA3. This vector contains a constitutively active human cytomegalovirus promoter, which is known to drive high levels of heterologous

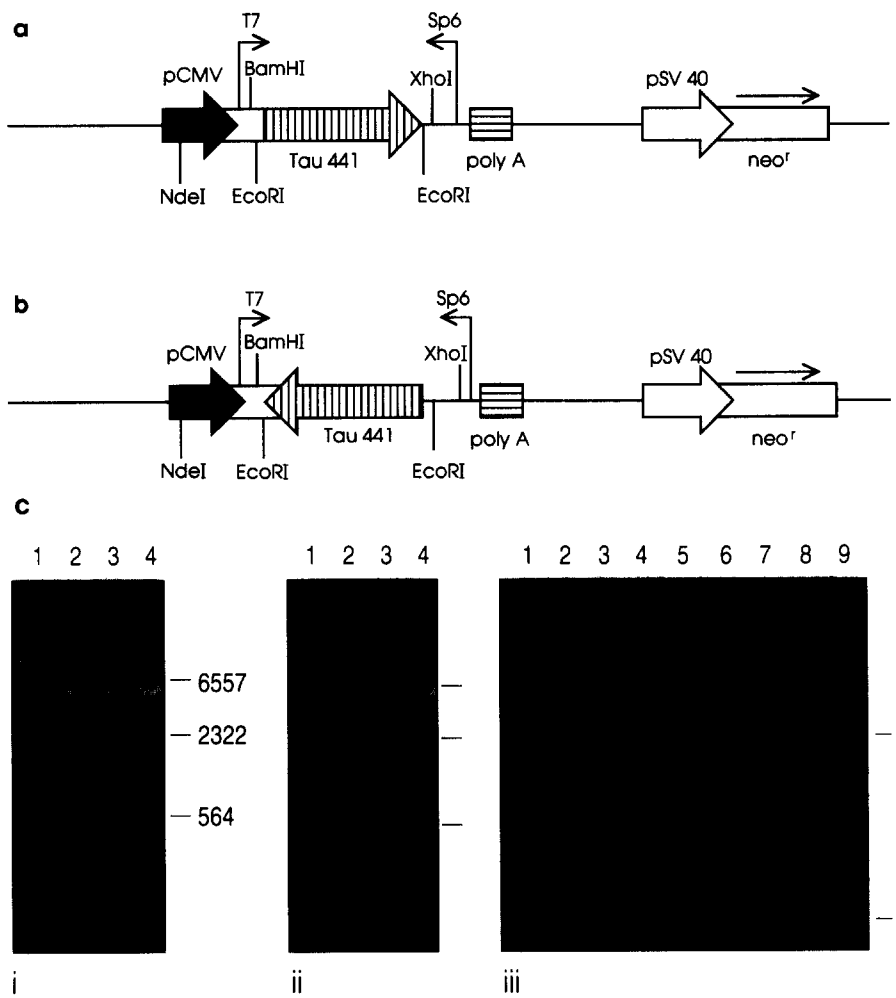

Fig. 1. Schematic diagram of tau pCDNA3 constructs and analysis of pCDNA3-tau_{441} clones. (a) Sense orientation; (b) Anti-sense orientation; (c) Selected miniprep DNAs containing the putative tau_{441} clones (lanes 2,3) and pCDNA-3 vector (lane 4) were analyzed for the presence of (i) *Eco*RI and (ii) *Nde*I and *Xho*I sites. The orientation of the clones (iii) was confirmed by PCR amplification of either, the complete CMV promoter (lanes 2–5) or CMV promoter and tau_{441} (lanes 6–9). Lanes 2,6, vector; lanes 3,7, sense; lanes 4,8, anti-sense; lanes, 5,9, control-pRC/CMV (lanes 2–5 CMV primers, lanes 6–9 CMV and tau_{441} primers).

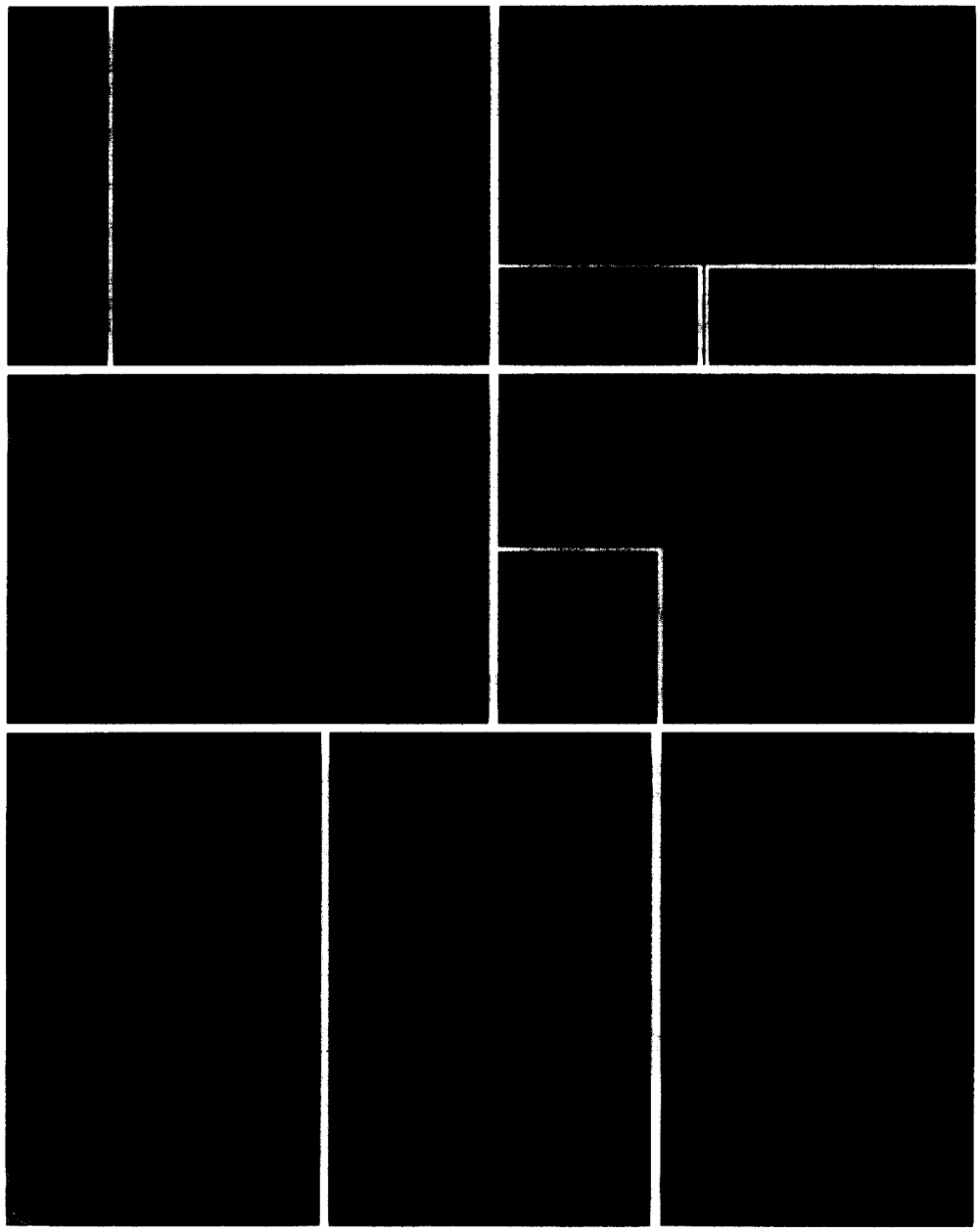

Fig. 2. Tau_{441} expression in CHO cells. Immunofluorescence staining of (a) transient and (b–g) stably transfected CHO cells was detected using (a,b) mAb Tau-1, (c) polyclonal antibody 92e and (d) mAb PHF-1. (e–g) Endogenous α-tubulin expression in CHO cells transfected stably with various constructs; tubulin expression was detected with mAb DM1A; (e) anti-sense to tau_{441}, (f) tau_{441} sense and (g) vector alone. Insert in: (a) shows a cell with a fine long process, (b) shows punctuate immunostaining of tau, and (b′) shows the binding of tau to the cytoskeleton in Triton X-100 extracted cells.

gene expression in a wide variety of mammalian cell lines. The full length 1.4 kb, four repeat isoform, of human tau was cloned and screened (Fig. 1) for a sense and an anti-sense clone.

We had previously observed that CMV-driven chloramphenicol acetyltransferase (CAT) gene constructs were extremely active in CHO cells 48 h following transfection (data not shown). Therefore, we initially examined whether human tau_{441} could be transiently expressed in CHO cells following the same protocol. Forty hours after transfection many cells were rounded though a small percentage had flattened. A few cells had small processes. Tau expression was not detected by immunoblotting but immunofluorescence studies showed punctuate staining with mAb Tau-1 (Fig. 2a) in tau_{441} transfected cells.

For stable transfection, cells were selected on the basis of their resistance to G418. Cells expressing tau_{441} were large and there were occasional giant cells with large nuclei and very long processes. These cells stained positive with tau antibodies. In general, tau expressing cells took a longer time to reach confluence but the expression of tau did not affect the viability of the cells. Immunofluorescence staining with antibodies to tau, as expected, did not show any immunoreactivity in untransfected CHO cells or cells transfected with the vector or anti-sense. Therefore, it was easy to screen the tau expressing cells.

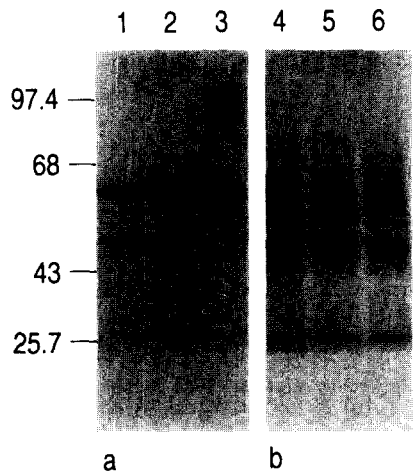

Fig. 3. Analysis of protein expression in CHO cells stably transfected with various constructs. Samples were electrophoresed and transferred to Immobilon. One half of the blot was stained with Coomassie blue (b); the other half was immunolabeled with mAb DM1A to tubulin (a). Twenty and 50 μg of protein/lane were applied for the Coomassie blue and immunostaining, respectively. Lanes 1 and 6, vector; lanes 2 and 5, anti-sense; lanes 3 and 4, sense.

Cells transfected with tau_{441} in the sense orientation were positively stained with tau antibodies, 92e (Fig. 2c), to total tau and Tau-1 (Fig. 2b) which only labels tau that is not phosphorylated at Ser-199/202 [7]. The intensity of labeling varied from one cell to another and was independent of the number of passages. Generally, the immunostaining was diffuse and punctuate. Some of the cells expressing tau showed fine elongated processes which were tau positive along with the cell body. Some cells showed more than one process while others had wider and shorter cytoplasmic extensions. Several cells were polynucleated. In addition to apparently normal tau, some cells also exhibited brightly immunofluorescent clusters in the cytoplasm when stained with antibodies AT8 (data not shown) or PHF-1 (Fig. 2d), indicating that tau in some selected cells is hyperphosphorylated at Ser-199/202 and/or Ser-396. The number of cells staining positively with mAb PHF-1 and AT8 increased with subsequent passages. However, the increase seen with mAb PHF-1 appeared considerably smaller than that with AT8. Monoclonal antibody DMIA, to α-tubulin did not show any detectable differences in staining the microtubule network between the control and the tau_{441} expressing cells (Fig. 2e–g). This was confirmed by immunoblot analysis (Fig. 3).

To assess whether any of the tau expressed in stably transfected CHO cells was bound to microtubules, the soluble cytoplasmic proteins were removed by Triton X-100 extraction prior to formaldehyde fixation. While strong staining of the microtubule network with tubulin antibodies was readily observed, labeling with Tau-1 was only observed in a few cells (Fig. 2b′). These immunocytochemical studies indicate that in the transfected cells the tau is expressed both unbound and bound to microtubules.

On immunoblots of cell lysates mAb Tau-1 stained one major band with an apparent M_r of around 66,000 and a series of minor bands, most probably degradative products of tau (Fig. 4). Dephosphorylation of the blot prior to application of the antibody did not result in an increase of the staining intensity. Only weak staining of the tau band was observed with antibodies PHF-1 (data not shown) and AT8 (Fig. 4).

4. Discussion

Previous studies have suggested that MAP2 and tau are both synthesized during neuronal differentiation [20] and are important for maintaining the microtubule array characteristic of neuronal morphology [21]. Transfection studies have demonstrated that expression of these proteins in non-neuronal cells stabilizes and, in some cases, promotes bundling of microtubules [12,22–25].

In the present study CHO cells were stably transfected with human tau_{441} cDNA. Tau expression was observed both immunocytochemically and by Western blotting after many cell passages. The detection of tau expression in cells transfected with tau_{441} in the sense but not the anti-sense-orientation, or cells transfected with vector alone demonstrates the successful tranfection of tau_{441} in CHO cells.

CHO cells are normally either round or spindle shaped and are relatively simple, non-differentiated cells. These non-neuronal cells express low levels of a heat stable MAP-4 like protein [26]. On transfection with tau_{441} the CHO cells developed one or more processes, some wide and short and some thin and several cell lengths long. The development of the processes by the transfected cells probably was due to increased stabilization of microtubules produced by a combination of the expressed tau and the endogenous MAPS. These findings are consistent with those of Esmaeli-Azad, et al. [13] who showed that the neurite outgrowth was directly proportional to the levels of tau expression in PC12 cells transfected with tau.

Immunocytochemical staining of the transfected cells, and these cells after extraction and removal of the cytoplasmic non-cytoskeletal proteins revealed a discrete labeling of the cytoskeleton by antibodies to tubulin. In contrast, identically treated transfected cells, when labeled with tau antibodies, revealed less discrete cytoskeletal staining, suggesting that only a small amount of the tau in the transfected cells was associated with microtubules. No increase in bundling of microtubules was apparent in the transfected cells as determined by immunocytochemical staining with antibodies to tubulin; the sense, anti-sense and the vector alone transfected cells looked similar. In a previous study transfection of Swiss 3T3 cells with tau also did not result in any increase in microtubule bundling [27].

Western blots of the cell lysates developed with tubulin antibodies failed to reveal any apparent increase in the expression of tubulin. Furthermore, both immunocytochemical and Western blot analysis of the tau_{441} transfected cells developed with monoclonal antibodies to phosphoepitopes of tau revealed that some of the expressed tau became phosphorylated at Ser-199/Ser-202 and/or Ser-396. The signal for the phosphorylated protein appeared to increase with increasing number of passages. It cannot be determined with the antibodies available to what degree the transfected tau might be phosphorylated at sites other than Ser-199/202 and Ser-396. These studies suggest that the autoregulation of microtubules in cells might not only involve the changes in the synthesis of tubulin as proposed by Cleveland [28], but also the phosphorylation of tau; hyperphosphorylated tau is known not to bind to tubulin/microtubules [5,6,11]. The tau in excess of that required for the mainte-

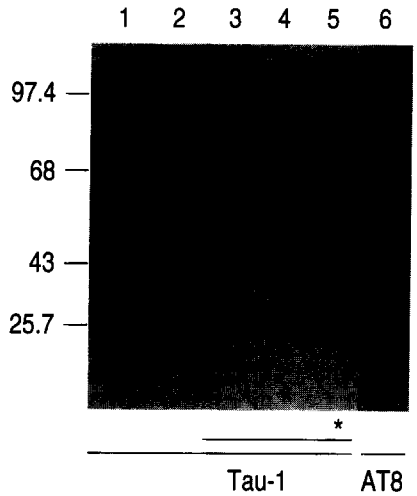

Fig. 4. Immunolabeling on Western blot of human tau_{441} stably transfected CHO cell lysates. The protein samples were electrophoresed on a 80 × 60 × 0.75 mm SDS-polyacrylamide gel (7.5–15% acrylamide gradient) and transferred to Immobilon and immunolabeled with antibodies Tau-1 and AT8. *Indicates blot that was treated with alkaline phosphatase prior to incubation with the antibodies, mAb Tau-1 only reacts with tau that is not phosphorylated at Ser-199/202 whereas AT8 recognizes tau epitopes phosphorylated at Ser-199/202. Lanes 2,3,6: cell lysate from transfected cells and lane 1 from untransfected control cells, 100 μg protein in lanes 1,2 and 3; 150 μg protein in lane 6. Lane 4: purified human brain tau, 2.5 μg protein. Lane 5: hyperphosphorylated AD tau, 0.5 μg protein. Like untransfected cells (lane 1), vector alone and antisense transfected cells were also immuno-negative for tau.

nance of microtubules may become phosphorylated and may accumulate without involving an increase in the synthesis of tubulin. While this manuscript was in preparation, Barlow, et al. [29] also reported an absence of any changes in the levels of tubulin in CHO cells transfected with tau or MAP4; these investigators did not study the state of phosphorylation of tau in the transfected cells.

The accumulation of tau unbound to microtubules in the tau_{441} transfected CHO cells appears to be consistent with the accumulation of abnormally hyperphosphorylated tau seen in affected neurons in the brain of patients with Alzheimer disease, and inconsistent with the autoregulatory model involving changes in the expression of tubulin.

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