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Cell Cycle-dependent Phosphorylation and Microtubule Binding of Tau Protein Stably Transfected into Chinese Hamster Ovary Cells

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Tau protein, a neuronal microtubule-associated protein, is phosphorylated in situ and hyperphosphorylated when aggregated into the paired helical filaments of Alzheimer's disease. To study the phosphorylation of tau protein in vivo, we have stably transfected htau40, the largest human tau isoform, into Chinese hamster ovary cells. The distribution and phosphorylation of tau was monitored by gel shift, autoradiography, immunofluorescence, and immunoblotting, using the antibodies Tau-1, AT8, AT180, and PHF-1, which are sensitive to the phosphorylation of Ser202, Thr205, Thr231, Ser235, Ser396, and Ser404 and are used in the diagnosis of Alzheimer tau. In interphase cells, tau becomes phosphorylated to some extent, partly at these sites; most of the tau is associated with microtubules. In mitosis, the above Ser/Thr-Pro sites become almost completely phosphorylated, causing a pronounced shift in M_r and an antibody reactivity similar to that of Alzheimer tau. Moreover, a substantial fraction of tau is found in the cytoplasm detached from microtubules. Autoradiographs of metabolically labeled Chinese hamster ovary cells in interphase and mitosis confirmed that tau protein is more highly phosphorylated during mitosis. The understanding of tau phosphorylation under physiological conditions might help elucidate possible mechanisms for the hyperphosphorylation in Alzheimer's disease.

INTRODUCTION

Microtubule-associated proteins (MAPs)[1] are involved in the rearrangement of the microtubular network, and their tissue- and development-specific stabilization of microtubules is presumably one of their most important functions (Drubin and Kirschner, 1986; Chapin and Bulinski, 1992; Lee, 1993; Hirokawa, 1994). Phosphorylation of MAPs appears to modulate the affinity for microtubules (Brugg and Matus, 1991; Buendia *et al.*, 1992; Biernat *et al.*, 1993; Lieuvin *et al.*, 1994) but in vivo it is still poorly understood. One class of mammalian brain MAPs is tau, which is specifically localized in axons of neuronal cells. In Alzheimer's disease tau protein becomes hyperphosphorylated and forms the paired helical filaments (PHFs). Hyperphosphorylation might therefore play a major role in the onset of neuronal degeneration by causing disorganization of the microtubule cytoskeleton, blocking axonal transport, and allowing deposition of hyperphosphorylated tau into PHFs. Most of the identified aberrant phosphorylation sites in PHF-tau are Ser/Thr-Pro motifs (Ishiguro *et al.*, 1991; Lee *et al.*, 1991; Biernat *et al.*, 1992; Gustke *et al.*, 1992; Hasegawa *et al.*, 1992; Morishima-Kawashima *et al.*, 1995). However, one cannot conclude that the kinases phosphorylating tau protein in vitro such as MAP kinase (Drewes *et al.*, 1992), GSK-3 (Hanger *et al.*, 1992; Mandelkow *et al.*, 1992), cdc2 kinase (Ledesma *et al.*, 1992; Vulliet *et al.*, 1992), or cdk5 (Baumann *et al.*, 1993; Paudel *et al.*, 1993) are necessarily the ones that have a strong influence on microtubule stability in vivo. A good candidate for regulating the dynam-

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[1] Abbreviations used: AD, Alzheimer's disease; CHO, Chinese hamster ovary; ECL, enhanced chemoluminescence; FCS, fetal calf serum; MAP, microtubule-associated protein; PHF, paired helical filaments.

ics and rearrangement of microtubules in cells via phosphorylation of tau and other MAPs is the novel microtubule-associated protein/microtubule affinity-regulating kinase (p110 mark) (Drewes *et al.*, 1995). This kinase phosphorylates tau on its KIGS and KCGS motifs in the repeat domain, a region within the carboxy-terminal half of the protein that contains three or four imperfect repeats of 31 or 32 residues and binds to microtubules (Lee *et al.*, 1988; Himmler *et al.*, 1989). In particular, phosphorylation of Ser262 in the first repeat abolishes tau's binding to microtubules and makes the microtubules dynamically unstable (Biernat *et al.*, 1993).

One way to identify phosphorylation sites of tau protein is to use antibodies with known epitopes that discriminate between "normal" tau and the hyperphosphorylated "pathological" forms (Binder *et al.*, 1985; Greenberg and Davies, 1990; Lee *et al.*, 1991; Hasegawa *et al.*, 1992; Mercken *et al.*, 1992). Most of these antibodies recognize epitopes containing Ser-Pro or Thr-Pro motifs. For example, AT8, a monoclonal antibody raised against PHF-tau, recognizes phosphorylated Ser202/Thr205 (Biernat *et al.*, 1992; Zheng-Fischhöfer, 1994) while the monoclonal antibody Tau-1 reacts with almost the same region of tau, but only if it is unphosphorylated (Kosik *et al.*, 1988; Biernat *et al.*, 1992; Szendrei *et al.*, 1993). Therefore, the increase of AT8 staining and the decrease of Tau-1 staining can be used to mark the appearance of PHF-like phosphorylation in tau protein.

Many studies have demonstrated that the dynamics of microtubules varies markedly during the cell cycle (McIntosh and Hering, 1991). During interphase, in which microtubules are responsible for intracellular transport and the maintenance of cellular architecture, the dynamics of growth and shrinkage is relatively slow, but increase abruptly at the entry to mitosis where microtubules form the spindle apparatus that is essential for chromosome segregation (Belmont *et al.*, 1990). Protein phosphorylation is a common mechanism utilized to regulate these cellular processes (Nigg, 1993), and in the case of microtubule dynamics it is likely to operate at the level of MAP phosphorylation (Buendia *et al.*, 1992). In the present study, we have stably transfected Chinese hamster ovary (CHO) cells, which normally do not express tau protein, to investigate the phosphorylation and dephosphorylation of tau in the cell. This has two advantages. First, it ensures a homogeneous population of tau protein in the cells, compared with the various isoforms found in the brain. Secondly, the behavior of tau is likely to be comparable to the endogenous MAP4 because these proteins have similar microtubule-interacting domains (Chapin and Bulinski, 1991; West *et al.*, 1991).

We observed that antibodies directed against phosphorylated Ser/Thr-Pro-motifs found in PHF-tau also labeled tau protein in transfected cells during mitosis. These findings imply that kinases belonging to the family of proline-directed Ser/Thr-kinases can phosphorylate tau during cell division and induce a mobility shift reminiscent of Alzheimer PHF tau. Thus, CHO cells stably transfected with a human tau isoform provide a useful system for observing the influence of phosphorylation on the interaction of tau protein with microtubules in vivo (Bramblett *et al.*, 1993).

MATERIALS AND METHODS

Antibodies

Several monoclonal antibodies were generous gifts of the following colleagues: Tau-1 clone from Dr. L. Binder (Northwestern University, Evanstown, IL); AT8 from Dr. A. Vandevoorde (Innogenetics, Ghent, Belgium); PHF-1 from Drs. S. Greenberg and P. Davies (Albert Einstein College of Medicine, Bronx, NY); T46 from Dr. V.M.-Y. Lee (University of Pennsylvania, Philadelphia, PA). Rat monoclonal anti-tubulin antibody YL1/2 (recognizing the C-terminus of tyrosylated α-tubulin; Kilmartin *et al.*, 1982) was obtained from Sera Lab (Sussex, England). The antibody epitopes on tau protein are shown in Figure 1. The tau antibodies Tau-1 (Binder *et al.*, 1985) and T46 (Kosik *et al.*, 1988) are directed against unphosphorylated epitopes; AT8 (Mercken *et al.*, 1992) and PHF-1 (Greenberg and Davies, 1990) recognize phosphorylated epitopes. The sites of Tau-1 and AT8 are nearly complementary to one another around residue 200; Tau-1 reacts when there is no phosphorylation in this region (Kosik *et al.*, 1988), and AT8 reacts when there are two phosphorylation sites, Ser202 and Thr205 in full-length tau (Biernat *et al.*, 1992; Goedert *et al.*, 1993; Szendrei *et al.*, 1993; Zheng-Fischhöfer, 1994). PHF-1 reacts with phosphorylated Ser396 (Lang *et al.*, 1992) but preferably when Ser404 is also phosphorylated (Otvos *et al.*, 1994); in this regard the antibody is similar to SMI31 (Lichtenberg-Kraag *et al.*, 1992). The polyclonal rabbit anti-tau antibody (Dako, Hamburg, Germany), which was used for immunoprecipitation, recognizes the C-terminal half of phosphorylated and nonphosphorylated tau protein.

Construction of a Vector Containing the cDNA of htau40

The methods used here were mostly described previously (Biernat *et al.*, 1992). Recombinant human tau protein (htau40) was derived from the cDNA clones of Goedert *et al.* (1989) and expressed in *Escherichia coli* using pNG2, a variant of the pET expression vector (Studier *et al.*, 1990). For direct subcloning of htau40 into the eukaryotic expression vector, pRc/CMV (Invitrogen, San Diego, CA) was modified, introducing a new multiple cloning site. The 1.3-kb cDNA of htau40 was inserted into the *NdeI*/*BglII* sites of the multiple cloning site. This plasmid containing the coding sequence of htau40 was used for stable transfection.

Cell Culture and Transfection

CHO cells were grown in HAM's F12 medium supplemented with 10% fetal calf serum (FCS; Biochrom, Berlin, Germany). Approximately 60% confluent cells were plated on 35-mm dishes for transfection or on coverslips for immunofluorescence analysis and incubated at 37°C with 5% CO_2. The cells were transfected with 2 μg of DNA and 9 μl of Lipofectamine according to the manufacturer's recommendations (Life Technologies, Eggenstein, Germany). Stably transfected cells were selected by growing them in the presence of 800 μg/ml of Geneticin (G-418). After incubation for an additional 2–3 wk, cells were cloned by limiting dilution and screened for htau40-expressing cells by immunofluorescence and polymerase chain reaction.

Immunofluorescence

Cells were washed in a stabilizing buffer [80 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid), 1 mM $MgCl_2$, 1 mM EGTA, 4% polyethylene glycol, pH 6.8] and fixed with either methanol at −20°C for 5 min or with 2% paraformaldehyde for 20 min following permeabilization with 0.2% Triton X-100 for 5 min. The fixed cells were treated with 5% nonfat dry milk for 1 h and incubated with the primary monoclonal mouse antibodies T46, Tau-1, AT8, and PHF-1 at 1:600, 1:50, 1:2000, and 1:2000 dilutions, respectively, and with the anti-tubulin antibody YL1/2 at 1:200 for 1 h at 37°C. For the secondary antibodies, fluorescein-conjugated goat anti-mouse or rhodamine-conjugated goat anti-rat antibodies (Dianova, Hamburg, Germany) were used at 1:300 dilution and incubated at 37°C for 30 min. The cells were examined with an Axioplan fluorescence microscope (Zeiss, Oberkochen, Germany) using filters optimized for double-label experiments and a 63× fluorescence objective.

Cell Extracts

Total cell extracts of CHO cells transfected with htau40 or with the vehicle only were prepared by lysing subconfluent cells on ice in buffer A containing 50 mM Tris (pH 7.4), 1% Nonidet P-40, 1 mM $MgCl_2$, 5 mM EGTA, 5 mM dithiothreitol, 120 mM NaCl, 20 mM NaF, 1 mM vanadate, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 10 mM benzamidine. Extracts were centrifuged immediately at 15800 × *g* for 10 min and the supernatants were used directly for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Extracts were also prepared from mitotically arrested cells following treatment with 0.4 μg/ml nocodazole (Sigma, Deisenhofen, Germany) for 5 h. Cells were detached by mechanical shake-off and lysed on ice in buffer A containing 500 mM NaCl. Extracts were boiled for 10 min and centrifuged at 15800 × *g* for 10 min. The soluble fraction containing heat-stable tau was applied to SDS-PAGE. Protein concentrations were determined by the method of Bradford (1976).

SDS-PAGE and Immunoblotting

Extract samples of both total and nocodazole-treated CHO cells were electrophoresed on 10% SDS-polyacrylamide gels (12 μg protein per lane) and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Eschborn, Germany). Residual protein-binding sites on the membrane were blocked with 5% nonfat dry milk in Tris-buffered saline following incubation with the monoclonal antibodies T46 (1:6000), Tau-1 (1:500), AT8 (1:4000), and PHF-1 (1:400). Bound antibody was detected with a peroxidase-conjugated goat anti-mouse antibody (Dianova, Hamburg, Germany) using diaminobenzidine as substrate, or the immunostaining was visualized using enhanced chemoluminescence (ECL) according to the manufacturer's instructions (Amersham, Braunschweig, Germany). For immunoblot analysis recombinant htau40 from *E. coli* was isolated by fast protein liquid Mono S (Pharmacia, Freiburg, Germany) chromatography, on the basis of its heat stability (for details see Hagestedt *et al.*, 1989). Phosphorylation reaction was carried out as described by Drewes *et al.* (1995).

^{32}Phosphate Labeling

Stably transfected CHO cells grown in a 75-cm^2 culture flask were incubated for 30 min in phosphate-free DMEM medium (Life Technologies) containing 5% FCS. The following incubation was carried out with phosphate-free DMEM supplemented with 10% dialyzed FCS, 25 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, and [^{32}P]orthophosphate (0.7 mCi/ml). After 1 h of preincubation, the cells were mitotically arrested and lysed as described above.

Immunoprecipitation

Mitotic and nonmitotic cell-extracts labeled with [^{32}P]orthophosphate were boiled for 10 min and centrifuged immediately at 15800 × *g* for 10 min. First, 5–10 μg of polyclonal rabbit anti-Tau antibody (Dako, Hamburg, Germany) was added to the supernatants, and then after 4 h at 4°C, 40 μl of protein-A/G-Sepharose beads (Dianova, Hamburg, Germany) was added. After an overnight incubation at 4°C the immune complexes were recovered by centrifugation and rinsed three times in immunoprecipitation buffer. The immunoprecipitated tau protein was resolubilized in SDS-sample buffer and boiled for 5 min. Electrophoresis was carried out on a 10% SDS-polyacrylamide gel and subjected to autoradiography. Before addition of the polyclonal anti-Tau antibody, protein concentrations of the mitotic and interphase cells were determined by the method of Bradford (1976). Equal amounts of antibody were added, and after immunoprecipitation the same amounts of protein (5.5 μg per lane) were loaded onto a 10% SDS gel. The gel was silver stained, dried, and subjected to autoradiography. Furthermore, the tau bands were cut out of the dried gel, and radioactivity was quantified by Cerenkov counting.

RESULTS

Proline-directed Phosphorylation of tau

Stably transfected CHO cells were examined by double-immunofluorescence for the uniformity of expression of htau40, the largest human isoform of tau (441 residues) using several anti-tau antibodies (Figure 1). The cells were fixed 36–48 h after plating on coverslips and stained with antibodies to tau protein and tubulin. As seen in Figure 2a, the monoclonal antibody T46, which reacts independently of tau's phosphorylation state, recognized all transfected cells equally well. The typical microtubular pattern was observed after staining with the antibody YL1/2 (Figure 2b). No influence on the viability of these cells was noticed, although the expression of tau slightly changed the morphology to a more rounded shape compared with the elongated form of the control cells. Nontransfected CHO cells, which do not contain endogenous tau protein, showed no reaction with the tau antibodies except the typical microtubular staining pattern with the antibody YL1/2 (our unpublished data). The antibody Tau-1 (which binds optimally when the region around residue 200 is completely unphosphorylated) recognized most transfected cells well (Figure 2c). However, we also noted that some of the transfected cells showed no discernible staining with the antibody Tau-1. Comparison with the microtubule network (Figure 2d) showed that this was correlated with the cell cycle stage: Tau-1 staining was never observed with transfected cells during mitosis (compare Figure 2, c and d, center), suggesting that the transfected tau became phosphorylated at the Tau-1 site during mitosis.

A notable effect of transfection with tau is the formation of microtubule bundles, often around the periphery of the cell (e.g. Figure 2, a and c). This effect has been observed with several MAPs (MAP2,

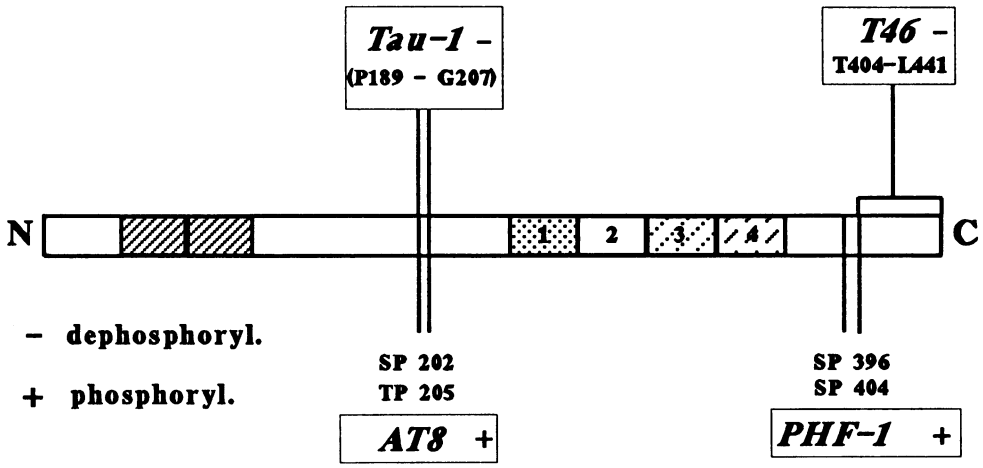

Figure 1. Diagram of the largest human tau isoform (htau40, 441 residues) showing the epitopes of tau antibodies Tau-1 (unphosphorylated region Pro189-Gly207), AT8 (phosphorylated Ser202, Thr205), PHF-1 (phosphorylated Ser396, Ser404), and T46 (Thr404-Leu441, phosphorylation independent). This human tau isoform contains four imperfect repeats near the carboxy terminus (shaded boxes, numbered 1–4), which constitute the microtubule-interacting domain and two 29 mer amino-terminal inserts (hatched boxes).

MAP2c, and tau; Kanai *et al.*, 1989; Weisshaar *et al.*, 1992; Brandt and Lee, 1993; Berling *et al.*, 1994) and can be explained by the increased concentration and nucleation capacity of tau in the cytoplasm.

To identify in vivo phosphorylation sites of tau protein, antibodies raised against Alzheimer PHFs that recognize specific phosphorylated epitopes of the tau protein were used. The antibody AT8 is directed against phosphorylated Ser202/Thr205 and PHF-1 recognizes phosphorylated Ser396/404. As a control, the cellular microtubules were visualized during all cell stages by staining with the anti-tubulin antibody YL1/2 (Figure 3, b and d). In contrast to Tau-1 immunoreactivity, both phosphorylation-dependent antibodies AT8 and PHF-1 reacted strongly with mitotic cells (compare Figure 3, a and b, with c and d). In nonmitotic cells, AT8 labeling was absent while PHF-1 showed a weak reaction (Figure 3c). These results indicate that the residues 202, 205, 396, and 404 of tau become preferentially phosphorylated during mitosis, all of them within Ser/Thr-Pro motifs, and that Ser396/Ser404 are phosphorylated to a small extent even during interphase. Because the longest human tau isoform (htau40, 441 residues) contains 17 Ser-Pro or Thr-Pro sites, many of which are readily phosphorylatable, it is likely that even more sites become phosphorylated that could be visualized by other antibodies (preliminary results point in this direction).

Phosphorylation and Microtubule Binding of Tau

Tau is normally thought to bind tightly to microtubules. However, in transfected cells this need not be the case; moreover, phosphorylation is thought to reduce the affinity of tau to microtubules. To check this point, we compared two fixation procedures. In the first set of experiments we used paraformaldehyde followed by detergent permeabilization, whereby all soluble and unsoluble proteins are cross-linked within the cells (Figure 3). In the second set, using methanol fixation (Figure 4), proteins were precipitated and resolubilized during the following washing steps with aqueous solutions, whereby proteins can be partly extracted (Fujiwara and Pollard, 1980; Melan and Sluder, 1992). Because MAPs are thought to be lost from cells during detergent extraction (Schliwa *et al.*, 1981; Chapin and Bulinski, 1994), we decided to use methanol fixation to clearly demonstrate the colocalization of tau protein to microtubules in the transfected cells.

The comparison allows one to distinguish between the two pools of tau: cytosolic and bound to microtubules. In cells fixed with paraformaldehyde (Figure 3), the bound fraction is largely concealed by the abundant unbound tau protein. Methanol fixation reveals mostly the colocalization of the bound protein with cellular microtubules (compare Figure 4, a and c). However, both of the fixation methods had no influence on tau phosphoepitopes in general, because bound and cytosolic tau protein showed similar reactivity with the phosphorylation-dependent antibodies. There was no Tau-1 staining observed in mitotic cells, while AT8 stained only mitotic cells and PHF-1 stained mostly mitotic cells (Figure 4, a and c). Both phosphorylation-dependent antibodies recognized tau still bound to the mitotic spindle. This phenomenon occurred in all mitotic cells investigated. This means that the phosphorylation of tau by proline-directed kinases does not necessarily lead to the detachment of tau from microtubules (consistent with the binding studies of Biernat *et al.*, 1993). This observation may be important with regard to Alzheimer PHFs, which are thought to form via phosphorylation of tau and release from microtubules (see DISCUSSION).

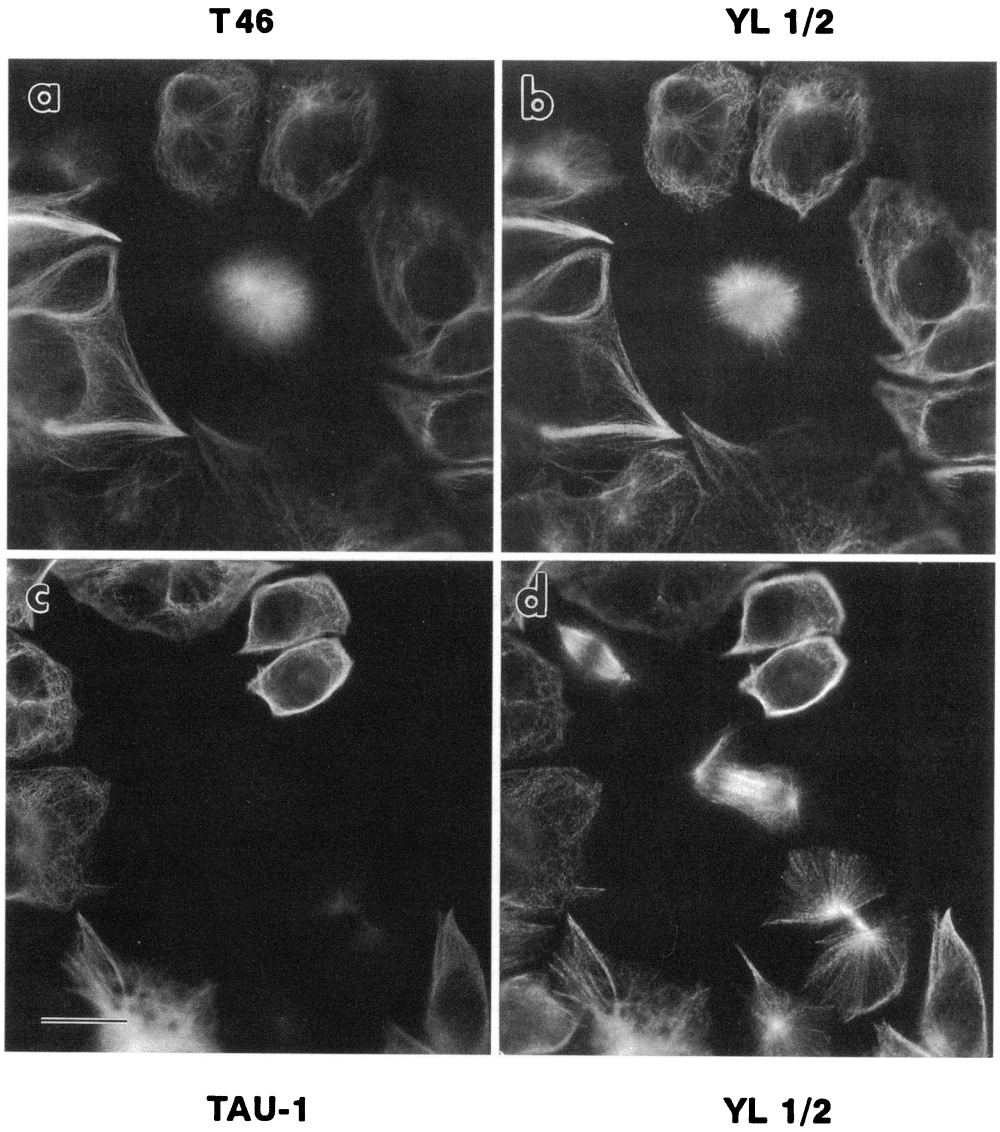

Figure 2. Double-label immunofluorescence of stably transfected htau40 protein and tubulin in CHO cells. Cells were fixed with methanol. Comparison between (a) the antibody T46 (unphosphorylated tau) and (c) antibody Tau-1 (no phosphorylation in region around residue 200). (b and d) Staining with the anti-tubulin antibody YL1/2, showing the cellular microtubule network. T46 stains all cells, whereas Tau-1 misses the mitotic cells (compare c and d, center, upper left, lower right). Note the pronounced microtubule bundles around the periphery of some cells. Bar, 5 μm.

Phosphorylation of Tau during the Stages of Mitosis

To specify the phosphorylation and dephosphorylation of tau protein in mitosis, immunofluorescence staining was carried out with the antibodies T46, Tau-1, AT8, and YL1/2 (Figure 5). The different cell stages were visualized by staining of the microtubule network with the anti-tubulin antibody YL1/2 (row 4 in the panels of Figure 5). The fixation was performed with methanol so that mainly tau protein bound to the microtubules was seen. The phosphorylation-independent antibody T46 recognized all cell stages equally well (Figure 5, row 1, panels a–h). Immunoreactivity of the monoclonal antibody AT8 was observed from early prophase (Figure 5, row 3, panel b) until early telophase (Figure 5, panel f). The phosphorylation of the AT8 epitope started at the centrosome. The intensity of the immunoreaction increased strongly in prophase (Figure 5, panel c), whereas during anaphase (Figure 5, panel e) most of the tau protein appeared to be detached from the microtubules [note weaker staining with the AT8 antibody (Figure 5, row 3) during anaphase compared with the previous cell cycle stages, whereas the staining with the tubulin antibody (Figure 5, row 4) showed no difference throughout the cell cycle]. At early telophase (Figure 5, panel f) the AT8 immunostaining was only seen in the midbody region and was no longer visible later in telophase (Figure 5, panel g). Similar results were obtained with the antibody PHF-1, showing that the phosphorylation at the respective sites rises and falls roughly in synchrony. As expected, Tau-1 (Figure 5, row 2) stained in a nearly complementary fashion to

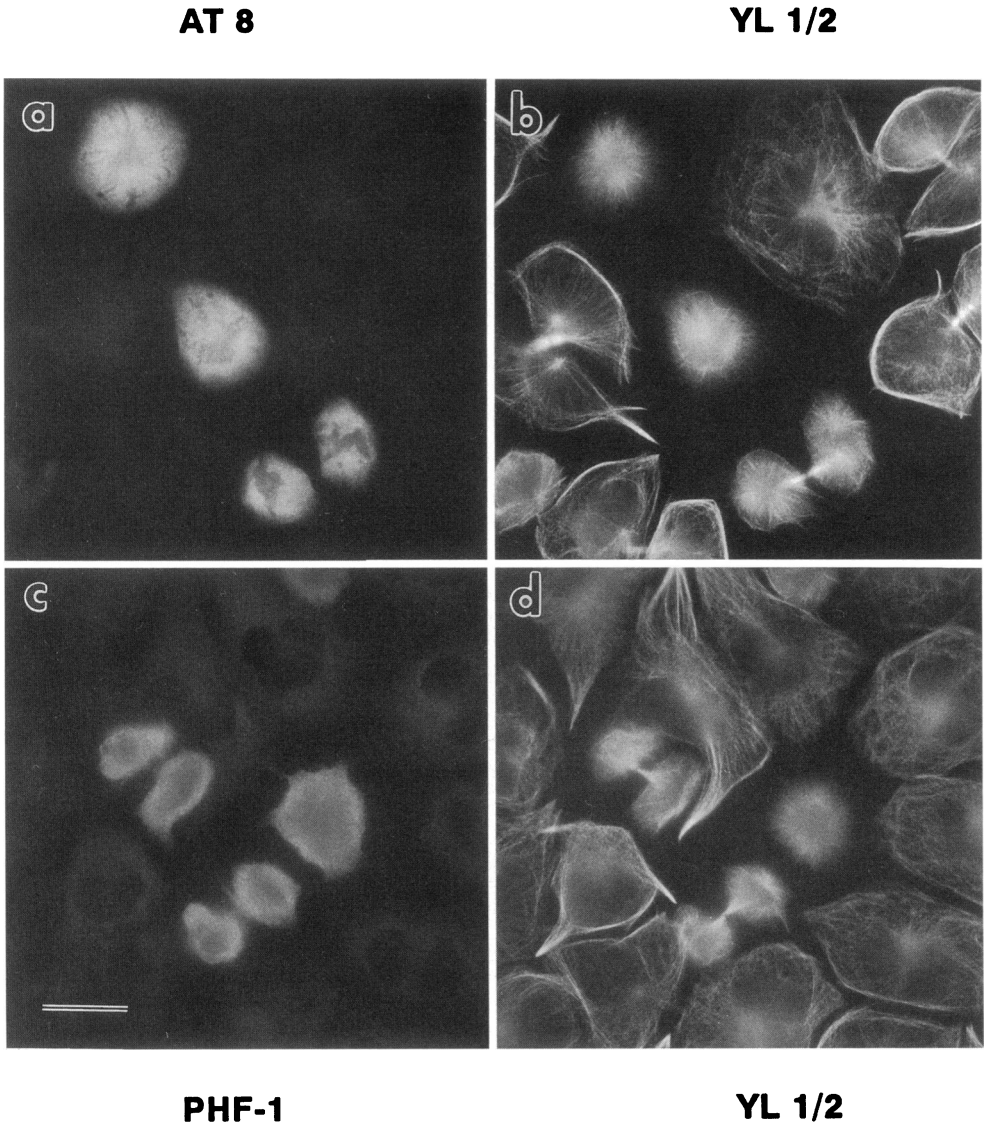

Figure 3. Immunofluorescence of tau-transfected CHO cells after paraformaldehyde fixation. Comparison between antibodies AT8 (a, phosphorylated Ser202/Thr205) and PHF-1 (c, phosphorylated Ser396/Ser404). Microtubule staining with YL1/2 (b and d). Both AT8 and PHF-1 select mitotic cells, whereas the tubulin antibody shows cells in all stages. Note that in addition to the stained microtubules there is cytoplasmic staining with the tau antibodies, showing that a substantial tau fraction is not bound to microtubules, yet is retained in the cytoplasm. Bar, 5 μm.

AT8. No staining was observed from early prophase (Figure 5, panel b) until early telophase (Figure 5, panel f), but Tau-1 staining increased strongly at the beginning of cytokinesis (Figure 5, panel g). These results illustrate the tight correlation between tau's phosphorylation and mitosis.

Multiple Phosphorylation of Transfected Tau

The antibodies provided a convenient tool for monitoring the phosphorylation of tau within cells, but these experiments left open questions such as the degree of phosphorylation and possible cross-reactions of antibodies with other proteins. We therefore isolated tau from interphase and mitotically arrested cell populations to check their phosphorylation state by SDS-PAGE and Western blotting, using the same set of antibodies. In the case of tau, SDS gels are remarkably informative about the type of phosphorylation, because the M_r depends sensitively on the phosphorylated sites (the shift in the SDS gel has long been recognized as one of the hallmarks of the Alzheimer state of tau (Grundke-Iqbal *et al.*, 1986; Lee *et al.*, 1991; Lichtenberg-Kraag *et al.*, 1992).

To demonstrate the increase in proline-directed phosphorylation during mitosis, we induced mitotic arrest with nocodazole, harvested the cells, and analyzed tau. The results from the heat-stable fractions of interphase and mitotic cell extracts are shown in Figure 6. As a reference, lane 1 shows the unphosphorylated isoform htau40 expressed in *E. coli* and

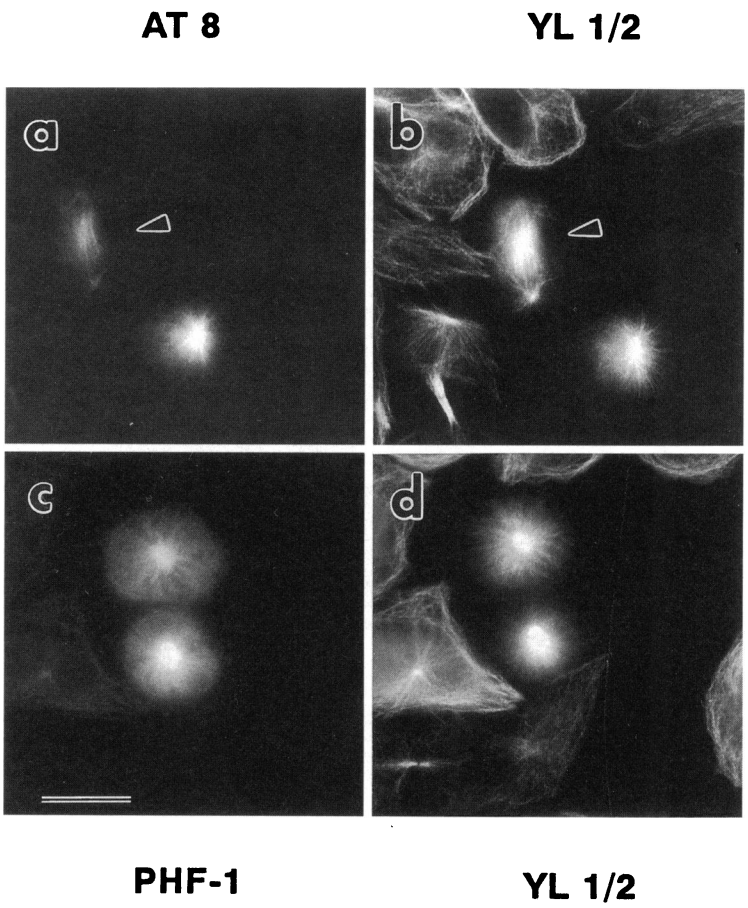

Figure 4. Similar experiment as in Figure 3 except fixation with methanol. (a) Antibody AT8, (c) antibody PHF-1, and (b and d) tubulin antibody YL1/2. The tau antibodies against phosphorylated epitopes recognize only the mitotic cells. The unbound tau protein has mainly disappeared, thus highlighting the phosphorylated tau still bound to the mitotic microtubules. Note the faint tau staining of the anaphase spindle in panel a (arrowhead) compared with the strong microtubule staining in panel b (arrowhead). Bar, 5 μm.

stained with antibody T46, and lane 2 shows htau40 phosphorylated with a mammalian brain extract also stained with T46; this protein contained about six phosphates and showed an M_r shift comparable to that of Alzheimer tau (for details see Gustke *et al.*, 1992). In blots with T46, the tau preparations from mitotic and nonmitotic cells both showed three main bands with the antibody T46, but tau protein from mitotic cells showed a much clearer upward shift in the SDS gel (Figure 6, compare lanes 3 and 4). Even though transfection was done with a single tau isoform (giving a single band when expressed in *E. coli*; Figure 6, lane 1), tau was modified in the CHO cells to give rise to at least three distinct bands. Such a heterogeneity is characteristic of native tau, independently of and in addition to the multiplicity of isoforms. Tau-1 staining overlapped with that of T46, but only in the nonmitotic cells, while mitotic cells showed negligible Tau-1 staining, indicating phosphorylation in the region around residue 200 (Figure 6, lanes 5 and 6). Conversely, AT8 stained the top bands of the mitotic extract, but only faintly stained the nonmitotic tau bands (Figure 6, lanes 7 and 8), showing that mitotic tau was almost completely phosphorylated at Ser202 and Thr205. Finally, PHF-1 recognized nonmitotic tau to some extent, but the reaction was much more pronounced with mitotic tau (Figure 6, lanes 9 and 10). These results taken together confirmed the immunofluorescence results that the affinity of Tau-1 antibody for phosphorylated tau was absent during mitosis, whereas immunoreactivity of the AT8 and PHF-1 antibodies increased strongly during cell division. This combination of high M_r and reactivity with AT8 or PHF-1 is most notable in Alzheimer tau, but evidently also occurs in transfected cells to a small extent.

The cross-reactivity of the antibodies with other cellular proteins was checked using cell extracts from CHO cells transfected with htau40 or with the vehicle only (Figure 7). All of the antibodies used showed no cross-reaction with other cellular proteins from CHO cells, demonstrating their specificity for the transfected tau protein. The phosphorylation-independent antibody T46 reacted with tau protein from mitotic and nonmitotic cells (Figure 7, lanes 3 and 4). Furthermore the antibody stained some lower molecular weight bands, possibly representing degradation prod-

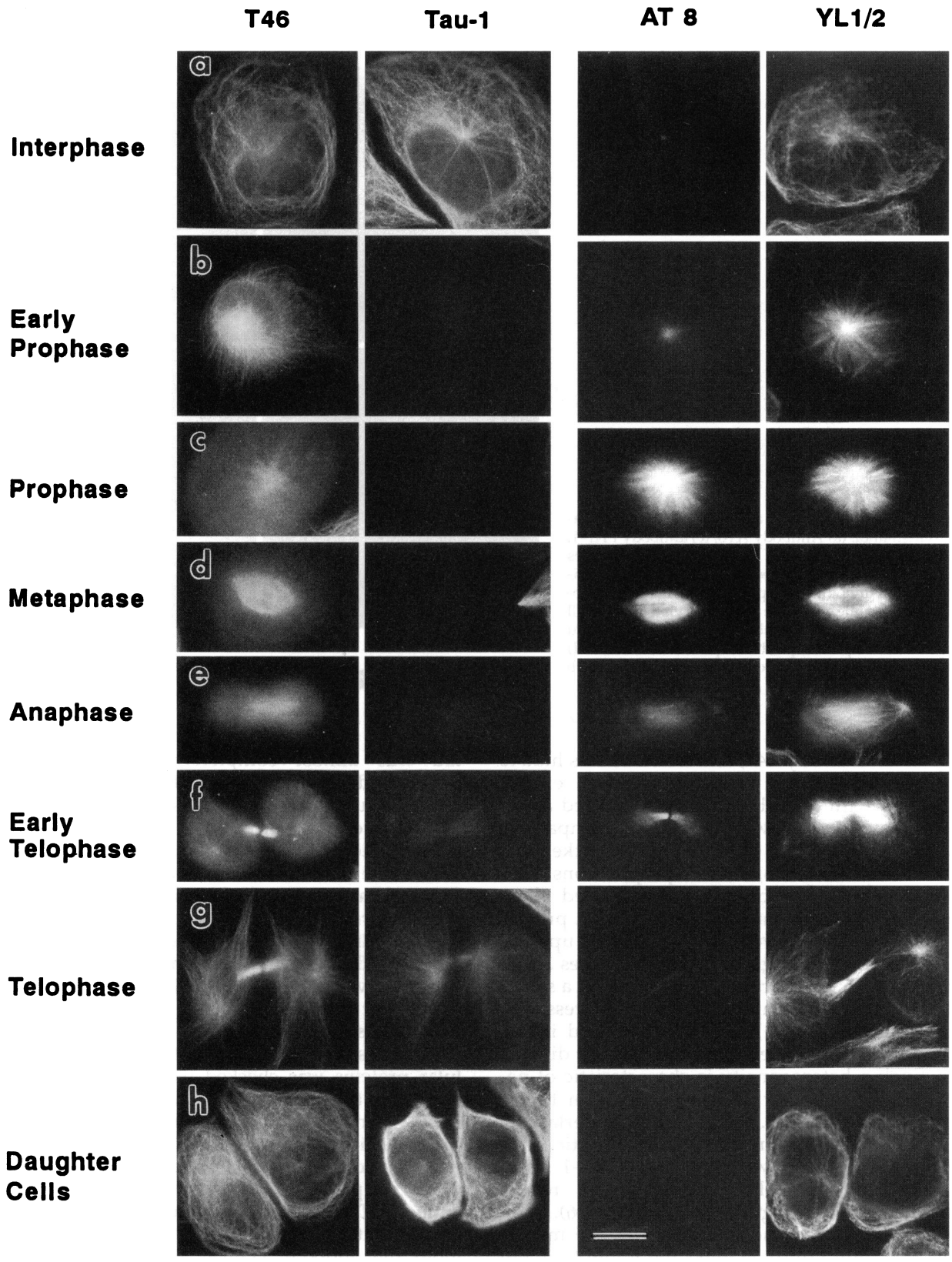
T46
Tau-1
AT 8
YL1/2
Interphase
Early Prophase
Prophase
Metaphase
Anaphase
Early Telophase
Telophase
Daughter Cells
a
b
c
d
e
f
g
h

Figure 5.

ucts of the tau protein. However, no reaction was seen with cellular proteins from mitotic and nonmitotic cells transfected with the vehicle only (Figure 7, lanes 1 and 2). Tau-1 reacted as shown in Figure 6; there was no Tau-1 staining in tau-transfected mitotic cells (Figure 7, lane 7), whereas tau protein from transfected interphase cells was recognized (Figure 7, lane 8). No Tau-1 reaction was observed with vehicle-transfected mitotic and nonmitotic cell extracts (Figure 7, lanes 5 and 6). The phosphorylation-dependent antibodies AT8 and PHF-1 reacted strongly with the tau fraction from mitotically arrested cells, showing the lowest electrophoretic mobility (highest M_r) (Figure 7, lanes 11 and 15). The antibody PHF-1 also stained various bands of lower and higher molecular weight besides tau protein, the latter still being the most prominent band, however. Because no PHF-1 reactivity was observed in mitotic vehicle–transfected CHO cells (Figure 7, lane 13), we assume that the additional low molecular bands represent degradation products of the transfected tau protein. The uppermost band of approximately 100 kDa probably corresponds to the dimerization of tau protein (Wille *et al.*, 1992). AT8 showed no reaction (Figure 7, lane 12) and PHF-1 showed only a hardly discernible reaction (Figure 7, lane 16) with tau protein from transfected interphase cells.

To see whether the increase in immunoreactivity of the phosphorylation-dependent antibodies AT8 and PHF-1 during mitosis correlates with a higher state of phosphorylation of tau protein, interphase and mitotically arrested cells were incubated for several hours in the presence of $^{32}P_i$. Labeled tau protein was isolated by immunoprecipitation and equal amounts of interphase and mitotic tau protein were loaded on an SDS gel. Figure 8 shows the autoradiography of the polyacrylamide gel. Clear radioactive bands are seen in interphase and mitotic cells that correspond to htau40 isolated from interphase and mitotic cells (compare Figure 7). Furthermore the labeled mitotic tau protein showed a pronounced upward shift in the gel. To quantify the labeled interphase and mitotic tau protein, bands were cut out of the gel and the radioactivity was determined by Cerenkov counting. The observed incorporation of $^{32}P_i$ into tau protein was sevenfold higher in mitotic cells than in interphase cells.

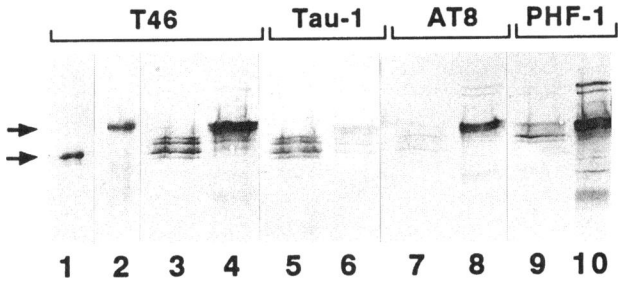

Figure 6. Phosphorylation of tau in mitotic and nonmitotic-transfected CHO cells. The heat-stable fractions of the cell extracts were incubated with different phosphorylation-dependent and independent antibodies. Lanes 1 and 2: recombinant htau40 before and after phosphorylation with mouse brain extract. Lanes 3, 5, 7, and 9: interphase extracts of transfected CHO cells; lanes 4, 6, 8, and 10 mitotic extracts of transfected CHO cells. Lanes 1–4 were probed with antibody T46; lanes 5–6 with antibody Tau-1; lanes 7–8 with antibody AT8; and lanes 9–10 with antibody PHF-1. The arrows indicate the shift in electrophoretic mobility of tau protein.

DISCUSSION

The onset of mitosis correlates with a wave of phosphorylation that affects, among others, MAPs and structures involved in microtubule nucleation and dynamics (Vandre *et al.*, 1991; Buendia *et al.*, 1992; Nigg, 1993). Because the normal function of MAPs is to stabilize microtubules, the phosphorylation of MAPs is believed to be the mechanism by which their binding to microtubules is weakened so that they become more dynamic and ready for the rearrangement of the cytoskeleton. The details of this hypothesis still have to be worked out; at present, the MAPs involved, the phosphorylation sites affected, and the kinases are known only in part or not at all. This illustrates the need for a model system. Our approach was to use tau protein transfected into CHO cells, for the following reasons: 1) Tau is by far the best characterized MAP in terms of phosphorylation sites and corresponding kinases and phosphatases; 2) a set of phosphorylation-dependent antibodies is available that enables one to follow the state of phosphorylation in cells; 3) tau is distinct from other MAPs yet shares homologous regions with some, especially the ubiquitous MAP4, implying similarity of function, e.g. in mitosis; 4) finally, tau is interesting in itself because it forms the basis for the PHFs of Alzheimer's disease (for reviews see Mandelkow and Mandelkow, 1993; Kosik and Greenberg, 1994; Trojanowski and Lee, 1994). Thus, by studying tau as an exogenous MAP in a dividing cell one hopes to gain information not only on the role of MAP phosphorylation during the cell cycle, but also on the mechanisms underlying the pathology of Alzheimer's disease.

Figure 5 (cont). Immunofluorescence staining of transfected CHO cells during mitosis. The fixation was done with methanol. First row: antibody T46; second row: antibody Tau-1; third row: antibody AT8; and fourth row: anti-tubulin antibody YL1/2. (a-h) Correlation between the appearance and disappearance of Tau-1 and AT8 staining during mitosis. Note that the phosphorylation-independent antibody T46 stained all cells equally well throughout the cell cycle. Phosphoepitopes of tau begin to appear at early prophase at the centrosome (b) and disappear at telophase (g). Bright AT8 staining is seen during prophase and metaphase (c and d), whereas during anaphase most tau seems to be detached from microtubules (note weaker staining with AT8 in e compared with c and d). Bar, 2 μm.

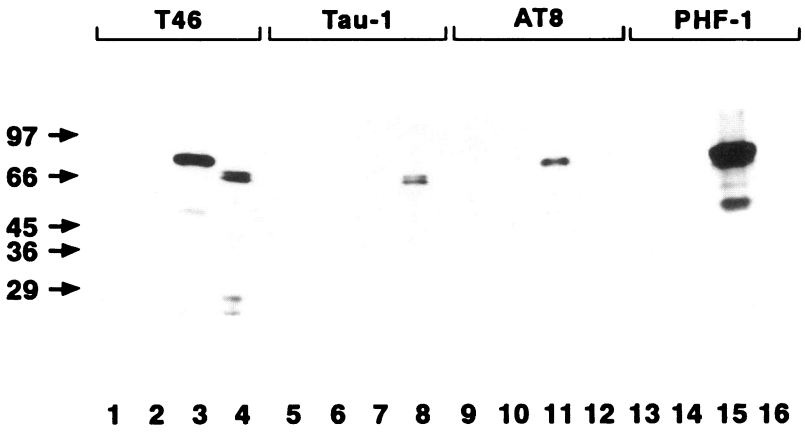

Figure 7. Immunoblot analysis (ECL) of mitotic and nonmitotic cell extracts of CHO cells transfected with htau40 and with the vehicle only. For each antibody the samples were loaded as follows: mitotic CHO cells (vehicle), interphase CHO cells (vehicle), mitotic CHO cells (htau40), and interphase CHO cells (htau40). Lanes 1–4, T46; lanes 5–8, Tau-1; lanes 9–12, AT8; and lanes 13–16, PHF-1. The positions of 97 kDa, 66 kDa, 45 kDa, 36 kDa, and 29 kDa molecular weight standards are shown at the left.

Many of the sites phosphorylated in Alzheimer tau turn out to be of the form Ser-Pro or Thr-Pro; there are several proline-directed kinases that can phosphorylate those sites, and several antibodies recognizing those sites are useful in analyzing Alzheimer neurofibrillary deposits (reviewed in Anderton, 1993; Mandelkow and Mandelkow, 1993). This includes the antibodies Tau-1 (Binder *et al.*, 1985), PHF-1 (Greenberg and Davies, 1990), and AT8 (Mercken *et al.*, 1992) used in this study (Figure 1). By using these antibodies some of tau's phosphorylation sites in cells can be analyzed.

The phosphorylation of Ser/Thr-Pro motifs was initially considered to point to a pathological state of tau. However, there is increasing evidence that at least some of the sites are phosphorylated in physiological conditions. This applies in particular to fetal tissue and differentiating cells (Kanemaru *et al.*, 1992; Bramblett *et al.*, 1993; Goedert *et al.*, 1993; Watanabe *et al.*, 1993; Matsuo *et al.*, 1994), raising the possibility that tau's hyperphosphorylation might be the result of the neuron's attempt to re-initiate proliferation. Subsequent microinjection and transfection studies revealed that even in nonneuronal cells certain Ser-Pro motifs of MAPs become phosphorylated by proline-directed kinases. This is particularly noticeable when the cells change their status, e.g. during differentiation or in response to toxic effects (Gallo *et al.*, 1992; Bramblett *et al.*, 1993; Lo *et al.*, 1993; Sygowski *et al.*, 1993; Berling *et al.*, 1994; Greenberg *et al.*, 1994; Kirby *et al.*, 1994). In particular, Pope *et al.* (1994) have observed mitotic staining of tau with antibody PHF-1 in a neuroblastoma cell line (containing the endogenous mixture of tau isoforms), and their data are in good agreement with ours.

The experiments described here were started to study the cellular phosphorylation of MAPs. CHO cells were stably transfected with the longest human isoform (htau40) to ensure a homogeneous population of tau protein expressed at a high level, which was considered necessary for further biochemical and structural analysis. When probing the cells with phosphorylation-dependent antibodies the following main results emerged: 1) Tau can be expressed without major changes in the cell's viability or behavior. 2) A small fraction of tau is constitutively phosphorylated at Ser-Pro motifs (mainly at the PHF-1 site); 3) however, a dramatic increase in phosphorylation occurs during mitosis, to the extent that almost all tau appears to be "Alzheimer-like" by the accounts of M_r shift and reactions with the diagnostic antibodies. 4) By Western blotting, the single tau isoform gives rise to three major species, presumably different in "basic" phosphorylation, but all capable of becoming highly phosphorylated during mitosis. 5) A substantial fraction of this hyperphosphorylated tau becomes detached from microtubules. 6) Metabolic labeling confirmed higher phosphorylation during mitosis.

In a related study, Bramblett *et al.* (1993) investigated CHO cells transfected with tau and observed the phosphorylation at Ser396 using the antibodies PHF-1 and T3P. They did not report any changes in PHF-1 immunostaining during the cell cycle, just a weak staining of CHO cells in general. By contrast, we find that the phosphorylation at both the PHF-1 and the

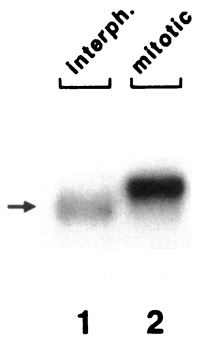

Figure 8. Autoradiography of $^{32}P_i$-labeled interphase and mitotic tau, immunoprecipitated with the rabbit polyclonal Tau antibody. Lane 1, interphase tau protein; lane 2, mitotic tau protein. The degree of phosphorylation increases sevenfold in the mitotic protein, and there is a pronounced upward shift in the gel.

AT8 epitopes are elevated during mitosis, as well as the overall extent of phosphorylation. The different conclusions can probably be explained by the fact that the complementary pair of antibodies AT8 and Tau-1 that we have used is a more sensitive marker of proline-directed phosphorylation; moreover, the PHF-1 staining shows a higher background throughout the cell cycle, indicating that this epitope is more easily phosphorylated. The conclusion of Bramblett *et al.* (1993) that the phosphorylation of Ser396 leads to a reduced binding of tau to microtubules is probably an overinterpretation, for two reasons: one is that the bound and unbound pools of tau do not differ significantly in terms of phosphorylation at the AT8 or PHF-1 epitopes; and second, in vitro studies showed that phosphorylation at most Ser-Pro sites has little influence on the tau-microtubule binding (Biernat *et al.*, 1993). The most plausible interpretation is that binding is controlled by additional phosphorylation sites, not necessarily seen through the narrow window of the available antibodies, Ser 262 being one likely candidate (Drewes *et al.*, 1995).

On the other hand, it is clear that the dynamics of microtubules increases dramatically during mitosis (Belmont *et al.*, 1990; McIntosh and Hering, 1991; Buendia *et al.*, 1992), and it is likely that the same kinases whose activities one can visualize (via the antibodies) affect other sites as well, including those that control the interaction between MAPs and microtubules. The nature of this interaction is not well understood at present. In contrast to the usual well-defined protein-protein interactions, tau interacts with microtubules through distributed weak binding sites (Butner and Kirschner, 1991), and tau itself has a structure approaching that of a random coil (Schweers *et al.*, 1994). Our current model is that tau interacts with microtubules by at least three subdomains, two of which form the targeting domains (the domains flanking the repeats as "jaws"), while the repeats convey the catalytic activity in terms of microtubule stabilization (Gustke *et al.*, 1994). These domains are phosphorylated by different kinases, e.g., the flanking regions by proline-directed kinases, and the repeats by p110 mark. It therefore is likely that regulation could occur on the level of targeting and/or catalytic activity. The relationship between these functions still needs further clarification.

Whether or not the transfected tau molecules influence the behavior of the host cell is not clear at present; apart from the bundling effect, the exogenous tau does not appear to override the control of microtubule dynamics by the endogenous MAPs such as MAP4 (in analogy with the observations of Barlow *et al.*, 1994). This would mean that the control of entry into mitosis lies at a higher level, such as the mitotic kinase(s), which affect tau and endogenous MAPs in a similar fashion. Thus the exogenous tau can be considered as a "flag" indicating the activity of kinases, or the lack of activity of the corresponding phosphatases. In the context of the cell cycle, the obvious candidate kinase is p34(cdc2), a proline-directed kinase (for review see Nigg, 1993). It is known to phosphorylate a number of substrates, including MAPs (Gotoh *et al.*, 1991; Vandre *et al.*, 1991; Buendia *et al.*, 1992; Lieuvin *et al.*, 1994), and in addition it activates–via a cascade of reactions–other kinases that are also known to phosphorylate MAPs, such as MAP kinase (Shiina *et al.*, 1992; Drewes *et al.*, 1992). The nonproline-directed sites Ser262 and Ser356 within the microtubule binding region have been reported to be significantly phosphorylated by p110 mark, a novel kinase purified from porcine brain (Drewes *et al.*, 1995). P110 mark phosphorylates tau on its KIGS or KCGS motifs in the repeat domain, whereas no significant phosphorylation outside this region was detected. The phosphorylation of Ser262 within the microtubule-binding region of tau is of particular interest because so far it is observed only in Alzheimer's disease (Hasegawa *et al.*, 1992) and because phosphorylation of this site alone dramatically reduces the affinity for microtubules in vitro (Biernat *et al.*, 1992; Drewes *et al.*, 1995). Studies are in progress to elucidate the function of this kinase in vivo.

Taken together, the results demonstrate that extensive phosphorylation of tau protein is a potent modulator of tau's affinity for microtubules in vivo. They help explain the high degree of phosphorylation of fetal tau (Bramblett *et al.*, 1993) because during embryogenesis nerve cells still undergo mitosis. Furthermore, this study supports a hypothesis stating that the pathological phosphorylation of Alzheimer tau may be the result of the neuron's attempt to respond to some insult, such as oxidative stress, ischaemia, β-amyloid toxicity, or another factor. The neuron behaves as if it wanted to divide, thus turning on the appropriate phosphorylation cascades, including the proline-directed kinases whose effect one observes on tau with phosphorylation-dependent antibodies (e.g. Tau-1, AT8, and PHF-1). A transfected CHO cell can divide without difficulty, but a postmitotic neuron cannot. Thus, turning on the wrong program may lead to cell death instead of rejuvenation. This picture is attractive, but it does not yet include two observations: Alzheimer tau aggregates into PHFs, and it ceases to bind to microtubules (Yoshida and Ihara, 1993; Bramblett *et al.*, 1993); in this regard it differs from fetal and part of the mitotic tau, even when they show phosphorylation at Ser-Pro motifs. The difference is probably due to other kinases and special phosphorylation sites, such as Ser262 and its kinase MARK (e.g. Biernat *et al.*, 1993; Drewes *et al.*, 1995). However, phosphorylation by itself might not be sufficient to allow tau to aggregate into PHFs, because no such filaments have been induced in cells so far. One possible explanation is that in dividing cells (express-

ing endogenous or exogenous tau) high phosphorylation only occurs during a short period of time and tau is dephosphorylated again before aggregation could take place. If we assume that postmitotic neurons turn on the wrong program as a reaction to some insult, dephosphorylation might be delayed or absent for unknown reasons and therefore tau could aggregate into PHFs.

The diagram of Figure 9 summarizes some of the observations discussed here. In interphase (A), tau is in a state of low phosphorylation and largely bound to microtubules. In mitosis (B), tau is more highly phosphorylated at Ser-Pro or Thr-Pro motifs, which are recognizable by their reaction with the diagnostic antibodies and partly dissociated from microtubules.

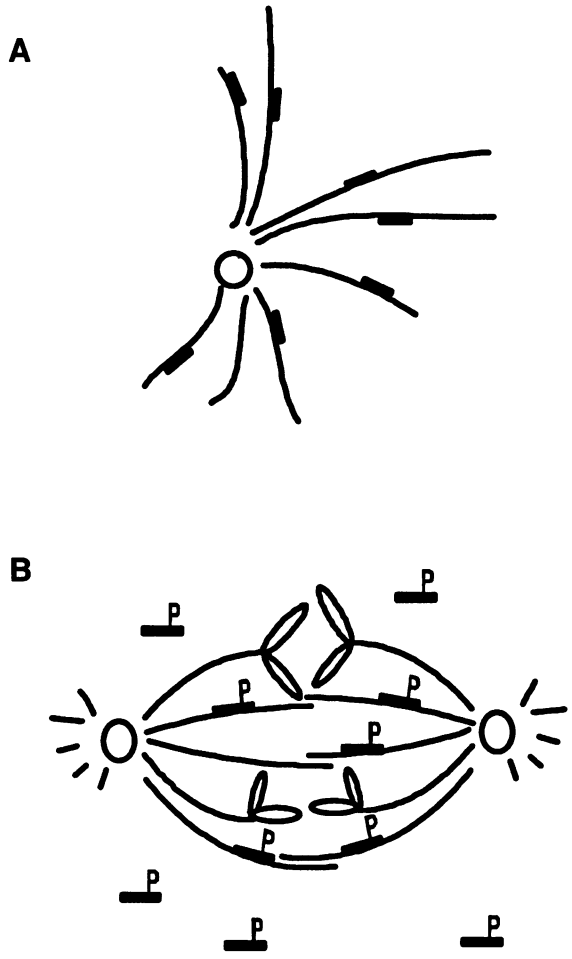

Figure 9. Diagram illustrating the phosphorylation of tau and its interaction with microtubules in transfected CHO cells. The circle indicates a microtubule-organizing center, the lines represent microtubules, with chromosomes attached (bottom), and tau molecules are shown as black bars. (A) In interphase most tau is bound to microtubules. In this situation tau has a low degree of phosphorylation, but not at the Ser/Thr-Pro motifs recognized by the diagnostic antibodies Tau-1, AT8, or PHF-1. (B) In mitosis tau becomes hyperphosphorylated (indicated as "P"), and part of tau becomes detached from the microtubules.

Note added in proof. Additional phosphorylation sites at Ser/Thr-Pro motifs that increase during mitosis include Thr231 and Ser235. They are recognized by antibody AT180 (a gift of E. Vanmechelen, Innogenetics, Ghent; for epitope mapping see Goedert *et al.*, 1994; Zheng-Fischhöfer, 1994).

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