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Expression and phosphorylation of a three-repeat isoform of tau in transfected non-neuronal cells

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The neuronal microtubule-associated protein, tau, is expressed as a set of isoforms containing either three or four tandemly repeated 31-amino-acid motifs in the *C*-terminal half of the molecule that can bind to microtubules. Three-repeat forms are the only ones expressed early in development. A single three-repeat isoform of tau has been stably expressed in non-neuronal cells which do not express endogenous tau. Chinese hamster ovary (CHO) cells were transfected with a full-length cDNA coding for the foetal form of human tau cloned downstream of the simian virus 40 (SV40) promoter, and a cell line constitutively expressing tau, CHO[pSVtau3], was isolated. Double-label immunofluorescence microscopy reveals that tau co-localizes with the microtubular network of normal or taxol-treated CHO[pSVtau3] cells, without inducing any dramatic change in cell morphology. Tau is expressed in CHO[pSVtau3] cells as three bands in SDS/PAGE recognized by antibodies to tau, the slow-migrating tau species being the most abundant. Tau also appears as three bands in a heat-stable fraction from CHO[pSVtau3] cells, but a single band of enhanced immunoreactivity is detected following treatment of this fraction with alkaline phosphatase. This single band co-migrates with the fast-migrating band of untreated fractions or whole-cell extracts. In conclusion, a three-repeat isoform of tau is capable of binding to microtubules in transfected non-neuronal cells; furthermore, in this system, the protein is phosphorylated in at least two different states inducing a reduced electrophoretic mobility.

INTRODUCTION

Neuronal polarity is highly correlated with the properties of microtubules present in axons and dendrites. For instance, microtubules are uniformly oriented in axons, e.g. their plus ends are directed distal to the cell body, whereas microtubules with mixed orientations are found in dendrites (Baas *et al.*, 1988, 1989). Microtubules in axons and dendrites also differ in their microtubule-associated proteins (MAPs): the high-molecular-mass MAP2 is found in dendrites and cell bodies (Matus *et al.*, 1981; DeCamilli *et al.*, 1984), whereas tau proteins are specifically axonal (Binder *et al.*, 1985; Dotti *et al.*, 1987; Brion *et al.*, 1988).

MAPs appear to have a role in neurite outgrowth, since experiments using antisense techniques have shown that MAP2 was responsible for neurite extension in embryonal carcinoma cells (Dinsmore & Solomon, 1991) and that tau was necessary for both differentiation and maintenance of axonal processes in cerebellar neurones in primary culture (Caceres & Kosik, 1990; Caceres *et al.*, 1991). These latter data, together with the earlier observation that tau and MAP2 do not segregate before a neurite has developed an axonal morphology (Kosik & Finch, 1987), suggest that tau functions after an initial period of growth. The role of tau in process growth is further emphasized by the extension of long processes induced by overexpression of tau in Sf9 insect cells infected with a recombinant baculovirus containing a tau cDNA insert (Knops *et al.*, 1991).

Tau proteins are a group of at least six isoforms generated by alternative RNA splicing (Goedert *et al.*, 1989*a*,*b*; Himmler *et al.*, 1989; Kosik *et al.*, 1989). Tau isoforms contain either three or four tandemly repeated similar 31-amino-acid sequences in its *C*-terminal half which are microtubule-binding sites (Aizawa *et al.*, 1989; Ennulat *et al.*, 1989; Himmler *et al.*, 1989; Lee *et al.*, 1989). The expression of tau isoforms is regulated during development (Francon *et al.*, 1982). Three-repeat isoform(s) are the only one(s) expressed until day 20 of embryonic growth in rat (Kosik *et al.*, 1989), and are the only forms present in human foetal brain (Goedert *et al.*, 1989*a*,*b*); thus they are likely to play a role in axonal elongation during development.

Tau is phosphorylated in brain and phosphorylation appears to regulate the binding of tau to microtubules, since dephosphorylation of tau increases its ability to promote microtubule assembly *in vitro* (Lindwall & Cole, 1984). Furthermore, a phosphorylated form of tau found in brains of people with Alzheimer's disease, referred to as A68, is not capable of binding to microtubules (Lee *et al.*, 1991). Individual bacterially expressed tau isoforms can be phosphorylated *in vitro* by purified kinases, including Ca^{2+}/calmodulin-dependent protein kinase and protein kinase C (Steiner *et al.*, 1990). However, an issue that remains unresolved by such studies is the pattern of phosphorylation of individual tau isoforms in a cellular environment. This can be directly assessed in non-neuronal cells, which do not express endogenous tau, transfected with cDNAs coding for single tau isoforms. Such an approach has been used by Kanai *et al.* (1989), who showed that a four-repeat form of rat tau bound to microtubules and was phosphorylated in transfected mouse L cells. In this paper, we report the stable expression of a three-repeat form of tau in transfected Chinese hamster ovary (CHO) cells which binds to microtubules in the cells, and we show that tau is phosphorylated in at least two different states in this system.

Abbreviations used: MAP, microtubule-associated protein; CHO, Chinese hamster ovary; PCA, perchloric acid; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; SV40, simian virus 40.

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EXPERIMENTAL

Construction of pSVtau3 expression plasmid

The isolation of clone p19, which contains a cDNA insert coding for the entire human foetal tau, has been described previously (Lee *et al.*, 1989). The vector was obtained from the plasmid pSV_2neo (Southern & Berg, 1982) by removing the aminoglycoside phosphotransferase (*neo*^{r}) gene. The plasmids p19 and pSV_2neo were digested with *Sal*I and *Hin*dIII, respectively, and blunt-ended with the Klenow fragment of *Escherichia coli* DNA polymerase I. The DNA fragments were then digested with *Eco*RI and the digestion products were separated by electrophoresis in 1% (w/v) low-melting-point agarose. The tau cDNA insert and the linearized vector were excised from the gel and purified by phenol and chloroform extractions (Sambrook *et al.*, 1989). The tau cDNA fragment and the vector were ligated at the *Eco*RI site with T4 DNA ligase and the resulting DNA was circularized by overnight incubation at 14 °C with T4 DNA ligase. The reaction mixture was used to transform competent *E. coli* HB101 cells. These manipulations were carried out according to standard protocols (Ausubel *et al.*, 1990). A clone containing the tau insert ligated to the vector was selected by restriction digestion analysis. Large-scale plasmid DNA purification was carried out on Qiagen pack 500 columns (Diagen G.m.b.H, Düsseldorf, Germany) according to the manufacturer's instructions.

Cell culture and transfection

CHO cells were maintained in α-minimal essential medium supplemented with 10% (v/v) foetal calf serum (Gibco/BRL, Paisley, Scotland, U.K.). A total of 10^6 cells were plated in 25 cm^2 flasks and co-transfected the following day with 7 μg of pSVtau3 and 0.35 μg of pSV_2neo by the calcium phosphate method (Graham & van der Eb, 1973). Cells were incubated for 6 h with the DNA/calcium phosphate precipitate and subsequently subjected to a 3 min glycerol shock. Transfected cells were selected by growing them in the presence of 500 μg of G-418/ml (Geneticin; Gibco/BRL) for 2 weeks. Cells were cloned by limiting dilution, and screening for tau-expressing cells was performed by indirect immunofluorescence with a polyclonal antibody to tau (see below). In some experiments, cells were treated overnight with 10 μmol of taxol/l (provided by the National Cancer Institute, Bethesda, MD, U.S.A.).

Antibodies

The mouse monoclonal anti-tau antibody, tau 1 (Binder *et al.*, 1985), was generously provided by Dr. Lester Binder (University of Birmingham, Birmingham, AL, U.S.A.), and the mouse monoclonal anti-tau antibody 5E2 was described previously (Kowal & Kosik, 1987). A mouse monoclonal antibody to β-tubulin was purchased from Amersham (Amersham, Bucks., U.K.). A polyclonal antibody to human tau was also prepared by immunizing a rabbit with a tau-enriched fraction prepared from post-mortem human brain by the perchloric acid (PCA) method (PCA tau) as described elsewhere (Baudier & Cole, 1987; Greenberg & Davies, 1990; Hanger *et al.*, 1991). For affinity purification, tau was further purified from PCA tau by phosphocellulose chromatography (Weingarten *et al.*, 1975) and coupled to an Aminolink column (Pierce, Oud-Beijerland, The Netherlands). This column was used to affinity purify antibodies to tau from the rabbit antiserum, according to the manufacturer's instructions.

Immunofluorescence microscopy

For immunofluorescence staining, cells grown on untreated glass coverslips were washed in Tris-buffered saline and fixed for 5 min in acetone at −20 °C. Primary antibodies were affinity-purified polyclonal anti-tau antibody (9 μg/ml) alone or mixed with anti-β-tubulin monoclonal antibody (diluted 1:100). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG (Nordic, Maidenhead, Berks., U.K.), diluted 1:35, were used as secondary antibodies.

Biochemical procedures

Control and transfected cells were harvested and homogenized in electrophoresis sample buffer [62.5 mmol/l Tris HCl, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.005% (w/v) Bromophenol Blue, pH 6.8]. Proteins were resolved by SDS/PAGE in 10% (w/v) polyacrylamide gels according to Laemmli (1970), and blotted on to nitrocellulose by the method of Towbin *et al.* (1979). Nitrocellulose sheets were incubated with anti-tau antibodies and antibody binding was detected by appropriate secondary biotinylated antibodies followed by either alkaline phosphatase- or peroxidase-conjugated streptavidin (Amersham). The monoclonal antibodies tau 1 and 5E2 were used as culture supernatants diluted 1:300 and 1:7.5 respectively; the affinity-purified polyclonal anti-tau antibody was used at 1 μg/ml. A heat-stable fraction from CHO[pSVtau3] cells was prepared by homogenizing cells from four subconfluent 80 cm^2 flasks into 240 μl of Mes/NaCl buffer [Mes 100 mmol/l, $MgCl_2$ 0.5 mmol/l, EGTA 2 mmol/l, NaCl 1 mol/l, dithiothreitol 2 mmol/l (pH 6.5)] and heated at 100 °C for 5 min. The suspension was centrifuged at 16 000 $g_{av.}$ for 15 min and the supernatant was recovered. The heat-stable fraction of CHO[pSVtau3] cells was dephosphorylated for 28 h at 37 °C with 30 units of *E. coli* alkaline phosphatase/ml by using the method described by Kanai *et al.* (1989). The enzyme was omitted from controls. The incubation mixture was then prepared for SDS/PAGE by adding 0.25 vol. of five times concentrated electrophoresis sample buffer.

RESULTS

Expression plasmid

The plasmid p19, isolated from λgt11 human foetal brain expression library (Lee *et al.*, 1989), was used for the purpose of expressing tau in CHO cells. p19 contains a 2.5 kb cDNA insert coding for the full-length sequence of a three-repeat isoform of tau, without an *N*-terminal insert, plus a 1.2 kb 3′ flanking sequence. *Sal*I cleaves this cDNA insert at an unique site, 28 bases upstream from the ATG translation initiation codon. The *Sal*I/*Eco*RI fragment of the cDNA insert has been subcloned downstream to the simian virus 40 (SV40) early promoter; the resulting 4.95 kb plasmid vector has been designated pSVtau3.

Transfection

CHO cells were co-transfected with pSVtau3 and pSV_2neo. Stably transfected cells were selected on the basis of their resistance to G-418. Immunofluorescence staining with antibodies to tau did not reveal any immunoreactivity in untransfected CHO cells, which is in agreement with the neuronal specificity of tau. Therefore an easy screening method for tau-expressing cells was immunofluorescence staining with antibodies to tau. Less than 10% of G-418-resistant cells exhibited a cytoplasmic filamentous pattern when stained with a polyclonal antibody to human tau. G-418 resistant cells were cloned by limiting dilution. Several rounds of cloning were necessary to obtain a clone in which the majority of the cells displayed positive staining for tau. This difficulty in cloning has also been

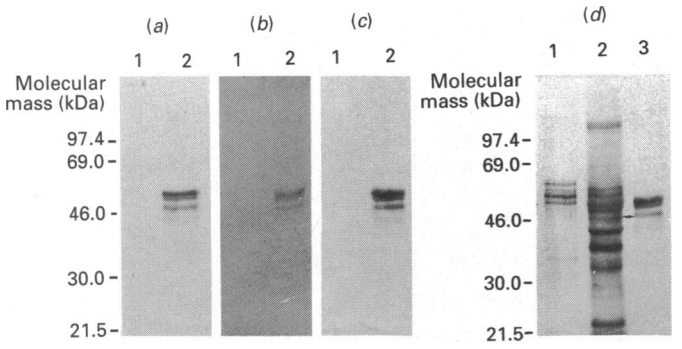

Fig 1. Immunoblot analysis of tau expression in CHO[pSVtau3] cells

(*a*)–(*c*) Total cell extracts from untransfected CHO cells (lane 1) and CHO[pSVtau3] cells (lane 2) were stained with the monoclonal antibodies tau 1 (*a*) or 5E2 (*b*), or with an affinity-purified polyclonal antibody to human tau (*c*). (*d*) Comparison of brain tau and tau expressed in CHO[pSVtau3] cells. Heat-stable MAPs from bovine brain (lane 1), total homogenate from human hippocampus (lane 2) and total cell extract from CHO[pSVtau3] cells (lane 3) were reacted with an affinity-purified polyclonal antibody to human tau. The high-mobility tau species of CHO[pSVtau3] cells co-migrated with the fast-migrating tau band of human brain (double arrow). The bands below the group of tau proteins in lane 2, including the two heavily stained ones, are degradation products of tau commonly found in post mortem material (Hanger *et al.*, 1991). Molecular mass markers were phosphorylase *b* (97.4 kDa), BSA (69.0 kDa), ovalbumin (46.0 kDa), carbonic anhydrase (30.0 kDa) and trypsin inhibitor (21.5 kDa).

reported for cells expressing MAP2 (Lewis *et al.*, 1989). The clone has been designated CHO[pSVtau3] and was used for further studies. The intensity of labelling varies from one cell to another, and this was not affected by successive clonings. The expression of tau affects the cell morphology slightly: whereas non-transfected CHO cells are rather elongated, CHO[pSVtau3] cells are more spread, and they do not reach confluence in culture. If the cells are allowed to overgrow, it appears that the few negative cells still present survive better than the positive ones. No tau immunoreactivity was present on cells transfected with the selection vector only (pSV_2neo).

Expression of tau in CHO[pSVtau3] cells

Western blot analysis confirmed that tau was expressed in CHO[pSVtau3] cells. Immunoblots of total extracts from untransfected CHO cells and CHO[pSVtau3] cells were stained with the well-characterized monoclonal antibodies, tau 1 and 5E2, to defined epitopes of tau (Figs. 1*a* and 1*b*), and with an affinity-purified polyclonal antibody to human tau (Fig. 1*c*). In each case, tau was expressed as a set of three immunoreactive bands. The slow-migrating species appears to be the most abundant, irrespective of the antibody used for detection. The high-mobility tau species of CHO[pSVtau3] cells co-migrates with the fast-migrating tau band of human brain (Fig. 1*d*, double arrow). This band represents the 352-amino-acid-long tau isoform that has been determined to have a true molecular mass of 36 760 Da and an apparent molecular mass of 48 000 Da (Goedert & Jakes, 1990).

Subcellular distribution of tau in CHO[pSVtau3] cells

An affinity-purified polyclonal antibody to human tau stained a filamentous network in the cytoplasm of CHO[pSVtau3] cells that radiated from the juxtanuclear region (Fig. 2*a*). In some cells, the centrioles can be clearly distinguished (Fig. 2*b*). Some mitotic spindles were also observed with the antibody (not shown).

Double immunofluorescence staining showed that the staining pattern obtained with antibodies to tau was indistinguishable from that with a monoclonal antibody to β-tubulin (Figs. 3*a*, and 3*c*). No bundles of microtubules similar to those described by Kanai *et al.* (1989) and Lewis *et al.* (1989) were observed. To confirm further the co-localization of tau and microtubules in CHO[pSVtau3] cells, the cells were treated with the microtubule-stabilizing drug taxol. Exposure of cultured cells to taxol induces the formation of microtubule bundles (Schiff & Horwitz, 1980). After an overnight incubation with 10 μmol of taxol/l, CHO[pSVtau3] cells were processed for double immunofluorescence staining. The taxol-induced microtubule bundles were clearly identified by the anti-β-tubulin antibody, and were also stained by anti-tau antibodies (Figs. 3*b* and 3*d*).

Tau is phosphorylated in CHO[pSVtau3] cells

Since CHO[pSVtau3] cells have been transfected with a single

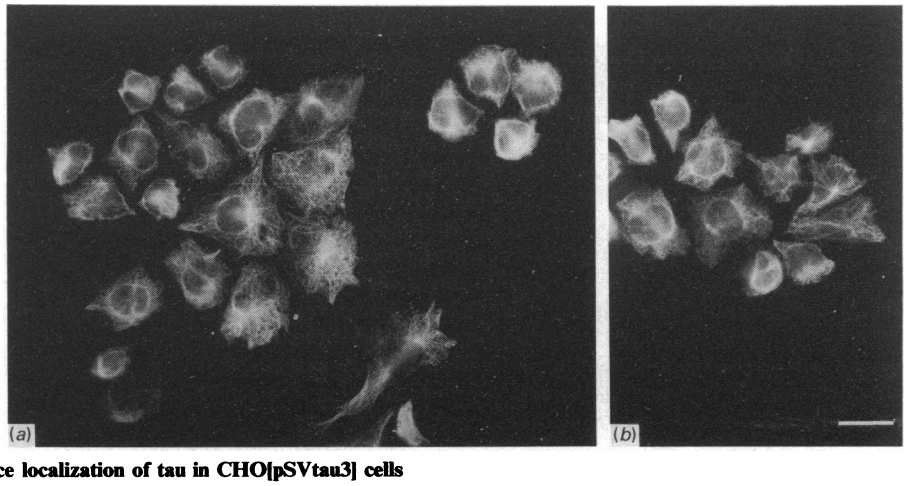

Fig 2. Immunofluorescence localization of tau in CHO[pSVtau3] cells

CHO[pSVtau3] cells were fixed and permeabilized with acetone and processed for immunofluorescence labelling with an affinity-purified polyclonal antibody to human tau. Note the variation in the level of tau immunoreactivity from one cluster of cells to another. Some stained centrioles can easily be observed in (*b*). Scale bar, 25 μm.

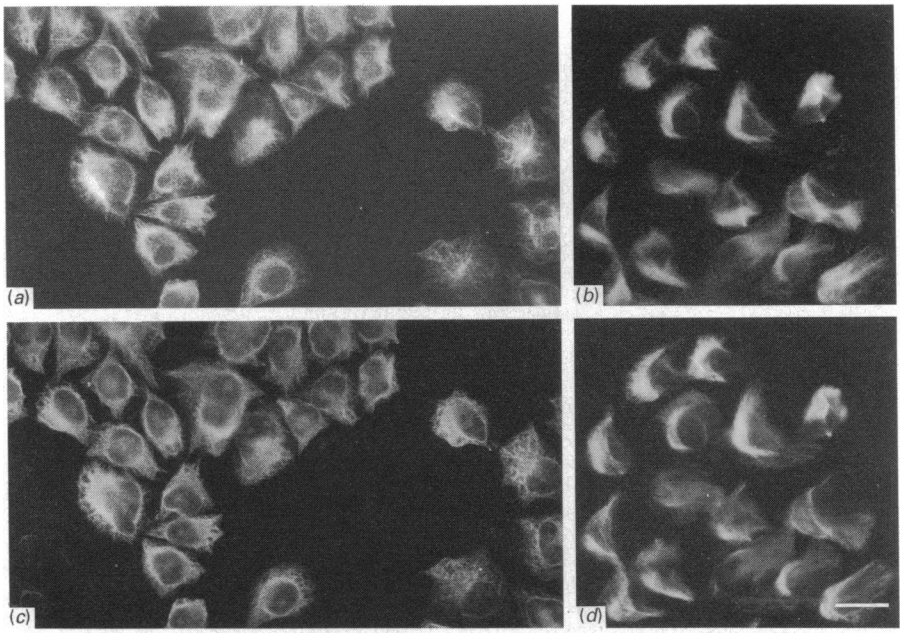

Fig 3. Co-localization of tau and microtubules in CHO[pSVtau3] cells by double-label immunofluorescence staining

Cells were stained with a rabbit affinity-purified polyclonal antibody to human tau, followed by FITC-conjugated goat anti-rabbit IgG (*a* and *b*), or by a mouse monoclonal antibody to β-tubulin followed by TRITC-conjugated goat anti-mouse IgG (*c* and *d*). Untreated cells (*a*, *c*) and cells treated overnight with 10 μmol of taxol/l (*b*, *d*), were used. Scale bar, 25 μm.

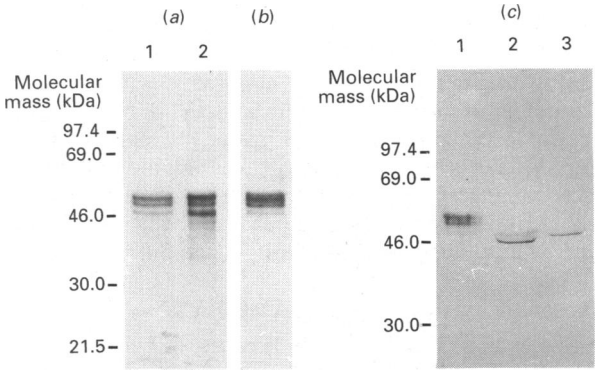

Fig 4. Phosphorylation of tau in CHO[pSVtau3] cells

(*a*) Analysis of the heat-stable fraction of CHO[pSVtau3] cells. Heat stable fraction (lane 1) and whole cell extract (lane 2) from CHO[pSVtau3] cells were blotted on to nitrocellulose and stained with the monoclonal antibody tau 1. (*b*) Same sample as in (*a*), lane 1, but stained with an affinity-purified polyclonal antibody to human tau. (*c*) Dephosphorylation of tau from CHO[pSVtau3] cells. The heat-stable fraction from CHO[pSVtau3] cells was incubated for 28 h at 37 °C without (lane 1) or with (lane 2) 30 units of *E. coli* alkaline phosphatase/ml, immunoblotted and reacted with the monoclonal antibody tau 1. The sample in lane 3 is the same as in lane 2, but at a lower loading on the gel.

cDNA, the multiple bands observed in Western blots are likely to result from post-translational modification(s) of tau. Tau is phosphorylated in brain, and some types of tau phosphorylation induce a decreased electrophoretic mobility (Baudier & Cole, 1987; Steiner *et al.*, 1990; Litersky & Johnson, 1992); a likely modification of tau in CHO[pSVtau3] cells is, therefore, phosphorylation. In order to compare the electrophoretic pattern of tau in extracts of CHO[pSVtau3] cells before and after dephosphorylation, a heat-stable fraction from CHO[pSVtau3] cells was prepared. This fraction contains the same three tau bands as whole cell extracts (Fig. 4*a*), stained by both tau 1 (Fig. 4*a*) and the polyclonal antibody to tau (Fig. 4*b*). The heat-stable fraction from CHO[pSVtau3] cells was treated for 28 h with 30 units of *E. coli* alkaline phosphatase/ml. This treatment resulted in the disappearance of the two slow-migrating bands and in an enhanced immunoreactivity of the fast-migrating band for both the monoclonal antibody tau 1 (Fig. 4*c*) and the polyclonal antibody to tau (results not shown). Lower loadings of the alkaline phosphatase-treated sample on a gel show that dephosphorylated tau co-migrates with the fast-migrating tau band of the untreated fraction (Fig. 4*c*, lane 3).

DISCUSSION

Tau proteins are predominantly expressed in neurones. Here we show that, under the control of the SV40 promoter, a tau cDNA can be expressed in CHO cells, which do not express endogenous tau. So far, transfection studies with tau have mainly been done in transiently transfected cells, partly because of the difficulty in obtaining stably transfected cell lines expressing MAPs (Lewis *et al.*, 1989). The only such cell line, other than CHO[pSVtau3], that has been reported expresses an adult form of rat tau (Kanai *et al.*, 1989).

Tau isoforms contain either three or four tandem repeats of 31-amino-acid sequences in the *C*-terminal half of the molecule (Goedert *et al.*, 1988, 1989*a*; Lee *et al.*, 1988; Kosik *et al.*, 1989) that are tubulin-binding sites (Aizawa *et al.*, 1989; Ennulat *et al.*, 1989; Himmler *et al.*, 1989; Lee *et al.*, 1989). *In vitro*, both three- and four-repeat isoforms of tau promote tubulin polymerization, but the rate of polymerization is up to three times faster with four-repeat forms than with three-repeat forms (Goedert & Jakes, 1990).

Our results show that the transfected three-repeat isoform of tau co-localizes with microtubules or taxol-induced microtubule bundles in CHO[pSVtau3] cells. Binding of tau to microtubules in non-neuronal cells has also been observed after microinjection in fibroblastic cells (Drubin & Kirschner, 1986) and transfection

of various cell types with three-repeat (Lewis *et al.*, 1989) and four-repeat (Kanai *et al.*, 1989) forms of tau.

In the latter systems, the expression of tau resulted in the formation of bundles of parallel arrays of microtubules. MAP2, which possesses three tubulin-binding domains homologous to their tau counterparts (Lewis *et al.*, 1988), also induces microtubule bundling in transfected cells (Lewis *et al.*, 1989). Microtubule bundling was essentially observed in transiently transfected cells, but a few microtubule bundles were present in a mouse L cell line stably transfected with a four-repeat isoform of rat tau (Kanai *et al.*, 1989). The absence of microtubule bundling in the cell line CHO[pSVtau3] described here might be due to the presence of only three tandemly repeated microtubule-binding sites in the isoform of tau used for transfection, which would not stabilize microtubules to the same extent as would a four-repeat isoform. These results therefore suggest that the number of tubulin-binding sites may contribute to the microtubule bundling properties of tau.

CHO[pSVtau3] cells have been transfected with a single cDNA; however, tau is expressed as three bands of different mobilities in SDS/PAGE. Treatment of CHO[pSVtau3] tau with *E. coli* alkaline phosphatase demonstrated that the two slow-migrating bands represent two phosphorylation states of tau. Kanai *et al.* (1989) performed similar experiments and found that a four-repeat form of rat tau was phosphorylated in mouse L-cells. In these authors' system, treatment with alkaline phosphatase gave rise to two bands instead of one, as in CHO[pSVtau3] cells. They hypothesized the occurrence of an as yet unidentified post-translational modification of tau different from phosphorylation. Such a post-translational mechanism appears not to occur in CHO cells, or does not modify the tau isoform used in our study.

In vitro, phosphorylation of tau by Ca^{2+}/calmodulin-dependent kinase induces an electrophoretic shift, on both previously dephosphorylated brain tau (Baudier & Cole, 1987) and bacterially synthesized recombinant tau (Steiner *et al.*, 1990). Such a shift has also been found with cyclic AMP-dependent protein kinase (Litersky & Johnson, 1992; J. Robertson, T. Loviny, M. Goedert, R. Jakes, K. Murray, B. Anderton & D. Hanger, unpublished work). A site phosphorylated *in vitro* by Ca^{2+}/calmodulin-dependent kinase has been identified as Ser-405 in a 430-amino-acid sequence of bovine tau, and phosphorylation at this site is sufficient to induce a shift in tau mobility (Steiner *et al.*, 1990). An explanation for the reduced electrophoretic mobility of some phosphorylated forms of tau is provided by electron microscopy observations showing a major change in tau structure, such that it becomes long and stiff after phosphorylation by Ca^{2+}/calmodulin-dependent kinase (Hagestedt *et al.*, 1989).

Here we show that a single three-repeat isoform of human tau can be phosphorylated *in vivo* in at least two different states of this type. The two observed bands might reflect a different extent of phosphorylation by the same kinase, possibly Ca^{2+}/calmodulin-dependent kinase, or the presence in CHO cells of at least two kinases inducing an electrophoretic shift of tau. Elucidating the regulatory mechanisms of this type of tau phosphorylation is important because of its occurrence in hyperphosphorylated tau in Alzheimer's disease (Flament & Delacourte, 1989; Hanger *et al.*, 1991; Lee *et al.*, 1991).

In conclusion, we have shown that a three-repeat isoform of human tau bound to microtubules and was phosphorylated in a stably transfected non-neuronal cell line. Furthermore, the properties of tau in CHO[pSVtau3] cells appear to differ from those previously reported in another system (Kanai *et al.*, 1989), and this suggests the occurrence of isoform-specific properties of tau, including phosphorylation and association with the microtubule network.

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