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Microtubule Binding and Clustering of Human Tau-4R and Tau-P301L Proteins Isolated from Yeast Deficient in Orthologues of Glycogen Synthase Kinase-3β or cdk5*

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Phosphorylation of Tau protein and binding to microtubules is complex in neurons and was therefore studied in the less complicated model of humanized yeast. Human Tau was readily phosphorylated at pathological epitopes, but in opposite directions regulated by kinases Mds1 and Pho85, orthologues of glycogen synthase kinase-3β and cdk5, respectively (1). We isolated recombinant Tau-4R and mutant Tau-P301L from wild type, Δ*mds1* and Δ*pho85* yeast strains and measured binding to Taxol-stabilized mammalian microtubules in relation to their phosphorylation patterns. Tau-4R isolated from yeast lacking *mds1* was less phosphorylated and bound more to microtubules than Tau-4R isolated from wild type yeast. Paradoxically, phosphorylation of Tau-4R isolated from kinase Pho85-deficient yeast was dramatically increased resulting in very poor binding to microtubules. Dephosphorylation promoted binding to microtubules to uniform high levels, excluding other modifications. Isolated hyperphosphorylated, conformationally altered Tau-4R completely failed to bind microtubules. In parallel to Tau-4R, we expressed, isolated, and analyzed mutant Tau-P301L. Total dephosphorylated Tau-4R and Tau-P301L bound to microtubules very similarly. Surprisingly, Tau-P301L isolated from all yeast strains bound to microtubules more extensively than Tau-4R. Atomic force microscopy demonstrated, however, that the high apparent binding of Tau-P301L was due to aggregation on the microtubules, causing their deformation and bundling. Our data explain the pathological presence of granular Tau aggregates in neuronal processes in tauopathies.

Transport along microtubules (MT)[3] is essential for normal neuronal functions by providing synapses with necessary protein complexes, vesicles, and organelles. MT-associated proteins (MAP) regulate the structural and functional organization of the MT network in relation to the rest of the cytoskeleton, to allow axonal transport. Tau protein is an important active MAP, as negatively exemplified by the diverse group of neurodegenerative diseases termed tauopathies, including frontotemporal dementia and Alzheimer disease (AD) (for reviews see Refs. 2–12).

In all tauopathies, hyperphosphorylation and aggregation of Tau protein appear invariably associated. Whereas it can be assumed that in normal conditions all Tau protein is bound to MT, it is not known whether detachment of Tau protein from MT is a prerequisite for phosphorylation to take place. Whereas Tau-MT binding depends critically on phosphorylation, the complexity and variability of the phosphorylation pattern of neuronal Tau precludes precise definition of the relative or absolute importance of individual phosphorylation sites.

We have generated humanized yeast strains by expressing human Tau protein, different isoforms and mutants, to develop a less complex, eukaryotic cell-based model (1). Phosphorylation of human Tau at specific residues, known to be pathological in the Tau aggregates in clinical tauopathies, was already important in normal yeast cultures probably due to the fact that human Tau did not bind appreciably to yeast MT. Significantly, phosphorylation was regulated in opposite directions by kinases Mds1 and Pho85, the functional homologues of GSK-3β and cdk5, respectively (1). Thereby this model recapitulated our observations *in vivo* in brain of transgenic mice expressing human Tau protein and Tau kinases GSK-3β and cdk5/p35 (5, 13–18).

Overexpression of wild type Tau-4R in neurons of transgenic mice triggered a severe axonopathy due to excessive binding of Tau-4R to MT. The resulting blockage of bidirectional axonal transport in turn caused axonopathy with severe wallerian degeneration and motor defects (13). GSK-3β, co-expressed with Tau-4R rescued the axonopathy as well as the motor impairment completely (14), illustrating the importance of phosphorylation of Tau-4R by GSK-3β for MT binding *in vivo*. The other major Tau kinase cdk5 was, however, not capable of phosphorylating Tau-4R or rescue the axonopathy, not even when co-expressed with its neuron-specific activator p35 in triple transgenic mice (15). This indication that cdk5 acted unlike GSK-3β, and actually completely opposite was underlined by the increased phosphorylation of cytoskeletal proteins

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[3] The abbreviations used are: MT, microtubules; MAP, microtubule-associated protein; AD, Alzheimer disease; AFM, atomic force microscopy; mAb, monoclonal antibody; GSK-3β, glycogen synthase kinase-3β; MES, 4-morpholineethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid.

in brain of $p35^{-/-}$ mice (18), and joins seamlessly with our observations in yeast (1).

Mutations in the microtubule-associated protein Tau gene coding for Tau protein on chromosome 17 cause a varied group of dominantly inherited diseases, collectively known as frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17). Both exonic and intronic mutations are known, giving rise to either mutant Tau protein or to augmented Tau-4R isoform expression, respectively, which apparently shares the propensity to become phosphorylated and aggregate into filaments and tangles, the common pathological defect in all tauopathies (for reviews see Refs. 3–12).

Conflicting information concerns the influence of expressed mutations in Tau protein on its binding to MT and self-assembly (19–23). Overexpression of different frontotemporal dementia mutants of Tau in transgenic mice results in the development of tauopathy (24–26). We generated Tau-P301L mice and extensively compared them to Tau-4R transgenic mice (16, 17). The contrast in pathology was evident because beyond 8 months of age, Tau-P301L mice developed a typical tauopathy, *i.e.* intra-neuronal aggregates and fibrils evolving into authentic NFT containing Tau with all major pathological epitopes (16).

Here we extrapolate studies on transgenic mice to humanized yeast cells expressing Tau-4R or mutant Tau-P301L. In addition, we isolated recombinant human Tau from wild type yeast and strains lacking yeast kinases Mds1 or Pho85, the orthologues of mammalian GSK-3β and cdk5, respectively (Ref. 1 and references therein). By an optimized filter assay, we measured binding of different Tau isoforms to Taxol-stabilized mammalian MT, in direct relation to the physiological phosphorylation status of Tau. The data demonstrate a close parallel between phosphorylation of Ser^{396}/Ser^{404} and Ser^{409}, recognized by monoclonal antibodies (mAbs) AD2 and PG5 (Refs. 1 and 16 and references therein), respectively, with loss of physiological binding to MT and pathological aggregation of Tau at the MT surface. Also clear, Mds1 and Pho85 control in opposite directions both the phosphorylation and MT-binding of Tau-4R.

Surprisingly, de-phosphorylated Tau-4R and Tau-P301L bound with equal high affinity to MT, proving that the mutation did not directly affect MT binding *per se*, but indirectly affected the phosphorylation of mutant Tau-P301L. Moreover, phosphorylated Tau-P301L actually aggregated on the MT surface as measured by the MT binding assays and observed directly by atomic force microscopy (AFM), including pronounced deformation and bundling of MT *in vitro*. This novel combination of cellular and *in vitro* modeling provide direct links of specific phosphorylation, aggregation, and MT binding of Tau protein, and we believe these models can now be adapted to analyze very precisely the effect of single phosphoepitopes on the biochemical behavior of human Tau protein.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—Yeast cells were grown exactly as described before (1). cDNA coding for the human Tau isoform 2N/4R (1) and human Tau-P301L 2N/4R (16) were inserted into the pYX212 plasmid containing the constitutive TPI promoter (R&D systems, Minneapolis, MN). Yeast transformants expressing Tau-4R or Tau-P301L were obtained in wild type, in Δ*mds1* and Δ*pho85* strains, and selected as described (1).

TABLE 1
Overview of the specificity of the monoclonal antibodies used in this project

Antibody	Specificity	Source
Tau-5	All isoforms	BD Pharmingen
AD-2	Phosphorylated Ser^{396}/Ser^{404}	Bio-Rad
AT8	Phosphorylated Ser^{202}/Ser^{205}	Innogenetics, Gent, Belgium
AT100	Phosphorylated Thr^{212}/Ser^{214}	Innogenetics, Gent, Belgium
AT180	Phosphorylated Thr^{231}/Ser^{235}	Innogenetics, Gent, Belgium
AT270	Phosphorylated Thr^{181}	Innogenetics, Gent, Belgium
PG-5	Phosphorylated Ser^{409}	Gift from P. Davies
Yol1/34	α-Tubulin	Abcam, Paris, France

Purification of Tau—Tau-4R and Tau-P301L were purified from wild type, Δ*mds1*, or Δ*pho85* yeast strains as described (1). Purified Tau was concentrated on 10-kDa centricon devices (Millipore Corp., Bedford, MA) equilibrated with 10 mM MES buffer (pH 6.4). The amount of Tau was quantified by Western blotting with mAb Tau-5 and recombinant His-tagged Tau (Merck, Darmstadt, Germany) as standard (1). Phosphorylation was assessed using phosphorylation-specific mAbs with specificity summarized in Table 1.

Dephosphorylation of Purified Tau—Purified Tau was concentrated in 50 mM Tris (pH 8.6) and quantified. Dephosphorylation was performed by incubating with alkaline phosphatase (Roche, Darmstadt, Germany), using 1.7 units/μg of Tau at 37 °C for 2 h.

Preparation of Taxol-stabilized MT—Pig tubulin was isolated as described previously (27). Tubulin aliquots were centrifuged (100,000 × *g*, 30 min, 4 °C) prior to analysis. MT were obtained by incubation of purified tubulin (10 μM) in assembly buffer (80 mM Pipes, 1 mM $MgCl_2$, 1 mM EGTA (pH 6.8)) adjusted gradually to 10 μM Paclitaxel, 1 mM GTP and incubated at 37 °C for 30 min.

Tau-MT Binding Filter Assay—Reaction mixtures in the same assembly buffer described above were prepared containing 10 nM Tau, which was centrifuged (100,000 × *g*, 30 min, 4 °C) prior to addition, with increasing concentrations of Taxol-stabilized MT (equivalent to up to 6 μM tubulin), further containing 0.05% bovine serum albumin and 1 mM GTP. After incubation at 37 °C for the indicated time periods, the mixture was transferred to pre-warmed 200-nm centrifuge devices (Nanosep MF, PALL, Michigan, MI) and centrifuged (4,000 × *g*, 10 min, 37 °C). Filtrates and retentates were adjusted and solubilized, respectively, in 50 mM Tris (pH 8.0), 10 mM β-mercaptoethanol, 2% SDS, 0.1% bromphenol blue, 10% glycerol, and boiled. SDS-PAGE and Western blotting (1) were used with mAb Tau-5 to detect Tau. The relative concentration of Tau was determined from a curve of optical density against dilution of a standard preparation of human Tau (10 nM). The amount of bound Tau and the concentration of tubulin were corrected for nonspecific binding of Tau to the membrane and the amount of unpolymerized tubulin, respectively. Curve fitting (SigmaPlot 9.0, Rockware Inc., Cureglia, Switzerland) yielded values for affinity constant and maximal binding (binding equation: $y = B_{max} \cdot x/(K_{d,app} + x)$, in which y is the

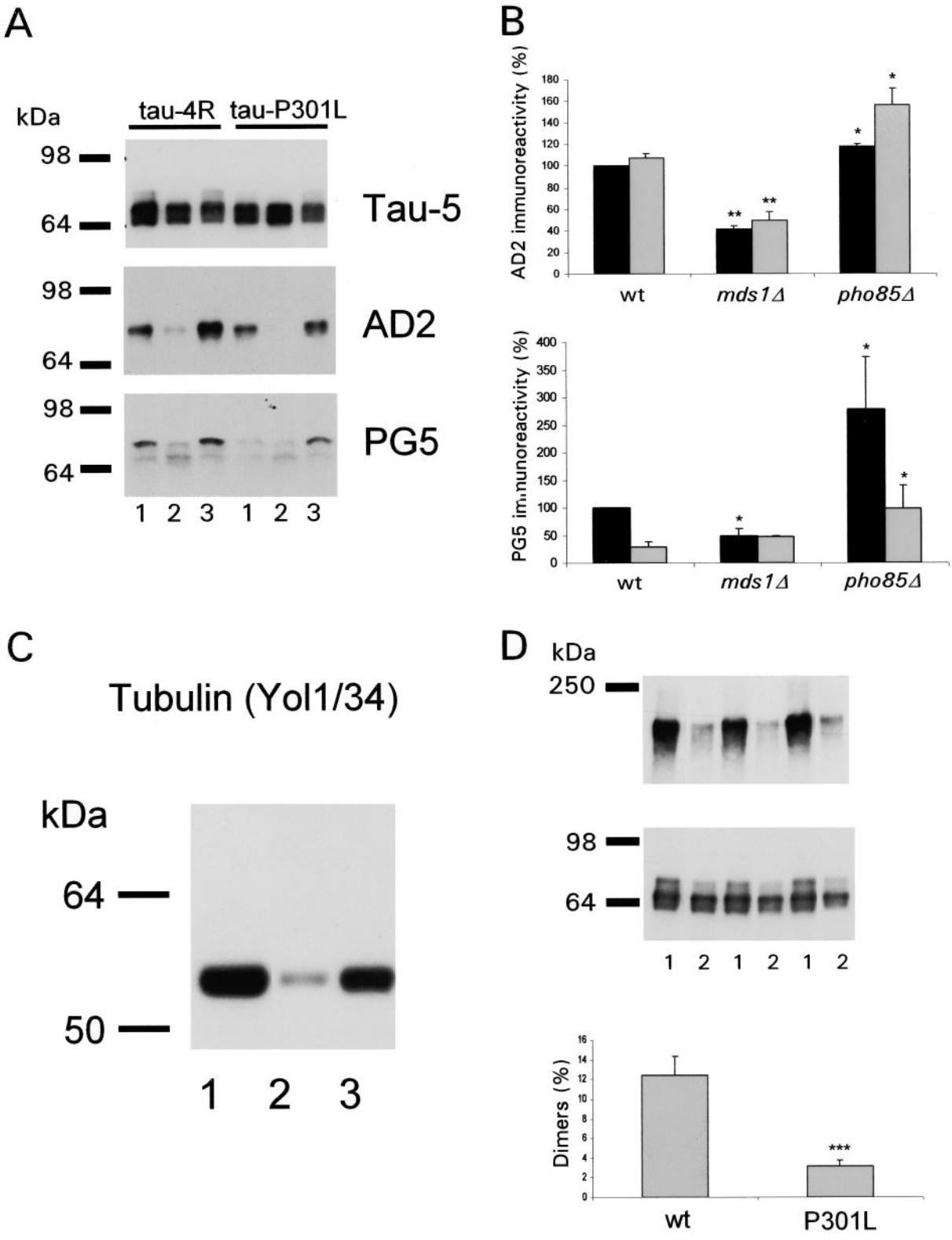

FIGURE 1. Western blots (*A*) and quantification (*B*) of phosphoepitope mapping of human Tau-4R (*black bars*) or Tau-P301L (*gray bars*), isolated from wild type (*wt*) (*lanes 1*), Δ*mds1* (*lanes 2*), and Δ*pho85* (*lanes 3*) yeast with mAbs as indicated (for specificity see Table 1), and (*C*) evaluation of the method to separate MT-Tau complexes from soluble Tau. Taxol-stabilized MT were prepared and separated as described under "Experimental Procedures." Total amounts of MT (*lane 1*), non-polymerized tubulin, passing the filter (*lane 2*), and MT retained on the filter (*lane 3*) were quantified by polyclonal antibody Yol1/34. *D*, differential dimerization of wild type Tau-4R (*lane 1*) and Tau-P301L (*lane 2*) expressed in and isolated from wild type yeast strains (see "Experimental Procedures") and blotted after SDS-PAGE with mAb Tau-5, separating monomeric (*middle panel*) and dimeric Tau (*upper panel*). Relative levels of dimeric Tau of wild type Tau-4R and Tau-P301L (P301L), as indicated, in the wild type yeast strain (*bottom panel*). All data are normalized for the amount of monomeric Tau determined with Tau-5, and mean ± S.E. of three independent experiments. *Asterisks* denote statistically significant difference (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

percentage binding and x is the concentration of MT expressed as tubulin monomer content). Statistical analysis was performed by one-way analysis of variance, followed by the multiple comparison test of Tukey.

Ultrastructural Analysis by AFM—Mixtures of 250 nM Tau and 1.5 μM MT were fixed with 2% glutaraldehyde and deposited on silanized silicon supports for AFM analysis as described previously (1). AFM low power images were taken with Point probes type FM cantilevers (Nanoworld, Neuchatel, Switzerland), whereas high power images were obtained with Data probe type cantilevers (DP18/HI'RES/AIBS) (MikroMasch, Madrid, Spain) (1).

RESULTS

Filter Assay of Binding of Recombinant Human Tau-4R Protein to Taxol-stabilized MT—Human Tau-4R protein was expressed in and isolated from wild type, Δ*mds1*, and Δ*pho85* yeast strains as described (1). As opposed to recombinant Tau isolated from bacterial sources, this procedure faithfully maintains the phosphorylation status of Tau protein as present intracellularly in wild type and manipulated yeast cells, a less complex but eukaryotic model (1). The quality and phosphorylation status of the used preparations of isolated Tau were authenticated by Western blotting (Fig. 1, *A* and *B*).

Binding of Tau protein to preformed, Taxol-stabilized MT was measured by varying the concentration of MT, expressed as the concentration of tubulin monomers measured spectrophotometrically and by Western blotting. A fixed relatively low concentration of recombinant Tau protein (10 nM) was used in these studies, allowing determination of the apparent dissociation constants of MT binding (28).

Binding of Tau-4R to MT was measured by an optimized filter assay (see "Experimental Procedures"). In brief, Tau/MT mixtures were incubated and centrifuged through disposable 200-nm filters, separating unbound Tau protein in the soluble pass-through fraction, although retaining all Tau-MT complexes. Bound and free Tau were measured in retentates and filtrates, respectively, by Western blotting with the pan-Tau monoclonal antibody Tau-5. This procedure allowed us to quantitatively differentiate MT-bound *versus* unbound Tau. In addition, as extra internal markers, the majority of tubulin retained on the filter as MT, and the small amount of monomeric tubulin in the filtrates, were measured by Western blotting with an anti-tubulin antibody (Fig. 1*C*).

Binding of Isolated Tau-4R to MT Is Affected in Opposite Directions by Mds1 and Pho85 Inactivation—We first measured binding of isolated Tau-4R to Taxol-stabilized MT, compared with dephosphorylated Tau-4R treated with alkaline phosphatase after isolation from the BY4741 wild type yeast strain. More than 90% of dephospho-Tau-4R bound to MT with an apparent affinity of 0.49 ± 0.09 μM (Fig. 2*A*, Table 2). The original phosphorylated Tau-4R preparation isolated from wild type yeast bound to MT with a lower apparent affinity of 2.15 ± 0.43 μM (Fig. 2*A*, Table 2).

We then compared MT binding of Tau-4R isolated from wild type, Δ*mds1*, and Δ*pho85* yeast strains. Tau-4R isolated from Δ*mds1* yeast bound to MT more extensively than Tau-4R isolated from wild type yeast (Fig. 2*A*, Table 2). In contrast, Tau-4R isolated from Δ*pho85* yeast strains showed a strongly reduced extent and affinity of MT binding (Fig. 2*A*, Table 2).

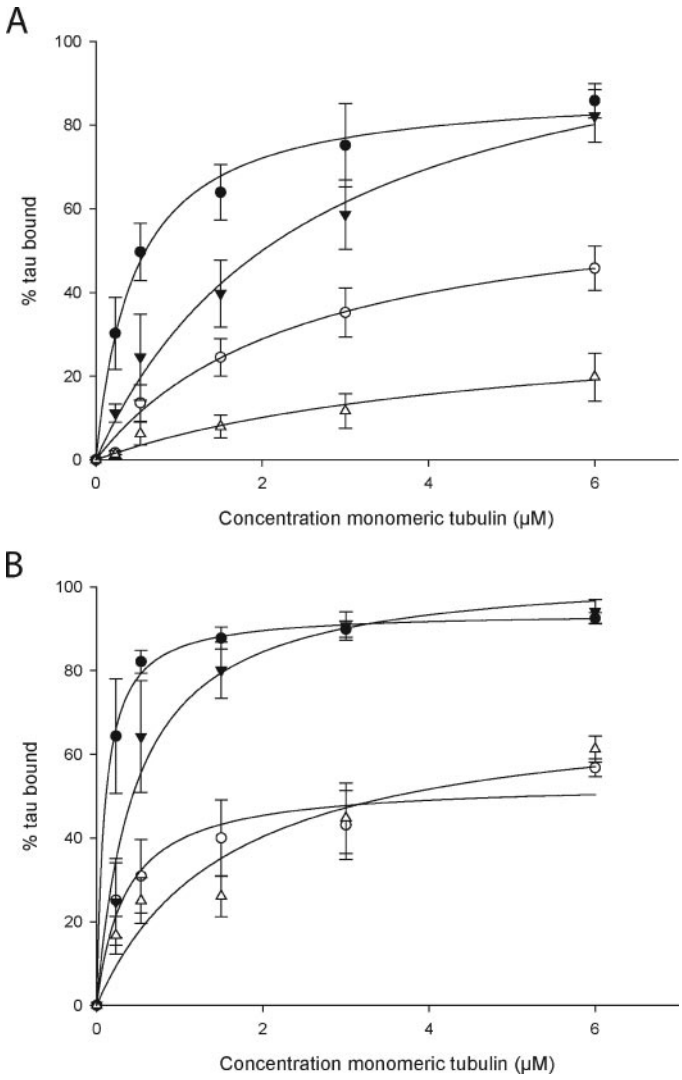

FIGURE 2. **Quantification of the *in vitro* binding of isolated Tau-4R monomers (*A*) or dimers (*B*) to Taxol-stabilized MT.** Relative amount of bound dephosphorylated Tau (*black circles*), BY4741-Tau (*open circles*), Δ*mds1*-Tau (*black triangles*), and Δ*pho85*-Tau (*open triangles*) were plotted to the concentration of monomeric tubulin to determine Tau binding characteristics (see "Experimental Procedures" and Table 2). All data are mean ± S.E. of 3 independent experiments.

Dimeric Tau-4R Protein Binds to MT with Higher Affinity—The preparations of recombinant Tau-4R isolated from yeast contained higher molecular weight species, tentatively identified and further referred to as Tau-4R dimers, based on their apparent M_r. Their origin or mode of apparent covalent linkage is unknown. Similar dimers were observed in the crude extracts from humanized Tau-4R yeast cultures (1), and moreover also in brain extracts from Tau-4R transgenic mice (13, 14, 16).

Tau-4R dimers, detected by Western blotting of bound and unbound fractions, were quantified in parallel to the Tau-4R monomers, eventually requiring somewhat longer exposure of the blots. Tau-4R dimers bound with higher apparent affinity to MT than monomeric Tau-4R (Fig. 2*B*, Table 2). Remarkably, Tau-4R dimers originating from Δ*pho85* yeast cultures bound

TABLE 2

$K_{d,app}$ (expressed in micromolar) of wild type and mutant Tau-4R monomers or dimers, isolated from different yeast strains

Data were analyzed by one-way analysis of variance followed by multiple comparison test of Tukey (see "Experimental Procedures"). Different superscript letters denote significantly different values at $p < 0.05$, data denoted by the same superscript letters are not significantly different.

	Monomer		Dimer, Tau-4R
	Tau-4R	Tau-P301L	
Dephosphorylation	0.49 ± 0.09^{a}	0.68 ± 0.07^{a}	0.08 ± 0.04^{d}
BY4741	2.15 ± 0.43^{b}	0.75 ± 0.27^{a}	0.83 ± 0.47^{a}
Δ*mds1*	2.48 ± 0.70^{b}	0.85 ± 0.15^{a}	0.63 ± 0.34^{a}
Δ*pho85*	$>10^{c}$	$1.23 \pm 0.45^{a,b}$	1.58 ± 0.51^{b}

to MT with much higher apparent affinity than the monomeric Tau-4R present in the same preparations (Fig. 2*B*, Table 2).

When no other factors are involved, homodimeric proteins are expected to bind with affinity that is the square of that of the corresponding monomers. The experimental data approach this theoretical value fairly closely, indicating that dimerization of Tau-4R did not increase the affinity by another mechanism. The dimers are unlikely to be heterodimers of Tau-4R with unknown proteins, given the fact that they are observed in these widely different organisms of transgenic mice and yeast.

Binding of Tau-4R to MT in Relation to Its Phosphorylation Status—Inspection of the Western blots developed with the pan-Tau antibody Tau-5 revealed important differences in electrophoretic mobility of Tau-4R present in bound and unbound fractions. Very conspicuously, electrophoretically faster migrating Tau subforms in all preparations bound preferably to MT, whereas electrophoretically slower migrating Tau isoforms were excluded from the MT-bound fractions.

Because in the heterologous yeast expression system the electrophoretic mobility of Tau-4R reflects its phosphorylation status (Ref. 1 and references therein) the data implied that phosphorylation of Tau-4R protein prevented or decreased the binding to MT. We tested first the isolated hyperphosphorylated Tau fraction, defined as hP-Tau/MC1 (1) and found it completely devoid of binding to MT (Fig. 3) as expected. The possibility was considered that the differences in apparent maximal binding of different preparations of recombinant Tau-4R described above were due to their relative content of hyperphosphorylated Tau protein, which does not bind at all.

Effect of Phosphorylation of the Tau-P301L Mutation on Binding to MT—The clinical mutant Tau-P301L was expressed in and purified from yeast cells, in parallel with wild type Tau-4R to allow their direct comparative analysis. In the heterologous yeast model neither Tau-P301L nor Tau-4R appeared to bind to the endogenous yeast microtubular system (results not shown), allowing us to relate the contribution of the single residue mutation directly to the phosphorylation and aggregation of human Tau-4R protein.

The clinical mutant Tau-P301L contained in the Tau-4R isoform was expressed constitutively from the same high copy number plasmid as wild type Tau-4R in BY4741 and W303-1A wild type yeast strains, as well as in the isogenic Δ*mds1* and Δ*pho85* mutant strains. Western blotting with the pan-Tau mAb Tau-5 demonstrated also a mixture of species varying in molecular mass from 64 to 72 kDa, which is overall similar to

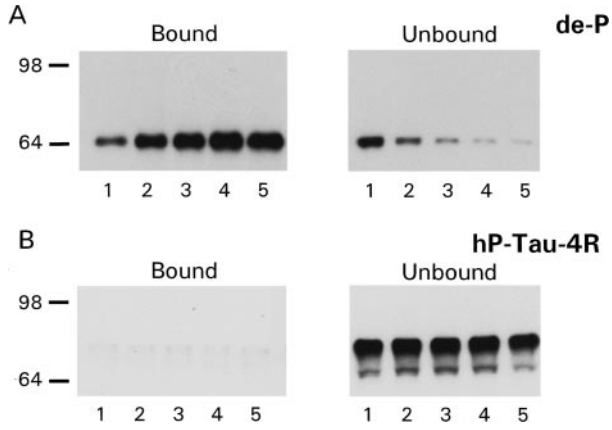

FIGURE 3. ***In vitro* binding of isolated hP-Tau/MC1 monomers on Taxol-stabilized MT determined by SDS-PAGE and Western blotting using pan-Tau mAb Tau-5.** *A* and *B*, bound and unbound fractions of dephosphorylated Tau-4R (*A*) and hP-Tau-4R (*B*) to 0 (*lane 1*), 0.8 (*lane 2*), 1.5 (*lane 3*), 3 (*lane 4*), and 6 μM MT (*lane 5*).

what we observed for wild type Tau-4R with some interesting differences (Fig. 1, *A* and *B*). After de-phosphorylation both wild type and mutant Tau protein migrated identically as single protein species (data not shown) (1), demonstrating that post-translational modifications besides phosphorylation were absent or at least not different for either wild type or mutant Tau protein heterologously expressed in wild type yeast.

Western blotting with the pan-Tau antibody Tau-5 demonstrated far less dimers to be formed by mutant Tau-P301L, *i.e.* less than 5% of total Tau-P301L present in a series of independent transformants. That contrasted with the amount of dimers formed by Tau-4R in yeast cells, and contained in preparations thereof, to about 15% of total Tau-4R protein present. Consequently, Tau-P301L dimers could only be visualized by much longer exposure of the Western blots (Fig. 1*D*) and this curtailed their inclusion in the analysis of binding to MT. Significantly, we observed the very same difference in formation of dimers *in vivo* in the brains of Tau-4R and Tau-P301L transgenic mice (16). These data are included here, as they further validate yeast as a heterologous expression system that recapitulates faithfully the biochemical behavior of Tau protein, as observed in mammalian neurons *in vivo*.

The characteristics of the MT binding of dephosphorylated Tau-P301L were very similar to those of de-phosphorylated Tau-4R, showing practically no difference in the extent of binding, and a small difference in apparent affinity, *i.e.* 0.68 ± 0.07 *versus* 0.49 ± 0.09 μM, respectively (Fig. 5*A*, Table 2).

Surprisingly, the analysis of binding to MT of mutant Tau-P301L isolated from wild type, Δ*mds1*, and Δ*pho85* yeast strains, demonstrated a far lesser influence of the cellular origin than observed for Tau-4R isolated from the same yeast strains (Figs. 4, 5, *B* and *C*, and Table 2). Actually, Tau-P301L isolated from all yeast strains bound to MT with very similar characteristics, approaching even those of the de-phosphorylated Tau preparations (Fig. 5*C*, Table 2). Moreover, Tau-P301L isolated from Δ*pho85* yeast cells bound to MT much more extensively than Tau-4R isolated from Δ*pho85*, and practically identically to Tau-4R isolated from wild type yeast (Fig. 5, *B* and *C*, Table 2). Remarkably, and similar to Tau-4R, the mutant Tau-P301L

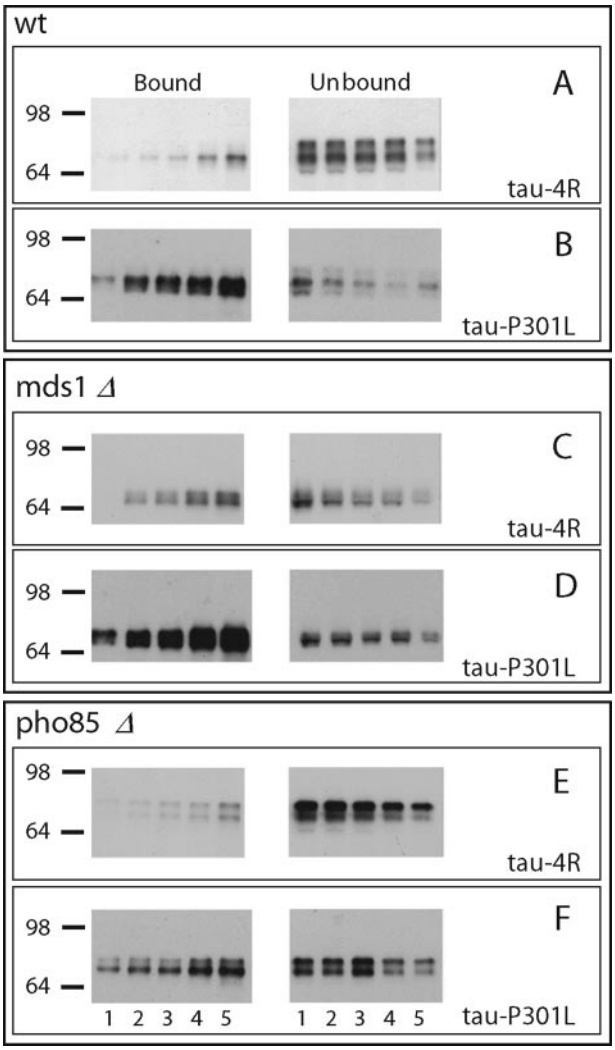

FIGURE 4. ***In vitro* binding of monomeric Tau-4R (*A*, *C*, and *E*) and Tau-P301L (*B*, *D*, and *F*) on Taxol-stabilized MT determined by SDS-PAGE and Western blotting using pan-Tau mAb Tau-5.** Bound and unbound fractions of BY4741-Tau (*A* and *B*), Δ*mds1*-Tau (*C* and *D*), and Δ*pho85*-Tau (*E* and *F*) to 0 (*lane 1*), 0.8 (*lane 2*) 1.5 (*lane 3*), 3 (*lane 4*), and 6 μM MT (*lane 5*).

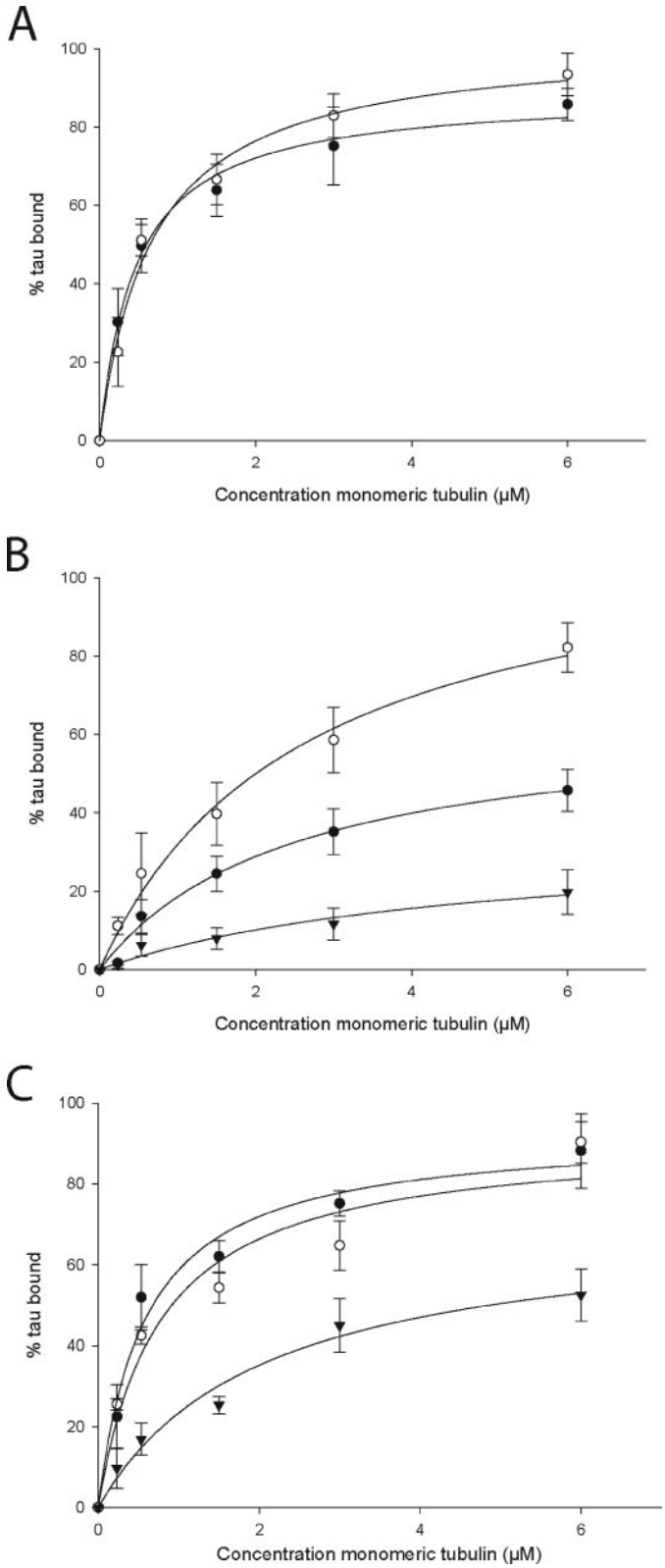

FIGURE 5. **Quantification of the *in vitro* binding of wild type Tau-4R and mutant Tau-P301L to Taxol-stabilized MT.** *A*, relative amount of bound dephosphorylated wild type Tau-4R (*black circles*) and Tau-P301L (*open circles*); *B*, BY4741-Tau-4R (*black circles*), Δ*mds1*-Tau-4R (*open circles*), and Δ*pho85*-Tau-4R (*black triangles*); and *C*, BY4741-Tau-P301L (*black circles*), Δ*mds1*-Tau-P301L (*open circles*), and Δ*pho85*-Tau-P301L (*black triangles*) were plotted to the concentration of monomeric tubulin to determine Tau binding characteristics (see "Experimental Procedures" and Table 2). All data are mean ± S.E. of 3 independent experiments.

isolated from Δ*pho85* yeast cells appeared to contain a fraction that was totally unable to bind to MT, as reflected by the low B_{max} (Fig. 5*C*, Table 3). The cause of this phenomenon is proposed to be the presence of hyperphosphorylated Tau protein in the MC1 configuration (hP-Tau/MC1) (1), which completely lost its MT-binding capacity as demonstrated above (Fig. 3).

Combined, our data demonstrate that the mutation in Tau-P301L did not affect markedly its binding to MT relative to Tau-4R, implying that the phosphorylation acquired in the yeast cells must be different, or that their mode of binding to MT must be different. Both options were analyzed, *i.e.* first by comparing the epitopes expressed on Tau-4R and Tau-P301L isolated from wild type and Δ*mds1* and Δ*pho85* yeast strains in relation to their binding to MT, and subsequently further by using AFM.

Phosphorylation and Aggregation of Tau-P301L in Cells and on MT—Epitope mapping was performed by Western blotting with mAbs AT8, AT100, AT180, AT270, AD2, and PG5 (Table 1) of Tau-4R and Tau-P301L expressed in wild type, Δ*mds1*,

TABLE 3

B_{max} (expressed in percentage) of wild type and mutant Tau-4R monomers or dimers, isolated from different yeast strains

Data were analyzed by one-way analysis of variance followed by multiple comparison test of Tukey (see "Experimental Procedures"). Different superscript letters denote significantly different values at $p < 0.05$; data denoted by the same superscript letters are not significantly different.

	Monomer		Dimer, Tau-4R
	Tau-4R	Tau-P301L	
Dephosphorylation	93.8 ± 6.5^a	97.7 ± 2.2^a	94.4 ± 4.2^a
BY4741	52.0 ± 2.3^b	97.7 ± 9.2^a	59.4 ± 1.8^b
Δ*mds1*	101.9 ± 6.2^a	93.8 ± 2.7^a	101.5 ± 4.6^a
Δ*pho85*	33.0 ± 2.8^c	61.6 ± 0.2^b	58.4 ± 3.6^b

and Δ*pho85* yeast strains (Fig. 1, *A* and *B*). Significant differences were observed only for epitopes of AD2 and PG5, fully consistent with our previous observations for Tau-4R (1) as outlined in the Introduction. The difference in phosphorylation between Tau-P301L and Tau-4R was actually restricted to the epitope of PG5, which was lower on Tau-P301L in wild type and Δ*pho85* yeast strains (Fig. 1, *A* and *B*). Consistently, less of the slow migrating isoform was evident in Western blotting with the pan-Tau mAb Tau-5 or with a polyclonal antibody (data not shown), additionally demonstrating that the slowest migration of hyperphosphorylated Tau subforms is mainly due to the presence of the epitopes of AD2 and PG5 (see Fig. 1 in Ref. 1).

Analysis by AFM demonstrated that MT, deposited in the absence of Tau or endogenous pig brain MAPs, were randomly distributed over the silicon surface (Fig. 6*A*). When MT were assembled from less purified tubulin preparations that still contain endogenous pig brain MAPs, the resulting MT were deposited in more regular patterns, most frequently spaced at distances of about 100 nm from each other (Fig. 6*B*) as observed by others (29). A very similar regular spacing was observed when MT were assembled from purified tubulin containing no endogenous MAPs, but incubated with wild type human Tau-4R. Tau-4R-MT complexes were deposited on the silica surfaces spaced mostly about 70–100 nm apart (Fig. 6, *C* and *D*). The binding of Tau-4R to MT with its 4 binding domains is envisaged to leave the projection domain free to interact with other MT-Tau-4R complexes, and thereby space them apart sufficiently to allow movement of motor proteins with their cargo *in vivo* (reviewed by Ref. 9).

In the presence of Tau-4R isolated from any yeast source, with and without de-phosphorylation, MT-Tau-4R complexes appeared as regular straight structures, with an apparent height of 8 nm and a width of ~40–50 nm. This appearance of MT observed by AFM operated in air is due to the flattening of the MT by the pressure exerted by the cantilever. Also MT complexed with dephosphorylated Tau-P301L analyzed by AFM appeared regularly spaced, with only some sporadic irregularities (results not shown).

In sharp contrast, MT binding of Tau-P301L isolated from wild type yeast was accompanied by extensive aggregation of Tau on the MT surface and by bundling of the MT (Fig. 6, *E–H*). In some regions, MT appeared to be fused by way of the Tau-P301L aggregates (Fig. 6, *F* and *H*). Note that MT bundles were also observed when MT were complexed with wild type Tau-4R isolated from the Δ*pho85* yeast strain, corroborating our observation that it was also more prone to aggregation into Sarkosyl-insoluble complexes inside the Δ*pho85* yeast cells (1).

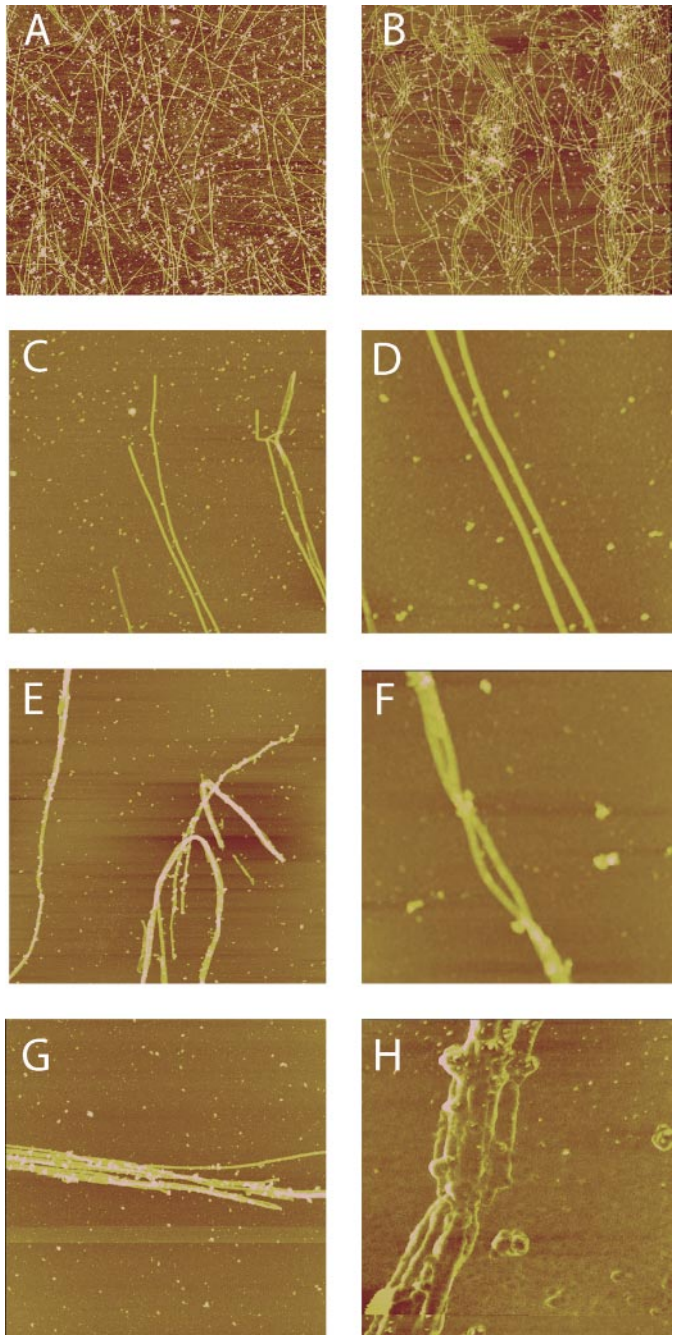

FIGURE 6. **Ultrastructural analysis of Taxol-stabilized MT incubated with or without different phosphorylated wild type or mutant Tau forms by AFM.** *A* and *B*, incubation of 10 μM MT without (*A*) and with (*B*) pig MAPs. Field widths are 20 μm. *C* and *D*, incubation of 1.5 μM MT with BY4741-Tau-4R displays no apparent changes in morphology of MT. Most MT are 8 nm in height and separately dispersed on the silicon surface, only coincidental MT crossing was observed and a complete absence of clustering on the MT surface. Field widths are 5 and 1.5 μm for *C* and *D*, respectively. *E–H*, incubation of 1.5 μM MT with BY4741-Tau-P301L display a drastic change in MT morphology causing clusters on the MT surface and causes MT to stick together and bend abnormally (*E* and *F*). At the end of these irregular structures, individual MT can be observed covered with Tau clusters (*G*) and high-resolution phase images clearly demonstrate a severely disturbed surface of MT with coagulated Tau-P301L (*H*). Field widths are 7, 1.5, 5, and 700 nm, respectively.

A major difference between Tau-4R and Tau-P301L appears to be kinetic in nature, whereby the P301L mutant tends to

aggregate much more readily in any condition. Both forms aggregated more extensively when more phosphorylated, as in the Δ*pho85* yeast strain and, moreover, also when presented with a suitable substrate like the anionic surface of MT. The results warrant the conclusion that mutant Tau-P301L aggregates faster than Tau-4R, even when lesser phosphorylated and bound to MT. Furthermore, this behavior explains that the higher extent of binding described in the previous section, is actually due to aggregation on the surface of the MT.

DISCUSSION

Recently, we introduced heterologous expression of human Tau protein in yeast as an eukaryotic cellular model, less complex than mammalian neurons or cell lines, to study the phosphorylation of Tau protein. Additionally, we demonstrated that yeast served as a well suited source of recombinant, physiologically phosphorylated human Tau protein, in contrast to bacterial sources. Here, we extended our studies to the biochemical and physiological characteristics of Tau protein, *i.e.* the binding to MT, and related these to its pathological role in the human tauopathies, known to involve phosphorylation and aggregation.

The inactivation of the specified yeast kinases Mds1 and Pho85 allowed us to specifically modify the phosphorylation pattern of the expressed and isolated human Tau protein at specific residues and to study the subsequent effect on binding to MT in a dedicated *in vitro* assay. Both GSK-3β and the atypical cell cycle kinase cdk5 are known as major Tau kinases although their exact contribution to the physiological function and pathological role are debated. In yeast, cdk5 can replace Pho85 in repressing acid phosphatase expression (30, 31). We previously demonstrated that human GSK-3β restored exogenous Tau phosphorylation in Mds1-deficient yeast (1).

The additional data on the generation of phosphorylated epitopes and the aggregation of human Tau-4R, especially in yeast lacking *pho85*, convincingly adds weight to the notion that Pho85 and Mds1 are the functional homologues of cdk5 and GSK-3β, respectively, with respect to phosphorylation of Tau protein (1). In other studies, recombinant human Tau protein is mainly expressed in and isolated from *Escherichia coli* to approach its characteristics of binding to MT (Refs. 28, 32, 33, reviewed in Ref. 34). The prokaryotic system lacks, however, protein phosphorylation and must be complemented with post-isolation phosphorylation *in vitro* with recombinant kinases or tissue extracts (32, 33, 35).

We present here the first study of isolated human Tau protein, comparing wild type and mutant Tau-4R isoforms, and phosphorylated in an eukaryotic cellular context. We determined the parameters of binding to MT in a well defined *in vitro* assay, taking into the equation the phosphorylation status of the recombinant Tau protein.

Binding of Wild Type Tau-4R—Results on the binding to MT of recombinant mutant human Tau isolated from bacterial sources are rather disparate (*e.g.* Refs. 22 and 36). Besides biochemical and biological aspects and reasons, the mathematical method of data analysis also appears important. We preferred to use a fixed low concentration of Tau in combination with increasing concentrations of MT, because unsaturable binding of Tau-4R was negligible and the data could be fitted to standard equation. The condition that the concentration of Tau should be much lower than $K_{d,\mathrm{app}}$ was satisfied, leaving us with the only drawback of not being able to determine the stoichiometry of the binding.

We conclusively demonstrate that Tau-4R purified from yeast is functional in its ability to bind to MT, whereas the extent and apparent affinity of binding is inversely related to the degree of phosphorylation. Particularly the phosphorylation of Tau at Ser^{396}/Ser^{404} (AD2) and Ser^{409} (PG5) was reduced in the absence of *mds1*, but was, paradoxically, increased by inactivation of kinase Pho85, thereby correlating very well with increased and decreased binding to MT, respectively. The findings corroborate the relation observed between GSK-3β activity and interaction of Tau with MT, suggested by observations *in vitro*, in cells and in transgenic mice on MT dynamics or morphology (14, 37, 38), although in none of these instances was Tau-MT binding affinity measured directly.

Binding of Hyperphosphorylated Tau—Tau isolated from yeast can roughly be divided in three fractions differing in electrophoretic mobility and state of phosphorylation. The most retarded fraction consists of hyperphosphorylated Tau in the MC1 configuration, named hP-Tau/MC1 (1). Formation of hP-Tau/MC1 required Mds1 activity, but not Pho85 activity, and correlated closely with phosphorylation of residues Ser^{396}/Ser^{404} (AD2) and Ser^{409} (PG5) (1). We isolated this fraction in its MC1 conformation from Δ*pho85* yeast cells, and demonstrated a close parallel in its tendency to aggregate, in its capacity as seed in the aggregation of wild type Tau-4R, and here also in its complete failure to bind to MT (Ref. 1 and this study). These properties of hP-Tau/MC1 are highly reminiscent of the hyperphosphorylated Tau forms isolated from the brain of AD patients that failed to interact normally with MT (Ref. 39 and references therein).

Both epitopes of AD2 and PG5 are almost exclusively present on the hP-Tau/MC1 isoform and are proposed to cause the conformational change and retarded electrophoretic migration. It is tempting to speculate that phosphorylation of Ser^{396}/Ser^{404} and Ser^{409} constitutes the needed biochemical push to convert Tau protein into the conformation defined by MC1, whereby the N terminus interacts with the third microtubule binding domain of Tau, thereby blocking its binding to MT completely. Whether this serves a physiological purpose is unlikely, and hP-Tau/MC1 must be considered the pathological seed that converts or drags less phosphorylated Tau-4R isoforms into the typical intraneuronal aggregates that are the pathological definition of all tauopathies.

Tau-4R Forms Dimers with Higher Binding Affinity for MT—Previously we demonstrated the presence of higher M_r Tau isoforms in the brain of Tau-4R transgenic mice, but not in Tau-P301L transgenic mice (13, 14, 16). Based on their molecular weight and immunoreactivity, we tentatively identified them as Tau-4R dimers. Remarkably, very similar dimers are formed in the heterologous yeast system expressing Tau-4R, and much less from Tau-P301L. The dimers bound to MT with higher affinity than monomeric Tau-4R.

Noteworthy, Tau-4R mice suffer from axonopathy due to blockage of axonal microtubular transport caused by excessive

binding of Tau to MT (13, 14, 16). Because axonopathy is totally absent in Tau-P301L transgenic mice, the correlation with the presence of Tau dimers is striking.

Binding and Aggregation of Tau-P301L on MT—The mechanism whereby mutations in Tau protein cause neuronal defects in tauopathy patients must be related to increased aggregation and/or reduced binding to MT. Either or both are presumed to disturb the microtubular transport system, although also other pathological actions must be considered. Increased tendency to aggregate appears an inherent characteristic of different Tau mutants (20–23), whereas at the same time they require a lesser degree of phosphorylation to aggregate (23).

To our knowledge, the influence of phosphorylation on the binding of mutant Tau to MT *in vitro* has not been reported. Indirect evidence indicates similar binding properties of mutant and wild type Tau to MT *in vitro* (22). Wild type and mutant Tau, when expressed in human cells, displayed similar localization with MT (38, 40, 41). Combined with our findings, the P301L mutation is proposed to have no major direct effect on the binding of Tau-P301L to MT, but nevertheless, to affect MT dynamics. Moreover, we demonstrate the peculiar characteristic that binding of mutant Tau-P301L to MT *in vitro* was much less sensitive to phosphorylation than wild type Tau-4R. This apparent controversy was explained by our observations with AFM that mutant Tau-P301L aggregated on the surface of MT. The clustering of mutant Tau also explains its observed non-saturable binding to MT (33). Moreover, we observed by AFM that clusters of Tau distorted the MT locally, and caused bundling and even local fusing of MT, into thicker, irregular filaments. In sharp contrast, wild type Tau-4R isolated from wild type yeast imposed a regular spacing of the MT (Ref. 29 and this study).

Because heavily phosphorylated Tau-4R isolated from Δ*pho85* yeast did not form large clusters on the MT, but even then disturbed their structure and organization, we conclude that this hyperphosphorylated form deposited more but as smaller aggregates that escaped detection by AFM. This behavior fits snugly with its much higher capacity to act as seed in the Tau-aggregation process inside yeast cells and *in vitro* (1). The enrichment of pathological phosphoepitopes in the bound Tau fraction of *pho85*Δ-Tau corroborates these conclusions (data not shown). Importantly, our results demonstrate that mutant Tau-P301L requires less phosphorylation to aggregate on the surface of MT than wild type Tau-4R, in line with observations on phosphorylation and aggregation of mutant Tau by brain extracts *in vitro* (23).

These data are proposed to recapitulate *in vitro*, the presence of granular inclusions in neurites in the brain of Tau-P301L transgenic mice, containing hyperphosphorylated Tau protein (16, 24). Granular inclusions were observed in neurites before neurofibrillary tangles were formed in the somata of the same neurons (16, 24). Moreover, in young Tau-P301L mice, only small aggregates of mutant Tau protein were observed immunohistochemically, whereas in older transgenic mice also large, intensely staining granules and clusters became evident (16). Because neurites are rich in MT, these *in vivo* observations corroborate the hypothesis that MT provide the actual substrate for the deposition of abnormally phosphorylated or mutant Tau. Although not well characterized, similar clusters of hyperphosphorylated Tau were observed in neurites surrounding amyloid plaques in APP transgenic mice that otherwise do not develop tauopathy (42, 43). Most recently, a "granular" form of Tau was isolated from the brain of AD patients, even before real neurofibrillary tangles were formed (44). Furthermore, tauopathy in brain of AD patients is preponderantly in the neuropil (85–90%) with only a minor fraction as neurofibrillary tangles in the somata (45). Finally, the concentration of soluble Tau aggregates, possibly interfering with MT in the spinal cord of Tau-P301L transgenic mice could be pharmacologically modified and shown to correlate with motoric defects, unlike the number of neurofibrillary tangle-bearing neurons that was unaffected (46).

Our results demonstrate directly that (i) Tau protein binds and aggregates on the surface of MT, (ii) mutant Tau aggregates more readily, and (iii) the degree of phosphorylation of Tau is critical. Moreover, Tau aggregates were observed to disturb the normal regular spacing of MT *in vitro*. *In vivo*, MT are spaced by MAP1b and MAP2 besides Tau protein (47, 48) to allow proper axonal transport and passage of motor proteins (reviewed by Ref. 2). We believe that the observed phenomena contributes to a disturbing transport along the microtubular network and thereby to neurodegeneration in tauopathies.

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