

Phosphorylation and Aggregation of Protein Tau in Humanized Yeast Cells and in Transgenic Mouse Brain

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Summary

Tau-4R and tau-P301L was expressed in neurons in the brain of transgenic mice and in the yeast *Saccharomyces cerevisiae*. Tau-4R was more phosphorylated than tau-P301L at three months of age in transgenic mice, but a fraction of tau became hyperphosphorylated only with age and onset of tauopathy. By contrast, a fraction of tau-4R became hyperphosphorylated in yeast as well, especially in a strain deficient in the yeast orthologue Pho85, while this fraction was not formed in a strain deficient in Mds1, the GSK-3 α orthologue. This hyperphosphorylated form of tau (designated hP-tau) was mainly cytosolic in yeast but Sarkosyl-insoluble in transgenic mice, while Sarkosyl-insoluble tau in yeast was phosphorylated at fewer sites than in brain of mice. Both hP-tau in yeast and in mice was conformationally altered, indicated by MC-1 reactivity. A comprehensive scheme for tau filament formation based on combined data of both models is presented. Phosphorylation by GSK-3 β is a first step for tau filament formation, causing and/or stabilising conformational change of tau, and promoting filament formation and additional phosphorylations.

Introduction

Tauopathies, like Alzheimer's disease, are characterized by aggregation of microtubule associated protein tau. Histological research of brains from patients suffering from tauopathies identified the presence of neu-

rofibrillary tangles as the main hallmark of this neurodegenerative disorder (Buée *et al*, 2000). Of the neurofibrillary tangles, consisting of filaments, hyperphosphorylated tau (hP-tau) is the building unit (Buée *et al*, 2000). In the human brain, six different isoforms of tau are formed by alternative splicing, differing in the number of N-terminal regions (none, 1 or 2) and of 3 or 4 C-terminal repeats. The C-terminal repeats code for microtubule-binding domains, and their binding to microtubules is regulated by dynamic phosphorylation (Buée *et al*, 2000). It has been demonstrated that hyper-phosphorylation is necessary to cause tau aggregation and that mutations in tau can cause hereditary forms of tauopathy (Buée *et al*, 2000) but many questions still remain regarding the exact contribution of the different phosphorylation sites, the kinases involved and the combined effect of phosphorylation and mutation. To address these issues different experimental models have been developed. Here we demonstrate that very different models like tau-transgenic mice and humanized yeast strains display similar biochemical features common to tauopathies. These models thereby are complementary in the elucidation of the molecular mechanisms leading to tau aggregation.

Results

We generated transgenic mice overexpressing wild-type tau-4R and mutant tau-P301L in the FVB/N background. In addition, we humanized the BY4741 yeast strain by expression of human tau-4R.

In transgenic mice at 3 months of age, tau-4R was more phosphorylated at many sites but not recognized by AT100 (Vandebroek *et al*, 2005). In contrast to tau-4R mice that developed axonopathy (Spittaels *et al*, 1999, 2000), tau-P301L mice developed tauopathy without any indications of axonopathy (Terwel *et al*, 2005). This is explained by reduced binding to microtubules of tau-P301L, allowing aggregation, as opposed to excessive binding of tau-4R, blocking axonal transport in transgenic mice (Terwel *et al*, 2005).

A fraction of tau became hyperphosphorylated with age in tau-P301L transgenic mice but not in tau-4R transgenic mice (Vandebroek *et al*, 2005). In yeast, phosphorylation patterns of tau-4R and tau-P301L were very similar (not shown). This difference between yeast and mice may be related to the fact that in transgenic mice, the ratio of tau to microtubules was much lower than in yeast.

On the other hand, in yeast pathological phospho-epitopes were present that required aging in order to be formed in tau-P301L transgenic mice, and that were never formed in tau-4R transgenic mice (Vandebroek *et al*, 2005). In mice and yeast overall phosphorylation patterns were similar but some differences were observed, i.e. AT100 reactivity was present in electrophoretically fast migrating tau isoforms in yeast, whereas in mice only on the slow-migrating hP-tau isoforms. Interestingly, for the

Table 1: Primary antibodies used in this study

Antibody	Epitope
HT-7	aa 159-163, phosphorylation independent
Tau-5	middle portion of tau, phosphorylation independent
AT8	P-Ser202/P-Thr205
AT100	P-Thr212/P-Ser214
AT180	P-Thr231
AT270	P-Thr181
AD2	P-Ser396/P-Ser404
AP422	P-Ser422
MC-1	aa 5-15/312-322, conformation dependent
PG5	P-Ser409

AD2 epitope this situation was reversed (Vandebroek *et al.*, 2005).

To define the kinases responsible for hyperphosphorylation of tau, genes coding for yeast orthologues of GSK-3 β and cdk5, respectively *mds1* and *pho85* were knocked out. Expression of tau in Mds1 deficient cells resulted in decreased phosphorylation at Ser 396/Ser404, a typical site phosphorylated by GSK-3 β *in vivo* (Spittaels *et al.*, 2000), as well as Ser409, detected by PG5. Inactivation of Pho85 paradoxically resulted in the increase of phosphorylation at Ser396/Ser404 and Ser409 and increase in the amount of hP-tau (Vandebroek *et al.*, 2005) indicating Pho85 to be a negative regulator of Mds1 activity in accordance with knockout of the cdk5 activator p35 in transgenic mice (Hallows *et al.*, 2003).

Conformational changes, defined as reactivity to monoclonal antibody (mAb) MC-1, have been shown to play an important role in the aggregation process of tau (Weaver *et al.*, 2000). Immunohistochemical analysis of brain sections from tau-P301L transgenic mice demonstrated that these mice develop MC-1-immunoreactive neurons by 9 months of age. Moreover, double stainings with MC-1 and polyclonal antibody (pAb) AP422, which recognizes a strictly pathological phosphorylation at Ser422, show essentially complete co-localisation (Vandebroek *et al.*, 2005). This co-localization between pathological phosphorylation and conformational change in tau could not be resolved temporally and spatially demonstrating a close association between these modifications in tau.

Cytosolic fractions of the different yeast strains expressing tau-4R were analysed for MC-1 reactivity with native PAGE. Only fractions of the Pho85 deficient strain expressing tau-4R were reactive with MC-1 (Vandebroek *et al.*, 2005). These results demonstrated a close relation-

ship between conformational change and phosphorylation of tau in yeast as was also observed in transgenic mice.

To address the effect of both modifications on the aggregation of tau, Sarkosyl-insoluble tau was isolated from the brains of transgenic mice and humanized yeast. In the transgenic mice, only tau-P301L formed Sarkosyl-insoluble tau (Vandebroek *et al*, 2005). Moreover, this fraction only consists of tau hyperphosphorylated at all sites examined, exclusively for those recognized by AT100 and PG5 and almost exclusively for AT8 (Terwel *et al*, 2005; Vandebroek *et al*, 2005). In contrast to mice, hP-tau in yeast was mainly present in the cytosolic fraction, while Sarkosyl-insoluble tau from the *pho85Δ* yeast strain, which forms more insoluble aggregates than in the wild-type strain, was hyperphosphorylated at AT100 and PG5 epitopes (Vandebroek *et al*, 2005).

Ultrastructural analysis of Sarkosyl-insoluble tau from tau-P301L transgenic mice by electron microscopy (EM) and atomic force microscopy (AFM) defined the tau-filaments as filaments of 10-20 nm wide and as straight or twisted. Immuno-EM also demonstrated that the filaments consist of hyperphosphorylated, conformationally altered tau. AFM demonstrated these tau filaments to be left-handed helical structures with a periodicity of 60 to 70 nm. Height measurements along the longitudinal axis of a filament revealed peaks of ~9 nm and valleys of ~7 nm (Terwel *et al*, 2005; Vandebroek *et al*, 2005). Sarkosyl-insoluble fractions obtained from total yeast cell extracts were not pure enough for detailed morphologic analysis by TEM or AFM, so we isolated tau from this yeast strain and let tau filaments assemble *in vitro*. The tau-aggregates present in the high-speed pellets were examined morphologically by EM and AFM, and were demonstrated to consist of structured tau filaments, about 5 nm in width, with a twisted, somewhat irregular appearance and differing in length.

Conclusion

Tau-4R-P301L expressed in mouse brain and tau-4R in *pho85Δ* yeast cells became hyper-phosphorylated and conformationally altered, and gave rise to tau aggregates. Despite the different situations in which tau aggregates are formed, these models revealed striking similarities. The fact that tau-4R in transgenic mice did not aggregate may be related to its higher affinity to microtubules. In yeast, the ratio tau to microtubules is higher than in neurons, ensuring that sufficient tau is available for aggregation. The difference in tau to microtubule ratio between neurons and yeast cells could also explain differences in phosphorylation patterns, besides different kinases and phosphatases that are active in these very different type of cells. Given these differences, the similarities in tau phosphorylation in both cell types are even more striking.

In yeast hyperphosphorylation of tau leads to formation of species of tau that serve as seed for filament formation. This form appears to be conformationally altered and its levels increase with kinase activity. While the temporal relation between phosphorylation, conformational change and filament formation could not be resolved in mice, it was possible in yeast. As presented schematically, hP-tau is formed first to become subsequently conformationally altered, and this conformer further serves as seed for tau filaments. Whether this includes the forcing of normal tau proteins into adoption of the same conformational change, is an attractive hypothesis, since this could lead to subsequent hyperphosphorylation, explaining why Sarkosyl-insoluble tau is always hyper-phosphorylated, in patients as well as in transgenic mice.

In the filament formation, drawn from the combined data obtained in transgenic mice and yeast (Vandebroek *et al*, 2005), the phosphorylation of tau by GSK-3 β is not sufficient but the necessary initial step in tau aggregation. Additional phosphorylation at Ser396/Ser404 and Ser409 is required, which can be due to inactivation of cdk5, resulting in hP-tau that folds into the pathological MC-1 conformation, stabilized by additional phosphorylation. Clearly, however, the formation of the MC-1 conformer must be controlled by cytosolic and/or membrane-associated proteins including chaperones that can either accelerate (X-type) or inhibit (Y-type) formation of the pathological conformation.

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