Functional Characterization of FTDP-17 tau Gene Mutations through Their Effects on Xenopus Oocyte Maturation*

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tau gene mutations cause frontotemporal dementia and parkinsonism linked to chromosomes 17 (FTDP-17). Here we have used Xenopus oocyte maturation as an indicator of microtubule function. We show that wild-type four-repeat Tau protein inhibits maturation in a concentration-dependent manner, whereas three-repeat Tau has no effect. Of the seven four-repeat Tau proteins with FTDP-17 mutations tested, five (G272V, ΔK280, P301L, P301S, and V337M) failed to interfere significantly with oocyte maturation, demonstrating a greatly reduced ability to interact with microtubules. One mutant protein (R406W) almost behaved like wild-type Tau, and one (S305N) inhibited maturation more strongly than wild-type Tau. With the exception of R406W, wild-type Tau and all the mutants studied were similarly phosphorylated during the Xenopus oocyte maturation, and this was independent of their effects on this process. Data obtained with R406W and S305N may be related to charge changes (phosphorylation and basic amino acids). Our results demonstrate variable effects of FTDP-17 mutations on microtubules in an intact cell situation. Those findings establish Xenopus oocyte maturation as a system allowing the study of the functional effects of tau gene mutations in a quantitative manner.

A variety of sporadic and familial neurodegenerative disorders, characterized clinically by dementia and/or motor dysfunction, demonstrate intracellular accumulations of filamentous material composed of the microtubule-associated protein Tau (1, 2). Six Tau isoforms are produced in the adult human brain by alternative mRNA splicing from a single gene (3). They differ from each other by the presence or absence of 29- or 58-amino acid inserts located in the amino-terminal half and an additional 31-amino acid repeat located in the carboxy-terminal half. Inclusion of the latter, which is encoded by exon 10, gives rise to the three isoforms with four repeats each; the other three isoforms have three repeats each. Similar levels of three-repeat (3R) and four-repeat (4R) Tau isoforms are found in normal cerebral cortex (4). These repeats constitute the microtubule-binding domains of Tau. They are made of imperfect 18-amino acids repeats separated by 13 or 14 amino acid inter-repeat (IR) regions (5, 6). Interestingly peptides corresponding to the first repeat (R1) and the first inter-repeat region (R1-R2 IR) alone are sufficient to suppress microtubule dynamics in a manner that is qualitatively similar to full-length Tau (6). Microtubule assembly also depends partially upon the phosphorylation state since phosphorylated Tau proteins are less effective than nonphosphorylated Tau proteins at promoting microtubule polymerization (for reviews, see Refs. 1 and 2). In Alzheimer’s disease, Pick’s disease, progressive supranuclear palsy, and corticobasal degeneration, abnormally phosphorylated Tau proteins aggregate into filaments. The discovery of tau gene mutations in familial frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) has shown that dysfunction of Tau protein can cause neurodegeneration and dementia (7–9); this has renewed interest in the mechanisms underlying the Tau pathology in Alzheimer’s disease and other tauopathies. Known tau mutations are either intronic mutations located close to the splice-donor site of the intron following exon 10 or missense, deletion, or silent mutations in the coding region (1, 2, 7–9).

At present, over 20 different coding region and intronic tau mutations are known in FTDP-17. Depending on their functional effects, these mutations can be divided into two largely nonoverlapping groups, namely those that have their primary effect at the RNA level and those that have their primary effect at the protein level (1, 2, 7–9). Mutations that act at the RNA level affect the alternative mRNA splicing of exon 10 of the tau gene, leading to a change in the ratio of 3R to 4R Tau isoforms and resulting in an overproduction of 4R Tau. This is the case of the intronic mutations located close to the 5′-splice site of the intron following exon 10 and of some mutations in exon 10 itself that influence exon 10 splicing enhancer and silencer sequences (1, 2, 8–10). Mutations that act at the protein level comprise missense and deletion mutations that are located in the microtubule-binding repeat region or close to it. Under in vitro conditions, these mutations have been shown to result in a reduced ability of Tau protein to interact with microtubules (10–16). However, the magnitude of the observed effects and the reported rank

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1 The abbreviations used are: 3R, three-repeat; 4R, four-repeat; FTDP-17, frontotemporal dementia and parkinsonism linked to chromosome 17; GVBD, germinal vesicle breakdown; MOPS, 4-morpholinepropanesulfonic acid; Wt, wild-type.
order of potency of individual mutations have been variable. Moreover, overexpression of mutant Tau in transfected cells has given inconsistent results as far as effects on microtubule binding and stability are concerned (17–19). Such studies are confounded by the problem that high expression of mutant Tau may override any effects that are present at more physiological levels.

In view of these uncertainties, we have used a cell system, maturation of the Xenopus oocyte induced by progesterone, to study the effects of seven tau gene mutations (G272V, AK280, P301L, P301S, S305N, V337M, and R406W) on microtubule function. During maturation, there is a dramatic reorganization of the microtubule cytoskeleton that can be assessed indirectly by the presence of a white maturation spot at the animal pole and directly by the anchoring of the meiotic spindle in the plasma membrane. Besides being an indicator of microtubule function, this system also enables quantitative studies through microinjection of recombinant Tau proteins of known concentration.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Wild-type and Mutated Tau Proteins**—Site-directed mutagenesis was used to change Gly-272 to valine, to delete Lys-280, and to change Pro-301 to either leucine or serine, Ser-305 to asparagine, Val-337 to methionine, and Arg-406 to tryptophan in the four-repeat 412-amino acid isoform of human Tau (expressed from cDNA clone htau46) (in the numbering of the 441-amino acid isoform of human Tau). Site-directed mutagenesis was used to change Arg-406 to tryptophan in the three-repeat 381-amino acid isoform of human Tau (expressed from cDNA clone htau37). All constructs were verified by DNA sequencing. Wild-type and mutated Tau proteins were expressed in *Escherichia coli* BL21(DE3) and purified as described previously (11, 20). Protein concentrations were determined using the BCA protein assay reagent (Pierce). In all experiments, wild-type and mutant Tau proteins were expressed and purified in parallel. These preparations are similar to those previously described in which circular dichroism spectra of wild-type and mutant three- and four-repeat Tau proteins indicate that they have random coil structures in phosphate-dichroism spectra of wild-type and mutant three- and four-repeat Tau proteins (21). Given the similarity of wild-type or mutant Tau protein to microinjected using a positive displacement digital microrpette (Nichiryo). With a diameter of 1.2–1.3 mm, the volume of the oocyte was estimated to be 0.9–1.2 μl. The quantities of protein injected were 25, 50, 100, and 200 ng and could be estimated to a final concentration within the oocyte of 0.6, 1.2, 2.5, and 5 μM for 3R Tau isoforms and 0.6, 1.2, 2.5, and 4.7 μM for 4R Tau isoforms. These concentrations are similar to those used in Chinese hamster ovary cells to study Tau-microtubule interactions (21). Given a cellular tubulin concentration of about 20 μM (22), tubulin dimers were severalfold in excess of Tau so that tubulin was not a limiting factor. Twenty oocytes were injected for each concentration of Tau protein. Control oocytes were injected with the same volume of buffer. After 1 h, maturation was induced by the addition of progesterone (10 μM) to the medium and assessed either by detection of the white maturation spot resulting from germinal vesicle breakdown (GVBD) and migration of the germinal vesicle and anchoring of the meiotic spindle or by microscopic examination of the meiotic spindles.

**Cytological Analysis**—At the end of the maturation process, the position of the meiotic spindles was determined as described previously (23). Brackets were fixed overnight, dehydrated in an ascending ethanol series, and placed overnight in butanol. They were then embedded in paraffin, and 7-μm sections were cut using a microtome (Reichert). Sections were stained with nuclear red to detect nuclei and chromosomes and with picro-indigo carmine, which reveals cytoplasmic structures.

**Gel Electrophoresis and Immunoblotting**—For biochemical analysis, oocytes were lysed and homogenized in pools of five in 50 μl of 25 mM MOPS, pH 7.2, containing 60 mM β-glycerophosphate, 15 mM para-nitrophenyl phosphate, 15 mM EGTA, 15 mM MgCl₂, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylphosphate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μM microcystin-LR, 100 μM benazolin, and 100 μM trypsin inhibitor, followed by a 5-min centrifugation at 13,000 × g to eliminate yolk platelets and the addition of 2 × sample buffer to the supernatant in a 1:1 volume ratio. Following 10% SDS-PAGE, immunoblotting was performed using anti-Tau antibodies AD2, AD2, AT180, AT270, Tau-1, and M19G as described previously (24). AD2 recognizes phosphorylated Ser in KXGS motif (AD2 and Ser-356), AD2 recognizes phosphorylated Ser and Ser-404 in Tau, AT180 recognizes phosphorylated Thr-231, AT270 recognizes phosphorylated Thr-181, and Tau-1 recognizes the dephosphorylated region (residues 189–207). Finally M19G recognizes amino acids 1–19 in Tau in a phosphorylation-independent manner. Oocyte homogenates were ultracentrifuged at 40,000 × g for 30 min (Optima TL ultracentrifuge, TLA 100.4 rotor, Beckman Coulter) to ascertain that there was no Tau aggregate after oocyte maturation. No Tau immunoreactivity was observed in the pellet for any injected Tau (wild type or mutant), whereas the full Tau immunoreactivity was observed in the supernatant. Both 13,000 and 40,000 × g centrifugations indicated that Tau proteins were in a soluble fraction.

**Quantitative Analysis of Tau Immunoreactivity**—All quantitative analyses were performed as described by Maillot et al. (24). A correction factor was introduced to adjust Tau immunoreactivity among samples and to obtain approximately equal strong immunoreactivity (if binding occurred at all) of the various samples based on binding of phosphorylation-independent antibody M19G to the various samples. Data are expressed as percentage of the four-repeat wild-type Tau immunoreactivity.

**RESULTS**

**Effects of Injected Tau on Oocyte Maturation**—By immunoblotting using antibody M19G, *Xenopus* oocytes did not express Tau protein in confirmation of previous findings (25). The injection of buffer alone resulted in a normal maturation of oocytes following stimulation by progesterone as assessed by the appearance of the white maturation spot at the animal pole (Fig. 1). Similar results were obtained using low concentrations (25 or 50 ng) of wild-type three- and four-repeat Tau. By contrast, at higher concentrations, wild-type 4R Tau interfered with maturation. White spot appearance was delayed upon injection of 100 ng and inhibited following injection of 200 ng of Tau. Wild-type 4R Tau thus interfered with oocyte maturation in a concentration-dependent manner. Conversely, high concentrations of the 3R Tau isoform (200 and 100 ng) did not affect the oocyte maturation. We next studied the effects of seven FTDP-17 tau gene mutations on *Xenopus* oocyte maturation (Fig. 1). Three-repeat Tau protein with the R406W mutation did not interfere with oocyte maturation at any concentration. Conversely, injection of the different 4R mutated Tau proteins displayed various effects on oocyte maturation. Five of the mutations studied (G272V, AK280, P301L, P301S, and V337M) failed to affect maturation whatever their concentration (25–200 ng). At low concentrations, Tau protein with the R406W mutation delayed maturation more severely than the wild-type protein, but its effect was less than that of wild-type Tau at the highest concentration studied (200 ng). Finally, 4R Tau with the S305N mutation inhibited oocyte maturation more severely than the wild-type protein. To ascertain that these effects were related to Tau mutations and not to phosphorylation, we investigated the levels of Tau phosphorylation during oocyte maturation.

**Tau Phosphorylation and Oocyte Maturation**—First, an experiment was performed with wild-type 4R Tau at a final concentration of about 1.2 μM (injection of 50 ng of Tau). A time course analysis of Tau phosphorylation was performed following progesterone addition. In a typical experiment, maturation occurred between 4 and 5 h (Fig. 2A). At that time (time 0) of induced maturation, 4R Tau exhibited an apparent molecular
mass of about 61 kDa and was detected with a polyclonal anti-Tau antibody (M19G) and the monoclonal antibody Tau-1 but not by any phosphorylation-dependent anti-Tau antibody (Fig. 2B). In metaphase II oocytes, at the end of the maturation process, it migrated at 69 kDa (Fig. 2B). In fact, these shifted forms of Tau, detected with both AD2 and M19G antibodies, occurred 4 h after hormone stimulation (Fig. 2B). However, Tau variants with a molecular mass higher than 61 kDa were detected by M19G as soon as 1 h after progesterone stimulation suggesting that Tau is already weakly phosphorylated at the beginning of the maturation process (Fig. 2B). Altogether these data suggested that Tau was phosphorylated during oocyte maturation. These Tau immunoreactivities were found for all Tau concentrations and were independent of the effect on oocyte maturation (data not shown).

We then quantified the phosphorylation of 3R and 4R wild-type and mutant Tau isoforms by immunoblotting, using different phosphorylation-dependent antibodies (AD2, 12E8, AT180, AT270, and Tau-1). Wild-type Tau and all the mutants studied were phosphorylated during Xenopus oocyte maturation independently of their effects on maturation. At the end of the oocyte maturation, the relative levels of phosphorylation were similar except for a few cases. No Tau-1 immunoreactivity was seen for any of the mutations. A weak loss (less than 15%) of AT180 and 12E8 immunoreactivities was observed for 4R Tau with the V337M and ΔK280 mutations. The 4R Tau with the R406W mutation displayed a 25% loss of phosphorylation compared with wild-type Tau for the AD2 and 12E8 epitopes (Fig. 3). The 3R Tau mutant R406W exhibited a severe loss of phosphorylation for the AD2 and 12E8 epitopes (about 50%) and the AT180 epitope (about 25%) (Fig. 3). These data are in agreement with previous results in other systems (17–19, 26, 27).

With the exception of the R406W Tau mutant, phosphorylation of wild-type and mutant Tau proteins was similar. Altogether data on white spot appearance and phosphorylation strongly suggest that the present observations are related to interactions between Tau (wild type and mutant) and microtubules independently of Tau phosphorylation. To further analyze the effects of these interactions in this system, we investigated the formation of meiotic spindles during oocyte maturation.
mutant) did not affect oocyte maturation or the organization of meiotic spindles. Wild-type 4R Tau blocked oocyte maturation at 100 and 200 ng: after GVBD, there was no formation of meiotic spindles. The mutant Tau proteins could be divided into three groups. The first group is made up of mutations (G272V, H9004K280, P301L, P301S, and V337M) that did not significantly interfere with the maturation process with normal meiotic spindles when 25 or 50 ng of mutant Tau was injected. Following the injection of 100 and 200 ng of mutant Tau, about 25–30% of spindles were ectopic for the G272V, P301L, P301S, and V337M mutations. This figure increased to 40% at 100 ng and 60% at 200 ng of Tau for the H9004K280 mutation. Mutation R406W made up the second group. It impaired oocyte maturation in a concentration-dependent manner, leading to the appearance of ectopic spindles and the lack of germinal vesicle translocation at 25 ng. With injections of 100 and 200 ng, GVBD occurred without germinal vesicle translocation and with no formation of meiotic spindles.

**DISCUSSION**

It is important to understand the in vivo role of Tau protein to dissect mechanisms leading to neurodegeneration in Alzheimer’s disease and other neurodegenerative disorders. Mutations in the tau gene have shown that dysfunction of Tau protein can cause neurodegeneration and dementia. In vitro studies demonstrated that missense and deletion mutations result in reduced function of Tau. However, in vivo effects have been more controversial. To avoid the variables inherent to transfection studies, *X. laevis* oocyte maturation was used to study the effects of Tau mutations on microtubule dynamics. Upon exposure to progesterone, oocytes undergo maturation, a
process that involves a dramatic reorganization of microtubules, allowing for GVBD, translocation of the germinal vesicle, and anchoring of meiotic spindles at the plasma membrane of the animal pole. A known amount of Tau protein (wild type or mutant) was microinjected into the oocyte, and the biological effect on microtubules was assessed.

Microinjection of wild-type 4R Tau into the oocyte perturbed the maturation process in a dose-dependent manner (from 0.6 to 4.7 μg), whereas 5 μg wild-type 3R Tau was without effect. Altogether these data suggest that there is a direct interaction between 4R Tau, but not 3R Tau, and microtubules. This is consistent with previous findings showing that four-repeat Tau has a higher affinity for microtubules than 3R Tau (4–6, 28, 29).

Regarding the functional effects of Tau mutants, three groups could be distinguished. The first includes mutations G272V, ΔK280, P301L, P301S, S305N, and V337M. These mutations allowed for normal oocyte maturation as visualized by the appearance of a white spot indicating that they failed to interact correctly with microtubules. Only the microinjection of high concentrations of mutant Tau led to abnormalities such as the formation of ectopic meiotic spindles. It was previously shown that these mutations cause a decreased ability of Tau to promote microtubule assembly in vitro, although there were discrepancies regarding their relative effects (11, 12). In the present study, mutations G272V, ΔK280, P301L, and P301S gave similar results to those shown for V337M (see Table I).

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**Table I**

Quantification of meiotic figures after cytology

| tau (ng) | 4R Wt | P301L | P301S | G272V | V337M |
|---------|-------|-------|-------|-------|-------|
| 25      | 100   | 100   | 100   | 100   | 100   |
| 50      | 100   | 100   | 100   | 100   | 100   |
| 100     | 100   | 100   | 100   | 100   | 100   |
| 200     | 100   | 100   | 100   | 100   | 100   |

| tau (ng) | ΔK280 | R406W | S305N | 3R Wt | 3R R406W |
|---------|-------|-------|-------|-------|----------|
| 25      | 100   | 100   | 100   | 100   | 100      |
| 50      | 100   | 100   | 100   | 100   | 100      |
| 100     | 100   | 100   | 100   | 100   | 100      |

**Fig. 5.** Average of meiotic figures after cytology. Wild-type (4R Wt and 3R Wt), 3R R406W mutant, and 4R mutant (G272V, ΔK280, P301L, P301S, S305N, V337M, and R406W) Tau proteins were microinjected at 25, 50, 100, and 200 ng. At the end of the maturation process, a cytological examination of the injected oocytes was performed, and the results are summarized as a histogram for each Tau concentration (n = 3). Four different phenotypes were observed: normal spindles (NS, in black), membrane-associated ectopic spindles (Mb ES, in dark gray), internal cytoplasmic ectopic spindles (Int ES, in light gray), and germinal vesicle breakdown without spindle formation (GVBD, in white). No perturbation was observed with 3R Tau isoforms. The effects of the mutant Tau proteins fell into three groups represented here by V337M, R406W, and S305N. Mutations G272V, ΔK280, P301L, and P301S gave similar results to those shown for V337M (see Table I).
tion. However, wild-type 3R and 4R Tau were found to be phosphorylated to the same extent as the G272V, R406W, and S305N Tau proteins. This indicates that the effect of S305N mutation is more pronounced in vitro than in vivo, suggesting that the in vivo Tau protein is more resistant to phosphorylation. Therefore, the presence of microtubules in cells can help to stabilize Tau and prevent it from being phosphorylated.

The study also shows that the presence of microtubules can help to stabilize Tau and prevent it from being phosphorylated. This is consistent with previous findings that microtubules can help to stabilize Tau and prevent it from being phosphorylated. Therefore, the presence of microtubules in cells can help to stabilize Tau and prevent it from being phosphorylated.

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