FEBS Letters 493 (2001) 40–44 FEBS 24704

Effects of FTDP-17 mutations on the in vitro phosphorylation of tau by glycogen synthase kinase 3β identified by mass spectrometry demonstrate certain mutations exert long-range conformational changes

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Received 29 January 2001; accepted 27 February 2001

First published online 7 March 2001

Edited by Jesus Avila

Abstract In vitro phosphorylation of recombinant wild-type 2N4R tau and FTDP-17 exonic mutant forms P301L, V337M and R406W by glycogen synthase kinase 3β (GSK3β) was examined by two dimensional phosphopeptide mapping analysis on thin layer cellulose plates. Comparison of these peptide maps with those generated from wild-type 1N4R tau isoform from which the phosphopeptide constituents and sites of phosphorylation had been determined previously, enabled us to monitor directly changes in phosphorylation of the individual tau proteins. No differences were found in the phosphorylation of wild-type, P301L or V337M tau by GSK3β but the R406W mutant showed at least two clear differences from the other three tau proteins. The peptides, identified by mass spectrometry corresponding to phosphorylation at both threonine 231 and serine 235 (spot 3), serines 396, 400 and 404 (spot 6a) and serines 195 and 199 (spot 6b) were absent from the R406W peptide map. The findings imply that the R406W mutation in tau exerts long-range conformational effects on the structure of tau.

Key words: Tau; Phosphorylation; Frontotemporal dementia with Parkinsonism linked to chromosome 17; Glycogen synthase kinase 3β; Two dimensional phosphopeptide mapping; Mass spectrometry

1. Introduction

Frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) is a group of inherited dementias characterised clinically by behavioural and cognitive changes accompanied by motor disorder. At autopsy, all patients with FTDP-17 have pronounced frontotemporal atrophy, with loss of neuronal cells, grey and white matter gliosis and superficial spongiform changes [1]. The discovery of up to 20 different exonic and intronic mutations in the gene encoding the microtubule-associated protein tau, in over 40 families with FTDP-17, has now confirmed the tau gene as the disease locus [1–5].

Neuropathologically FTDP-17 is one of a number of neurodegenerative diseases collectively known as 'tauopathies' including Alzheimer's disease, Pick's disease, progressive supranuclear palsy and corticobasal degeneration that are characterised by the presence of intracellular accumulations of tau protein in a hyperphosphorylated state [6,7]. Tau mutations in FTDP-17 fall into two major categories, exonic missense mutations which are close to the mictrotubule binding domains and intronic mutations in the intron adjacent to exon 10. There is some genotype–phenotype correlation in FTDP-17 as the site of the mutations in exon 10 or in the intron following exon 10 leads to a filamentous neuronal and glial cell tau pathology [8–10]. In exon 10 mutations, the filaments are narrow twisted ribbons consisting predominantly of tau isoforms with four microtubule binding repeats [10] whereas in the case of the intronic mutations, the filaments are wide twisted ribbons containing exclusively four repeat tau isoforms [8]. On the other hand missense mutations outside exon 10 lead to a predominantly neuronal pathology and the tau filaments are paired helical filaments (PHFs) and straight filaments consisting of all six tau isoforms [11,12].

The role of tau in promoting microtubule growth and stability has been well documented [13,14] and hyperphosphorylation of tau is known to reduce the ability of tau to bind to and stabilise microtubules [15]. Several candidate kinases have been identified which can phosphorylate tau in vitro [16] including the proline-directed kinase glycogen synthase kinase 3β (GSK3β) [17–20]. GSK3β has also been shown to phosphorylate tau in cells [21] and in neurones inhibition of GSK3β with lithium or via insulin signalling reduces tau phosphorylation, providing evidence that GSK3β is a physiological, if not the only, tau kinase [22–24].

Previous reports using immunoblotting suggested that one mutation in tau (R406W) had the effect of reducing tau phosphorylation in cells [25,26,32]. This was surprising given that the aggregated tau in FTDP-17 is highly phosphorylated. In order to examine this further we therefore studied the effects of three of the FTDP-17 missense mutations of tau, namely P301L, V337M and R406W, on the in vitro phosphorylation

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Abbreviations: FTDP-17, frontotemporal dementia with Parkinsonism linked to chromosome 17; GSK3β, glycogen synthase kinase 3β; PHF, paired helical filament; nanoES-MS, nanoelectrospray mass spectrometry; DTT, dithiothreitol; PMSF, phenylmethylsulphonylfluoride; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide electrophoresis

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PII: S0014-5793(01)02267-0

of the largest isoform of tau (2N4R) by GSK3β using two dimensional phosphopeptide mapping and nanoelectrospray mass spectrometry (nanoES-MS) to directly monitor changes in phosphorylation.

2. Materials and methods

2.1. Expression and purification of recombinant human tau

Recombinant full-length human wild-type (wt) and FTDP-17 mutant tau cDNA with two N-terminal inserts and four repeat domains, 2N4R wt, P301L, V337M, R406W, in pET30a bacterial expression vectors were used to transform *Escherichia coli* BL21-DE3 competent cells. Tau protein expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 2 h at 37°C and purified essentially by the method previously described [27]. Bacterial cells were collected by centrifugation at $6000\times g_{av}$ for 15 min at 4°C and the cell pellet washed once in 50 mM MES buffer pH 6.5. Washed cell pellet was resuspended in 50 mM MES buffer pH 6.5 containing 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonylfluoride (PMSF), 1 mM EDTA, 10 mM NaF and 20 mM sodium pyrophosphate by hand homogenisation before lysing the cells by sonication (VibraCell Sonics and Materials, USA) for 6×30 s with 30 s on ice between sonications. Unbroken cells and cell debris were pelleted by centrifugation at $15\,000\times g_{av}$ for 20 min at 4°C. The supernatant was adjusted to 0.5 M NaCl and boiled for 10 min before centrifuging at $100\,000\times g_{av}$ for 2 h at 4°C to collect the precipitated protein. Tau-enriched supernatant was saturated to 45% (w/v) by the addition of ammonium sulphate, stirring on ice for 1 h to precipitate the tau protein. Tau protein was collected by centrifugation at $18\,000\times g_{av}$ for 20 min at 4°C and the precipitated protein resuspended in 50 mM MES buffer pH 6.5 containing 1 mM DTT and 1 mM PMSF prior to dialysing overnight at 4°C against at least two changes of the same buffer. Anion exchange chromatography on a Mono S FPLC column was then used as the final step to purify the tau protein. Aliquots of the various major purification steps were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (10% w/v acrylamide SDS–PAGE) followed by transfer of the resolved proteins onto nitrocellulose paper and protogold staining (British Biocell International, UK).

Purified tau protein was quantified by 10% w/v acrylamide SDS–PAGE followed by Coomassie brilliant blue staining against a bovine serum albumin standard of known concentration.

2.2. In vitro phosphorylation of recombinant human tau (wt, P301L, V337M and R406W) with GSK3β

Purified recombinant human tau (wt, P301L, V337M and R406W) protein was phosphorylated by rat GSK3β as described below. The rat GSK3β was supplied by J.R. Woodgett, Ontario Cancer Institute, Canada, in a recombinant baculovirus and purified by the method of Hughes et al. [28] incorporating the modification of Utton et al. [29].

1–2 μg of tau protein was phosphorylated overnight by incubation at 30°C in 40 μl of 20 mM HEPES buffer pH 7.4 containing 10 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 10 μM leupeptin, 10 μM pepstatin, 1 μM aprotinin, 5 μM okadaic acid, 10 μM sodium orthovanadate, 0.1 mg/ml heparin and 0.32–0.64 μg of GSK3β. The reaction was started by the addition of ATP (final concentration 3 mM) containing 10 μCi of [γ-^{32}P]ATP. Phosphorylation was terminated by boiling in Laemmli sample buffer for 5 min.

2.3. Two dimensional phosphopeptide mapping of human tau (wt, P301L, V337M and R406W)

Radiolabelled human tau (wt, P301L, V337M and R406W) in electrophoresis sample buffer was resolved by 10% (w/v) acrylamide SDS–PAGE, then transferred onto Immobilin P membrane (Millipore, UK) at 15 V for 45 min. Protein was visualised by staining of the membrane with 0.1% (w/v) amido black solution [30] and radiolabelled protein identified by phosphorimage analysis on a Fujix Bas 1000 imager. The phosphorylated tau bands were excised, counted by Cerenkov radiation then destained in 200 μl of 200 μM NaOH solution for 2×30 min at 37°C. Destained membrane was washed with distilled H_2O then blocked in 0.04% (v/v) polyvinylpyrrolidone 40 in 100 mM acetic acid for 1 h at 37°C. Membrane was washed again in distilled H_2O prior to placing in 200 μl of 0.2 M NH_4HCO_3 pH 8.0 containing trypsin (1:20 (w/v) trypsin:tau) and incubated for ~15 h at 37°C. A second addition of trypsin (1:20 (w/v) trypsin:tau) was made at this point and the incubation continued for a further 4 h at 37°C. Tryptic peptide solution was removed from the PVDF membrane strip and prepared for phosphopeptide mapping analysis by drying under vacuum on a Savant SC199 Speed-vac to remove all of the water and NH_4HCO_3 pH 8.0 and then resuspended twice in distilled H_2O and twice in pH 1.9 electrophoresis buffer (water/acetic acid/formic acid: 90/7.8/2.2 (v/v/v)), each time lyophilising the samples to dryness. Each dried sample was resuspended in 5 μl of pH 1.9 electrophoresis buffer and loaded onto a thin layer cellulose chromatography plate (20×20 cm). Two dimensional separation was achieved by electrophoresis at pH 1.9 for 1.25 h in the first dimension, then, after air-drying, by ascending phospho-chromatography in the second dimension (*n*-butanol/pyridine/acetic acid/water: 37.5/25/7.5/30 (v/v/v/v)) for 18 h. The plate was removed from the chromatography tank, air-dried and then exposed to phosphorimage analysis and autoradiography at −70°C to enable visualisation of phosphopeptides.

2.4. NanoES-MS analysis of phosphopeptide spots

Phosphopeptide spots were scraped from the thin layer chromatography plates, the phosphopeptide constituents eluted in 20% (v/v) acetonitrile in water and analysed by nanoES-MS as described previously [31].

3. Results

3.1. Expression and purification of wt, P301L, V337M and R406W 2N4R tau

The locations of the three exonic missense FTDP-17 mutations of tau used in this study are illustrated in Fig. 1A,B. Two of the mutations lie within the microtubule binding domains of the largest isoform of tau (P301L and V337M) whilst the third mutation is found close to the C-terminus of the protein (R406W). The P301L mutation is only found in four repeat tau as this mutation occurs in exon 10 which is spliced out during generation of three repeat tau mRNA. Wt 2N4R tau and the three exonic FTDP-17 mutants, P301L, V337M and R406W 2N4R tau forms, were expressed in *E. coli* as described and all proteins purified in the exact same manner. Fig. 2 illustrates a protogold-stained blot of the various purification stages for the wt 2N4R tau isoform. In all cases, the tau protein was eluted from the Mono S anion exchange column at essentially the same part of the salt gradient (~250 mM NaCl), indicating that the single amino acid mutation changes had little or no effect on this physical property of the individual tau proteins (lane 7).

3.2. In vitro phosphorylation of wt tau and the exonic FTDP-17 mutated tau proteins

Wt 2N4R tau and the P301L, V337M and R406W FTDP-

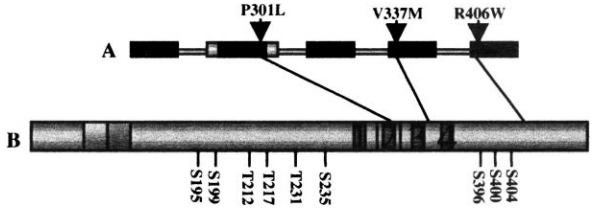

Fig. 1. FTDP-17 tau mutations and tau phosphorylation sites numbered according to the largest tau. A: Schematic diagram of the tau gene from exon 9 to exon 13 showing the three FTDP-17 mutations, P301L, V337M and R406W. B: Full-length 2N4R tau with the serine and threonine phosphorylation sites found to be phosphorylated by GSK3β in this study (identified by nanoES-MS analysis of GSK3β phosphorylated 1N4R tau) and the positions of the three missense FTDP-17 mutations relative to the phosphorylation sites.

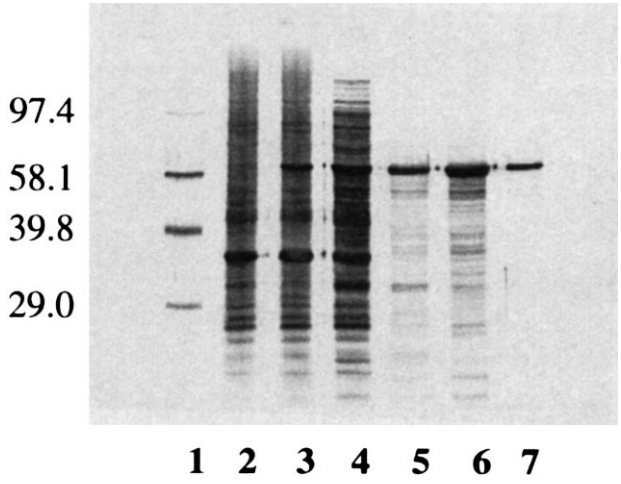

Fig. 2. Purification profile of *E. coli* expressed wt tau (2N4R). Recombinant wt tau (2N4R) was purified from *E. coli* according to the procedure described in Section 2. 10% (w/v) acrylamide SDS–PAGE analysis of the purification fractions followed by transfer onto nitrocellulose paper and protogold staining of the membrane according to the manufacturer's instructions is shown: lane 1, M_r standards; lane 2, bacterial cell pellet (uninduced); lane 3, bacterial cell pellet (induced); lane 4, sonication supernatant; lane 5, heat/stable supernatant; lane 6, 45% ammonium sulphate precipitate and lane 7, Mono S purified fraction.

17 mutant proteins were phosphorylated in vitro by the proline-directed kinase GSK3β in the presence of radiolabelled [γ-^{32}P]ATP overnight at 30°C. The labelled proteins were separated by 10% w/v acrylamide SDS–PAGE and then subjected to two dimensional phosphopeptide mapping analysis following trypsin digestion of the proteins as described. Individual phosphopeptide spots were visualised by phosphorimage analysis. Fig. 3 compares the pattern of phosphopeptides found for wt, P301L, V337M and R406W tau phosphorylated by GSK3β. There were no differences in the general phosphopeptide mapping patterns of wt, P301L and V337M 2N4R tau (Fig. 3A–C), each consistently showing six well resolved spots. However, the R406W tau phosphopeptide map demonstrated clear differences from the other three maps with at least two major phosphopeptide spots being absent (Fig. 3D). To determine the phosphopeptide constituents and the actual sites of phosphorylation of each individual spot (numbered 1–6), we used the previously identified sites found in 1N4R wt tau as the peptide maps generated following GSK3β phosphorylation of the 1N4R and 2N4R wt tau isoforms were identical (Fig. 4 and Table 1). Phosphopeptide spots 3 (residues 231–240) and 6a (residues 386–404) and a second peptide component of spot 6 (spot 6b) covering residues 191–209 were absent from the R406W peptide map (Fig. 3D). The actual sites of phosphorylation reduced were determined to be Thr231 and Ser235 for spot 3 and Ser396, 400 and 404 for the first peptide constituent of spot 6 (6a) and Ser195 and 199 for spot 6b (Table 1). Interestingly, spot 5 (residues 226–240) is unaffected by the R406W mutation even though it includes phosphorylated Ser231 and at least one other phosphorylation site. Unfortunately to date we have been unable to identify the phosphopeptide constituents of spots 1 and 4 by nanoES-MS.

4. Discussion

The discovery of a group of diseases called the 'tauopathies'

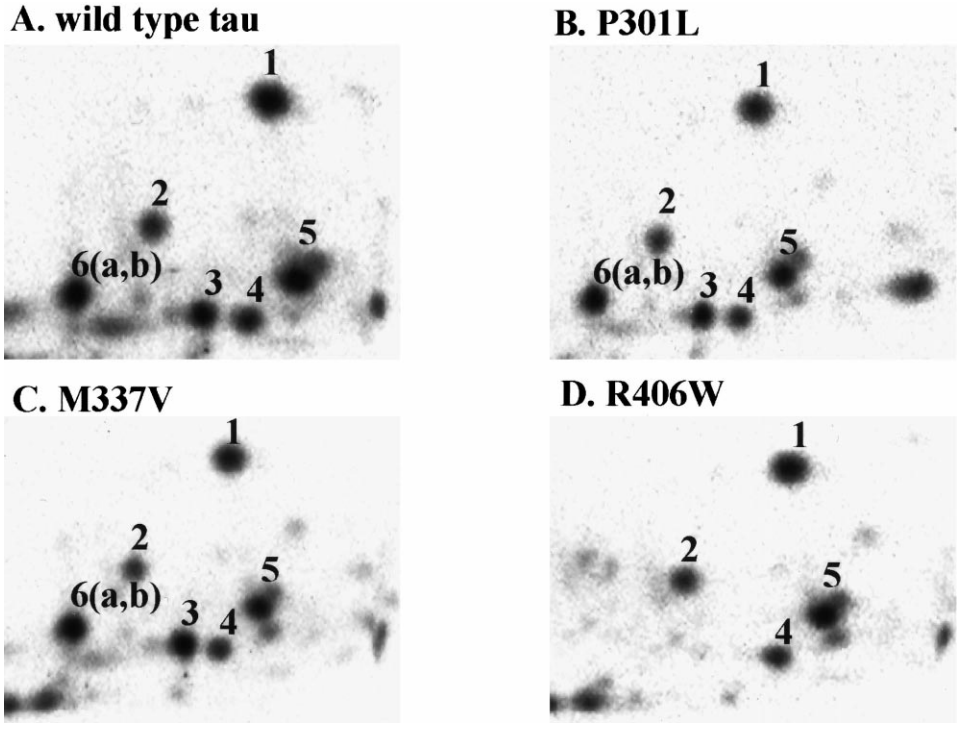

Fig. 3. Two dimensional phosphopeptide map analysis of the phosphorylation of recombinant wt, P301L, V337M and R406W tau (2N4R) by GSK3β. 1–2 μg of wt, P301L, V337M and R406W tau (2N4R) was phosphorylated by GSK3β in the presence of [γ-^{32}P]ATP and two dimensional phosphopeptide mapping analysis performed as described in Section 2. Identical phosphopeptide maps were generated with the wt, P301L and V337M forms of tau. However, phosphopeptide spots 3 and 6a and 6b were absent from the R406W phosphopeptide mapping analysis.

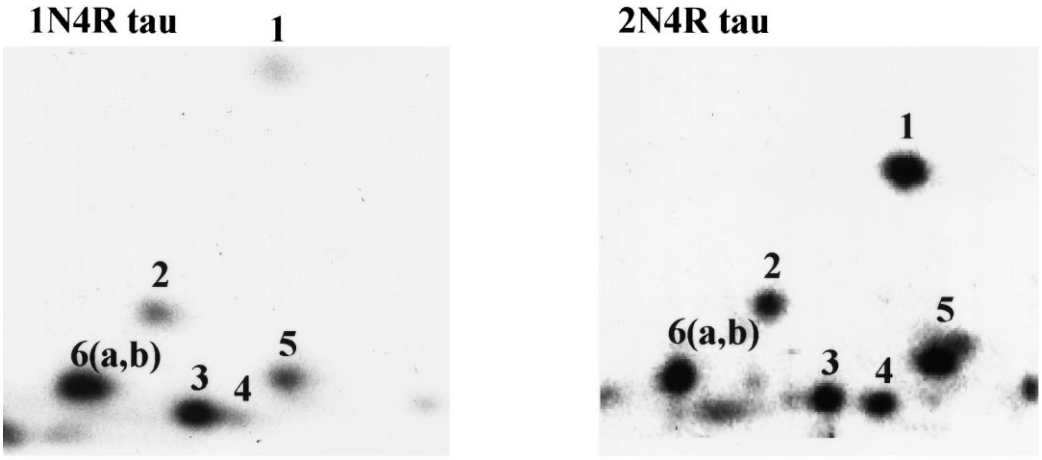

Fig. 4. Two dimensional phosphopeptide map analysis of the phosphorylation of recombinant 1N4R and wt 2N4R tau by GSK3β. 1–2 μg of 1N4R and wt 2N4R tau were phosphorylated by GSK3β in the presence of [γ-^{32}P]ATP and two dimensional phosphopeptide mapping analysis performed as described in Section 2. Identical phosphopeptide maps were generated with these two tau isoforms.

in which the primary pathological lesion is an aggregation of hyperphosphorylated tau into filamentous forms led us to investigate the effects of various mutations of the tau gene found associated with FTDP-17 on the in vitro phosphorylation of the recombinant 2N4R tau isoform by GSK3β. We were able to directly monitor changes in sites of phosphorylation of wt, P301L, V337M and R406W tau by GSK3β using the highly sensitive techniques of two dimensional phosphopeptide mapping and nanoES-MS.

Several recent reports in cellular systems [25,26,32] using Western blotting to monitor changes in tau phosphorylation surprisingly suggested that the R406W mutation actually results in a lower state of phosphorylation of tau protein compared to the wt protein, particularly at the PHF-1 (Ser396 and Ser404) and AT180 sites (Thr231 and Ser235). We have confirmed these earlier findings in this paper where we clearly demonstrate that only the R406W mutant tau peptide map shows a reduced number of phosphopeptide spots (spots 3 and 6a and 6b absent) when compared to the wt, P301L and V337M phosphopeptide maps. One would have expected at least one difference in the R406W phosphopeptide map compared to the other tau proteins analysed in this study. Phosphopeptide 6a covering residues 386–406 would be affected by the Arg to Trp mutation, resulting in the loss of its C-terminal trypsin cleavage site, a central feature of the two dimensional phosphopeptide mapping analysis. The removal of this potential trypsin cleavage site results in the generation of a very large C-terminal tryptic peptide covering residues 386–437. A peptide of this size would probably be expected to remain close to the origin of separation of the sample in this analytical procedure. However, it was difficult to determine whether this peptide had indeed remained at or near the origin of separation as there was always much poorly resolved material in this region of the plate (Fig. 3). It is however quite likely that the R406W mutation markedly reduced phosphorylation at serines 396, 400 and 404 as previously reported above [25,26,32]. Furthermore, in a recent study by Vogelsberg-Ragaglia et al. [35] they illustrated by immunoblotting a reduction in phosphorylation at Ser396 and Ser404 in stably expressing CHO cells harbouring the R406W mutant tau but no other changes in phosphorylation.

Interestingly, in addition to spot 6a being absent from the R406W peptide map, spot 6b (residues 191–209, phosphorylated at Ser195 and 199) and spot 3 (residues 231–240, phosphorylated at Thr231 and Ser235) were also missing. This potential loss of phosphorylation at Thr231 and Ser235 is consistent with the Western blotting analysis of cell lysates prepared from transiently transfected CHO cells [25] and stably expressing CHO cells harbouring the R406W tau mutant protein [26]. However this technique reveals that spot 5 (residues 226–240), phosphorylated at Ser231, plus at least one other site is unaffected by the R406W mutation. Potential reasons for this discrepancy are that in vitro phosphorylated wt 2N4R tau has the potential to exist in at least two different conformational states that affect trypsin cleavage. This selective cleavage results in the generation of either spot 3 or spot 5 on the wt map. The R406W mutation could shift the equilibrium between conformations so as to further bias trypsin cleavage, resulting in the exclusive production of spot 5 over spot 3 on the R406W map. Spot 5 could also contain at least one other peptide component that is unaffected by the R406W mutation. Finally, the mutation may reduce phosphorylation

Table 1
Identification of phosphopeptides and sites of phosphorylation of 2N4R tau after in vitro phosphorylation by GSK3β

Phosphopeptide spot number	Residues	Sequence	Site(s) identified
2	210–221	SRTpPSLPTpPPTR	212, 217
3[a]	231–240	TpPPKSpPSSAK	231, 235
5	226–240	VAVVRTpPPKSPSSAK (+1 p)	231, (235, 237 or 238)
6a[a]	386–406	TDHGAEIVYKSpPVVSpGDTSpPR	396, 400, 404
6b[a]	191–209	SGDRSpGYSSpPGSPGTPGSR	195, 199

2N4R tau phosphopeptides and sites of phosphorylation were assigned from the equivalent spots on 1N4R tau (data not shown).
[a]Represents the spots missing from the R406W peptide map. Sp and Tp indicate phosphorylated serine or threonine residues.

at Ser235 only, altering the migration pattern of spot 3. This could result in its co-migration with an unaffected phosphopeptide. In this interpretation spot 5, potentially phosphorylated at two other sites, remains unaffected by the mutation.

Although it is possible for the R406W mutation to alter the activity of trypsin via conformational changes, this detailed chemical analysis of tau phosphorylation has enabled us to demonstrate that the R406W mutation of tau does in fact cause changes in phosphorylation distant from the mutational site. Clearly, the change of a basic amino acid residue Arg for a hydrophobic amino acid Trp results in a change in the conformation of this tau protein molecule which not only renders sites of phosphorylation close to the mutation inaccessible (Ser396, 400 and 404) to GSK3β but also sites of phosphorylation far removed from the mutation site (Ser195, 199 and 235 and Thr231). The fact that the in vitro phosphorylation at Thr231 and Ser235 of the R406W mutant tau was similar to that observed in cells [25,26] demonstrates that this effect of the R406W mutation on the conformation is robust and not just an in vitro observation.

The reduced phosphorylation of tau by the R406W mutant is not the only experimental system where this mutant behaves differently from other known FTDP-17 mutants. In a number of in vitro and cellular assays when compared to wt tau and other FTDP-17 mutants, the R406W tau protein only shows a small loss of function [25,31,33,34]. All of the findings to date may contribute to why this mutation causes late onset of disease relative to other known FTDP-17 mutants and may account for the relatively mild phenotype found in families with this mutation.

Acknowledgements: This work was supported by The Wellcome Trust.

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