# The role of the VQIVYK peptide in tau protein phosphorylation

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## Abstract

Although it remains unclear whether they are related to one another, tau aggregation and phosphorylation are the main pathological hallmarks of the neuronal disorders known as tauopathies. The capacity to aggregate is impaired in a variant of the tau 3R isoform that lacks residues 306–311 (nomenclature for the largest CNS tau isoform) and hence, we have taken advantage of this feature to study how phosphorylation and aggregation may be related as well as the role of this six amino acid peptide (VQIVYK). Through these analyses, we found that the phosphorylation of the tau variant was higher than that of the complete tau protein and that not only the deletion of these residues, but also the interaction of these residues, in tau 3R, with thioflavin-S augmented tau phosphorylation by glycogen synthase kinase 3. In addition, the binding of the peptide containing the residues 306–311 to the whole tau protein provoked an increase in tau phosphorylation. This observation could be physiologically relevant as may suggest that tau–tau interactions, through those residues, facilitate tau phosphorylation. In summary, our data indicate that deletion of residues VQIVYK, in tau protein produces an increase in tau phosphorylation, without tau aggregation, because the VQIVYK peptide, that favors aggregation, is missing. On the other hand, when the whole tau protein interacts with thioflavin-S or the peptide VQIVYK, an increase in both aggregation and phosphorylation occurs.

Keywords: aggregation, phosphorylation, tau, tauopathies, VQIVYK.

*J. Neurochem.* (2007) 103, 1447–1460.

Alzheimer's disease (AD) is characterized by the presence of two aberrant structures in the brain of patients: senile plaques and neurofibrillary tangles (NFTs: Alzheimer 1907). NFTs are intracellular inclusions comprised of paired helical filament aggregations (PHFs: Kidd 1963). About 20 years ago, it was discovered that the microtubule-associated protein tau was a component of PHFs (Grundke-Iqbal *et al.* 1986a; Ihara *et al.* 1986; Kosik *et al.* 1986; Wood *et al.* 1986; Binder *et al.* 2005) and that purified tau was able to assemble into fibrillar polymers resembling PHFs *in vitro* (Montejo de Garcini *et al.* 1986). As hyperphosphorylated tau protein is present in PHFs (Grundke-Iqbal *et al.* 1986b) and phosphotau was found to be the main component of isolated PHFs (Goedert *et al.* 1988; Wischik *et al.* 1988), it was assumed that a modified form of tau is an essential component of PHFs. A number of other human diseases are characterized by an age-related increase of abnormal protein deposits. This includes a group of diseases, termed 'tauopathies', where the microtubule-associated protein tau aggregates in a highly phosphorylated form. Nevertheless, the best-known tauopathy is still AD, where tau deposits in the brain accumulate together with aggregates of the Ab peptide (Selkoe 2004) and frontotemporal dementia and parkinsonism linked to chromosome 17, where mutant forms of tau accumulate abnormally in the absence of other protein aggregates (Lee *et al.* 2001).

As indicated above, the assembly of the tau protein into fibrillar polymers resembling the PHF found in the brain of AD patients was first described *in vitro* in 1986 (Montejo de Garcini *et al.* 1986). However, a large amount of tau was required to produce polymerization *in vitro* (Montejo de Garcini *et al.* 1986; Crowther *et al.* 1992; Wille *et al.* 1992). The minimum tau concentration required for assembly could be lowered in the presence of inducers, an effect attributed to distinct elements that facilitate tau polymerization *in vitro*

such as sulfoglycosaminoglycans (e.g. heparin: Goedert *et al.* 1996; Perez *et al.* 1996), quinones (Santa-Maria *et al.* 2004), or fatty acids (Gamblin *et al.* 2000). The minimum region required for tau self-assembly has been mapped to the third tubulin-binding motif of the tau molecule (Perez *et al.* 1996; von Bergen *et al.* 2000) and within this motif, two peptides have been implicated in self-assembly. These are a peptide containing residues 306–311 (peptide P1, VQIVKY) that is able to self-assemble in the absence of any additional compounds (von Bergen *et al.* 2000; Santa-Maria *et al.* 2005), and a peptide containing residues 317–335 (peptide P2: Perez *et al.* 1996). The characterization of tau variants lacking either of these peptides has indicated that while peptide P1 facilitates tau assembly, it is not essential for polymerization in the presence of quinones, although it appears to be important for the polymerization in the presence of heparin (Santa-Maria *et al.* 2005; Li and Lee 2006). In contrast, the presence of peptide P2 is a requisite for tau polymerization in the presence of quinones (Santa-Maria *et al.* 2005). A model to explain the influence of both peptides in promoting tau filament formation has recently been proposed (Gamblin 2005). In this model, it was suggested that tau will only assemble after these two peptides undergo a conformational change, P1 forming a b-sheet structure and P2 an a-helix (Gamblin 2005). Accordingly, there are tau variants with a diminished capacity for self-assembly (Santa-Maria *et al.* 2005; Khlistunova *et al.* 2006; Li and Lee 2006), as well as tau variants with an increased capacity to form aggregates (Khlistunova *et al.* 2006), the expression of which is toxic to neuroblastoma cells. This toxicity is unrelated to the changes observed in tau phosphorylation, but it could be related to prior tau fragmentation (Park and Ferreira 2005; Khlistunova *et al.* 2006). In contrast, less is known about how these aggregation deficient tau variants affect phosphorylation (Khlistunova *et al.* 2006). In this work, we have studied the phosphorylation of a tau variant lacking peptide (VQIVKY) *in vitro* and in cultured cells. An increase in the phosphorylation of this variant was observed when compared with the full tau protein and more significantly, the binding of the VQIVKY peptide to the full tau protein, which mimics tau aggregation, facilitates tau phosphorylation.

## Materials and methods

### Antibodies and chemicals
The antibodies used in this study were: the phosphorylation-independent 7.51 monoclonal antibody (1 : 100; kindly provided by Dr C. M. Wischik, UK); Tau-5, a monoclonal antibody against the unphosphorylated form of tau (1 : 1000; Calbiochem, San Diego, CA, USA); T14, which recognizes an epitope in human tau in the region comprising residues 83–120 (Zymed, San Francisco, CA, USA); 12E8, a monoclonal antibody against tau phosphorylated at serine 262 (1 : 1000; Elan Pharmaceuticals, San Francisco, CA,

USA, kindly provided by Dr Seubert: Seubert *et al.* 1995); Tau-1, a monoclonal antibody against unphosphorylated tau (1 : 1000; Chemicon (Millipore iberica S.A), Madrid, Spain); PHF-1, a monoclonal antibody against PHF tau recognizing tau phosphorylated at serine residues 396 and 404 (1 : 100; kindly provided by Dr P. Davies, USA: (Greenberg and Davies 1990); Alz-50, a conformational antibody against tau (1 : 10; a gift of Dr P. Davies, USA: Jicha *et al.* 1999); antibody 422, raised against serine 422 phosphorylated tau (obtained from Biosource International Inc., Camarillo, CA, USA); AT180, a monoclonal antibody against PHF tau (1 : 100; Innogenetics, Zwijndrecht, Belgium). The monoclonal antibodies directed against a-actin and b-tubulin (Sigma, St Louis, MO, USA) were used as internal controls for protein quantification, and the antibody against a-tubulin YL1/2 was purchased from Serotec (Oxford, UK).

Glycogen synthase kinase 3 (GSK3b) and the catalytic subunit of PKA were purchased form Calbiochem (San Diego) and Sigma, respectively. Thioflavin-S (Th-S) was purchased from Sigma. The VQIVKY peptide was obtained from Neomps (Strasbourg, France) and human brain samples were from the Netherlands Brain Bank (kindly provided by Dr R. Ravid and Dr P. van't Klooster).

### Construction of tau expression plasmids

#### Nomenclature
The human tau isoforms encoding tau with three or four tubulin-binding repeats are indicated as tau 3R and tau 4R. Tau 3R protein lacking the residues VQIVKY is indicated as tau 3R $D_{VQIVKY}$. Mainly, tau 3R protein was used and that tau protein is indicated as Tau and if it is lacking residues VQIVKY is indicated as $TauD_{VQIVKY}$. Phosphorylated tau is indicated as Tau-P and phosphorylated $TauD_{VQIVKY}$ as $TauD_{VQIVKY}$-P.

The human tau isoforms encoding tau with three tubulin-binding repeats (tau 3R) and the tau protein lacking the residues VQIVKY (first six residues of the third repeat) of tau ($tau3RD_{VQIVKY}$), were cloned by PCR using: the 5¢ A6 primer (CGGGATCCATAATGG-CTGAGCCC), which includes the initiation codon as well as *Bam*HI restriction site; and the 3¢ A8 primer (GCGAATTCTCACAAACCC-TGCTTGG) which includes a stop codon as well as a downstream *Eco*RI site. Plasmids pRKT30 and pRKT30DV6K (Santa-Maria *et al.* 2005) were used as templates (Santa-Maria *et al.* 2006) and the resulting cDNAs were inserted into the pcDNA3 vector (Current version of pcDNA3 is pcDNA3.1 (+/).), Invitrogen, Paisley, UK). All the constructs were confirmed by DNA-sequencing analysis.

### Cell culture and transfection
African green monkey kidney fibroblast (COS-7) cells were grown in Dulbecco's modified Eagle's medium (GibcoBRL, (Invitrogen), Carlsbad, CA, USA), supplemented with 10% (v/v) fetal calf serum, in a humidified atmosphere with 5% $CO_2$. Cells at 50–70% confluency were transiently transfected with the different constructs using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen).

### Immunofluorescence microscopy
Cells plated on polylysine-coated coverslips were fixed with 4% formaldehyde or methanol at )20°C, permeabilized with 0.1% Triton X-100 and then blocked with 1% bovine serum albumin for

15 min. The cells were labeled with the primary antibodies diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin for 1 h and the coverslips were then rinsed three times with PBS and incubated for 30 min with Oregon Green- or Texas Red-conjugated secondary antibodies (diluted 1 : 400; Molecular Probes, (Invitrogen), Madrid, Spain). After a final rinse with PBS the coverslips were mounted with FluorSave (Calbiochem, La Jolla, CA, USA) and the cells were analyzed using a Zeiss epifluorescence microscope. Th-S staining was performed as reported previously (Santa-Maria *et al.* 2006).

*Binding of tau to microtubules in cultured cells*
Cells were incubated in buffer (80 mmol/L Pipes, pH 6.8, 1 mmol/L MgCl$_2$, 5 mmol/L EGTA, and 0.5% Triton X-100) for 30 s, which is sufficiently brief to avoid altering the cells during the extraction procedure. The detergent-soluble fraction with the proteins that were not bound to the cytoskeleton was harvested first and the detergent-insoluble components, corresponding to cytoskeleton-bound proteins, were subsequently resuspended in buffer (80 mmol/L Pipes, pH 6.8, 1 mmol/L MgCl$_2$, 5 mmol/L EGTA). Equal volumes of Triton-soluble and Triton-insoluble fractions, were then analyzed by immunoblotting with b-tubulin (Sigma) and Tau-5 antibodies.

*Biochemical analysis*
Cells were harvested in ice-cold PBS, resuspended, and homogenized in buffer containing 50 mmol/L HEPES (pH 7.4), 10 mmol/L EDTA, 0.1% Triton X-100, 10 mmol/L NaF, 0.1 mmol/L sodium ortho-vanadate, 1 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitors cocktail (Roche Diagnostics, Mannheim, Germany). Lysed cells were centrifuged at 10 000 *g* for 10 min at 4°C and boiled for 5 min in electrophoresis sample buffer. The protein concentration in each homogenate was determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out using 10% gels, which were subsequently transferred to nitrocellulose. Immunoreactive proteins were probed with peroxidase-conjugated anti-rabbit or anti-mouse antibodies (DAKO, Glostrup, Denmark) and visualized by chemiluminescence (ECL kit; Pierce).

For Dot Blot analysis, samples were applied to nitrocellulose using a Bio-Dot microfiltration unit (Bio-Rad, Madrid, Spain) prior to antibody detection. All nitrocellulose bound samples were incubated in 5% non-fat milk-T-PBS (T-PBS: mixture of PBS and 0.05% Tween 20) to block non-specific binding for 1 h at 25°C. Primary antibodies were then added [Alz-50 (1 : 10), Tau-5 (1 : 1000)] in blocking solution and incubated for 1 h. Unbound primary antibodies were removed by washing in several changes of T-PBS. Peroxidase-conjugated secondary antibodies were then added at 1 : 1000 dilution in blocking solution and incubated for 1 h. The blots were then washed as above and visualized by chemiluminiscence (ECL kit).

Recombinant tau proteins were purified and characterized as previously described (Perez *et al.* 1996). For a further quantitation of tau protein, the reaction of the isolated protein, by western blot, with ab Tau-5 was achieved. For biochemical analysis the same amount of tau and its variant were used.

*Measurement of aggregated or polymerized protein*
Polymerization of tau protein in the presence of quinones was previously reported (Santa-Maria *et al.* 2004). Assembly of tau protein in the presence of Th-S was carried out by incubation of tau protein (0.4 mg/mL) at 37°C for 24 h in the presence of 100 lmol/L Th-S. In some experiments, the assembly conditions as previously indicated (Chirita *et al.* 2005) were used, yielding similar results.

To quantify the amount of polymerized protein, samples were centrifuged for 30 min at maximum speed in an Airfuge (Beckman (GMI, Inc.), Ramsey, MN, USA). The protein present in the supernatant and pellet was analyzed by western blotting using the 7.51 tau antibody. The amount of protein was quantified by densitometry.

*In vitro microtubule-binding assay*
Temperature-dependent assembly of tubulin from mouse brain was performed by the procedure of Shelanski *et al.* (1973) using one polymerization cycle in the absence of 20% glycerol but adding 5 lmol/L of taxol (Scheele and Borisy 1976). The recombinant tau proteins were expressed in the *Escherichia coli* strain BL21 (DE3: Perez *et al.* 1996) and the polymerized protein was separated from the unpolymerized protein by centrifugation for 20 min in a Beckman Airfuge at maximum speed at 25°C. The polymerized protein as well as the supernatant was further analyzed by western blotting with b-tubulin and tau antibodies, or by direct Coomassie blue staining in the case of tubulin.

*Tau phosphorylation by PKA and by GSK3*
The phosphorylation of Tau and the Tau variant (TauD$_{VQIVKY}$) by PKA and GSK3 was performed as described previously (Stambolic and Woodgett 1994; Arrasate *et al.* 1997; Martin *et al.* 2002). Protein phosphorylation by PKA kinase (25 units/assay) or GSK3 (5 units/assay) was performed in 50 mmol/L Tris–HCl, pH 7.5, 10 mmol/L MgCl$_2$, 1 mmol/L EGTA, 1 mmol/L EDTA, and 1 lmol/L okadaic acid buffer at 37°C. Briefly, PKA or GSK3 and 1–4 1g of substrate (Tau or TauD$_{VQIVKY}$) were mixed for the times indicated in a 15 1L total volume of buffer solution containing 20 lmol/L ATP and 0.2 1Ci of (c-$^{32}$P) ATP.

The presence of similar amounts for tau protein and its variant, for the tests, was confirmed by western blot using ab Tau-5. For PKA, p24 was also used as substrate (Martin *et al.* 2002), whereas the GS-1 peptide was used as an additional GSK3 substrate (Stambolic and Woodgett 1994). In other experiments, phosphorylation was carried out for 30 min in the presence of variable concentration of ATP (up to 200 lmol/L). The modified protein was characterized by gel electrophoresis followed by autoradiography. $^{32}$P incorporation, was determined by densitometry of the labeled proteins bands in autoradiographs. The assays performed in the presence of Th-S or VQIVYK peptide were carried out with 20 lmol/L ATP for 1 h. The phosphorylation sites in the tau protein were identified by mass spectrometry following the method of Reynolds *et al.* (2000).

## Results

### Expression of Tau and its TauD$_{VQIVYK}$ variant in COS-7 cells
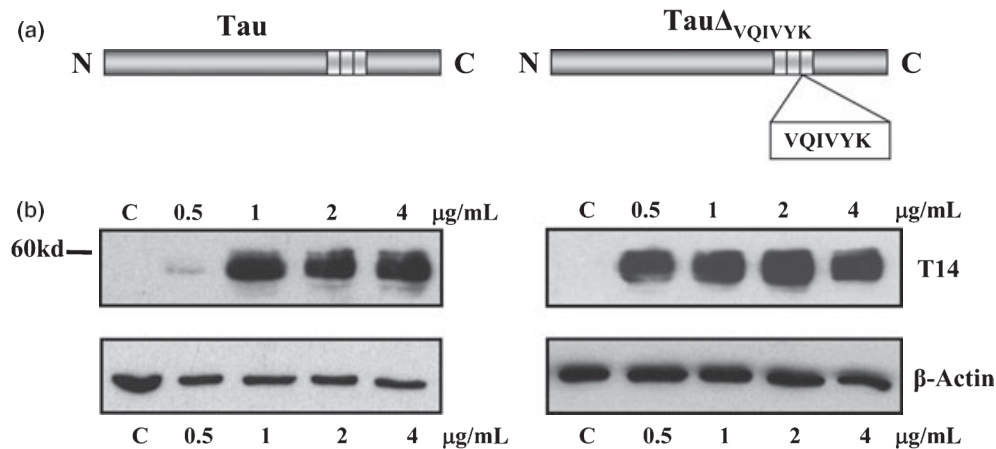The tau constructs expressed in COS-7 cells were the human tau cDNA containing three repeats (tau 3R) and the human

Fig. 1 Expression of Tau and TauD$_{VQIVYK}$ variant in African green monkey kidney fibroblast (COS-7) cells. (a) A schematic representation of the human tau cDNA constructs used in this experiment. The tubulin-binding domain is indicated in the boxes and the location of the VQIVYK peptide in the tau molecule is shown. (b) Tau wild-type (Tau, left) and tau variant (TauD$_{VQIVYK}$, right) were transfected at increasing concentrations in COS-7 cells and, 48 h after transfection, cell lysates were prepared. The extracts were analyzed by immunoblotting and probed with the T14 ab (that recognizes only with human tau) and actin as protein-loading control.

Tau cDNA of tau 3R variant lacking the VQIVYK residues (Tau D$_{VQIVYK}$; Fig. 1a). We have chosen the tau 3R isoform, because it is the simplest of the tau isoforms and thus, the possible function of peptides like VQIVYK cannot be complemented by other similar peptides present in tau 4R (like VQIINK) but not in tau 3R. The VQIVYK residues correspond to residues 306–311 in the tau molecule containing four repeats (tau 4R) or residues 275–281 in the tau containing three repeats (tau 3R) and potential roles for these residues have been indicated previously (von Bergen et al. 2000; Santa-Maria et al. 2005; Li and Lee 2006). The expression of the tau proteins was determined in western blots (Fig. 1b) after transfecting $10^5$ cells with 0.5, 1, 2, and 4 1g of either cDNA and waiting for 48 h. Accordingly, all the following experiments were performed by transfecting 2 1g of human Tau cDNA and 1 1g of TauD$_{VQIVYK}$ cDNA, and the transfected cells were collected 48 h after transfection.

Microtubule network in transfected cells
To examine the interaction of tau with microtubules, both full tau and its TauD$_{VQIVYK}$ variant, we expressed the corresponding cDNAs in COS-7 cells as these cells lack endogenous tau protein. The microtubule network was visualized in untransfected (control) and transfected cells by staining the cells with the tubulin YL1/2 (Ab Tb) or tau antibody (Ab Tau, Fig. 2). A higher proportion of cells bearing microtubule bundles were found in those cells transfected with tau than those transfected with its variant (see arrow in Fig. 2b). This difference could be due to less binding of the tau variant to microtubules than the full tau protein, which could either be due to a conformational change of the variant, to its modification (e.g. phosphoryla-

tion), or both. To test for the first possibility, we further studied the binding of each tau isoform to microtubules.

Changes in microtubule binding of tau and its variant
Soluble (S) or microtubule-bound tau protein (P) was isolated from COS-7 cells transfected with tau (Tau) or with its variant (TauD$_{VQIVYK}$: Fig. 3a). There was a similar proportion of polymerized tubulin in both types of cells, but a higher proportion of the full tau protein was associated to microtubules when compared with the tau variant (TauD$_{VQIVYK}$: Fig. 3b). In addition, we found that the full tau protein (Tau) shows a higher capacity to bind to microtubules in vitro than the tau variant (TauD$_{VQIVYK}$) when these tau isoforms were mixed with taxol-induced polymerized microtubules (Fig. 3c). After incubation with the assembled microtubules, 95% ($\pm$10%) of tau was associated to microtubules (Tau) compared with only 65% ($\pm$7%) of the tau variant (TauD$_{VQIVYK}$). As tau phosphorylation may also prevent the binding of the modified protein to microtubules, we tested whether the degree of phosphorylation differed for the tau variant.

The tau variant (TauD$_{VQIVYK}$) is expressed as a hyperphosphorylated isoform in COS-7 cells
The levels of phosphorylation of Tau and its variant TauD$_{VQIVYK}$, were assessed at different sites in the molecule that can be recognized by specific antibodies (Fig. 4a). These antibodies recognize sites modified by proline-directed protein kinases (PDPK) or by other non-proline-directed protein kinases (NPDPK). The sites recognized by antibodies PHF-1, AT180, and 422 are among those modified by PDPK (PDPK sites), while the Tau-1 antibody recognizes one such epitope in its unphosphorylated state. On the other hand, the
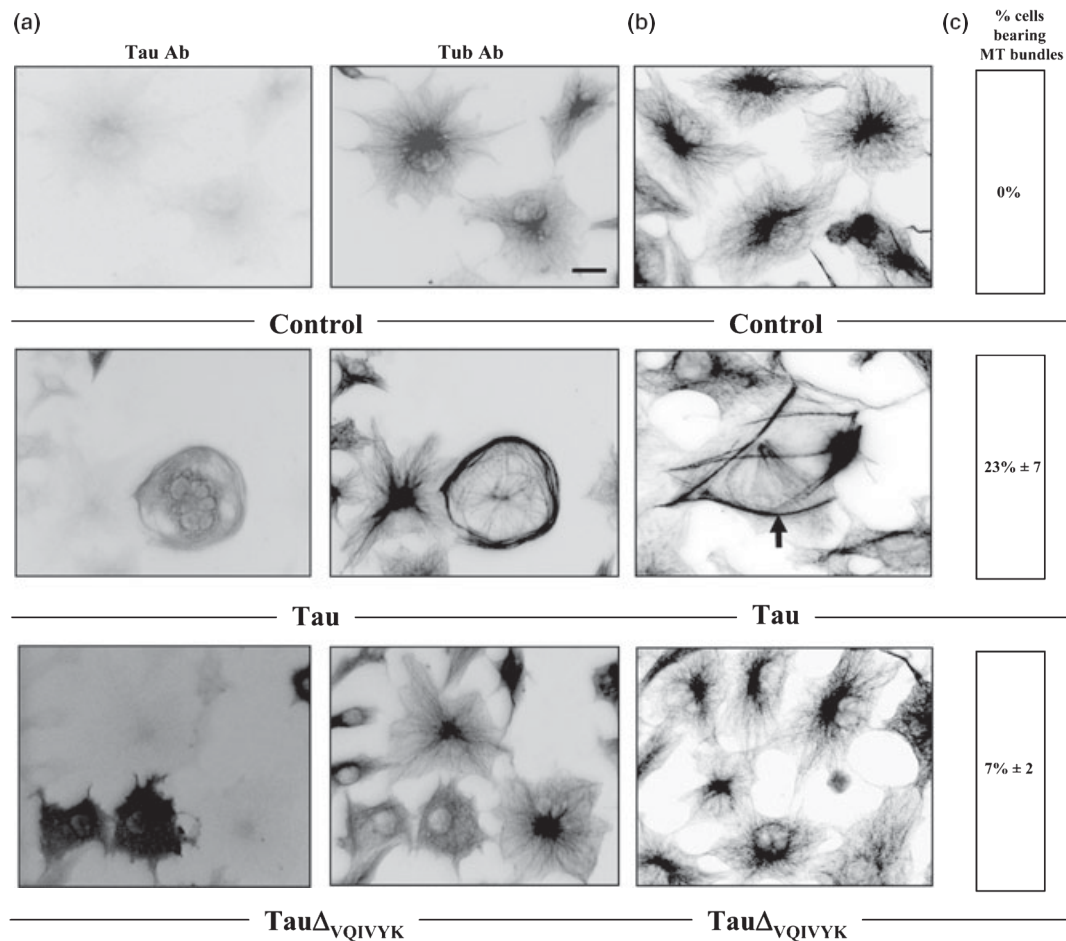
Fig. 2 The microtubule network in tau transfected cells. (a) African green monkey kidney fibroblast (COS-7) cells were transfected with mock (control), wild-type tau cDNA (TAU) or variant tau cDNA (TauD$_{VQIVYK}$), and the microtubule network was visualized using the YL1/2 antibody (anti-tubulin antibody; Tub Ab) or with a Tau-1 antibody (anti-tau antibody; Tau Ab). In the same field transfected (stained for tau) or untransfected (only stained for tubulin) cells can be observed. Bar indicates 10 lm. (b) Control (upper right image), Tau (middle right image) and tau variant TauD$_{VQIVYK}$ (lower right image) transfected cells were stained with tubulin antibody. Bar indicates 10 lm. (c) A total of 50 cells were counted and the proportion of cells bearing microtubule bundles is indicated (see the arrow indicating one of these bundles). The experiment was repeated three times and the average from those experiments is shown.

antibody 12E8 recognizes tau phosphorylated sites modified by NPDPK.

Different levels of tau phosphorylation were observed at the sites recognized by these antibodies in the full Tau protein and in its variant (TauD$_{VQIVYK}$: Fig. 4b–f). Furthermore, other antibodies were used as controls such as Tau-5 to determine the total tau expression independent of phosphorylation (Fig. 4g), or against actin as a control for protein loading (Fig. 4h). Figure 4i shows the quantitative results of the data shown in Fig. 4b–f, normalized by the reaction of the samples with ab Tau-5.

These results might suggest that the hyperphosphorylated tau variant in the transfected cells may affect the organization of the microtubule network (Fig. 2). Indeed, it has been shown that phosphorylation of tau by PDPK and NPDPK diminishes its binding to microtubules (For a review, see Avila *et al.* 2004). Moreover, NPDPK modification at phosphoserine 262, as recognized by the 12E8 antibody (Seubert *et al.* 1995), clearly impairs the interaction of tau with microtubules (Biernat *et al.* 2002). Thus, the increased phosphorylation of the tau variant could affect its binding to microtubules, as indicated in Figs 2 and 3.

The Tau variant is a better *in vitro* substrate than Tau protein for PDPK (GSK3) or NPDPK (PKA) phosphorylation

To determine if the increase in the phosphorylation of the tau variant when compared with the full tau protein, was due to possible conformational changes in the protein promoted by the deletion of the VQIVYK residues, and to test whether this change facilitates phosphorylation, the
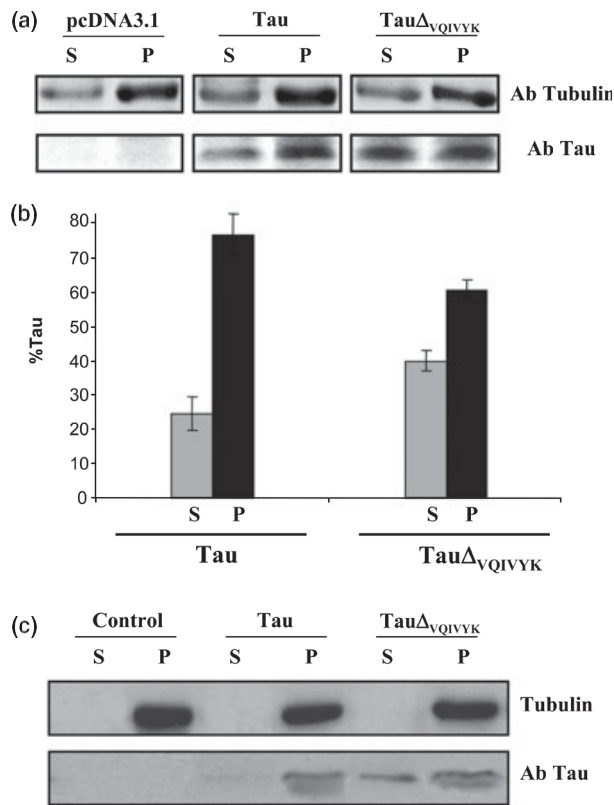
Fig. 3 TauD$_{VQIVYK}$ expressed in African green monkey kidney fibroblast (COS-7) cells binds to microtubules with a lower affinity than Tau expressed in COS-7 cells. (a) COS-7 cells were transfected with either an empty plasmid (pCDNA3.1), tau cDNA (Tau) or variant tau cDNA (TauD$_{VQIVYK}$) and after 2 days, the cells were incubated for 30 s with an extraction buffer containing 0.5% Triton X-100 (see Materials and methods for details). Homogenates were fractionated into the detergent-soluble supernatant fraction (S), and the pellet (P) containing polymerized cytoskeletal proteins and associated components. After sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting, each protein extract was probed with the Tau-5 antibody, a tau-specific antibody that measures the total tau (Ab Tau), and with a tubulin antibody (Ab Tubulin) as a control of protein loading. (b) The proportion of tau protein found in the supernatant (S) and in the pellet (P) was quantified ($n = 3$). Error bars represent standard deviations of the mean value. Asterisks (*) indicate statistical significance ($p < 0.05$). (c) Assembled microtubules were obtained by incubating (20 min/37°C) phosphocellulose-purified tubulin (5 mmol/L) with 10 lmol/L of taxol and they were then incubated with 0.2 mmol/L tau (Tau) or tau variant (TauD$_{VQIVYK}$: 5 min/37°C). After centrifugation to fractionate the assembled and unassembled protein, the presence of tubulin in the pellet (P) and supernatant (S), was determined by Coomassie blue staining (Tubulin) after copolymerization in the presence or absence (control) of tau protein (Tau) and in the presence of its variant (TauD$_{VQIVYK}$). Again, the presence of tau proteins in the supernatant and pellet was determined by the interaction of the Tau-5 antibody (Ab Tau).
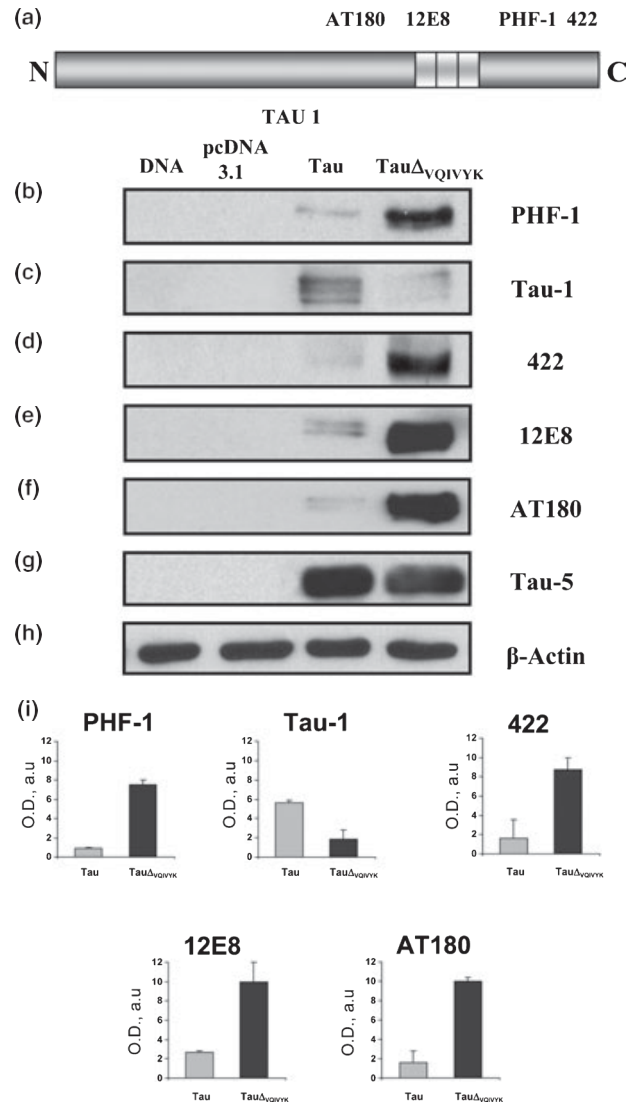
Fig. 4 Tau expression and phosphorylation state in transfected African green monkey kidney fibroblast (COS-7) cells. (a) Scheme of the tau molecule and the phosphorylation sites analyzed in this work. Untransfected COS-7 cells and COS-7 cells transfected with wild-type (Tau) or tau variant (TauD$_{VQIVYK}$) were collected, and the protein lysates were probed in western blots with: Tau-5 antibodies to determine total tau (g); Tau-1 that recognizes unphosphorylated tau (c); or the phospho-dependent tau antibodies paired helical filament aggregations-1 (PHF-1) (b), 422 (d), 12E8 (e), AT180 (f). Actin is shown as a loading control (h). The variant TauD$_{VQIVYK}$ is more heavily phosphorylated in transfected COS-7 cells at the PHF-1, 422, AT180, and 12E8 epitopes and there is less phosphorylation at the site recognized by Tau-1 than in Tau expressing COS-7 cells. (i) Shows the data from Fig. 4b–f quantified by densitometry, after being normalized by the reaction of the samples with ab tau 5. The units for the ordinate are optical density arbitrary units. The quantitation ($n = 3$) of the reaction of tau antibodies with Tau and its variant TauD$_{VQIVYK}$ is shown.
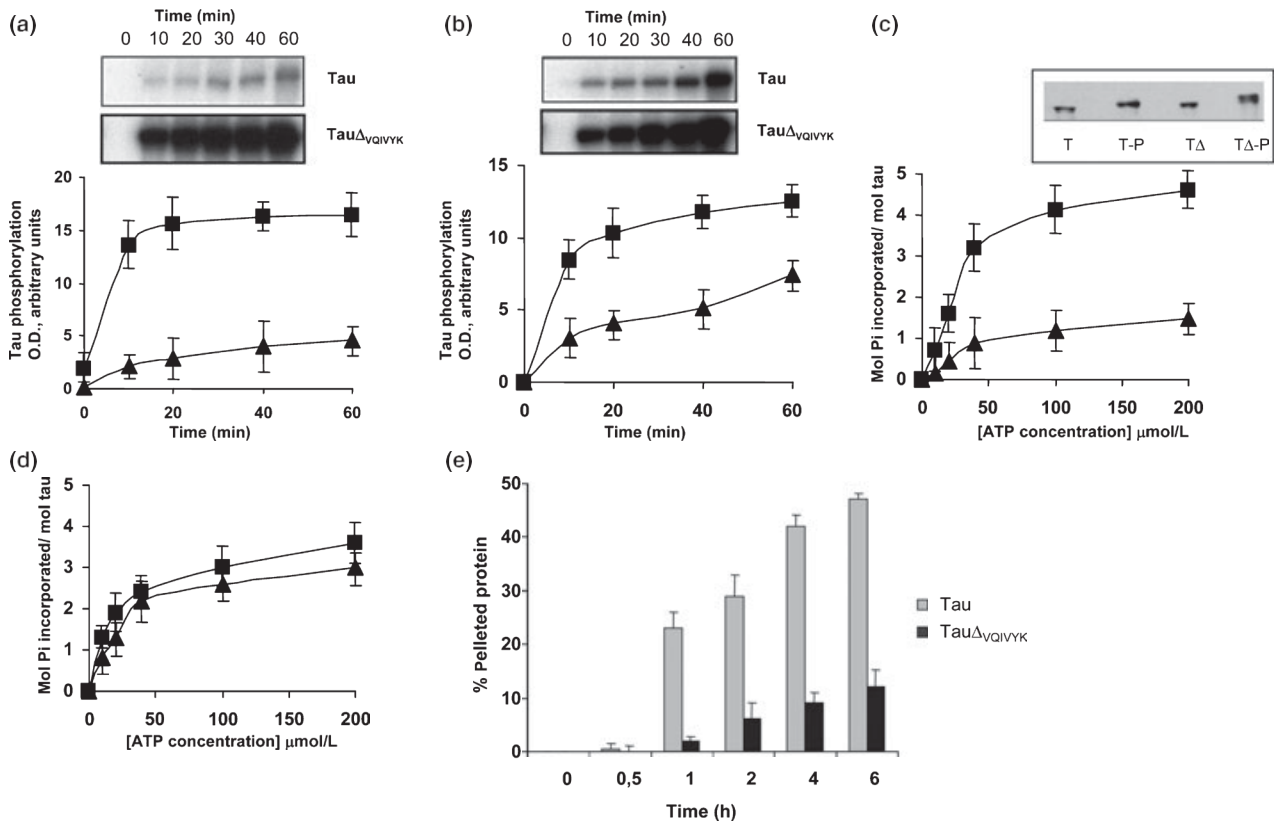
Fig. 5 *In vitro* tau phosphorylation, but not aggregation, augments after deletion of residues 306–311 from the tau molecule. Tau (▲) or TauD$_{VQIVYK}$ (■) phosphorylated by glycogen synthase kinase 3 (GSK3) (a and c) or protein kinase A (PKA) (b and d), in the presence of $^{32}$PcATP was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and characterized by densitometry of the bands obtained in autoradiographs (a and b, densitometry values are presented as arbitrary units). Also, the effect of the ATP concentration on the phosphorylation by GSK3 or PKA was measured (c and d). (e) Tau protein (■) or TauD$_{VQIVYK}$ (■) were mixed at a concentration of 1 mg/mL with coenzyme Q$_0$ (2 mmol/L), and the amount of assembled polymers was measured after centrifugation (see Materials and methods). Panel (e) shows the decrease in protein polymerization when tau lacks residues VQIVYK, when compared with the full tau protein.

phosphorylation of tau by GSK3 and by PKA was assayed *in vitro*.

Accordingly, TauD$_{VQIVYK}$ proved to be a better substrate for both GSK-3 and PKA than the full tau protein (Fig. 5a and b). Indeed, the incubation of Tau and TauD$_{VQIVYK}$ with increasing amounts of ATP did not change the $K$m values for ATP with both kinases (about 25 lmol/L for GSK-3 and PKA with both tau forms), while a 68% reduction in $V$max was detected for GSK-3 and 20% for PKA (Fig. 5c and d). These data suggest that TauD$_{VQIVYK}$ is a better substrate for both kinases than Tau while the affinity of the kinases for ATP is not altered in the presence of Tau or TauD$_{VQIVYK}$. Also, a decrease in electrophoretic mobility was observed for both tau isoforms upon phosphorylation by GSK3 (inset Fig. 5c).

We defined the sites modified by GSK3 after 5 h in the presence of the kinase by mass spectrometry. Some of the tryptic peptides detected augmented their molecular mass by 80 upon phosphorylation, suggesting that these tryptic peptides were phosphorylated only once. In other cases, the mass of the peptides increased by 160, indicative of the incorporation of two phosphates into the peptide. Thus, the primary sequences of some of the modified peptides from the Tau protein and its variant, and the number of phosphates incorporated into the peptides upon incubation with GSK3 was examined (Figs 6 and 7). In agreement with previous data (Reynolds *et al.* 2000), we found that the peptide containing residues 195–209/211 (following the numeration for the largest tau isoform), is modified in both Tau and its variant (TauD$_{VQIVYK}$), although an extra phosphorylation site was observed in the variant in the used conditions. In addition, phosphorylation of the peptide containing residues 210–212/221 was detected in both proteins (Figs 6 and 7). Using nanoelectrospray mass spectrometry we identified Ser199 and Thr212 as the sites of phosphorylation, which correspond to some GSK3-phosphorylated tau sites

**(a)**
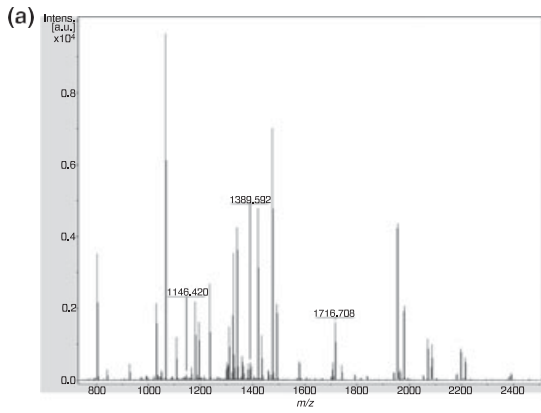
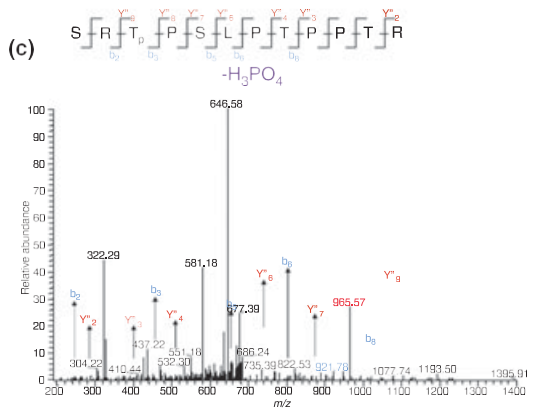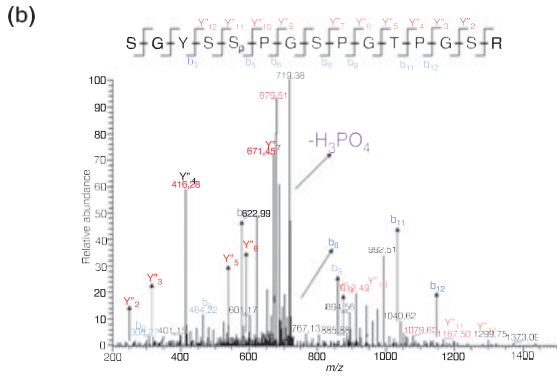| Tau-P | | | |
|---|---|---|---|
| Residues | Sequence | Mass | Phosphorytable sites |
| 195-211 | SGYSSPGSPGTPGSRSR | 1716.7 | 199 |
| 212-211 | TPSLPTPPTR | 1146.4 | 212 |
| 210-211 | SRTPSLPTPPTR | 1389.5 | 212 |

**(b)**

**(c)**

Fig. 6 Identification of phosphorylation sites on glycogen synthase kinase 3 (GSK3) phosphorylated tau. (a) The molecular mass of the peptides of tau modified by GSK3 in the tau protein, together with their sequence. The phosphopeptides of the major protein peaks were analyzed further, by nanoelectrospray (b and c) confirming the phosphorylation of serine 199 and threonine 212. These results support those shown in Fig. 4 as the phosphorylation at residue 199 could affect the interaction of tau protein with Tau-1 Ab.

(Reynolds *et al.* 2000). Furthermore, in the tau variant the peptide 386/406 was also modified (Fig. 7).

Thus, some extra sites are phosphorylated by GSK3 in the tau variant when compared to those modified in the full tau protein. In contrast, after exhaustive phosphorylation with PKA the same numbers of sites were modified in both tau proteins, one of which is Ser262 (Fig. 4).

These results suggest a possible conformational change in the tau protein upon deletion of residues VQIVYK that may facilitate its phosphorylation by tau kinases like GSK3 or PKA. Curiously, the capacity for self-aggregation (induced by quinones: Santa-Maria *et al.* 2005) is impaired by this deletion (Fig. 5e), suggesting that this tau variant adopts a conformation that facilitates its phosphorylation but avoids its aggregation, because of the lack of the interacting peptide VQIVKY.

The conformational change that may occur upon deletion of the VQIVKY peptide could be identified by a conformational antibody like Alz-50 (Carmel *et al.* 1996; Jicha *et al.* 1999). Alz-50 only binds efficiently to tau when its N-terminal end comes into contact with the microtubule-binding region (Brion *et al.* 1985; Carmel *et al.* 1996; Jicha *et al.* 1999). Furthermore, the Alz-50 conformation of tau appears first in pre-tangle neurons, although it is also present in neurons containing NFTs as well (Tabaton *et al.* 1988). In fact, Alz-50 binds to PHF tau (Carmel *et al.* 1996) or to other tau aggregates in other tauopathies (Tabaton *et al.* 1988). When we performed experiments with this antibody we observed that Alz-50 binds with a higher affinity to recombinant tau lacking the VQIVYK peptide than to the full tau protein (Fig. 8b). As control we also tested extracts from the brain of AD patients or from a non-demented control (Fig. 8a).

The presence of Th-S facilitates tau phosphorylation

It is thought that the peptide comprising residues VQIVYK is able to bind to Th-S or related compounds (Santa-Maria *et al.* 2005). In this way, there was less staining with Th-S of polymers assembled from the tau variant TauD$_{VQIVYK}$ lacking the peptide, while polymers assembled from the isolated VQIVYK peptide are indeed heavily stained with Th-S (Santa-Maria *et al.* 2006). It has also been suggested that binding of Th-S or related compounds to unpolymerized tau protein may favor tau fibrillization (Chirita *et al.* 2005). Thus, the interaction of Th-S with the VQIVYK peptide in the tau molecule may favor tau aggregation,
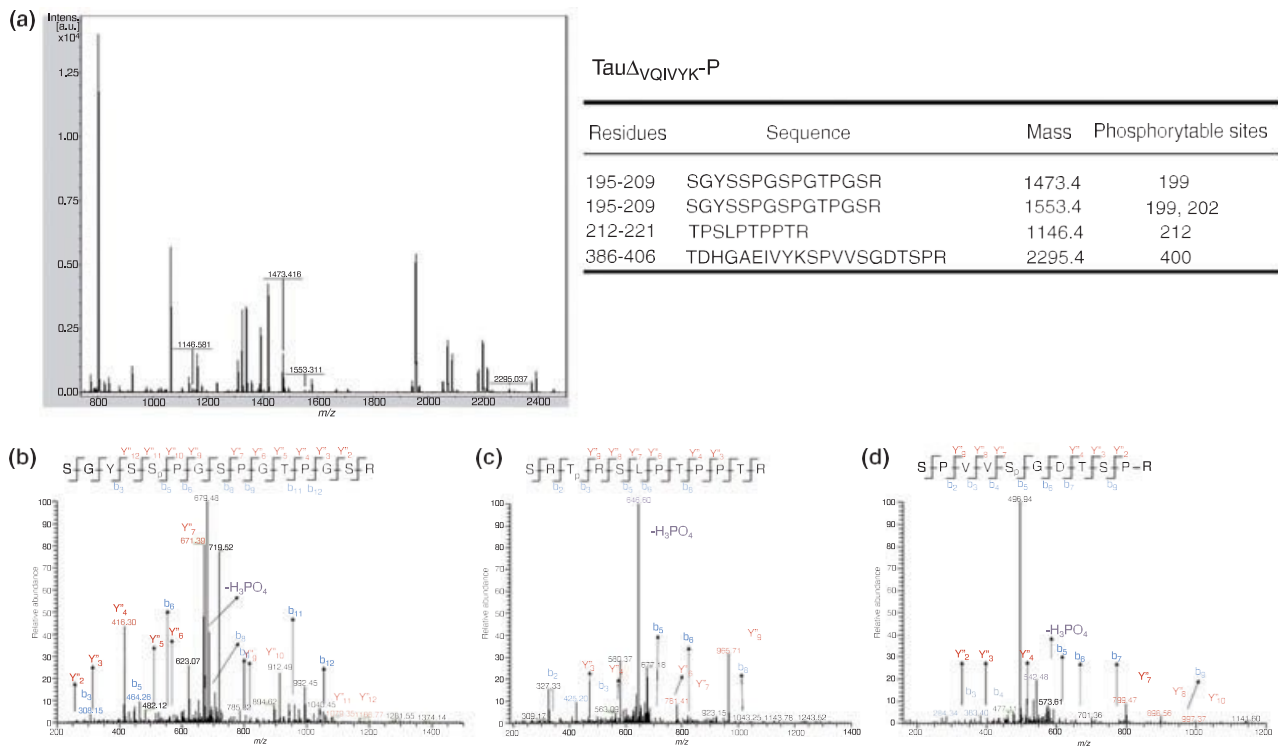
(a)

| TauΔ$_{VQIVYK}$-P | | | |
| --- | --- | --- | --- |
| Residues | Sequence | Mass | Phosphorytable sites |
| 195-209 | SGYSSPGSPGTPGSR | 1473.4 | 199 |
| 195-209 | SGYSSPGSPGTPGSR | 1553.4 | 199, 202 |
| 212-221 | TPSLPTPPTR | 1146.4 | 212 |
| 386-406 | TDHGAEIVYKSPVVSGDTSPR | 2295.4 | 400 |

(b) (c) (d)

Fig. 7 Identification of phosphorylation sites on glycogen synthase kinase 3 (GSK3) phosphorylated TauD$_{VQIVYK}$. (a) The molecular mass of the peptides of TauD$_{VQIVYK}$ modified by GSK3 in the tau protein, together with their sequence. The phosphopeptides of the major protein peaks were analyzed further, by nanoelectrospray (b–d) con- firming the phosphorylation of serine 199, threonine 212, and serine 400. These results support those shown in Fig. 4 as the phosphory- lation at residue 199 could affect the interaction of tau protein with Tau-1 antibody and that phosphorylation at residue 400 could favor the interaction of this tau variant with the PHF-1 antibody.

although little is known about its effect on tau phosphor- ylation.

We tested whether the interaction of Th-S with tau might affect phosphorylation of the tau protein. First, we examined the effect of Th-S on tau phosphorylation by adding different concentrations of the dye. The effect of Th-S on GSK3 activity was measured using the GS1 peptide as substrate and that on PKA activity was evaluated using the p24 protein as a substrate (see Materials and methods). Below 30 lmol/L, Th-S slightly diminished GSK3 activity (inset Fig. 9), whereas <10% of the initial PKA activity remained in the presence of 10 lmol/L Th-S (data no shown), indicating that Th-S is more toxic to PKA than to GSK3. After determining the effect of Th-S on GSK3 activity, the phosphorylation of the tau protein by GSK3 in the presence of increasing concentrations of Th-S was measured. In the presence of Th-S, there is a clear increase in Tau phosphorylation by GSK3 (Fig. 9) while, no such increase was found for TauD$_{VQIVYK}$ phosphorylation by GSK3. Hence, the interaction of the tau VQIVYK peptide, with Th-S produces a similar effect on tau phosphorylation as deleting that peptide from tau molecule.

In addition, we have tested for a possible effect of Th-S on the binding of tau to microtubules. A slight decrease (15%) was found for the binding of tau to microtubules in the presence of Th-S (data not shown). This is not surprising, as the sequence of tau with the highest capacity to bind to microtubules is that contained within the first repeat, the following inter region, and the second repeat (Goode *et al.* 1997).

It should be also indicated that the addition of 100 lmol/L Th-S, as also previously indicated by Chirita *et al.* (2005), results in the aggregation of tau protein 15% ± 2 of the total protein was sedimented by centrifugation, in the presence of 100 lm Th-S (Fig. 10c). Tau aggregation was confirmed by electron microscopy (Fig. 10a and b).

The addition of the VQIVYK peptide to tau protein favors tau phosphorylation

It was previously suggested that two molecules of tau could interact with each other, in part through their VQIVYK motifs (von Bergen *et al.* 2000). Thus, the interaction of tau with tau peptide VQIVYK may also modulate tau phosphor- ylation. When we carried out experiments to confirm this, we observed that tau phosphorylation increased in the presence of increased amounts of VQIVYK peptide (Fig. 11). As this interaction may take place through the binding of VQIVYK peptide with its counterpart present in tau molecule, the experiment was performed with the tau variant lacking that
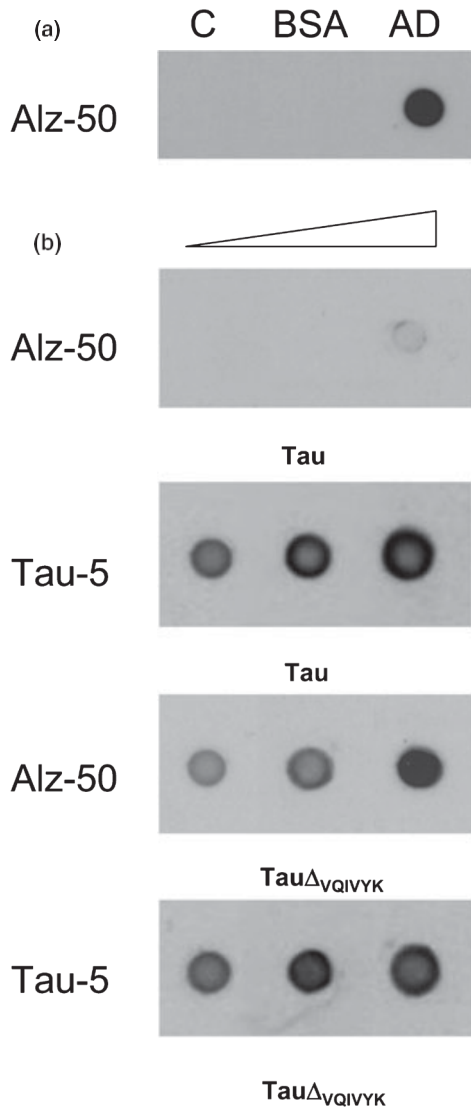
**Fig. 8** Interaction of TauD$_{VQIVYK}$ with the Alz-50 Ab. Equal amounts of an extract from the brain of Alzheimer's patient (AD) or a health control (C), or 2 1g of bovine serum albumin (BSA) were dot blotted together with increasing amounts (0.01, 0.1, and 1 1g) of Tau protein and TauD$_{VQIVYK}$, and probed with ab Alz-50. As indicated, the Ab reacted more strongly with TauD$_{VQIVYK}$ than the full Tau protein. Similar loading of each protein was confirmed by the reaction with the Tau-5 antibody.

peptide. The addition of the peptide VQIVYK to the tau variant TauD$_{VQIVYK}$ did not provoke any increase in GSK3 phosphorylation of this isoform (Fig. 11). Thus, the deletion of VQIVYK from the tau molecule, or an interaction of tau protein with Th-S or VQIVYK, resulted in an increase of tau phosphorylation. The peptide VQIVYK, or peptide P1, is involved in a type of tau–tau interaction. However, peptide P2 may also play a role in that tau self-assembly. Thus, we tested the role of peptide P2 in tau phosphorylation. In this case, we have found that, at difference of the effect found for
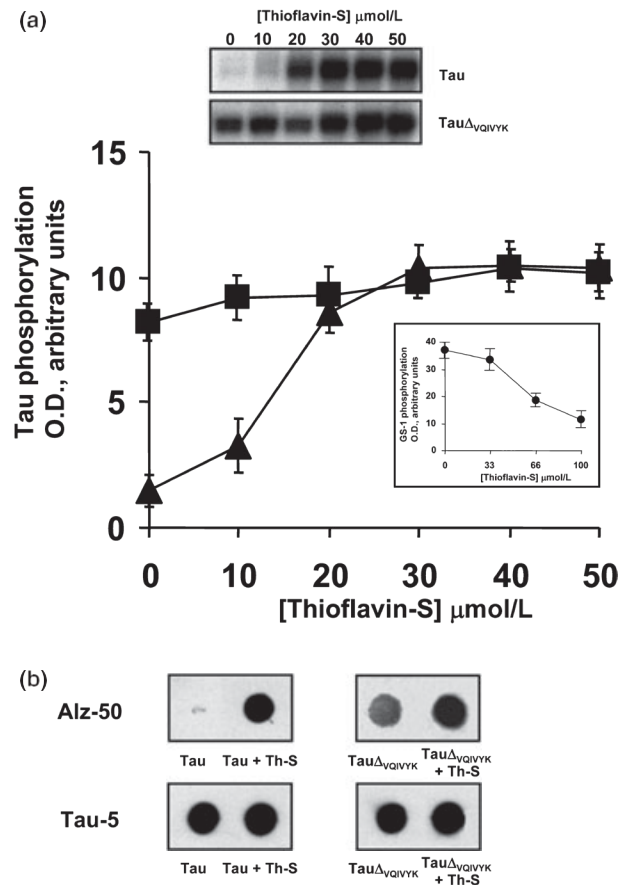


**Fig. 9** Tau and TauD$_{VQIVYK}$ phosphorylation in the presence of thioflavin-S (Th-S). (a) Tau (▲) or TauD$_{VQIVYK}$ (■) phosphorylation *in vitro* by GSK3 in the presence of $^{32}$PcATP and different concentrations of Th-S was characterized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and densitometry of the bands obtained in autoradiograms (densitometry values presented as arbitrary units). The inset indicates the phosphorylation of the peptide GS-1 at increasing Th-S concentrations. (b) The interaction of Alz-50 and Tau-5 antibodies with Tau and TauD$_{VQIVYK}$ in the absence or presence of Th-S, analyzed by Dot Blot, are shown.

VQIVYK peptide, peptide P2 does not increase tau phosphorylation (Fig. 11b).

To analyze if the differences observed for peptides P1 and P2 are due to differences in tau conformation promoted or not for these peptides, the reaction of tau with antibody Alz-50, in the presence of either peptide, was studied. Figure 11(c and d) shows that peptide VQIVYK, but not peptide P2, favors the conformational change of tau protein, allowing its reaction with Alz-50 antibody.

## Discussion

The abnormal aggregation and hyperphosphorylation of tau are hallmarks of different tauopathies including AD. Although a possible relationship between these features has
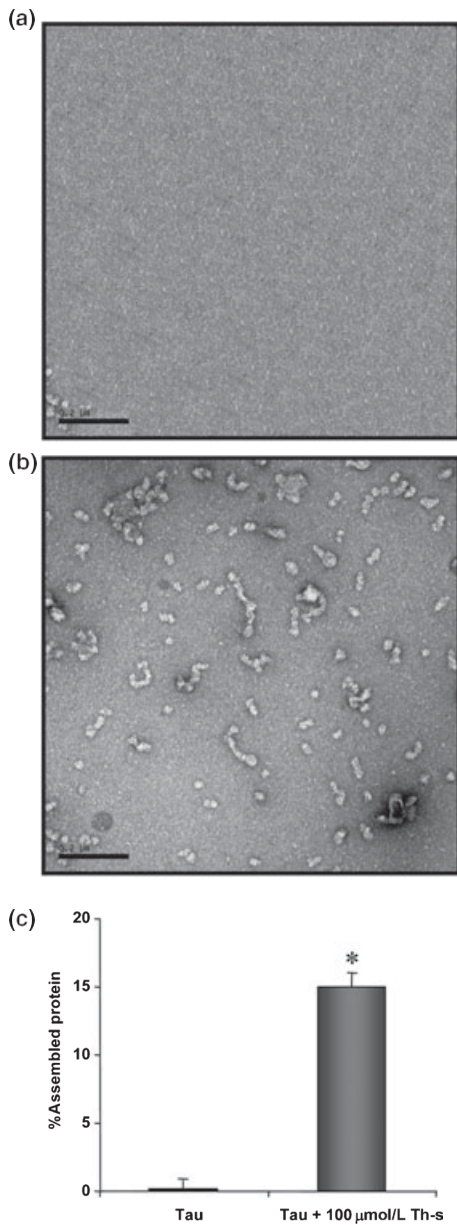
(a)

(b)

(c)

Fig. 10 Assembly of tau in the presence of thioflavin-S (Th-S). Tau protein (1 mg/mL) was incubated in the absence (a) or presence (b) of 100 lmol/L Th-S and the assembled polymers were visualized by electron microscopy and quantitated (*n* = 3) (c) after centrifugation (see Materials and methods). Error bars represent standard deviations of the mean value. Asterisks (*) indicate statistical significance (*p* < 0.01).

been suggested in some models (Perez *et al.* 2003; Noble *et al.* 2005), it is not clear whether tau phosphorylation is necessary for the latter and thus, it would be of interest to have models to analyze both these physiological processes. In this work, we have characterized the unexpected observation that a tau variant showing diminished capacity for aggregation (von Bergen *et al.* 2000; Santa-Maria *et al.*

2005; Khlistunova *et al.* 2006; Li and Lee 2006), can be readily hyperphosphorylated by tau PDPK (such as GSK3) and by NPDPK (such as PKA). Thus, in this model tau phosphorylation can occur in the absence of tau aggregation, although the failure to aggregate could be due to the lack of VQIVYK peptide. Indeed, this peptide appears to be needed for tau assembly in the presence of heparin (Li and Lee 2006) and it facilitates tau polymerization in the presence of quinones (Santa-Maria *et al.* 2005). Significantly, this phosphorylated tau protein bound less to microtubules (see also Alonso *et al.* 1996).

Tau phosphorylation has been extensively studied because it could be toxic *in vivo* (Jackson *et al.* 2002; Engel *et al.* 2006b; Fulga *et al.* 2007), a toxicity that could be manifested before tau aggregation (Lucas *et al.* 2001; Perez *et al.* 2003; Noble *et al.* 2005; Santacruz *et al.* 2005; Engel *et al.* 2006a). In our studies an increased phosphorylation of the tau variant by GSK3 when compared with that of the full tau protein was observed, and it could be due to the exposure of additional sites that are modified in the variant but not in tau protein. Several sites are phosphorylated by GSK3 or PKA in tau protein (Morishima-Kawashima *et al.* 1995). While few residues phosphorylated by PKA have been well characterized, like residues 214 or 409 and serine 262 (see for example Liu *et al.* 2006), at least 10 sites modified by GSK3 have been identified in tau protein (Reynolds *et al.* 2000). Indeed, some of these residues phosphorylated by GSK3 were seen to be modified in tau and in the tau variant by mass spectrometry.

Recently, it was indicated that tau aggregation could be toxic to cultured cells (Khlistunova *et al.* 2006), and that such aggregation might be unrelated to the changes observed in tau phosphorylation (Khlistunova *et al.* 2006). Thus, tau phosphorylation and tau aggregation could be independently toxic to cells. However, in the case of tau aggregation this toxicity could be related to the fact that tau fragmentation precedes tau aggregation in that model, and some of the resulting tau fragments could be toxic to the cell (Park and Ferreira 2005; Khlistunova *et al.* 2006).

Our experiments show that different events could induce tau phosphorylation: (i) deletion of the VQIVYK peptide, which would lead to phosphorylation but not tau aggregation; and (ii) interaction of Th-S or VQIVYK with tau protein producing both aggregation and tau phosphorylation. The latter might be suggestive of a relationship between both events, as observed in some animal models (Perez *et al.* 2003; Noble *et al.* 2005). In this way, our results suggest that binding of the VQIVYK peptide to tau mimics tau aggregation and will facilitate tau phosphorylation. Thus, deletion of VQIVYK or the interaction of this peptide in the tau molecule with other molecules like Th-S or the peptide itself, will result in an increase in phosphorylation and it may be the consequence of a previous conformational change. However, aggregation is facilitated when the peptide VQIVYK is not actually deleted, as it plays an important role in tau
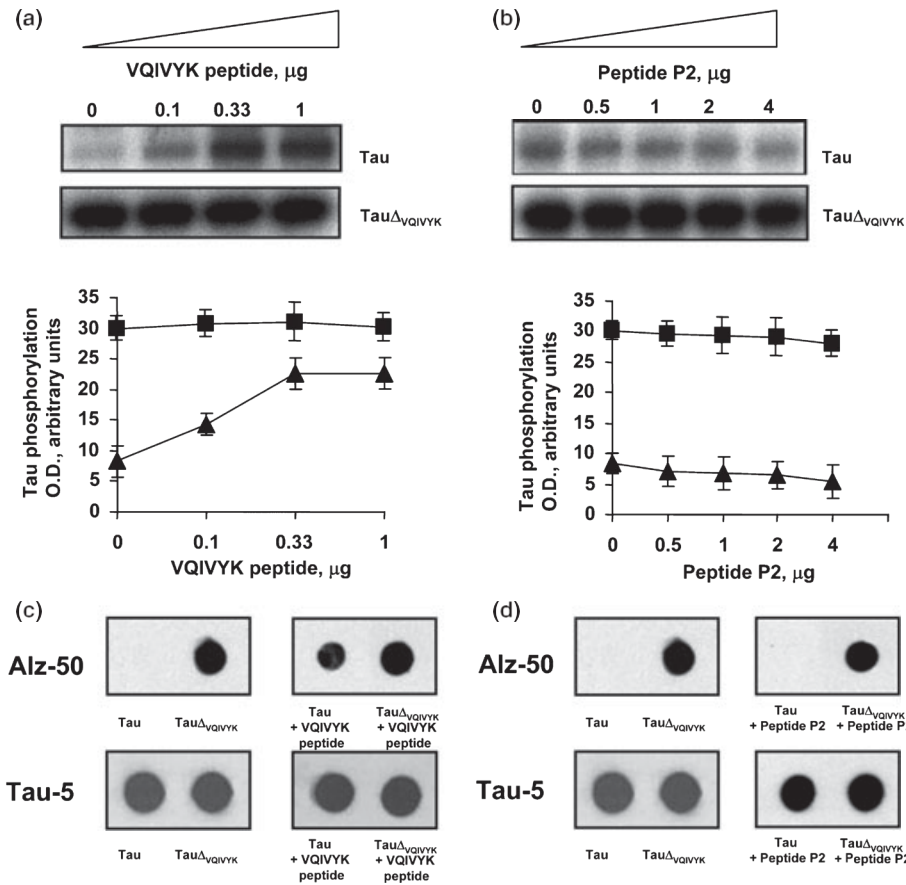
Fig. 11 Tau phosphorylation in the presence of the VQIVYK peptide and in the presence of peptide P2. Tau(▲) or TauD$_{VQIVYK}$ (■) modified by glycogen synthase kinase 3 (GSK3) in the presence of different amounts of VQIVYK peptide (peptide P1) (a) or peptide P2 (b) were isolated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and characterized by densitometry of the bands obtained by autoradiography (densitometry values presented as arbitrary units). The interaction of Alz-50 and Tau-5 antibodies with tau (c) and its variant (d), analyzed by Dot Blot, in the presence of P1 or P2 peptides are shown.

aggregation (von Bergen *et al.* 2000; Santa-Maria *et al.* 2005). Thus, a model for the events that occur in tau pathologies could be proposed in which a conformational change will occur that results in subsequent, tau phosphorylation, aggregation, or both. In this conformational change peptide VQIVYK plays an important role. This conformational change could be induced *in vivo* by different signals, an example could be oxidative stress, that is known to induce a conformational change that makes tau accessible to the Alz-50 antibody (Takeda *et al.* 2000). According to our model, this conformational change recognized by Alz-50 antibody (Binder *et al.* 2005) would pre-dispose tau to phosphorylation, aggregation or both, depending on the different affinities of tau for the different kinases present in the neuron, or on the concentration of modified tau. These differences will explain the different types of tau pathologies occurring in different neurons.

At present, we do not know if the expression of phosphotauD$_{VQIVKY}$ is more toxic than that of the tau protein. However, it is noteworthy that while we have isolated stable transfectants of Tau cDNAs, we have not yet been able to repeat this with TauD$_{VQIVKY}$ cDNA. Thus, while it is possible that the permanent expression of this tau variant could be toxic to the cell, more work must be carried out to study this phenomenon further.

It will also be of interest to search for compounds like Th-S or the VQIVYK peptide that facilitates tau phosphorylation by interacting with the tau protein through VQIVYK peptide. Interestingly, it is known that presenilin-1 associates with tau through, or close to the region containing the VQIVYK peptide leading to an increase in tau phosphorylation by GSK3. Indeed, such phosphorylation increases in the presence of presenilin-1 (PS-1) that bears some mutations found in AD (Takashima *et al.* 1998). This might explain the effect of these PS-1 mutations on tau phosphorylation in AD. However, it has not yet been tested whether the binding of PS-1 to tau protein could change the conformation of tau. In addition, the increase in GKS3 phosphorylation of tau,

induced by PS-1 mutations also could be due to an alternative mechanism (Baki *et al.* 2004).

Also, further studies should analyze whether there are some tau-associated proteins in different tauopathies that influence pathological tau phosphorylation, or that interact with the tau molecule through VQIVYK peptide (or close to it), producing a similar effect on tau phosphorylation to that of PS-1. In any case, much work remains to be carried out to identify the causes of the increase in tau phosphorylation in different tauopathies.

In summary, deletion of the VQIVYK peptide in tau results in a conformational change that decreases the binding of tau to microtubules and favors tau phosphorylation. The interaction of the VQIVYK peptide with tau, mimicking a type of tau aggregation, also favors tau phosphorylation, suggesting that tau aggregation could favor the phosphorylation of tau at certain specific sites.

## Acknowledgements

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