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ORIGINAL ARTICLE

Involvement of puromycin-sensitive aminopeptidase in proteolysis of tau protein in cultured cells, and attenuated proteolysis of frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) mutant tau

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Abstract

In tauopathies, tau protein is hyperphosphorylated, ubiquitinated, and accumulated in the brain; however, the mechanisms underlying this accumulation remain unclear. To gain an understanding of the role of proteases in the metabolism of tau protein, in the present study we evaluated the effects of protease inhibitors in SH-SY5Y human neuroblastoma cells and COS-7 cells transfected with the tau gene. When cells were treated with 0.1–10 μmol/L of lactacystin and 1.0–20 μmol/L of MG-132 (inhibitors of proteasome), 0.1–10 μmol/L of CA-074Me (a cathepsin inhibitor), and 0.1–2 μmol/L of puromycin (a puromycin-sensitive aminopeptidase (PSA) inhibitor) for up to 24 h, there were no significant changes in tau protein levels. However, pulse-chase experiments demonstrated that the proteolysis of tau protein in SH-SY5Y cells was attenuated following treatment of cells with 200 nmol/L puromycin. Increased tau protein levels were also observed in SH-SY5Y cells treated with short interference (si) RNA to PSA to inhibit the expression of PSA. These data suggest that PSA is a protease that catalyses tau protein predominantly in SH-SY5Y cells. The protein metabolism of tau-containing mutations of frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) was also investigated using pulse-chase experiments. The results indicate attenuated proteolysis of tau in cells transfected with mutant tau genes after 48 h. Further immunocytochemical analysis and subcellular fractionation experiments revealed that the mutations did not alter the intracellular distribution of tau and suggested that impaired accessibility of tau to PSA is unlikely to account for the attenuated proteolysis of tau protein. Western blotting with phosphorylation-dependent antibodies revealed that phosphorylation levels of tau at Thr^{231}, Ser^{396}, and Ser^{409} were increased in cells transfected with V337M, R406W, and R406W mutant tau genes, respectively. Together, the data suggest that attenuated proteolysis of FTDP-17 mutant tau may be explained by increased phosphorylation levels, resulting in resistance to proteolysis.

Key words: *Tau protein, proteolysis, phosporylation, puromycin-sensitive aminopeptidase, FTDP-17.*

INTRODUCTION

The microtubule-associated protein tau is a major component of neurofibrillary tangles (NFTs) in the brains of patients with tauopathies, including Alzheimer disease (AD),[1,2] frontotemporal dementia,[3] corticobasal degeneration,[4,5] and progressive supranuclear palsy. Furthermore, the hereditary disease frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) is caused by mutations of a gene coding tau protein.[6] Therefore

one part of neurodegenerative dementia is caused by tau. Tau protein is predominantly expressed in neurons, where it is believed to play major regulatory roles in microtubule assembly for the organization and integrity of the cytoskeletal network. Tau protein can promote microtubule assembly, an activity that is regulated by phosphorylation, which impairs microtubule assembly.[7]

In tauopathies, tau protein is hyperphosphorylated, ubiquitinated, and accumulated in the form of NFTs; however, the mechaisms underlying the accumulation of tau protein remain unclear. Previous studies have examined the expression of tau mRNA and found increased tau mRNA levels in the hippocampus of AD brain.[8] In addition, the proteolysis of tau protein has been investigated and tau has been shown to be processed *in vitro* by various proteases, including calpains,[9,10] cathepsins,[11] caspases,[12] thrombin,[13] and proteasome, although the protein degradation systems responsible for tau metabolism are not well characterized.

The ubiquitin proteasome system is one of the major systems for protein quality control in eukaryotes[14,15] and neurodegenerative diseases are characterized by aggregates and inclusions of aberrant proteins. Therefore, proteasome may be one of the most important factors in the degradation of aberrant proteins. Thus, the mechanisms underlying the degradation of tau protein have been investigated using several protease inhibitors, including proteasome inhibitors. SH-SY5Y cells have been treated with MG-132 and lactacystin and tau protein levels analyzed by western blotting. After treatment of cells with MG-132 or lactacystin, tau protein levels did not increase.[16,17] These studies suggest that, in cultured cells, tau may be degraded by mechanisms other than proteasome.

The cathepsin family is one of the most prominent proteases for the degradation of aberrant proteins and works in organelles called lysosomes. Lysosomes are known to fuse with autophagic vesicles, in which aged organelles and aberrant proteins have been accumulated.[18] A dysfunction of cathepsins and autophagy has been reported to cause neurodegeneration; however, its relevance to tauopathies is unclear.[19–21]

A recent genetic screening study using *Drosophila* identified puromycin-sensitive aminopeptidase (PSA) as a potent modifier of tau-induced pathology and suggested PSA as a possible tau-degrading enzyme.[22] In addition, PSA was shown to digest recombinant human full-length tau *in vitro*, with this activity hindered by puromycin.[23] These results suggest that PSA may be involved in the proteolysis of tau protein in living cells and thus the regulation of tau protein levels.

In FTDP-17, which is caused by mutations of the tau gene, tau is hyperphosphorylated, ubiquitinated, and accumulated in diseased brains. The microtubule assembly promoting activity of mutated tau is impaired and there is an increased tendency for aggregation. The effects of the FTDP-17 mutation of tau have been shown in cells transfected with tau genes and the degradation of some mutated tau proteins is impaired.[24] However, the relevance of these mutations to proteolysis remains unclear. Therefore, in the present study, we investigated the involvement of several proteases in the proteolysis of tau protein and the effects of mutation of tau in cultured cells.

METHODS

Preparation of gene constructs

The tau gene (the longest isoform: tau441), a generous gift from Dr M. Goedert (Medical Research Council, Cambridge, UK), was cut out with *Nde*I and *Eco*RI restriction enzymes and inserted with a linker into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). The FTDP-17 mutated tau constructs (valine 337 to methionine (V337M) and arginine 406 to tryptophan (R406W)) were constructed using site-directed mutagenesis with a Quick Change kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions.

Cell lines, culture conditions, and transfections

The SH-SY5Y human neuroblastoma cells were kindly donated by Dr J. L. Biedler (Sloan Kettering Institute, New York, NY, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco BRL, Rockville, MD, USA) containing 5% fetal bovine serum (FBS; JRH Biosciences, Leneza, KS, USA). The COS-7 cells were cultured in DMEM (Gibco BRL) and 5% FBS. One day prior to transfection, the COS-7 cells were plated on dishes (10 cm diameter) and were grown overnight to obtain 90–95% confluence at the time of transfection. Cultured cells were transfected with the pcDNA3.1 vector containing wild-type tau gene (tau441) and mutant genes (V337M, and R406W) using Lipofectamine 2000 (Invitrogen) according to

the manufacturer's instructions. Briefly, cells were incubated with a complex of each DNA construct and Lipofectamine 2000 in the absence of antibiotics for 5 h; after that time, the medium was exchanged for normal growth medium.

Treatment of cell cultures with protease inhibitors

Cell cultures were treated for up to 20 h with: (i) the proteasome inhibitors lactacystin (0–10 μmol/L; Calbiochem, San Diego, CA, USA) and MG-132 (0–20 μmol/L; Sigma-Aldrich Japan, Tokyo, Japan); (ii) the cathepsin inhibitor CA-074Me (0–10 μmol/L; Calbiochem); and (iii) the PSA inhibitor puromycin (0–2 μmol/L; Sigma-Aldrich Japan). Cell viability was evaluated using the lactate dehydrogenase (LDH) cytotoxicity assay kit (Medical and Biological Laboratory, Nagoya, Japan) to identify any non-specific toxic effects of each of the drugs tested.

Treatment of cell cultures with short interference RNA

SH-SY5Y cells were cultured in DMEM/F12 (Gibco BRL). One day prior to transfection, cells were plated into six-well plates with 2 mL growth medium in the absence of antibiotics and were cultured to 30–50% confluence at the time of transfection. For short interference (si) RNA treatment of cells, 200 pmol siRNA oligomer was incubated with 250 μL Opti-MEM I Reduced Serum Medium (Gibco BRL) at room temperature for 5 min before the oligomer was incubated for a further 20 min at room temperature with Lipofectamine 2000. The oligomer–Lipofectamine 2000 complexes were added to each of the wells containing cells and medium and cells were incubated in the presence of the oligomer for 36 h at 37°C in a CO_2 incubator until harvesting.

Western blotting

Cells were lysed with RIPA buffer containing 50 mmol/L Tris-HCl, pH 8, 150 mmol/L NaCl, 20 mmol/L EDTA, 1% v/v Nonidet-P40, 50 mmol/L natrium fluoride, 20 mmol/L *N*-ethyl maleimide, and 100 mmol/L sodium orthovanadate (Sigma-Aldrich), supplemented with protease inhibitor cocktail (Calbiochem) and phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich Japan), on ice for 1 h. The lysates were centrifuged for 30 min at 12 000 *g* to remove cellular debris, and protein concentrations in the supernatant were determined using the BCA Protein Assay Kit (Pierce, Cheshire, UK). Equal amounts of protein were applied to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Blots were blocked with 5% (w/v) ECL blocking agent (GE Healthcare, Uppsala, Sweden) in TBS-T (50 mmol/L Tris HCl, pH 7.4, 150 mmol/L NaCl, 0.5% Tween-20). Membranes were probed overnight at 4°C with: (i) anti-tau phosphorylation-independent monoclonal antibody Tau-5 (Calbiochem; 1:1000 dilution); (ii) polyclonal antibody Tau(H-150) (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1000 dilution); (iii) anti-tau phosphorylation-dependent antibodies PT231, PS396, and PS409 (Biosource, Nivelles, Belgium), which recognize phosphorylated threonine 231, serine 396, and serine 409 on tau, respectively; and (iv) anti-PSA goat polyclonal antibody PSAP(N-20) (Santa Cruz). Then, peroxidase-labeled anti-mouse, anti-rabbit, and anti-goat IgG antibodies were applied as secondary antibodies. The reaction products were visualized using an ECL kit (Amersham Biosciences, Buckinghamshire, UK).

[^{35}S]-Methionine pulse-chase experiments

Cells were rinsed in phosphate-buffered saline (PBS) and incubated for 1 h in 4 mL Met-free medium (Gibco) for starvation. Afterward, cells were rinsed with PBS and incubated for 3 h in 5 mL Met-free DMEM containing 1.5 MBq of [^{35}S]-methionine-*trans*-labeled methionine (EXPRE35S 35S Protein Labeling Mix; Perkin Elmer Japan, Kanagawa, Japan). Cells were then rinsed twice with 2 mL PBS and incubated for chase intervals of 0 and 24 h in 5 mL non-radioactive growth medium supplemented with 5% FBS containing each chemical reagent. Cells lysates were harvested with RIPA buffer and precipitated with Tau-5 monoclonal antibody overnight at 4°C. The antibody–antigen complex was extracted from the lysate by incubating with Protein G–Sepharose (GE Healthcare) for 1 h at 4°C, followed by centrifugation for 1 min at 12 000 *g*. Protein G–Sepharose pellets were suspended in lysis buffer and washed six times with lysis buffer and once with washing buffer (50 mmol/L Tris, pH 8.0). The samples were then electrophoresed in 10% Tris-Glycine gel (Invitrogen) and vacuum dried. Radiolabeled tau bands were visualized and quantified densitometrically.

Subcellular fractionation

Subcellular fractionation was performed using the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem) according to the manufacturer's instructions. Briefly, cells were carefully washed twice with cold washing buffer before buffer I (supplied in the kit) with Protease Inhibitor Cocktail was added to the cells and the samples were rotated in 10 r.p.m. for 10 min at 4°C. Samples were centrifuged at 500 *g* for 10 min and the supernatant was collected as Fraction I, whereas the pellets were subjected to further fractionation. Similarly, the Fractions II and III were obtained by using buffers II and III (supplied in the kit), respectively. Fractions I, II, and III correspond to the cytosol, membrane/organelle, and nuclear fractions, respectively.

Immunocytochemistry

Tau-transfected COS-7 cells were plated onto Lab-Tek plates (Nunc, Roskilde, Denmark). Cells were rinsed with PBS and were fixed with ice-cold methanol for 5 min. After fixation, cells were rinsed with 2 mL PBS three times and were then treated with 5% bovine serum albumin (BSA)/TBS-T for 10 min before being probed with the Tau-5 (1:100 dilution) and PSAP(N-20) (1:100 dilution) antibodies overnight at 4°C. Cells were washed in 2% BSA/TBS-T three times before the secondary antibodies fluorescein isothiocyanate (FITC) anti-mouse IgG (Cappel, Philadelphia, PA, USA) and Alexa FluorR 633 Rabbit Anti-goat IgG (Molecular Probes, Eugene, OR, USA; 1:1000 dilution) were applied for 30 min at 37°C. After plates had been washed with PBS at least five times, cells were observed under confocal microscopy.

RESULTS

To evaluate the involvement of proteases in the metabolism of tau protein, different protease inhibitors were applied to SH-SY5Y human neuroblastoma cells and COS-7 cells transfected with the tau gene. First, cell viability was determined and essentially no cytotoxic effects were observed when SH-SY5Y human neuroblastoma cells were treated with the proteasome inhibitors lactacystin (0–10 μmol/L) and MG-132 (0–20 μmol/L), the cathepsin inhibitor CA-074Me (0–1 μmol/L), and the PSA inhibitor puromycin (0–2 μmol/L) for up to 20 h (data not shown). Although these conditions have been reported to be effective in reducing each of the protease activities,[25–28] no significant changes were seen in tau protein levels (data not shown). The effects of these same protease inhibitors were also evaluated in COS-7 cells transfected with the tau gene (tau441). Essentially, no cytotoxic effects were observed when COS-7 cells were treated with lactacystin (0–10 μmol/L), CA-074Me (0–10 μmol/L), or puromycin (0–2 μmol/L) for up to 20 h (data not shown). Western blot analysis showed no significant changes in tau protein levels (n = 3; Fig. 1a–c). These data suggest that tau protein is not catalysed predominantly by one of those proteases and that it is likely to be catalysed by other proteases.

To investigate the involvement of those proteases in the catalysis of tau further, [^{35}S]-methionine pulse-chase experiments were performed to detect subtle changes in tau protein levels. These pulse-chase experiments showed that tau protein in SH-SY5Y cells was degraded gradually and only 30–40% of tau protein remained after 24 h. When SH-SY5Y cells were treated with lactacystin (0–8 μmol/L), MG-132 (0–15 μmol/L), and CA-074Me (0–1 μmol/L), there were no significant changes in tau protein levels (n = 4) (Fig. 2a–c); however, significant increases were observed in tau protein levels when SH-SY5Y cells were treated with puromycin (200 nmol/L; n = 4; Fig. 2d). Increased tau protein level were not observed in cells treated with much higher concentrations of puromycin in this pulse-chase experiment; we assumed that this was due to an inhibitory effect of puromicin against ribosome-attenuated protein synthesis, which interfered with the effects of puromycin on tau protein levels.

On the basis of these results, experiments were performed investigating the inhibition of PSA expression using siRNA against PSA. In these experiments, increased tau protein levels were observed in SH-SY5Y cells treated with siRNA to PSA, but not in cells treated with siRNA against glyceroaldehyde 3-phosphate dehydrogenase (GAPDH) as an experimental control (Fig. 3). These data suggest that PSA is a protease that catalyses tau protein predominantly in SH-SY5Y cells.

Based on the results obtained in the siRNA experiments, we further investigated the protein metabolism of tau with FTDP-17 mutations using pulse-chase experiments. In these experiments, COS-7 cells were

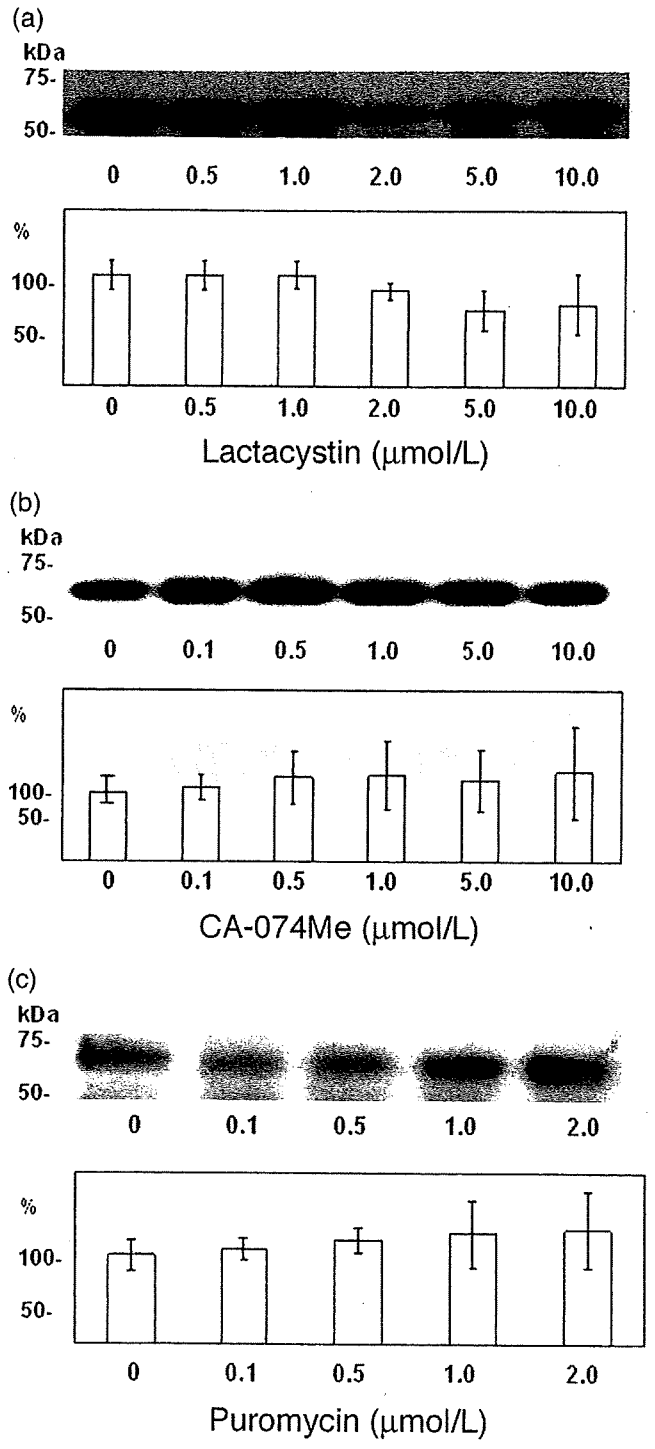

Figure 1 Effects of protease inhibitors on proteolysis of tau in COS-7 cells transfected with a tau gene. Wild-type tau (tau441) was transfected into COS-7 cells before cells were treated with different concentrations of (a) lactacystin, (b) CA-074Me, and (c) puromycin for 20 h, as indicated. Tau protein levels were examined by western blotting using Tau-5, a phosphorylation-independent antibody, before band intesities were determined densitometrically. Data are the mean ± SEM of three independent experiments. Tau levels in control cells were set at 100%, and levels under other conditions were normalized accordingly. No significant changes were seen in tau protein levels.

transfected with wild-type and mutant type (V337M and R406W) tau genes and tau protein levels were chased up to 48 h. No changes were observed in cells transfected with these tau genes after 24 h; however, attenuated proteolysis of tau was observed in cells transfected with the mutant tau genes after 48 h (n = 4; Fig. 4).

To clarify the effects of FTDP-17 mutations on the topological relevance of tau and PSA, immunocytochemistry and subcellular fractionation were performed to investigate the accessibility of tau to the PSA. In COS-7 cells transfected with tau genes, immunocytochemical studies showed that tau was located in the cytoplasm and nucleus, presumably because tau is overexpressed by the transfection (Fig. 5a), whereas PSA was located only in the cytoplasm (Fig. 5b). Tau protein and PSA were colocalized in the cytoplasm (Fig. 5c). No changes were observed in the localization of tau in cells transfected with the wild-type and mutant tau genes. Furthermore, subcellular fractionation revealed that tau was found predominantly in Fraction I (cytoplasm) and that faint tau was observed in Fraction III (nucleus; Fig. 5d). In contrast, PSA was observed only in Fraction I (cytoplasm; Fig. 5e). There were no changes in the intracellular distribution of tau and PSA among cells transfected with wild-type and mutant tau genes. These results suggest that attenuated proteolysis of FTDP-17 mutant tau is not explained by the accessibility of tau to PSA.

Finally, phosphorylation levels of tau protein were investigated in cells transfected with tau genes. First, tau expression levels were normalized using western blots stained with Tau-5, a phosphorylation-independent antibody, to ensure equal levels of protein loading. Three antibodies were used and, compared with results obtained for wild-type tau, significant increases were observed in the phosphorylation of tau at Thr^{231}, Ser^{396}, and Ser^{409} in cells transfected with V337M and R406W mutant tau genes (n = 4; Fig. 6a–c). These results suggest that attenuated proteolysis of FTDP-17 mutant tau may be

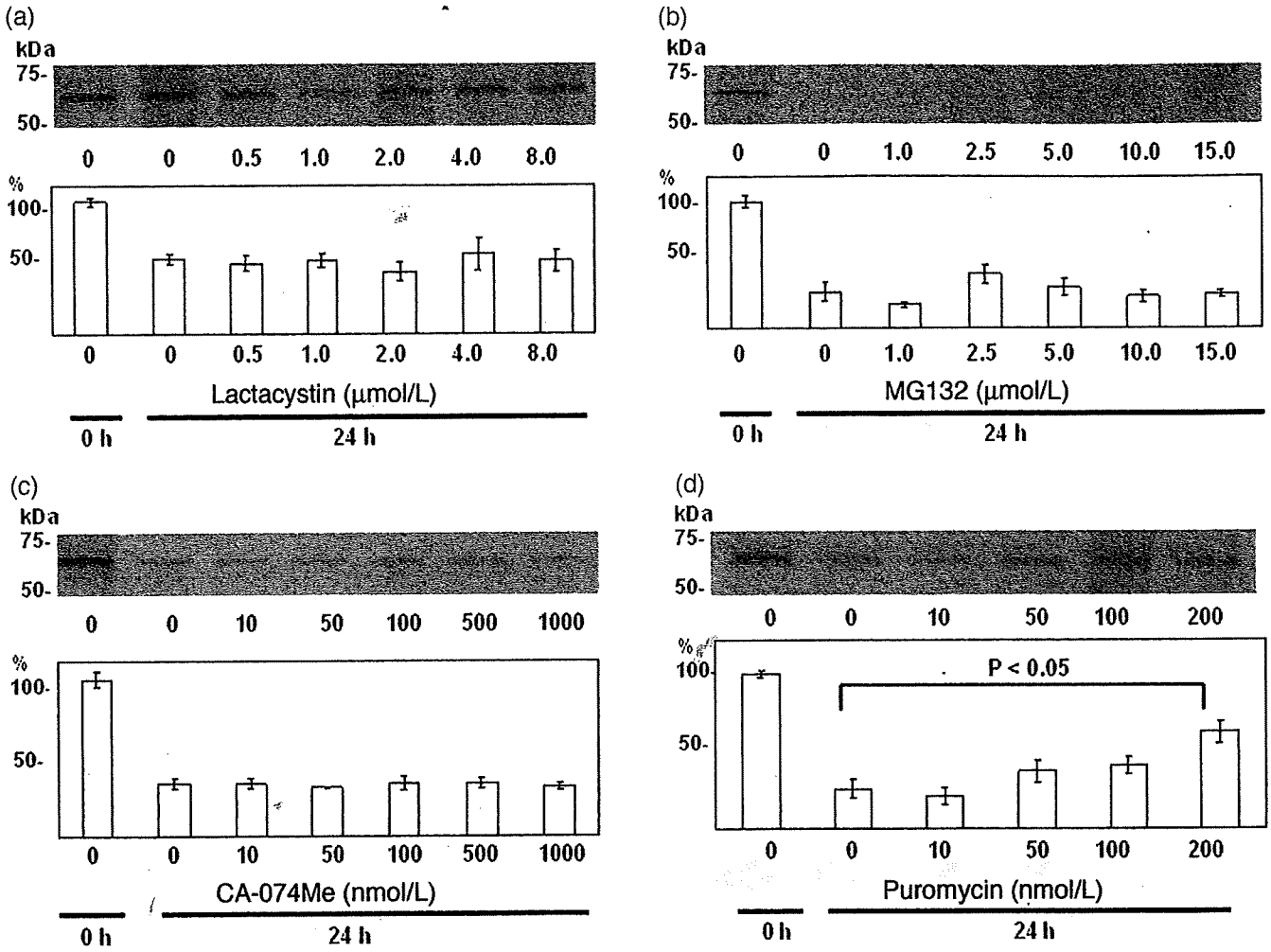

Figure 2 Pulse-chase analysis of the effects of protease inhibitors on the proteolysis of tau in SH-SY5Y cells. SH-SY5Y human neuroblastoma cells were labeled with [^{35}S]-methionine for 3 h before being incubated in unlabeled chase medium for intervals up to 24 h. Cells were harvested at the times indicated. The amount of residual ^{35}S-labeled tau protein in SH-SY5Y cells was normalized against that present immediately after pulse labeling (0 h), taken to be 100%. Data are the mean ± SEM of four independent experiments. Significant increases in tau protein were seen following treatment with 200 nmol/L puromycin (d), but not after treatment with lactacystin (a), MG132 (b), or CA-074 (c).

explained by increased phosphorylation levels resulting in resistance to proteolysis.

DISCUSSION

In tauopathies, tau protein is hyperphosphorylated, ubiquitinated, and accumulated in the form of NFTs; however, the mechaisms underlying this accumulation remain unclear. In the present study, the involvement of several proteases in tau protein metabolism was investigated, and only PSA was found to be a protease that predominantly regulates tau protein levels in cultured cells. This is the first study to report that the effect of inhibition of PSA by puromycin in an increase of tau protein in cultured cells.

Previously, the involvement of proteasomes in tau degradation has been investigated. Feuillette *et al.* reported that, in SH-SY5Y cells treated with different proteasome inhibitors, including MG132 and lactacystin, instead of an increase in tau protein, an unexpected decrease in tau protein levels was observed.[16] These authors also used mutant alleles of the 20S proteasome in *Drosophila* and found that genetic inactivation of the proteasome resulted in a decrease of tau levels in *Drosophila*. Delobel *et al.* have

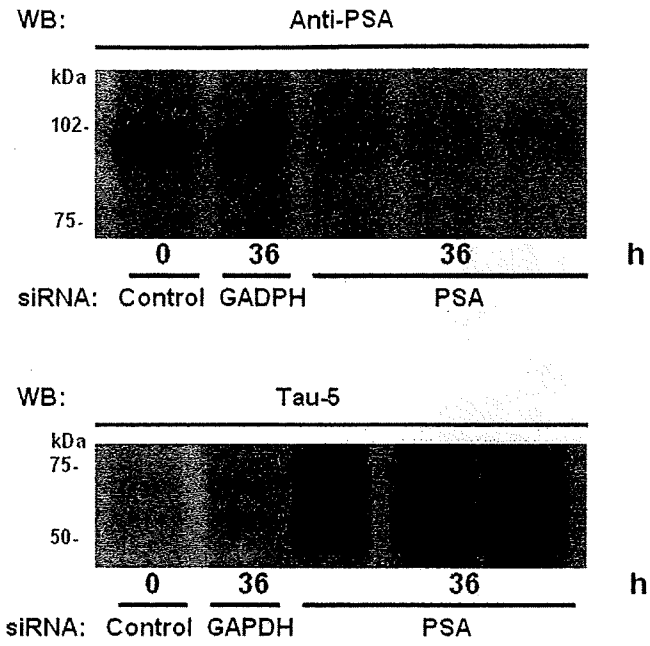

Figure 3 Effects of short interference (si) RNA against puromycin-sessitive aminopeptidase (PSA) on tau in SH-SY5Y cells. Oligomers of siRNA to PSA and glyceroaldehyde 3-phosphate dehydrogenase (GAPDH), as an internal control, were used. The oligomer–Lipofectamine 2000 complexes were added to cells and cells were incubated at 37°C in a CO_2 incubator for 36 h. Western blot (WB) analysis revealed that PSA expression was decreased by siRNA to PSA and that tau expression increased comaprd with control. The siRNA to GAPDH had no significant effect.

reported that inhibition of the proteasome leads to a decrease in tau levels via an increase in calpain levels, which result in accelerated degradation of tau protein.[17] The results of the present study indicate no change in tau levels in cells treated with 0–15 μmol/L MG132; this discrepancy may be explained by the culture conditions or the concentration of the inhibitors used. Both studies used MG132 at low concentrations of 0–0.5 μmol/L and decreases in tau following inhibition of the proteasome may be observed under limited conditions. However, the interaction between several proteases should be taken into consideration. Cathepsin is a plauseible candidates for the degradation of tau protein; however, in the present study, no changes were observed in tau levels in cells treated with a cathepsin inhibitor. This enzyme is known to react with substrates in the lysosome and, in normal cell cultures, tau may not be degraded via incorporation into lysosome or autophagic vesicles.

Karsten *et al.* first reported PSA as a tau-degrading enzyme following genomic screening using *Droso-*

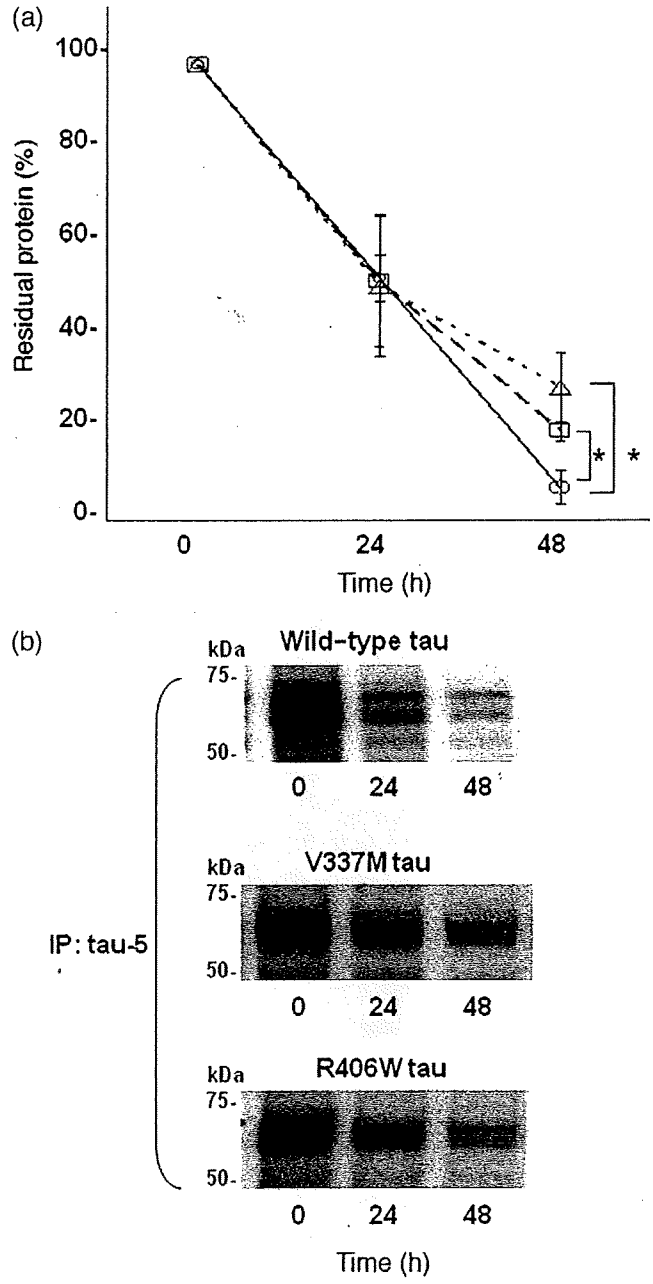

Figure 4 Effects of the frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) mutation on proteolysis of tau in COS-7 cells transfected with tau genes. Pulse-chase experiments were performed in COS-7cells transfected with wild-type (○) or the mutant V337M (△) and R406W (□) tau. Cells were labeled with [^{35}S]-methionine for 3 h, were incubated in unlabeled chase medium for intervals up to 48 h, and were harvested at 0, 24, and 48 h. The amount of residual ^{35}S-labeled wild-type, V337M and R406W mutated tau proteins in COS-7 cells is normalized against the amount of label measured immediately after pulse labeling (0 h), taken as 100%. Data are the mean ± SEM of four independent experiments. Attenuated proteolysis of tau was observed in the cells transfected with the mutant tau (V337M and R406W) genes after 48 h ($P < 0.05$). IP, immunoprecipitation.

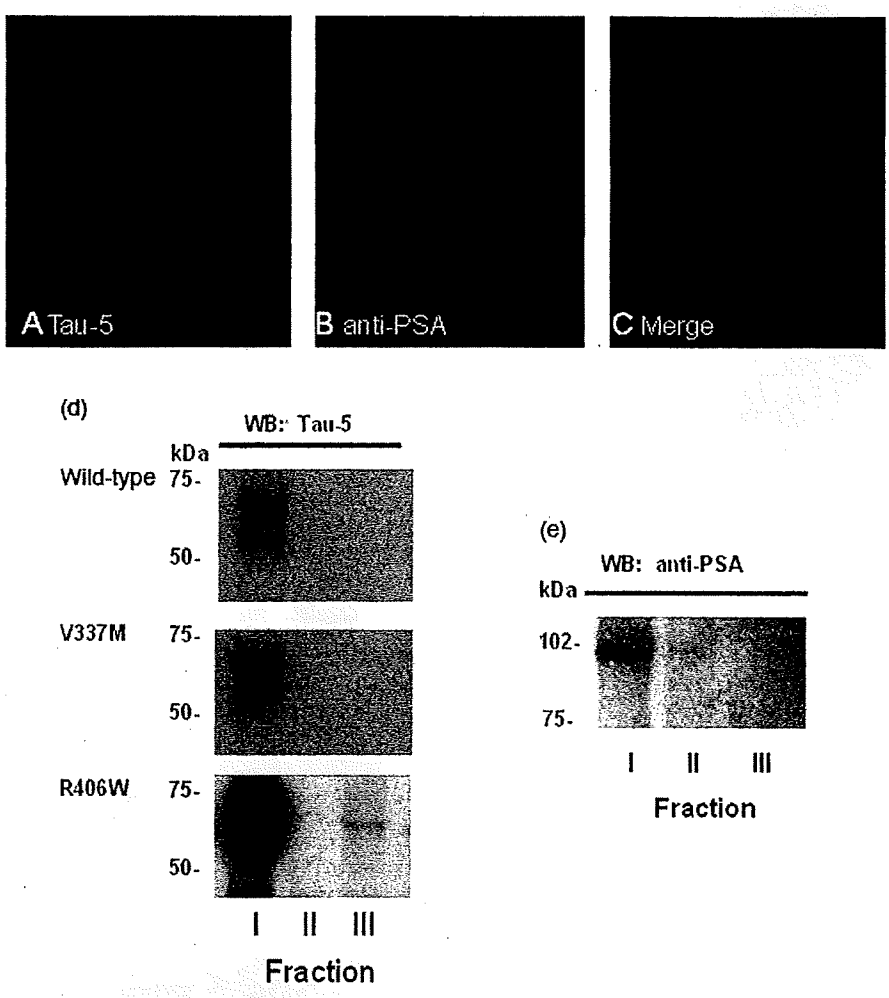

Figure 5 Intracellular localization of tau and puromucin-senstitive aminopetidase (PSA) in COS-7 cells transfected with tau genes. In COS-7 cells transfected with tau genes, tau and PSA were stained immunocytochemically. When COS-7 cells were transfected with wild-type tau (tau441), tau was observed in the cytoplasm and nucleus (a), whereas PSA was observed only in the cytoplasm (b). Tau protein and PSA were colocalized in the cytoplasm (c). When COS-7 cells were transfected with mutant type tau (V337M and R406W), similar results were observed. Subcellular fractionation was performed, with Fractions I, II, and III corresponding to cytosolic, membrane/membranous organelle, and nuclear fractions, respectively. (d) Tau protein was found predominantly in Fraction I (cytoplasm) and faintly in Fraction III (nucleus). (e) In contrast, PSA was observed only in Fraction I (cytoplasm). No change in the intracellular distribution was observed between cells transfected with wild-type and mutant tau genes.

phila.[22] In that study, the authors used a cross-species functional genomic approach to analyze gene expression in multiple brain regions in the mouse in parallel with validation in *Drosophila* to identify tau modifiers. They reported that PSA protected against tau-induced neurodegeneration *in vivo*. Furthermore, Senguputa *et al.* reported that tau is degraded by PSA *in vitro*;[23] therefore, it is likely that PSA is involved in the metabolism of tau protein in living cells. However, the effects of PSA inhibition in cultured cells had not been examined. In the present study, we showed that tau levels were increased in cells treated with 200 nmol/L puromycin, but that higher concentrations of puromycin were not effective because of inhibition of protein synthesis. The effect of PSA inhibition on tau protein levels was also confirmed by siRNA leading to decreased expression of PSA. Therefore, our results suggest that PSA functions as the predominant regulator of tau protein levels in normally cultured cells.

Next, we tried to evaluate the delayed proteolysis of FTDP-17 mutated tau in COS-7 cells and studied the topological relevance of the mutated tau and PSA. We found attenuated tau proteolysis in cells transfected with the mutant tau (V337M and R406W) genes after 48 h compared with wild-type tau. However, the mutation did not result in differences in the intracellular distribution of tau and impaired accessibilities of tau to PSA is unlikely. Therefore, phosphorylation levels of tau protein were examined and both mutations resulted in increased phosphorylation of tau protein at at least one Ser/Thr site. Previously, it was repoted that phosphorylation induced resistance of tau to proteolysis[29] and that FTDP-17 mutations induced hyperphosphorylation of tau in certain cells.[30] These changes may explain the delayed degradation of tau protein in mutant tau-transfected cells. Hyperphosphorylation of tau could induce breakdown of microtubules,[31] and this may be one of the causes of

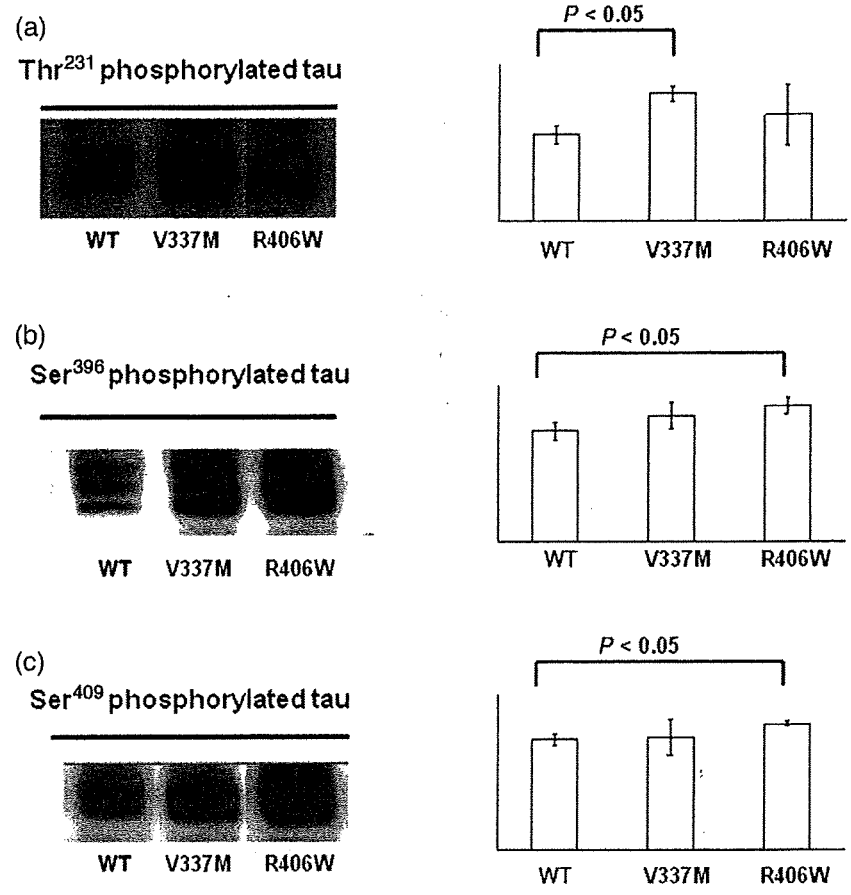

Figure 6 Effects of the frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) mutation on phosphorylation of tau in COS-7 cells transfected with tau genes. Phosphorylation levels of tau protein were studied in cells transfected with wild-type (WT) tau and mutant tau (V337M and R406W). To compare the phosphorylation levels of tau protein, variations in expression levels of tau protein in each culture were normalized by western blotting with tau-5, a phosphorylation-independent antibody, and the same amounts of tau protein were applied to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Three phosphorylation-dependent antibodies were used and the levels of mutant tau were compared against those of WT tau. (a) Phosphorylated tau at Thr^{231} was significantly increased in V337M mutant tau ($P < 0.05$); (b) phosphorylated tau at Ser^{396} was significantly increased in R406W mutant tau ($P < 0.05$); and (c) phosphorylated tau at Ser^{409} was significantly increased in R406W mutant tau ($P < 0.05$).

neuronal cell death in these neurodegenerative diseases. Further studies on the effects of phosphorylation and the FTDP-17 mutation on direct proteolysis of tau by PSA are required.

Conclusions

The protein metabolism of tau was investigated using several protease inhibitors and, in the present study, PSA was found to be the predominant regulator of tau protein levels in normally cultured cells. The FTDP-17 mutation delayed the proteolysis of tau and increased the phosphorylation of tau. Both tau and PSA are colocalized in the cytoplasm and PSA may be involved in the mechanism underlyng the delayed proteolysis of tau seen with the mutations.

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Differential interaction and aggregation of 3-repeat and 4-repeat tau isoforms with 14-3-3ζ protein

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ABSTRACT

Tau isoforms, 3-repeat (3R) and 4-repeat tau (4R), are differentially involved in neuronal development and in several tauopathies. 14-3-3 protein binds to tau and 14-3-3/tau association has been found both in the development and in tauopathies. To understand the role of 14-3-3 in the differential regulation of tau isoforms, we have performed studies on the interaction and aggregation of 3R-tau and 4R-tau, either phosphorylated or unphosphorylated, with 14-3-3ζ. We show by surface plasmon resonance studies that the interaction between unphosphorylated 3R-tau and 14-3-3ζ is ~3-folds higher than that between unphosphorylated 4R-tau and 14-3-3ζ. Phosphorylation of tau by protein kinase A (PKA) increases the affinity of both 3R- and 4R-tau for 14-3-3ζ to a similar level. An *in vitro* aggregation assay employing both transmission electron microscopy and fluorescence spectroscopy revealed the aggregation of unphosphorylated 4R-tau to be significantly higher than that of unphosphorylated 3R-tau following the induction of 14-3-3ζ. The filaments formed from 3R- and 4R-tau were almost similar in morphology. In contrast, the aggregation of both 3R- and 4R-tau was reduced to a similar low level after phosphorylation with PKA. Taken together, these results suggest that 14-3-3ζ exhibits a similar role for tau isoforms after PKA-phosphorylation, but a differential role for unphosphorylated tau. The significant aggregation of 4R-tau by 14-3-3ζ suggests that 14-3-3 may act as an inducer in the generation of 4R-tau-predominant neurofibrillary tangles in tauopathies.

Introduction

In neurodegenerative diseases with dementia, like Alzheimer's disease (AD), abnormal accumulation of aggregative products, i.e., amyloid beta and tau protein, has been characterized thoroughly. However, the mechanisms of pathological processes are still unclear [1,2]. Tau is a microtubule-associated protein expressed predominantly in neurons, where its major known biological function is to stimulate microtubule (MT) assembly and to stabilize the MT network. Tau comprises a family of six isoforms generated by alternative mRNA splicing from a single gene [3,4]. They fall into two groups, one of which contains four C-terminal imperfect repeat domains and the other, three such repeat domains. Phosphorylation [5] and expression of the different isoforms [6] are two important mechanisms by which tau regulate microtubule polymerization and stabilization of microtubules during development. Whereas in the fetal brain, tau is phosphorylated at multiple sites and only 3R-tau is expressed, in adults tau is phosphorylated at only a few sites and both 3R- and 4R-tau are expressed in almost equal proportion. *In vitro* studies have shown that phosphorylated tau and 3R-tau isoforms bind and stabilize the microtubule more weakly than unphosphorylated tau and 4R-tau isoforms [7], thus indicating that the regulation of phosphorylation and expression of tau isoforms is required for microtubule dynamics during development. Despite its physiological role, tau is central to the pathogenesis of tauopathies as it becomes abnormally hyperphosphorylated and aggregated in these diseases. The predominant aggregation of either 3R- or 4R-tau is another characteristic to certain tauopathies [8]. For example, in Pick's disease (PiD) 3R tau is predominantly accumulated, but in progressive supranuclear palsy (PSP) and in frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), accumulation of 4R-tau is predominant. Genetic mutations in tau alter the 3R/4R tau ratio in FTDP-17 due to splicing of exon 10 [6]. However, in other tauopathies such as PiD, PSP and corticobasal degeneration (CBD), where no mutations have been reported, the exact causes and mechanisms leading to the altered 3R-/4R-tau ratios remain elusive.

Tau is a very soluble protein and does not assemble readily *in vitro* even at high concentrations. Increasing the levels of tau in both animal and cell culture models through transgenic expression of wild-type human tau at concentrations that saturate the endogenous MT also failed to result in robust tau assembly [9]. However, cofactors, also called exogenous inducers, such as

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heparin, RNA, glycosaminoglycans, etc. have been found associated with neurofibrillary tangles (NFTs) and promote the aggregation of tau independently of phosphorylation [10–12]. Therefore, it seems reasonable to consider that the assembly of tau to form inclusions *in vivo* also requires the presence of cofactors that might be specific in certain tauopathies and may differentially induce tau aggregation. The identification of such cofactors may provide new insights into the pathogenesis of tauopathy.

14-3-3 is a family of highly conserved abundant regulatory proteins found in all eukaryotes and involved in many intracellular processes such as cell cycle control, apoptosis, signal transduction cascades and cytoskeletal reorganization [13]. It is a phosphoprotein binding protein that generally binds to its partner in a phosphorylation dependent manner. Tau is a phosphoprotein and we have recently reported that 14-3-3ζ binds to tau regulated by phosphorylation of tau at Ser214 by PKA [14]. The binding is of high affinity which greatly reduces tau aggregation. Since tau is highly phosphorylated at Ser214 and its complex with 14-3-3ζ is found at greater levels in the fetal brain, the interaction of tau with 14-3-3 may underlie the reorganization of the microtubule cytoskeleton during development. Despite a possible role in development, 14-3-3 has been found to be associated with neurofibrillary tangles (NFTs) in several tauopathies, including AD, PiD [15–17], and able to interfere with the steps of tau pathology. It modulates phosphorylation of tau at sites that are hyperphosphorylated in AD [18] and promotes aggregation of tau into filaments by binding to its repeat domain independently of phosphorylation [19]. However, the role of 14-3-3 in normal and abnormal tau action is only incompletely understood.

As 3R- and 4R-tau isoforms are differentially involved in neuronal development and in tauopathies and as 14-3-3 proteins are found to be associated with tau in both of the physiological and pathological conditions, investigating the interaction and aggregation of tau isoforms with 14-3-3 may provide further understanding of the possible role of 14-3-3 in the physiological and pathological tau action.

Materials and methods

Antibodies and Western blotting. Tau-5 monoclonal antibody, which reacts equally with both the phosphorylated and nonphosphorylated tau, was purchased from BioSource International (Camarillo, CA, USA). CP-3 antibody, which recognizes phosphorylated Ser214 was a gift from Dr. Peter Davis (Albert Einstein University, New York, NY, USA). Western blotting was performed as previously described [14].

Protein kinases. The catalytic subunits of protein kinase A (PKA) were purchased from Sigma (Saint Louis, MI, USA). One unit of PKA was defined as the amount of enzyme which catalyzed the incorporation of 1 pmol of phosphate into the synthetic peptide LRRASLG in 1 min.

Recombinant proteins. Recombinant 14-3-3ζ was prepared following the method described earlier [18]. Human tau with 3- or 4-repeats (411- or 441-residue isoforms, Fig. 1) were subcloned into pET-22b for bacterial expression and transformed into *E. coli* BL21 (DE3) for expression of the histidine-tagged protein. The protein was purified as described previously [14] and purity was assessed on sodium dodecyl sulphate–polyacrylamide gel (SDS–PAGE, 10%) stained with Coomassie brilliant blue (CBB).

In vitro phosphorylation of tau. Phosphorylation was carried out by incubating tau (0.2 mg/ml) at 30 °C for 6 h in a reaction mixture containing 50 mM Tris–HCl (pH 7.4), 1 mM DTT, 10 mM $MgCl_2$, and

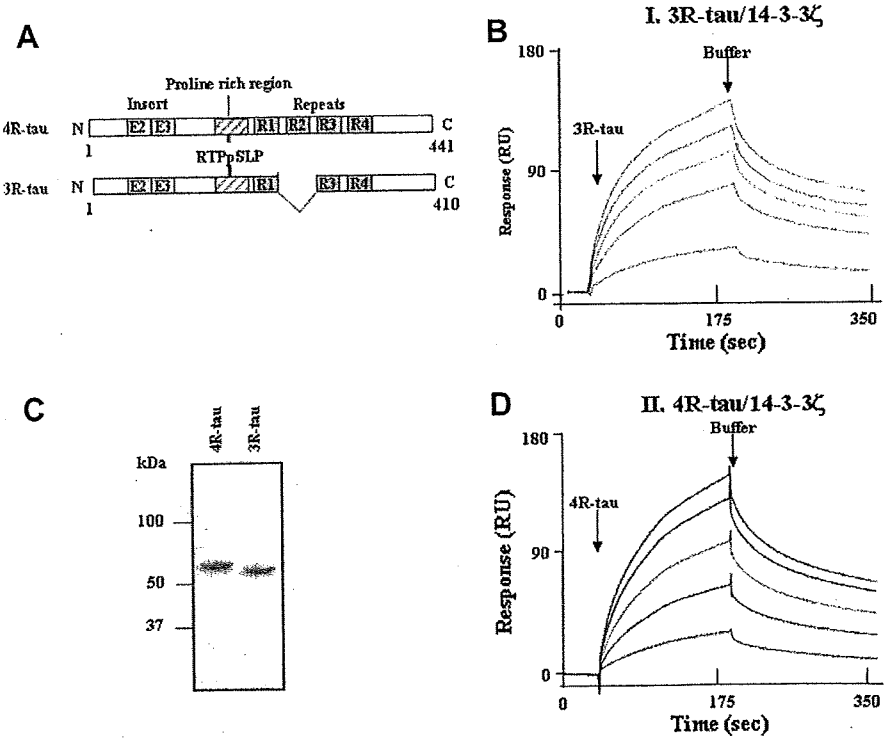

Fig. 1. 14-3-3ζ binds to unphosphorylated 3R-tau with higher affinity than unphosphorylated 4R-tau. (A) A schematic diagram of the longest human 4R- and 3R-tau isoforms employed. The amino acid residues numbers are according to tau441. 4R-tau contains two alternatively spliced inserts near the N-terminus (E2 and E3) and 4-repeats (~31 residues each, R1–R4) in the C-terminal half. 3R-tau differs from 4R-tau only by the absence of R2, due to alternative splicing of exon 10. The positions of the phosphorylation-dependent binding motif of 14-3-3, 211-RTPpSLP-216 in 4R- and 3R-tau, are highlighted. (B) CBB-stained SDS–polyacrylamide gel (10%) of purified recombinant 4R- and 3R-tau proteins. (C) Overlay of SPR sensograms resulting from the injection of 50, 100, 150, 200, and 250 nM (from bottom to top) unphosphorylated tau over immobilized 14-3-3ζ. I, 3R-tau; II, 4R-tau. The arrows represented by 3R- or 4R-tau and buffer indicated the start of the association and dissociation curves, respectively.

Table 1
Kinetic constants for the interaction between tau and 14-3-3ζ obtained by SPR.

	k_a (M^{-1} s^{-1})	k_d (s^{-1})	k_A (M^{-1})	K_D (M)
4R-tau	$3.66 \pm 0.17 \times 10^3$	$1.17 \pm 0.16 \times 10^{-3}$	$3.07 \pm 0.32 \times 10^6$	$3.25 \pm 0.44 \times 10^{-7}$
3R-tau	$2.40 \pm 0.38 \times 10^4$	$2.68 \pm 0.33 \times 10^{-3}$	$0.90 \pm 0.06 \times 10^7$	$1.13 \pm 0.06 \times 10^{-7}$
PKA-phospho 4R-tau	$6.27 \pm 0.30 \times 10^4$	$1.93 \pm 0.28 \times 10^{-3}$	$3.30 \pm 0.61 \times 10^7$	$2.91 \pm 0.61 \times 10^{-8}$
PKA-phospho 3R-tau	$5.86 \pm 0.08 \times 10^4$	$1.26 \pm 0.16 \times 10^{-3}$	$4.63 \pm 0.34 \times 10^7$	$2.16 \pm 0.34 \times 10^{-8}$

2 mM ATP with 100 U/ml of PKA. The reactions were stopped by boiling for 5 min and heat stable tau protein was removed from the denatured kinases by centrifugation (10,000g for 10 min).

Surface plasmon resonance (SPR) studies of 14-3-3ζ and tau. The affinity of unphosphorylated or phosphorylated tau with 14-3-3ζ was measured by surface plasmon resonance (SPR) spectroscopy using a Biacore 2000 (Biacore, Inc., Uppsala, Sweden) as described [14]. In brief, 14-3-3ζ was immobilized to the desired level on one flow cell (FC2) of a CM5 sensor chip by primary amine coupling, according to the manufacturer's instructions. A blank surface (FC1) was made by ethanolamine deactivation of the activated dextran surface. Purified tau, unphosphorylated tau or phosphorylated, at various concentrations (50–250 nM) was injected over the flow cells (FC1 and FC2) at a rate of 40 μl/min and the bound analytes were removed by washing with buffer after the injection. The first flow cell (FC1) was the minus tau protein control channel and was subtracted from the sample channel (FC2) during the run. The bulk refractive index contributions were therefore expected to be zero or not significant in the reference subtracted sensograms. To correct for nonspecific binding, blank runs were performed with HBS-EP buffer on both surfaces before and after each binding analysis and this value was subtracted prior to the kinetic analysis. Equilibrium association and dissociation rate constants were calculated using the Langmuir (1:1) binding model with the BIA evaluation 2.1 software supplied by the manufacturer.

In vitro aggregation and fluorescence spectroscopy. Aggregation was induced by incubating tau at a concentration of 20 μM in 50 mM Tris–HCl (pH 7.4) buffer, 150 mM NaCl and 4 mM DTT at 37 °C for different time periods, and mixing it with 14-3-3ζ in an equimolar ratio. At each time point, the aggregation of tau was monitored by measuring the fluorescence of Thioflavine S (ThS) using a spectrofluorometer (Perkin-Elmer Japan, Kanagawa, Japan) with an excitation filter of 430 nm and an emission filter of 520 nm. Measurements were carried out at room temperature in Tris–HCl buffer with 10 μM ThS and typically done in triplicate. Background fluorescence and light scattering of the sample without ThS was subtracted when needed. Curves show average values.

Electron microscopy. Polymerization samples were applied to a 300 mesh carbon-coated grid and negatively stained with 2% (w/v) uranyl acetate as described earlier [14]. Grids were analyzed using a JEOL JEM-1220 EM instrument at 50 kV.

Results and discussion

Alternative splicing of exon 10 gives rise to two major types of tau isoforms, 3R- and 4R-tau that differ by the absence or presence of the R2 repeat, respectively [6]. They show key differences in binding with their partners as well as their biological functions. 14-3-3 binds to the repeat domain of tau independently of phosphorylation [14,18]; however, the extent of binding with the different tau isoforms is unknown. To determine whether 14-3-3 exhibits any differences in interaction with tau isoforms independently of phosphorylation, we used the 4R- and 3R-tau with full N-terminal insertions (Fig. 1) and assessed their interaction with 14-3-3ζ by surface plasmon resonance (SPR) spectroscopy, using a Biacore 2000 as described earlier [14]. Real time interaction between unphosphorylated tau and 14-3-3ζ was measured at 25 °C by injecting tau onto a sensor chip with immobilized 14-3-3ζ.

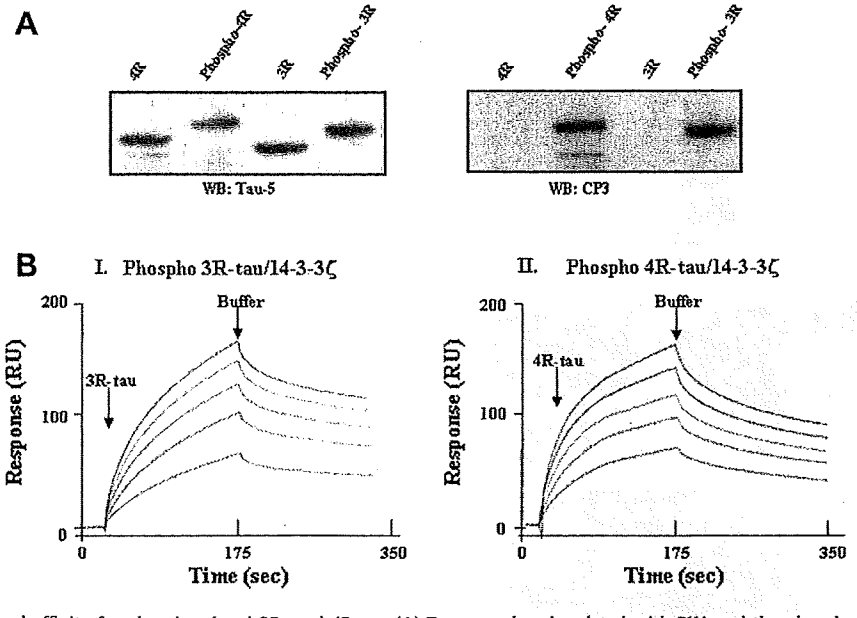

Fig. 2. 14-3-3ζ shows almost equal affinity for phosphorylated 3R- and 4R-tau. (A) Tau was phosphorylated with PKA and the phosphorylation sites were analyzed by Western blotting with the indicated phosphorylation-independent and -dependent antibodies. B, An overlay of SPR sensograms resulting from the injection of 50, 100, 150, 200, and 250 nM (from bottom to top) tau over immobilized 14-3-3ζ. I, PKA-phosphorylated 3R-tau; II, PKA-phosphorylated 4R-tau. The arrows represented by 3R- or 4R-tau and buffer indicated the start of the association and dissociation curves, respectively.

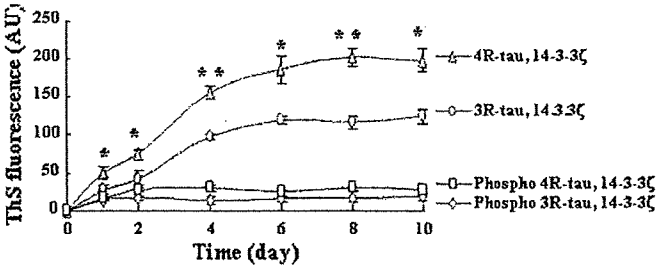

Fig. 3. Unphosphorylated 4R-tau is assembled more readily than unphosphorylated 3R-tau by the induction of 14-3-3ζ in vitro. The rate of aggregation of unphosphorylated 4R-tau (△) and 3R-tau (○) or phosphorylated 4R-tau (□) and 3R-tau (◇) were measured by ThS fluorescence. The intensity of ThS (AU) is plotted as a function of induction time. The values are means ± SD, $n = 3$, from a single microplate. $^{*}P < 0.05$, $^{**}P < 0.01$ when compared with 3R-tau. Data were analyzed by Student's t-test.

The binding of 14-3-3ζ to tau caused an increase in mass at the surface of the chip which was reflected by an increase in SPR response over time (Fig. 1). Different concentrations of tau ranging from 50 to 250 nM were used to quantitatively determine the association and dissociation constants (Table 1). The equilibrium dissociation constant (K_D) for the interaction of unphosphorylated 3R-tau with 14-3-3ζ was ~113 nM, whereas the K_D between 14-3-3ζ and unphosphorylated 4R-tau was 325 nM. Approximately 3-folds more affinity was observed between 3R-tau and 14-3-3ζ in comparison to 4R-tau, which suggests that the R2 domain has an impact on the affinity of 4R-tau with 14-3-3ζ. The differences in interaction between 3R- and 4R-tau could be caused by isoform specific folding as suggested from earlier studies with microtubules [7]. These results are consistent with the reported evidence that Src family nonreceptor tyrosine kinases, fyn and Src, interact with 3R-tau at higher affinity than 4R-tau [20]. The impact of the R2 repeat is apparent, even though fyn and Src interact with tau at the proline rich domain which is close to the N-terminus of repeats.

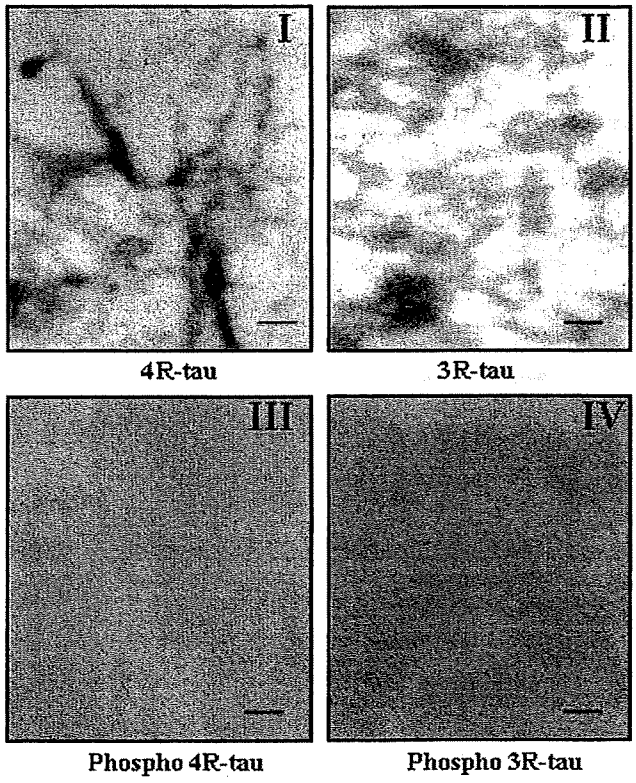

Fig. 4. Negative stain electron micrographs of filaments of 3R- and 4R-tau. Filaments of unphosphorylated 4R-tau (I) and 3R-tau (II) or phosphorylated 4R-tau (III) and 3R-tau (IV) assembled in the presence of 14-3-3ζ (same filament preparations as for the kinetic data at the endpoint of assembly). The size bar for 3R- and 4R-tau represent 330 and 500 nm, respectively.

Our previous study demonstrated that 14-3-3ζ binds to tau with higher affinity when tau is phosphorylated by PKA at Ser214 [14]. The primary structure of 4R-tau at Ser214 (209-RSRTPpSLP-216) is very similar to the canonical binding motif of 14-3-3 (RS/TXpSXP) [13]). In 3R-tau, Ser214 as well as the residues that compose the motif are conserved. To determine whether 14-3-3ζ exhibits any differences in interaction between 3R- and 4R-tau mediated by phosphorylation, the interaction was measured by SPR after phosphorylating tau at Ser214. 3R- and 4R-tau were phosphorylated by incubating them with PKA in a reaction mixture described earlier [14] and phosphorylation of tau at Ser214 was confirmed by immunoblotting with phosphorylated-Ser214 dependent CP3 antibody (Fig. 2A). The K_D values for the interaction of 3R- and 4R-tau with 14-3-3ζ were estimated to be 21 and 29 nM, respectively (Fig. 2B, and Table 1), thus suggesting that 14-3-3ζ has almost similar affinity for both 3R- or 4R-tau when tau is phosphorylated at Ser214. These results indicate that phosphorylation at Ser214, which is conserved in all the isoforms, might induce a drastic change in the conformation of tau such that 14-3-3 displays a similar strong affinity for both the isoforms. Recently, Sluchanko et al. [21] reported the interaction between 14-3-3ζ and the smallest 3R-tau isoform with or without phosphorylation. Our results are in accordance with their conclusion that phosphorylation of 3R-tau by PKA increases its interaction with 14-3-3ζ, but differ in the equilibrium dissociation constant, presumably due to experimental procedures, such as the method of determining the constants or the state of phosphorylation and oligomerization of tau that also might affect the tau/14-3-3 interaction.

14-3-3ζ facilitates tau aggregation by binding to its repeat domain [14,19]. Since 14-3-3ζ displays a higher affinity for 3R-tau than for 4R-tau, aggregation of both 3R- and 4R-tau was examined in the presence of 14-3-3ζ using the ThS fluorescence method. Equimolar amounts of 3R- and 4R-tau were incubated with 14-3-3ζ and the rate of aggregation was monitored by measuring samples at different time periods. The results (Fig. 3) show a significant increase in the aggregation of 4R-tau compared to 3R-tau under the same conditions. Tau aggregates were checked for filaments by negative stain electron microscopy (EM) and the results were very consistent with the results from ThS fluorescence assay. 4R-tau produced more filaments than 3R-tau (Fig. 4), but most filaments from both 4R- and 3R-tau were long and straight, thus suggesting that the aggregation was based on similar structural principles.

Phosphorylation of tau at Ser214 by PKA has been shown earlier to drastically reduce the aggregation of tau following the induction by 14-3-3ζ [14]. To determine the difference in aggregation between phosphorylated 3R- and 4R-tau, tau was phosphorylated by PKA at Ser214 and aggregation of phosphorylated tau was measured by ThS fluorescence method after incubation with 14-3-3ζ. Results (Fig. 3) showed that both 4R- and 3R-tau isoforms aggregated to a similar low level, suggesting that phosphorylation reduces the aggregation of both tau isoforms presumably in a similar manner. The results of ThS fluorescence studies were confirmed by negative stain electron microscopy (EM) and no filaments were found (Fig. 4).

Our results demonstrate that 14-3-3ζ binds to and induce the assembly of 3R- and 4R-tau isoforms differentially when tau is unphosphorylated, but similarly when tau is phosphorylated at Ser214 by PKA. Although the differential affinity of 3R- and

4R-tau for 14-3-3ζ is consistent with their differential aggregation by induction of 14-3-3ζ, the high affinity of 3R-tau relative to 4R-tau but the weak aggregation of 3R-tau than 4R-tau tempts us to speculate that like arachidonic acid- or heparin-induced aggregation of tau, the binding of 14-3-3ζ to tau is probably an obligate step preceding tau-tau interaction for aggregation [12,22]. The assembly behavior of 3R- and 4R-tau isoforms by induction of 14-3-3ζ appears to be very similar to the polyanion inducer heparin or fatty acid inducer arachidonic acid, which induces more aggregation of 4R-tau than 3R-tau [12,22–23]. In contrast, the similar level of high affinity and low aggregation of 3R- and 4R-tau isoforms after phosphorylation at Ser214 by PKA suggest that upon Ser214 phosphorylation, both isoforms might adopt a similar conformation for which they show similar higher affinity for 14-3-3ζ and resist aggregation to a similar extent by induction of 14-3-3ζ. Altogether, these results showed that 14-3-3ζ exhibits a similar role for tau isoforms in phosphorylated form, but a differential role in unphosphorylated form, which might have significance in tauopathy.

Tauopathies are mostly sporadic, and it is possible that different cofactors may function to induce the differential aggregation of tau isoforms that accumulate in inclusions observed in AD, PSP, CBD, and PiD. Some inducers may be effective in promoting the assembly of any tau isoforms, whereas others may prefer 4R- or 3R-tau. In this regard, *in vitro* studies have shown that tau isoforms can be differentially aggregated by cofactors other than 14-3-3, such as heparin, arachidonic acid, RNA. However, their involvement in the formation of NFT *in vivo* remains debated. For example, heparin is primarily extracellular, whereas tau protein is exclusively intracellular. Similarly, RNA is intracellular and present at high concentration, but is heavily complexed with proteins and not clear of its availability in free form in a sufficient amount to induce tau filament formation. The level of free cytoplasmic arachidonic acid is quiet low in the normal brain, whereas a high concentration of arachidonic acid is required for tau polymerization [24]. In comparison with the cofactors identified to date, 14-3-3 appears to be a potential candidate in the generation of NFTs in tauopathies for several reasons: first, 14-3-3 is an abundant cytosolic protein, which accounts for more than 1% of the total soluble protein. Second, 14-3-3 is a natural partner of tau and it facilitates tau aggregation by binding to its repeat domain, and third, 4R-tau is more aggregated than 3R-tau in presence of 14-3-3ζ. The observation of preferential interaction of 14-3-3ζ with 3R-tau than with 4R-tau, and induction of predominant aggregation of 4R-tau in comparison with 3R-tau may explain the association of 14-3-3 with NFTs found in tauopathies [15–17,25]. Therefore, 14-3-3 is probably the most important cofactor, identified to date, involved in the generation of NFTs predominantly of 4R-tau and our study will certainly be valuable in future research to clarify the role of 14-3-3 as a physiological inducer *in vivo*.

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Phosphorylation of tau at Ser214 mediates its interaction with 14-3-3 protein: implications for the mechanism of tau aggregation

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Abstract

The microtubule associated protein tau is a major component of neurofibrillary tangles in Alzheimer disease brain, however the neuropathological processes behind the formation of neurofibrillary tangles are still unclear. Previously, 14-3-3 proteins were reported to bind with tau. 14-3-3 Proteins usually bind their targets through specific serine/threonine – phosphorylated motifs. Therefore, the interaction of tau with 14-3-3 mediated by phosphorylation was investigated. In this study, we show that the phosphorylation of tau by either protein kinase A (PKA) or protein kinase B (PKB) enhances the binding of tau with 14-3-3 *in vitro*. The affinity between tau and 14-3-3 is increased 12- to 14-fold by phosphorylation as determined by real time surface plasmon resonance studies. Mutational analyses revealed that Ser214 is critical for the phosphorylation-mediated interaction of tau with 14-3-3. Finally, *in vitro* aggregation assays demonstrated that phosphorylation by PKA/PKB inhibits the formation of aggregates/filaments of tau induced by 14-3-3. As the phosphorylation at Ser214 is up-regulated in fetal brain, tau's interaction with 14-3-3 may have a significant role in the organization of the microtubule cytoskeleton in development. Also as the phosphorylation at Ser214 is up-regulated in Alzheimer's disease brain, tau's interaction with 14-3-3 might be involved in the pathology of this disease.

Keywords: 14-3-3 protein, aggregation, Alzheimer disease, microtubule-associated protein tau, phosphorylation, tauopathy.

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Tau protein belongs to the family of microtubule associated proteins and is a major component of the neurofibrillary tangles (NFTs) in the brains of patients with Alzheimer disease (AD) and related disorders 'tauopathies'; however, the neuropathological processes involved in the formation of NFTs are still unclear (for a review, see Iqbal *et al.* 2005). Tau is predominantly expressed in neurons, where it is believed to play major regulatory roles in microtubule assembly for the organization and integrity of the cytoskeletal network. Tau is a cytosolic phosphoprotein and phosphorylated *in vitro* by an array of proline- and non-proline-directed protein kinases including MAPK, cyclin-dependent kinase (CDK) 5, glycogen synthase kinase-3 (GSK-3), cAMP-dependent protein kinase (PKA), PKB, and microtubule affinity-regulated kinase (for review, see Tanaka *et al.* 1995a; Buée *et al.* 2000; Avila *et al.* 2002). The stoichiometry of phosphorylation in tau is high in the fetal brain and decreases substantially in the adult brain (Watanabe *et al.* 1993). Tau in fetal brain has been shown to promote microtubule assembly less efficiently than tau in adult brain (Goedert and Jakes 1990) and the elevated level of phosphorylated tau in fetal brain correlates with the presence of dynamic microtubules during periods of high plasticity in the developing mammalian brain (Brion *et al.* 1994). In addition, hyperphosphorylated tau is observed in neuroblastoma cells and the regulation of phosphorylation of tau via a balance of activities of kinases/phosphatases is suggested

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Abbreviations used: AD, Alzheimer's disease; CDK, cyclin-dependent kinase; CKI, casein kinase I; FC, flow cell; GSK, glycogen synthase kinase; GST, glutathione *S*-transferase; NFTs, neurofibrillary tangles; PKA, protein kinase A; SPR, surface plasmon resonance; ThS, thioflavine S.

(Tanaka *et al.* 1995b, 1998). Also tau isolated from patients with AD is highly phosphorylated similar to the modifications during fetal development (Morishima-Kawashima *et al.* 1995). It is therefore suggested that an increased state of phosphorylation of tau (hyperphosphorylation) represents both an early event during development and a critical event of tau pathology. To understand these processes, both normal and pathological, it is important to investigate the functional implications of the phosphorylation of tau.

Because the hyperphosphorylation of tau and disruption of microtubules are central points in the etiopathology of AD and taupathies, the role of tau's phosphorylation in the regulation of microtubules has been extensively studied over the past few years. Phosphorylation, in general, has been shown to negatively regulate the ability of tau to bind and stabilize microtubules. Studies *in vitro* and *in situ* have demonstrated that the phosphorylation of certain sites plays a significant role in tau–microtubule interaction (Mandelkow and Mandelkow 1998; Johnson and Stoothoff 2004). Among these sites, Ser262 is considered critical to tau–microtubule interaction as it is located in the microtubule-binding domain and its phosphorylation is potent to detach tau from the microtubule (Drewes *et al.* 1995). However, the precise role of each site in the normal and aberrant regulation of microtubules still remains elusive.

Apart from regulating the microtubules, phosphorylation of tau appears to be important for binding with the regulatory proteins. Lu *et al.* (1999) first demonstrated that Pin-1, a prolyl isomerase, specifically binds with Thr231 on the tau protein when the site is phosphorylated. It catalyzes isomerization and thereby increases the accessibility of tau to the protein phosphatase 2A, leading to the dephosphorylation of the tau protein. Shimura *et al.* (2004) showed that HSP27 preferentially binds with phosphorylated tau, but not unphosphorylated tau. The formation of a complex alters the conformation of pathological hyperphosphorylated tau and reduces its concentration in the cell by facilitating its degradation and dephosphorylation, thus HSP27 contributes to a neuroprotective role in AD. These findings imply a role for phosphorylation in tau's interaction and modulation with regulatory proteins. The identification of such proteins may provide new insight into the molecular mechanisms of tau's regulation and function.

The 14-3-3 proteins are a family of highly conserved abundant regulatory proteins found in all eukaryotes. They consist of at least seven isoforms encoded by separate genes (β, γ, ε, η, σ, θ, and ξ). The 14-3-3 proteins bind to a wide variety of intracellular proteins and regulate diverse cellular processes such as intracellular signal transduction, the cell cycle, and cell survival (for review, see Fu *et al.* 2000; Berg *et al.* 2003). The binding of 14-3-3 proteins with most of their partners mainly depends on phosphorylation of the Ser or Thr residue. For example, BAD, a pro-apoptotic protein, is inactivated by the binding of 14-3-3 after its phosphorylation (Zha *et al.* 1996). Hashiguchi *et al.* (2000) reported that the 14-3-3ζ isoform is associated with tau in brain extract and that the association accelerates the PKA-catalyzed phosphorylation of tau. In their study, effects of binding to 14-3-3 protein on the phosphorylation of tau were focused; however, effects of phosphorylation of tau on its affinity for 14-3-3 were analyzed insufficiently. Therefore, in this study, we investigated the effects of phosphorylation on the interaction of tau with 14-3-3 protein and its functional significance in relation to the pathogenesis of AD.

Materials and methods

Antibodies and western blot

Polyclonal 14-3-3 antibody was obtained from Upstate Biotechnology (Charlottesville, VA, USA), and polyclonal antibodies for the ζ (C-16), β (K-19), and ε (T-16) 14-3-3 isoforms were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal anti-Tau antibody that cross-reacts with phosphorylated and non-phosphorylated tau equally was from Santa Cruz Biotechnology. The phosphorylation-independent monoclonal antibody Tau-5 and site-specific phosphorylation-dependent tau antibodies pT212, pS262, and pS356 were purchased from BioSource International (Camarillo, CA, USA). CP-3 and PHF-1 antibodies, which recognize phosphorylated Ser214 and Ser396/404, respectively, were gifts from Dr Peter Davis (Albert Einstein University, New York, NY, USA). Western blotting was performed as previously described (Tanaka *et al.* 1998).

Protein kinases

Recombinant GSK-3β, CDK2 – cyclin A, and casein kinase I (CKI) were purchased from New England Biolabs (Beverly, MA, USA). GSK-3β was assayed by phosphorylation of the phosphopeptide KRREILSRRPSpYR. One unit of GSK-3β was defined as the amount of enzyme which incorporated 1 pmol of phosphate into the phosphopeptide in 1 min. The activity of CDK2-cyclin A was assayed based on the phosphorylation of histone H1. One unit of CDK2-cyclin A was the amount of enzyme which incorporated 1 pmol of phosphate into histone H1 in 1 min. The activity of CKI was assessed using the phosphorylation of the phosphopeptide KRRALSpVASLPGL. One unit of CKI was the amount of enzyme which catalyzed the incorporation of 1 pmol of phosphate into the phosphopeptide in 1 min. The catalytic subunits of PKA were purchased from Sigma (St Louis, MI, USA) and PKBα was purchased from Upstate Biotechnology (Temecula, CA, USA). One unit of PKA was defined as the amount of enzyme which catalyzed the incorporation of 1 pmol of phosphate into the synthetic peptide LRRASLG in 1 min. One unit of PKBα was defined as the amount which incorporated 1 pmol of phosphate into 1 nmol of CROSStide substrate peptide (GRPRTSSFAEG) in 1 min.

Plasmids

Rat 14-3-3ζ, η, β, and ε cDNA were a gift from Dr T. Ichimura of Tokyo Metropolitan University, Japan. An *Eco*R1–*Bam*H1 cDNA encoding the coding region of each 14-3-3 isoform was amplified by PCR, cloned into pGEX-5X-1 and transformed into *E. coli* JM109

for expression of the glutathione *S*-transferase (GST) fused 14-3-3 protein. Likewise, tau and the deletion mutants, tau-(1–244) and tau-(245–441), were prepared by PCR amplification using cDNA for the longest isoform of human tau as a template, cloned into pET-22b to produce the histidine-tagged protein, and transformed into *E. coli* BL21(DE3). Mutations at Ser214 and Thr212, substitutions with alanine, were introduced using site-directed mutagenesis with a Quick Change kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions and this was followed by DNA sequencing.

Preparation of recombinant proteins

Recombinant GST-14-3-3ζ, η, β, and ε were purified from bacterial lysates. An overnight culture was used to inoculate a 500 mL culture that was grown to an optical density of 0.6–0.8 at 600 nm. GST-14-3-3 protein expression was then induced with 0.1 M isopropyl-β-D-thiogalactopyranoside for 3 h. Following this induction, the bacterial cultures were harvested, and the pellets were suspended in ice-cold phosphate-buffered saline (150 mM NaCl, 10 mM Na_2HPO_4, and 10 mM NaH_2PO_4, pH 7.4) containing 1 mM dithiothreitol, 2 mM EDTA, 1% Tween 20, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, 1 μg/mL aprotinin, and 1 μg/mL pepstatin. The suspension was then sonicated for 30 s on ice and centrifuged at 12 000 *g* for 10 min at 4°C. The supernatant was dialyzed against lysis buffer for 3 h and mixed with glutathione–sepharose beads (GE Healthcare, Uppsala, Sweden) pre-equilibrated with lysis buffer containing 0.5% Nonidet P-40. The beads were shaken end-over-end for 3 h and packed in a glass column. The column was washed with a two column volume of lysis buffer and eluted with 10 mM reduced glutathione. The eluted GST-14-3-3 was concentrated and dialyzed against lysis buffer. For preparation of 14-3-3ζ protein, GST was removed from GST-14-3-3ζ by digestion with Factor Xa (Sigma) for 9 h at 23°C, and 14-3-3ζ was further purified by gel filtration chromatography (Superdex 200; GE Healthcare). For purification of recombinant tau and the deletion mutants, tau-(1–244) and tau-(245–441), tau synthesis was first induced by isopropyl-β-D-thiogalactopyranoside as described above for GST-14-3-3 and the protein was purified under non-denaturing conditions according to the manufacturer's instructions. In brief, cells were lysed in 500 mM NaCl, 10 mM imidazole, and 10 mM Tris–HCl, pH 7.4, and sonicated. Lysates were centrifuged at 12 000 *g* and purified over a Ni–NTA agarose column (GE Healthcare).

Immunoprecipitations

Brain extracts from adult mouse (6 weeks) and fetal mouse were prepared as previously described (Andorfer and Davies 2000). For immunoprecipitation, 1–5 μg of anti-14-3-3 antibody was added to each brain extract (0.5 mL, ~500 μg protein) in buffer A (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 μM Na_3VO_4, 1 mM NaF, 1 μM okadaic acid, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/mL each of leupeptine, aprotinine, and pepstatin) and shaken for 1 h at 4°C. After shaking, immobilized protein G sepharose beads (20 μL) (Pierce, Rockford, IL, USA) were added to each brain extract and the mixture was further shaken for 4 h at 4°C. The beads were collected by centrifugation (2300 *g*), washed five times with ice-cold buffer A, and immunoblotted with the indicated antibodies.

His-pulldown and GST-pulldown assays

For the His-pulldown assay, tau proteins were coupled to nickel beads (50 μL) in buffer C (50 mM Tris–HCl, pH 7.4, 50 mM NaCl, and 1 mM EDTA) and added to recombinant 14-3-3ζ or isoforms of GST-14-3-3 (20 μg/mL). Similarly, for the GST-pulldown assay, GST-fused 14-3-3 proteins were coupled to glutatione–sepharose beads (50 μL) in buffer C and added to tau (20 μg/mL). The mixture was then shaken for 3 h at 4°C and centrifuged (2300 *g*) to separate the beads. The beads were washed three times with ice-cold buffer C, mixed with 50 μL of sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer, boiled, and centrifuged (2300 *g*), and 5 μL of supernatant was analyzed by western blotting with the indicated antibody. The intensity of the 14-3-3 band was quantified by scanning densitometry using a Fluor Chem V3.0 (Alpha Inntech Corporation, San Leandro, CA, USA). For semi-quantitative analysis of the effect of phosphorylation on the interaction between 14-3-3 and tau, equimolar amounts of each of unphosphorylated and phosphorylated tau coupled to nickel beads were used and all pulldown assays were done in triplicate.

In vitro phosphorylation of tau

The phosphorylation was carried out by incubating tau (0.2 mg/mL) at 30°C for 6 h in a reaction mixture containing 50 mM Tris–HCl, pH 7.4, 1 mM dithiothreitol, 10 mM $MgCl_2$, and 2 mM ATP with 2 units/mL of PKB, and 100 units/mL of PKA, PKB, GSK-3β, CDK2, or CK1. The reactions were stopped by boiling for 5 min and the heat-stable tau protein was removed from denatured proteins by centrifugation (10 000 *g* for 10 min).

Surface plasmon resonance studies of 14-3-3 and Tau

The affinity between tau or phosphorylated tau and 14-3-3 was measured by surface plasmon resonance (SPR) spectroscopy using a Biacore 2000 (Biacore, Inc., Uppsala, Sweden). Tau or phosphorylated tau was immobilized on one flow cell (FC2) of a CM5 sensor chip by primary amine coupling according to the manufacturer's instructions. Briefly, the chip surface was activated with a 0.2 M *N*-ethyl-*N*-(3-diethylamino-propyl)-carbodiimide, 0.05 M *N*-hydroxy-succinimide solution followed by the injection of ligand at a concentration of between 5 and 50 μg/L in acetate buffer, pH 5.0. When the desired level of binding was achieved, unreacted *N*-hydroxy-succinimide ester groups were blocked with 1 M ethanolamine hydrochloride. A blank surface (FC1) was made by ethanolamine deactivation of the activated dextran surface. All measurements were performed using 10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.05% surfactant P-20 buffer. Purified 14-3-3ζ at various concentrations (200–1000 nM) was injected over FC1 and FC2 at a rate of 40 μL/min and the bound analytes were removed by washing with buffer after the injection. After each run, the sensor chip was regenerated with 10 mM glycine, pH 1.5 for 30 s. The FC1 was the minus 14-3-3ζ protein control channel and was subtracted from the sample channel (FC2) during the run. Bulk refractive index contributions were therefore expected to be zero or not significant in reference subtracted sensograms. To correct for non-specific binding, blank runs were performed with 10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.05% surfactant P-20 buffer on both surfaces before and after each binding analysis, and this value was subtracted prior to the kinetic analysis. Equilibrium association and dissociation rate constants were

calculated using the Langmuir (1 : 1) binding model with the BIA evaluation 2.1 software supplied by the manufacturer.

In vitro polymerization and electron microscopy

Recombinant tau was first phosphorylated by the indicated kinase and then purified by the methods as described above. Unphosphorylated tau, which was used as control, was treated the same way in parallel, except that kinase was replaced with the same amount of bovine serum albumin. Aggregation was induced by incubating tau at a concentration of 20 μM in 50 mM Tris–HCl, pH 7.4, buffer at 37°C for different time periods and mixing it with 14-3-3ζ in an equimolar ratio. The aggregation of tau was monitored by measuring the fluorescence of thioflavine S (ThS) using a spectrofluorometer (Perkin-Elmer Japan, Kanagawa, Japan) with an excitation filter of 430 nm and an emission filter of 520 nm. Measurements were carried out at 23°C in Tris–HCl buffer with 10 μM ThS. Background fluorescence and light scattering of the sample without ThS was subtracted when needed. Measurements were typically done in triplicate. Curves show average values.

After 10 days of incubation, polymerization samples were applied to a 300 mesh carbon-coated grid and negatively stained with 2% (w/v) uranyl acetate as described earlier (Kopke *et al.* 1993). Grids were analyzed using a JEOL JEM-1220 electron microscopy instrument at 50 kV (JEOL, Tokyo, Japan).

Results

Phosphorylation enhances the interaction of tau with 14-3-3

The 14-3-3 protein binds to unphosphorylated tau (Hashiguchi *et al.* 2000). Because 14-3-3 is a phosphoprotein-binding protein and tau is phosphorylated at many sites *in vivo*, we asked whether the phosphorylation has any effect on the interaction between tau and 14-3-3. We prepared tau by over-expressing it in bacteria and phosphorylated it in a reaction *in vitro* with PKA, PKB, GSK-3β, CDK2, and CK1, the kinases implicated in the phosphorylation of almost all sites of tau that are found in normal and AD brain (for review, see Tanaka *et al.* 1995a; Buée *et al.* 2000; Avila *et al.* 2002). Phosphorylation of tau was evident by an upward shift in mobility upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Fig. 1a) because of the incorporation of phosphates. To examine the interaction between 14-3-3 and tau, we employed a His-pulldown assay wherein equimolar amounts of tau or phosphorylated tau coupled to nickel beads were used and incubated with recombinant 14-3-3ζ, an isoform that binds to unphosphorylated tau. Beads were washed and immunoblotted with anti-14-3-3 antibodies (Fig. 1a). Quantification of blot band intensities (Fig. 1b) indicated that ~2.5-fold more 14-3-3 came down with tau phosphorylated by PKA and PKB compared with unphosphorylated tau. There was no significant change in the binding of tau with 14-3-3 because of phosphorylation with GSK-3β, CDK2, and CK1. These

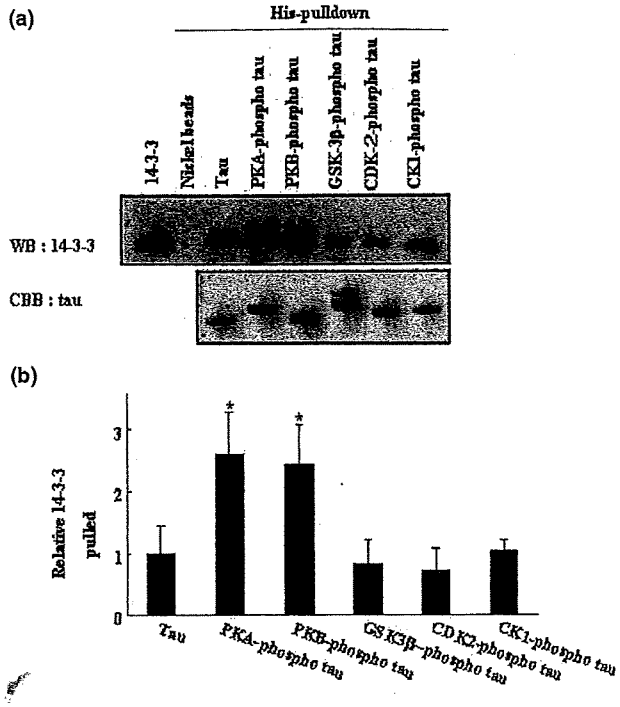

Fig. 1 Phosphorylation increases the binding of tau and 14-3-3. Tau unphosphorylated or phosphorylated by PKA, PKB, GSK-3β, CDK2, or CK1, coupled to nickel beads in equimolar amounts, were incubated with recombinant 14-3-3ζ, washed and immunoblotted with anti-14-3-3 antibody. The intensity of 14-3-3 bands was analyzed densitometrically. (a) Upper panels, immunoblot of beads with anti-14-3-3 antibody; lower panels, coomassie blue staining of gels to demonstrate the loading of tau proteins. (b) Quantification of pulled 14-3-3, with the value for unphosphorylated tau as one. The results are expressed as mean ± SEM ($n = 3$). *$p < 0.01$ when compared with unphosphorylated tau. Data were analyzed by one way analysis of variance, followed by Student's *t*-test.

results indicated that the phosphorylation of tau enhanced its association with 14-3-3.

Phosphorylation increases the affinity of tau for 14-3-3

To obtain quantitative data on the phosphorylation-mediated interaction between tau and 14-3-3, we performed SPR studies using a Biacore 2000. The real-time interaction between tau and 14-3-3ζ was measured at 25°C by injecting 14-3-3ζ onto a sensor chip with immobilized tau. The binding of 14-3-3ζ and tau caused an increase in mass at the surface of the chip which was reflected by an increase in SPR response over time (Supplementary Fig. S1). Different concentrations of 14-3-3ζ ranging from 200 to 1000 nM were used to quantitatively determine the association and dissociation constants. Prior to the kinetic analysis, the 14-3-3ζ alone control response was subtracted from the tau plus 14-3-3ζ response. The kinetic constants are shown in Table 1. The equilibrium dissociation constant (K_d) for the interaction of PKA and PKB-phosphorylated

Table 1 Kinetic constants for the interaction between tau and 14-3-3ζ obtained by SPR

	k_a (per M/s)	k_d (s^{-1})	K_A(M^{-1})	K_D (M)
14-3-3ζ and unphosphorylated tau	$8.42 \pm 2.65 \times 10^{-3}$	$2.52 \pm 0.01 \times 10^{-3}$	$3.38 \pm 1.10 \times 10^{6}$	$3.12 \pm 1.02 \times 10^{-7}$
14-3-3ζ and PKA-phosphorylated tau	$1.38 \pm 0.26 \times 10^{-4}$	$3.78 \pm 0.51 \times 10^{-4}$	$3.64 \pm 0.17 \times 10^{7}$	$2.74 \pm 0.13 \times 10^{-8}$
14-3-3ζ and PKB-phosphorylated tau	$2.24 \pm 0.94 \times 10^{-4}$	$5.11 \pm 1.36 \times 10^{-4}$	$4.28 \pm 0.72 \times 10^{7}$	$2.24 \pm 0.57 \times 10^{-8}$

SPR, surface plasmon resonance; PKA, protein kinase A; PKB, protein kinase B.

tau with 14-3-3 was 27 and 22 nM, respectively, whereas the K_d between 14-3-3 and unphosphorylated tau was 312 nM. Approximately 12–14 times more affinity was observed between tau and 14-3-3 because of phosphorylation. This data revealed that 14-3-3 has higher affinity for the phosphorylated tau.

Phosphorylation by PKA or PKB generates a 14-3-3-binding site in tau

Tau contains two main functional domains: an N-terminal projection domain and a C-terminal microtubule binding domain (Goedert *et al.* 1989). 14-3-3 binds to tau through the microtubule binding domain in a phosphorylation-independent manner (Hashiguchi *et al.* 2000). To locate the domain responsible for the phosphorylation-mediated interaction of tau/14-3-3, we used two deletion mutants, tau-(1–244) and tau-(245–441), that span the projection domain and microtubule binding domain, respectively. These mutants were prepared by over-expressing them in bacteria, and phosphorylated with PKA or PKB *in vitro*. Their binding with 14-3-3ζ was then evaluated. As shown in Fig. 2, full-length tau, tau-(1–441) bound to 14-3-3ζ and the binding was increased after phosphorylation as expected. Under the same experimental conditions, tau-(1–244) did not bind to 14-3-3ζ when it was unphosphorylated, but bound after phosphorylation by PKA or PKB (Fig. 2) demonstrating that phosphorylation generates a 14-3-3-binding site in the projection domain of tau. Consistent with a previous report (Hashiguchi *et al.* 2000), tau-(245–441) bound to 14-3-3ζ and phosphorylation by PKA or PKB did not enhance the binding (Fig. 2), clearly indicating that this region of tau is not involved in the phosphorylation-mediated interaction of tau and 14-3-3. These results suggested that (i) tau has at least two regions bound to 14-3-3 protein and (ii) possibly the projection domain of tau is associated with phosphorylation-mediated interaction with 14-3-3.

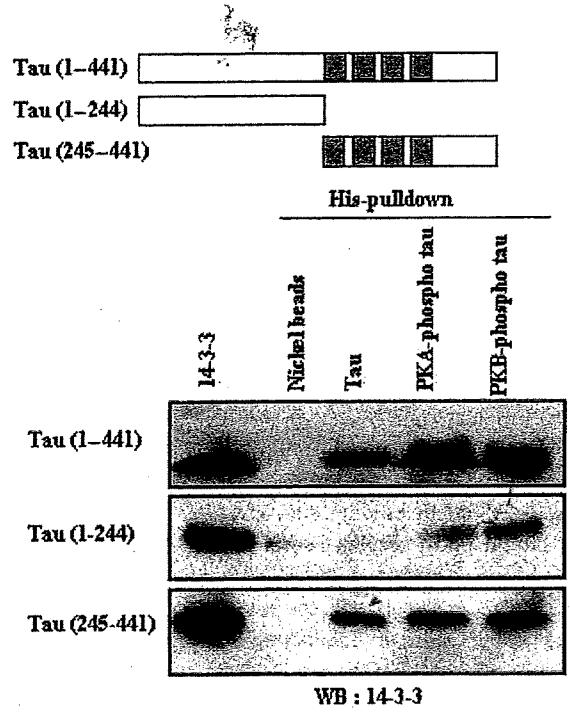

Fig. 2 Location of phosphorylation-mediated binding region of 14-3-3 within tau. Nickel beads (50 μL) coupled to the indicated tau species either unphosphorylated or phosphorylated with PKA or PKB was mixed with recombinant 14-3-3ζ, washed, and immunoblotted with anti-14-3-3 antibody. (a) A schematic sketch of various tau species used in the assay. (b) immunoblot analyses of beads representing full-length tau (WT), tau-(1–244), and tau-(245–441).

Ser214 is critical for the interaction of tau/14-3-3 mediated by phosphorylation

It is reported that tau is phosphorylated at many sites by PKA, but only at few sites by PKB (Scott *et al.* 1993; Ksiezak-Reding *et al.* 2003; Tanaka *et al.* 2003; Hanger *et al.* 2007). To learn about the phosphorylation sites of tau under our experimental condition, we mapped the sites by western blot analysis developed with antibodies that recognize tau only when it is phosphorylated at the specific individual phosphorylation sites, which is a well established and commonly used method for mapping the phosphorylation sites. We found that PKA phosphorylated tau at Ser214, Ser262, and Ser356, but not at Thr212 or Ser396/404 and that PKB phosphorylates tau at Thr212 and Ser214 (Fig. 3a). This finding is consistent with the previous reports that identified by mass spectrometry (Ksiezak-Reding *et al.* 2003; Hanger *et al.* 2007). Therefore, the common site of phosphorylation by PKA and PKB was found to be Ser214. To avoid the complexity of multiple phosphorylation, PKB was first employed for experiments to identify the phosphor-

