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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

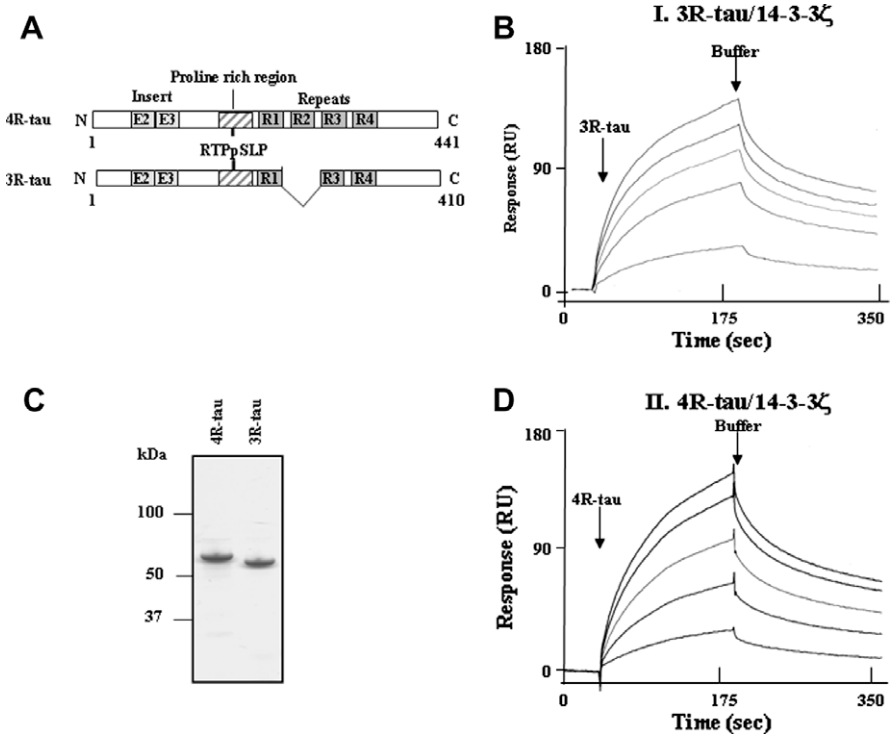

Fig. 1. 14-3-3ζ binds to unphosphorylated 3R-tau with higher affinity than unphosphorylated 4R-tau. (A) A schematic diagram of the longest human 4R- and 3R-tau isoforms employed. The amino acid residues numbers are according to tau441. 4R-tau contains two alternatively spliced inserts near the N-terminus (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 (10,000*g* 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.

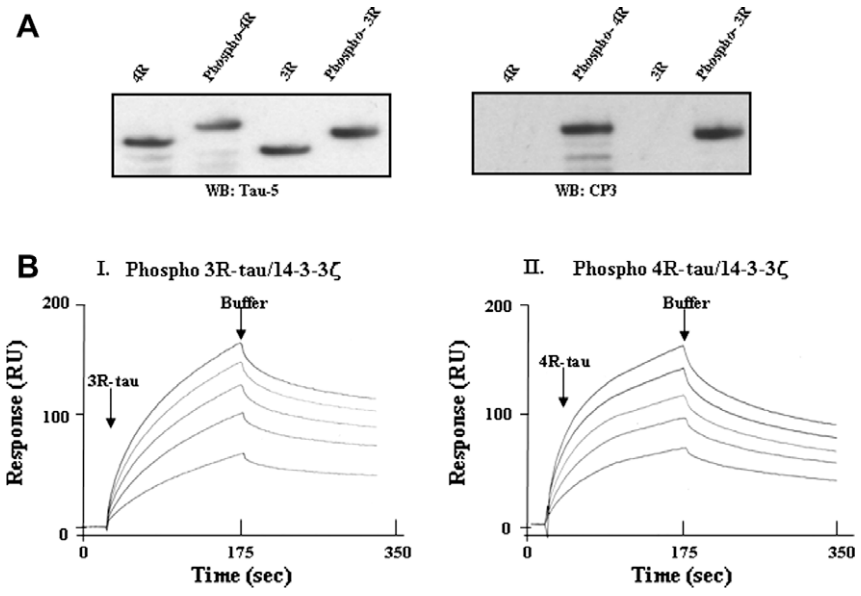

Fig. 2. 14-3-3ζ shows almost equal affinity for phosphorylated 3R- and 4R-tau. (A) Tau was phosphorylated with PKA and the phosphorylation sites were analyzed by Western blotting with the indicated phosphorylation-independent and -dependent antibodies. B, An overlay of SPR sensograms resulting from the 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.

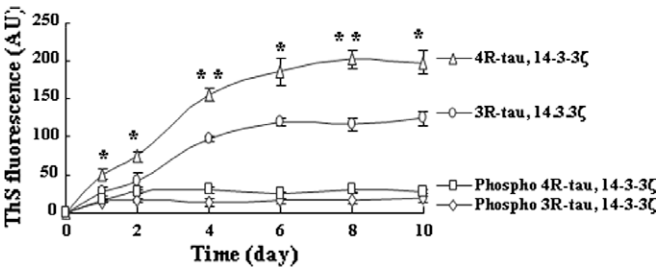

Fig. 3. Unphosphorylated 4R-tau is assembled more readily than unphosphorylated 3R-tau by the induction of 14-3-3ζ in vitro. The rate of aggregation of unphosphorylated 4R-tau (△) and 3R-tau (○) or phosphorylated 4R-tau (□) and 3R-tau (◇) were measured by ThS fluorescence. The intensity of ThS (AU) is 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.

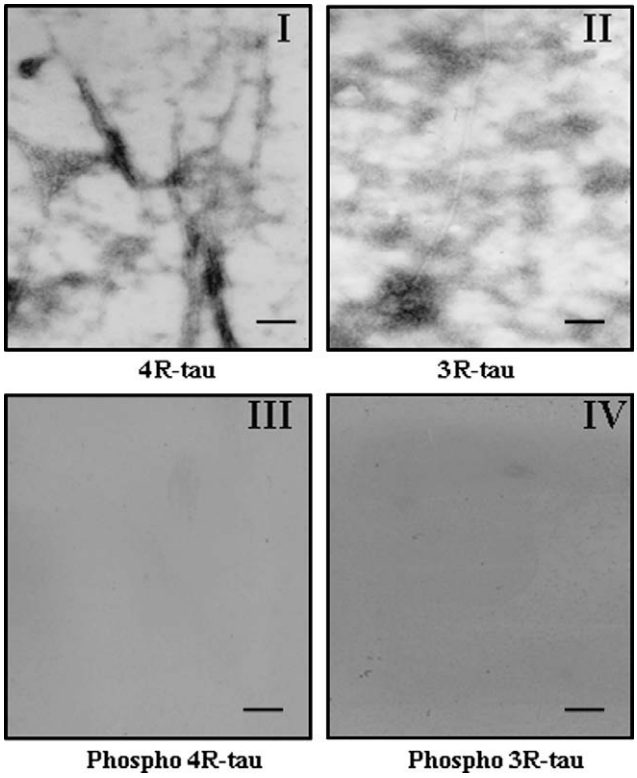

Fig. 4. Negative stain electron micrographs of filaments of 3R- and 4R-tau. Filaments of unphosphorylated 4R-tau (I) and 3R-tau (II) or phosphorylated 4R-tau (III) and 3R-tau (IV) assembled in the presence of 14-3-3ζ (same filament preparations as for the kinetic data at the endpoint of assembly). The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

Acknowledgments

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