Activation of Glycogen Synthase Kinase 3β Promotes the Intermolecular Association of Tau

THE USE OF FLUORESCENCE RESONANCE ENERGY TRANSFER MICROSCOPY*

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Tau is hyperphosphorylated and undergoes proteolysis in Alzheimer disease brain. Caspase-cleaved tau efficiently forms fibrillar structures in vitro and in situ. Glycogen synthase kinase 3β (GSK3β) phosphorylates tau and induces the aggregation of caspase-cleaved tau in situ. Given the hypothesis that increased association of tau precedes the formation of fibrillar structures, we generated a cell model to quantitate the extent of tau association in situ using fluorescence resonance energy transfer (FRET) microscopy. The cyan and yellow fluorescent proteins were attached to full-length (T4) and caspase-cleaved (T4C3) tau at either the N or C termini, and a pair of cyan and yellow fluorescent protein-tagged tau were co-transfected into human embryonic kidney cells. The FRET efficiency was examined in the presence of a constitutively active or a kinase-dead GSK3β. Active GSK3β significantly increased FRET efficiency with both T4 and T4C3, indicating that GSK3β activation resulted in an increase in the self-association of both T4 and T4C3, but interestingly only T4 is efficiently phosphorylated by GSK3β. There was no significant difference in FRET efficiency between T4 and T4C3, although only T4C3 in the presence of active GSK3β leads to the formation of Sarkosyl-insoluble inclusions. These FRET studies demonstrate that GSK3β facilitates the association of T4 and T4C3, and the presence of caspase-cleaved tau is necessary for the evolution of tau oligomers into Sarkosyl-insoluble inclusions even though it is not extensively phosphorylated. These data imply that increased association of tau should not be regarded as a direct indicator of the formation of insoluble tau aggregates.

The accumulation of polymeric filaments of the microtubule-associated protein tau as the intracellular neurofibrillary tangles (NFTs) is one of the major neuropathological features of several diseases known as “tauopathies,” which include Alzheimer disease (AD) and frontotemporal dementia with parkinsonism linked to chromosome 17, a group of autosomal dominant neurodegenerative diseases caused by mutations in the tau gene (1). NFTs are mainly composed of paired helical filaments (PHFs), which are formed from abnormally hyperphosphorylated tau (2, 3). Although in AD, the abundance of NFTs correlates positively with the severity of cognitive impairment (4), the role of NFTs as toxic mediators of neuronal dysfunction and death is still not clear. Several animal models show cognitive deficits and impaired axonal transport in the absence of NFTs (5–7). Furthermore, suppression of tau expression in a transgenic mouse model restored memory function and stabilized neuronal cell populations, whereas NFTs continued to accumulate (8). These studies suggest that NFTs are not sufficient to cause cognitive decline or neuronal death, and small soluble oligomers may be the toxic species (9). Therefore, elucidating the early steps in the process of tau oligomerization is of fundamental importance.

Post-translational modifications of tau such as aberrant phosphorylation have been demonstrated to play a significant role in tau aggregate formation (10–15). Tau is hyperphosphorylated at numerous sites in NFTs of AD brains (16), and in vitro studies showed that hyperphosphorylated tau can self-assemble into PHFs (17, 18). Phosphorylation at specific sites can significantly increase the tendency of tau to aggregate. For example, pseudophosphorylation of Ser396 and Ser404, as well as Ser205, Thr205, and Thr212, makes tau more fibrillogenic (19–21). There is emerging evidence that glycogen synthase kinase 3β (GSK3β) may play a major role in regulating tau phosphorylation in pathological conditions (22, 23). Increased expression of GSK3β results in increased tau phosphorylation at pathological sites, including the PHF-1 epitope (Ser(P)396/404) (24, 25). Furthermore, there is evidence for increased activation of GSK3β in AD brain (26–28).

Along with aberrant phosphorylation, caspase cleavage has been also reported to play a role in the aggregation of tau (29–31). It has been demonstrated that caspases play a critical role in AB-induced neuronal apoptosis (32) and are activated in apoptotic neurons in AD brain (33). Although tau in AD is present predominantly as an intact full-length molecule (16), tau in AD NFTs has been shown to be truncated at Asp623 (29). Truncated tau, albeit not a large amount, may play a significant role in the neuronal cell death and PHF formation, given that truncated tau has been shown to be associated with apoptosis in cultured...
cells (34, 35) and has been demonstrated to play a significant role in the nucleation-dependent filament formation of tau (35). Therefore, it is important to investigate how GSK3β-mediated phosphorylation and caspase cleavage of tau contribute to the initiation of the tau aggregation process. In a previous study, it was demonstrated that tau truncated at Asp421 (T4C3), but not full-length tau (T4), partitioned into a Sarkosyl-insoluble fraction and formed thioflavin S-positive aggregates when co-expressed with GSK3β (36). This indicates that a combination of phosphorylation and cleavage of tau may coordinate in regulating tau aggregation.

Fluorescence resonance energy transfer (FRET) is a process by which energy is transferred from a donor fluorophore (CFP) to an acceptor fluorophore (YFP) in a distance-dependent manner (typically 20–60 Å). FRET is one of the few tools available for measuring nanometer scale distances and changes in distances, both in vitro and in vivo (37–39). Because FRET efficiency decreases as the inverse sixth power of distance, small distance and orientation changes between the donor and acceptor fluorophores can dramatically affect the FRET efficiency (40). A number of applications using FRET to understand protein-protein interactions have been reported (38, 41–43). For example FRET has been used to detect the dimerization of a protein (44), indicating that FRET can be used to evaluate the self-association of a protein.

This study was undertaken to understand the intermolecular association of tau proteins in response to GSK3β activation and/or caspase cleavage. To achieve this goal FRET microscopy was used with full-length tau (T4) and caspase-cleaved (Asp421) tau (T4C3) to which CFP and YFP were linked. To evaluate different tau orientations during the intermolecular interactions of tau, N-terminally linked CFP (CFP-tau) was co-transfected with N-terminally linked YFP (YFP-tau) or C-terminally linked YFP (tau-YFP) for both T4 and T4C3 in the absence or presence of active GSK3β, and subsequent to quantitating FRET efficiency. The data from these studies demonstrate that activation of GSK3β results in an increase in the intermolecular association of both T4 and T4C3 tau. However, increased intermolecular association did not necessarily lead to the formation of insoluble tau aggregates, suggesting that although the increased intermolecular interaction is likely an early event in the formation of insoluble tau aggregates, tau may remain in an oligomeric state and not progress into an aggregate in the absence of other contributing cellular processes.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—To make the FRET constructs, PCR products of pECFP-C1 (Clontech) and pEYFP-C1 (Clontech) were cloned into Nhel and KpnI sites of pcDNA3.1(+) (Invitrogen) to make the N-terminally linked CFP and YFP vectors, nCFP and nYFP vector, respectively. The PCR product of pEYFP-N1 (Clontech) was cloned into XbaI and Apal sites of pcDNA3.1(+) (Invitrogen) to make a C-terminally linked YFP vector, cYFP vector. To make a positive FRET control construct, a PCR product of pEYFP-N1 was cloned into XbaI and Apal sites of nCFP vector (nCFP-YFP vector), and then a highly flexible linker domain, which consisted of Gly-Ser-Asp-Gly-Gly-Ser-Gly-Gly-Ser-Thr-Ser (45), was introduced into the vector to make nCFP-Linker-YFP. T4 containing human tau with four microtubule-binding repeats but without exons 2 and 3 and T4C3 with the last 20 amino acids deleted and thus mimicking caspase-cleaved tau (36) were introduced into KpnI and XbaI sites of the pcDNA3.1(+) FRET vectors to yield nCFP-tau, nYFP-tau, and tau-cYFP constructs. To make the tau-cYFP vectors, the stop codons of T4 and T4C3 were replaced with GGA before cloning into FRET vectors. All the constructs were confirmed by DNA sequencing analysis.

GSK3β-S9A-HA, which is a constitutively active form of GSK3β, was constructed in pcDNA3.1, as described previously (46), and the kinase-dead (kd)-GSK3β-HA construct, in which lysine residues at 85 and 86 were mutated to alanines, was a generous gift from G. Meares (University of Alabama at Birmingham, Birmingham, AL) (47).

**Cell Culture and Transient Transfection**—Human embryonic kidney (HEK) cells were grown in Dulbecco’s modified Eagle’s medium/F-12 medium (Cellgro, Herndon, VA) supplemented with 5% bovine growth serum (HyClone, Logan, UT), 2 mM l-glutamine, 10 units/ml penicillin, and 100 units/ml streptomycin (Cellgro). Cells at 50–70% confluency were transiently transfected using FuGENE 6 (Roche Applied Science) according to the manufacturer’s protocol. 48 h after transfection, the cells were collected and processed as described below for the different assays.

**Coverslip Preparation for FRET Microscopy**—Forty eight hours after transfection, cells on the coverslips were washed with PBS and fixed with 3% paraformaldehyde in PBS for 15 min. The cells were washed with PBS and mounted. Coverslips were sealed with clear nail polish.

**FRET Microscopy**—Intermolecular interaction of tau proteins was determined by the Acceptor Photobleaching method of FRET detection (37–39). A Leica SP2 confocal microscope (Leica) was used to record the fluorescence of CFP and YFP both before and after selective photobleaching of 70% of the YFP acceptor fluorophore. CFP was excited by 458 nm light, and the emission was collected through a 465–500 nm band-pass filter (Chroma Technology, Brattleboro, VT). YFP was excited by 514 nm light and the emission was collected through a 525–600 nm bandpass filter (Chroma Technology, Brattleboro, VT). Selective photobleaching of YFP was performed by repeatedly scanning a region of the specimen with the 514 nm laser line set at maximum intensity to photobleach at 70% of the original acceptor fluorescence. The fluorescence emission from the donor and the acceptor were collected using the Leica software. A Timed Bleach protocol was utilized to automate the acquisition of pre-bleach images, perform acceptor photobleaching of the acceptor, and acquisition of post-bleach images. The pre- and post-bleach images are saved as a single file that can be analyzed using measurement functions resident in the Leica software. Average fluorescence intensities of the donor are measured before and after bleaching, and the efficiency of FRET was calculated by $E_T = 1 - (I_{DA}/I_D)$ where $I_{DA}$ and $I_D$ represent the steady state donor fluorescence in the presence and the absence of the acceptor.

**Immunoblotting**—Cells were collected in lysis buffer (150 mM NaCl, 10 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium vanadate, 0.5% Nonidet P-40), containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium vanadate, 0.5% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride (PMSF).
RESULTS

Generation and Expression of FRET Tau Constructs—To determine the intermolecular interaction of tau proteins using FRET microscopy, CFP and YFP constructs, which were linked to full-length (T4) or tau truncated at Asp421 (T4C3) that mimics caspase-3 cleavage, were generated. To detect different orientations for the tau-tau interactions, YFP fluorophore was linked to the N terminus or C terminus of tau, whereas the CFP fluorophore was linked only to the N terminus of tau protein (Fig. 1). As a positive control for FRET, a flexible linker domain consisting of 12 amino acids was introduced between CFP and YFP (CFP-Linker-YFP in Fig. 1). HEK cells were transfected with these FRET constructs and immunoblotted to determine their expression levels (Fig. 2). The T4 CRET constructs migrated faster than the T4 FRET constructs. For molecular weight comparisons, naive T4, which was not linked to a fluorophore, was also included in these blots.

FIGURE 1. Diagram of tau and positive control constructs for FRET microscopy. Full-length tau (T4) and caspase-cleaved tau (T4C3) were linked to CFP or YFP fluorophores as indicated at either C terminus or N terminus of tau. The T4 tau construct, which is a human tau, contains four microtubule binding repeats but without exons 2 and 3, and T4C3 contains the same as T4 except the last 20 amino acids were removed, which mimics caspase-cleaved tau. The CFP fluorophore was only linked to the N terminus of T4 and T4C3, and the YFP fluorophore was linked to either the N terminus or C terminus of tau as indicated. To link YFP fluorophore at the C terminus of tau proteins, the stop codon of the tau constructs was mutated to GGA, which codes for glycine. The top bar shows a positive control FRET construct where the CFP fluorophore was linked with the YFP fluorophore using a flexible linker domain, composed of 12 amino acids as follows: Gly-Ser-Asp-Gly-Gly-Ser-Gly-Gly-Ser-Thr-Ser.

FIGURE 2. Cellular expression of FRET constructs. Fluorophore-linked tau and positive control constructs were transiently transfected into HEK cells. Blots were probed with GFP (top panel) and 5A6 (bottom panel) antibodies. Representative immunoblots with these antibodies showed that the different tau constructs were expressed at approximately equal levels, and linking of a fluorophore to tau reduced the electrophoretic mobility of the tau proteins as expected. The T4 CRET constructs migrated faster than the T4 FRET constructs. For molecular weight comparisons, naive T4, which was not linked to a fluorophore (far right band on blots), and CFP-linker-YFP, the positive FRET control construct (far left band on blots), were included in these blots.
Active GSK3β Increased Tau Intermolecular Interactions

To determine the intermolecular association of T4 or T4C3, CFP-tau was transiently co-transfected with YFP-tau or tau-YFP in the presence of active GSK3β or kd-GSK3β, and then FRET efficiency was measured. As a positive control in FRET microscopy, the CFP-linker-YFP construct was used (54). To determine the FRET efficiency, approximately half of a cell was selected as the region of interest to bleach, and the other half of the cell was used as background to obtain pure FRET efficiency. The net FRET values were calculated using the following equation: net FRET efficiency = FRET efficiency in acceptor-bleached area − FRET efficiency in the other half non-bleached area. The FRET efficiency in the nonbleached area was negligible in all groups. Representative FRET images are shown in Fig. 3, and quantitative analyses of FRET efficiency, which were obtained from three independent experiments, are presented in Fig. 4. The positive control CFP-Linker-YFP showed a FRET efficiency of 15.81 ± 1.46, and images of CFP and YFP with this construct completely overlapped as expected. Expression of active GSK3β resulted in significantly increased FRET efficiency with both T4 and T4C3 compared with kd-GSK3β, which indicates that active GSK3β increased intermolecular associations of both T4 and T4C3 (Fig. 3 and Fig. 4). The arbitrary FRET efficiencies (mean ± S.E.) were as follows: 3.61 ± 0.21 and 5.89 ± 0.30 for CFP-T4 with YFP-T4 in the presence of active and kd-GSK3β, respectively; 3.63 ± 0.29 and 5.73 ± 0.52 for CFP-T4 with T4-YFP in the presence of active and kd-GSK3β, respectively; 2.94 ± 0.28 and 4.83 ± 0.68 for CFP-T4C3 with YFP-T4C3 in the presence of active and kd-GSK3β, respectively; 2.98 ± 0.50 and 5.29 ± 0.70 for CFP-T4C3 with T4C3-YFP in the presence of active and kd-GSK3β, respectively (Fig. 4).

However, there were no significant differences in the FRET intensity between YFP-tau and tau-YFP, which suggests that there is equivalent parallel and anti-parallel self-association of tau in situ. Furthermore, no significant differences were observed between T4 and T4C3 either in basal conditions or in the presence of active GSK3β. No FRET efficiency was observed with either CFP-tau or YFP-tau alone (data not shown).

GSK3β Phosphorylates T4 More Efficiently than T4C3—

To determine the relative effectiveness of GSK3β-mediated phosphorylation of these FRET tau constructs, samples were immu-
FRET Microscopic Analysis of Tau-tau Interactions

FIGURE 4. Quantitative FRET efficiencies of intermolecular association of tau by active GSK3β. FRET efficiency was calculated using area averages for donor (D) before and after bleaching, FRET = (D after - D before)/D after. To show a positive FRET efficiency, donor (CFP) intensity after acceptor (YFP) bleaching should be significantly increased compared with that of prebleaching donor (CFP) upon acceptor (YFP) photobleaching. In this study, the acceptor (YFP) was bleached to 30% of its original intensity, and the mean values of FRET efficiencies were calculated from at least five cells from three independent experiments using the Leica FRET software and displayed as mean ± S.E. The presence of active GSK3β resulted in a significant increase in the FRET efficiencies of both T4 and T4C3 compared with those in the presence of kd-GSK3β. However, no significant differences between the two orientation groups were observed. Furthermore, there was no significant difference between T4 and T4C3 either in the presence of active or kd-GSK3β. * and ** represent YFP- and -YFP, respectively. *, p < 0.05.

noblotted with the phosphospecific antibodies PHF-1 (Ser(P)396/404) or AT-180 (Thr(P)231) (Fig. 5). Co-expression of active GSK3β with T4 resulted in a significant decrease in electrophoretic mobility compared with the mobility when kd-GSK3β was used indicating that the phosphorylation state of tau was increased with active GSK3β (Fig. 5, Total tau). In contrast to what was observed with T4, the electrophoretic mobility shift of T4C3 was negligible in the presence of active GSK3β (Fig. 5, Total tau). The extent of phosphorylation of T4 by active GSK3β at both the PHF-1 and AT-180 epitopes was substantially greater than that of T4C3 (Fig. 5). This is consistent with a previous finding showing that truncation of tau at Asp421 attenuates GSK3β-mediated phosphorylation (36).

GSK3β Causes T4C3 but Not T4 to Form Sarkosyl-insoluble Aggregates—To examine the role of GSK3β on the formation of Sarkosyl-insoluble aggregates of these tau constructs used for the FRET, the partitioning of fluorophore-tagged tau into the Sarkosyl-insoluble fractions was investigated. Lysates were prepared from cells expressing the tau constructs in the presence of either active or kd-GSK3β and then separated into RAB, RIPA, Sarkosyl-soluble, and Sarkosyl-insoluble fractions (Fig. 6). In the presence of kd-GSK3β, none of the tau FRET constructs localized to the Sarkosyl-insoluble fractions. In accordance with previous findings (10, 36), expression of active GSK3β resulted in the presence of tau in the Sarkosyl-insoluble fractions with the fluorophore T4C3 constructs but not the T4 constructs (Fig. 6). Interestingly, T4C3 was more abundant in the RIPA fraction than T4, which indicates T4C3 is less soluble in basal conditions compared with T4.

DISCUSSION

This study demonstrates for the first time using FRET microscopy that the kinase activity of GSK3β results in increased intermolecular association of tau, which is likely an early step in the process of the formation of insoluble tau aggre-
gates. In addition, the data suggest that although increased interactions of tau may be an initial step, it does not necessarily predict the efficiency of tau aggregate formation.

Although PHFs are one of the characteristic hallmarks of AD, the role of aggregates as toxic mediators of neuronal dysfunction and death is still not clear. Indeed, the correlation between NFT presence and the incidence of disease does not necessarily dictate a causal relationship (5–8), and therefore the focus has been shifting toward identifying the toxic tau species during the entire fibrillogenic process from the soluble monomers through oligomers to the insoluble mature tangles (NFTs). Whether the monomers, oligomers, or NFTs are the important toxic mediators, the question of what causes tau to aggregate and the kinetic profile of this process must be addressed. Therefore, in this study we used FRET microscopy to examine the initial steps of tau self-association, which then subsequently contribute to aggregate formation.

Although the confocal images of co-expressed CFP-tagged and YFP-tagged tau proteins appeared to be overlapping in the presence of kd-GSK3β, FRET efficiency data confirmed that in the basal condition CFP-tagged and YFP-tagged tau proteins were not within close proximity, and therefore a mere overlap of fluorescence is not necessarily indicative of an interaction. However, expression of active GSK3β resulted in an increase in FRET efficiency, indicative of an increased association between the tau proteins, accompanying the apparent overlapping images of the two proteins. Therefore, FRET microscopy provides a significant advantage in obtaining information about protein-protein interactions compared with confocal microscopy (55). To detect different orientations of intermolecular association of tau, N-terminally CFP-tagged tau protein (CFP-tau) was co-transfected with either N-terminally YFP tagged tau (YFP-tau) or C-terminally YFP tagged tau (tau-YFP). Active GSK3β resulted in increased FRET efficiencies in with both orientations of T4 and T4C3, and there was no significant difference in FRET efficiency between the two orientations, suggesting that either orientation occurs when tau self-associates in situ. Although the intermolecular interaction of tau was significantly increased with GSK3β activation, the FRET efficiency was not increased as high as the positive control with both T4 and T4C3, indicating that intermolecular association of tau protein at this stage may not be as tight as that of the mature aggregates.

Activation of GSK3β resulted in the increased intermolecular association of both full-length (T4) and caspase-cleaved tau (T4C3) in the present study, an initial step in the oligomerization and aggregate formation of tau. It has been suggested that the fibrillogenic pathway of tau can be subdivided based on key steps (56). First, the microtubule binding function of tau must be neutralized so that tau protein accumulates in an assembly-competent form (57), which suggests that aggregation of tau may be concentration-dependent. Second, tau molecules self-associate through their microtubule binding repeat regions to form the β-sheet-enriched filaments. When the critical concentration of tau molecules is achieved, unfolded monomer tau molecules, which have no substantial secondary structures, oligomerize leading to a conformational change to a β-sheet enriched structure (57, 58). The earliest secondary structure detectable with fluorescent dyes corresponds to tau aggregates associated with membranous structures (59), suggesting that the folding of tau protein into β-sheet-containing species may be facilitated by interaction with intracellular membranes and organelles. The final step involves the nucleation of tau filaments and formation of mature NFTs. In the transition, NFTs undergo proteolytic modifications (57) and become highly insoluble (58).

It has been suggested that post-translational modifications of tau such as aberrant site-specific phosphorylation and caspase cleavage contribute to the formation of NFTs in the earliest stages. Several studies have provided evidence that phosphorylation of key sites on tau has a strong impact on the normal function of tau and likely contributes to its pathological role, including the tendency of aggregate formation. The role of phosphorylation on tau assembly was previously hypothesized (60). According to their hypothesis, tau self-assembles mainly through the microtubule binding domains; however, regions of tau molecule N-terminal and C-terminal to the microtubule binding domains inhibit tau association in the naive state. Hyperphosphorylation of tau neutralizes these inhibitory domains enabling self-association of tau. In accordance with this hypothesis, phosphorylation at both Ser396/Ser404 and Ser422 enhances tau aggregation (11). It also has been reported that pseudophosphorylation of tau at Ser205, Thr205, and Thr212 enhances polymerization of tau into filaments (20). In addition, phosphorylation of tau at Thr231 (46) or Ser262 (61) negatively regulates tau-microtubule interactions. However, phosphorylation at Ser262 may decrease the ability of tau to polymerize (11, 15, 19), suggesting that abnormal phosphorylation of this site on tau does not play a facilitatory role in the pathological aggregation into PHFs (11). The data in this study show that extensive phosphorylation, including phosphorylation at the PHF-1 epitope, does not necessarily lead to the aggregate formation of full-length tau (T4), whereas the aggregates were observed with truncated tau (T4C3), which was not phosphorylated extensively, suggesting that hyperphosphorylation is not sufficient to cause tau to form insoluble aggregates.

In addition to aberrant phosphorylation, caspase cleavage of tau has been reported to influence fibril formation of tau (62). Tau is a substrate for caspase-3 in vitro, and tau truncated at Asp421, the caspase-3 cleavage site in tau, aggregates more rapidly than full-length tau (29, 34, 35). In addition, the presence of Asp421-truncated tau in pretangle neurons suggests a role for caspase cleavage in the initiation of polymer formation (63). In accordance with previous reports, the present data demonstrated that T4C3 formed Sarkosyl-insoluble aggregates in the presence of active GSK3β. Given the present data, either phosphorylation or caspase cleavage alone may not be sufficient to cause tau aggregation. Although T4 was extensively phosphorylated by active GSK3β and intermolecular interaction was significantly increased as shown in FRET microscopy, no T4 was present in the Sarkosyl-insoluble fractions indicating that T4 did not form aggregates. In contrast, even though T4C3 is minimally phosphorylated by GSK3β, T4C3 formed Sarkosyl-insoluble aggregates in the presence of active GSK3β, an event that was concurrent with an increase in the intermolecular association of T4C3 as determined by FRET microscopy. Given
Asp421-truncated tau is not efficiently phosphorylated by GSK3β.

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