Phosphorylation by Neuronal cdc2-like Protein Kinase Promotes Dimerization of Tau Protein in Vitro*  

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In Alzheimer’s disease, the microtubule-associated protein tau forms paired helical filaments (PHFs) that are the major structural component of neurofibrillary tangles. Although tau isolated from PHFs (PHF-tau) is abnormally phosphorylated, the role of this abnormal phosphorylation in PHF assembly is not known. Previously, neuronal cdc2-like protein kinase (NCLK) was shown to phosphorylate tau on sites that are abnormally phosphorylated in PHF-tau (Paudel, H. K., Lew, J., Ali, Z., and Wang, J. H. (1993) J. Biol. Chem. 268, 23512–23518). In this study, phosphorylation by NCLK was found to promote dimerization of recombinant human tau (R-tau) and brain tau (B-tau) purified from brain extract. Chemical cross-linking by disuccinimidyl suberate (DSS), a homobifunctional chemical cross-linker that specifically cross-linked R-tau dimers, and a Superose 12 gel filtration chromatography revealed that R-tau preparations contain mixtures of monomeric and dimeric R-tau species. When the structure of NCLK-phosphorylated R-tau was studied by a similar approach, DSS preferentially cross-linked the phosphorylated R-tau over the nonphosphorylated R-tau, and the phosphorylated R-tau eluted as a dimeric species from the gel filtration column. Phosphorylated R-tau became resistant to DSS upon dephosphorylation and was recovered as a monomeric species from the gel filtration column. In the presence of a low concentration of dithiothreitol (1.65 μM), R-tau formed disulfide cross-linked R-tau dimers. When compared, phosphorylated R-tau formed more disulfide cross-linked dimers than the nonphosphorylated R-tau. B-tau also was specifically cross-linked to dimers by DSS. When B-tau and NCLK-phosphorylated B-tau were treated with DSS, phosphorylated B-tau was preferentially cross-linked over nonphosphorylated counterpart. Taken together, these results suggest that phosphorylation by NCLK promotes dimerization and formation of disulfide cross-linked tau dimers, which is suggested to be the key step leading to PHF assembly (Schweers, O., Mandelkow, E. M., Biernat, J., and Mandelkow, E. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8463–8467).

Senile plaques and neurofibrillary tangles are two characteristic pathological lesions found in the brains of patients suffering from Alzheimer’s disease (AD)† for a review, see Ref. 1. Neurifibrillary tangles develop in nerve cells that undergo degeneration and contain paired helical filaments (PHFs) as the major fibrous component. Tau, a neuronal microtubule-associated protein (MAP) required for the stability of axonal microtubules, is the major component of PHFs (2, 3). There does not appear to be any AD-specific mutation in the tau gene, and studies indicate that tau mRNA level does not change in AD brain (4). Thus, it is puzzling how tau, a highly soluble axonal protein, becomes insoluble PHF in degenerating neurons during the ontogeny of AD. Various investigators have attempted to reconstitute PHF to elucidate the biochemical steps involved in PHF assembly (5–8). Interestingly, recombinant tau fragments derived from microtubule binding repeats of tau spontaneously dimerize (5, 6). These dimers, when treated with buffers containing high salt and/or low pH, aggregate into PHF-like filaments (5–7). The full-length tau molecules, on the other hand, have a reduced tendency to dimerize under similar conditions (5, 6). These observations suggest that dimerization of tau is a key event leading to PHF assembly and that full-length tau molecules may require some post-translational modification and/or an additional factor to promote their dimerization.

PHF-tau (tau found in PHFs) is abnormally phosphorylated (i.e. contains more phosphate than normal tau (1, 2)). It has a reduced mobility on SDS-PAGE and is incapable of binding to microtubules and promoting microtubule assembly. Upon dephosphorylation, PHF-tau regains its normal mobility on SDS-PAGE and the ability to bind and regulate microtubule dynamics (9, 10). It is thought that in AD brain, abnormal phosphorylation prevents tau from performing its microtubule-related functions, leading to an unstable cytoskeleton and neurodegeneration (1). However, some recombinant tau constructs can assemble into PHF-like filaments without phosphorylation (5–7). As has been pointed out (11), juvenile tau, which shares many abnormal phosphorylation sites with PHF-tau, does not exist as PHFs (12). These observations raised the question as to whether the abnormal phosphorylation observed in PHF-tau is required for normal tau molecules to be converted into PHFs or whether it is merely a secondary event occurring during PHF assembly.

In a previous report, we showed that neuronal cdc2-like
kinase (NCLK), also known as brain proline-directed protein kinase, CDK5, tau kinase II, and PSSLRE kinase co-purifies with microtubules from brain extract (13, 14). In neurons, NCLK is highly expressed and is the major tau kinase activity (15). In brain extract, tau and NCLK are associated with each other, forming a large complex. More importantly, NCLK phosphorylates S-195, S-202, T-205, T-231, S-235, S-396, and S-404 of tau (numbered according to the longest isoform of human tau (4)) (14). Since sites S-202, T-231, S-235, S-396, and S-404 are phosphorylated in PHF-tau (3), NCLK was suggested to be one of the kinases that participates in the abnormal phosphorylation of tau occurring in AD brain (13, 14). In this study, NCLK was used to phosphorylate a recombinant human tau (R-tau) and tau purified from bovine brain homogenate (B-tau), to study the effect of phosphorylation on tau self-association. Herein, it is reported that phosphorylation by NCLK promotes self-association of tau molecules, leading to dimerization.

**MATERIALS AND METHODS**

**Proteins—**R-tau was purified from lysates of Escherichia coli that overexpressed the longest isoform of human tau (htau 40) (16). B-tau was purified from bovine brain extract using perchloric acid (17). NCLK was purified from a fresh bovine brain homogenate (18). Calmodulin and phosphoprotein phosphatase 2B (PP2B) were generous gifts from Dr. Jerry H. Wang (University of Calgary). Polyclonal antibody against bovine tau was prepared in the Medical Research Council, Signal Transduction Antibody Facility of the University of Calgary.

**Phosphorylation and Dephosphorylation—**The phosphorylation mixture contained 25 mM Hepes (pH 7.2), 0.1 mM EDTA, 0.2 mM DTT, 0.1 M NaCl, 0.5 mM [gamma-32P]ATP, 10 mM MgCl2, 2 mg/ml R-tau, and 300 units/ml NCLK (14). R-tau and phosphorylated R-tau to generate Figs. 3, 4, and 5 were prepared by incubating two vials containing all of the components of the phosphorylation mixture, except that one contained buffer and another contained NCLK. After a 4-h incubation at 30 °C, phosphorylated R-tau (R-tau incubated with NCLK) contained 4.9 mol of phosphate/mol of R-tau. Samples in both vials were dialyzed against 25 mM Hepes (pH 7.2), 0.1 mM EDTA, 0.2 mM DTT, 0.1 M NaCl at 4 °C for 14 h in a same vessel. B-tau and phosphorylated B-tau, to generate Figs. 7, B and D, were also prepared as above. After 4 h at 30 °C, B-tau was found to contain 3.5 mol of phosphate/mol of B-tau.

The dephosphorylation mixture contained 25 mM Hepes (pH 7.2), 0.1 mM EDTA, 0.2 mM DTT, 0.1 M NaCl, 0.5 mM CaCl2, 1 mM MnCl2, 10 μg/ml calmodulin, 0.5 mg/ml [gamma-32P]-phosphorylated R-tau, and 25 μg/ml PP2B. To generate Fig. 6, phosphorylated R-tau was divided into two equal halves. Both halves were treated with all the components of the dephosphorylation mixture except one-half was incubated with PP2B and another half with buffer. After 2 h at 30 °C, phosphorylated R-tau in one half containing PP2B was found to be almost completely dephosphorylated (data not shown).

**FPLC Gel Filtration—**Unless otherwise stated, gel filtration was carried out at 4 °C on a Pharmacia FPLC system using a Superose 12 HR 10/30 column (1 × 30 cm) in 25 mM Hepes (pH 7.2), 0.1 mM EDTA, 0.2 mM DTT, and 0.1 M NaCl at a flow rate of 0.25 ml/min. Sample (0.5 ml) was injected into the column, and 250-ml fractions were collected. The column was calibrated using the high molecular weight gel filtration kit (Pharmacia Biotech Inc.), and two types of calibration curves were generated using marker proteins included in the kit. In the first type, Kav (elution volume of the protein – void volume)/total bed volume – void volume) values of the marker proteins were plotted against the log of their molecular weights. In the second type, log(Kav) values of the marker proteins were plotted against their known Stokes radii. The Kav value of R-tau was then determined by passing R-tau through the above column. Based on this determined Kav value, the molecular weight and Stokes radius of R-tau were estimated using first and second calibration curves, respectively.

**Chemical Cross-linking—**Unless otherwise stated, the cross-linking of R-tau and B-tau by disuccinimidyl suberate (DSS) (Pierce) was carried out at room temperature by the addition of 1 μl of DSS stock solution in N,N-dimethyl formamide (DMF) to a vial containing 59 μl of the rest of the components of the cross-linking reaction. The cross-linking mixture contained 25 mM Hepes (pH 7.2), 0.1 mM EDTA, 0.2 mM DTT, 0.1 M NaCl, 1.7% DMF, 166 μM DSS, and 1 mg/ml R-tau or 0.1 mg/ml B-tau. At various time points, aliquots were withdrawn, mixed with an equal volume of SDS-PAGE sample buffer (100 mM Tris-HCl (pH 6.8), 40 mM DTT, 0.2% bromphenol blue, 2% SDS, and 25% glycerol) and analyzed by SDS-PAGE or immunoblotting using antibody against tau. To generate Figs. 2C, 3A, 3B, 5, 6A, 7B, and 7D, the concentrations of two tau species were normalized, and then the cross-linking was carried out at the same time under identical conditions. Cross-linking in Figs. 3A, 3B, 5, 6A, 7B, and 7D were quantitated from the densitometric scans of the blots using personal densitometer SI (Molecular Dynamics). Percent dimer cross-linked was calculated by dividing the optical density of the cross-linked R-tau dimer in a lane of the blot containing DSS-treated R-tau by the optical density of the control R-tau (same amount but treated with the solvent) in that blot.

DSS-treated R-tau to generate Figs. 4, A and D, was prepared by treating R-tau (1.0 mg) with DSS as above for 15 min. To purify DSS-cross-linked R-tau, R-tau (0.5 mg) was treated with DSS. After 15 min of treatment, the entire reaction mixture was passed through a FPLC Superose 12 column. The cross-linked R-tau dimer eluted before noncross-linked R-tau from the column.

**Other Methods—**Gel electrophoresis under nondenaturing conditions was performed in continuous 5% polyacrylamide gels (11). Immunoblotting was performed as described (19). Concentrations of R-tau and B-tau were determined spectrophotometrically (20). Concentrations of phosphorylated R-tau and phosphorylated B-tau were determined by Bio-Rad protein assay using R-tau and B-tau as standards, respectively. Concentrations of all other proteins were determined by Bio-Rad protein assay using bovine serum albumin as standard. The amount of NCLK is based on its activity (18).

**RESULTS**

**Chemical Cross-linking of R-tau—**When R-tau was incubated with DSS, a homobifunctional chemical cross-linker with a spacer arm of 11.4 Å, a progressive increase in the intensity of a protein band heavier than R-tau was observed with increasing time (Fig. 1A, lanes 2–5). When a similar experiment was carried out and the proteins were immunoblotted with antibody against tau protein, both R-tau and the heavier band displayed cross-reactivity (Fig. 1B, lanes 1–6). The molecular weights of R-tau and the cross-linked band were determined in an SDS-PAGE system using standard protein markers (Fig. 1C). Consistent with the previous report (21), R-tau migrated as a 65-kDa band. The apparent molecular mass of the cross-linked band was determined to be 151 kDa, which is ~2.3 times larger than R-tau. Thus the size of the cross-linked band is that of a dimeric R-tau. These observations are consistent with previous reports (11, 22) and indicate that R-tau exists in dimeric form.
FIG. 1. Chemical cross-linking of R-tau by DSS. R-tau was incubated with DSS as described under “Materials and Methods.” At various times aliquots were withdrawn from the reaction mixture and subjected to SDS-PAGE. Gels were either stained for proteins or immunoblotted with antibody against tau protein. A, protein-stained gel. Lane 1, R-tau control (5 μg) incubated with solvent DMF alone for 15 min; lanes 2–5, R-tau (5 μg each) incubated with DSS for 5, 10, 15, and 20 min, respectively. B, immunoblot. Lane 1, R-tau control (5 μg) incubated with solvent DMF alone for 20 min; lanes 2–6, R-tau (5 μg/ lane) incubated with DSS for 2.5, 5, 10, 15, and 20 min, respectively. C, determination of the molecular weight of DSS-cross-linked R-tau dimer. The SDS-PAGE standard curve used to estimate the molecular weight of DSS-cross-linked R-tau dimer. The SDS-PAGE standard curve used to estimate the molecular weight of DSS-cross-linked R-tau dimer. The SDS-PAGE standard curve used to estimate the molecular weight of DSS-cross-linked R-tau dimer.

Since tau is a protein with an asymmetric structure (20, 23), the above molecular weights and Stokes radii of R-tau species determined by a gel filtration column calibrated with globular proteins do not reflect the actual molecular sizes of R-tau species with the asymmetric shapes. Therefore, which peak in Fig. 2A represented R-tau dimer could not be determined from the above gel filtration data. However, the gel filtration data does indicate that the size of R-tau species in peak 1 is approximately double that of the one in peak 2. This means that if peak 1 R-tau is a dimer, peak 2 R-tau is a monomer. Similarly, if peak 2 R-tau is a dimer, then peak 1 R-tau is a tetramer.

To determine which peak is the dimeric R-tau, R-tau species from peak 1 and peak 2 were treated with DSS, and the products were analyzed. As shown in Fig. 2C, DSS readily cross-linked the R-tau species in peak 1 (lanes 6–8). In contrast, the R-tau species in peak 2 cross-linked poorly, and very little cross-linked dimer was apparent, even after treatment with DSS for 15 min (Fig. 2C, lanes 2–4). Since peak 1 and peak 2 overlap, a small amount of cross-linking of peak 2 R-tau species observed in Fig. 2C (lane 4) could be due to contamination from peak 1. These results suggest that peak 1 and peak 2 represent R-tau dimer and monomer, respectively.

To confirm the above suggestion, R-tau species from peak 1, peak 2, and purified DSS cross-linked R-tau dimer (see “Materials and Methods” for purification) were subjected to polyacrylamide gel electrophoresis. As shown in Fig. 2D, on an SDS gel, DSS cross-linked R-tau dimer migrated as a 151-kDa band (lane 2). R-tau species from peak 1 (lane 3) and peak 2 (lane 4) migrated with mobilities similar to that of control R-tau (lane 5). However, on a nondenaturing gel, R-tau species in peak 1 (Fig. 2E, lane 3) and DSS-cross-linked R-tau dimer (Fig. 2E, lane 2) migrated with similar mobilities, whereas R-tau species in peak 2 (Fig. 2E, lane 4) migrated faster than DSS-cross-linked R-tau dimer (Fig. 2E, lane 2). These observations indicate that R-tau in peak 1 is a dimeric species, and because the...
**Fig. 3. Chemical cross-linking and FPLC gel filtration of R-tau and phosphorylated R-tau.** R-tau and phosphorylated R-tau were prepared as described under “Materials and Methods” and subjected to chemical cross-linking and gel filtration. A, chemical cross-linking by DSS. R-tau and phosphorylated R-tau were treated with DSS with concentrations of R-tau and phosphorylated R-tau of 50 μg/ml each. After indicated time points, a 9-μl sample was withdrawn from each cross-linking mixture and immunoblotted with antibody against tau. Lane 1, R-tau control treated with solvent DMF for 15 min; lanes 2–6, R-tau treated with DSS for 1, 2, 5, 10, and 15 min, respectively; lane 7, phosphorylated R-tau control treated with solvent DMF for 15 min; lanes 8–12, phosphorylated R-tau treated with DSS for 15 min. B, comparison of cross-linking of R-tau and phosphorylated R-tau by DSS in three independent experiments. Cross-linking was carried out as in A for 15 min. For each experiment, different R-tau preparation was used. % dimer cross-linked was calculated as described under “Materials and Methods.” C, FPLC gel filtration of R-tau and phosphorylated R-tau. Gel filtration was carried out as in Fig. 2A. D and E, SDS gels of fractions representing R-tau and phosphorylated R-tau, respectively, from C. From the indicated column fractions, a 20-μl sample was removed and electrophoresed.

size of R-tau in peak 2 is approximately one-half the size of R-tau in peak 1, the R-tau species in peak 2 must be the monomeric R-tau.

**Effect of Phosphorylation on the Structure of R-tau—**When R-tau and phosphorylated R-tau were treated with DSS and the products were analyzed, a time-dependent increase in the formation of the 151-kDa dimer in both R-tau (Fig. 3A, lanes 2–6) and phosphorylated R-tau (Fig. 3A, lanes 8–12) was observed. When the gel in Fig. 3A was autoradiographed, both phosphorylated R-tau and the cross-linked bands in lanes 7–12 were found radiolabeled (data not shown), indicating that the cross-linked bands in lanes 8–12 were derived from phosphorylated R-tau. Interestingly, DSS-cross-linked phosphorylated R-tau dimer was visible within 1 min of incubation of phosphorylated R-tau with DSS (Fig. 3A, lane 8), whereas DSS cross-linked R-tau dimer was apparent only after 5 min under identical conditions (Fig. 3A, lane 4). Furthermore, the densitometric quantitation of various bands in Fig. 3A indicated that at 2-, 5-, 10-, and 15-min time points (lanes 9–12), phosphorylated R-tau dimer cross-linked to 8.5, 12.0, 14.4, and 18.3%, respectively. R-tau dimer at 10 and 15 min (lanes 5 and 6) cross-linked to 3.9 and 6.6%, respectively. Thus, DSS cross-linked phosphorylated R-tau dimer ~3-fold more than R-tau dimer. To confirm this finding, three different R-tau preparations were phosphorylated by NCLK. Phosphorylated and non-phosphorylated species from each preparation were treated with DSS under identical conditions, and the cross-linking was evaluated. In all three preparations tested, DSS preferentially cross-linked phosphorylated R-tau dimers over its nonphosphorylated counterpart (Fig. 3B).

**Phosphorylation Promotes Dimerization of R-tau—**To explain the preferential cross-linking by DSS of phosphorylated R-tau over R-tau, the following possibilities were considered. Because R-tau preparations contain a mixture of monomeric and dimeric R-tau species (Fig. 2), phosphorylation may cause monomeric R-tau molecules to dimerize, resulting in an increased number of dimers. Alternatively, phosphorylation may simply make R-tau molecules more accessible to DSS, leading to higher levels of cross-linking irrespective of their oligomeric structures. To discriminate between these two possibilities, R-tau and phosphorylated R-tau were passed through a FPLC Superose 12 column as in Fig. 2A, and their elution profiles were compared. As shown in Figs. 3, C, and D, R-tau eluted as peak 1 and peak 2 representing R-tau dimer and R-tau monomer, respectively. Under identical conditions, phosphorylated R-tau eluted as peak 1 representing R-tau dimer, with a concomitant decrease in the size of the peak 2 representing R-tau monomer (Figs. 3, C, and E). These observations suggest that phosphorylated R-tau is a dimer and enhanced cross-linking by DSS of R-tau upon phosphorylation (Fig. 3A) is due to phosphorylation promoting the dimerization of R-tau.

To substantiate the above idea, DSS-treated R-tau and phosphorylated R-tau were passed through a FPLC Superose 12 gel filtration column, and their elution profiles were compared. As shown in Fig. 4, A and B, DSS-cross-linked R-tau dimer eluted within fractions 38–44, representing peak 1 with peak fraction 40. When passed through the same column under identical conditions, phosphorylated R-tau was also recovered within fractions 38–44, with peak fraction 40 (Figs. 4, A, and C). These observations indicate that the size of phosphorylated R-tau is that of the DSS-cross-linked R-tau dimer and are consistent with phosphorylated R-tau being in dimeric form.

To further substantiate the notion that phosphorylated R-tau is a dimer, DSS treated R-tau, R-tau, and phosphorylated R-tau were treated with SDS to disrupt their oligomeric structures and then were subjected to gel filtration under denaturing condition. As shown in Fig. 4D, DSS-treated R-tau eluted as a broad peak from a Superose 12 gel filtration column equilibrated and eluted with the buffer containing 0.1% SDS. Immunoblot analysis revealed that DSS cross-linked R-tau dimer was present within fractions 26–32, with peak fraction 28 (Figs. 4, D, and E). Under identical conditions, R-tau eluted as a symmetric single peak from the column and was recovered within fractions 30–36 with peak fraction 34 (Figs. 4, D, and F). Note that this behavior of R-tau is different than that observed when subjected to gel filtration under native condition where it elutes as peak 1 and peak 2, representing R-tau monomer and R-tau dimer (Fig. 2A). Since under denaturing condition dimers are expected to dissociate into monomers, all R-tau in Fig. 4D must be in monomeric form. When passed through the same column under identical conditions, phosphorylated R-tau also eluted as a single symmetric peak in a manner similar to R-tau and was present within fractions 30–36 with peak fraction 34.
equilibrated and eluted with 25 mM Hepes (pH 7.2), 0.1 mM EDTA, 0.2 mM DTT, 0.1 M NaCl, and 0.1% SDS. The chromatography was carried out at room temperature with a flow rate of 15 ml/h. Fractions (1.5 ml each) were subjected to FPLC gel filtration as in Fig. 2A, representing DSS-treated R-tau, R-tau, and phosphorylated-R-tau, respectively, from the cross-linking mixture were 25 mM Hepes (pH 7.4), 0.1 mM EDTA, 3.3 μM DTT, 0.1 M NaCl, and 33.3 μg/ml R-tau or phosphorylated R-tau or 3.3 units/ml NCLK. After incubation, 10 μl was withdrawn from each sample, mixed with an equal volume of SDS-PAGE sample buffer without or with 20 mM DTT as indicated.

Effect of Dephosphorylation on the Structure of R-tau—To examine if dephosphorylation could reverse the phosphorylation-enhanced dimerization of R-tau, phosphorylated R-tau was dephosphorylated by PP2B. The phosphorylated R-tau and dephosphorylated R-tau were treated with DSS, and the cross-linking was evaluated. As shown in Fig. 6A, phosphorylated R-tau dimers were cross-linked by DSS as expected (lanes 3–7). Under identical conditions, however, dephosphorylated R-tau cross-linked very poorly even after treatment with DSS for 15 min (lane 13). Thus, dephosphorylation decreased the reactivity of phosphorylated R-tau toward DSS. These results suggest that the dephosphorylated R-tau sample contains a lower number of dimers than its phosphorylated counterpart.

To substantiate the above observation, phosphorylated and dephosphorylated R-tau species were passed through a FPLC gel filtration column, and the effluent fractions were analyzed. Phosphorylated R-tau eluted in peak 1, representing R-tau dimers as expected (Figs. 6, B and C). The dephosphorylated R-tau, on the other hand, was recovered in peak 2, representing R-tau monomers (Figs. 6, B and D). These results further support the suggestion that phosphorylated R-tau dimers dissociate into monomers upon dephosphorylation.

Chemical Cross-linking of B-tau—If dimerization of tau occurs in vivo, tau dimers would be expected to be present in brain extracts. When B-tau purified from bovine brain extract was treated with DSS, a progressive increase in the formation of a cross-linked species with the size of R-tau dimer was indicated that the heavier band is a disulfide cross-linked R-tau dimer. When R-tau and phosphorylated R-tau were diluted in a buffer containing no reducing agent and electrophoresed under low concentrations of DTT (1.65 μM), both R-tau (Fig. 5, lane 4) and phosphorylated R-tau (Fig. 5, lane 6) formed disulfide-cross-linked dimers. Densitometric quantitation of bands in Fig. 5 indicated that the amount of disulfide-cross-linked R-tau dimer in lane 4 was 13% that of control (lane 5). Similarly, the amount of disulfide-cross-linked-phosphorylated R-tau dimer (lane 6) was 45% that of control (lane 7). These observations are consistent with phosphorylation enhancing the dimerization of R-tau.

Phosphorylation Enhances the Formation of Disulfide Cross-linked Dimers of R-tau—When subjected to SDS-PAGE under low concentrations (3 μM) DTT, R-tau formed a heavier band that migrated with a mobility similar to that of DSS-cross-linked R-tau dimer (Fig. 5, lane 2). When the concentration of DTT was increased to 20 mM in the SDS-PAGE sample buffer, this heavier band disappeared (Fig. 5, lane 3). These results suggest that the dephosphorylated R-tau sample contains a lower number of dimers than its phosphorylated counterpart.

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preparations (Fig. 2). These observations are consistent with previous reports (11, 22) and suggest that B-tau contains monomeric and dimeric forms of R-tau, respectively. Similarly, B-tau dimers were also specifically cross-linked by DSS (Fig. 7A). These observations are consistent with previous reports (11, 22) and indicate that tau contains dimeric structure.

DISCUSSION

When tau was first examined by a Sepharose 6B gel filtration column, it eluted as a major peak containing monomeric tau and a heavier shoulder peak (24). The heavier shoulder peak, although not characterized in that study, may have been an oligomeric form of tau. Subsequent studies confirmed that tau exists as oligomers and at least one of the oligomeric form is the dimeric structure (11, 22). In this study, a high resolving FPLC Superose 12 gel filtration column separated R-tau into 195- and 310-kDa species (Fig. 2A). Chemical cross-linking by DSS and gel electrophoresis under nondenaturing conditions revealed that the 195- and 310-kDa species represent mono-
In the presence of a low concentration of DTT (1.65 μM), phosphorylated R-tau formed more disulfide-cross-linked dimers than R-tau (Fig. 5). These observations are consistent with a previous report in which phosphorylated tau isolated from brain extract was shown to contain more disulfide-cross-linked dimers than its nonphosphorylated counterpart (22). Thus phosphorylation of tau also promotes formation of disulfide-cross-linked tau dimers under low concentrations of DTT. It should be noted here that since DTT was included in all experiments except in Fig. 5 as indicated, disulfide cross-linking is not required for dimerization of R-tau, but instead dimerization may precede and facilitate the disulfide cross-linking.

In this study, it is shown that phosphorylation promotes tau dimerization and conversely, dephosphorylation causes dimers to dissociate into monomers. At the present moment, it is not known if self-association of tau occurs in vivo. However, when treated with DSS, B-tau dimers specifically cross-linked and phosphorylation of B-tau by NCLK enhanced this cross-linking (Figs. 7, A-D). Phosphorylated tau dimers were detected in the brain tau prepared by a combination of metal chelating and gel filtration chromatographies (22). PHFs present in the brain of the AD patients are considered to be the result of abnormal self-association of tau molecules because of an aberrant phosphorylation of tau (1). These PHFs dissociate into individual tau molecules upon dephosphorylation by phosphoprotein phosphatases (10). Finally, transfection of cultured cells with tau cDNA caused microtubule bundling (25, 26), which was suggested to occur as a result of tau-tau interactions and tau-induced cross-linking of adjacent microtubules (26). These observations suggest that self-association of tau occurs in vivo and may be regulated by phosphorylation/dephosphorylation of tau. Notably, other neuronal MAPs such as MAP2 and MAP2C also associate to homodimers (26, 27). Thus, it appears that dimerization of MAPs may be a general phenomenon.

Two lines of evidence suggest that dimerization of tau is involved in the formation of PHFs. First, PHF consists of a protease-sensitive outer coat and a protease-resistant inner core. The outer coat can be removed by Pronase, leaving an intact inner core that releases a 12-kDa fragment derived from microtubule-binding repeats of tau upon disruption with formic acid (28, 29). This 12-kDa fragment has a high tendency to microtubule-binding repeats of tau. Notably, other neuronal MAPs such as MAP2 and MAP2C also associate to homodimers (26, 27). Thus, it appears that dimerization of MAPs may be a general phenomenon.

In vitro, phosphorylation by NCLK promotes dimerization and formation of disulfide-cross-linked R-tau dimers under low concentrations of DTT (Fig. 5). Finally, disulfide-cross-linked tau constructs assemble into PHF-like filaments, and the blocking of disulfide cross-linking inhibits the PHF-like filament assembly from these constructs (6).

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