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Heparin-induced Conformational Change in Microtubule-associated Protein Tau as Detected by Chemical Cross-linking and Phosphopeptide Mapping*

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In Alzheimer's disease, microtubule-associated protein tau becomes abnormally phosphorylated and aggregates into paired helical filaments. Sulfated glycosaminoglycans such as heparin and heparan sulfate were shown to accumulate in pretangle neurons, stimulate *in vitro* tau phosphorylation, and cause tau aggregation into paired helical filament-like filaments. The sulfated glycosaminoglycan-tau interaction was suggested to be the central event in the development of neuropathology in Alzheimer's disease brain (Goedert, M., Jakes, R., Spillantini, M. G., Hasegawa, M., Smith, M. J., and Crowther, R. A. (1996) *Nature* 383, 550–553). The biochemical mechanism by which sulfated glycosaminoglycans stimulate tau phosphorylation and cause tau aggregation remains unclear. In this study, disuccinimidyl suberate (DSS), a bifunctional chemical cross-linker, cross-linked tau dimers, tetramers, high molecular size aggregates, and two tau species of sizes 72 and 83 kDa in the presence of heparin. In the absence of heparin only dimeric tau was cross-linked by DSS. Fast protein liquid chromatography gel filtration revealed that 72- and 83-kDa species were formed by intramolecular cross-linking of tau by DSS. These observations indicate that heparin, in addition to causing aggregation, also induces a conformational change in tau in which reactive groups are unmasked or move closer leading to the DSS cross-linking of 72- and 83-kDa species. Heparin-induced structural changes in tau molecule depended on time of heparin exposure. Dimerization and tetramerization peaked at 48 h, whereas conformational change was completed within 30 min of heparin exposure. Heparin exposure beyond 48 h caused an abrupt aggregation of tau into high molecular size species. Heparin stimulated tau phosphorylation by neuronal cdc2-like kinase (NCLK) and cAMP-dependent protein kinase. Phosphopeptide mapping and phosphopeptide sequencing revealed that tau is phosphorylated by NCLK on Thr^{212} and Thr^{231} and by cAMP-dependent protein kinase on Ser^{262} only in the presence of heparin. Heparin stimulation of tau phosphorylation by NCLK showed dependence on time of heparin exposure and correlated with the heparin-induced conformational change of tau. Our data suggest that heparin-induced conformational change exposes new sites for phosphorylation within tau molecule.

Paired helical filaments (PHFs),[1] the major fibrous component of the neurofibrillary tangles associated with Alzheimer's disease (AD), are composed mainly of microtubule-associated protein tau (1, 2; for review, see Ref. 3). PHF-tau (tau isolated from PHFs) has retarded mobility on an SDS-gel, is highly insoluble, is abnormally phosphorylated (*i.e.* contains more phosphate than normal tau), and is functionally inactive (1–3). After dephosphorylation, PHF-tau migrates as normal tau on an SDS-gel and regains the ability to bind to, and regulate, microtubule dynamics (4, 5). Abnormal phosphorylation may prevent tau from performing microtubule-related functions, resulting in cytoskeleton instability, loss of axonal transport, and PHF formation (3). Tau is a natural phosphoprotein, and sites that are phosphorylated in normal adult brain (6) are also phosphorylated in PHF-tau (2). Fetal tau (6), much like PHF-tau (2), is also hyperphosphorylated. Strikingly, both normal adult tau and fetal tau do not form PHFs. Furthermore, PHF-like filaments can be reconstituted from tau molecules that do not contain any phosphate (7–9). These observations have raised the possibility that abnormal phosphorylation alone may not be sufficient, and another factor(s) may be involved in converting tau to PHFs.

There are 19 phosphorylation sites within PHF-tau (2). Aberrant activation of tau-specific kinase (s) has been suggested to lead to the abnormal phosphorylation of tau in AD brain (3), because adult tau is phosphorylated only on four sites (6). Therefore, considerable effort is being made by many investigators to identify kinases that phosphorylate tau. A number of proline-directed and non-proline-directed kinases phosphorylate tau *in vitro* (10–22). Surprisingly, none of these kinases has been shown to be activated in AD brain. Furthermore, the above-mentioned kinases normally phosphorylate a diverse group of proteins in neurons, but in AD brain only phosphorylation of tau is significantly up-regulated (3). Therefore tau, in AD brain, may be phosphorylated either by a kinase (s) that is yet to be identified or by a known kinases in the presence of a tau-specific substrate modulator, which renders tau more susceptible to phosphorylation.

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[1] The abbreviations used are: PHF, paired helical filament; AD, Alzheimer's disease; DMF, *N,N*-dimethyl formamide; DSS, disuccinimidyl suberate; FPLC, fast protein liquid chromatography; mAb, monoclonal antibody; NCLK, neuronal cdc2-like protein kinase; A kinase, cAMP-dependent protein kinase; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

Sulfated glycosaminoglycans (such as heparin, heparan sulfate, chondroitin sulfate, and dermatan sulfate) are sulfated copolymers of glucosamine and uronic acid residues (23). Several studies have indicated the presence of glycosaminoglycans in senile plaques and neurofibrillary tangle (9, 24–27). Recently, heparan sulfate was shown to accumulate in pretangle neurons (9), to stimulate *in vitro* tau phosphorylation by various kinases (28–32), to prevent tau from binding to microtubules, and to cause tau to aggregate into PHF-like filaments (9). An increase in the sulfated glycosaminoglycans within the nerve cells was suggested to trigger the hyperphosphorylation of tau, destabilization of microtubules, and assembly of PHFs (9). The interaction of sulfated glycosaminoglycan and tau was suggested to be the central event in the development of neuropathology in AD (9, 33). However, the biochemical mechanism by which glycosaminoglycans enhance tau phosphorylation and cause tau to aggregate into PHFs remains unclear.

In this study, we have investigated the effect of heparin on the structure and phosphorylation of tau by chemical cross-linking and phosphopeptide mapping. Herein we report that heparin, in addition to causing aggregation of tau, also changes tau's conformation, exposing new sites within the tau molecule for kinase phosphorylation.

MATERIALS AND METHODS

Proteins and Peptides—Tau protein used in this study was purified from extracts of *Escherichia coli* overexpressing the longest isoform of human tau (htau 40) as described (19), except effluent from Q-Sepharose column was chromatographed through an S-Sepharose column. Neuronal cdc2-like protein kinase (NCLK) was purified from fresh bovine brain extract as described previously (34). cAMP-dependent protein kinase (A kinase), catalytic subunit of A kinase (C subunit), trypsin, and thermolysin (protease type X), and Kemptide (LRRASLG) were from Sigma. Preparations of polyclonal antibody against bovine brain tau and synthetic peptide substrate of NCLK (KTPKKAKKPKT-PKKAKKL) were described previously (19). The concentration of tau was estimated spectrophotometrically (35). Amounts of A kinase, C subunit, and Kemptide were based on their dry weights. Concentration of synthetic peptide NCLK substrate was determined by amino acid analysis. The amount of NCLK was estimated by enzymatic activity (34).

Kinase Assay—Unless otherwise stated, NCLK activity was measured as described previously (19) in an assay mixture containing 25 mM Hepes (pH 7.2), 0.1 mM EDTA, 0.2 mM dithiothreitol, 60 mM NaCl, 0.5 mM [^{32}P]ATP, 10 mM $MgCl_2$, 50 μM peptide substrate, or 0.5 mg/ml tau and 400 units/ml NCLK. The assay was initiated by the addition of 5 μl of kinase to a 20-μl mixture containing the rest of the assay mixture components. After 20 min at 30 °C, aliquots were withdrawn and analyzed for the amount of radioactivity incorporated into the substrate by phosphocellulose strip assay. Activity of C subunit was determined as above, except Kemptide was used as the peptide substrate, and the concentration of C subunit was 10 μg/ml. A kinase was assayed in a manner similar to that described above for C subunit, except the assay mixture also contained 10 μM cAMP.

Chemical Cross-linking—Chemical cross-linking of tau by disuccinimidyl suberate (DSS), a homobifunctional chemical cross-linker with an 11.4-Å spacer arm, was performed essentially as described previously (36) in a mixture containing 1.5 mg/ml tau, 0.1 mM EDTA, 0.2 mM dithiothreitol, 60 mM NaCl, 1 mM DSS (Pierce) and 2% *N,N*-dimethyl formamide (DMF). The reaction was initiated by the addition of 1 μl of DSS stock solution in DMF to 49 μl of mixture containing the rest of the cross-linking mixture components. After various time points at room temperature, aliquots were removed, mixed with an equal volume of SDS-PAGE sample buffer (0.1 M Tris-HCl, pH 6.8, 25% glycerol, 0.2% bromphenol blue, 10% β-mercaptoethanol, and 2% SDS), boiled, and electrophoresed on a 7.5% Laemmli SDS-gel. The amounts of cross-linked bands were quantitated by scanning the gels using a Molecular Dynamics SI personal densitometer. Band intensities were determined by dividing the optical density of each cross-linked band with the band intensity of tau control (treated with the solvent). The amount of tau that was not recovered in the gel was expressed as the higher molecular size species that did not enter the gel after DSS cross-linking. Apparent molecular weights of various cross-linked species were determined essentially as described previously (36).

Phosphopeptide Mapping and Purification of Phosphopeptides—Tau (0.5 mg), phosphorylated for 6 h by NCLK or A kinase, was digested with trypsin and subjected to HPLC C_{18} reverse phase chromatography essentially as described previously (19). To purify phosphopeptide 1, peak 1 fractions depicted in Fig. 5*A* were combined, concentrated to ~0.5 ml, and chromatographed through a Sephadex G-25 column (0.5 × 25 cm) preequilibrated and eluted with 0.1% trifluoroacetic acid. The effluent fractions (0.5 ml each) were collected. Only one radioactive peak eluted from the column. Fractions containing radioactivity were combined, concentrated, and injected into an HPLC column as described above. The column was eluted with an acetonitrile gradient of 0–30% in 50 min. Phosphopeptide 3b was purified from peak 3 (see Fig. 5*B*) fractions and is shown in Fig. 6*B*. To purify phosphopeptide 4, peak 4 fractions (see Fig. 5*B*) were vacuum dried and redissolved in 0.2 ml of 50 mM NH_4HCO_3 (pH 8.0) containing 25 μg/ml thermolysin. The sample was then incubated at 37 °C for 3 h and then injected into an HPLC column. The peptide was then eluted from the column by a linear gradient of acetonitrile (0–40%) in 50 min. To purify phosphopeptide e, peak e fractions (see Fig. 7*B*) were combined, vacuum dried, dissolved in 500 μl of 50 mM NH_4HCO_3 containing 25 μg/ml thermolysin, and incubated at 37 °C for 3 h. After incubation, the sample was loaded onto a ~1-ml DEAE-Sephacel (Sigma) column pre-equilibrated in 25 mM Hepes (pH 7.0). The column was washed with 10 ml of equilibration buffer and eluted with 0.25 M NaCl in equilibration buffer. Effluent fractions (0.2 ml each) were collected. Fractions containing radioactivity were combined and concentrated to ~0.2 ml, and the phosphopeptide was purified by HPLC as above using acetonitrile gradient 0–40% in 50 min. Phosphopeptides were sequenced using a gas phase amino acid sequencer (19) at the Department of Biochemistry and Microbiology, University of Victoria.

RESULTS

Chemical Cross-linking of tau—When tau was incubated with DSS and the product was analyzed by SDS-PAGE, there was a time-dependent formation of a heavier species with a concomitant decrease in the tau band intensity (Fig. 1*A*, *lanes 2–4*). The molecular size of the heavier band on an SDS-gel was estimated to be ~153 kDa. In a previous study we have shown that the tau isoform used in this study that migrates as a 65-kDa band on the SDS-gel is a mixture of tau monomers and dimers when purified from bacterial lysate. These dimers, when cross-linked by DSS, migrate with a size of ~151 kDa on an SDS-gel (36). Thus, the cross-linked band in Fig. 1*A*, *lanes 3* and *4*, is the dimeric tau.

Cross-linking of tau under identical conditions in the presence of heparin showed four major differences when compared with tau cross-linked alone (Fig. 1). First, the intensity of the dimeric band was higher in tau cross-linked in the presence of heparin (Fig. 1, compare *lanes 3, 4* and *6–8*). Second, a band with size of ~246 kDa was formed in the presence, but not in the absence, of heparin. Because the molecular size of tau is ~65 kDa (Fig. 1*A*), this 246-kDa band is ~3.8 times heavier than tau and must therefore be tetrameric tau. Third, a protein band that migrated as a streak on the top portion of the gel was formed only when heparin was present in the cross-linking mixture (Fig. 1, *lanes 7* and *8*). This band must be a heavy molecular size tau aggregate cross-linked by DSS. Fourth, in the presence of heparin, two species of molecular sizes 72 and 83 kDa were also cross-linked by DSS (Fig. 1, *lanes 6–8*). When immunoblotted using an anti-heparin monoclonal antibody (mAb; Chemicon), none of the above cross-linked bands displayed immunoreactivity (data not shown), indicating that the DSS cross-linked bands were not formed by tau-heparin cross-linking. Densitometric quantitation of various bands in Fig. 1*A* indicated that at the 15-min time point 3.5% dimeric tau was cross-linked by DSS in the absence of heparin, whereas in the presence of heparin 4.1, 6.2, 10.5, 7.5, and 3.0% tau was cross-linked into 72-kDa, 83-kDa, dimer, tetramer, and higher molecular size species, respectively.

The cross-linking of dimeric, tetrameric, and higher aggregates by DSS in Fig. 1 suggested that heparin promotes dimer-

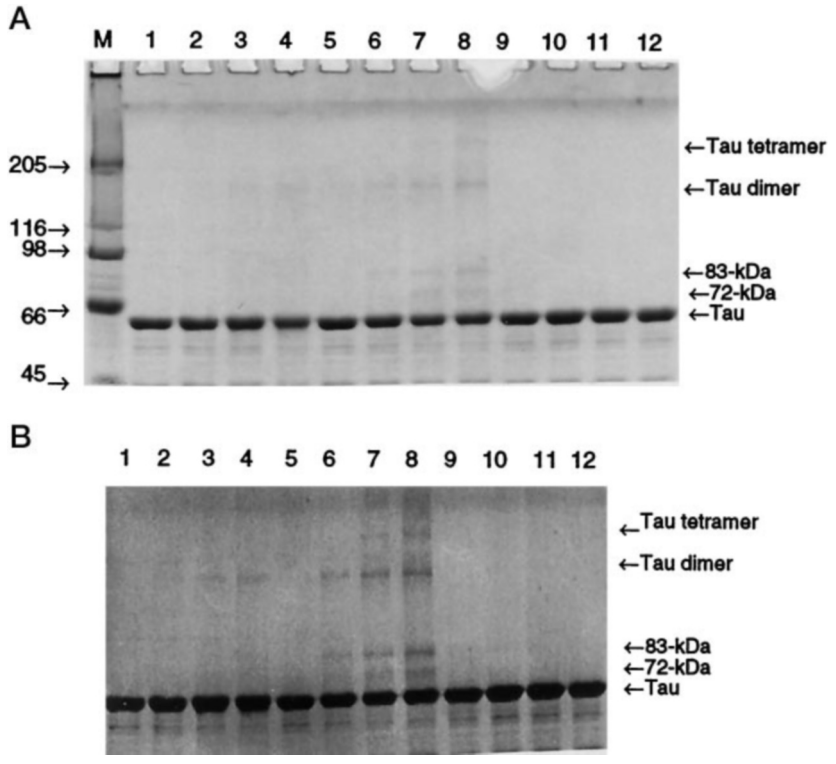

FIG. 1. **Chemical cross-linking of tau by DSS in the presence and absence of heparin.** Tau was preincubated for 30 min at room temperature with heparin or water in a mixture containing 25 mM Hepes (pH 7.2), 0.1 mM EDTA, 0.2 mM dithiothreitol, 60 mM NaCl, 0.2 mg/ml heparin, and 1.5 mg/ml tau. DSS was added to each preincubated sample to initiate the cross-linking. After various time points at room temperature, 10 μl was removed from each reaction mixture and mixed with 20 μl of SDS-PAGE sample buffer, and 10 μl was subjected to SDS-PAGE. Gels were either stained for proteins or subjected to immunoblotting using antibody against tau. *A*, protein stained gel; *B*, immunoblot; *M*, molecular size marker. *Lanes 1–4*, tau preincubated with water and treated with DSS for 1, 2.5, 5, and 10 min, respectively; *lanes 5–8*, tau preincubated with heparin and treated with DSS for 1, 2.5, 5, and 10 min, respectively; *lanes 9–12,* tau preincubated with heparin and treated with solvent (DMF) for 1, 2.5, 5, and 10 min, respectively. The immunoblot in *B* shows that all the cross-linked bands are derived from tau.

ization and causes the formation of tetrameric and higher molecular sized tau species. These observations are consistent with previous reports and indicate that heparin causes tau aggregation (9, 32). To interpret the cross-linking of 72- and 83-kDa bands by DSS in the presence of heparin (Fig. 1), we wished to know whether these two cross-linked bands were formed by cross-linking of tau intermolecularly or intramolecularly. Because DSS cross-linked dimer and tetramer migrated with molecular sizes of 153 and 246 kDa, respectively, on an SDS-gel (Fig. 1*A*), the sizes 72 and 83 kDa are too small to be tau dimer, trimer, or tetramer that would have been formed if tau were cross-linked intermolecularly.

To substantiate the idea that 72- and 83-kDa bands are formed by intramolecular cross-linking, we cross-linked tau (0.75 mg) with DSS in the presence of heparin as described under "Materials and Methods." The cross-linked tau was then fractionated through a fast protein liquid chromatography (FPLC) Superose 12 gel filtration column (Fig. 2*A*), and various column fractions were immunoblotted using anti-tau antibody (Fig. 2*C*). Tau tetramers were recovered within fractions 32–36 with peak fraction 34. Tau dimers were present within fractions 36–40 with peak fraction 38. The 72- and 83-kDa species were present within fractions 40–44 with peak fraction 42. Importantly, tau chromatographed through the same column under identical conditions eluted with peak fraction 42 (Fig. 2*B*). Thus, the sizes of 72- and 83-kDa bands correspond to monomeric tau. As shown in Fig. 1, 72- and 83-kDa species are cross-linked by DSS only in the presence of heparin. These observations indicated that heparin causes a conformational change that exposes groups reactive to DSS within tau molecule leading to intramolecular covalent cross-linking of tau by DSS. These cross-linked tau species migrate slightly slower than tau on SDS-gels.

Effect of Preincubation Time on Chemical Cross-linking—In a previous study (9), heparin-induced aggregation of tau was reported to be dependent on time of incubation, and PHF-like filaments were formed after incubating tau with heparin for >48 h. We therefore preincubated tau with heparin for various time points. Aliquots were removed from the preincubation mixture, treated with DSS, and subjected to SDS-PAGE. The intensities of various bands on the gel were quantitated. The amount of tau that was not recovered on the gel was regarded as the high molecular size aggregate, which, after cross-linking, did not enter the SDS-gel.

As shown on Fig. 3, *A* and *B*, the intensities of the dimeric and tetrameric bands increased with increasing time of preincubation, peaked at 48 h (Fig. 3*A*, *lane 11*) and diminished significantly at 72 and 96 h. The formation of the higher aggregate tau, which was slow until 48 h, sharply increased with concomitant decrease in the intensities of all other cross-linked and monomeric tau bands when incubated beyond 48 h (Fig. 3*B*).

Like the dimeric band, 72- and 83-kDa bands were visible within a few min of preincubation with heparin (Fig. 3*A*, *lane 3*) and displayed a biphasic effect with respect to preincubation time (Fig. 3*B*). However, these two bands peaked at the 30-min time point (Fig. 2*A*, *lanes 6* and *B*), remained constant for several hr, and then declined very slowly until 48 h (*lane 11*). At 72 and 96 h, both bands progressively faded.

In conclusion, the dimerization and tetramerization of tau increased with the increase in heparin incubation time until 48 h. The formation of higher molecular size aggregate was slow during the initial period of heparin incubation until 48 h. Incubation with heparin beyond 48 h converted almost all tau species into the higher molecular size aggregate. Heparin-induced conformational change of tau, as detected by DSS cross-linking of 72- and 83-kDa species, completed within ~30 min of heparin exposure.

Effect of Heparin on tau Phosphorylation—In addition to causing aggregation, heparin is known to stimulate tau phosphorylation by various kinases (28–32). To further investigate this phenomenon, we measured the activity of NCLK in the presence of heparin. Heparin stimulated the tau phosphorylation activity of NCLK but not of a synthetic peptide substrate (Fig. 4*A*). To test whether heparin affects tau phosphorylation by other kinases also, we examined tau phosphorylation by A

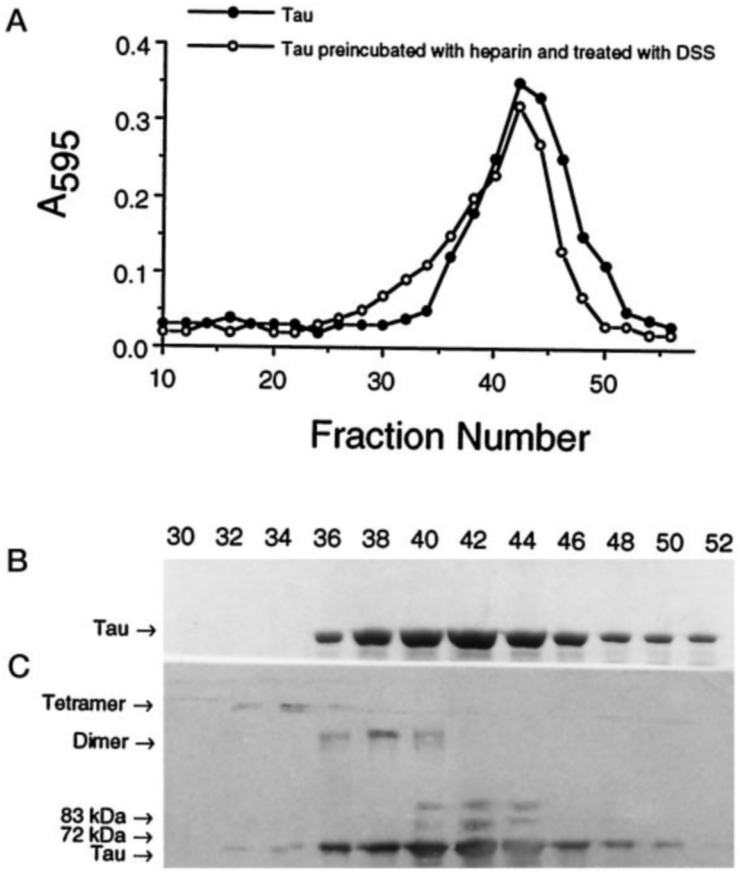

FIG. 2. **FPLC gel filtration of tau and tau preincubated with heparin and treated with DSS.** Tau (0.75 mg) was preincubated with heparin in a total volume of 0.5 ml as described in the legend to Fig. 1. After 30 min of preincubation, 1 μl of stock DSS solution was added to initiate the cross-linking. After 15 min at room temperature, 10 μl of 1.5 M Tris-HCl (pH 8.8) was added to quench the cross-linking, and then the entire sample injected into a gel filtration column. Gel filtration was carried out on an Amersham Pharmacia Biotech FPLC Superose 12 HR 10/30 column (1 × 30 cm) at a flow rate of 0.5 ml/min. Fractions (0.5 ml each) were collected, and 20 μl from indicated fraction was analyzed by SDS-PAGE. *A*, FPLC gel filtration. *B* and *C*, SDS-gels of column fractions representing tau and tau preincubated with heparin and treated with DSS, respectively.

kinase (Fig. 4*B*). Heparin was stimulatory of tau phosphorylation activity of A kinase and had no effect on the phosphorylation of Kemptide, a synthetic peptide substrate of A kinase. These results suggested that heparin is a substrate modulator acting through tau.

Phosphopeptide Mapping—To gain further insight into the effect of heparin on tau phosphorylation, tau was phosphorylated by NCLK in the presence and absence of heparin under identical conditions. Phosphorylated tau species were digested with trypsin and fractionated through an HPLC reverse phase column. As shown in Fig. 5, tau phosphorylated in the absence of heparin showed three radioactive peaks (Fig. 5*A*, *peaks 1–3*). Phosphopeptide map of tau phosphorylated in the presence of heparin (panel *B*) showed three differences when compared with that of tau phosphorylated in the absence of heparin (Fig. 5*A*). First, *peak 1* present in Fig. 5*A* was absent in Fig. 5*B*. Second, *peak 4* was present in Fig. 5*B* but not in Fig. 5*A*. Third, *peak 3* in Fig. 5*B* was larger than *peak 3* in Fig. 5*A*.

The presence of *peak 1* (Fig. 5*A*) only in the phosphopeptide map of tau phosphorylated in the absence of heparin indicated that heparin may have altered the digestibility of tau by trypsin leading to the disappearance of peak 1, or the site (s) corresponding to peak 1 was either lost or altered in the presence of heparin. To determine this site(s), peak 1 fractions were combined, concentrated, and chromatographed through a Sephadex G 25 followed by HPLC. Only one phosphopeptide designated as peptide 1 was isolated (data not shown). This peptide was subjected to 10 cycles of Edman degradation using a gas phase amino acid sequencer. As shown in Table I, the amino acid sequence of peptide 1 is TPPKXPSSAK, where X is the unidentified PTH amino acid indicating that the 5th residue is phosphorylated. This idea is further confirmed by the release of radioactivity during the fifth cycle. Based on these results, and the known sequence of tau protein (37), peptide 1 is determined to extend from residues 231–240 of tau, and Ser^{235} (numbered according to the longest isoforms of human tau; Ref. 37) is the phosphorylation site.

The presence of *peak 4* only in the map of tau phosphorylated in the presence of heparin (Fig. 5*B*) indicated that either heparin affects the cleavage of tau by trypsin, causing the appearance of peak 4 or NCLK phosphorylates tau on novel site (s) in the presence of heparin. To determine this site (s), fractions containing peak 4 from the phosphopeptide map of tau phosphorylated in the presence of heparin were combined and digested with thermolysin. The digested peptide was chromatographed through HPLC. Only one phosphopeptide, designated peptide 4, was recovered. As shown in Table I, the amino acid sequence of peptide 4 is VVRXPPKXPSS, where X represents the unidentified PTH amino acid. This peptide released radioactivity during the fourth and eighth cycles (Table I). Based on these observations and the sequence of tau (37), peptide 4 was determined to extend from residues 228–238 of tau, and Thr^{231} and Ser^{235} are the phosphorylation sites.

The above phosphopeptide analysis data showed that NCLK phosphorylates tau on Thr^{231} and Ser^{235} in the presence of heparin. However, Ser^{235} is also phosphorylated by NCLK in the absence of heparin (Table I, phosphopeptide 1). Therefore, in the presence of heparin a new site, Thr^{231}, is phosphorylated by NCLK.

Peak 3 in the phosphopeptide map of tau phosphorylated by NCLK, in the presence of heparin, (Fig. 5*B*) is larger than the *peak 3* phosphorylated in the absence of heparin (Fig. 5*A*). We reasoned that in the presence, as opposed to the absence, of heparin NCLK either phosphorylates the site (s) corresponding to peak 3 more completely, or NCLK phosphorylates tau on an additional site(s). To discriminate between these two possibilities, fractions containing peak 3 from the phosphopeptide maps of tau phosphorylated in the presence and absence of

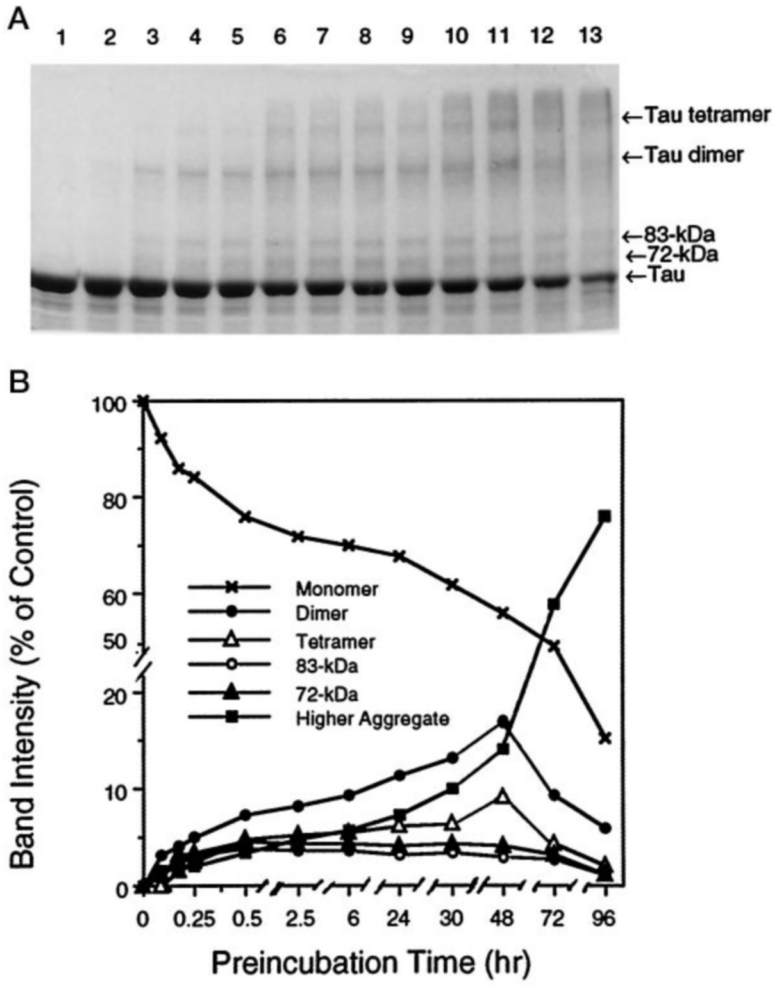

FIG. 3. **Chemical cross-linking of tau preincubated with heparin for various time points.** Tau was preincubated with heparin as described in the legend to Fig. 1. Each preincubated sample was treated with DSS for 15 min, and the was product analyzed by SDS-PAGE. *A*, SDS-gel. *Lane 1*, tau (7.5 μg) preincubated with heparin for 96 h; *lane 2*, tau (7.5 μg) preincubated with DMF for 96 h and treated with DSS; *lanes 3–13*, tau (7.5 μg each) preincubated with heparin for 0.1, 0.17, 0.25, 0.5, 2.5, 6, 24, 30, 48, 72, and 96 h, respectively, and treated with DSS. *B*, intensities of various bands in *A*. Band intensities were determined as described under "Materials and Methods" and are expressed as % of control tau on *lane 1*.

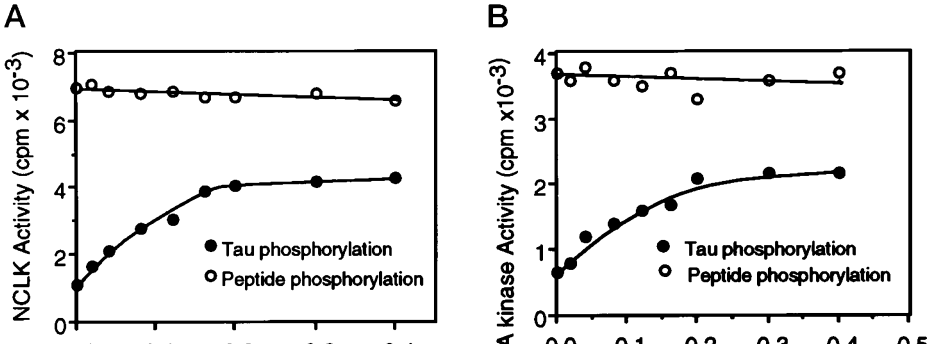

FIG. 4. **Effect of heparin on the activities of NCLK (*A*) and A kinase (*B*).** Activities of NCLK and A kinase were measured for 20 min in the presence of indicated amounts of heparin using either peptide or tau as the substrate.

heparin (Fig. 5, *A* and *B*) were passed through a Sephadex G-25 column and subsequently subjected to HPLC peptide mapping. As shown in Fig. 6*B*, peak 3 of tau phosphorylated in the presence of heparin contained two phosphopeptides, *3a* and *3b*. Peak 3 of tau phosphorylated in the absence of heparin contained only one phosphopeptide, *3a* (Fig. 6*A*), indicating that NCLK phosphorylates tau on additional sites in the presence of heparin. The amino acid sequence of phosphopeptide 3b is shown in Table I. This peptide extends from tau residues 210–221, and Thr^{212} is the phosphorylation site. Thus, Thr^{212} is phosphorylated by NCLK only in the presence of heparin.

An HPLC tryptic phosphopeptide map of tau phosphorylated by A kinase in the absence of heparin contained four radioactive peaks, designated *a–d* (Fig. 7*A*). The tryptic phosphopeptide map of tau phosphorylated by A kinase under identical conditions, but in the presence of heparin, in addition to *peaks a–d* also contained *peak e* (Fig. 7*B*, *arrow*). These observations indicated that peak e may have resulted from heparin either altering the cleavage of tau by trypsin or A kinase phosphorylating tau on an additional site(s) in the presence of heparin.

To determine the site(s) corresponding to *peak e* (Fig. 7*B*), peak e fractions were combined, vacuum dried, and digested with thermolysin. The digest was subjected to DEAE-Sephacel followed by HPLC chromatography. Only one phosphopeptide was recovered. This phosphopeptide was designated peptide e. The sequence of peptide e is IGXTEN (Table I). This peptide is

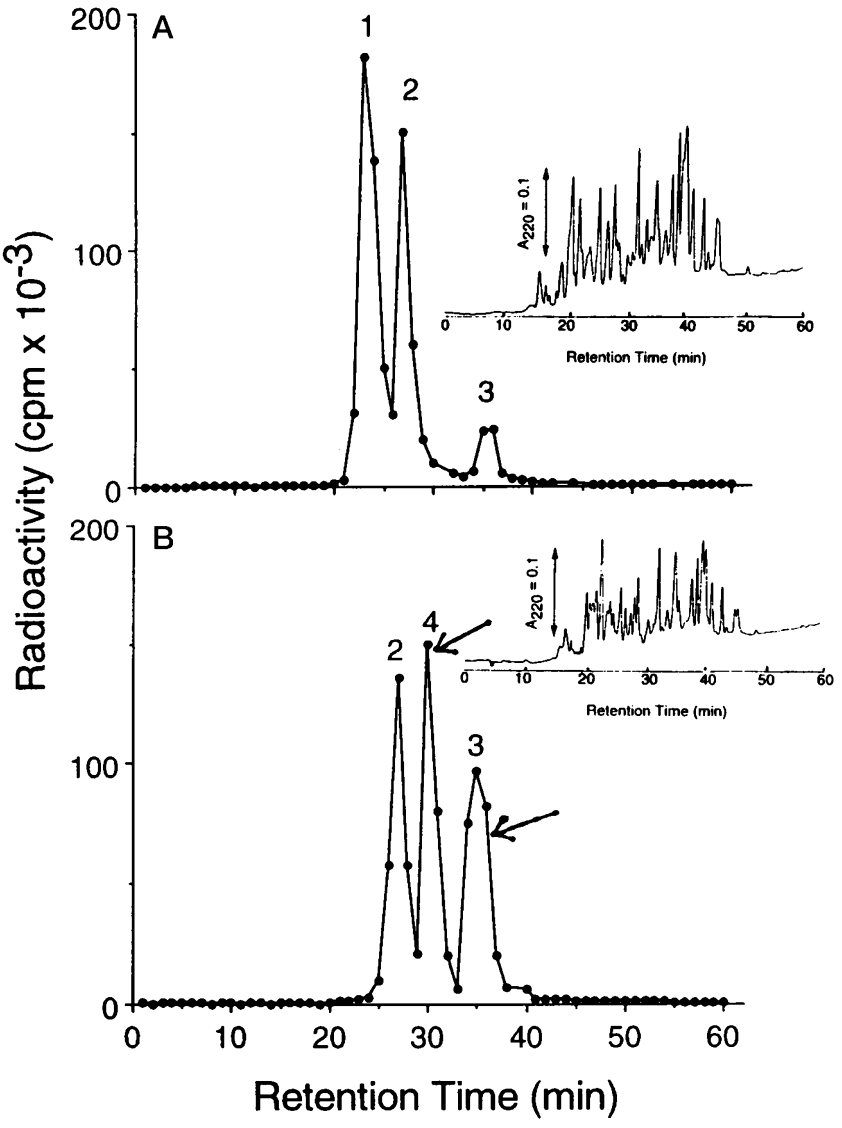

FIG. 5. **HPLC tryptic phosphopeptide maps of tau phosphorylated by NCLK in the absence (*A*) and presence (*B*) of heparin.** Two tau species (0.5 mg each) phosphorylated by NCLK in the presence and absence of heparin for 6 h at 30 °C were digested with trypsin under identical conditions and subjected to HPLC reverse phase chromatography through a Delta Pak 5-μm 100-A C_{18} reverse phase column (Millipore) using a Waters HPLC system at a flow rate of 0.5 ml/min. The column was eluted with a linear gradient of 0–40% acetonitrile in 50 min. Fractions (0.5 ml each) were collected, and 10 μl from each fraction was counted in a liquid scintillation counter. *Insets*, HPLC profiles.

TABLE I
Sequence determination of phosphopeptides

The amino acid sequence of each phosphopeptide was determined by Edman degradation using a gas phase sequencer. AA, PTH amino acid identified after each cycle; Yield, pmol of PTH amino acid released after each cycle; cpm, amount of radioactive released in each cycle; X, amino acid whose PTH derivative could not be identified. Refer to the text for the nomenclature of phosphopeptides.

Cycle	1			4			3b			e		
	AA	Yield	cpm	AA	Yield	cpm	AA	Yield	cpm	AA	Yield	cpm
1	Thr	141	250	Val	81	181	Ser	12	100	Ile	82	111
2	Pro	134	300	Val	79	492	Arg	16	1066	Gly	227	2372
3	Pro	139	730	Arg	45	753	Xaa		4057	Xaa		2795
4	Lys	64	810	Xaa		2258	Pro	11	3612	Thr	55	2222
5	Xaa		4992	Pro	21	1702	Ser	9	2292	Glu	56	896
6	Pro	81	2877	Pro	28	914	Leu	9	1776	Asn	19	664
7	Ser	39	1960	Lys	24	860	Pro	6	914			
8	Ser	43	574	Xaa		1761	Thr	7	615			
9	Ala	76	437	Pro	5	1178	Pro	6	332			
10	Lys	7	300	Ser	3	960	Pro	5	350			
11				Ser	2	800	Thr	4	310			
12							Arg	2	312			

derived from tau residues 260–265, and Ser^{262} is phosphorylated. Previously, Ser^{262} was shown not to be phosphorylated by A kinase (16). Based on these observations, and complete absence of peak e in the phosphopeptide map of tau phosphorylated by A kinase in the absence of heparin (Fig. 7*A*), we concluded that A kinase phosphorylates Ser^{262} only in the presence of heparin.

Our data indicated that NCLK phosphorylates tau on Ser^{212} and Thr^{231} only in the presence of heparin. Similarly, A kinase phosphorylates Ser^{262} only when heparin is present in the phosphorylation mixture. To confirm that these effects are caused specifically by heparin, and not by any other unknown component(s) present in the phosphorylation mixture, we prepared a series of tau species phosphorylated under different conditions. Using heparin from two different sources (Sigma and Fisher), tau was phosphorylated by NCLK and A kinase. We also phosphorylated tau in the presence and absence of heparin using NCLK from two different preparations. Similarly, we prepared tau phosphorylated by A kinase and catalytic subunit of A kinase in the presence and absence of heparin. Finally, tau from two independent preparations was phosphorylated by NCLK and A kinase in the presence and absence of heparin. These phosphorylated tau species were digested with trypsin, and HPLC phosphopeptide maps, as in Figs. 5 and 7, were generated and compared. All tau species phosphorylated by NCLK in the absence of heparin contained three radioactive peaks corresponding to *peaks 1-3* in Fig. 5*A*, and tau species phosphorylated by NCLK in the presence of heparin had radioactive peaks corresponding to *peaks 2-4* in Fig. 5*B*. On all occasions, the size of peak 3 was larger in the

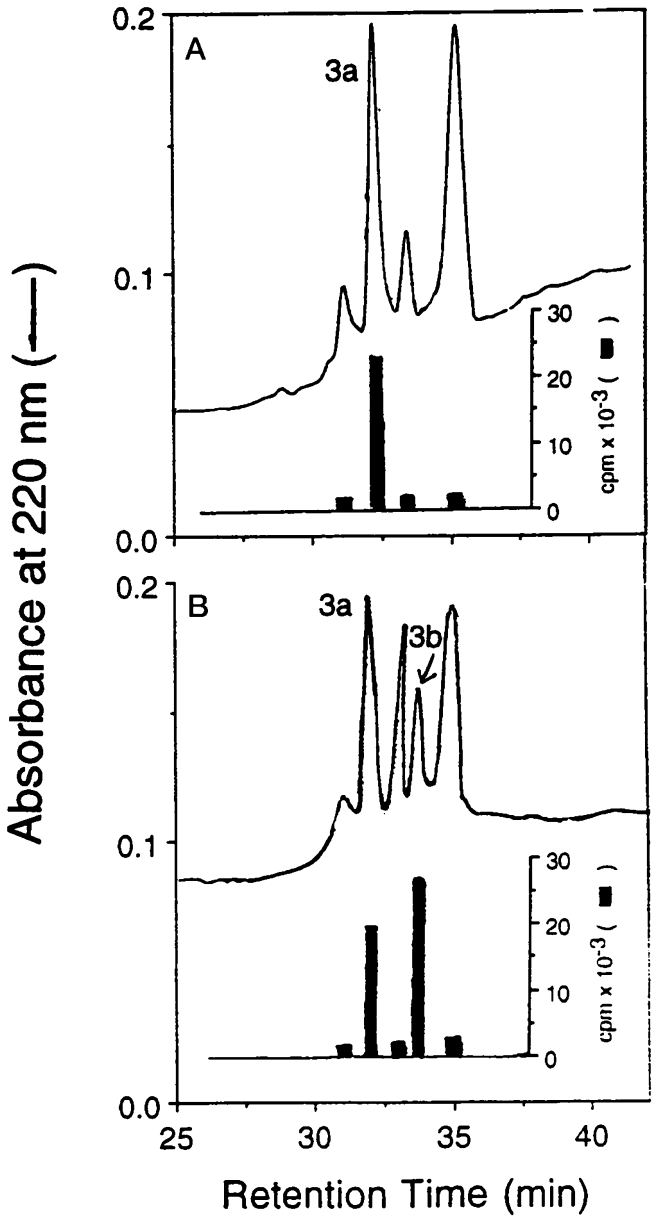

FIG. 6. **HPLC peptide maps of peak 3 fractions from Fig. 5, *A* and *B*.** Peak 3 fractions from Fig. 5, *A* and *B* were pooled separately and fractionated through a Sephadex G 25 column. Effluent fractions containing radioactivity were pooled, lyophilized, redissolved in 0.2 ml of 0.1% trifluoroacetic acid, and rechromatographed by HPLC. All the chromatographic conditions were same as in Fig. 5, except the acetonitrile gradient was 0–45% in 50 min. Effluent-containing peptide peaks were manually collected, and 10 μl was counted in a scintillation counter. *A*, HPLC profile of *peak 3* from Fig. 5A; *B*, HPLC profile of *peak 3* from Fig. 5*B*. Note that phosphopeptide *3b* (*arrow*) is present only in *B*. This peptide was subjected to amino acid sequencing.

map of tau phosphorylated in the presence of heparin (data not shown).

Similarly, all tau species phosphorylated by A kinase and C subunit in the absence of heparin had identical phosphopeptide maps and contained radioactive peaks corresponding to *peaks a–d* in Fig. 7*A*. Tau species phosphorylated by A kinase or C subunit in the presence of heparin contained five peaks corresponding to *peaks a–e* in Fig. 7*B* (data not shown).

Cross-linking and Activation—To determine whether stimulation of tau phosphorylation by heparin is caused by heparin-induced tau aggregation and/or change in tau conformation, we preincubated tau with heparin for various time points, and the product was subjected to DSS cross-linking or phosphorylation by NCLK. Stimulation of tau phosphorylation rapidly increased within a few min of heparin exposure, peaked in 30 min, and slowly declined until 48 h (Fig. 8*B*, *filled circles*). At 72 h stimulation of phosphorylation sharply declined and completely vanished at 96 h. These observations suggest that the structural change responsible for enhancing tau phosphorylation (Fig. 8*B*) is completed within 30 min of heparin exposure.

As shown in Fig. 8, *A* and *B*, the dimeric and tetrameric bands rapidly appeared, and their intensities increased continuously until 48 h. The intensity profiles of both the 72- and 83-kDa bands increased rapidly and peaked in 30 min (Fig. 8*A*, *lane 5*) in a manner similar to phosphorylation (Fig. 8*B*). Like phosphorylation, the intensity profiles of both bands declined slowly until 48 h. Thus we observed a correlation between formation of 72- and 83-kDa bands and stimulation in tau phosphorylation by NCLK with respect to heparin preincubation time (Fig. 8*B*). These experiments were repeated two more times using two different tau preparations and heparin from two different sources. In all occasions identical results were obtained. These correlative data strongly support the notion that heparin induces a conformational change, making tau a better substrate for kinase phosphorylation.

DISCUSSION

The two characteristic features of PHFs are tau aggregation and abnormal phosphorylation (1–3). However, the cellular and biochemical mechanisms by which tau becomes abnormally phosphorylated and aggregates into PHFs is not clear. The presence of sulfated glycosaminoglycan in pretangle neurons (9, 24–27) and the ability of heparin to cause tau to aggregate into PHF-like filaments (9, 32) and to stimulate *in vitro* tau phosphorylation by various kinases (28–32) have recently generated considerable interest. The interaction of tau with sulfated glycosaminoglycan was suggested to be the key event leading to the abnormal phosphorylation and aggregation of tau that occurs in degenerating neurons of patients with AD (9, 33). Among all sulfated glycosaminoglycans tested, heparin was found to be one of the most effective in causing tau's aggregation (9, 32).

In this study, we investigated the effect of heparin on tau structure. Our cross-linking data indicate that in the presence of heparin, tau forms dimers, tetramers, and high molecular size aggregates in a time-dependent manner. Dimers are the first oligomers formed during the heparin-induced oligomerization of tau, followed by tetramers and the higher molecular size aggregates (Fig. 3). Although not analyzed in this study, but based on previous reports (9, 32), these high molecular size species are likely to be PHF-like filaments. These observations are consistent with the hypothesis that dimerization of tau precedes PHF assembly (7).

The optimal aggregation of tau into PHF-like filaments was reported to require exposure of tau to heparin over 48 h (9). In this study, we found that when tau was exposed to heparin, dimers followed by tetramers were formed, and formation of these two species displayed a biphasic effect with respect to time of heparin exposure. Amounts of both species increased with exposure time and peaked at exposure time of 48 h (Fig. 3). Exposure beyond 48 h caused loss of monomers, dimers, and tetramers. The formation of high molecular size aggregate was slow during the first part of the exposure period. Even at 48 h, only ~16% tau aggregated into higher molecular size species. At exposure time 72 h, however, ~60% tau was lost as higher molecular size species. Thus, such an abrupt transition of tau into high molecular size species indicates that heparin-induced aggregation of tau is a cooperative process as described for protein aggregation (38, 39), and 48 h is the threshold time point for this aggregation.

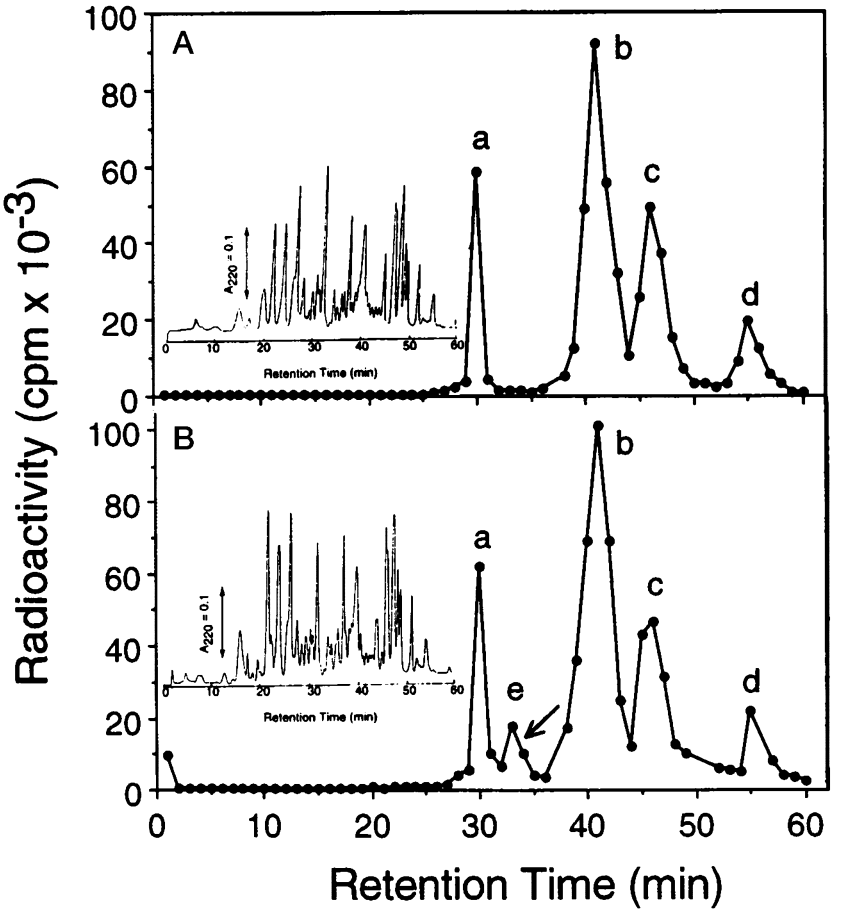

FIG. 7. **HPLC tryptic phosphopeptide maps of tau phosphorylated by A kinase in the absence (*A*) and presence (*B*) of heparin.** Tau species (0.5 mg each) phosphorylated by A kinase in the absence and presence of heparin were trypsinized and subjected to phosphopeptide mapping as in Fig. 5. *Insets*, HPLC profiles.

In addition to cross-linking dimers, tetramers, and high molecular size species, DSS cross-linked tau into 72- and 83-kDa species. FPLC Superose 12 gel filtration chromatography determined that these two species are monomeric tau formed by intramolecular cross-linking of tau molecules. Because 72- and 83-kDa species were not formed when heparin was excluded from the cross-linking mixture (Fig. 1, *lanes 2–4*), these observations indicate that heparin causes a conformational change in the tau molecule in which groups reactive to DSS become accessible and approach within 11.4 Å, the distance of the spacer arm of DSS. Because tau is a naturally denatured protein with no apparent folding (35, 40), such conformational change will induce polypeptide chain folding within the tau molecule.

As shown in Figs. 3 and 8, heparin-induced dimerization and tetramerization become maximum at an exposure time of 48 h, whereas formation of 72- and 83-kDa species plateau at 30 min. Based on the intensities of various bands in Fig. 3, at 15 min, amounts of dimer and tetramers are ~35% of the maximum, whereas formation of 72- and 83-kDa species are ~80% of the maximum. Thus the polypeptide chain folding of tau caused by heparin occurs more rapidly than tau's dimerization and tetramerization. Although yet to be confirmed, heparin-induced polypeptide chain folding of tau may precede tau's oligomerization. If true, heparin-induced aggregation of tau may follow a sequential mechanism. Polypeptide chain folding may expose docking surfaces for interchain interaction between tau molecules, leading to the formation of dimers, tetramers, and high molecular size aggregates. Such a model has been proposed for aggregation of proteins from unfolded polypeptides (38).

Heparin, in addition to causing aggregation of tau, also stimulates tau phosphorylation by various kinases (28–32). Because heparin's stimulatory effect is not observed when tau is replaced by synthetic peptide substrates, (Fig. 4), these observations are consistent with the idea that heparin is a substrate modulator acting through tau (28). Our cross-linking data show that heparin induces a conformational change in tau, which can be detected by the DSS cross-linking of 72- and 83-kDa species. More importantly, our data in Fig. 8 show that cross-linking of 72- and 83-kDa species correlates with the stimulatory effect of heparin on tau phosphorylation. Furthermore, phosphopeptide mapping of tau phosphorylated in the presence and absence of heparin revealed that both NCLK and A kinase phosphorylated tau on sites that are not accessible for phosphorylation when heparin is excluded from the phosphorylation mixture (Figs. 5 and 7). Together, these observations indicate that the heparin-induced conformational change in tau exposes new sites for phosphorylation by NCLK and A kinase. Interestingly, these new sites are located within a stretch of 50 amino acid residues within the tau sequence (37). This 50-amino acid stretch composed of residues 212–262 may be one of the regions that becomes exposed during heparin-induced conformational change of tau.

Tau has been suggested not to have a unique folded conformation but to exist as a random coil (35, 40). In fact, tau can be boiled and dissolved in acid without losing its microtubule binding ability, indicating that folded conformation may not be necessary for tau-microtubule interaction (41), and, in solution, tau behaves as a disordered polypeptide without any compact folding (40). In this study, however, by chemical cross-linking and phosphopeptide mapping we showed that heparin induces a conformational change in tau, exposing new sites for kinase phosphorylation. Thus, our data suggest that tau may have a defined conformation that can be affected by ligands such as heparin.

In a previous study (10), NCLK phosphorylated tau on seven

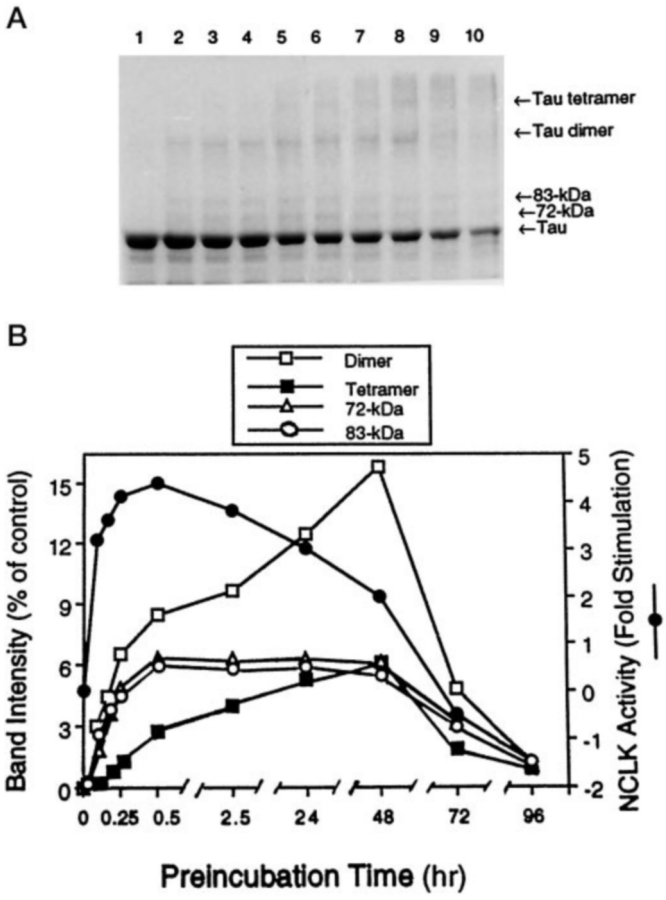

FIG. 8. **Effect of preincubation with heparin on DSS cross-linking and phosphorylation of tau.** Tau was preincubated with heparin as described in the legend to Fig. 1. After various time points, aliquots were withdrawn and either subjected to DSS cross-linking or used as substrate for NCLK activity assay. *A*, SDS-gel of DSS cross-linking. To each vial containing 97 μl of tau preincubated for various time points with heparin, 3 μl of DSS stock solution was added. After 15 min at room temperature, 10 μl was withdrawn from each vial and mixed with an equal volume of SDS-PAGE sample buffer, and 10 μl was electrophoresed. *Lane 1*, tau (7.3 μg) preincubated with water for 96 h and treated with DMF; *lanes 2–10*, tau (7.3 μg each) preincubated with heparin for 0.1, 0.17, 0.25, 0.5, 2.5, 24, 48, 72, and 96 h, respectively, and treated with DSS. *B*, comparison of phosphorylation and formation of cross-linked dimer, tetramer, and 72- and 83-kDa species in *A*. NCLK activity assay was carried out as described under "Materials and Methods." To each vial containing 97 μl of tau preincubated with heparin or water for various time points, an aliquot of 3 μl containing [γ-^{32}P]ATP/Mg^{2+} and NCLK was added to initiate the reaction. After 15 min at 30 °C, 20 μl of sample was withdrawn and analyzed for the amount of ^{32}P incorporated into tau. NCLK activity is expressed as the fold stimulation (*i.e.* activity of NCLK against tau preincubated with heparin divided by activity of NCLK against tau preincubated with water for same amount of time as with heparin). Intensities of various bands were estimated by scanning the gel in *A* and are expressed as % of control tau (*lane 1*).

sites, including a partial phosphorylation of Thr^{231}. In this study, we found that NCLK phosphorylates Thr^{231} only when heparin is included in the phosphorylation mixture. The reason for this discrepancy is very likely attributable to the type of tau used in the two studies. In the previous study (10), tau purified form brain extract was used as the substrate as opposed to the present work, which used bacterially expressed recombinant tau. Moreover, we found that phosphorylation by NCLK on this site can be induced by a substrate modulator such as heparin. A fraction of brain tau used in the previous study (10) may have contained some substrate modulator, and that may have led to the phosphorylation of Thr^{231} by NCLK.

mAb 12E8 was raised against a phosphopeptide corresponding to tau residues 257–270 containing phosphate on Ser^{262} (42). This mAb has been used by various investigators to determine the phosphorylation of tau on Ser^{262}. Recently, tau phosphorylated by NCLK in the presence of heparin was reported to be immunolabeled by this mAb (32). Similarly, this mab was reported to cross-react with tau phosphorylated by A kinase (43). Based on these data NCLK was suggested to phosphorylate tau on Ser^{262} in the presence of heparin (32), whereas A kinase phosphorylates tau on Ser^{262} in the absence of any effector (43). In this study, we found that NCLK does not phosphorylate tau on Ser^{262} in the presence of heparin, and A kinase phosphorylates Ser^{262} only when heparin is included in the phosphorylation mixture. Consistent with our data, a previous study using direct sequence analysis of phosphopeptides showed that A kinase does not phosphorylate tau on Ser^{262} (16). Furthermore, protein kinase C and calcium/calmodulin kinase II (17, 18) do not phosphorylate tau on Ser^{262}, but tau phosphorylated by both of these kinases displays immunoreactivity to mAb 12E8 (44). These observations suggest that mAb 12E8 immunoreactivity may not truly reflect the phosphorylation state of tau on Ser^{262}. There are other widely used phosphorylation-sensitive mAbs against tau, such as PHF1, tau 1, AT8, SMI31, SMI33, SMI34, and AP422. These mAbs have all been found to be unreliable in determining the site-specific phosphorylation state of tau (45).

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