Alzheimer-like Changes in Microtubule-associated Protein Tau Induced by Sulfated Glycosaminoglycans

INHIBITION OF MICROTUBULE BINDING, STIMULATION OF PHOSPHORYLATION, AND FILAMENT ASSEMBLY DEPEND ON THE DEGREE OF SULFATION

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The paired helical filament (PHF) and the related straight filament (SF) are the major components of the neurofibrillary deposits that form a defining neuropathological characteristic of Alzheimer's disease and a number of other neurodegenerative disorders. We have recently shown that full-length recombinant tau assemblies into Alzheimer-like filaments upon incubation with heparin. Heparin also promotes phosphorylation of tau by a number of protein kinases, prevents tau from binding to taxol-stabilized microtubules, and produces rapid disassembly of microtubules assembled from tau and tubulin. Here, we have used the above parameters to study the interactions between tau protein and a number of naturally occurring and synthetic glycosaminoglycans. We show that the magnitude of the glycosaminoglycan effects is proportional to their degree of sulfation. Thus, the strongly sulfated glycosaminoglycans dextran sulfate, pentosan polysulfate, and heparin were the most potent, whereas the non-sulfated dextran and hyaluronic acid were without effect. The moderately sulfated glycosaminoglycans heparan sulfate, chondroitin sulfate, and dermatan sulfate had intermediate effects, whereas keratan sulfate had little or no effect. These in vitro interactions between tau protein and sulfated glycosaminoglycans reproduced the known characteristics of paired helical filament-tau from Alzheimer's disease brain. Sulfated glycosaminoglycans are present in nerve cells in Alzheimer's disease brain in the early stages of neurofibrillary degeneration, suggesting that their interactions with tau may constitute a central event in the development of the neuronal pathology of Alzheimer's disease.

Hyperphosphorylated microtubule-associated protein tau is the major proteinaceous component of the paired helical and straight filaments which constitute a defining neuropathological characteristic of Alzheimer's disease and a number of other neurodegenerative disorders. We have recently shown that full-length recombinant tau assemblies into Alzheimer-like filaments upon incubation with heparin. Heparin also promotes phosphorylation of tau by a number of protein kinases, prevents tau from binding to taxol-stabilized microtubules, and produces rapid disassembly of microtubules assembled from tau and tubulin. Here, we have used the above parameters to study the interactions between tau protein and a number of naturally occurring and synthetic glycosaminoglycans. We show that the magnitude of the glycosaminoglycan effects is proportional to their degree of sulfation. Thus, the strongly sulfated glycosaminoglycans dextran sulfate, pentosan polysulfate, and heparin were the most potent, whereas the non-sulfated dextran and hyaluronic acid were without effect. The moderately sulfated glycosaminoglycans heparan sulfate, chondroitin sulfate, and dermatan sulfate had intermediate effects, whereas keratan sulfate had little or no effect. These in vitro interactions between tau protein and sulfated glycosaminoglycans reproduced the known characteristics of paired helical filament-tau from Alzheimer's disease brain. Sulfated glycosaminoglycans are present in nerve cells in Alzheimer's disease brain in the early stages of neurofibrillary degeneration, suggesting that their interactions with tau may constitute a central event in the development of the neuronal pathology of Alzheimer's disease.

In the present study we show that the degree of sulfation of glycosaminoglycans is of crucial importance for their ability to induce tau filament formation, to prevent tau from binding to microtubules, and to promote microtubule disassembly. Of those tested, dextran sulfate, pentosan polysulfate, and heparin are the most sulfated, followed by dermatan sulfate, heparan sulfate, chondroitin sulfate, and keratan sulfate. Hyaluronic acid and dextran are not sulfated (see Table I for degrees of sulfation). We also show that heparin prevents tau from binding to microtubules and promotes microtubule disassembly. Heparan sulfate and hyperphosphorylated tau have been found to co-exist in nerve cells in Alzheimer's disease brain at the earliest known stages of neurofibrillary pathology (28). Moreover, phosphorylation of tau by cdc28, cAMP-dependent protein kinase, GSK3, and several SAP kinases is markedly stimulated by heparin (11, 15, 30–32). Taken together, these findings suggest that an interaction between tau protein and sulfated glycosaminoglycans may be a central event in the development of the neurofibrillary pathology of Alzheimer's disease.

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The paired helical filament (PHF) and the related straight filament (SF) are the major components of the neurofibrillary deposits that form a defining neuropathological characteristic of Alzheimer's disease and a number of other neurodegenerative disorders (reviewed in Ref. 1). They are composed of microtubule-associated protein tau, in a hyperphosphorylated state. Mass spectrometry and immunological studies have identified a large number of phosphorylation sites in PHF-tau (2–11). Some of these sites are not phosphorylated in tau from normal brain, whereas others are phosphorylated to a greater extent in PHF-tau than in tau from normal brain. Many phosphorylated sites are serine/threonine-prolines. Consequently, tau protein can be phosphorylated in vitro at many of these sites by proline-directed protein kinases, such as mitogen-activated protein (MAP) kinase (12–14), stress-activated protein (SAP) kinases (15, 16), glycogen synthase kinase-3 (GSK3) (17–19), and neuronal cdc2-like kinase (NCLK) (20, 21). Moreover, cAMP-dependent protein kinase and Ca<sup>2+</sup>/calmodulin-dependent protein kinase II phosphorylate tau at specific sites in vitro, some of which are also phosphorylated in PHF-tau (22–24). Hyperphosphorylation of tau results in its inability to bind to microtubules and is believed to precede PHF assembly (25–27). However, it is unclear whether hyperphosphorylation of tau is either necessary or sufficient for PHF formation.

We have recently shown that a phosphorylation-independent interaction between recombinant tau and sulfated glycosaminoglycans leads to the formation of Alzheimer-like filaments under physiological conditions in vitro (28, 29). Three repeat-containing tau isoforms gave rise to paired helical-like filaments, whereas four repeat-containing isoforms formed straight filaments, thus suggesting an explanation for the two tau assemblies present in Alzheimer’s disease brain. We also showed that heparin prevents tau from binding to microtubules and promotes microtubule disassembly. Heparan sulfate and hyperphosphorylated tau have been found to co-exist in nerve cells in Alzheimer’s disease brain at the earliest known stages of neurofibrillary pathology (28). Moreover, phosphorylation of tau by cdc28, cAMP-dependent protein kinase, GSK3, and several SAP kinases is markedly stimulated by heparin (11, 15, 30–32). Taken together, these findings suggest that an interaction between tau protein and sulfated glycosaminoglycans may be a central event in the development of the neurofibrillary pathology of Alzheimer’s disease.

In the present study we show that the degree of sulfation of glycosaminoglycans is of crucial importance for their ability to induce tau filament formation, to prevent tau from binding to microtubules, and to promote microtubule disassembly. Of those tested, dextran sulfate, pentosan polysulfate, and heparin are the most sulfated, followed by dermatan sulfate, heparan sulfate, chondroitin sulfate, and keratan sulfate. Hyaluronic acid and dextran are not sulfated (see Table I for degrees of sulfation). We also show that the phosphorylation of tau by MAP kinase, NCLK, and GSK3β is markedly stimulated in the presence of heparin, at heparin concentrations lower than those required for tau filament formation. Phosphorylation of tau by MAP kinase, NCLK, and GSK3β is also stimulated by heparan sulfate, dextran sulfate, and pentosan polysulfate, but
not by dextran and hyaluronic acid, with the magnitude of stimulation of tau phosphorylation being proportional to the degree of glycosaminoglycan sulfation. Tau phosphorylation by MAP kinase, NCLK, and GSK3 is also stimulated in the presence of nucleic acids and tubulin. Nucleic acids had little effect on the binding of tau to microtubules. However, incubation of tau with tRNA led to the formation of filaments, in confirmation of a recent report (33).

These results raise the possibility that an interaction between tau protein and negatively charged polymers with a sugar backbone, as found in sulfated glycosaminoglycans and nucleic acids, results in a conformational change in tau that induces polymerization of tau molecules via the microtubule-binding repeats of individual tau molecules, resulting in the formation of filaments like those present in Alzheimer’s disease and other neurodegenerative disorders.

### EXPERIMENTAL PROCEDURES

**Materials**—The 3-repeat 381-amino acid (htau37) and the 4-repeat 441-amino acid (htau40) isoforms of human tau (34) were expressed in *Escherichia coli* 441-amino acid (htau40) isoforms of human tau (34) were expressed in *Escherichia coli* and purified as described (29). The tau mutants htau24S262A, htau24S356A, and htau24S262AS356A have been described previously (9, 24). Activated recombinant p42 MAP kinase was prepared as described (14). GS3kβ3 was purified from rabbit skeletal muscle, as described (35). Active NCLK was reconstituted from recombinant cyclin-dependent protein kinase-5 and a recombinant fragment of the brain-specific activator p35 (36). Antiserum 134 recognizes the carboxyl terminus of tau in a phosphorylation-independent manner (34). AT8 is a phosphorylation-dependent monoclonal antibody which recognizes tau phosphorylated at Ser-202 and Thr-205 (37). The monoclonal antibody 12E8 recognizes tau protein phosphorylated at Ser-202 and Thr-205 (8). Heparin (from bovine intestinal mucosa), dextran, dextran sulfate, pentosan polysulfate, and poly-L-glutamic acid were purchased from Sigma. Heparan sulfate (from bovine kidney), chondroitin-4-sulfate (from bovine trachea), dermatan sulfate (from porcine skin), keratan sulfate (from bovine cornea), and hyaluronic acid (from human umbilical cord) were obtained from Fluka Chemie AG. Tubulin was purchased from Cytoskeleton Inc. Double-stranded DNA was obtained from CLONTECH and tRNA from Boehringer Mannheim. Oligonucleotides (30–60 mers) were made on an Applied Biosystems DNA synthesizer.

**Phosphorylation Assays**—Phosphorylation assays (0.050 ml) were carried out at 30 °C and comprised 25 mM Tris-HCl, pH 7.4, 0.1 mM EGTA, 0.1 mM sodium orthovanadate, 2.5 μM PKI (a specific inhibitor of cyclic AMP-dependent protein kinase), protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 5 μM aprotinin, 5 μM leupeptin, and 0.5 μM pepstatin), tubulin protein (4 μM), 10 mM magnesium acetate, 2 mM γ-[32P]ATP (approximately 10⁶ cpm/nmol), and 1 unit/ml activated p42 MAP kinase, 1 unit/ml GS3kβ3, or 5 units/ml recombinant reconstituted NCLK. Reactions were initiated with ATP and aliquots were removed at various times ranging from 10 min to 24 h and used for SDS-polyacrylamide gel electrophoresis and immunoblotting. Immunoblots were performed as described (15). Alternatively, incorporation of [32P]radioactivity was measured after adsorption to Whatman P-81 paper, as described (14). Glycosaminoglycans and nucleic acids were included in the assays at 50 μM and tubulin at 20 μM.

**Microtubule Binding and Assembly**—For microtubule binding, recombinant htau40 (4 μM, 0.18 mg/ml) was incubated with different concentrations of glycosaminoglycans and nucleic acids (10, 50, 100, 500, and 1,000 μg/ml) in assembly buffer (80 mM PIPES, 1 mM MgCl₂, 2 mM EGTA, 0.1 mM sodium orthovanadate, 2.5 μM PKI, 1 mM GSK3b, or 5 units/ml recombinant reconstituted NCLK in the presence or absence of 50 μg/ml heparin. 1 mM EGTA, 1 mM dithiothreitol, 1 mM GTP, pH 6.8) for 10 min at 37 °C, then added to 10 μM taxol-stabilized microtubules and incubated for a further 20 min. Following ultracentrifugation, aliquots of supernatants (free tau) and pellets (microtubule-bound tau) were subjected to SDS-polyacrylamide gel electrophoresis. Protein concentrations were estimated by scanning the gels with a Molecular Dynamics computing densitometer (Model 300 A), and were expressed as percentage of tau bound to microtubules in the absence of glycosaminoglycans and nucleic acids (taken as 100%). For microtubule assembly, recombinant htau40 (2 μM) was incubated with tubulin (10 μM) in assembly buffer at 37 °C. After 5 min, glycosaminoglycans and nucleic acids (100 μg/ml) were added and incubated for a further 5 min. Polymerization and depolymerization of microtubules were monitored by measuring the turbidity at 350 nm.

**Tau Filament Assembly**—Purified recombinant htau37 (40 μM) or htau40 (40 μM) was incubated with various concentrations of glycosaminoglycans and nucleic acids (ranging from 5 to 20 μM). In 25 μl of 30 mM MOPS, 1 mM 4-(2-aminoethyl)benzenesulfonylfluoride (Calbiochem), pH 7.4, at 37 °C for 48 h, as described (28). In some experiments various concentrations of CaCl₂, MgCl₂, ZnCl₂, and AlCl₃ were added. Control experiments consisted of using tau alone, glycosaminoglycans alone, tau plus di- and trivalent cations, and glycosaminoglycans plus di- and trivalent cations. Samples were examined by electron microscopy, as described (28).

### RESULTS

**Effects of Glycosaminoglycans on Phosphorylation of Tau by NCLK**—Recombinant htau40 was incubated for various times (ranging from 10 min to 24 h) with 5 units/ml recombinant reconstituted NCLK, in the presence or absence of 50 μg/ml heparin. After 24 h tau incorporated 3.8 mol of phosphate/mol of protein in the absence of heparin and 11.4 mol of phosphate/mol of protein in the presence of heparin (Fig. 1). The stimulation of tau phosphorylation by heparin was apparent throughout the incubation period and was particularly evident at early time points (Fig. 1). A dose-response curve showed that the effect was maximal at 30 μg/ml heparin. Phosphorylation of tau by NCLK in the presence of heparin produced the epitopes of phosphorylation-dependent anti-tau antibodies which recognize (S/T)P sites in tau, as shown in Fig. 2 for antibody AT8 which recognizes tau phosphorylated at Ser-202 and Thr-205 (8). Moreover, tau was also immunoreactive for antibody 12E8 which recognizes the phosphorylated non-(S/T)P sites Ser-262 and/or Ser-356 (9). Phosphorylation of htau24S262A, htau24S356A, and htau24S262AS356A by NCLK plus heparin showed that Ser-262, but not Ser-356 was phosphorylated (Fig. 2). The effects of glycosaminoglycans (50 μg/ml) other than heparin on tau phosphorylation by NCLK were investigated
Hyaluronic acid (HA), chondroitin 4-sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), and DNA were without a significant effect. Addition of 50 μg/ml nucleic acids also stimulated tau phosphorylation by NCLK, with tRNA producing a larger effect than DNA (Fig. 3). Incubation of tau with NCLK in the presence of 20 μM tubulin led to an approximately 50% stimulation of tau phosphorylation (Fig. 3).

Effects of Glycosaminoglycans on Phosphorylation of Tau by GSK3β and MAP Kinase—Recombinant httau40 was incubated for 18 h with 1 unit/ml GSK3β purified from skeletal muscle or 1 unit/ml activated recombinant p42 MAP kinase, in the presence or absence of 50 μg/ml glycosaminoglycans, nucleic acids, and 20 μM tubulin. As shown before, phosphorylation of tau by GSK3β was strongly stimulated by heparin. Dextran sulfate had a similar effect, with an almost 5-fold stimulation of phosphorylation (Fig. 3). Addition of heparan sulfate, chondroitin sulfate, pentosan polysulfate, and dermatan sulfate resulted in a 2.0–3.0-fold stimulation of tau phosphorylation by GSK3β, whereas keratan sulfate, hyaluronic acid, dextran, and poly-L-glutamic acid were without a significant effect. RNA and DNA produced a 2.5–3.0-fold stimulation of tau phosphorylation by GSK3β; an effect of similar magnitude was obtained upon addition of detergent (Fig. 3). Phosphorylation of httau40 by MAP kinase was stimulated 1.5–3.0-fold by heparin, heparan sulfate, pentosan polysulfate, dextran sulfate, and DNA (Fig. 3). Chondroitin sulfate, dermatan sulfate, keratan sulfate, hyaluronic acid, dextran, poly-L-glutamic acid, and DNA were without a significant effect. Incubation of tau with GSK3β or MAP kinase in the presence of 20 μM tubulin led to a 3-fold stimulation of tau phosphorylation (Fig. 3).

Effects of Glycosaminoglycans on the Binding of Tau to Microtubules—Recombinant httau40 was incubated with different concentrations (50, 100, 250, 500, and 1,000 μg/ml) of glycosaminoglycans or nucleic acids and added to taxol-stabilized microtubules, followed by ultracentrifugation to separate unbound tau from microtubule-bound tau. The addition of heparin, dextran sulfate, and pentosan polysulfate resulted in a dose-dependent inability of tau to bind to microtubules, with IC₅₀ values of approximately 100 μg/ml (Fig. 4). Heparan sulfate and dermatan sulfate produced a smaller, but significant, reduction which amounted to 40% at 1 mg/ml (Fig. 4). Only small reductions in the ability of tau to bind to microtubules were observed upon addition of RNA and DNA. Hyaluronic acid, chondroitin sulfate, keratan sulfate, and poly-L-glutamic acid had no significant effect, even at high concentrations (Fig. 4).

Effects of Glycosaminoglycans on Tau-promoted Microtubule Stability—Recombinant httau40 was incubated with tubulin and microtubule assembly monitored by an increase in turbidity. After 5 min, when assembly was maximal, glycosaminoglycans or nucleic acids (100 μg/ml) were added and microtubule disassembly monitored for a further 5 min by a decrease in turbidity. Addition of heparin, pentosan polysulfate, dextran sulfate, and DNA caused rapid and complete microtubule disassembly (Fig. 5). Heparan sulfate and dermatan sulfate had an intermediate effect (Fig. 5). RNA and poly-L-glutamic acid produced only a small effect on microtubule disassembly, whereas addition of hyaluronic acid, chondroitin sulfate, keratan sulfate, and dextran had no significant effect (Fig. 5).

Glycosaminoglycan-induced Assembly of Tau into Paired Helical-like Filaments—Incubation of the three repeat-containing tau isoform httau37 with glycosaminoglycans led to bulk assembly into twisted filaments with a morphology similar to the PHFs from Alzheimer’s disease brain (Table II, Figs. 6 and 7). As observed before (28, 29), incubation of four repeat-containing tau isoforms with glycosaminoglycans gave straight fila-
ments with a morphology similar to the SFs from Alzheimer’s disease brain (data not shown). Incubation of htau37 with heparin of molecular masses ranging from 3 to 30 kDa gave large numbers of twisted filaments (Fig. 6, A and B). A second class of filament appearing as thinner, wavy structures was also observed (shown in Fig. 6C). These may correspond to half-twisted filaments, as they are sometimes seen extending from the ends of twisted filaments. This would imply that the filaments formed in vitro are two-stranded, like Alzheimer filaments (37). The relative numbers of each type of filament were strongly dependent on the tau:heparin ratios, with a 2-fold change in the ratios being sufficient to switch from a predominance of half-filaments to a preponderance of twisted filaments. This would imply that the filaments formed in vitro are two-stranded, like Alzheimer filaments (37). The relative numbers of each type of filament were strongly dependent on the tau:heparin ratios, with a 2-fold change in the ratios being sufficient to switch from a predominance of half-filaments to a preponderance of twisted filaments. This would imply that the filaments formed in vitro are two-stranded, like Alzheimer filaments (37). The relative numbers of each type of filament were strongly dependent on the tau:heparin ratios, with a 2-fold change in the ratios being sufficient to switch from a predominance of half-filaments to a preponderance of twisted filaments. This would imply that the filaments formed in vitro are two-stranded, like Alzheimer filaments (37). The relative numbers of each type of filament were strongly dependent on the tau:heparin ratios, with a 2-fold change in the ratios being sufficient to switch from a predominance of half-filaments to a preponderance of twisted filaments. This would imply that the filaments formed in vitro are two-stranded, like Alzheimer filaments (37). The relative numbers of each type of filament were strongly dependent on the tau:heparin ratios, with a 2-fold change in the ratios being sufficient to switch from a predominance of half-filaments to a preponderance of twisted filaments. This would imply that the filaments formed in vitro are two-stranded, like Alzheimer filaments (37). The relative numbers of each type of filament were strongly dependent on the tau:heparin ratios, with a 2-fold change in the ratios being sufficient to switch from a predominance of half-filaments to a preponderance of twisted filaments.

Effects of glycosaminoglycans and nucleic acids on the binding of tau to microtubules. Taxol-stabilized microtubules were incubated with recombinant htau40 (441-amino acid isoform of human tau) in the presence of varying concentrations of hyaluronic acid (HA), chondroitin-4-sulfate (CS), keratan sulfate (KS), dermatan sulfate (DS), heparan sulfate (HS), heparin (H), dextran (Dex), poly-L-glutamic acid (PGA), DNA, RNA, dextran sulfate (Dex S), and pentosan polysulfate (PPS). Ultracentrifugation was used to separate tau bound to microtubules from unbound tau. The results are expressed as the percentage of tau bound in the absence of glycosaminoglycans or nucleic acids (taken as 100%). A typical experiment is shown. Similar results were obtained in at least three separate experiments.

Table II

| Additive         | Relative amount of htau37 assembly with different added glycosaminoglycans or nucleic acids |
|------------------|------------------------------------------------------------------------------------------|
| No additive      | 0                                                                                      |
| Dextran sulfate  | +++                                                                                     |
| Pentosan polysulfate | +++                                                                                   |
| Heparin          | +++                                                                                     |
| Heparin sulfate  | +++                                                                                     |
| Dermatan sulfate | +                                                                                       |
| Chondroitin sulfate | +                                                                                      |
| Keratan sulfate  | 0                                                                                       |
| Hyaluronic acid  | 0                                                                                       |
| Dextran          | 0                                                                                       |
| tRNA             | ++                                                                                      |
| DNA              | 0                                                                                       |

Effects of tRNA and both single-stranded and double-stranded DNA on tau filament formation (Table II). Whereas DNA of various sizes failed to induce filament formation reproducibly, addition of tRNA to the triple-repeat tau isoform htau37 resulted in the formation of twisted filaments with a similar morphology to PHFs (Fig. 8A). Addition of tRNA to the four-repeat tau isoform htau40 gave straight filaments (Fig. 8B).

DISCUSSION

Abnormal tau filaments in the form of PHFs and SFs constitute one of the defining neuropathological characteristics of
Alzheimer’s disease. We have recently shown that addition of heparin to full-length recombinant tau induces bulk assembly of tau into filaments that closely resemble the PHFs and SFs of Alzheimer’s disease (28, 29). Similar results have been reported with partial tau sequences encompassing the microtubule-binding repeat region (38). We also showed that tau is a heparin-binding protein and that heparin inhibits binding of tau to taxol-stabilized microtubules and induces rapid disassembly of tau-stabilized microtubules (28, 29). Previous experiments have shown that phosphorylation of tau by a number of protein kinases is stimulated by heparin (11, 15, 30–32). In the present study we have carried out an analysis of the effects of different glycosaminoglycans on stimulation of tau phosphorylation by the proline-directed protein kinases NCLK, GSK3β, and MAP kinase, on the ability of tau to bind to microtubules, on disassembly of tau-stabilized microtubules (28, 29). Previous experiments have shown that phosphorylation of tau by a number of protein kinases is stimulated by heparin (11, 15, 30–32). In the present study we have carried out an analysis of the effects of different glycosaminoglycans on stimulation of tau phosphorylation by the proline-directed protein kinases NCLK, GSK3β, and MAP kinase, on the ability of tau to bind to microtubules, on disassembly of tau-stabilized microtubules, and on assembly of tau-containing filaments. We have found that the degree of sulfation is an important variable that determines the Alzheimer-like interactions of glycosaminoglycans with tau protein. Thus, dextran sulfate was the most potent of the glycosaminoglycans tested, whereas dextran was without effect. In general, dextran sulfate, pentosan polysulfate, and heparin were the most effective, followed by heparan sulfate, chondroitin sulfate, and dermatan sulfate. Keratan sulfate, hyaluronic acid, and dextran were the least effective. Some of the differences in the effects of the moderately sulfated glycosaminoglycans cannot be accounted for by sulfation alone, indicating that sequence differences between individual glycosaminoglycans also play a role.

Heparin markedly stimulated the phosphorylation of tau by NCLK, resulting in a 3-fold stimulation of phosphate incorporation after an incubation of 18 h. The effect of heparin was most marked at early time points and it was maximal at 30 μg/ml. Similar results were obtained with MAP kinase, adding NCLK and MAP kinase to the list of protein kinases whose ability to phosphorylate tau is stimulated by heparin. This list also includes cdk28, GSK3, cAMP-dependent protein kinase, SAPK1, SAPK3, and SAPK4 (11, 15, 30–32). Phosphorylation of tau by NCLK in the presence of heparin produced the epitopes of phosphorylation-dependent anti-tau antibodies that recognize (S/T)P sites, in accordance with the known substrate specificities of NCLK. Unexpectedly, tau also became immunoreactive with antibody 12E8 which recognizes the non-(S/T)P sites Ser-262 and/or Ser-356 located in the microtubule-binding...
repeat region (9). By using tau mutants we could show that Ser-262, but not Ser-356, became phosphorylated. Hyperphosphorylation of tau at multiple (S/T/P) sites and at Ser-262 is a pathological hallmark of PHF-tau. It has been suggested that phosphorylation of tau at Ser-262 plays an important role in regulating the ability of tau to bind to microtubules (39). Several non-proline-directed protein kinases have been shown to phosphorylate tau at Ser-262 (24, 40, 41). The present findings indicate that in the presence of sulfated glycosaminoglycans the proline-directed NCLK phosphorylates tau at multiple (S/T/P) sites, as well as at Ser-262.

The study of the influence of glycosaminoglycans on phosphorylation of tau by NCLK, MAP kinase, and GSK3β showed that heparin, dextran sulfate, pentosan polysulfate, and heparan sulfate were the most effective, whereas keratan sulfate, hyaluronic acid, dextran, and poly-L-glutamic acid had intermediate effects, whereas hyaluronic acid, chondroitin sulfate, and dextran sulfate were the most effective, whereas keratan sulfate, hyaluronic acid, chondroitin sulfate, and dextran sulfate had little or no effect. Chondroitin sulfate and dermatan sulfate had intermediate effects when tau was phosphorylated by NCLK and GSK3β, but no effect when tau was phosphorylated by MAP kinase.

RNA stimulated the phosphorylation of tau by NCLK, GSK3β, and MAP kinase, whereas DNA stimulated the phosphorylation of tau by NCLK and GSK3β, suggesting that nucleic acids bind to tau in a similar manner to sulfated glycosaminoglycans. Tubulin similarly stimulated phosphorylation of tau by NCLK, GSK3β, and MAP kinase. A previous study showed that tubulin stimulates phosphorylation of tau by GSK3β (42). We show that this is also true of phosphorylation by NCLK and MAP kinase. Tau binds through its positively charged repeat region to the negatively charged carboxyl terminus of tubulin (43, 44). This may result in a conformational change in tau that renders some phosphorylation sites more accessible to protein kinases. The stimulation of tau phosphorylation by tubulin may represent a mechanism for regulating the binding of tau to microtubules in nerve cells.

The degree of glycosaminoglycan sulfation also influenced the ability of tau to bind to microtubules. Heparin, dextran sulfate, and pentosan polysulfate had a strong negative effect, with intermediate effects for heparan sulfate and dermatan sulfate. Hyaluronic acid, chondroitin sulfate, keratan sulfate, dextran, poly-L-glutamic acid, DNA, and tRNA had little or no effect. DNA had a strong negative effect toward tau-promoted microtubule assembly, similar to heparin, dextran sulfate, and pentosan polysulfate. Heparan sulfate, dermatan sulfate, and tRNA had intermediate effects, whereas hyaluronic acid, chondroitin sulfate, keratan sulfate, dextran, and poly-L-glutamic acid had little or no effect.

Heparin induced bulk assembly of three-repeat recombinant tau into twisted filaments similar to PHFs. As noted before (28), the molar tau:heparin ratio was of crucial importance, being optimal at approximately 4:1. When suboptimal, a preponderance of thin, wavy filaments was observed, with only few twisted filaments. The morphology of the thin, wavy filaments suggests that they may correspond to half-twisted filaments. Under non-optimal tau:heparin ratios, no filaments were formed. Dextran sulfate and pentosan polysulfate, two highly sulfated synthetic analogues of glycosaminoglycans, induced the formation of large numbers of filaments when incubated with the three-repeat tau isoform htau37 at a tau:glycosaminoglycan ratio of approximately 4:1. Unlike the filaments formed after addition of heparin, these filaments were short, suggesting that a high degree of glycosaminoglycan sulfation may affect the ratio of nucleation to growth. Incubation of three-repeat tau with heparan sulfate at a molar ratio of 4:1 led to the formation of twisted filaments with a similar morphology to the filaments formed after addition of heparin. The number of heparan sulfate-induced filaments was smaller, in keeping with its lower degree of sulfation. Addition of ZnCl₂ significantly stimulated the formation of tau filaments induced by heparan sulfate. Addition of MgCl₂ had a small stimulatory effect, whereas addition of NaCl, CaCl₂, and AlCl₃ was without effect. The effective zinc concentration was 1–10 μM, in the same range as that which has been shown to stimulate Aβ amyloid aggregation (45). Zinc is present at high concentrations in cerebral cortex and hippocampal formation (46), two brain regions which are particularly prone to develop the neuropathology of Alzheimer’s disease. Moreover, high concentrations of zinc have been found in cytoc-derived flour which has been linked to the high incidence of the amyotrophic lateral sclerosis/Parkinsonism-dementia complex on the island of Guam, a condition characterized by an extensive fibrillary tau pathology (47, 48). Incubation of the three-repeat tau isoform htau37 with chondroitin sulfate and dermatan sulfate led to the formation of small numbers of twisted filaments with a similar morphology to those formed after addition of heparin and heparan sulfate. No filaments were formed after addition of keratan sulfate, hyaluronic acid, dextran, and poly-L-glutamic acid.

A recent study has shown the formation of tau filaments after incubation of recombinant tau with tRNA (33). We have observed a similar effect, with the formation of twisted filaments after incubation of the three-repeat tau isoform htau37 with tRNA. Similar to our previous results with sulfated glycosaminoglycans, the four-repeat tau isoform htau40 gave straight filaments when incubated with tRNA. By acridine orange staining, RNA has been shown to be sequestered in the neurofibrillary lesions of Alzheimer’s disease (49).

Sulfated glycosaminoglycans and RNA share a repeat sugar backbone and negative charges in the form of sulfates or phosphates. This may be of relevance for the mechanism underlying tau filament formation. Tau protein is thought to be an extended molecule with little secondary structure which becomes partially structured upon binding to microtubules (50). Binding of sulfated glycosaminoglycans or RNA to tau may induce or stabilize a conformation of tau that brings the microtubule-binding repeats of individual tau molecules in close proximity, creating sites which favor polymerization of tau into filaments.

Immunohistochemical studies have shown the presence of heparan sulfate in nerve cells in Alzheimer’s disease brain in the early stages of neurofibrillary degeneration which are characterized by the presence of hyperphosphorylated tau (28, 51–53). Chondroitin sulfate and dermatan sulfate proteoglycans have also been found in association with the neurofibrillary pathology of Alzheimer’s disease (54, 55). Sulfated glycosaminoglycans stimulate tau phosphorylation at lower concentrations than those required for tau filament formation. The pathological presence of heparan sulfate within the cytoplasm of nerve cells, perhaps as a result of leakage from membrane-bound Golgi compartments (56), could first lead to hyperphosphorylation of tau, resulting in its inability to bind to microtubules. At higher heparan sulfate concentrations tau could then assemble into PHFs and SFs.

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