American Journal of Pathology, Vol. 151, No. 4, October 1997

Role of Glycosaminoglycans in Determining the Helicity of Paired Helical Filaments

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It is known from previous work that tau is the main component of paired helical filaments (PHFs) and that it can assemble *in vitro* into polymers resembling PHFs when high concentrations of protein are used. In the search for molecules that can facilitate tau polymerization, a component of neurofibrillary tangles, heparan sulfate (or its more sulfated form, heparin), and other glycosaminoglycans have been tested. Glycosaminoglycans, in the sulfated but not in the unsulfated form, facilitate not only tau assembly but also the formation of polymers resembling PHFs. Conversely, PHFs were found to contain heparan sulfate and chondroitin sulfate. Heparinase or chondroitinase treatment of PHFs results in the formation of straight structures. All of these results suggest a role for sulfated glycosaminoglycans in determining the helicity of PHFs. *(Am J Pathol 1997, 151:1115–1122)*

Alzheimer's disease (AD) is a senile dementia characterized by the presence of two aberrant structures: senile plaques and neurofibrillary tangles (NFTs). As the severity of dementia has been correlated with accumulation of NFTs in different brain regions,[1] it is of interest to understand the mechanism of NFT formation.

In a pioneer work, Kidd[2] described that NFTs are composed of bundles of paired helical filaments (PHFs). The structure and morphology of these filaments have been analyzed,[3,4] and by using atomic force microscopy, it was suggested that PHFs are composed of two 10-nm filaments that are wound into a helix with a maximal diameter of 20 nm and a half period of 65 to 80 nm.[4] Also, a lower proportion of another type of filament, termed straight filament (SF), was detected (see for example Ref. 5). SFs, in contrast to PHFs, did not show the variations in width (along their length) found in PHFs. In later studies at a molecular level, the microtubule-associated protein tau was shown to be a major component of both PHFs and SFs.[6–13] Subsequently, tau was shown to be able to polymerize, *in vitro*, yielding fibrillar polymers that can resemble SFs or even, in some cases, PHFs.[10,14–22] The region of tau involved in self-aggregation corresponds to the microtubule-binding region of the protein,[14,19,22–24] and the formation of fibrillar polymers can be achieved with only an 18-residue-long peptide corresponding to the third motif of the microtubule-binding region of tau protein, if anionic compounds are added.[24]

As the *in vitro* polymerization of tau usually requires a large amount of protein and because such quantities are usually not present in physiological conditions,[10,14,16–18,20,22] it has been suggested that *in vivo* there are probably other molecules that facilitate tau assembly into fibrillar polymers. Likely candidates are sulfated glycans, as these molecules have been shown to be present in NFTs.[25–29] Glycosaminoglycans (GAGs) are sugar molecules that are usually covalently linked to proteins to form proteoglycans, components of the extracellular matrix.[30] Four major GAGs have been described: the unsulfated hyaluronic acid, chondroitin sulfate (and dermatan), heparan sulfate (and its more extensively sulfated form, heparin), and keratan sulfate.[30] Sulfated glycans are also present in senile plaques.[31–33] Additionally, one of these sugars (heparin) facilitates the *in vitro* assembly of tau protein.[24,26]

In this work we have analyzed the characteristics of the heparin-tau interaction or that of tau with other sulfated glycans to test for the possible involvement of GAGs in the formation of structures such as SFs or PHFs.

Materials and Methods

Materials

Two monoclonal antibodies, against heparin and chondroitin sulfate, were obtained from Seikagaku Corp. (Japan). Calmodulin was a generous gift of Dr. Díaz Nido.

Heparinase and chondroitinase ABC were purchased from Sigma Chemical Co. (St. Louis, MO). These enzymes did not show protease activity when they were tested on recombinant tau.

Supported by the Spanish CICYT, Fundación Caja Madrid, and an institutional grant of Fundación Ramón Areces. M. Pérez was supported by Fundación Caja Madrid and M. Arrasate by Fundación Ferrer Investigación.

Accepted for publication July 9, 1997.

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Isolation of Paired Helical Filaments

Brain samples, supplied by Dr. Ravid (Netherlands Brain Bank), from AD patients were used as a source to isolate PHFs by following the procedure of Greenberg and Davies.[34]

Protein Isolation

Recombinant human tau, or fragments (N, 3RC, 4R, and 3R, containing the amino-terminal region, the tubulin-binding domain, and the carboxyl-terminal region or the tubulin-binding domain of tau, respectively) were purified as previously indicated.[24,35]

The proteins were characterized by gel electrophoresis as described.[24]

Polymerization of Tau into Filaments

Recombinant tau protein or its fragments were polymerized in hanging drops by the vapor diffusion often used for protein crystallization.[15] The protein concentration and buffer conditions used in the assay have been described previously.[24] The assay was done in the presence of increasing amounts (0.5 to 5 mg/ml) of heparin or chondroitin sulfate.

Heparin-Sepharose Chromatography

Twenty micrograms of tau protein or its fragments N, 3RC, 4R, or 3R were chromatographed on a heparin-Sepharose column (with a volume of 0.5 ml), equilibrated in 0.01 mol/L MES, pH 6.4, 0.05 mmol/L $MgCl_2$, 0.2 mmol/L EGTA (buffer A:10 or binding buffer). In the same conditions, calmodulin was also chomatographed. The absorbed protein was eluted stepwise by washing the column with 0.1, 0.2, 0.5, and 1.0 mol/L NaCl in buffer A:10. The fractionated protein was characterized by gel electrophoresis.

In some experiments, solutions of 1 mg/ml heparin or hyaluronic acid in buffer A:10 were used as elution buffers.

Treatment of PHFs with Heparinase or Chondroitinase

A solution of PHFs (0.5 mg/ml) was incubated in the presence of increasing amounts (25 to 100 U/ml) of heparinase or chondroitinase for different times from 2.5 to 24 hours at 37°C. After the treatment, the PHFs samples were visualized by electron microscopy.

Electron Microscopy

Samples were incubated on a carbon-coated grid for 2 minutes and then stained with 2% (w/v) uranyl acetate for 1 minute. Transmission electron microscopy was performed in a JEOL model 1200EX electron microscope operated at 100 kV. Electron micrographs were obtained at a magnification of 40,000 on Kodak SO-163 film. Micrographs were digitized using an Eikonix IEEE-499 camera with a pixel size equivalent to 7 Å in the specimen plane. Processing and measurements were performed using the Digital Micrograph 2.1 software from Gatan. Several standards were used for the control of measurements.

Immunoelectron microscopy was performed after absorption of the samples to electron microscopy grids and incubation with the first antibody (anti-heparan or anti-chondroitin) for 1 hour at room temperature. After extensive washing, the grids were incubated with the secondary antibody conjugated with 10-nm-diameter gold particles. Finally, the samples were negatively stained as described above.

Results

Tau Filaments with Variable Width Are Formed in the Presence of Glycosaminoglycans

Heparin has been demonstrated to facilitate the assembly of tau into filaments, some of which resemble PHFs.[24,26] As not only heparin[27] but also other GAGs, such as chondroitin,[25] have been described in NFTs, other glycans were tested for their ability to promote tau polymerization. Figure 1A shows that chondroitin sulfate promotes polymerization, but hyaluronic acid, an unsulfated glycan, is unable to promote polymerization (Figure 1B).

These results are compatible with a physiological role for glycans not only in facilitating tau aggregation but also in facilitating the formation of PHF-like structures. Thus, heparin or chondroitin sulfate could be present in PHFs.

Paired Helical Filaments Contain Heparin Sulfate and Chondroitin Sulfate

In a previous report,[27] an association between heparin sulfate (HS) proteoglycan and the neurofibrillary (inter- and extracellular) tangles of Alzheimer's disease was demonstrated. As PHFs are the major components of NFTs, the presence of HS in dispersed PHFs was tested using an antibody raised against that glycan. Figure 2A shows that this antibody reacts with some of the PHFs. This reaction appears to be specific as no reaction was observed when the antibody was previously mixed with a heparin solution (10 mg/ml) for its adsorption (Figure 2B). Additionally, a reaction was also observed when an antibody raised against chondroitin sulfate was tested (Figure 3A). No reaction was found when the filaments were previously treated with chondroitinase (Figure 3B). A similar result was obtained for heparinase-treated filaments when the reaction with anti-heparan was tested (data no shown). Nevertheless, it should be indicated that not every filament reacts with anti-chondroitin or with anti-heparan antibodies.

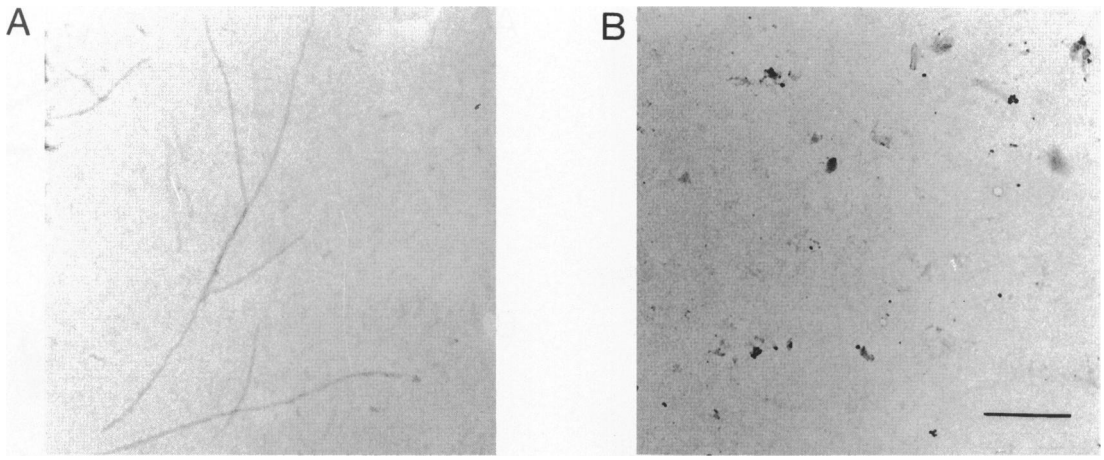

Figure 1. Effect of chondroitin sulfate and hyaluronic acid in the assembly of tau protein. **A:** Tau polymers assembled in the presence of chondroitin sulfate (0.5 mg/ml). **B:** In the presence of hyaluronic acid (0.5 mg/ml), tau protein (1 mg/ml) is unable to form fibrillar polymers. Bar, 200 nm.

Treatment of PHFs with Heparinase or Chondroitinase

To test whether HS (or its more extensively sulfated form, heparin) plays a role in PHFs morphology, samples of PHFs were treated with heparinase and their morphology observed by electron microscopy. Figure 4 shows that, on exhaustive heparinase treatment, an increase in the straight filaments (or a decrease of twisted polymers) was observed. This effect was observed at different heparinase or chondroitinase concentrations (Figure 4) and quantified (Table 1), indicating how the increase in enzyme concentration added to the PHFs preparation correlates with the decrease in the twisted (or paired) structures.

Figure 5 shows the effect of increasing incubation times in the presence of heparinase (Figure 5, A–C) or chondroitinase (Figure 5, D–F) on the morphology of PHFs. It shows how, in the presence of either glycanase, PHFs became straight filaments in which their width was essentially constant along their length. Using short treatment times (2.5 hours), the increase in the pitch (a measurement of untwisting PHFs) correlated with glycanase concentration. Also, a dependence on incubation time in the presence of enzyme was observed (Table 1). At the highest concentration (125 U) tested and at the longest incubation time (24 hours), mainly straight filaments of 10 nm width were found. This suggests that, in these conditions, untwisting of PHFs has occurred. This untwisting could be followed by the state of events depicted in Figure 5 in which the presence of two unrolled filaments is suggested (see Figure 5D). Also, it was observed that the action of chondroitinase appears to be more efficient than that of heparinase on the untwisting of PHFs.

Figure 6, A and B, indicates, at higher magnification, the morphology of untreated and chondroitinase-treated PHFs. The arrows show filaments with a similar length. In Figure 6A, a PHF is shown, whereas in Figure 6B, the arrow indicates a straight filament.

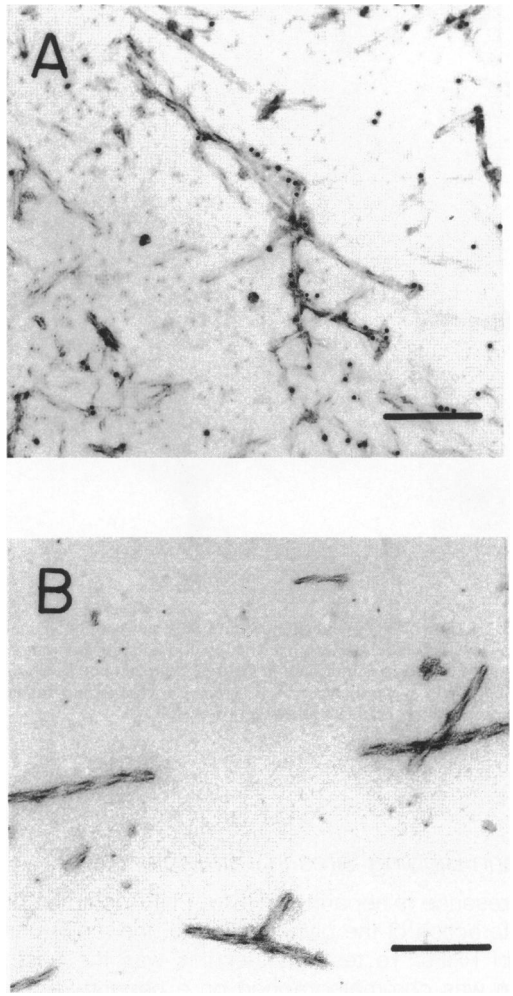

Figure 2. Labeling of PHFs with heparan sulfate antibody. **A:** Samples of PHFs were layered on an electron microscopy grid and were labeled with a heparan sulfate antibody for 45 minutes, followed by incubation with the secondary antibody conjugated with 10-nm-diameter gold particles for another 30 minutes. The electron micrograph of the incubated sample is shown. Bar, 200 nm. **B:** A sample of PHFs was analyzed as in **A**, but the heparan sulfate antibody was previously mixed with a heparin solution (10 mg/ml). The presence of heparin could also favor the integrity of the PHFs structure.

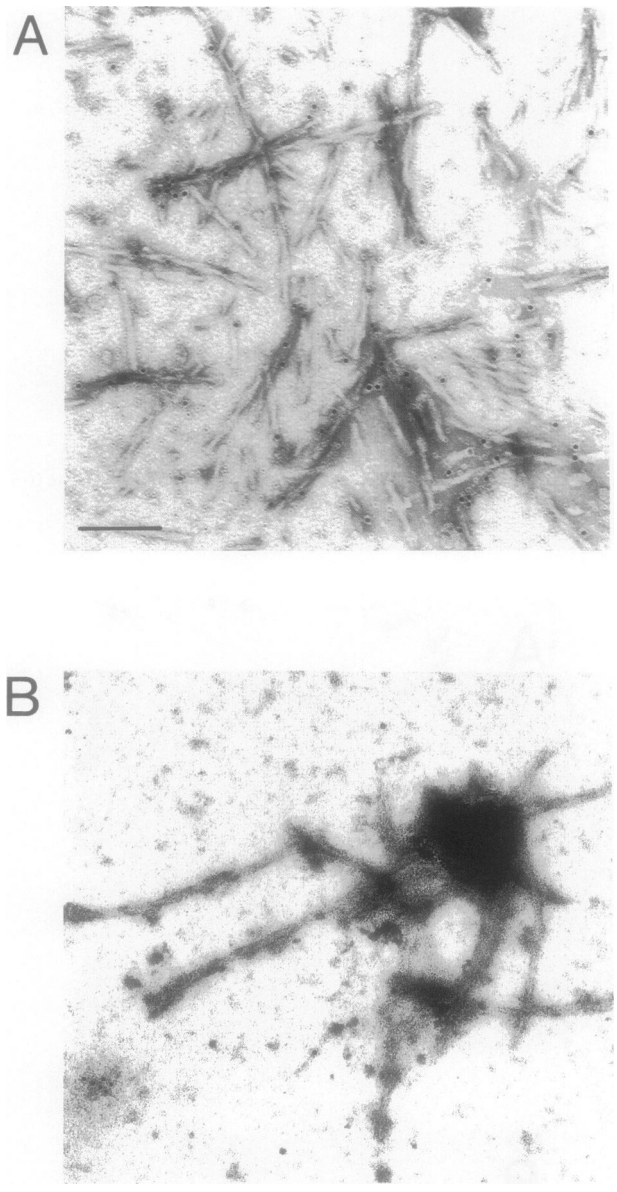

Figure 3. Labeling of PHFs with chondroitin sulfate antibody. **A:** A sample of PHFs was layered on an electron microscopy grid and labeled with a chondroitin sulfate antibody as indicated in Figure 3. Bar, 200 nm. **B:** Chondroitinase-treated PHFs were analyzed as in **A** using a chondroitin sulfate antibody. In this case no gold labeling was observed.

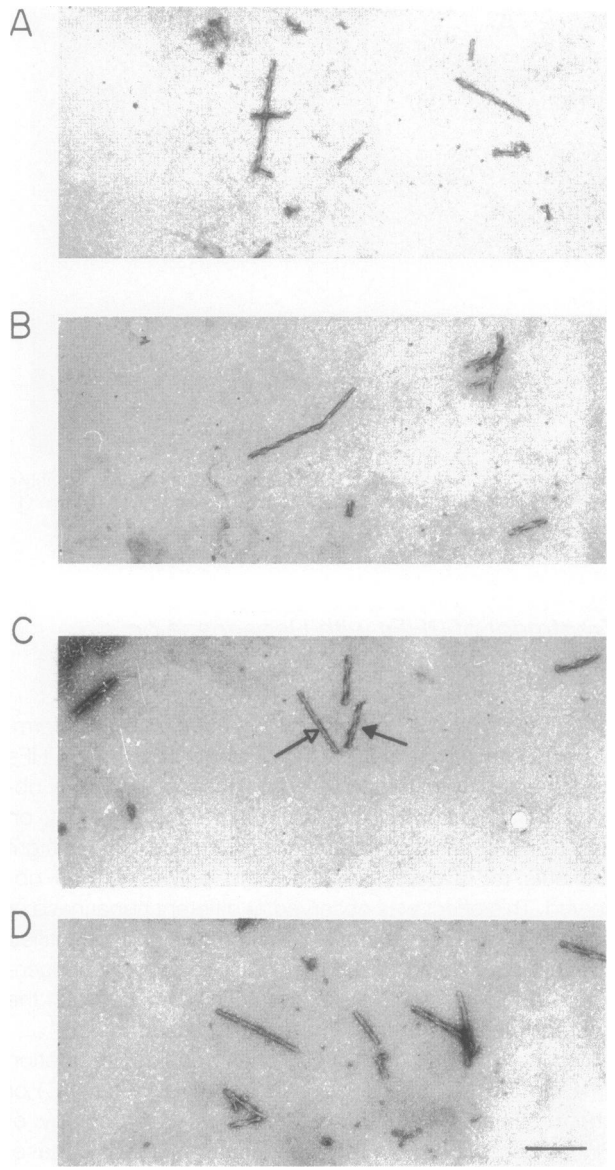

Figure 4. Effect of heparinase concentration on the helicity of PHFs. **A:** Control PHFs sample. **B** to **D:** PHFs samples incubated with heparinase (25 to 100 U) for 2.5 hours at 37°C. Polymers containing a twisted and an untwisted region can be found (**B**). In the same preparation, twisted and untwisted polymers are observed (see the **arrows** in **C**). This can be also observed in **D**. Bar, 200 nm.

Heparin Binding Sites in Tau Molecule

The presence of heparin (or HS) in PHFs could be due to the interaction of the glycan with tau, the major component of PHFs. To test whether this was the case, tau protein was chromatographed on a heparin-Sepharose column to analyze whether the protein binds to it. Figure 7 shows that, indeed, the whole tau protein, but not calmodulin (tested as a negative control), binds to the column and is eluted by adding 1 mol/L NaCl to the binding buffer (see Materials and Methods). Alternatively, tau was eluted from the heparin-Sepharose column by the addition of heparin (1 mg/ml) but not by the addition of hyaluronic acid (1 mg/ml; data no shown). These data suggest that tau preferentially binds to sulfated GAGs.

To determine the localization of heparin-binding sites on the tau molecule, different fragments of the protein (see Figure 8) were chromatographed on heparin-Sepharose. Figure 7 shows that those fragments containing the tubulin-binding region of the tau protein and also those containing the amino-terminal half of the molecule were able to bind to heparin, implying that tau protein contains at least two heparin-binding sites (see Figure 9).

Table 1. Width of PHFs on Heparinase and Chondroitinase Treatment

	Sample	Pitch (Å)	Observation
A	Untreated PHF	645 ± 60	
	Heparinase, 25 U	696 ± 50	
	Heparinase, 100 U	788 ± 70	
B	Untreated PHF	648 ± 60	
	Chondroitinase, 25 U	766 ± 40	
	Chondroitinase, 125 U	803 ± 60	Many SFs with a constant width
C	Untreated PHF	660 ± 60	
	Chondroitinase, 2.5 hours	792 ± 40	
	Chondroitinase, 5 hours	890 ± 70	Many SFs
	Chondroitinase, 24 hours		SFs

PHFs usually have a width that alternates from 10 to 20 nm with a periodicity (pitch) of ~650 ± 50 Å. In the table, the average pitch values for untreated or glycanase-treated PHF samples are shown. The samples were incubated 2.5 hours at 37°C, in the case of A and B. In C, 50 U of chondroitinase was used for each treated sample, at the incubation times indicated.

Discussion

The molecular characterization of the two major aberrant structures present in AD, senile plaques and NFTs, has indicated that, despite the differences between the two major components (Aβ and tau) present in these structures, there are common molecules, such as negatively charged GAGs present in both types of structures.[27–29,32] These GAGs could facilitate, in the case of the tau protein, the formation of fibrillar polymers resembling SFs and PHFs[24,26] in a way similar to that indicated for negatively charged sugars in the formation of fibrillar polymers from other proteins, such as type VI collagen.[36]

The influence of negatively charged carbohydrates, such as heparin, in the assembly of tau into fibrillar polymers, is probably due to the anionic nature of these sugars as uncharged carbohydrates such as hyaluronic acid did not facilitate tau assembly. Additionally, the presence of anions other than GAGs can mimic the effect of the negatively charged glycans.[24] However, the fact that these anionic carbohydrates and not other anions are located in NFTs[25,27] and in PHFs suggests that these sugars could also play a role *in vivo*, a role that has been suggested could be related to AD pathogenesis.[37] Additionally, the morphological changes observed in PHFs from AD patients on heparinase or chondroitinase treatment also support a role for heparin, chondroitin sulfate, or negatively charged carbohydrates in facilitating PHFs

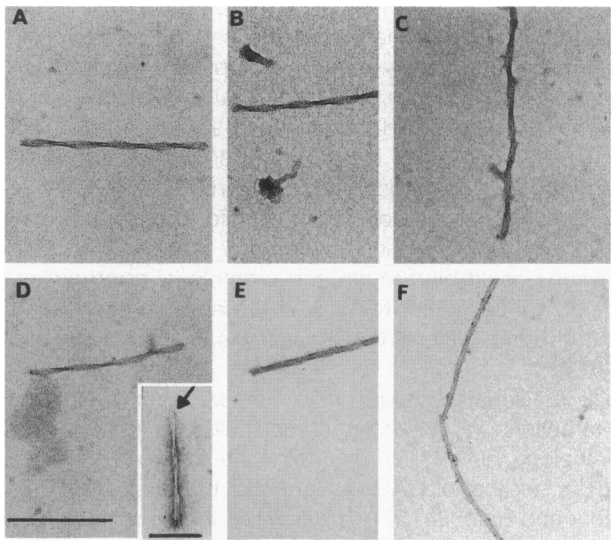

Figure 5. Effect of time of incubation with heparinase and chondroitinase on the helicity of PHFs. **A:** to **C:** Samples incubated with heparinase (100 U) for 5, 7, and 24 hours at 37°C, respectively. **D** to **F:** Samples incubated with chondroitinase (50 U) in the same conditions for 5, 7, and 24 hours. In the **inset**, the **arrow** shows the beginning of untwisting of one PHF in more detail. Bar, 250 nm and 125 nm (**inset**).

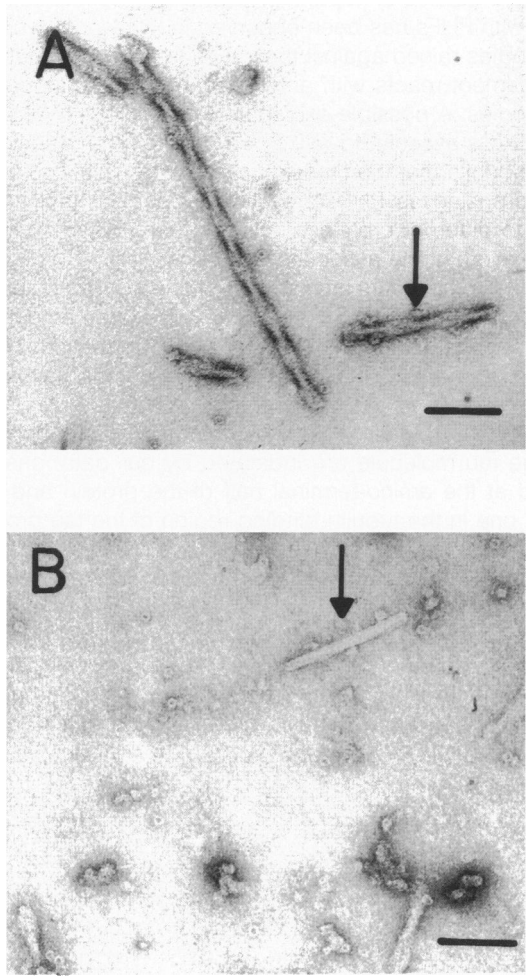

Figure 6. Treatment of PHFs with chondroitinase. PHFs were incubated in the absence (**A**) or the presence (**B**) of chondroitinase (50 U) for 12 hours, and the morphology of the samples was visualized by electron microscopy. The **arrows** show filaments with similar length. Bar, 160 nm.

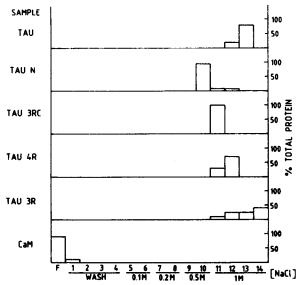

Figure 7. Heparin binding of tau or fragments of tau protein. Twenty micrograms of the whole recombinant tau molecule (tau), or the fragments (see Figure 7) containing the amino-terminal region (tau N), three tubulin-binding motifs and the carboxyl-terminal region (tau 3RC), four or three tubulin-binding motifs (4R or 3R), and calmodulin (CaM) (tested as negative control), were chromatographed on a 0.5-ml heparin-Sepharose column under the conditions indicated in Materials and Methods. The protein absorbed to the column was eluted stepwise by increasing the NaCl concentration. The eluted protein was characterized by gel electrophoresis, and the proportion of eluted protein at each step was measured.

formation and influencing the helicity of these filaments. With respect to this point, it is noteworthy that tau from PHFs is glycosylated and deglycosylation results in the formation of SFs.[38]

The presence of heparin or chondroitin sulfate associated with PHFs has been observed in this work by using antibodies raised against these GAGs. However, not every filament reacts with anti-heparan or anti-chondroitin antibodies. A possible explanation for this observation is that GAGs associated with PHFs bind to other additional tau subunits that then mask these GAGs. This would not be surprising as PHFs are sticky structures that could bind to different proteins, including unmodified tau[39] or other microtubule-associated proteins.[40]

PHF-like polymers have been obtained *in vitro* by using only recombinant tau.[14,22,24] However, a large amount of recombinant tau is required for polymer formation. Thus, *in vivo* heparin or other sulfated glycans could favor tau polymerization at lower concentrations by interacting with the protein. At least two putative binding sites of heparin on the tau molecule are indicated by our data: one located at the amino-terminal half of the protein and the other one in the tubulin-binding region of the tau protein (Figure 9).

A motif for heparin-binding sites with the consensus sequence BBXB (B for basic amino acid) has been proposed by Cardin and Weintraub.[41] On examination of the tau protein sequence,[42] residues 140 to 143 (present in the amino-terminal half) were observed to contain a heparin-binding motif (KKAK). Interestingly, in the β-amy-

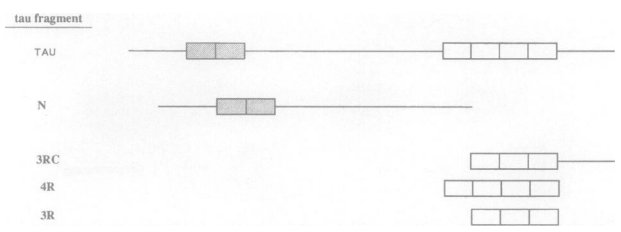

Figure 8. Schematic diagram of tau fragments. The structure of the different fragments used in Figure 7 is shown. Extra exons at the amino region are shaded and the tubulin-binding repeats are shown as open boxes.

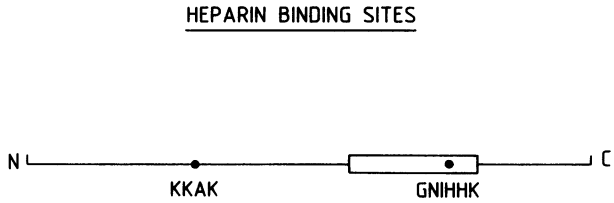

Figure 9. Heparin-binding sites on the tau molecule. The possible localization of two heparin-binding sites on the tau molecule are indicated. One, located at the amino-terminal half of the molecule, contains the sequence KKAK (residues 141–144[42]) and follows the consensus motif indicated in Ref. 41. The other one, present in the third tubulin-binding motif of tau protein,[42] shows a partial homology with that described for Aβ[31] (see text).

loid peptide, the sequence RHDS*GYEVHHQK*LV has been proposed as a heparin-binding site.[31] Comparison of this sequence with the putative heparin-binding site VTSKCGSL*GNIHHK*PGGG located in the tubulin-binding region of tau[24] reveals some similarity.

In every case, the binding of heparin (an anion) to tau could be through basic (positive) protein residues. A possible role for heparin (or sulfated glycans) in the formation of extracellular NFTs could be proposed as sulfated glycans are components of the extracellular matrix. However, heparin sulfate has been also reported to be present in intracellular NFTs.[27,29] The formation of these intracellular structures requires further analysis, as PHFs formation may require components other than tau.

It is not known whether tau can bind to GAGs in the cell cytoplasm. However, the possibility that tau binds to heparin (or other sulfated GAGs) in the cytoplasm could be similar to that of the binding of apolipoprotein E (apoE) to tau in the same compartment. Indeed, apoE-enriched lipoprotein could first be associated with cell surface proteoglycans and subsequently transferred to low-density lipoprotein (LDL)-receptor-related protein (LRP).[23] This complex could then be internalized via the endocytic pathway that normally leads to lysosomal degradation.[43] However, it has been suggested that part of apoE (and perhaps GAGs) could escape that pathway[43] and accumulate, little by little, in the cytoplasm[44] where it could bind to tau. If this process also occurs with GAGs, these compounds could slowly accumulate in the cytoplasm until a critical GAG concentration is reached, and then glycans could bind to tau and promote its aggregation, yielding intracellular NFTs. This possibility is consistent with the presence of GAGs, such as HS, in intracellular NFTs.[27,29]

It is also interesting to note that GAGs show different interactions with the different molecules implicated in the pathology of AD; for example, the binding of HS to Aβ[31,37] or that of GAGs to tau have been reported (this work and Refs. 24–27). Additionally, the heparan sulfate proteoglycan-LDL receptor could be involved in apoE binding[45] or APP binding.[46] Finally, the synthesis of these glycans takes place in cellular compartments (Golgi complex)[47] where presenilins (related with the early onset of AD) may play a role.

Acknowledgments

We thank Drs. E. Montejo de Garcini and P. Bonay for helpful discussions, Dr. Ravid (Nederlands Hersebank) for providing brain samples to isolate PHFs, and Dr. F. Lim for critical reading and comments on the manuscript.

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