In Vitro Assembly of Tau Protein: Mapping the Regions Involved in Filament Formation†

M. Pérez,*,§ M. Arrasate,§ E. Montejo de Garcini,§ V. Muñoz,‡ and J. Avila*,§

Centro de Biología Molecular "Severo Ochoa", Universidad Autónoma de Madrid, 28049-Madrid, Spain, and Department of Chemistry and Biochemistry, and Center for Biomolecular Structure and Organization, University of Maryland, College Park, Maryland 20742, USA

Received December 29, 2000; Revised Manuscript Received February 28, 2001

ABSTRACT: Unraveling the mechanism of self-assembly of the protein tau into paired helical filaments (PHFs) is a crucial step toward the understanding of Alzheimer's and other neuropathological diseases at the molecular level. In an effort to map the role of different regions of tau in the mechanism of self-assembly, we have studied the polymerization ability of different tau fragments using an in vitro assay. Our results indicate that the N-terminal domain interferes with tau's ability to polymerize in vitro. The effect seems to be size dependent. Particularly, an isoform of tau from the peripheral nervous system, which has a much larger N-terminal domain, was found unable to form filaments in our in vitro assay. This finding can explain why in Alzheimer's patients PHFs only accumulate in the neurons from the central nervous system. We also report that a short segment of tau located in the third microtubule binding repeat (residues 317 to 335, peptide 1/2R) is probably the minimal segment of that region able to grow into filaments in vitro and in the presence of heparin. In contrast with whole peptide 1/2R, peptides corresponding to either the N-terminal or C-terminal halves of this segment were unable to form filaments. Finally, our polymerization studies of peptides from the C-terminal domain reveal a short sequence spanning residues 391 to 407 that grows into filaments in vitro. This tau segment forms filaments regardless of whether is incubated with heparin. Moreover, such filaments differ in diameter and morphology, suggesting a different mechanism of self-assembly.

The histopathological analysis of brain tissue from Alzheimer's disease (AD)[1] patients reveals two aberrant structures: neurofibrillary tangles (NFTs) and senile plaques (*1*). The senile plaques accumulate in the extracellular matrix, while NFTs remain in the interior of the affected neurons (*2*). These neurons are always from the central nervous system (CNS), while the peripheral nervous system (PNS) is not affected by AD. Interestingly, formation of NFTs appears to cause the symptoms of senile dementia that are associated with AD. This was suggested by the finding of a correlation between the level of dementia displayed by AD patients and the number of NFTs accumulated in their brains (*3*). Moreover, structures similar to NFTs appear associated with other neurological diseases that also produce symptoms of dementia, such as frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17), Pick's disease, progressive supranuclear palsy, and corticobasal degeneration (*4*). To understand the relationship between NFTs formation and neurological diseases it is, therefore, imperative to unravel the high-resolution structure of these particles and the mechanism of assembly.

In the sixties, it was found that NFTs are not unique particles but rather, aggregates of fibrillar structures (*5*). These newly discovered fibers were termed paired helical filaments (PHFs), based on their ultrastructural properties under the electron microscope (*5*). PHFs are basically polymers of the microtubule associated protein tau, although other molecules can be present in minor amounts (*6*−*13*). In the CNS, there are six different isoforms of tau protein, which are produced from the same gene by alternative splicing (*14*). These isoforms have molecular masses ranging between 50 and 60 kDa, and are all constituents of the PHFs isolated from brains of AD patients (*14*). In the PNS, however, only one tau isoform is expressed. The PNS isoform has a much larger size (110−120 kDa) than its CNS counterparts (*15*). An attractive proposition that arises from this observation is that neurons from PNS could not be affected in AD because the large tau isoform cannot form PHFs.

In the last few years, there have been many efforts to develop assays to study the formation of PHFs in vitro. Using hanging drop assays similar to those utilized in crystallization trials, it has been possible to grow filamentous particles from solutions of tau protein (*16*−*20*). In these experiments, tau

† This work was supported by grants from the Spanish CICYT, Comunidad de Madrid, La Caixa and an institutional grant from Fundación Ramón Areces.

* Corresponding author. E-mail: Javila@cbm.uam.es; fax: 34-91-397 47 99.

§ Universidad Autónoma de Madrid.

‡ University of Maryland.

[1] Abbreviations: sGAGs, sulfated glycosaminoglycans; AD, Alzheimer's disease; CNS, central nervous system; PNS, peripheral nervous system; NFTs, neurofibrillary tangles; FTDP-17, frontotemporal dementia and Parkinsonism linked to chromosome 17; PHFs, paired helical filaments.

10.1021/bi002961w CCC: $20.00
Published on Web 04/25/2001

was obtained by either purification from healthy brains or by recombinant techniques. The ultrastructure of the filaments produced in vitro, as determined by electron microscopy, resembles that of PHFs isolated from AD patients. This suggested that the scaffold of the PHFs is probably constituted by tau only, in agreement with the protease resistance patterns that have been observed after digestion treatment of PHFs (*12*). Moreover, the success of the hanging drop assay indicates that it might be possible to understand the mechanism of PHFs formation by studying the polymerization process in vitro. The assay is very inefficient, however, and too large, i.e., nonphysiological, concentrations of tau are needed to grow significant amounts of fibers.

To circumvent this potential limitation of the assay, several groups have investigated a variety of additional factors that could be acting as facilitators of the process in vivo. One possibility is a role in PHF formation of tau's postranslational modifications, such as phosphorylation (*7*), glycation (*21*, *22*), oxidation (*23*−*25*), and/or truncation (*26*). Alternatively, the facilitator could be another molecule that copolymerizes with tau in vivo. Among these, particularly appropriate candidates are the sulfated glycosaminoglycans (sGAG), like heparin (*27*, *28*). sGAG are present in NFT (*2*), and their polyanionic nature can counteract the large excess of positive charges from lysines and arginines occurring in the central region of tau. Following a similar rationale, other polyanions, such as RNA or fatty acids, have been investigated (*29*, *30*).

The in vitro assembly of tau and fragments in the presence of sGAG has been extensively studied. Such experiments have resulted in the identification of the microtubule binding repeats as the region of tau that is mainly involved in polymer formation (*16*, *20*, *28*, *31*), again, in agreement with PHFs protease resistance experiments (*12*). In one of these studies, it has been found that a short 19-residue peptide (1/2R) corresponding to a segment of the third microtubule binding repeat (residues 317 to 335) is able to self-assemble into fibrillar polymers when incubated in the presence of heparin (*28*). To date, this is the smallest segment of tau that has been reported to be able to form filamentous polymers by itself in the hanging drop assay. A small peptide also from the microtubule binding domain has recently been shown to accelerate the polymerization of other tau fragments by coincubation (*32*). This peptide was not able to form filaments in the hanging drop assay. On the other hand, tau protein can be divided in different regions that could have different roles in its binding to microtubules (*33*, *34*).

In a similar fashion, in this work we attempt to further map the role that different regions of tau, i.e., the N-terminal domain, microtubule binding repeats, and C-terminal domain, have on the ability of this protein to polymerize into fibrillar structures. To investigate these issues, we use the hanging drop assay with different tau molecules, fragments, and peptides. The structure of the in vitro polymers is studied by transmission electron microscopy, and the amount of polymer produced is quantified by sedimentation and Western blot analysis. By comparing the ability to polymerize into filamentous structures displayed by each of these molecules we address three specific questions related to PHFs formation: (i) does a larger N-terminal domain, such as the one of the PNS isoform, decrease tau's ability to form PHFs-like structures; (ii) is the region between residues 317 and 335, indeed, the minimal segment of tau capable of independently forming filamentous polymers; and (iii) are there any segments of the C-terminal domain involved in tau self-assembly to form PHFs.

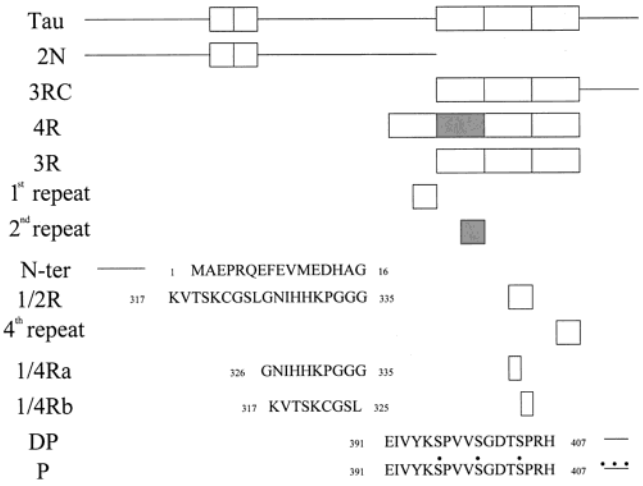

FIGURE 1: Different tau fragments used in this work. The localization of the different tau fragments in whole tau is indicated schematically; (●) indicates that the peptide is phosphorylated. The residue number is for the biggest tau CNS isoform (*14*). Some variants of these peptides (see text) were also used.

MATERIALS AND METHODS

Protein and Peptide Preparation. Tau protein was isolated from adult rat dorsal root ganglion (*35*), rat brain, or rat cerebellum. The tissue was homogenized in 0.1 M MES, 0.5 mM $MgCl_2$, and 2 mM EGTA (buffer A). After centrifugation of the sample, the supernatant was heat treated as described (*36*) to remove the particulate fraction. The heat-resistant protein was precipitated with ammonium sulfate and purified by FPLC after resuspension in buffer A. Purified tau protein was then characterized by gel electrophoresis and Western blotting. Recombinant tau human tau (isoform with three tubulin binding motifs and two extra exons in the N-terminal domain), tau fragment 2N, which contains the amino-terminal half of tau protein, tau fragment 3RC, which contains the three tubulin binding motifs and the carboxyl-terminal region, and tau fragments 4R, 3R, i.e., four or three tubulin binding motifs, were isolated as previously described (*28*) (see Figure 1 for a schematic representation).

In this work, we use the following peptides, which have been synthesized and purified as previously reported (*37*). Briefly, the peptides were synthesized on an automatic solid-phase peptide synthesizer (type 430A, Applied Biosystems) and purified by reverse-phase HPLC on a Nova Pak c18 column. Peptide 1/2R (third repeat): KVTSKCGSLGNIHHKPGGG; peptide 1/2R-P: with serine 8 in phosphorylated form, prepared as indicated in ref *37*; peptide 1/2R (third repeat) without the first lysine: VTSKCGSLGNIHHKPGGG; peptide 1/4Ra: GNIHHKPGGG; peptide 1/4Rb: KVTSKCGSL; peptide DP: EIVYKSPVVSGDTSPRH; peptide LDP: EIFYKSPVVSGDTSPRHLSNVSSTGSIDMVDSP; peptide P: DP with all serines in phosphorylated form; peptide N-terminal: MAEPRQEFEVMEDHAG; peptide first repeat: VKSKIGSTENLKM Q PGGG; peptide second repeat: VQSKCGSKDNIKH V PGGG; peptide fourth repeat: VQSKIGSLDNITH V PGGG.

Assembly of Tau Peptides into Filaments. Filaments were grown by vapor diffusion in hanging drops, as previously

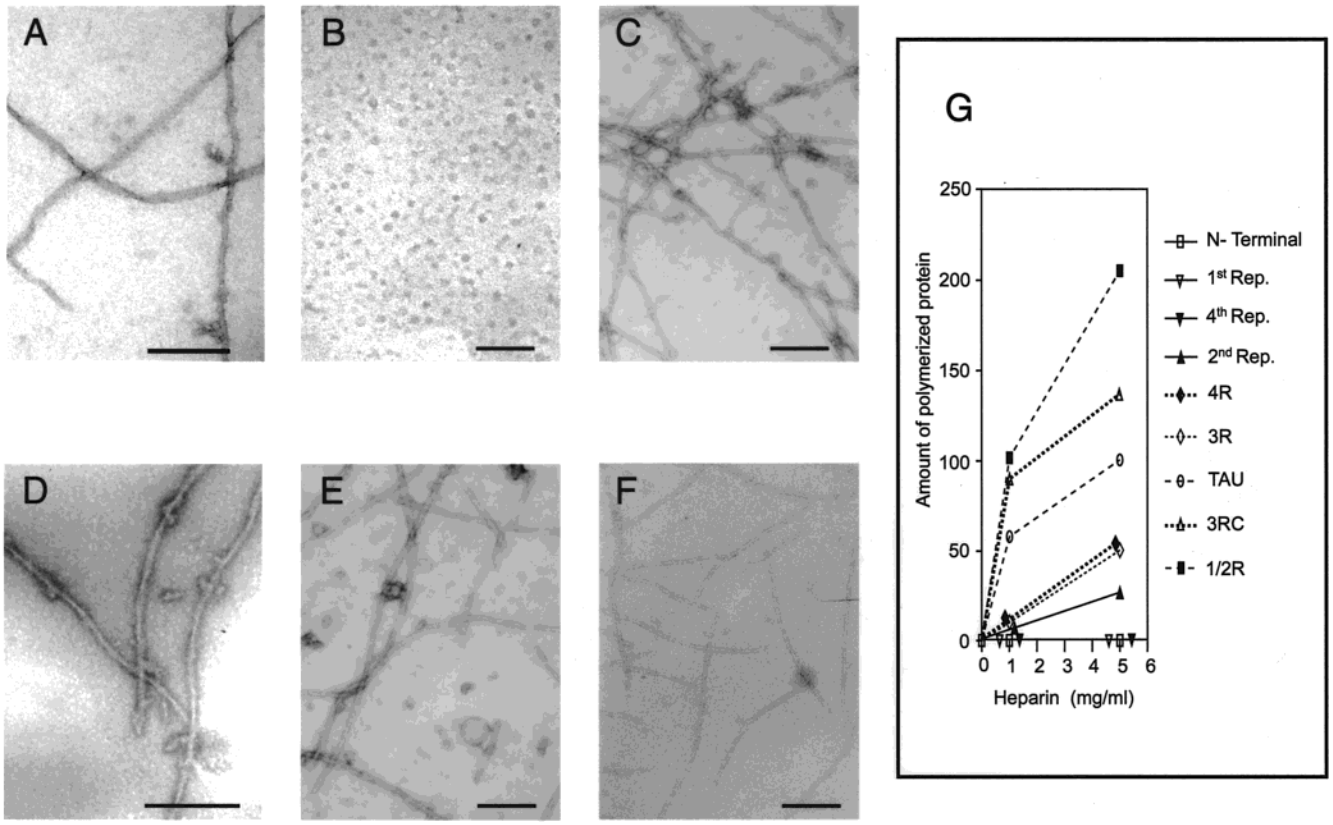

FIGURE 2: In vitro polymerization of tau fragments. (A) Electron microscopy image of the hanging drop assay with whole tau molecule (see Figure 1); (B) N-terminal region; (C) 3RC peptide; (D) 4R peptide; (E) 3R peptide. (F) 1/2 R peptide. (G) All previous fragments and those similar to 1/2 R peptide present in the first, second, and fourth tau repeats (see Materials and Methods and text) were incubated with increasing amounts of heparin in hanging drop assays. Their ability to assemble into filamentous polymers was measured by sedimentation using centrifugation and quantitation of Coomassie blue staining in SDS−PAGE gels. Bars in panels B, C, E, and F represent 200 nm and represent 100 nm in panels A and D.

described (*28*). Protein polymerization was tested in the presence or absence of heparin at different concentrations (0.5−5 mg/mL) (high molecular weight heparin was purchased from Rovi, Spain) (*28*) or in its absence. Protein or peptide concentration was set to 1 mg/mL in final volumes between 20 μL and 1 mL. To quantify the amount of polymerized protein, samples were centrifuged for 30 min at maximum speed in an Airfuge (Beckman). The protein present in supernatant and pellet was analyzed by gel electrophoresis followed by Coomassie Brilliant Blue staining. The amount of protein was quantified by densitometry using bovine serum albumin as reference. The presence of tau protein was determined by Western blotting using antibody 7.51, (a kind gift of Dr. C. Wischik, UK).

Electron Microscopy. The samples were visualized as previously indicated (*28*).

RESULTS

In Vitro Polymerization of Tau and its Fragments. Whole recombinant tau can be induced to self-assemble into filaments by slowly concentrating the protein using a hanging drop assay in which heparin has been added in ~equimolar amounts (*28*). The structure of these filaments, as observed by negative staining electron microscopy, closely resembles PHFs (Figure 2, panel A). The hanging drop assay in the presence of heparin can also be used to investigate the ability to form filaments in vitro of tau fragments that lack different regions of the protein. Incubation with heparin of a fragment of tau that includes the N-terminal domain and proline-rich region (2N, see Figure 1) does not produce measurable amounts of polymer. This is the case whether we investigate polymerization by electron microscopy (Figure 2, panel B) or using a sedimentation assay (Figure 2, panel G). The region that corresponds to the other segment of tau (3RC), i.e., the three microtubule binding repeats and the C-terminal domain, forms polymers in amounts that are measurable by the sedimentation assay (Figure 2, panel G) and have filamentous structure (Figure 2, panel C). This experiment indicates that, probably, the last half of the protein is the one responsible for self-assembly in vitro. If the polymerization assay is carried out with a smaller fragment that only includes the four (4R) or three repeats (3R) we still observe filamentous polymers (Figure 2, panels D and E). The sedimentation assay reveals, however, that these fragments render significantly less amount of filamentous polymer than 3RC or whole recombinant tau. The smallest region that has been found to self-assemble into filaments when incubated with heparin is a fragment including roughly half of the third microtubule repeat (1/2R) (*28*) (Figure 2). Not only this fragment is the smallest fragment that forms polymers, but forms them quite efficiently. As can be seen in Figure 2, panel G, fragment 1/2R forms more polymeric material in this assay than any other tau fragment or even the whole recombinant tau. The straight filaments assembled from 1/2R peptide show a diameter of 11 ± 1 nm, slighter thinner than those assembled from the whole tau protein (*28*). Fragment

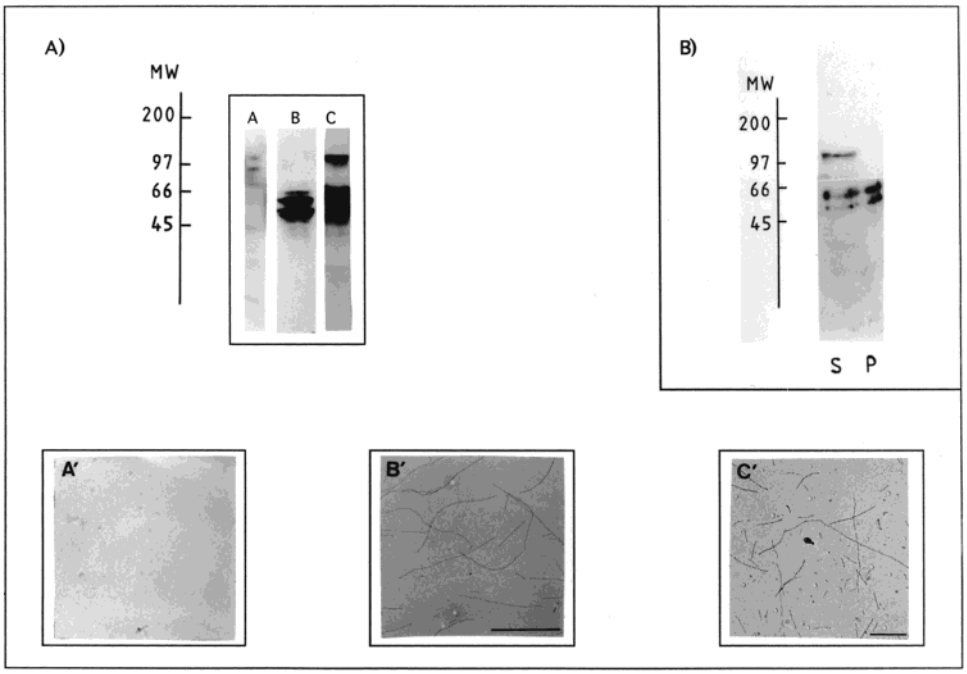

FIGURE 3: Characterization and polymerization assays in the presence of heparin of tau protein from different sources. (A) Tau protein was isolated from rat dorsal root ganglion (A, A′); rat brain (B, B′); and rat cerebellum (C, C′). Tau was characterized by Western blot using tau antibody 7.51. (B) In C and C′ the polymers formed by cerebellum tau (P) were separated from the unassembled protein (S) by centrifugation, the presence of big and small tau were analyzed by Western blot using 7.51. To avoid a very strong reaction of ab 7.51 with small tau, the paper where the proteins were blotted was divided in two parts to carry out the reaction of big and small tau with ab 7.51 independently. Bars indicate 1 μm in B′ and 500 nm in C′.

1/2 R has some sequence similarities to the other peptides present in the first, second, or fourth tau repeats (see Materials and Methods). However, when the polymerization of these peptides was assayed only that corresponding to the second repeat is able to form polymers, but in a much lower amount (Figure 2, panel G, and see also ref *31*). In support of the previous data, recently it has been indicated that the presence of the fourth repeat could be dispensable for tau polymerization (*38*).

Effect of the N-terminal Domain on Tau Polymerization. The results of the previous section show that the fragment 3RC is more prone to form polymers than whole recombinant tau (Figure 2). This suggests that the N-terminal half of tau could be interfering with tau self-assembly to form filaments. The tau molecule used in these experiments derives from the central nervous system. In the peripheral nervous system, a larger isoform (molecular mass 110 kDa) is normally expressed (*35*). The larger size of this molecule is due to the addition of an alternatively spliced exon to the N-terminal domain. To study the ability of this molecule to form filaments in vitro, we have isolated it from adult rat dorsal ganglion, where it is the predominant tau isoform (*35*). A Western blot analysis of the isolated protein shows a typical pattern of tau protein from this tissue (Figure 3, panel A), which indicates that in this preparation we primarily have the large isoform. The hanging drop assay in the presence of heparin for the large isoform of tau does not show any detectable amounts of filamentous material (Figure 3A′). To determine whether the different polymerization behavior of this sample in our assay was due to differences in the amino acid sequence between human (experiments from the previous section) and rat tau, we also carried out a hanging drop assay using tau isolated from rat brain. In our assay, this sample forms filamentous polymers similar to those produced by human tau (Figure 3B′), which is consistent with previous studies (*27*, *28*). In an isolation of tau from rat cerebellum, however, we found the presence of both small and large tau isoforms (Figure 3). The second one is quite possibly the result of a contamination of the sample. We have taken advantage of this circumstance to investigate the copolymerization ability of the two isoforms. The hanging drop assay of the mixture of both isoforms does, indeed, render filamentous polymers (see photograph C′ in Figure 3). These polymers are only composed of the smaller tau isoform, as indicated by Western blot analysis of the polymeric fraction isolated by centrifugation (Figure 3, panel B). All these results indicate that a larger N-terminal domain in tau protein interferes with its ability to grow into filaments in vitro.

Is 1/2R the Smallest Intra-Repeat Region of Tau Able to Self-Assemble into Filaments in the Presence of Heparin? 1/2R fragment is able to polymerize into filamentous structures with higher efficiency than bigger tau fragments or whole tau (Figure 2, panel F). Moreover, it has been proposed that this segment constitutes the minimal region required for protein assembly (*28*). To test this idea, we have synthesized two peptides that include the sequence of the N-terminal (peptide 1/4Rb) and C-terminal (peptide 1/4Ra) halves of 1/2R. We incubated each of the peptides independently with increasing amounts of heparin (Table 1) in hanging drop assays to determine their ability to form filaments. Under these conditions, no signs of self-assembly into either filaments or amorphous aggregates are observed for peptide 1/4Ra (see also Figure 4, panel B). Peptide 1/4Rb self-assembles, but it does so by forming amorphous aggregates instead of filaments (see Figure 4, panel C). Moreover, coincubation of each of these two peptides with fragment 1/2R decreases the amount of filamentous polymer recovered after the hanging drop assay performed in the

Table 1: Polymerization of 1/2R, 1/4Ra, 1/4Rb Peptides (1 mg/mL) into Filamentous Polymers in the Presence of Different Heparin Concentrations, Determined by Electron Microscopy Analysis[a]

hep (mg/mL)	1/2R	1/4Ra polymerization?	1/4Rb
0	−	−	−
0.1	−/+	−	−
0.5	+	−	−
1	+	−	−
5	++	−	−
10	+++	−	−
15	+++	−	−

[a] The number of + symbols indicates an approached measurement of the amount of polymers observed.

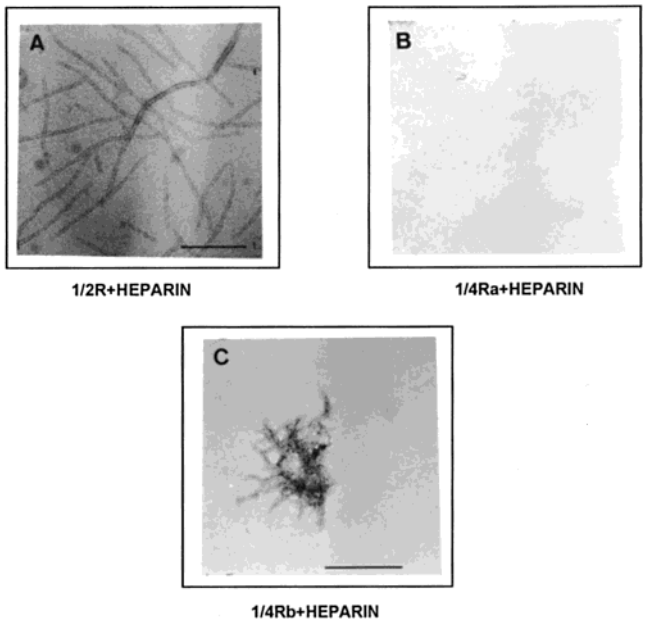

FIGURE 4: The smallest tau peptide that could form fibrillar polymers. (A) peptide 1/2R; (B) peptide 1/4Ra; (C) peptide 1/4Rb. The peptides were tested for assembly using the hanging drop assay in the presence of heparin. Only 1/2R peptide assembles into fibrillar polymers, whereas peptide 1/4Rb only results in the formation of a few protein aggregates. Bars indicate 200 nm.

presence of heparin (Table 2). This is probably caused by direct competition between these peptides and 1/2R for binding to heparin.

Modifications on 1/2 R Peptide and Their Effects on its Assembly. In Figure 2, panel F, and in a previous report (*31*), it is shown that the peptide KVTSKCGSLGNIHHKPGGG (1/2R peptide) can form polymers in the presence of sGAG. Since sGAG are polyanions, a role for basic residues such as lysine in the binding to sGAG could be proposed. On the other hand, the presence of acidic residues such as glutamatic or aspartatic acid should decrease the interaction with sGAG. 1/2R peptide contains three lysine residues, the second one, number 5, is conserved in the different repeats. Removal of the first lysine in 1/2 R peptide does not prevent its assembly into filaments (*28*). Additionally, glycine located at position 10 is replaced by an acidic residue in the similar peptides present in the other repeats. Thus, we tested if a peptide containing the sequence of 1/2R peptide but changing glycine 10 by aspartic acid and lysine 15 by valine is able to assemble. Our results (by electron microscopy analysis) indicated a lack of polymerization of that peptide. However, a similar one to this mutated peptide is present in the second

Table 2: Polymerization of 1/2R Peptide (0.3 mM) in the Presence of Heparin at 0.5 mg/mL and Different Amounts of Peptides 1/4Ra and 1/4Rb Determined by Electron Microscopy Analysis

(A)		(B)	
1/4Ra (mM)	1/2R polymerization?	1/4Rb (mM)	1/2R polymerization?
0.175	+	0.175	+
0.375	+	0.375	+/−
0.750	+	0.750	−
1.5	+	1.5	−
3	+/−	3	−
4	−	4	+/−[a]
6	−	6	+[a]

[a] Amorphous aggregates.

repeat and is able to polymerize, although in a lower amount (see Figure 2, panel G), the main difference between both peptides being the presence in the second repeat peptide of a lysine at its C-terminal region. Thus, we can suggest that the presence of the lysine close to the C-terminal of 1/2 R peptide is a positive factor for peptide polymerization whereas, that of an acidic residue in position 10, could act as a negative factor.

Another specific characteristic for 1/2R peptide and for that peptide present in the second repeat (the only two peptides that polymerize in vitro in the presence of sGAG) is the presence of a cysteine. Thus, we tested the influence of that cysteine residue in the assembly of 1/2R peptide. In this way, we analyze the assembly of 1/2R peptide (and also the whole tau protein) in the absence or presence of 1, 5, and 20 mM DTT. No differences in polymerization, as determined by electron microscopy analysis, were observed in the different samples (not shown). This result agrees with a recent report (*39*).

Finally, we tested if phosphorylation of tau serine 324 (residue number 8 in 1/2R peptide) could modify 1/2R peptide assembly. To do that, we isolated phospho 1/2R peptide, as described by ref *37*, containing 0.75 mol of phosphate/mol of peptide. Comparison of polymer formation by modified and unmodified peptide, by electron microscopy, indicates a dramatic decrease in filament formation for phosphorylated 1/2R peptide as compared with unmodified 1/2R peptide. This result could agree with that reported by ref *40*, using different conditions, to study the influence of phosphorylation of tau repeats in the assembly of tau facilitated by polyanions.

Assembly of Tau C-Terminal Peptides. Experimental results from many different groups, including us, indicate that different fragments of the microtubule-binding repeat region of tau are able to form filaments in vitro (*16*, *20*, *28*, *31*). This suggests that this is the main region involved in formation of PHFs. However, a region from the C-terminal domain of tau [termed long depolymerized peptide (LDP), see Materials and Methods] has been found to self-assemble into filaments under certain conditions (*41*). The polymerization experiments that we have performed with different fragments of tau also indicate that fragments that contain the microtubule binding repeats and the C-terminal domain form filaments more efficiently than the repeats alone (Figure 2). Therefore, it is possible that both microtubule binding repeats and C-terminal domain regions are involved in the formation of PHFs. The peptide LPD, which encompasses

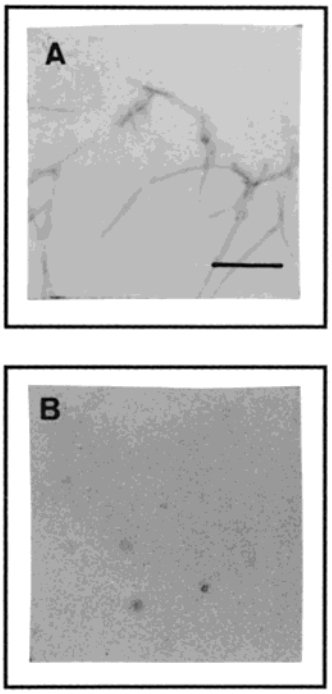

FIGURE 5: The assembly of DP tau peptide. (A) peptide DP; (B) peptide P. Bars indicate 200 nm.

residues 391 to 423 [nomenclature for the longest CNS tau isoform (*14*) is also able to form filaments in hanging drop assays in the presence of heparin; data not shown]. Interestingly, a shorter version of this peptide including only residues 391 to 407 (depolymerized peptide, DP) forms filaments (Figure 5). The amount of polymer recovered in these two experiments is, however, significantly less than the material recovered when peptide 1/2R is incubated in the presence of heparin. The morphology and thickness of the DP filaments are independent of coincubation with heparin. Moreover, DP filaments have different morphology by electron microscopy and are significantly thinner (6−7 nm versus 10−12 nm) than the straight filaments formed by peptide 1/2R in the presence of heparin. This suggests that each peptide polymerizes using a different mechanism that results on a different filament structure. Additionally, and as a control experiment, a peptide comprising the first 16 residues present at the N-terminus of the molecule was also tested to know if it could polymerize in the presence or absence of heparin. No polymers were obtained when it was assayed in either condition. Finally, we find that peptide DP does not form filaments in our assay if all serines present in its sequence (residues 396 and 404) are phosphorylated (peptide P, see Materials and Methods).

DISCUSSION

One of the most intriguing properties of tau protein is its ability to self-assemble into filamentous structures that accumulate inside CNS neurons causing AD-related dementia. While at this point it is impossible to directly study PHF formation in vivo, there are simple in vitro assays that induce formation of fibrillar polymers of tau (*16*). These assays are being used widely, because they permit the investigation of the mechanism of tau self-assembly in a carefully controlled environment. Using these experiments, it has been possible to identify the microtubule binding repeats as the tau region that is mainly involved in self-assembly (*16*, *20*, *28*). The role of different posttranslational modifications has also been investigated by carrying out in vitro polymerization assays with different forms of tau (*7*, *21*−*26*). In this work, we have used the same in vitro assays to investigate the possible role of different domains of tau protein on the formation of PHFs.

Our investigation of the role of the N-terminal domain of tau indicates that this region of the protein is not involved in self-assembly. In the conditions of our experiments, in which tau forms filaments, the N-terminal half, i.e., the N-terminal domain and proline-rich region, alone is not able to polymerize. More importantly, tau fragments that lack the N-terminal half form significantly more filamentous polymers than the complete tau. These results clearly suggest an inhibitory role in tau polymerization for the first half of the protein. Such hypothesis is further supported by our polymerization experiments with the PNS isoform of tau. PNS tau is almost twice as big as CNS tau, with all the difference in size arising from an insertion in the N-terminal domain. We have invariably failed to produce significant amounts of PNS tau polymers in vitro. Furthermore, when we coincubated short and long tau isoforms we obtained polymers that were only made of short tau molecules. An interesting question arises from these observations: how does the N-terminal half decrease tau's ability to polymerize if it does not chemically interact with the regions of tau involved in self-assembly? An explanation to this effect can be proposed based on simple ideas derived from polymer physics. The proline-rich region, with unusually high contents of prolines, constitutes a very rigid segment of the protein. In normal conditions, most of these prolines will be in trans conformation, resulting in a lollipop structure in which the, either globular or random-coiled, N-terminal domain is connected to the self-interacting C-terminal half by an almost rodlike segment. This structure will result in a very large excluded volume, which should decrease the number of productive encounters between tau monomers, thereby decreasing the probability of forming complexes that grow into polymers. Interestingly, this theory predicts that the larger the segment of N-terminal chain connected to the proline-rich region, the stronger the inhibitory effect, which is in agreement with our results on PNS tau polymerization. Therefore, it is possible that it is the larger N-terminal domain of its predominant tau isoform what protects PNS neurons from accumulating PHFs. This model also provides a simple explanation for the possible role in PHF formation of the prolyl-isomerase Pin-1 and phosphorylation of the N-terminal half (*42*−*44*). Serine/threonine phosphorylation could affect the isomerization state of adjacent prolines, either directly or through Pin-1, which would, in turn, change the excluded volume of the N-terminal half by modifying its shape and flexibility.

On the other hand, it has been proposed a role for the N-terminal half of tau molecule in the interaction of the protein with cell membrane components (*45*, *46*). The region of tau that has most consistently been found to form filamentous polymers in hanging drop assays encompasses the microtubule binding repeats.

From the four different tubulin binding repeats present in the tau molecule, mainly the third one appears to be involved in self-assembly. No polymerization was observed for the first and fourth repeat [recently, it has been indicated that

the fourth repeat is dispensable for polymerization (*38*)]. Only the second repeat could form also polymers but in a much lower amount than the third repeat containing 1/2R peptide. It has been suggested that in different pathologies (tauopathies), such as progressive supranuclear paralysis, corticobasal degeneration (*47*, *48*), or Pick's disease (*49*, *50*), there are tau filaments exclusively composed by a single set of tau isoforms, those containing four or that containing three repeats. In our assays, in the presence of sGAG, we did not find main differences between the polymerization of tau peptides containing different three or four repeats. Thus, the presence of those filaments in those pathologies could be explained by the presence in a higher proportion of a tau isoform with a specific number of repeats, in the damaged neurons.

In a previous attempt to map (in tau molecule) the self-interacting region more precisely, a 19-residue peptide from that region (1/2R) was found to form filaments when coincubated with heparin (*51*). These polymers have similar dimensions (10−12 nm diameter) and morphology to others grown by incubating larger fragments of tau (Figure 2). 1/2R peptide is very interesting, because it seems to be the core of the region involved in self-assembly (*28*). The question is then what makes this peptide form fibrillar polymers with heparin, and whether it is possible to form filaments with an even shorter sequence. 1/2R does not have significant hydrophobic patches that could facilitate self-assembly. It does have, however, three lysines that confer this peptide with a net positive charge of 3 at neutral pH. The positive charges are probably responsible for the assembly of this peptide into copolymers with the polyanion heparin. The three lysines are spread throughout the sequence with spacing that would place all of them on one face of a putative β-sheet conformation. The β-sheet could be stabilized by salt-bridges between the lysines in one face of the structure and the negative charges of heparin. Interestingly, both parallel and antiparallel β-sheets would result in a similar structure, although with slightly different charge distribution patterns. To test this hypothesis, we have synthesized two peptides that correspond to the two halves of 1/2R (1/4Ra and 1/4Rb). These peptides are not able to form such structures because they are too short and do not have the appropriate pattern of positive charges. Therefore, if the in vitro filaments are produced by addition of monomers in β-strand conformation to a preformed β-sheet structure, which is stabilized by heparin, we should expect 1/4Ra and 1/4Rb to be unable to grow into polymers. Indeed, when we incubated either 1/4Ra or 1/4Rb with heparin we did not find filamentous polymers. Incubation of 1/4Rb produced aggregates with amorphous shape as revealed by electron microscopy (Figure 4, panel C). Despite their inability to form filaments these peptides interfere with the formation of 1/2R filaments. Coincubation of either of these peptides with 1/2R resulted in less filamentous polymers than incubation of 1/2R alone, presumably, because peptides 1/4Ra and 1/4Rb cannot contribute to the growth of the filament but can compete for the heparin binding sites. All these results are compatible with a β-sheet-heparin sandwich model for filament formation. Moreover, they suggest that 1/2R is very close to be the minimal segment of the microtubule binding repeat region of tau that can grow into fibrillar polymers in the presence of heparin.

Other sulfated glycosaminoglycans (sGAG) present in brain could also promote tau assembly (*52*). However, the physiological relevance of this feature is discussed. In this way, it has been suggested that in pathological conditions, such as AD, sGAG could be present in the cytosol as a result of a leakage from membrane compartments where sGAG and proteoglycans are normally present (*53*). On the other hand, the formation of tau polymers is also promoted in vitro by other membrane components that could be damaged in AD. An example could be fatty acids (*54*) or other compounds derived from them (*55*).

Additionally, we have tested if modifications on peptide 1/2 R could prevent its capacity for polymerization. We tested the role of polar residues, cysteine oxidation, or phosphorylation at serine 313. Our results agree with the recent report (*39*) with respect to cysteine's role in fiber assembly and with those of ref *40* about phosphorylation at the tubulin binding repeats and its consequences for assembly in the presence of heparin.

Finally, we have investigated the role on filament formation of the C-terminal domain of tau. Polymerization experiments with fragments of tau reveal that fragments with both microtubule binding repeats and C-terminal domain grow into filaments more efficiently than fragments including the repeats only (Figure 2, panel G). This is in contrast with the observation for the N-terminal domain and suggests a stimulation of filament growth by the C-terminal domain. Moreover, it has been reported that a peptide termed LDP, corresponding to a fragment of the C-terminal domain, can facilitate the assembly of the whole tau molecule and form polymers by itself (*41*). In this work, we have confirmed these last results and investigated the polymerization ability of shorter peptides from that region. We have found a 17-residue peptide (DP) that forms filaments in the hanging drop assay without the addition of heparin. These results are important, because they indicate that the ability to self-associate into filaments is not unique to tau's microtubule binding repeats. The C-terminal domain can also polymerize into filamentous structures. It is probably the cooperation between the self-assembly of microtubule binding repeats and the C-terminal domain which makes tau fragments that include both regions more prone to form filaments in vitro. Filaments grown from DP peptide show, however, different morphology under the electron microscope (Figure 5) and have a smaller diameter. The differences in morphological properties between DP and 1/2R filaments suggest a different molecular structure and mechanism of formation. This argument is further supported by the fact that peptide DP also forms filaments in the presence of heparin, but with the same morphology as those raised without heparin. A different mechanism of polymerization could also explain why phosphorylation of this region seems to have a negative effect on in vitro fiber formation and in some cases on in vivo PHF accumulation. We have found that DP peptide does not grow into filaments when its serines are phosphorylated. In vivo, it has been reported that in some taupathies, such as in FTDP-17, bearing the mutation R406W PHFs have low levels of tau's C-terminal phosphorylation (*55*, *56*). The fact that both microtubule binding repeats and the C-terminal domain are able to grow into filaments with different structure and, probably, distinct mechanisms of formation indicate that PHF formation is more complex than initially

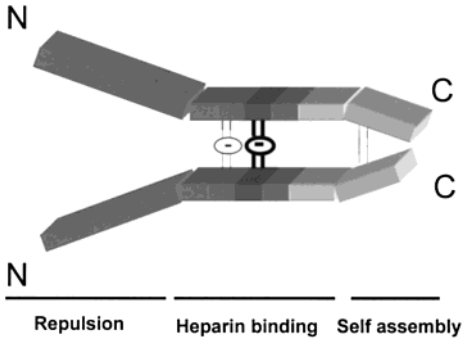

FIGURE 6: Involvement of different tau regions in tau assembly. In the figure, it is indicated that the N-terminal region has a negative effect on tau assembly, whereas the effect of the C-terminal region is positive. The protein motif required for polymerization in the presence of sGAGs is present in the tubulin binding motifs, mainly at the third one (−). The scheme is not following the real dimensions of each region on the molecule.

assumed. It is, therefore, possible that during PHF assembly different segments of tau self-associate independently and into diverse structures. This highlights the importance of in vitro self-assembly experiments with tau fragments to fully understand the physical basis of PHF formation in the cell.

CONCLUSIONS

Using the data presented in this work we propose a very low-resolution structural model of tau assembly (Figure 6). The model defines the role that different tau regions have in tau polymerization. The N-terminal region decreases the ability of tau to self-assemble with an effect that increases with its molecular size. The microtubule binding repeats are probably the scaffold of tau polymerization into PHFs, as it has been proposed by several groups (*12*, *16*, *20*, *28*), its assembly being facilitated by the presence of anions (*27*−*30*). Finally, the C-terminal region increases tau's ability to polymerize, even in the absence of heparin, by providing a self-associating surface through its 392−407 region.

REFERENCES

1. Alzheimer, A. (1911) *Z. Ges Neurol. Psychiat. 4*, 356−385.
2. Perry, G., Siedlak, S. L., Richey, P., Kawai, M., Cras, P., Kalaria, R. N., Galloway, P. G., Scardina, J. M., Cordell, B., Greenberg, B. D., Ledbetter, S. R., and Gambetti, P. (1991) *J. Neurosci. 11*, 3679−3683.
3. Arriagada, P. V., Growdon, J. H., Hedley-Whyte, E. T., and Hyman, B. T. (1992) *Neurology 42*, 631−639.
4. Spillantini, M. G., and Goedert, M. (1998) *Trends Neurosci. 21*, 428−433.
5. Kidd, M. (1963) *Nature 197*, 192−193.
6. Brion, J. P., Passasiro, H., Nuñez, J., and Flament-Durand, J. (1985) *Arch. Biol. 95*, 229−235.
7. Grundke-Iqbal, I., Iqbal, K., Quinlan, M., Tung, Y. C., Zaidi, M. S., and Wisniewski, H. M. (1986) *J. Biol. Chem. 261*, 6084−6089.
8. Ihara, Y., Nukina, N., Miura, R., and Ogawara, M. (1986) *J. Biochem. Tokyo 99*, 1807−1810.
9. Kosik, K. S., Joachim, C. L., and Selkoe, D. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4044−4048.
10. Nieto, A., Correas, I., Montejo de Garcini, E., and Avila, J. (1988) *Biochem. Biophys. Res. Commun. 154*, 660−667.
11. Wischik, C. M., Novak, M., Thogersen, H. C., Edwards, P. C., Runswick, M. J., Jakes, R., Walker, J. E., Milstein, C., Roth, M., and Klug, A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4506−4510.
12. Wischik, C. M., Novak, M., Edwards, P. C., Klug, A., Tichelaar, W., and Crowther, R. A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4884−4888.
13. Wood, J. G., Mirra, S. S., Pollock, N. J., and Binder, L. I. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4040−4043.
14. Goedert, M., Spillantini, M. G., Jakes, R., Rutherford, D., and Crowther, R. A. (1989) *Neuron 3*, 519−526.
15. Couchie, D., Mavilia, C., Georgieff, I. S., Liem, R. K., Shelanski, M. L., and Nuñez, J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 4378−4381.
16. Crowther, R. A., Olesen, O. F., Jakes, R., and Goedert, M. (1992) *FEBS Lett. 309*, 199−202.
17. Crowther, R. A., Olesen, O. F., Smith, M. J., Jakes, R., and Goedert, M. (1994) *FEBS Lett. 337*, 135−138.
18. Montejo de Garcini, E., Carrascosa, J. L., Correas, I., Nieto, A., and Avila, J. (1988) *FEBS Lett. 236*, 150−154.
19. Montejo de Garcini, E., Serrano, L., and Avila, J. (1986) *Biochem. Biophys. Res. Commun. 141*, 790−796.
20. Wille, H., Drewes, G., Biernat, J., Mandelkow, E. M., and Mandelkow, E. (1992) *J. Cell. Biol. 118*, 573−584.
21. Ledesma, M. D., Bonay, P., Colaço, C., and Avila, J. (1994) *J. Biol. Chem. 269*, 21614−21619.
22. Yan, S. D., Chen, X., Schmidt, A. M., Brett, J., Godman, G., Zou, Y. S., Scott, C. W., Caputo, C., Frappier, T., Smith, M. A., Perry, G., Yen, S. H., and Stem, D. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 7787−7791.
23. Schweers, O., Mandelkow, E. M., Biernat, J., and Mandelkow, E. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 8463−8467.
24. Smith, M. A., Sayre, L. M., Monnier, V. M., and Perry, G. (1996) *Mol. Chem. Neuropath. 28*, 41−48.
25. Troncoso, J. C., Costello, A., Watson, A. L., Jr., and Johnson, G. V. (1993) *Brain Res. 613*, 313−316.
26. Wischik, C. M., Lai, R. Y. K., and Harrington, C. R. (1997) in *Brain Microtubule Associated* (Avila, J., Brandt, R., Kosik, K. S., Eds.) pp 185−241, Harwood Academic Publ GmbH, Chur, Switzerland.
27. Goedert, M., Jakes, R., Spillantini, M. G., Hasegawa, M., Smith, M. J., and Crowther, R. A. (1996) *Nature 383*, 550−553.
28. Pérez, M., Valpuesta, J. M., Medina, M., Montejo de Garcini, E., and Avila, J. (1996) *J. Neurochem. 67*, 1183−1190.
29. Kampers, T., Friedhoff, P., Biernat, J., Mandelkow, E. M., and Mandelkow, E. (1996) *FEBS Lett. 399*, 344−349.
30. Wilson, D. M., and Binder, L. I. (1995) *J. Biol. Chem. 270*, 24306−24314.
31. Arrasate, M., Pérez, M., Armas-Portela, R., and Avila, J. (1999) *FEBS Lett. 446*, 199−202.
32. von Bergen, M., Friedhoff, P., Biernat, J., Heberle, J., Mandelkow, E. M., and Mandelkow, E. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 5129−5134.
33. Brandt, R., and Lee, G. (1993) *J. Biol. Chem. 268*, 3414−3419.
34. Goode, B. L., Denis, P. E., Panda, D., Radeke, M. J., Miller, H. P., Wilson, L., and Feinstein, S. C. (1997) *Mol. Biol. Cell 8*, 353−365.
35. Oblinger, M. M., Argasinski, A., Wong, J., and Kosik, K. S. (1991) *J. Neurosci. 11*, 2453−2459.
36. García de Ancos, J., Correas, I., and Avila, J. (1993) *J. Biol. Chem. 268*, 7976−7982.
37. Correas, I., Díaz-Nido, J., and Avila, J. (1992) *J. Biol. Chem. 267*, 15721−15728.
38. Abraha, A., Ghoshal, N., Gamblin, T. C., Cryns, V., Berry, R. W., Kuret, J., and Binder, L. I. (2000) *J. Cell. Sci. 113*, 3737−3745.
39. Gamblin, T. C., King, M. E., Kuret, J., Berry, R. W., and Binder, L. I. (2000) *Biochemistry 39*, 14203−14210.
40. Schneider, A., Biernat, J., von Bergen, M., Mandelkow, E., and Mandelkow, E. M. (1999) *Biochemistry 38*, 3549−3558.
41. Yanagawa, H., Chung, S. H., Ogawa, Y., Sato, K., Shibata-Seki, T., Masai, J., and Ishiguro, K. (1998) *Biochemistry 37*, 1979−1988.
42. Carmel, G., Mager, E. M., Binder, L. I., and Kuret, J. (1996) *J. Biol. Chem. 271*, 32789−32795.
43. Kosik, K. S. (1992) *Science 256*, 780−783.

44. Lu, P. J., Wulf, G., Zhou, X. Z., Davies, P., and Lu, K. P. (1999) *Nature 399*, 784−788.
45. Brandt, R., Leger, J., and Lee, G. (1995) *J. Cell. Biol. 131*, 1327−1340.
46. Arrasate, M., Perez, M., and Avila, J. (2000) *Neurochem. Res. 25*, 43−50.
47. Delacourte, A., and Buee, L. (1997) in *International Review of Cytology* (Jeon, K. W., Ed.) 167−224, pp, Academic Press Inc., San Diego, CA.
48. Ksiezak-Reding, H., Tracz, E., Yang, L. S., Dickson, D. W., Simon, M., and Wall, J. S. (1996) *Am. J. Pathol. 149*, 639−651.
49. Sergeant, N., David, J. P., Lefranc, D., Vermersch, P., Wattez, A., and Delacourte, A. (1997) *FEBS Lett. 412*, 578−582.
50. Delacourte, A., Robitaille, Y., Sergeant, N., Buee, L., Hof, P. R., Wattez, A., Laroche-Cholette, A., Mathieu, J., Chagnon, P., and Gauvreau, D. (1996) *J. Neuropathol. Exp. Neurol. 55*, 159−168.
51. Pérez, M., Wandosell, F., Colaço, C., and Avila, J. (1998) *Biochem. J. 335*, 369−374.
52. Arrasate, M., Pérez, M., Valpuesta, J. M., and Avila, J. (1997) *Am. J. Pathol. 151*, 1115−1122.
53. Goedert, M. (1999) *Philos. Trans. R. Soc. London B. Biol. Sci 354*, 1101−1118.
54. Wilson, D. M., and Binder, L. I. (1997) *Am. J. Pathol. 150*, 2181−2195.
55. Pérez, M., Lim, F., Arrasate, M., and Avila, J. (2000) *J. Neurochem. 74*, 2583−2589.
56. Dayanandan, R., VanSlegtenhorst, M., Mack, T. G. A., Ko, L., Yen, S. H., Leroy, K., Brion, J. P., Anderton, B. H., Hutton, M., and Lovestone, S. (1999) *FEBS Lett. 446*, 228−232.

BI002961W