Mutations of Tau Protein in Frontotemporal Dementia Promote Aggregation of Paired Helical Filaments by Enhancing Local \(\beta\)-Structure*

The microtubule-associated protein tau is a natively unfolded protein in solution, yet it is able to polymerize into the ordered paired helical filaments (PHF) of Alzheimer’s disease. In the splice isoforms lacking exon 10, this process is facilitated by the formation of \(\beta\)-structure around the hexapeptide motif PHF6 (*P301L* and *P301S*) encoded by exon 11. We have investigated the structural requirements for PHF polymerization in the context of tau isoforms containing four repeats (including exon 10). In addition to the PHF6* motif there exists a related PHF6* motif (\(\text{VQIINK}_{280}\)) in the repeat encoded by the alternatively spliced exon 10. We show that this PHF6* motif also promotes aggregation by the formation of \(\beta\)-structure and that there is a cross-talk between the two hexapeptide motifs during PHF aggregation. We also show that two of the tau mutations found in hereditary frontotemporal dementias, AK280 and P301L, have a much stronger tendency for PHF aggregation which correlates with their high propensity for \(\beta\)-structure around the hexapeptide motifs.

Pathological protein aggregation is increasingly recognized as a common denominator underlying a variety of diseases, especially in diseases of the brain where the disposal of non-functional components and the regeneration of cells is slow or absent. This applies to inappropriately processed amyloid protein or hyperphosphorylated tau protein in Alzheimer’s disease, huntingtin with expanded polyglutamine tracts in Huntington’s disease, prion protein with altered conformations in Creutzfeld-Jacob’s disease, or accumulated \(\alpha\)-synuclein in Parkinson’s disease, or mutated tau-protein in certain frontotemporal dementias, among others (1–5). There is a debate on whether the aggregates per se are detrimental to the cells because the extent of aggregation, visible by postmortem autopsies, does not always correlate with the severity of the disease. In the case of transgenic mouse models, removal of aggregates by vaccination or gene regulation has been shown to reverse the pathological effects of protein aggregation (6, 7). It is therefore of major interest to understand the pathway of abnormal protein aggregation in the brain and to find methods to prevent it or at least to slow it down.

Despite the diversity of proteins involved in the diseases, the process of aggregation appears to rest on a remarkably uniform principle, the formation of intermolecular \(\beta\)-sheets by locally unfolded segments of the polypeptide chain (for review see Ref. 8). The proteins tend to aggregate into fibers such that the \(\beta\)-strands run roughly perpendicular to the fiber axis. This “cross-\(\beta\)” structure can be recognized by fiber x-ray diffraction (9–11). In addition, the fibers can be stained with dyes that interact preferentially with \(\beta\)-sheets, such as Congo Red, thioflavine S, and others, a fact that can be exploited for neuropathological diagnosis.

In the case of tau aggregation in Alzheimer’s disease, the recognition of the principles of aggregation was complex. First, tau is an unusually hydrophilic protein, containing many polar and charged residues, and remains in solution even after heat and acid treatment (12, 13). This slowed down the attempts to find conditions for aggregation in vitro and for studying the nature of the fibers. Certain buffers, certain domains of tau, and certain cofactors were necessary to make the aggregation process experimentally accessible. Thus, the repeat domain aggregates more readily than full-length tau (14); this domain also forms the core of Alzheimer PHFs (15). Aggregation can be enhanced when tau is dimerized by oxidation (14, 16), by addition of polyanions such as heparin or RNA (17–19), or by addition of fatty acids (20, 21). A second problem was to monitor tau aggregation in real time and in solution, rather than just by electron microscopy; this has been overcome by fluorescence or light scattering assays (22, 23). But perhaps the biggest conceptual obstacle was the realization that tau had very little if any tendency to form ordered secondary structure, as seen by spectroscopic or x-ray scattering methods (12, 24), consistent with its hydrophilic nature. The CD spectra of both soluble and aggregated tau were dominated by a random coil pattern. X-ray fiber patterns of aggregates isolated from Alzheimer brain showed some component with cross-\(\beta\)-structure (10), but this could have been caused by minor contributions from amyloid fibers, and later studies with Alzheimer fibers or recombinant aggregated tau did not reproduce the result (24, 25). It was therefore unclear whether the aggregation of tau was based on \(\beta\)-sheet formation or some other structural principle.

The puzzle was partially answered recently when it was realized that a small fraction of tau, the hexapeptide motif

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* This work was supported by a grant from the Deutsche Forschungsgemeinschaft. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PHF, paired helical filament; FTIR, Fourier transform infrared spectroscopy; FTD-17, frontotemporal dementia with parkinsonism linked to chromosome 17; TFE, trifluoroethanol; ThS, thioflavine S.
PHF6 (309VQIVYK311), is capable of inducing tau aggregation via formation of a β-sheet interaction (26). For intact tau, the increase in β-structure during aggregation is minor, which explains why the structures of PHFs made from whole tau are still dominated by the random coil conformation. However, the shift toward β-structure becomes visible with smaller constructs, such as the repeat domain or shorter peptides, because here the β-structure occupies a larger fraction of the protein. These results, however, left open the issue of the different tau isoforms. Alzheimer PHFs contain a mixture of all six isoforms of tau in the central nervous system (27, 28). The repeat domain is involved in binding to microtubules (together with the flanking domains, Fig. 1); the repeats also form the core of PHFs that may contain either all four repeats (R1, R2, R3, and R4; Fig. 1) or only three (R1, R3, and R4 (29, 30)). The assembly-promoting hexapeptide motif PHF6 lies in repeat R3, and the earlier results had been obtained with tau domains and peptides lacking R2. The open question was, therefore, whether 4-repeat tau would obey similar principles of aggregation. In addition, because the extra repeat R2 can modify the rate of aggregation in different ways, depending on aggregation conditions (16, 21), we asked how the two hexapeptide motifs would interact with each other. In the present study we show that 4-repeat tau isoforms contain another hexapeptide motif, similar to PHF6, which promotes β-structure formation and aggregation.

A second open question was the role of tau mutations of frontotemporal dementias (FTDP-17). Most of the known mutations affect the C-terminal domain around the repeats, either because they change the splicing pattern (affecting the relative amount of 3-repeat versus 4-repeat tau) or because they introduce point mutations (for review see Ref. 31). Because the repeat domain participates both in microtubule binding and in PHF aggregation, one can argue that the disease-causing effect of the mutations could be a change in either of these functions (for review see Ref. 32). Previous experiments had shown that point mutations tended to accelerate PHF aggregation in vitro, and two mutations had a particularly pronounced effect, P301L and ΔK280 (23, 33–35). Because these two mutations were near or in the assembly-promoting hexapeptide motifs, we suspected that the mutations could increase the propensity for β-sheet formation and aggregation. We show that this is indeed the case. In fact, the tendency of the ΔK280 for β-sheet formation is so strong that one can achieve aggregation of the repeat domain within minutes, and the mutant ΔK280 is able to polymerize even in the absence of the polyamionic cofactors commonly used to promote aggregation. These features might explain the toxic effect of these mutants in frontotemporal neurodegeneration.

MATERIALS AND METHODS

Chemicals and Proteins—Heparin (average M₉ of 3000) and thioflavin S were obtained from Sigma. Human tau isoforms and constructs (Fig. 1) were expressed in Escherichia coli as described (36). The numbering of the amino acids is that of the isoform htau40 containing 441 residues (37). The protein was expressed and purified as described elsewhere making use of the heat stability and Mono S fast protein liquid chromatography (Amersham Biosciences) (38). The purity of the proteins was analyzed by SDS-PAGE. Protein concentrations were determined by the Bradford assay. Synthetic peptides were obtained from fluorometry, using concentrations of at least 500 μM in the absence of heparin, centrifuged at 100,000 × g. The pellets were placed into the wide end of a borosilicate capillary tube with a diameter of 0.5 mm and pushed down using a 2% agarose cushion. The samples were dried at room temperature. Diffraction patterns were recorded on a rotating anode x-ray generator (Rigaku U2000) operated at 50 kV, 100 mA, λ = 1.5418 Å (CuK), and equipped with an image plate detector (MarResearch, Hamburg, Germany).

RESULTS

Polymorization of Tau or FTDP-17 Mutants into PHFs Is Accompanied by the Formation of β-Structure in the Repeat Domain—Tau in the human central nervous system occurs in six isoforms; the two inserts near the N terminus and the second of four imperfect repeats in the C-terminal half may be absent because of alternative splicing (Fig. 1A). The repeat domain, in combination with its proline-rich flanking domains, is important for microtubule binding (38, 39); it forms the core of PHFs in Alzheimer’s disease (15) and also assembles more readily than full-length tau into bona fide PHFs in vitro (14). For studying the principles of assembly, we therefore used recombinant full-length tau isoforms and tau constructs comprising either four repeats (K18, Fig. 1B) or three repeats (repeats 1, 3, and 4, construct K19, corresponding to fetal tau, Fig. 1C). As shown previously, in 3R-tau the hexapeptide motif PHF6 (309VQIVYK311, Fig. 1, D and E) is crucial for PHF assembly by inducing local β-structure (26). A comparison of the four repeats shows that a homologous motif is present in the second repeat (termed PHF6”, 27VQIVNK280, Fig. 1E) but not in repeats 1 and 4. We noted that the hexapeptide motifs were close to point mutations in tau described for certain frontotemporal dementias such as G272V, N279K, K312N, and L346V (23, 33, 34, 40–42) with some variation in the results.

Preparation of Samples for FTIR Analysis—Soluble tau proteins were precipitated by acetone, and the pellets were washed with 90% ethanol in water and dried by vacuum, and the protein solutions were dissolved in D₂O (Sigma). Preformed filaments were centrifuged at 100,000 × g. The supernatant was discarded, and the pellet was resuspended in D₂O and incubated for at least 2 h before measuring.

Fourier Transform Infrared Spectroscopy—FTIR experiments were performed on a Jasco FT-IR410 instrument (Jasco, Groß-Umstadt, Germany). Atmospheric water vapor was removed by flushing the spectrometer with nitrogen. Interferograms were recorded between 1700 and 1600 cm⁻¹ at a spectral resolution of 1 cm⁻¹, and 128 spectra were averaged. They were acquired in the transition mode using CaF₂ cells, separated by spacers of different thicknesses (usually 25 μm). After recording a reference spectrum of the instrument and of the used D₂O lot, the protein solutions were applied, and the absorbance spectrum of the sample was measured. The D₂O spectra and sample spectra were first corrected for the water vapor background before subtracting the D₂O spectra from the sample spectrum. To facilitate comparison the spectra were then normalized with respect to their maxima. Spectra were recorded repeatedly for every sample (up to 6 times); there was no significant difference between different preparations of a protein or different authors performing the experiments.

PHF Assembly—Aggregation was induced by incubating varying concentrations of tau isoforms or tau constructs and fragments (typically in the range of 1–100 μM) in volumes of 20–50 μl at 37 °C in 50 mM ammonium acetate, pH 7.0, or phosphate-buffered saline, pH 7.4, 1 mM dithiothreitol containing anionic cofactors (heparin) as described (22, 33). Incubation times varied between minutes up to several days. The formation of aggregates was ascertained by thioflavin S fluorescence and electron microscopy as described (22, 33).
random coil conformation by x-rays and CD (24, 35, 47, 48). By using FTIR as a sensitive method to detect β-structure, a similar dominance of random coil structure is observed for all tau constructs in solution which we have studied thus far (Fig. 2 and 3). The random coil conformation of the soluble protein is in marked contrast to the regular appearance of the paired helical filaments found in Alzheimer’s disease and other tauopathies.

PHFs assembled from full-length tau show only a weak increase in β-structure (Fig. 2). However, this becomes more clearly detectable with tau constructs comprising roughly the repeat domain (which also forms the core of Alzheimer PHFs), in particular when using FTIR spectroscopy which is more sensitive for β-structure than CD. In the experiments described here the sensitivity was enhanced by first polymerizing the protein and then pelleting the aggregates and measuring the pellet and supernatant separately. Fig. 2A shows the spectra of K18 in the soluble and in the polymerized state (solid and dashed lines). The monomeric protein exhibits a peak of absorbance in the amide I band around 1645 cm⁻¹, indicating mostly random coil structure (49). The spectrum of the polymerized protein exhibits a maximum around 1630 cm⁻¹ indicating an increased content of β-sheet. The same is true for the construct K18 containing four repeats (Fig. 2B); the maximum shifts from 1645 to 1625 cm⁻¹ upon polymerization. For comparison this procedure was also performed with the full-length tau isoforms htau23 and htau40 (Fig. 2, C and D); in these cases the polymerization induces only a shoulder at 1625 cm⁻¹ or some broadening of the peak, consistent with the fact that only a small fraction of the full-length protein is involved in forming β-structure during aggregation.

Next we wanted to determine whether mutated forms of tau occurring in frontotemporal dementias (FTDP-17) undergo similar structural transitions during PHF aggregation. Judging by their CD spectra, all full-length tau proteins containing one of the FTDP-17 mutations showed a structure similar to normal full-length tau in solution, dominated by the random coil conformation (33). To enhance the signal and the rate of aggregation, we cloned and expressed the mutants G272V, N279K, ΔK280, P301L, and V337M on the basis of the 4-repeat construct K18. Fig. 3 shows the FTIR traces obtained before and after assembly of the K18 mutants in the presence of heparin. In all cases, the spectra of the unpolymerized samples had a maximum around 1645 cm⁻¹ (typical of random coil), and assembly induced a shift of the maximum to 1620–1625 cm⁻¹ (indicating more β-structure). A special case was the mutant K18/ΔK280 whose FTIR spectrum showed a distinct shoulder at 1625 cm⁻¹ even without inducing aggregation with heparin (arrows in Fig. 3; see also assembly studies below). This can be explained by the high intrinsic tendency for aggregation which results in the spontaneous formation of oligomers, as confirmed by gel filtration (data not shown) and the higher initial ThS fluorescence level (Fig. 3A).

**PHFs Assembled from the Repeat Domain Show a Cross-β X-ray Diffraction Pattern**—The most direct demonstration of β-structure within PHFs is x-ray fiber diffraction. Earlier experiments with PHFs isolated from Alzheimer brain tissue did not show evidence for β-structure (24). However, because the overall β-content in PHFs aggregated from full-length tau is low, we used the construct K18 which has a higher fraction of β-structure, and we tried to align the PHFs in the sample by stretching and applying magnetic orientation that had been successful for orienting fibrous proteins, including fibers from β-amyloid (50). As shown in Fig. 4, the degree of orientation remained low but was sufficient to detect the characteristic reflections from a cross-β-structure (meridional 0.47 and equatorial 1 nm reflections). This means that the building blocks responsible for PHF aggregation have their β-strands oriented mainly perpendicularly to the PHF axis. This arrangement is reminiscent of other amyloid fibers (for review see Ref. 51).

The Hexapeptide Motif PHF6 (275VQINK280) in the Second Repeat Is Involved in the Transition from Random Coil to β-Sheet During Aggregation—To analyze the structural properties of the protein sequence centered around the hexapeptide PHF6 (275VQINK280), we synthesized peptides from this region of tau (R2/wt) and from the various FTDP-17 mutations located in this area (such as N279K, R2/N279K, ΔK280, R2/ΔK280). To prevent uncontrolled aggregation and β-sheet formation, the synthesized peptides were first dissolved in 100% TFE, a helix-inducing agent. FTIR spectra were taken in TFE; thereafter the peptides were dissolved in phosphate buffer, and aggregation reactions were performed in the presence or absence of heparin.

The peptides representing the wild-type sequence (R2/wt) and the mutation N279K (R2/N279K) exhibit an FTIR maxi-
Differences in the propensity for aggregation but requires heparin for aggregation. This means that in the pre-polymeric state (ΔK280 mutants being particularly pronounced) but disappear when the proteins or peptides are fully polymerized (see also Fig. 3).

For studying the aggregation of the ΔK280 peptide (R2/ΔK280) in the presence of heparin, it was first dissolved in hexafluoroisopropanol to suppress β-sheet formation (53). Prior to the experiment it was diluted into phosphate buffer in the presence of heparin. The FTIR spectrum is shown in Fig. 5C, together with that of the aggregated wild-type peptide. Both have a maximum at about 1615–1620 cm\(^{-1}\) indicating a high content of β-sheet structure, even without heparin in the case of the R2/ΔK280 peptide. Peptide R3/wt containing the PHF6 motif in the third repeat also acquires a high content of β-structure but requires heparin for aggregation. This means that differences in the propensity for β-structure are present in the pre-polymeric state (ΔK280 mutants being particularly pronounced) but disappear when the proteins or peptides are fully polymerized (see also Fig. 3).

Proline scanning mutagenesis had shown previously that a change of any residue within the PHF6 motif into a proline severely inhibited the aggregation into PHFs because proline interrupts the extended chain conformation necessary for β-sheet interactions (26). As a control for the subsequent experiments, we synthesized a peptide corresponding to the hexapeptide in the third repeat where the proline just downstream of the motif (R3/ΔP312) is deleted. When this peptide is allowed to aggregate in phosphate buffer, it shows a pronounced shift to β-structure, as expected because the hexapeptide motif was not interrupted (Fig. 5C, open circles).

This result can be compared with the mutation P301L, located upstream of the hexapeptide motif PHF6 in the third repeat. This FTDP-17 mutation induces a pronounced increase in PHF assembly from tau (33). Because the PHF6 motif is a strong determinant of PHF assembly, we analyzed the interplay between the P301L mutation and the PHF6 motif on the basis of the peptide R3/P301wt and its P301L mutant R3/P301L. The FTIR spectra of the peptides dissolved in TFE (Fig. 5D, filled and open circles) show a maximum around 1650 cm\(^{-1}\), consistent with random coil and/or α-helical structure induced by the solvent. After aggregation in the presence of heparin (Fig. 5D, filled and open squares) frequencies around 1625 cm\(^{-1}\) become dominant, indicative of pronounced β-structure. In the aggregated state there is no significant difference between wild-type peptide and peptide carrying a mutation.

In summary, both hexapeptide motifs PHF6* (in the second repeat) and PHF6 (in the third repeat) enhance β-interactions when aggregating in the form of short peptides, consistent with the assumption that these motifs also enhance the aggregation of full-length tau with concomitant formation of cross-β-structure. Furthermore, in both cases mutations occurring in FTDP-17 are fully capable of aggregation by the same structural principles, notably mutation ΔK280 (near the PHF6* motif) and P301L (near PHF6).

Mutation of ΔK280 within the PHF6* Motif in the Second Repeat Strongly Enhances PHF Assembly Even Without Polya-
Our earlier studies with htau40 and construct K18 had shown that FTDP-17 mutations H9004K280 and P301L tended to enhance the rate of PHF aggregation in the presence of polyanions (33). We asked whether mutations in construct K18 were capable of promoting aggregation even without polyanions. In this case, the aggregation of K18 or its mutants, G272V, N279K, P301L, and V337M, is extremely slow and does not give rise to appreciable increase in ThS fluorescence over a period of several days (Fig. 6, bottom curve). The surprising exception to this rule was K18/H9004K280. The solution of K18/H9004K280 polymerized with a half-time of 10 h (Fig. 6, top curve). Here, too, the presence of heparin leads to a further acceleration with a half-time of 20 min.

Electron microscopy of the assembly products revealed that the ΔK280 mutant formed highly regular filaments with the typical PHF structure. This was true both in the presence and absence of heparin (Fig. 7, A and B). By contrast, shorter peptides aggregated either into fibers without the twisted morphology of PHFs (e.g., peptide R2wt containing the PHF6 motif, Fig. 7D) or formed amorphous aggregates (Fig. 7, E and F). This means that although the motifs PHF6 or PHF6* act as nuclei of aggregation by inducing β-structure, they are not sufficient for obtaining the morphology of PHFs.

**Interactions between Hexapeptide Motifs PHF6 and PHF6* during Aggregation**—As shown above, the deletion of residue Lys-280 strongly promotes the formation of PHFs, whereas introducing prolines into the PHF6 motif suppresses PHFs by suppressing β-structure (26). Because the 4-repeat domain K18 contains two such motifs, PHF6 and PHF6*, we asked whether they interfere with each other and whether the ΔK280 mutation could override a proline mutation. We therefore generated mutant constructs where the conserved residues Ile-277 (in PHF6*) or Ile-308 (in PHF6) were replaced by prolines, either singly or both. Fig. 6B shows the aggregation kinetics in the presence of heparin. Construct K18 aggregates with a half-time of ~10 h (Fig. 6A, top curve). Here, too, the presence of heparin leads to a further acceleration with a half-time of 20 min.

**Fig. 3.** FTIR spectra of FTDP-17 mutations introduced into the 4-repeat construct K18. Note that in all cases there is a shift toward β-structure upon aggregation (dashed line), compared with the soluble protein (solid line). Polymerization was performed in the presence of heparin, except where noted (K18/H9004K280-heparin). From top to bottom: K18wt (control), G272V, N279K, ΔK280 with heparin, ΔK280 without heparin, P301L, and V337M. wt, wild type.

**Fig. 4.** X-ray diffraction pattern of PHFs derived from tau construct K18. A, x-ray pattern of a partially oriented sample of PHFs. The mean orientation of the fiber axis is roughly vertical, but due to the wide spread of orientations, the reflections are smeared out into arcs. The white disc at the center is caused by the lead backstop intercepting the primary beam. There is a sharp meridional arc at 0.47 nm spacing and a broader equatorial arc at about 1.0 nm spacing, both hallmarks of a cross-β-structure (67). The diffuse equatorial scattering intensity near the backstop arises from the contrast of the fibers and their (inhomogeneous) separation in the sample. B, diagram of a cross-β-structure where β-strands within the sheets run roughly perpendicular to the fiber axis (separated by 0.47 nm), and the sheet planes are separated by ~1 nm.
of ~4 h, but either of the proline mutations I277P and I308P in the hexapeptide motifs essentially suppressed PHF formation. No filaments were observed by electron microscopy, and no shift to a β-conformation was observed by FTIR (data not shown). This means that 4-repeat forms of tau require both hexapeptide motifs to adopt β-structure to induce PHFs. Conversely, a non-β-conformation in one of the motifs is enough to disrupt the aggregation potential of the other. This behavior of 4-repeat constructs is in contrast to 3-repeat constructs where elements outside the hexapeptide motifs alone is not sufficient to generate fibers with PHF appearance. Short model peptides assemble into conglomerates of short fibrous fragments or at best thin smooth fibrils. A PHF-structure is correspondingly larger, indicating that it already contains β-structure, presumably due to oligomerization, even in TFE. B, same peptides as in A, immediately after dissolving them in phosphate buffer. R2/wt and R2/N279K show largely random coil structure with a maximum around 1640 cm⁻¹; R2/ΔK280 shows a pronounced peak corresponding to β-structure due to aggregation, even without heparin. C, peptides in phosphate buffer after inducing aggregation with heparin. Peptides R2/wt (filled squares), R2/ΔK280 (open diamonds), R3/wt (filled circles), and R3/ΔP312 (open circles) show pronounced β-structure after aggregation. D, the 15-mer peptide R3/P301wt (covering the transition between R2 and R3 containing the hexapeptide motif PHF6 and the P301L mutation, open circles) dissolved in TFE exhibits a peak at 1650 cm⁻¹ and a small shoulder at about 1675 cm⁻¹. The peptide with the wild-type sequence (filled circles) in TFE exhibits a more pronounced shoulder at 1675 cm⁻¹, indicating a substantial content of turn structure. After aggregation in the presence of heparin both peptides show a sharp maximum at about 1625 cm⁻¹ (wt, filled squares; P301L, open squares), corresponding to a shift toward β-structure.

Turning now to K18/ΔK280, this construct aggregates much more rapidly and to a higher extent (half-time ~1.5 h, Fig. 6A, top curve). Disruption of the PHF6* motif by the I277P mutation just upstream of the ΔK280 mutation abrogates PHF formation. Surprisingly, however, the mutation I308P in the PHF6 motif does not suppress PHFs (Fig. 6B, 2nd curve from top). In other words, the PHF6* motif with the ΔK280 mutation is so potent that it can induce aggregation even when the PHF6* motif in the third repeat cannot participate in nucleating β-structure. This is reminiscent of the situation in 3-repeat tau (K19) where a single competent hexapeptide motif suffices for aggregation.

**DISCUSSION**

**Aggregation of Tau Leads to Cross-β-structure in PHFs**—Pathological aggregation via β-sheet formation is now recognized as a recurrent theme in a number of diseases, often termed “amyloid” or “conformational” diseases (for review see Ref. 54). The aggregates usually take the form of smooth fibers with diameters around 10 nm and no pronounced substructure. The underlying building principle appears to be remarkably similar, namely a cross-β-structure, formed by β-strands running roughly perpendicular to the fiber axis (10). In the case of PHFs, the detection of this feature has been difficult because full-length tau has a mostly random-coil structure that remains dominant even when it is polymerized. In addition it has so far not been possible to obtain well oriented fiber samples from PHFs. Thus, PHFs from Alzheimer brains show little or no evidence for β-structure by x-ray diffraction (10, 24), consistent with the results from CD spectroscopy. However, in fibers assembled from shorter constructs, such as the repeat domain, the fraction of β-structure is correspondingly larger and becomes visible. This is demonstrated in the fiber pattern of Fig. 4, in agreement with similar results reported recently (25), and with the results of FTIR and CD spectroscopy. On the other hand, the induction of β-structure by the hexapeptide motifs alone is not sufficient to generate fibers with PHF appearance. Short model peptides assemble into conglomerates of short fibrous fragments or at best thin smooth fibrils. A PHF-like appearance requires at least 3 or 4 repeats (constructs K19 or K18; Fig. 7). Thus, elements outside the hexapeptide motifs are needed for building and shaping the PHF structure.

**Some FTDP-17 Mutations Accelerate Aggregation by Enhancing β-Structure**—PHFs are found not only in Alzheimer’s disease but also in other neurodegenerative diseases, notably hereditary frontotemporal dementias caused by mutations in the tau gene (FTDP-17, for review see Ref. 32). Several mutations in coding or non-coding regions affect the splicing pattern of tau such that 4-repeat forms are increased (e.g. mutation N279K and the intronic mutations favor 4R-tau and ΔK280 favors 3R-tau, whereas G272V, P301L, V337M, and R406W leave the ratio of 4R- to 3R-tau roughly equimolar, as in normal brain (31)). Although mutant tau forms tend to bind to microtubules somewhat less tightly, it is doubtful whether this explains toxic effects of tau in brain degeneration because most carriers of FTDP-17 are heterozygous and therefore have one
normal tau gene, and second, the increase of 4R isoforms would counteract the destabilizing effect of the mutations. A more appealing explanation would therefore be if mutant tau protein had a higher tendency to aggregate into PHFs. In this case, if aggregation were toxic for the neuron, the mutations would constitute a gain of toxic function. The data on this issue are not uniform. Some (but not all) mutations introduced into tau isoforms or constructs show a faster rate of assembly; this is not uniform. Some (but not all) mutants have a higher tendency to aggregate into PHFs. In this case, if the motif PGGG would tend to form a β-structure because of their flexibility, and the proline at residue 301 positively disrupts the extended conformation; in fact the motif PGGG would tend to form a β-turn. Replacing Pro-301 with a leucine removes the negative constraint so that now a longer stretch can contribute to β-structure. In this case, no change in the overall charge distribution is evident, and indeed the P301L mutants still require polyanions as countercations for aggregation in vitro (33). Consistent with these considerations, FTDP-17 patients carrying the P301L mutation show a preferential incorporation of the mutated tau into PHFs (58). In this context we also note that Pro-312 following the PHF6 motif would be expected to limit the extent of β-structure, and indeed when one removes it the aggregation becomes enhanced (data not shown).

**Note that under these conditions only K18/ΔK280 shows aggregation (filled circles, with heparin; open circles, without heparin), and all others remain soluble (open triangles and squares, not resolved on this scale). This indicates that the propensity of K18/ΔK280 for β-structure and aggregation is much higher than the other constructs (which require polyanions for aggregation). B, solid curves: polymerization of K18 wt (filled circles) and its proline mutants K18/I308P (filled squares), K18/I308P (filled triangles), and K18/I308P (filled diamonds). Dashed curves, polymerization of K18/ΔK280 (open circles) and its proline mutants K18/ΔK280/I277P (open triangles), K18/ΔK280/I308P (open squares), and K18/ΔK280/I277P/I308P (open diamonds). Note that the proline mutations in the hexapeptide motifs abrogate the ability to polymerize, except for the case of K18/ΔK280/I308P. wt, wild type.**

**FIG. 6. Polymerization of K18/ΔK280 without polyanions and interference between hexapeptide motifs. A, time course of the aggregation of construct K18 and its mutants (G272V, N279K, ΔK280, P301L, and V337M) in the absence of heparin in phosphate-buffered saline, 1 mM dithiothreitol, measured by the ThS fluorescence assay. Note that under these conditions only K18/ΔK280 shows aggregation (filled circles, with heparin; open circles, without heparin), and all others remain soluble (open triangles and squares, not resolved on this scale). This indicates that the propensity of K18/ΔK280 for β-structure and aggregation is much higher than the other constructs (which require polyanions for aggregation). B, solid curves: polymerization of K18 wt (filled circles) and its proline mutants K18/I308P (filled squares), K18/I308P (filled triangles), and K18/I308P (filled diamonds). Dashed curves, polymerization of K18/ΔK280 (open circles) and its proline mutants K18/ΔK280/I277P (open triangles), K18/ΔK280/I308P (open squares), and K18/ΔK280/I277P/I308P (open diamonds). Note that the proline mutations in the hexapeptide motifs abrogate the ability to polymerize, except for the case of K18/ΔK280/I308P. wt, wild type.**

**FIG. 7. Electron micrographs of PHFs. K18/ΔK280 forms PHFs in the presence (A) and in the absence (B) of heparin. Both show the typical twisted morphology. C, the proline mutant K18/ΔK280/I308P is still capable of forming typical PHFs in the presence of heparin. D, the peptide R2/wt, centered around the PHF6 motif of the second repeat, aggregates into thin filaments in the presence of heparin, but not into bona fide PHFs. E and F, by contrast, the peptide R2/ΔK280 forms irregular aggregates both with heparin (E) or without heparin (F). wt, wild type.**

**Note that under these conditions only K18/ΔK280 shows aggregation (filled circles, with heparin; open circles, without heparin), and all others remain soluble (open triangles and squares, not resolved on this scale). This indicates that the propensity of K18/ΔK280 for β-structure and aggregation is much higher than the other constructs (which require polyanions for aggregation). B, solid curves: polymerization of K18 wt (filled circles) and its proline mutants K18/I308P (filled squares), K18/I308P (filled triangles), and K18/I308P (filled diamonds). Dashed curves, polymerization of K18/ΔK280 (open circles) and its proline mutants K18/ΔK280/I277P (open triangles), K18/ΔK280/I308P (open squares), and K18/ΔK280/I277P/I308P (open diamonds). Note that the proline mutations in the hexapeptide motifs abrogate the ability to polymerize, except for the case of K18/ΔK280/I308P. wt, wild type.**

**Note that under these conditions only K18/ΔK280 shows aggregation (filled circles, with heparin; open circles, without heparin), and all others remain soluble (open triangles and squares, not resolved on this scale). This indicates that the propensity of K18/ΔK280 for β-structure and aggregation is much higher than the other constructs (which require polyanions for aggregation). B, solid curves: polymerization of K18 wt (filled circles) and its proline mutants K18/I308P (filled squares), K18/I308P (filled triangles), and K18/I308P (filled diamonds). Dashed curves, polymerization of K18/ΔK280 (open circles) and its proline mutants K18/ΔK280/I277P (open triangles), K18/ΔK280/I308P (open squares), and K18/ΔK280/I277P/I308P (open diamonds). Note that the proline mutations in the hexapeptide motifs abrogate the ability to polymerize, except for the case of K18/ΔK280/I308P. wt, wild type.**
Trated on the lower surface (Fig. 9).

**Fig. 8.** Model of β-sheet formation by the hexapeptide motifs PHF6* or PHF6 and suggested effect of the FTDP-17 mutations ΔK280 and P301L. Both hexapeptide motifs PHF6* and PHF6 contain local clusters of hydrophobic residues and residues with high β-propensity. The hexapeptide motifs are arranged in β-strand formation, amino acid residues are shown in single letter code. Residues present in the peptides are color-coded as follows: basic, blue (K); acidic, red (D); hydrophobic, gray (V, I, and L); and polar uncharged, yellow (Q and N). In the cross-β-structure of the PHFs, with the fiber axis pointing up the plane of the paper (as in Fig. 4), the side chains would alternatively point above and below the plane of the paper. A, sequence around hexapeptide PHF6* of repeat 2, starting at Lys-274 (last residue of repeat 1, exon 9). Hydrophobic side chains (gray) point to the top and bottom of the β-sheet, generating local patches with elevated hydrophathy. B, the deletion ΔK280 causes a flip of the second part of the motif so that almost all of the hydrophobic side chains point to the bottom, thus increasing the hydrophathy and the tendency for aggregation on this face of the sheet. C, sequence around hexapeptide PHF6 of repeat 3, starting at Pro-301 (near the end of repeat 2, exon 10). Most hydrophobic side chains point to one face of the β-sheet and thus cause the tendency for aggregation. The PGGG motif located before the PHF6 motif in normal tau represents a typical β-turn motif that presumably hampers the extension of the β-conformation in the chain and thus counteracts the tendency for aggregation. D, the P301L mutation reduces the propensity for β-turn and thus relieves the constraints against β-sheet formation by the PHF6 motif.

Two-dimensional views of the β-sheet surfaces are shown for the case of the PHF6* peptide without or with the ΔK280 mutation (Fig. 9). Four strands are arranged in an antiparallel fashion, consistent with the observed cross-β-structure of the PHFs. Side chains are color-coded as in Fig. 8, i.e., hydrophilic (yellow), positively charged (blue), negatively charged (red), and hydrophobic (gray). In the case of wild type PHF6*, both the top and bottom surfaces appear with mixed colors, indicating a mixture of hydrophilic and hydrophobic residues (Fig. 9, A and B). By contrast, in the sheet made from the ΔK280 hexapeptide the hydrophobic amino acids (gray) are concentrated on the lower surface (Fig. 9, C and D). This asymmetric distribution would strongly favor the stacking of sheets and thus promote aggregation. In the case of the repeat domain (K18), PHFs are formed even in the absence of polyamines, whereas full-length tau isoforms polymerize very slowly without these cofactors, even with the ΔK280 mutation (33, 34). This illustrates the general observation that the regions of tau outside the repeat domain tend to be inhibitory for the pathological function of PHF formation, although they are important for the physiological function of microtubule binding of tau (38). It is also interesting to note that the sequence around PHF6*, especially the double lysine motif Lys-280 and Lys-281, is particularly important for the interaction between tau and microtubules (39, 57), but in this case the requirements for the conformation of tau are not known.

**Fig. 9.** Two-dimensional representation of the PHF6* peptide arranged into a 4-stranded antiparallel β-sheet, without (A and B) and with the ΔK280 mutation (C and D). Side chains are color-coded as follows: basic, blue (K); acidic, red (D); hydrophobic, gray (V, I, and L); and polar uncharged, yellow (Q and N). In the cross-β-structure of the PHFs the fiber axis would lie in the plane of the paper pointing up. Bold letters indicate the side chains facing the observer, and faint letters indicate residues on the hidden surface. The wild type sequence (A and B) generates surfaces with mixed side chains; the ΔK280 mutant (C and D) segregates polar and apolar side chains on different surfaces.

** Tau during PHF Aggregation—** Although 3R forms of tau have only one hexapeptide motif (PHF6 in the third repeat), which initiates β-structure and PHF aggregation, 4R forms have two (PHF6 in the third and PHF6* in the second repeat). Initially, this would suggest that 4R-tau should polymerize more readily than 3R-tau, but this is not supported by experimental evidence in our assembly conditions. To test the relationship between the two hexapeptide motifs we used two tools, assembly-promoting mutants (ΔK280) and assembly-inhibiting mutants (I277P, I308P; Fig. 6B). When only one hexapeptide motif is present (e.g. in the 3-repeat construct K19), a simple model describes the situation. The motif PHF6 promotes aggregation, any β-breaking mutant in PHF6 disrupts it (such as I308P). When two hexapeptide motifs are present (e.g. in the 4-repeat construct K18) their contributions to aggregation can be analyzed by single and double probe mutations. Interestingly, both probe mutations in the PHF6 or PHF6* motifs inhibit PHF formation. Thus, one assembly-promoting hexapeptide is not enough to induce aggregation when the other is “poisoned” by mutation. However, the inhibition by the I308P in PHF6 mutation can be overcome by turning PHF6* into a strong assembly promoter via the ΔK280 mutation.

Several scenarios can be envisaged to explain this. One is that the two hexapeptide motifs interact with each other and thus change their overall capacity for aggregation. In this case, disrupting one motif by a probe mutation would inactivate both of them with regard to aggregation. The possibility of direct interference would be consistent with earlier observations from fluorescence transfer experiments that cysteines 291 and 322, downstream of the PHF6* and PHF6 motifs, are in close proximity in solution (16). In this picture, strengthening the β-propensity in PHF6* (by the ΔK280 mutation) might keep the whole molecule assembly-competent, even when PHF6 is poisoned by a probe mutation. A second model is that the hexapeptide motifs interact for a critical sub-step of aggregation, such as dimerization which is known to be an important prerequisite (14, 16, 22). For example, poisoning the hexapeptide motifs by a probe mutation might still allow dimer formation but prevent the subsequent conformational change required for filament elongation. A similar model was postulated for the aggregation of Aβ peptides (59). A third model is that the two hexapeptide motifs contribute independently of each other to PHF growth. Each aggregation-competent β-strand could attach to a growth point and present an attachment site.
for the next molecule. In this scheme, poisoned motifs (containing a proline mutation) could become incorporated by "ping-pong" backacting on competent motifs, but accumulation of too many poisoned sites would slow down or stop aggregation eventually. This is reminiscent of the limited incorporation of tubulin subunits poisoned by colchicine into microtubules (60).

In the case of FTDP-17, one important aspect of tau is the change in the alternative splicing pattern. Normal tau is a roughy even mixture of all isoforms, but most mutations around exon 10 shift the balance toward 4R isoforms, except AK280 where 3R isoforms predominate, and mutations away from exon 10 have the normal mix of isoforms (41, 63–65). The shift in splicing has been explained by the effect of the mutations (in the nucleotide sequence) on the regulation of splicing (64, 65). Because some of the mutations are silent on the protein level (e.g., those in the stem loop following exon 10), it has been suggested that the increase in 4R isoforms per se is responsible for causing the disease, independently of any change in the properties of tau. This issue is currently not resolved, but the occurrence of splicing shifts in opposite directions (toward 4R or 3R isoforms) speaks against a simple explanation, and in addition only one allele of the tau gene is usually affected. Another possibility is that the mutations change the overall level of tau expression. This could be significant because an elevation of tau can cause transport defects in neurons more reliably, but the observation illustrates how a change in splicing with regard to the tendency to aggregate is so strong that even small intracellular cofactors could cooperate in starting the pathological aggregation of tau. This order may be reversed in the presence of tubulin subunits poisoned by colchicine into microtubules (60). This is reminiscent of the limited incorporation of tubulin subunits poisoned by colchicine into microtubules (60).

**Acknowledgments**—We thank Karin Blume and Brigitte Krüger for excellent technical assistance in cloning and preparation of the protein and Peter Friedhoff (University of Giessen) for insightful suggestions.

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