In vitro tau fibrillization: Mapping protein regions

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Abstract

We have investigated the propensity to form fibrillar aggregates of a variety of fragments and variants of the tau protein under the influence of a tau fibrillization inducer: coenzyme Q_0. To better identify fibrillization hotspots, we compare the polymerization propensity of tau fragments containing the sequence of putative hotspots with that of tau variants with that same sequence deleted. We also investigate the effects of biologically occurring modifications such as phosphorylation and deamidation. We found that residues 305 to 335 are essential for in vitro tau fibrillization. Residues 306 to 311 facilitate in vitro assembly, but are not sufficient to mimic the in vivo fibrillization of tau. Furthermore, the propensity of the 306–311 sequence to form fibrils is highly decreased by chemical modifications of tyrosine 310 that are commonly found in vivo.
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1. Introduction

Alzheimer's disease is characterized by the appearance of two aberrant structures in the brain of patients: senile plaques (SP) and neurofibrillary tangles (NFTs) [1]. NFTs are intracellular inclusions made of clumps of protein fibers termed paired helical filaments (PHFs) [2]. About 20 years ago it was found that the microtubule associated protein tau is a major component of PHFs [3–9] and that purified tau was able to assemble in vitro into fibrillar polymers that resemble the morphology of ex vivo PHFs (from here on we use the term PHF exclusively to denote in vivo produced material) [10]. Subsequent studies revealed that PHFs were rich in a phosphorylated protein [11], which turned out to be a hyperphosphorylated form of tau [12,13]. Aberrant phosphotau polymers also appear associated with other neurological diseases like Pick's disease, frontotemporal dementia linked

to chromosome 17, corticobasal degeneration, and progressive supranuclear palsy [14]. Interestingly, all these disorders are characterized by the presence of dementia symptoms [15].

Intriguingly, and in contrast to the β-amyloid peptides [16], tau protein has very low propensity to aggregate. This property has made difficult the development of in vitro procedures that faithfully mimic PHF formation. For instance, the hanging drop assay, as used for protein crystallization, renders tau fibrils with PHF-like ultra-structure, but requires extremely high protein concentrations [17]. Such limitation is often overcome by enzymatically modifying tau, or with the addition of compounds that facilitate tau assembly (i.e., inducers). Protein phosphorylation [18], glycation [19,20], deamination [10,21] and truncation by protein cleavage [22], have all been reported to increase the propensity of tau to fibrillize in vitro. Some examples of molecules that act as inducers are sulfated glycosaminoglycans (e.g., heparin [23,24]), other polyanions [25], fatty acids such as arachidonic acid [26,27], or products resulting from the oxidation of arachidonic acid like hydroxinonenal (HNE) [28]. More recently, Coenzyme Q_0 and other

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quinones have been reported to act as inducers of tau fibrillization [29]. In all these cases the concentration of tau required for its assembly is dramatically decreased (for a review see [30]).

Tau is also a large protein (i.e., 441 residues for the human full-length tau) with 6 different isoforms produced by alternative splicing [31,32], thus suggesting that only a fraction of the molecule should be involved in its fibrillization. Indeed, analysis of the PHF core showed that only a fragment of ∼100 residues from the tubulin binding domain of tau is involved in forming the PHF scaffold [33]. The tubulin binding domain of tau comprises three (tau 3R) or four (tau 4R) repeated regions with similar aminoacid sequences [34,35]. It has later been proposed that it is the third repeat region of the tubulin binding domain the one primarily involved in tau fibrillization, with the second repeat playing a surrogate role in the assembly reaction [24]. Following these ideas, in this work we investigate the in vitro fibrillization of tau using Coenzyme Q_0, as inducer for polymerization of different tau fragments and/or variants with the goal of identifying the regions of tau that participate in PHF formation.

2. Materials and methods

2.1. Materials

2,3-dimethoxy-5-methyl-1,4-benzoquinone (coenzyme Q_0, ref no. D-9150), p-Benzoquinone (Ref. B-1266) was obtained from Aldrich (Steinheim, Germany). Acrylamide/bisacrylamide solution was supplied by Bio-Rad. Synthetic oligonucleotides were purchased from Isogen (Belgium) and peptides were obtained from NeoMPS (Strasbourg, France).

2.2. Construction of expression plasmids of three different tau deletion mutants

The plasmid pRKT42 [24], which encodes a human 4-repeat tau isoform with two N-terminal exons, was used a template. To obtain other constructs, mutagenesis was carried out in the parent plasmid pRKT42 using the polymerase chain reaction (PCR). Oligonucleotides R2D1 (AAACACGTCCCGGGAGGCGGCCAGGTG-GAAGTAAAATCTGAGAAGCTTGACGTCAAGCTTCTCAGATTT-TACTTCCACCTGGCCGCCTCCCGGGACGTGTTT),R2D2 (AAA-CACGTCCCGGGAGGCCCAGTTGACCTGAGCAAGGTGACCTCCG-GAGGTCACCTTGCTCAGGTCAACTGGGCCGCCTCCCGGGZCGTGTTT) and R2D3 (TACAAACCAGTTGACCTGAGCCAGGTGGAAGTAAAATCT-GAGAAGCTTGACGTCAAGCTTCTCAGATTTTACTTCCACCTGGCT-CAGGTCAACTGGTTTGTA) were used as 3′ primers.

Oligonucleotide A6 (CGGGATCCATAATGGCTGAGCCC), which includes the initiation codon as well as BamHI and NdeI sites for a proper cloning onto the vector, was used as 5′ primer. The obtained fragment was used as template for a second PCR. Oligonucleotide A8 (GCGAATTCT-CACAAACCCTGCTTGG), which includes a stop codon as well an EcoRI site downstream, was used as the 3′ primer. The fragments generated by PCR amplification were digested with NdeI and EcoRI and the ligated into NdeI–EcoRI-digested pRK172. A similar approach was done to isolate tau deletion mutants using the plasmid pRKT30, which encodes a human three-repeat tau isoform.

2.3. Protein and peptide preparation

Recombinant human tau (whole molecule), tau fragment 1 (containing the amino-terminal half of tau protein), tau fragment 2 (containing the four tubulin binding motifs and the carboxyl-terminal region), tau fragment 3 (containing the four tubulin binding motifs), tau fragment 4 (containing the second and third

tubulin binding motifs), and tau fragment 5 (containing the carboxy-terminal region), were isolated as previously described, [29] (see Fig. 7 for a scheme of the tau fragments used here). Among, tau variants we have used tau protein with the four tubulin binding repeats (tau 4R) but lacking residues 306–311 (tau $4R\Delta_{306-311}$), or tau protein with three tubulin binding repeats (tau 3R), lacking the equivalent residues (first six residues located at the 3rd repeat) of tau 4R (tau$3R\Delta_{275-281}$). Also, we have used those tau proteins lacking the whole 3rd repeat (tau $4R\Delta_{305-335}$) or residues 321–335 (tau $4R\Delta_{321-335}$). In this work, also we use the following peptides, which have been synthesized with solid-phase methods and purified as previously reported [36]. Peptide I (first repeat): QTAPVPMPDLKNVKSKIGSTENLKHQPGGGK; peptide II (second repeat): VQIINKKLDLSNVQSKCGSKDNIKHVPGGGS; peptide IIa: VQIINK; peptide IIb: NVQSKCGSKDNIKHVPGGGS; peptide III (third repeat): VQI-VYKPVDLSKVTSKCGSLGNIHHKPGGGQ; peptide IIIa: VQIVYK (also tested with tyrosine in phosphorylated form, or replaced by phenylalanine); peptide IIIi: PVDLSKVTS; peptide IIIb: KCGSLGNIHHKPGGGQ and peptide IV (four repeat): VEVKSEKLDFKDRVQSKIGSLDNITHVPGGGN.

2.4. Assembly of Tau peptides into filaments

Filaments were grown by vapor diffusion in hanging drops, as previously described [24]. For assembly of peptides in the presence of quinones, tau protein (0.5–2 mg/ml) in final volume between 20 µl and 1 ml, was incubated at 4 °C, in a buffer containing 0.1 M MES pH 6.4, 0.5 mM $MgCl_2$, 2 mM EGTA (buffer A) plus 50 mM NaCl, in the absence, or the presence, of 0.25 mM to 4 mM of
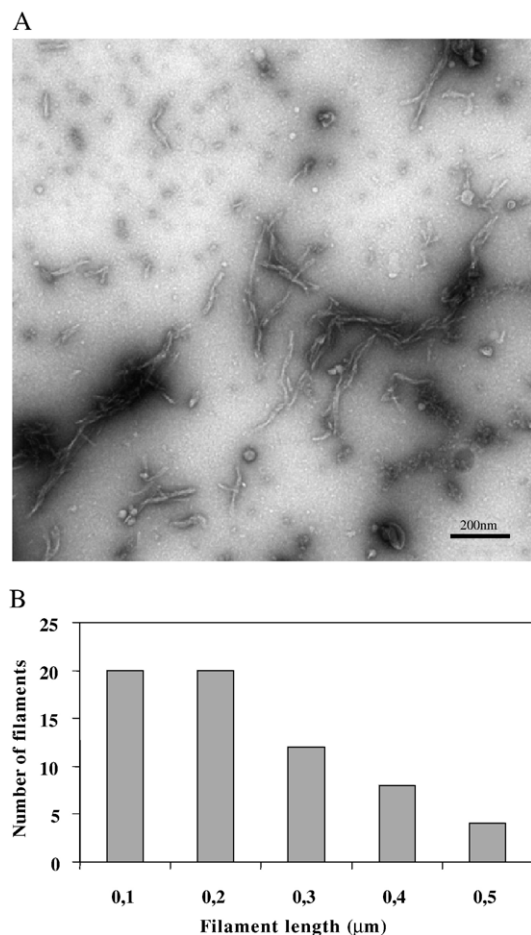

Fig. 1. Assembly of tau in the presence of quinones. Tau protein (1 mg/ml) was mixed with Coenzyme Q_0 (1 mM) and the assembled polymers from tau were visualized by electron microscopy (A). The length distribution of the polymers is shown in panel B.

coenzyme Q_0. The reservoir for the hanging drop assay [37] contained 0.2 M NaCl in buffer A.

2.5. Measurements of aggregated or polymerized protein

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 western blot using tau antibody 7.51 (a kind gift of Dr. C. Wischik, UK). The amount of protein was quantified by densitometry.

2.6. Electron microscopy

The samples were prepared and visualized under the transmission electron microscope as previously indicated [24].

2.7. Immunofluorescence studies

Tau fibrillar polymers were visualized by immunofluorescence, using a variation of the technique employed for microtubule visualization [38,39], by a novel technique [40]. Briefly, a suspension of tau polymers was placed in a corex tube containing a glass coverslip. After centrifugation at 1500 rpm in a HB4 rotor for 60 min at room temperature, the protein sedimented on the coverslip was fixed with 4% paraformaldehyde, and processed for double immunofluorescence [38] using an antibody raised against tau (T14)(1:500) and 0.01% thioflavin S. With this technique, tau filaments, that cannot be seen under the optic microscopy, can be visualized under a fluorescent microscope. Fluorescence microscopy was used to measure fluorescence intensity. Fibrillar polymers were observed with a Zeiss Axiovert200 fluorescent microscope. T14 antibody (obtained from Zymed, CA, USA) and thioflavin-S fluorescence images were captured through a 100× objective

on a high-resolution CCD camera (SPOT RT Slider, Diagnostic). The images were saved for later analysis and quantification. Camera exposure and light settings were kept constant during each experiment.

The fluorescence intensity measurements were carried out using the image analysis software Metamorph 6.1 r6 (Universal Imaging).

2.8. Iodination of tau peptide IIIa

The peptide was labeled with iodine using the chloramine T technique. The peptide was further purified by gel filtration as described previously (Antibodies. A laboratory manual. Chapter 9. by Ed. Harlow and D Lane. Cold Spring Harbor Laboratory).

3. Results and discussion

3.1. Tau fibrillization induced by quinones

As we have described previously [29], tau readily fibrillizes in vitro in the presence of quinones (see Fig. 1A). The length distribution of these polymers is shown in Fig. 1B. An average length of 0.23 μm was found. This average length was different to that described for heparin-promoted tau polymers (around 0.8 μm), using similar assembly conditions to those used in the presence of quinones [24]. Also, assembly of longer polymers was found in the presence of heparin but using a different assembly buffer [37]. In that case also very tiny filaments ("dashed minifilaments") were observed together with the long filaments. About the diameter of the filaments, a range from
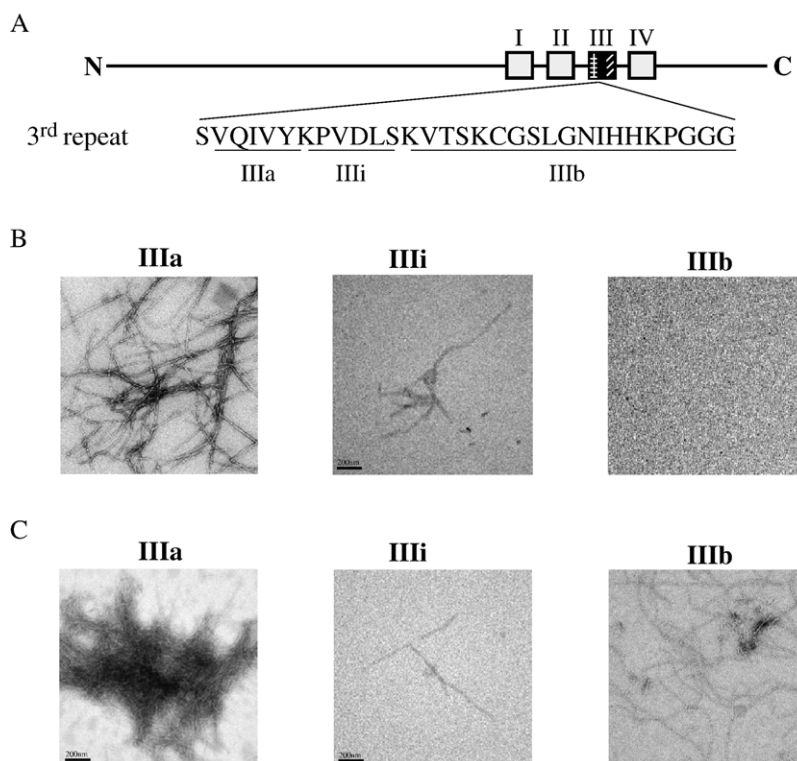

Fig. 2. Assembly of the different peptides comprising the 3rd tubulin binding repeat, in the presence of quinones. (A) A scheme of tau molecule showing the 3rd tubulin binding repeat, divided in three peptides, IIIa, IIIi, IIIb, is shown. (B) Assembly of the three peptides (1 mg/ml) in the absence of any added compound. (C) Assembly of the three peptides in the presence of Coenzyme Q_0 (see Materials and methods).

9 nm to 20 nm was described for heparin-induced tau filaments [24,37], and range from 10 nm to 15 nm was observed for quinone-induced filaments.

3.2. The role of the tubulin binding repeats II and III in tau assembly

Several reports have shown that peptides corresponding to the isolated sequences of repeats II or III can fibrillize in vitro [17,24,41,42]. Here, we perform a complementary experiment. The experiment consists in investigating the fibrillization ability of a variant of tau with repeats II and III deleted. If repeats II and III are, indeed, the hotspots for fibrillization of full-length tau, a variant with both repeats deleted should prove unable to fibrillize in vitro. To thoroughly test this idea, we

attempted to fibrillize this tau variant with quinones. Quinones failed to induce fibrillization of the tau variant without repeats II and III even at relatively high concentrations (i.e., 2 µg/µl) (data not shown). These results indicate that, in addition to being able to fibrillize independently, repeats II and III comprise the sequence critical for inducing tau fibrillization in vitro. Interestingly, a tau isoform that naturally lacks repeat II is able to fibrillize in vitro [17], thus suggesting that the fibrillization hotspot can be mapped more precisely as part of repeat III. To analyze this region of the whole tau (tau 4R) we divided it in three short segments: peptide IIIa (residues 306 to 311), peptide IIIi (residues 312 to 320) and peptide IIIb (residues 321 to 335) (see Fig. 2A). Peptides encompassing each of these three sequences were able to fibrillize in the presence of quinones (Fig. 2C). However, peptide IIIi
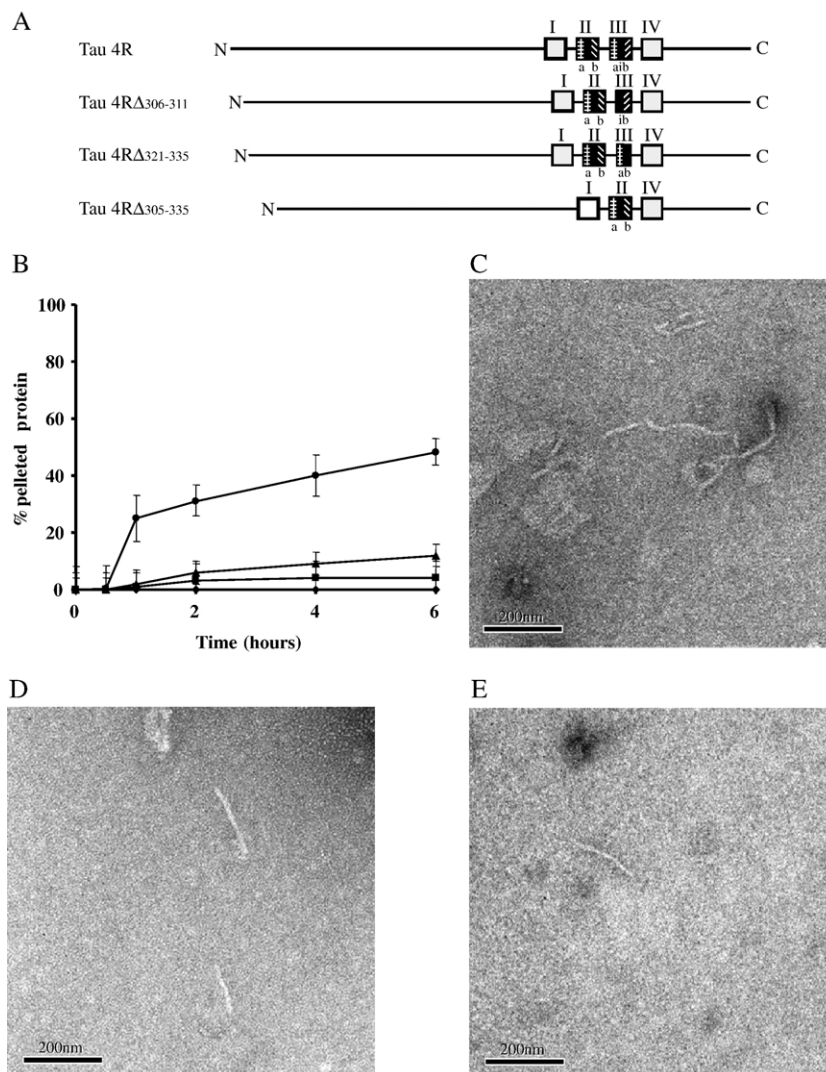

Fig. 3. Assembly of tau protein variants lacking some residues from the 3rd tubulin binding region. (A) Scheme of tau 4R protein and its variants tested in this experiment. (B) The whole tau protein (●) or variants lacking the whole third tubulin binding repeat (residues 305–335) (♦), lacking the residues comprising peptide IIIb (317–335) (■), or lacking the residues comprising peptide IIIa (306–311) (▲) were mixed at a concentration of 1 mg/ml with Coenzyme Q₀ (2 mM), and the amount of assembled polymers was measured after their centrifugation (see Materials and methods). Panel B shows a decrease in protein polymerization when tau lacks segments IIIa, or IIIb and it is not found in the absence of the whole repeat. Panels C–E show examples of the fibrils assembled from tau, tau lacking peptide IIIa and tau lacking peptide IIIb, respectively.
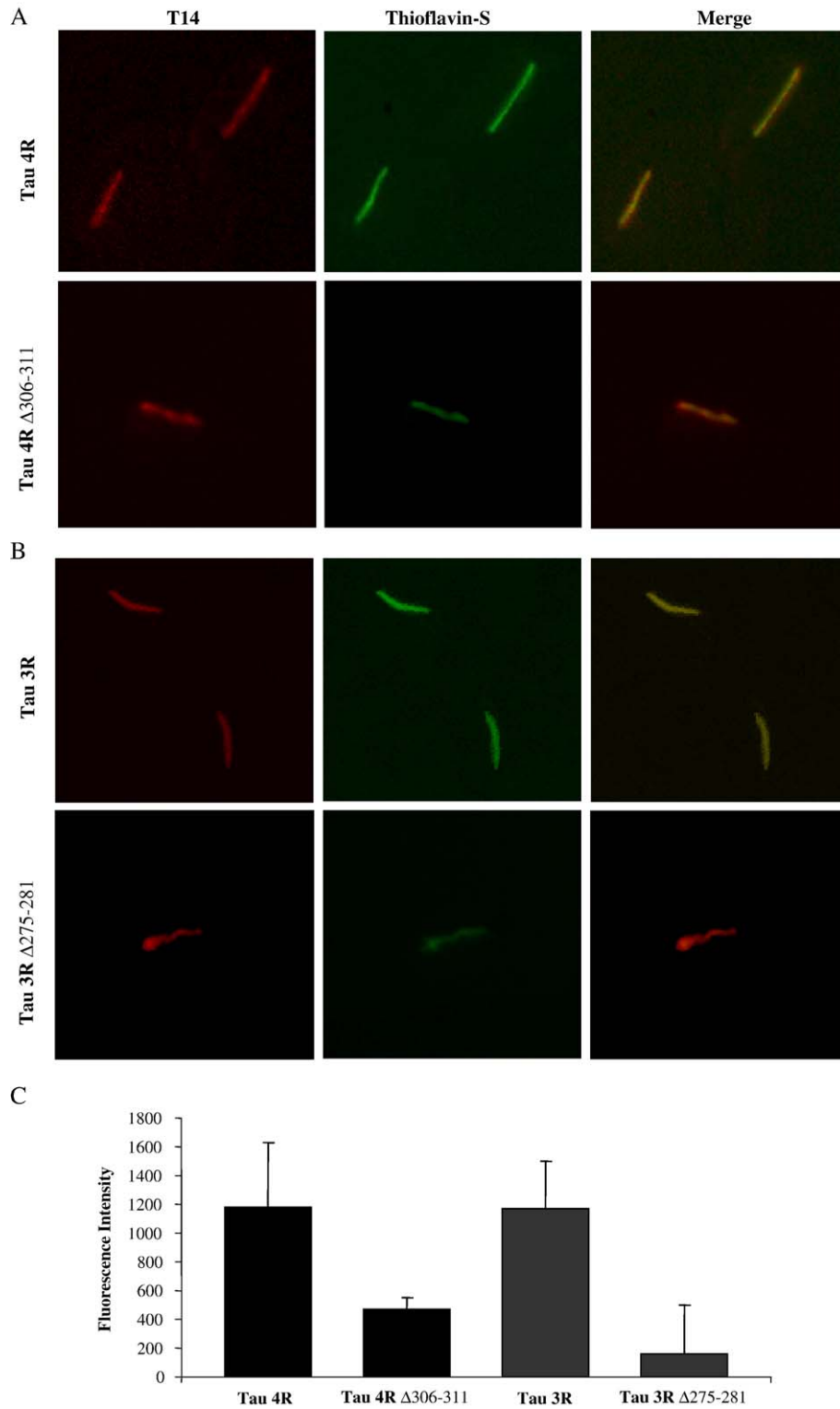
Fig. 4. Thioflavin-S fluorescence of tau and tau (lacking peptide IIIa) polymers. (A) The whole tau containing the four tubulin binding repeats (tau 4R) and tau 4R variant lacking peptide IIIa (residues 306–311 in tau 4R)(tau 4R$\Delta_{306-311}$) were assembled and visualized by immunofluorescence (see Materials and methods) using tau antibody T14 and/or thioflavin-S. The obtained results and the merge of T14 plus thioflavin-S images are shown. (B) As in (A) but testing tau 3R and tau 3R variant lacking the equivalent peptide IIIa (residues 275–281)(tau 3R$\Delta_{275-281}$) from tau 4R. (C) Quantification of the thioflavin fluorescence observed by fluorescence microscopy. The fluorescence intensity of the filament area was obtained by background subtraction. Tau variants lacking peptide IIIa show a significantly reduced fluorescence intensity ($n=10$, $P<0.01$ as compared with tau 4R or with tau 3R).

fibrillized with significantly lower efficiency than the other two. Peptide IIIa, which is only 6 residues long, fibrillizes readily even in the absence of any added inducer (Fig. 2B). The high intrinsic tendency to fibrillize of peptide IIIa could indicate that this short sequence contains the fibrillization hotspot for tau. At this level of dissection (from over 400 residues down to 6) it is important to exert caution, however, because short unstructured peptides have high intrinsic propensity to aggregate into β-amyloid fibrils [43]. Indeed, a tau variant lacking this segment of six residues displays a reduced propensity to fibrillize in the presence of quinones, but fibrillar polymers can still be found. Fig. 3A shows the polymers from tau 4R and Fig. 3B, those assembled from tau variant lacking those six residues (tau $4R\Delta_{306-311}$). Additionally, Fig. 3C and D shows two examples of those polymers assembled from tau variant lacking residues 317–335 (tau $4R\Delta_{317-335}$). The few fibrils observed are comprised of tau molecules, as observed by immunofluorescence with tau antibody T14 (Fig. 4A), and display decreased thioflavin-S fluorescence (Fig. 4A, and C for the quantitative analysis). This result was found by testing both tau 4R (Fig. 4A) or tau 3R (Fig. 4B) variants lacking that segment of six residues.
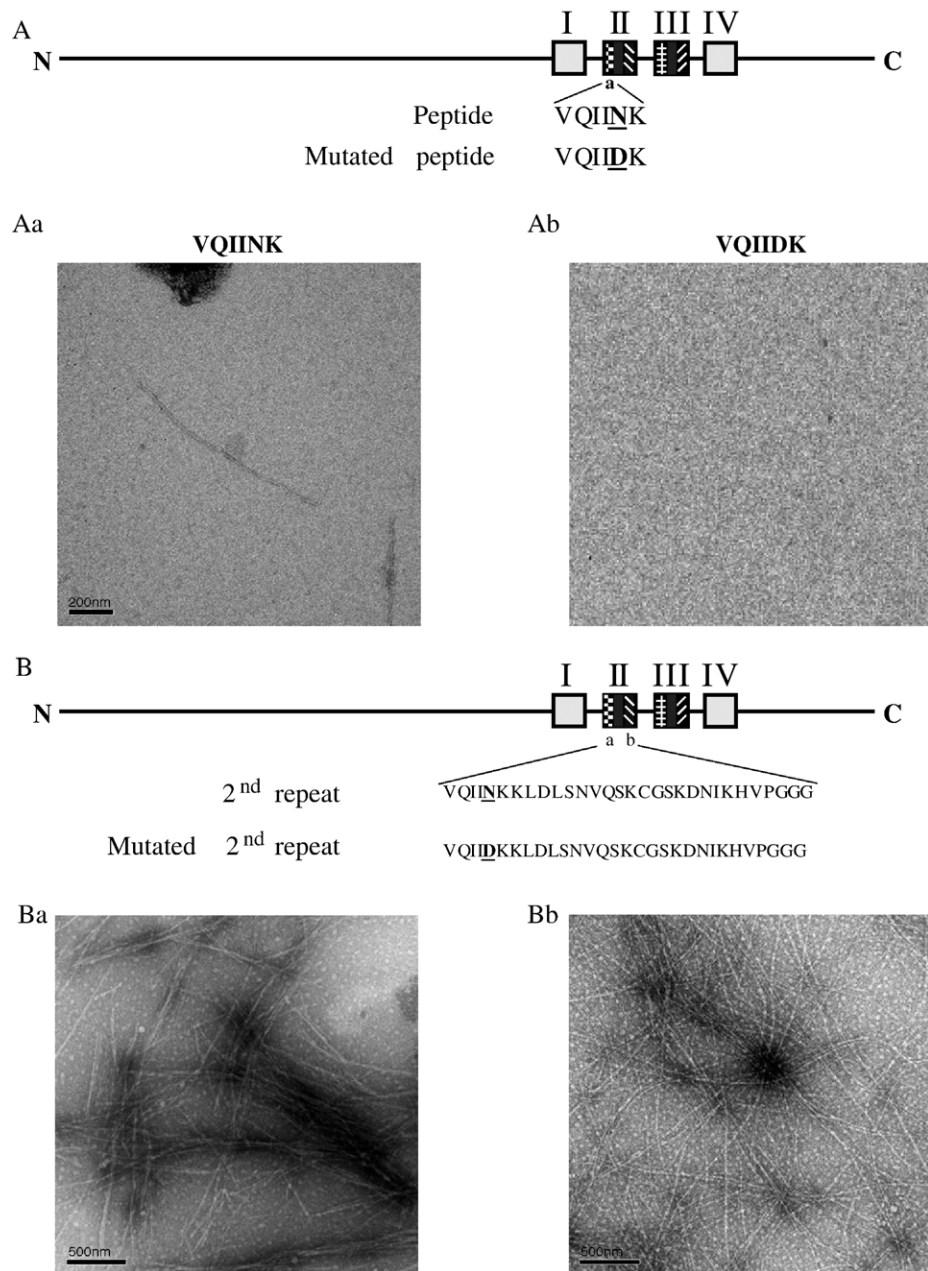

Fig. 5. Assembly of peptide VQIINK and its variants. Assembly of the second tubulin binding repeat peptide. (A) A scheme of the localization of tau peptide VQIINK in tau molecule is shown. Also, the variant of that peptide where aspartic acid replaces asparagine is indicated. (Aa) Few fibrils from VQIINK peptide were found in the absence of any added compound. (Ab) No fibrils were observed for VQIIDK. (B) Scheme of the second tubulin binding repeat peptide and its variant whose asparagine was mutated to aspartic acid. (Ba) Assembly of the second repeat peptide in the presence of quinone. (Bb) Assembly of the mutated second repeat peptide in the presence of quinone.

3.3. Role of chemical modifications in segments IIa and IIIa

Tau protein extracted from Alzheimer's disease patients often has the asparagine of peptide IIa (VQIINK) deamidated to aspartic acid [21]. To investigate the potential role in fibrillization of this chemical modification we first analyzed the propensity to fibrillize of 6-mer peptides of sequence VQIINK and VQIIDK. In the absence of any inducer, the original sequence (VQIINK) fibrillizes, while no fibrils are observed for the peptide mimicking the deamidation product (Fig. 5A). These results are incompatible with the frequent observation of deamidation in Alzheimer's patients [21]; once more giving a warning on the relevance of the fibrillization behavior of such short peptides. Furthermore, quite different results are obtained when the same chemical modification is investigated using peptides encompassing the complete repeat II. Repeat II requires inducers to fibrillize in vitro, and, under those conditions, no differences are observed between the fibrillization propensity of the original sequence and the deamidated product (Fig. 5B).

The equivalent sequence in repeat III (i.e., fragment IIIa with sequence VQIVYK) is also a potential target of chemical modifications affecting its tyrosine, which could be either iodated [44] or phosphorylated. Fragment IIIa is particularly interesting because it has been postulated by other authors as the "nucleus" for the fibrillization reaction of tau [44]. In the experiments described in the previous section we find that this fragment might too small to play such a critical role, but does seem to be part of the fibrillization hotspot in vitro (see above). Following the same rationale applied above, we investigated the effect caused by the chemical modifications on the in vitro fibrillization of this short peptide. Fig. 6B and C show that iodation of peptide IIIa does not significantly affect its propensity to self-assemble into fibrils. Fig. 6D shows that the phosphorylation of tyrosine 310, on the other hand, abolishes the formation of peptide IIIa fibrils in our experimental
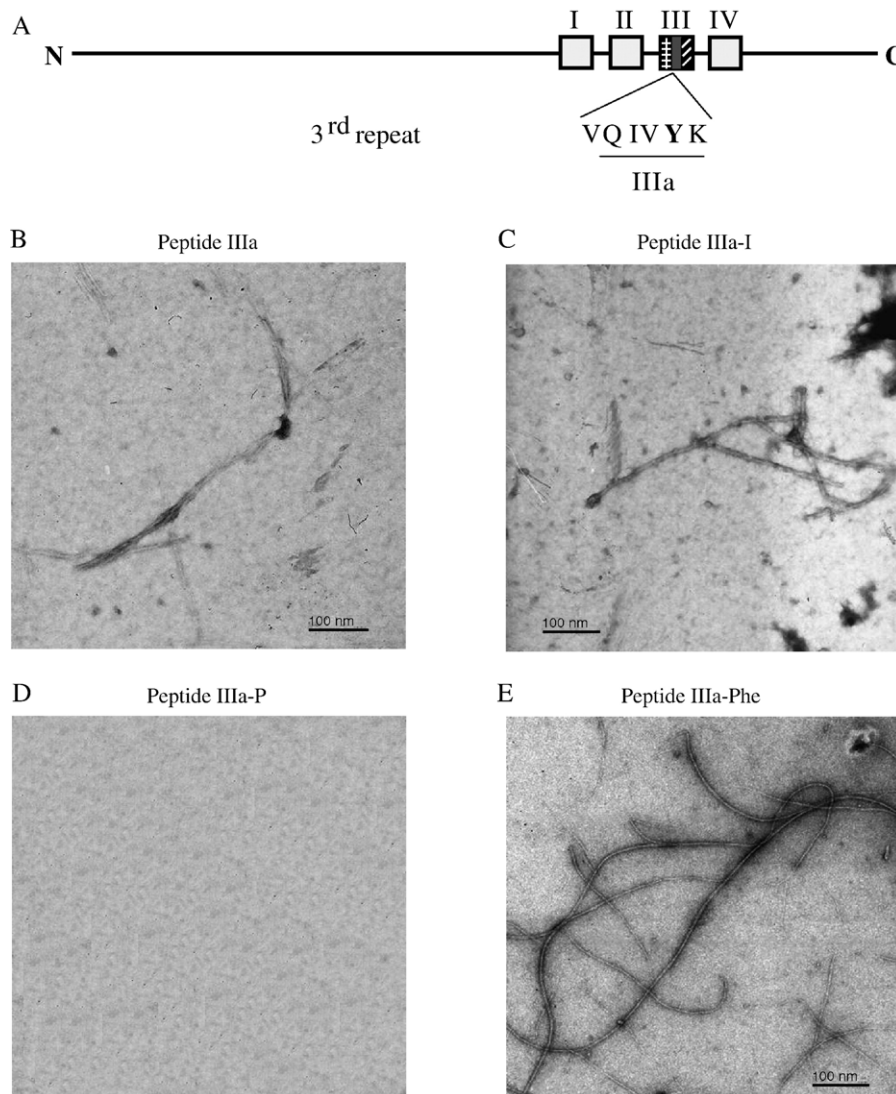

Fig. 6. Assembly of peptide VQIVYK and its variants. (A) Scheme of the localization of peptide IIIa (VQIVYK) in tau molecule. (B) Fibrils assembled from peptide IIIa in the absence of any added compound. (C) Fibrils assembled from peptide IIIa with its tyrosine iodated. (D) Absence of fibrils of peptide IIIa with its tyrosine phosphorylated. (E) Assembled fibrils of peptide IIIa with its tyrosine replaced by phenylalanine.

conditions. As a control for the specificity of the reaction, we also investigated the consequence of replacing tyrosine 310 by phenylalanine in peptide IIIa. This mutation increases the hydrophobicity of the sequence, and is not related to Alzheimer's disease. Fig. 6E shows that this peptide has a slightly higher propensity to form fibrils, which are more regular and longer. From these results it is clear that non-biological modifications that increase the hydrophobicity of the sequence also increase formation of fibrils in vitro, while potentially biological modifications, such as phosphorylation, can eliminate the formation of fibrils. This result is consistent with the previous finding of Pawar et al. [45]. The combination of these observations strongly suggests that the in vitro fibrillization of peptide IIIa is not specific of PHF formation,

but a logical consequence of its short sequence and hydrophobic character.

4. Conclusions

In this work we have analyzed the specificity of tau fibrillization reaction in the presence of inducers such as quinones. Additionally, we have mapped the in vitro fibrillization hotspot of tau onto the third repeat of its microtubule binding domain.

Fig. 7 summarizes present and previous results on our efforts to map the fibrillization hotspot of tau. Looking at Fig. 7 it is possible to conclude that tau variants containing the second and third tubulin binding repeats fibrillize in vitro in the presence of

A

Tau fragment (residues)		Asembly alone	Asembly + heparin	Asembly* + inducer	References
Whole molecule	(1-441)	−	+	+	(24, 29)
1	(1-251)	−	−	−	(36)
2	(251-441)	−	+	+	(36)
3	(250-367)	−	+	+	(29, 36)
4	(250-335) deleted (274-304)	−	++	++	(24, 29)
5	(367-441)	+/−	−	−	(36)
I	(250-273)	−	−	−	(24)
II	(274-304)	−	+	+	(24, 29)
IIa	(275-280)	+	+	+	(this work)
IIb	(286-304)	−	+	+	(this work)
III	(305-335)	−	+	+	(24, 29, 36)
IIIa	(306-311)	++	+	+	(this work)
IIIi	(312-320)	+/−	+/−	+/−	(this work)
IIIb	(321-335)	−	+	+	(this work)
IV	(336-367)	−	−	−	(24)

* The assembly increases upon phosphorylation (see ref 29)

B

Tau fragment (residues)		Asembly alone	Asembly + heparin	Asembly* + inducer	References
Whole molecule	(1-441)	−	++	++	(this work)
Tau 4R Δ306-311	(1-441) deleted (306-311)	−	NA	+/−	(this work)
Tau 4R Δ321–335	(1-441) deleted (321-335)	−	NA	+/−	(this work)
Tau 4R Δ305-335	(1-441) deleted (305-335)	−	NA	+/−	(this work)

Fig. 7. Summary of the assembly characteristics of tau peptides located along tau molecule. (A) Summary of the data from this and previous works on the mapping of tau fibrillization hotspot. The propensity to fibrillize in the absence or presence of polymerization inducers is shown using a semi-quantitative scale. (B) Fibrillization propensity of tau and its variants, indicated in Fig. 3A. NA means not analyzed.

quinones. A tau fragment containing residues 305 to 335 displays a high propensity to fibrillize in vitro, although it requires the presence of inducers. Furthermore, deleting sections of this sequence severely hampers the ability of tau to fibrillize in vitro. Several experiments have also tested the fibrillization propensity of smaller fragments within this area of tau. A 6-mer peptide comprising residues 306 to 311 (VQIVYK) is able to fibrillize in the absence of any added compound. Other groups have described that even a tripeptide containing residues 309 to 311 (AcVYK) can fibrillize in vitro without inducers [46]. The equivalent 6-mer from repeat II, comprising residues 275 to 280 (VQIINK), can also self assemble in the absence of any added compound. Our results, however, raise serious concerns regarding whether these short peptides retain the fibrillization behavior of full-length tau, or are simply forming a non-specific cross beta sheet conformation in vitro. These concerns are further substantiated by the analysis of tau chemical modifications related to Alzheimer's disease. Some of these chemical modifications alter the fibrillization behavior of the short peptides in patterns that are incompatible with their biological role. In the context of the whole protein, the short fragment 306–311 (peptide IIIa) seems to facilitate tau fibrillization in vitro, but it is not required for fibrillization in the presence of quinones, consistently with Gamblin's model for tau fibrillization [47]. From all these observations we conclude that the region corresponding to repeat III is the minimal hotspot for tau fibrillization in vitro.

Acknowledgements

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