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RNA stimulates aggregation of microtubule-associated protein tau into Alzheimer-like paired helical filaments

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Abstract The microtubule-associated protein tau is the main component of the paired helical filaments (PHFs) of Alzheimer's disease, the most common senile dementia. To understand the origin of tau's abnormal assembly we have studied the influence of other cytosolic components. Here we report that PHF assembly is strongly enhanced by RNA. The RNA-induced assembly of PHFs is dependent on the formation of intermolecular disulfide bridges involving Cys^{322} in the third repeat of tau, and it includes the dimerization of tau as an early intermediate. Three-repeat constructs polymerize most efficiently, two repeat constructs are the minimum number required for assembly, and even all six full-length isoforms of tau can be induced to form PHFs by RNA.

Key words: Paired helical filament; Alzheimer's disease; Microtubule-associated protein; RNA

1. Introduction

A characteristic feature of brains afflicted with Alzheimer's disease is the abnormal deposition of two types of proteins, the amyloid peptide Aβ, and the microtubule-associated protein tau. The latter loses its affinity for the natural partner (microtubules) and instead self-assembles into PHFs which in turn aggregate into neurofibrillary tangles. These filaments have the appearance of two intertwined strands of 10–20 nm diameter, with a repeat distance around 75–80 nm [29]. PHF tau is modified in several ways, most noticeably by phosphorylation, and it is tempting to speculate that the modifications are related to the abnormal aggregation. On the other hand, recombinant tau can aggregate even in an unmodified form when the ionic strength is increased [5,26]. This tendency is particularly pronounced with tau constructs comprising three of the internal repeats (see Table 1). This agrees well with the observation that the repeat domain constitutes the protease-resistant core of Alzheimer PHFs [30]. One explanation is that the repeat domain is capable of forming dimers which in turn promote PHF assembly. This process can be further enhanced by intermolecular disulfide bridges involving Cys^{322} in the third repeat [22,27].

On the other hand, tau constructs containing either an additional repeat (no. 2), the domains flanking the repeats, or whole tau isoforms, hardly assemble into PHFs, as if the additional domains acted as inhibitors of the aggregation [22]. This contrasts with the fact that Alzheimer PHFs contain all six tau isoforms of the human CNS [12], suggesting that the neuron may contain factors that overcome the assembly barrier for full-length tau. We therefore started a search for such factors in the cytoplasm. An initial hint was provided by the tubulin molecule, the natural partner of tau. Tubulin associates with tau, polymerizes into microtubules, and thus prevents tau's interaction with itself. Tubulin's C-terminus, to which tau binds [15], is unusually acidic, suggesting that tau might respond to other polyanionic molecules. Other prominent polyanions in the cytosol are the various RNA species. It turns out that these molecules have the capacity of promoting PHF assembly. In this respect, they are similar to polyanions of the extracellular matrix, such as heparin or heparan sulfate, whose effect on PHF assembly has been reported recently [8,19]. While it is conceptually difficult to imagine how components of the extracellular matrix might interact with cytosolic proteins, the potential role of cytosolic polyanions seems straightforward, making the RNA-PHF connection an attractive model for further investigation.

2. Materials and methods

2.1. Preparation of recombinant tau protein

Constructs of the tau protein (see Table 1) were designed and expressed in *E. coli* as described [1]. The numbering of the amino acids is that of the isoform htau40 containing 441 residues [9]. The proteins were expressed and purified as described elsewhere making use of the heat stability and FPLC Mono S (Pharmacia) chromatography [10]. The purity of the proteins were analyzed by SDS-PAGE.

2.2. Assembly of PHF-like filaments from recombinant tau

Varying concentrations of tau isoforms or tau constructs (typically in the range of 40–400 μM) in volumes of 15–500 μl were incubated at 37°C in 100 mM Tris-HCl, pH 6.8 containing various anionic cofactors: total RNA from yeast (Boehringer) or bovine liver (Sigma), tRNA from bovine liver (Sigma), rRNA from bovine liver (Sigma), or heparin (Sigma) were varied between 0.05 and 0.5 mg/ml. Incubation times varied between 2 h and up to several days. Assembly reactions without polyanions were carried out as described in [22].

2.3. Electron microscopy

Protein solutions were placed on 600-mesh carbon-coated copper grids and negatively stained with 2% uranyl acetate. The specimen were examined in a Philips CM12 electron microscope at 100 kV.

2.4. Light scattering

Microtubule assembly was monitored by light scattering in a Kontron Uvikon 810 spectrophotometer by absorption at 350 nm. 10 μM tubulin dimers (purified as described in [17]) were incubated in 80 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT, 1 mM GTP, pH 6.8 with or without the addition of 0.2 mg/ml RNA in a 10 mm cuvette. Polymerization was started at 37°C by adding a small volume of tau to a final concentration of 2 μM.

3. Results

3.1. Assembly of PHFs from tau protein is stimulated by RNA, a cellular polyanion

To assess the effect of different polyanions on the assembly

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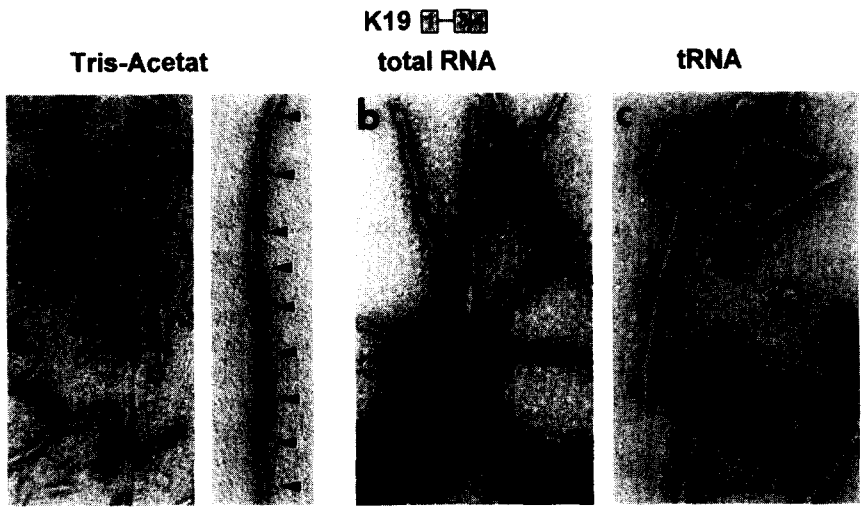


Fig. 1. PHFs assembled from 3-repeat construct K19. (a) 0.2 M Tris-HCl, 0.2 M Na acetate, 2 mM K19, 7 weeks; (b) total RNA (0.5 mg/ml), 200 μM K19, 14 h; (c) tRNA (0.5 mg/ml), 200 μM K19, 14 h.

of tau into PHFs, we initially chose constructs consisting essentially of the repeats. Construct K12 (= 3 repeats plus a C-terminal extension, Table 1) was shown earlier to have a high tendency to form PHFs because it readily forms dimers and higher aggregates [26], and it contains only one cysteine (Cys^{322}) which favors the formation of intermolecular disulfide bridges [22]. Construct K19 (= 3 repeats only) assembles even better (Fig. 1). The assembly can be driven by increasing the buffer concentration (e.g. up to 0.4 M Tris-HCl, pH 6.8, 0.4 M Na-acetate); the effect becomes particularly evident by allowing the buffer to evaporate slowly and thereby increasing the protein and salt concentrations. The filaments have the typical PHF-like appearance, with widths varying between 10 and 20 nm, and a cross-over repeat of about 75 nm.

The fact that tau normally interacts with tubulin's acidic C-terminal region prompted the question of whether other polyanions would bind to tau. As tau is a cytoskeletal protein we were particularly interested in cytosolic components. Prime candidates are ribonucleotides. RNA is abundant in the cytoplasm, about 10 mg/ml. It consists mainly of rRNA (80%), tRNA (18%), mRNA (< 2%). Several RNA species (total RNA from yeast or bovine liver, rRNA, tRNA) were able to promote the assembly of K19 noticeably, reducing the assembly times from days to hours, and again forming the typical PHF-like structures (Fig. 1). The effects are analogous to those described for polyanions of the extracellular matrix such as heparin (Fig. 1; see [8,19]).

3.2. *Most tau isoforms or constructs assemble into PHFs in the presence of RNA*

Our next question was which tau isoforms or constructs would assemble under the influence of RNA. As noticed earlier, it is difficult to obtain PHFs from constructs containing domains outside the repeats or the second repeat, at least under conditions where assembly is driven by high ionic strength and high protein concentration [5,26]. This would suggest that some non-repeat domains of tau prevent PHF formation. The same tendency is observed here again, but now we find that the inhibition can be easily overcome by RNA in most cases. Fig. 2 shows examples of PHFs made from the construct K10 where we have added the entire C-terminal tail to the 3-repeat domain. This construct slowly develops PHFs when incubated in high salt (0.4 M Tris-HCl, pH 6.8, 0.4 M Na-acetate) and at high protein concentration (about 1 mM) over the course of several days (data not shown). By contrast, with tRNA (0.5 mg/ml) one obtains filaments rapidly, within hours, (Fig. 2a). The next step was to take 3 repeats plus the N-terminal domain (construct K44); this also readily forms filaments in the presence of tRNA (Fig. 2b). Finally the full-length three repeat isoform htau39 was tested. In high salt, this isoform is hardly capable of forming

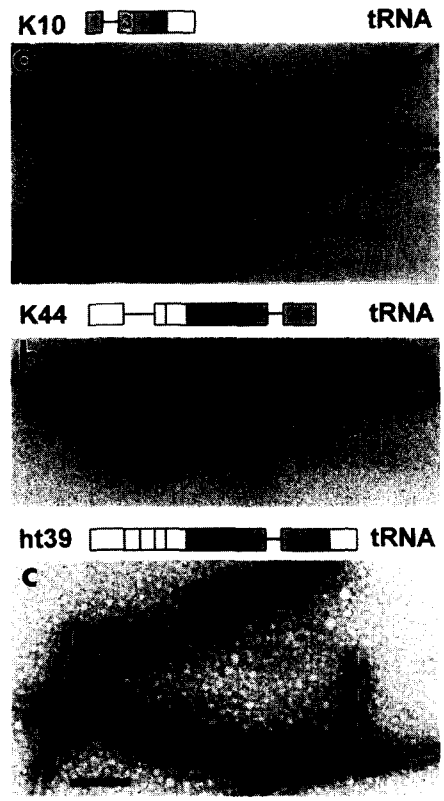


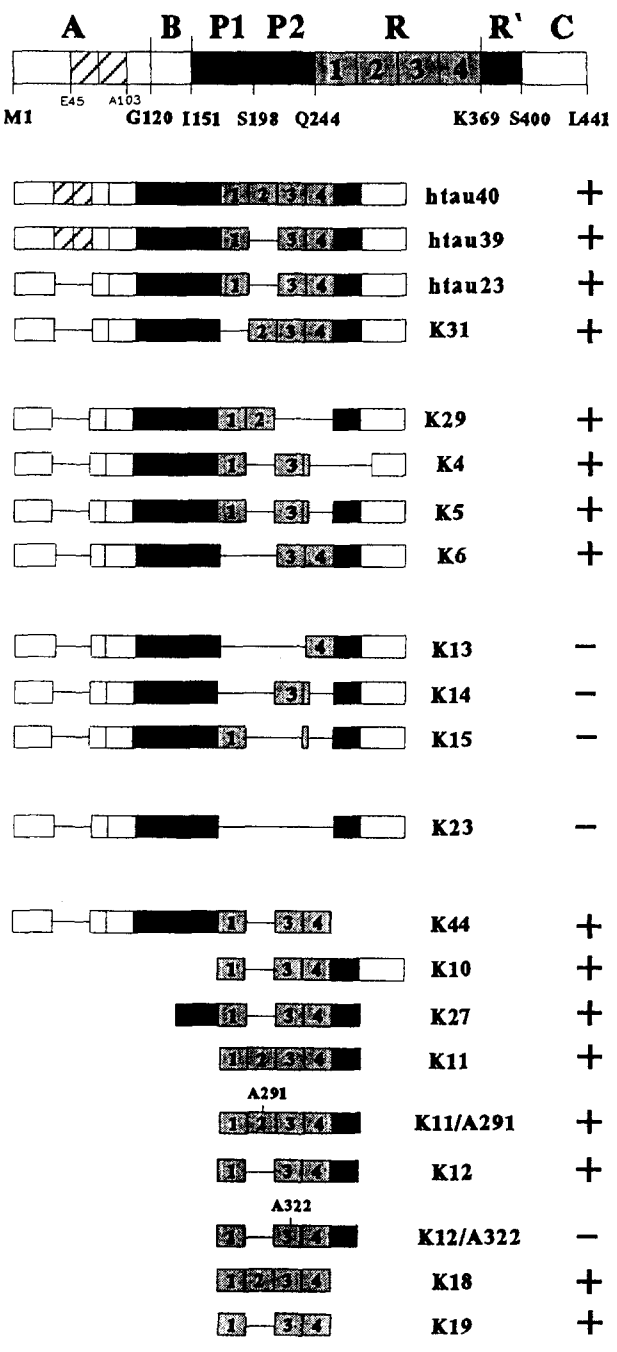
Fig. 2. PHFs assembled from constructs containing 3 repeats and extensions in the presence of 0.5 mg/ml tRNA. (a) 40 μM K10 (3 repeats plus C-terminal tail); (b) 40 μM K44 (3 repeats and N-terminal domain); (c) 40 μM htau39 (the second-largest tau isoform).

PHFs, but with tRNA one obtains PHFs in hours even at low protein concentration (Fig. 2c). Note however that the aggregation of htau39 is not as fast and efficient as with the 3-repeat construct alone (K19).

In our earlier studies we had found that 4-repeat isoforms are inefficient in PHF assembly because the two cysteines can form intramolecular disulfide bonds which stabilize the 'compact' monomer and prevent dimerization. Since dimers are building blocks of PHFs, the extra repeat (no. 2) effectively acted as a PHF inhibitor [22]. However, this inhibition can be overcome by tRNA. The four-repeat domain K11 forms filaments, part of which have the authentic twisted appearance while others are straight (Fig. 3a). If Cys^{291} in the second repeat is mutated into Ala (leaving only the single Cys^{322} in the third repeat) the assembly of PHFs becomes highly efficient again (Fig. 3c,d). Some of these filaments show a supercoil of diameter 40–100 nm and pitch 150–200 nm. Extending K11 in the N- and C-terminal direction is equivalent to the largest tau isoform htau40. In this case, even with tRNA it is difficult to obtain bona fide PHFs. Instead one observes a mixture of polymorphic filaments, including thin straight filaments, twisted filaments, and 'spiny' filaments with protrusions at ~20 nm intervals (Fig. 3e,f).

Since repeats were considered important for PHF assembly we asked how the removal of repeats would affect the process. We made several constructs derived from the isoform htau40 in which the number of repeats was reduced to 3, 2, 1 or 0. The loss of repeats lead to a reduction in PHF assembly, even in the presence of tRNA. Constructs with two repeats were less efficient while constructs with only one repeat (K13, K14, K15) or no repeat (K23) did not form any PHF-like filaments (Fig. 4a–c, Table 1).

Table 1
Diagrams of tau isoforms and constructs used in this study, and their propensity to form PHF-like filaments in standard buffer with 0.5 mg/ml tRNA



3.3. Structure and kinetics of PHF assembly

Perhaps the most remarkable feature of PHF assembly from different tau constructs is the similarity in the resulting structure. The majority of the fibers have the appearance of two strands twisted around one another, with widths of 10–20 nm and cross-over repeats on the order of 75 nm. However, there was also a population of filaments with cross-over periodicity of about 120 nm (100–130 nm). The type and composition of the constructs, or the agent promoting the assembly, seem to matter only in a second approximation. The simplest interpretation is that PHFs are built on a common principle. The smallest construct from which we have obtained PHFs is the construct K19 (3 repeats), and therefore it is likely that PHF assembly is based on the interactions between at least one (probably several) of the repeats. Although the PHF preparations are dominated by twisted fibers there is usually a fraction which appear straight, but of comparable width (20 nm). Similar straight filaments have been observed in other assembly conditions of tau (e.g. [6,13,28]), and even in Alzheimer PHFs [4]. We did not observe a defined influence of tau domains on the straight or twisted fraction of filaments. In this regard our results differ somewhat from those reported earlier with heparin. Goedert et al. [8] observed only straight filaments with 4-repeat isoforms while we find straight and twisted filaments (Fig. 3d, arrow). Perez et al. [19] reported mostly untwisted filaments with both 3- and 4-repeat tau constructs. Since a straight filament may gradually convert into a twisted one, and vice versa, it is likely that the two appearances are closely related (as noted in [4]).

As shown before, the rate of PHF assembly is enhanced if tau monomers are first allowed to form dimers stabilized by intermolecular disulfide bonds involving Cys^{322}. We therefore asked whether disulfide bridge formation has an influence on the RNA-induced assembly of PHFs. Indeed, when disulfide bridges were prevented by reducing agents (such as DTT), fiber formation was strongly reduced. The same effect was achieved by mutating Cys into Ala. These observations are

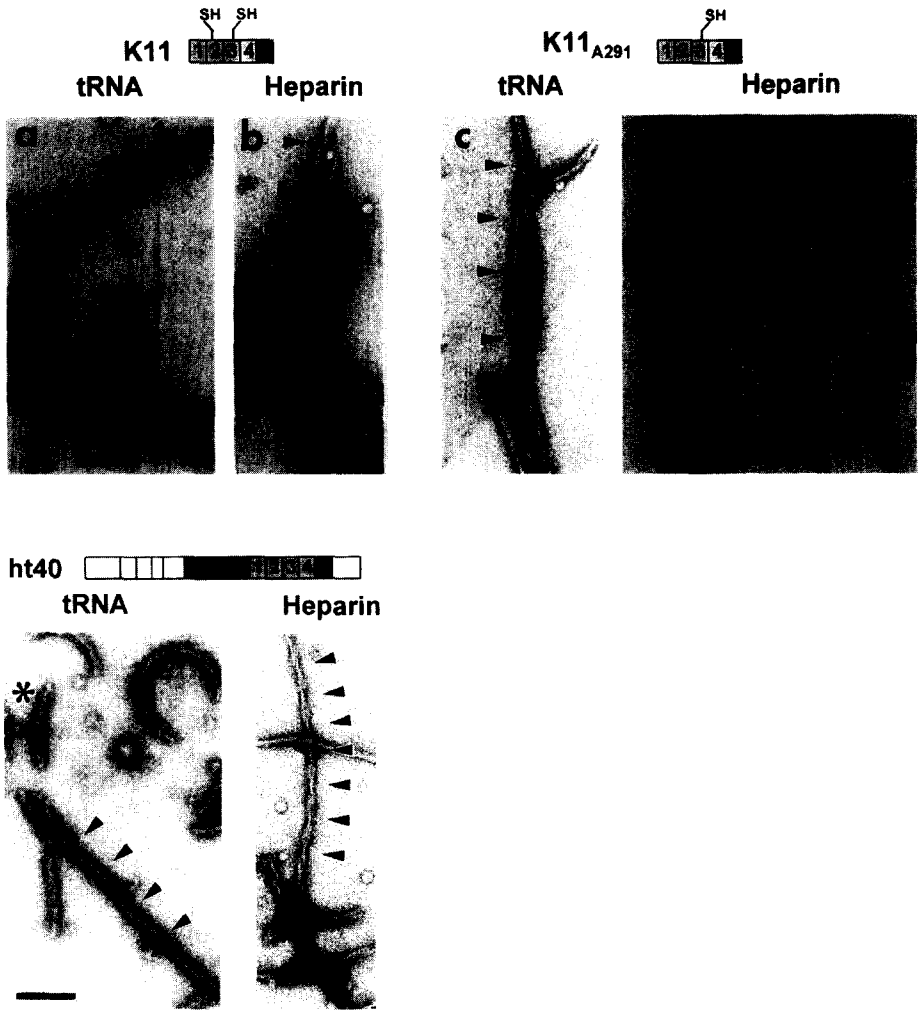


Fig. 3. PHFs assembled from 4 repeat tau constructs or isoforms. K11 (400 μM) with (a) 0.5 mg/ml tRNA and (b) 10 μM heparin, or K11 mutant $Cys^{291}Ala$ (400 μM) with (c) 0.5 mg/ml tRNA and (d) 10 μM heparin. (e) 40 μM htau40 (largest tau isoform) with 0.5 mg/ml tRNA or (f) 40 μM htau40 with 10 μM heparin. Note that full-length tau assembles much less readily than the repeat domain, and that the filaments show more polymorphism.

in agreement with the assembly model proposed earlier based on disulfide-crosslinked tau dimers [22]. On the other hand, this model also postulated that only constructs with one cysteine would form dimers (and thus PHFs), while others with two cysteines (Cys^{291} and Cys^{322} in repeats 2 and 3) would form intramolecular disulfide bridges, leading to a 'compact' formation of tau which would not contribute to PHF assembly. The present data show that even 4-repeat tau (with 2 cysteines) can assemble into PHFs in the presence of RNA, albeit with low efficiency (Fig. 3). The simplest explanation is that RNA prevents the compact conformation leading to intramolecular bonds, at least for part of the molecules, so that intermolecular dimerization is possible. Consistent with this interpretation, the mutant $Cys^{291}Ala$ shows abundant PHF assembly since the lone Cys^{322} can only enter intermolecular disulfide bonds (Fig. 3c,d).

The role of RNA as a 'scavenger' of tau can be demonstrated most directly by a microtubule assembly assay. In the experiment of Fig. 6 (upper curve), microtubule assembly was monitored by light scattering. The concentration of tubulin (10 μM) was chosen such that it would not self-assemble but required tau for nucleation and stabilization. However, when RNA was added as well, tau was competed away so that microtubule assembly was inhibited (lower curve).

4. Discussion

Formally speaking, the assembly of tau and tubulin can be described in complementary terms: Tubulin (a polyanion) self-assembles with the help of a polycation (tau), and tau (a polycation) self-assembles with the help of a polyanion (tubulin). The complete microtubule can be described as a heteropolymer: a core filament (poly-tubulin) and an outer coat (poly-tau). In this system, the interaction between tubulin molecules determines the appearance of the structure, while tau seems to be restricted to a helper function. But in principle one could also conceive the reverse situation – a polymeric structure determined by tau, with a tubulin-like molecule in a helper function. This function can be fulfilled by RNA. Indeed, both tubulin and RNA compete for the pool of tau in the cell [3] and in vitro (Fig. 5). We know very little about the structural requirements of the tubulin-tau interaction, but it is possible that the tau-tau interaction, or the tau conformation, on the surface of a microtubule resembles that in a tau poly-

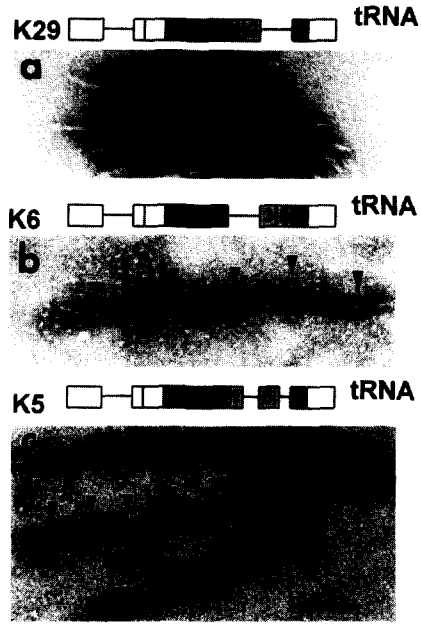


Fig. 4. Assembly experiments of tau constructs with two repeats under the influence of tRNA. (a) K29 600 μM (repeats 1,2), (b) K6 200 μM (repeat 3,4), (c) K5 400 μM (repeats 1,3). Removal of the repeats decreases tau's tendency to form PHFs.

mer, i.e. in the PHF. This would help to explain why tau self-assembles once the underlying tubulin core is lost (as in Alzheimer's disease).

The analogy can be carried one step further: The self-assembly of tubulin can be induced by other polycations, such as DEAE dextran [7], and the self-assembly of tau can be induced by other polyanions such as RNA or heparin. The case of tubulin is instructive because Erickson and Voter [7] showed that the assembly of tubulin by DEAE-dextran could be likened to complex coacervation of polyelectrolytes, such that the effective concentration of the anionic protein (tubulin) is increased on the surface of the polycation so that the nucleation barrier is overcome. A similar situation might apply for the assembly of tau; this would be compatible with the different potencies of polyanions to induce PHFs (compare tRNA, total RNA, heparin, etc.). Independently of this general function of RNA there is the possibility that tau interacts specifically with a particular RNA structure. For example, tau mRNA is transported to the axon hillock in a complex with ribosomes, adaptor and motor proteins so that there is a high local synthesis of tau protein which is destined for slow transport down the axon [21]. The elevated local concentration of RNA and tau protein in the proximal axon could initiate local PHF assembly which would interfere with the axonal transport. This would be compatible with the 'dying back' of axons observed in Alzheimer's disease [2].

The comparison of different tau domains shows that RNA-induced assembly of PHFs works best with the 3-repeat construct K19, while adding domains outside the repeats or the repeat no. 2 have an inhibitory effect which must be counteracted by higher protein concentration and longer incubation times. The data can be summarized by the model of Fig. 6 which is an extension of the dimerization model proposed earlier. The domain consisting of repeats 1, 3, and 4 dimerize most easily on account of the single Cys^{322} in repeat no. 3 which can enter intermolecular disulfide bonds. The resulting antiparallel dimers have a high tendency to interact with others to form PHFs, and the process can be inhibited by reducing agents such as DTT. The repeat no. 2 is inhibitory because its extra Cys^{291} can form an intramolecular disulfide bond, making the molecule compact (as judged from its migration on native gels [22]) and unable to dimerize. In practice, whether or not a disulfide bond is intra- or intermolecular will depend on the protein concentration, the molecular collision frequencies, the rate of oxidation and other parameters. This would explain why even 4 repeat domains can form dimers at higher concentrations, albeit less readily.

The inhibitory effect of the N- and C-terminal tails could be explained by their conformation. Both tails are acidic and therefore could fold back onto the repeat domain. Such an interaction would be consistent with the flexible nature of the polypeptide chain [23], with the reactivity of certain antibodies [14], and with electron microscopic or fluorescence energy transfer experiments [22,26]. In the model we assume that the folded-back tails somehow protect the repeat domain, making it unavailable for dimerization and PHF assembly. However, the folded state can be 'forced open' by polyanions such as RNA. If this happens, dimerization is possible again, and once the dimers are stabilized by intermolecular disulfide bonds they can be stably incorporated into PHFs. This would explain why larger tau constructs assemble less efficiently in the absence of polyanions, and that their assembly is prevented by DTT, pointing to the role of disulfides. In this model, the 'open' conformation of tau can interact with other tau molecules. In addition, it is possible that the same conformation is also the one that interacts with different polyanions, particularly microtubules. Thus, the open conformation could be viewed as the physiologically active one, while the folded conformation would represent an inactive storage form.

Alzheimer tau differs from normal tau not only in terms of aggregation, but also by an abnormally high degree of phosphorylation (for review, see [16]). It is attractive to speculate that a high degree of phosphorylation predisposes tau for aggregation into PHFs. However, the evidence thus far does not support this, since PHF-like fibers can be formed from unphosphorylated recombinant tau, at least in vitro. Phosphorylation, particularly of the repeat domain, clearly has a role in regulating tau-microtubule interactions [11]. Whether or not phosphorylation has an additional effect on tau-tau interactions remains to be determined.

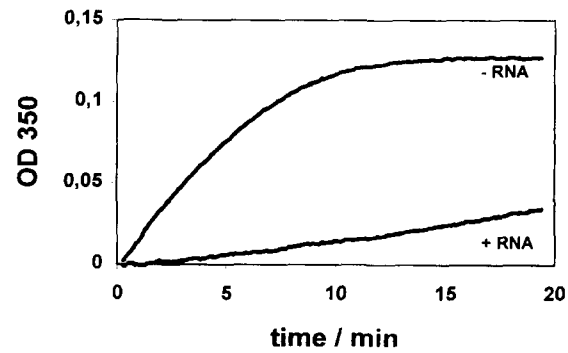


Fig. 5. Assembly of microtubules (10 μM) in the presence of htau40 (2 μM), without or with 0.2 mg/ml total RNA. Note the inhibition of microtubule assembly by RNA which competes with tubulin for tau protein.

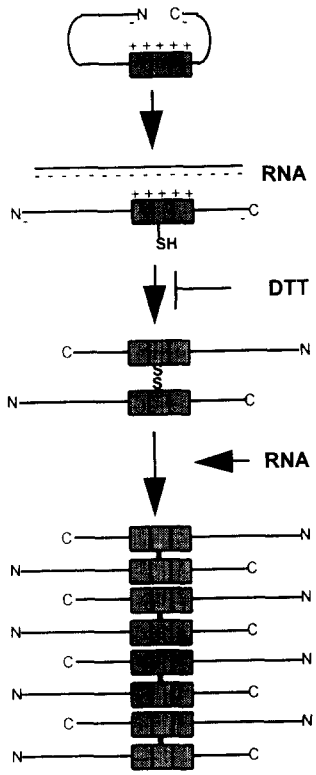


Fig. 6. Model of the influence of RNA or other anions on the assembly of PHFs from tau protein. To form PHFs, tau molecules initially dimerize with their repeat domains [26]. The regions of tau flanking the repeats on either side (particularly the acidic N-terminal and C-terminal tails) are normally folded over the repeats, thus preventing dimerization and subsequent PHF assembly. Polyanions counteract the folded conformation, opening the repeats up to dimerization and PHF assembly.

The emphasis in our discussion has been on cytosolic factors that might affect the aggregation of the cytosolic tau protein. However, tau is not exclusively cytosolic: Binder and colleagues showed that mRNA transcripts and tau isoforms occur in the nucleus, and particularly in nucleoli [24,25]. The role of nuclear tau is not clear, but it does not function as a MAP because there are no nuclear microtubules. It is interesting to note that nuclear tau localizes to regions rich in RNA (mostly rRNA and tRNA). Given the results described above one could speculate that nuclear tau and RNA contribute to, or maybe even initiate abnormal assembly of PHFs in Alzheimer neurons. We also found that certain tau constructs can translocate into nucleoli after microinjection or transfection of cells [20]. This is particularly apparent for highly basic tau constructs which lack the more acidic N- and C-terminal tails. Since proteolysis of tau is thought to play a role in the initial stages of PHF assembly [18], one scenario is that truncated tau species migrate from the cytosol to the nucleoli where they aggregate under the influence of RNA.

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