

Effects of different anti-tau antibodies on tau fibrillogenesis: RTA-1 and RTA-2 counteract tau aggregation

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Abstract Tau is the major antigenic component of neurofibrillary pathology in tauopathy, including Alzheimer's disease. Although conversion of soluble tau to an insoluble polymerized fibrillar form is a key factor in the pathogenesis of tauopathy, the mechanism of the change is unclear and no inhibitors of fibril formation are available. Monoclonal antibodies against the 1st or 2nd repeat of the microtubule binding domain, but not the C-terminal 16 residues, completely inhibited tau aggregation into PHF. Furthermore, they did not inhibit tau-induced tubulin assembly. Thus, they are useful to investigate tau protein conversion and will be useful therapeutic lead materials.
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1. Introduction

The appearance of insoluble neurofibrillary tangles (NFTs) in the diseased brain is an important pathological feature of tauopathy, including Alzheimer disease (AD) [1]. NFTs are composed of bundles of paired helical filaments (PHFs), which are abnormal aggregates of tau protein [2–5]. Tau is one of the main, although not exclusive, neuronal microtubule-associated proteins. The most important function of tau is the modulation of microtubule organization during morphogenesis and process outgrowth in neurons. The discovery of mutations in the tau gene in familial frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) has provided a clear link between tau dysfunction and dementing disease [6–10]. Although the number of NFTs has been reported to be

correlated with the degree of dementia [11], the direct connection between NFT formation and neuronal loss causing dementia is still a matter of debate, and it is important to understand the mechanism of tau aggregation.

The core of tau aggregation is composed mainly of the microtubule-binding domain (MBD), which is comprised of three or four repeats of 31 or 32 amino acids [12], and this domain also shows seeding effects on PHF assembly in vitro [13,14]. Recently, we determined the structure of the 2nd (2MBD) and 3rd repeat (3MBD) of MBD in water and trifluoroethanol (TFE) solutions by NMR spectroscopy [14,15]. Both 2MBD and 3MBD showed that the Leu10-Leu20/Lys20 sequence take helical structures with an amphipathic-like distribution of the respective side-chains, whereas the C-terminal regions are both flexible. On the other hand, at the N-terminal Val1-Lys6 sequence, 2MBD shows a helical conformation, whereas 3MBD shows an extended conformation. The amphipathic behavior would be necessary for self-association of MBD into helical filaments, because such filaments are stabilized by the alternating hydrophilic and hydrophobic interactions. The assembly process can be enhanced by the oxidation of SH groups [13] or polyanions, such as heparin [16–18].

Although there have been a number of reports concerning PHF formation [14,19], it remains to be determined how to prevent self-assembly. Here, we report that RTA-1 and RTA-2, monoclonal antibodies against first or second repeat of MBD, respectively, but not RTA-C, a monoclonal antibody against the C-terminal 16 amino acid residues, completely inhibited in vitro tau aggregation into PHF. Furthermore, they did not exert any inhibitory effects on tau-induced tubulin assembly. Thus, RTAs would provide useful tools to investigate the mechanism of the conversion of tau protein from the soluble to the insoluble state and may also be useful as therapeutic lead materials.

2. Materials and methods

2.1. Production of RTAs

The peptide sequences used as antigens are shown in Fig. 1(a). Peptides were synthesized with additional N-terminal cysteine residues, to which the carrier protein, keyhole limpet hemocyanin (KLH), was coupled. The Freund's complete and incomplete adjuvant system was used to inoculate Balb/C mice. Three mice were injected once with complete

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Abbreviations: ThS, thioflavin S; AD, Alzheimer disease; NFT, neurofibrillary tangle; PHF, paired helical filament; FTDP-17, frontotemporal dementia and Parkinsonism linked to chromosome 17; MBD, microtubule-binding domain; KLH, keyhole limpet hemocyanin; ELISA, enzyme-linked immunosorbent assay; MAP, microtubule-associated protein

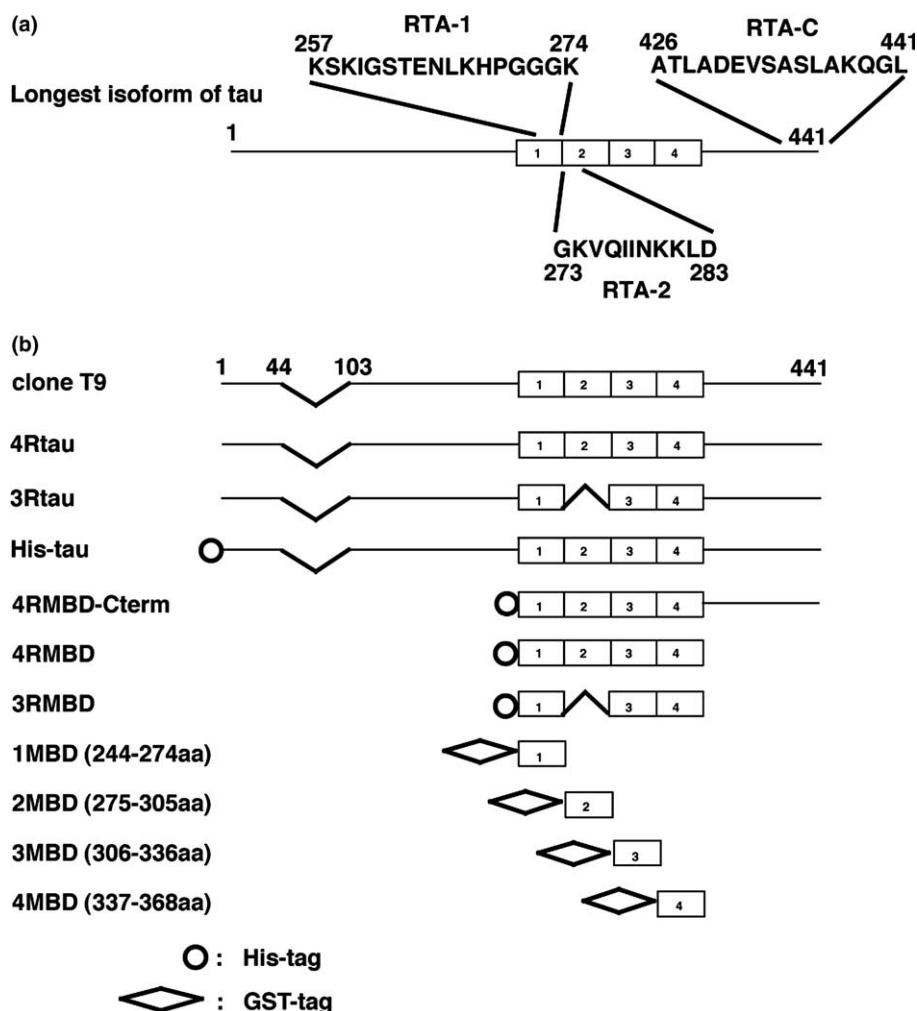


Fig. 1. The peptide sequences of antigens and schematic representation of various tau fragments. (a) A schematic drawing of the longest tau isoform is shown with the peptide sequences used as antigens. (b) The tau isoform (clone T9) is shown at the top and various tau fragments are aligned below. 1, 2, 3, and 4 indicate 1st (244–274 aa), 2nd (275–305 aa), 3rd (306–336 aa), and 4th (337–368 aa) repeat of MBD, respectively. The numbering of the aa residues refers to the longest isoform of human tau (441 residues).

adjuvant and four to eight times with incomplete adjuvant every 2 weeks. Seven days after the last injection, spleen cells were harvested and fused with mouse myeloma cells by standard procedures. Following several cycles of cloning and screening by enzyme-linked immunosorbent assay (ELISA), stable hybridoma clones (RTA-1, RTA-2 and RTA-C) were selected for antibody production. Purification of the antibody was performed as follows. Supernatant of hybridoma culture was chromatographed sequentially at 4 °C on columns of Q-Sepharose FF (Amersham Biosciences) equilibrated in buffer (HEPES–NaOH, pH 7.0) and Phenyl Toyopearl 650S (Tosoh) equilibrated in buffer (Succinic acid, pH 6.0) and Toyopearl HW-55S (Tosoh) equilibrated in buffer (Tris–HCl, pH 8.0). Protein concentration was monitored by measuring absorbance at 350 nm. The purity of the antibodies eluted from the gel filtration column (Toyopearl HW-55S) were >95% determined by SDS–PAGE followed by CBB staining.

2.2. Recombinant proteins, cell lysates and immunoblotting

All tau constructs used in the present study (Fig. 1(b)) were prepared by polymerase chain reaction amplification using a cDNA (clone T9) encoding the human tau (383 residues) as the template [20]. Schematic representations of tau fragments are shown in Fig. 1(b). 1MBD, 2MBD, 3MBD and 4MBD indicate the 1st, 2nd, 3rd and 4th repeat of MBD, respectively. Preparation of recombinant tau proteins and cell lysates and Western blotting were performed as described in [21].

2.3. *In vitro* tau aggregation and electron microscopy

Four-repeat MBD (4RMBD) at 15 μ M was mixed with 3.8 μ M heparin in 50 mM Tris–HCl, pH 7.5, containing 1 mM DTT and incubated at 37 °C for 100 min. A 600-mesh copper grid was used for negative staining electron microscopy. A drop of the protein solution was placed on the grid along with a drop of 2% uranyl acetate. After 2 min, excess fluid was removed from the grid. Negative-staining electron microscopy was performed with an electron microscope (Hitachi H-600) operated at 75 kV.

2.4. Monitoring of PHF formation by ThS fluorescence

Three-repeat MBD (3RMBD), 4RMBD and 4RMBD-C terminal (4RMBD-Cterm) were adjusted to a concentration of 15 μ M using 50 mM Tris–HCl, pH 7.5, containing 10 μ M thioflavin S (ThS) dye. Aggregation without or with the indicated RTA (7.5 μ M) was induced by adding heparin (final concentration = 3.8 μ M) to the reaction solution and mixed with a pipette prior to fluorescence measurement. The time scan of the fluorescence intensity was carried out using a JASCO FP-6500 instrument with a 2-mm quartz cell maintained at 25 °C using a circulating water bath.

2.5. *In vitro* microtubule assembly assay

Tau-induced tubulin assembly was performed using recombinant His-tau and bovine brain tubulin (Cytoskeleton) as described in [21]. Briefly, either all or one of His-tau (50 μ g, 3.1 μ M), tubulin (100 μ g,

5 μ M) (Cytoskeleton) and one of the RTAs (50 μ g, 0.8 μ M) was/were added to 400 μ l of assembly buffer (100 mM PIPES, pH 6.8, 1.25 mM EGTA and 1.25 mM GTP) as indicated and incubated at 37 $^{\circ}$ C. Polymerizations of microtubules at 20 min and 24 h were monitored by measuring absorbance at 350 nm [22,23].

3. Results

3.1. Characterization of RTAs

RTA-1 and RTA-C antibodies bound to both CHO-3Rtau and CHO-4Rtau (Fig. 2(a)), although RTA-2 binds to only CHO-4Rtau. RTA-1 bound specifically to 1MBD and RTA-2 to 2MBD (Fig. 2(b)). RTA-1 bound to both 3RMBD and 4RMBD, although RTA-2 bound 4RMBD but not 3RMBD (Fig. 2(c)). RTA-C did not bind to 1MBD, 2MBD, 3MBD, 4MBD, 3RMBD or 4RMBD.

3.2. MBD aggregation into PHF-like filaments

It has been reported that the MBD assumes the core structure of PHF [24] and promotes tau assembly in vitro [13]. We showed recently that 4RMBD aggregates into filaments with a typical PHF-like ultrastructure [25]. These filaments have a double-stranded twisted appearance with a crossover repeat of \sim 80 nm (Fig. 3).

3.3. Inhibitory effects of RTAs on PHF formation

Tau aggregation into PHF formation in solution can be monitored using the reporter dye ThS [26,27]. Both RTA-1 and RTA-2 completely blocked 4RMBD aggregation (Fig. 4(a)). On the other hand, RTA-1 completely blocked 3RMBD aggregation, while RTA-2 showed only a partial inhibitory ef-

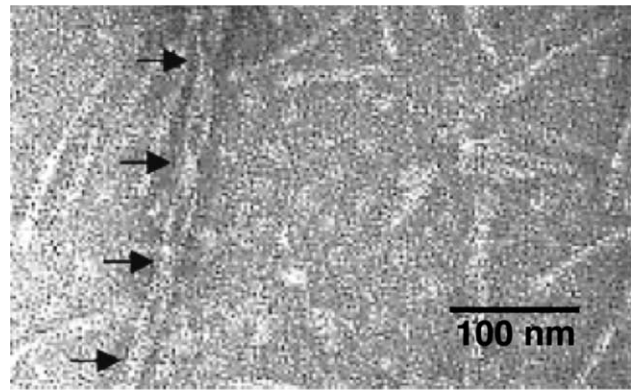


Fig. 3. In vitro polymerization of 4RMBD into PHF formation. The sample was negatively stained with 2% uranyl acetate and analyzed by electron microscopy. Arrows indicate the typical PHF ultrastructure, the double-stranded twisted appearance with a crossover repeat of \sim 80 nm. The bar indicates 100 nm.

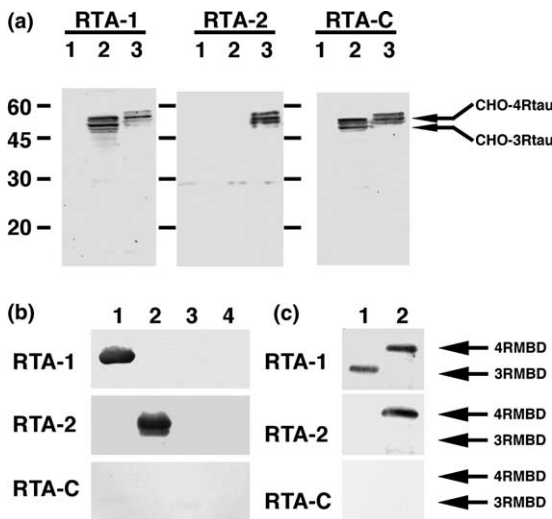


Fig. 2. Characterization of RTAs. (a) Aliquots of 10 μ g of total cell lysates from CHO (lane 1), CHO expressing 3Rtau (CHO-3Rtau) (lane 2) and CHO expressing 4Rtau (CHO-4Rtau) (lane 3) were subjected to SDS-PAGE followed by Western blotting analysis with RTA-1, RTA-2 and RTA-C. (b) Aliquots of 1 μ g of purified GST-tagged 1MBD (lane 1), 2MBD (lane 2), 3MBD (lane 3) and 4MBD (lane 4) were subjected to SDS-PAGE followed by Western blotting analysis with RTA-1, RTA-2 and RTA-C. (c) Aliquots of 1 μ g of purified His-tagged 3RMBD (lane 1) and 4RMBD (lane 2) were subjected to SDS-PAGE followed by Western blotting analysis with RTA-1, RTA-2 and RTA-C.

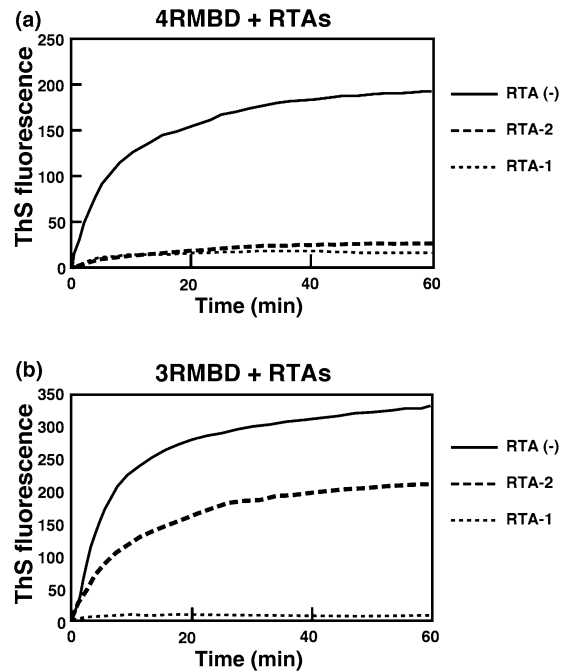


Fig. 4. Inhibition of PHF formation by RTA-1 and RTA-2. (a) PHF formation of 4RMBD in solution was monitored using ThS. RTA-1 and RTA-2 completely inhibited 4RMBD aggregation into PHF. (b) PHF formation of 3RMBD in solution was monitored using ThS. RTA-1 completely, while RTA-2 partially inhibited 3RMBD aggregation into PHF.

fect (Fig. 4(b)), indicating that the striking inhibitory effects of RTA-1 and RTA-2 were dependent on binding to MBD.

3.4. Inhibitory effects of RTAs are dependent on binding to MBD

Next, we examined whether the inhibitory effects of RTAs were dependent on binding to MBD or whether any antibodies capable of binding to regions of tau other than MBD could also inhibit PHF formation. In this experiment, we used MBD-C-term, because it also aggregated into filaments and formed PHF (Fig. 5, line RTA(-)). RTA-C detected the C-ter-

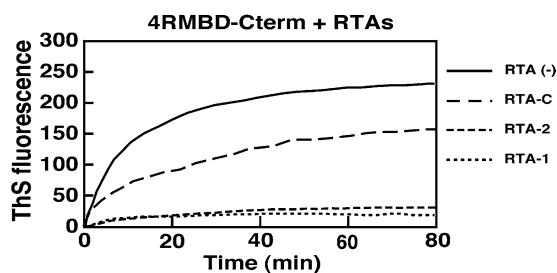


Fig. 5. The inhibitory effects of RTAs depend on binding to MBD. PHF formation of MBD-Cterm was monitored using ThS. RTA-1 and RTA-2 completely, while RTA-C partially inhibited 4RMBD-Cterm aggregation into PHF.

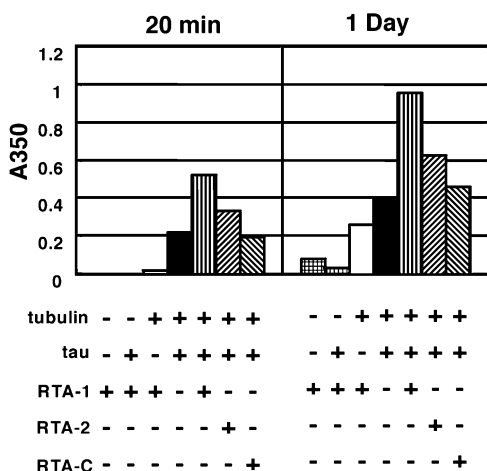


Fig. 6. Effects of RTAs on tau-induced tubulin assembly. Tau-induced tubulin assembly was analyzed with or without one of the RTAs. Polymerization of microtubules was monitored by measuring the absorbance at 350 nm. RTA-1, RTA-2 and RTA-C did not inhibit tau-induced tubulin assembly, but RTA-1 and RTA-2 promoted tau-induced tubulin assembly.

minal 16 amino acids and bound to MBD-Cterm. RTA-C showed a partial inhibitory effect, although RTA-1 and RTA-2 completely blocked MBD-Cterm aggregation as monitored using ThS (Fig. 5). These observations further supported the conclusion that the striking inhibitory effects of RTAs were dependent on binding to MBD.

3.5. RTAs did not inhibit tau-induced tubulin assembly

Tau is a microtubule-associated protein (MAP) and is involved in the promotion of tubulin assembly into microtubules and their stabilization. As expected, when recombinant His-tau was co-incubated with tubulin, it induced tubulin assembly. To examine the effects of RTAs on the tau-induced tubulin assembly, we added each RTA to the reaction. RTAs did not exert any inhibitory effects on the tau-induced tubulin assembly (Fig. 6). Interestingly, RTA-1 and RTA-2, but not RTA-C, promoted tau-induced tubulin assembly.

4. Discussion

AD and some other dementing diseases, collectively known as tauopathies, are characterized by abnormal protein deposits

in the brain. They are formed by fibrous assemblies called PHFs, which consist of tau protein. In the adult human brain, six tau isoforms are expressed by alternative splicing from a single gene located on chromosome 17. They differ in the presence of three or four repeats of MBD in conjunction with 0-, 29-, and 58-amino acid inserts located in the amino-terminal region.

Mutations in the tau gene directly cause neurodegeneration in FTDP-17, demonstrating that abnormalities of tau can be an important part of the neurodegenerative process [6–10]. The major categories of functional defects caused by tau mutations are decreased microtubule binding, increased propensity for self-polymerization, increased hydrophobicity, structural modifications, and altered isoform ratios [28]. Most mutations lie within MBD and those outside MBD are very close to MBD or lie in regions known to interact with MBD. Intronic mutations alter isoform expression of three-repeat versus four-repeat tau. All mutations found to date have some relevance to MBD [28].

MBD is composed of three or four microtubule-binding repeat containing a highly conserved repeat region of 18 amino acids that is known to interact with microtubules and less well-conserved 13–14 amino acid interrepeat regions that also interact with microtubules and greatly influence tau binding [29]. There is accumulating evidence that MBD would be vital to the polymerization of tau. Polyanions, such as heparin, can induce MBD polymerization. The resulting filaments have the morphological characteristics of PHFs, twisting with a regular periodicity of approximately 80 nm (Fig. 3) [25]. The 306-VQI-VYK-311 residues in the third microtubule-binding repeat are required for the induction of tau polymerization [30]. The stretch has been shown to form a β -strand structure in hydrophobic environments in vitro [14]. This β -strand probably represents the minimum unit required for tau–tau interactions, as a peptide composed of repeats of this sequence was demonstrated to be capable of self-polymerization [30]. Recently, we showed that phosphorylation of serine residues in MBD by PKN reduces the ability of tau to bind to tubulin or to stabilize microtubule organization [21]. Furthermore, phosphorylation in or near MBD protects tau aggregation into filaments [31] and reduces some proline-directed phosphorylation, one of the hallmarks observed in the brain of AD patients [21,32,33]. These findings further confirmed that MBD plays important roles in the pathogenesis of tauopathy.

In the present study, we showed that RTA-1 and RTA-2, which bind the 1st repeat and 2nd repeat of MBD, respectively, completely blocked 4RMBD and 4RMBD-Cterm aggregation into filaments (Figs. 4 and 5), but RTA-2 and RTA-C partially inhibited 3RMBD and 4RMBD-Cterm aggregation, respectively. Our findings indicate that when the antibody (RTA-1 and -2) binds to MBD, it completely blocks tau aggregation, although any antibody to tau would have some partial inhibitory effect on aggregation. The partial inhibitory effects of antibodies seem to be independent of their binding to tau, as RTA-2, which did not bind to 3RMBD, still showed a partial inhibitory effect on 3RMBD aggregation (Fig. 4(b)), and RTA-C also showed an inhibitory effect on 3RMBD and 4RMBD aggregation to the same extent as RTA-2 on 3RMBD aggregation (data not shown). MBD has been shown to assume an α -helical structure with amphipathic behavior [14,15], which would be necessary for self-association of MBD into helical filaments, because such filaments are sta-

bilized by the alternating hydrophilic and hydrophobic interactions. The Fc portion of an antibody is very hydrophobic and may inhibit the stabilization reaction by disturbing hydrophobic interactions of MBD. On the other hand, the Fc portion may promote microtubule assembly [34]. It should be emphasized that RTA-1 and RTA-2, but not RTA-C, increased the ability of tau to promote microtubule assembly rather than interrupting the interaction of tau with tubulin, indicating that they reduce the pathological effects but augment the physiological function of tau.

Protein–protein interactions have a key role in most biological processes, both physiological and pathological, and offer attractive opportunities for therapeutic intervention. However, the development of antagonists that modulate protein–protein interactions is difficult due to issues such as the lack of well-defined binding pockets. Here, we confirmed that MBD is a key binding domain of tau–tau interaction involved in PHF formation and we also showed that RTAs completely inhibit tau aggregation. Thus, RTAs may provide useful tools for therapeutic intervention.

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