Assembly of Alzheimer-like Filaments

Although the microtubule-binding regions (MTBRs) of both Tau and MAP2 can undergo self-assembly into straight filaments (SFs) in vitro, only the Tau MTBR forms paired helical filaments (PHFs). Moreover, Tau appears to be the exclusive building block of the neuropathic filaments observed in Alzheimer’s disease and certain frontotemporal dementias (FTDs). Despite significant conservation in the MTBR sequences, there are two persistently different stretches of amino acids (designated here as Module-A and Module-B) between Tau and MAP2 from a number of organisms. To evaluate the role of charged residues in these modules as potential morphology-specifying elements, we used site-directed mutagenesis to replace selected residues within the MAP2 MTBR by residues at corresponding positions in Tau. We then employed electron microscopy to determine the frequency of occurrence of SF and PHF morphology in filaments assembled from these mutant microtubule-binding regions. Our experimental results indicate that a very small number of residues are especially significant determinants of filament morphology; this inference is also supported by the observation that site-directed substitutions of individual Tau residues into MAP2 Module-B likewise result in the formation of PHF-like structures. Because the Module-B in Tau contains two naturally occurring FTD mutations, residues in this region may play a critical role in neuropathic filament assembly.

The mechanism of nerve cell degeneration/death in Alzheimer’s disease (AD)1 and related Chromosome-17 tauopathies remains uncertain. Dysfunctional β-amyloid protein processing appears to be significant in AD progression (1), culminating in cellular oxidative stress as well as formation of plaques comprised of amyloid β-protein. AD brain also exhibits extensive pathology, chiefly in the form of neurofibrillary tangles (NFTs), the abnormal cytoplasmic fibrinous aggregates associated with dead or dying neurons in the hippocampus and amygdala. Kidd (2) and Terry (3) found that NFTs consist mainly of paired helical filaments (PHFs) and straight filaments (SFs). Wischik et al. (4) made the seminal finding that the brain microtubule-associated protein Tau is the basic building block of AD filaments. Tau and other structurally related MAPs use their highly conserved microtubule-binding regions (MTBRs) to interact with assembled microtubules (5–7), and the MTBR of Tau comprises the core structure of PHF. Other NFT-producing neurodegenerative diseases result from point mutations in or near the MTBR of Tau (8–10), underscoring the concept that NFT formation stems from modifications within the MTBR of Tau.

Crowther et al. (11) first described the in vitro assembly of Alzheimer-like filaments from a 99-residue Tau fragment containing the microtubule binding repeats, and Wilson and Binder (12) demonstrated that full-length Tau also forms filaments. Schweers et al. (13) advanced our understanding of Tau polymerization by describing how disulfide-linked dimers more readily assemble into PHF-like structures. Our laboratory made the unprecedented finding that the MTBR of MAP2 as well as full-length MAP2c form straight filaments in vitro (14, 15), indicating that there is no clear explanation for why AD neurofibrillary pathology strictly involves Tau. Despite numerous attempts to modify solution conditions to favor the assembly of paired-helical filaments, we consistently observed that wild-type MAP2 MTBR could not form PHF structures. By contrast, Tau MTBR readily adopts both SF and PHF morphologies, leading to our hypothesis that one or more residues within the Tau microtubule-binding region play a major role in determining filament morphology. Although the amino acid sequences of the Tau and MAP2 MTBRs are highly related (5–7, 16), we noticed differences, especially with respect to charged residues that were clustered within two short stretches of residues. To evaluate the structural significance of these modules, we employed site-directed mutagenesis to replace certain MAP2 residues in each of these stretches by the residues at the corresponding locations in Tau. We also investigated the impact of single site substitutions. Our findings indicate that a small number of residues play a critical role in determining Tau filament morphology. While assessing intracellular turnover of Tau filaments is not currently feasible in humans, our mutagenesis studies suggest that a few morphology-conferring regions may help to explain the enhanced accumulation of neurofibrillary tangles in certain naturally occurring Chromosome-17 tauopathies.

EXPERIMENTAL PROCEDURES

Materials

We used the pETH-3b vector, a derivative of pBR-322, for both cloning and expression (16). Synthetic single-stranded oligonucleotides were purchased from Genemed Synthesis Inc. (San Francisco, CA). Invitrogen (Carlsbad, CA) One-Shot competent cells or DM1 competent cells (Invitrogen) were used for cloning, and Escherichia coli BL21 (DE3) pLYS S competent cells (Novagen, Inc., Madison, WI) were used for protein expression. Agar, tryptone, and yeast extract were obtained from Difco. Ampicillin, DNase, and isopropyl-β-thiogalactopyranoside...
were products from Sigma. DNA high-melt agarose, Wizard MiniPreps, dNTPs, λ DNA markers, and certain restriction enzymes were purchased from Fisher Scientific. Most restriction enzymes were purchased from New England Biolabs (Beverly, MA), while T4 DNA ligase, Taq DNA polymerase, and chloramphenicol were purchased from Roche Molecular Biochemicals. Situating BrdU labeling BrdU crystallization cells were obtained from Hampton Research (Laguna Niguel, CA). Paired helical filaments from Alzheimer brain tissue were the generous gift from Dr. Peter Davies of Albert Einstein College of Medicine.

Methods

All MAP2 MTBRs were cloned into pETh-3b using the 5′ Ndel site and the 3′ EcoRV site and expressed as described elsewhere (14–16). MAP2 and Tau MTBRs were expressed and purified by using a protocol presented elsewhere (16).

Module-A Mutants—The cDNA sequence corresponding to the 99-residue MAP2-MTBR was mutated to code for the specified Tau Module-A residues by use of cassette mutagenesis. The mutation sites are flanked by a BamHI site on their 5′ side and an AflII site on their 3′ side. Oligonucleotides were designed that annealed to one another, coded for the mutation of interest, had a blunt cassette mutagenesis. Module-B is flanked on the 5′ and 3′ sides by the HindIII site on their 5′ and 3′ sides, respectively, and破坏s the HindIII site when inserted into the MAP2 backbone. The PCR reaction product was digested with NdeI and EcoRI and cloned into the pETh-3b plasmid that had been digested with the same restriction enzymes and purified. Plasmids from colonies were screened for the addition of the HindIII site, and positives were grown and sequenced.

Individual point mutations in Module-B were also generated using cassette mutagenesis. Module-B is flanked by the StuI site and on the 3′ side by the Hind III site introduced for screening in the MAP2–123 Module-B mutant. Oligonucleotides were designed that annealed to one another, coded for the mutation of interest, had a blunt 5′ end, created a 3′ overhang, which complemented the Hind III sticky end, and destroyed the HindIII site when inserted into the MAP2–123 cassette. Plasmid containing MAP2–123 (Module-B) digested with NdeI and CfoI was gel-purified to remove the released fragment from the plasmid by gel electrophoresis, and ligating the annealed oligonucleotides into the plasmid. Transformed Invitrogen One-Shot competent cells were plated, and colonies were screened for their plasmids.

Module-B Mutants—The nucleotide sequence encoding Module-B of MAP2-MTBR was mutated to include the Gin, Glu, Lys, and Glu residues of Tau using four-primer PCR. Complementary internal primers were designed to contain the mutations and to provide a silent mutation site for Hind III on the 3′ side of the mutations to facilitate screening. These primers were used individually with primers for the ends of the clone that contained restriction sites for cloning. The PCR products for the front and back of the molecule were purified by gel electrophoresis and used as template in a second reaction with the primers for the ends of the molecule. The PCR reaction product was digested with NdeI and EcoRI and cloned into the pETh-3b plasmid that had been digested with the same restriction enzymes and purified. Plasmids from colonies were screened for the addition of the HindIII site, and positives were grown and sequenced.

30 s. Nonadsorbed sample was then wicked off and the grid was negatively stained by floating it on a drop of 1% uranyl acetate or 1% phosphotungstic acid for 30 s. Grids were examined after 15 min on a Hitachi H-7000 transmission electron microscope at 75 kV.

We used two parameters to determine the frequency of SF and PHF formation by examining negatively stained electron micrographs of assembled filaments. The transmission electron microscope was calibrated by using a high-magnification grating replica cross-ruled at 2160 lines/mm (Electron Microscopy Sciences, Inc., Fort Washington, PA). To avoid inherent limitations on calibrations of over- or underfocused micrograph prints, we only used the actual negatives. The bars presented in the micrographs therefore correspond to the measurements made on the actual negatives.

Finally, based on the distinctive morphologies of SFs and PHFs, we counted the SFs and PHFs on a series of randomly chosen micrographs. Because Crowther (21) previously demonstrated that SF and PHF structures have virtually identical masses per unit length, we estimated the relative abundance of protein in the SF and PHF structures by determining the total length of SFs and PHFs in each set of electron micrographs.

RESULTS

Tau and MAP2 each possess a projection arm, hinge domain, and MTBR, the latter consisting of three or four nonidentical 31-residue repeats (Fig. 1). Although the amino acid sequences of their MTBRs are in fact very similar (with only 16 nonconservative differences), a surprising fact is that the MTBR of Tau forms both straight filaments and paired helical filaments (see Fig. 1, inset, for typical morphologies), whereas the MTBR of MAP2 only forms straight filaments. Because bacterially expressed MAP2 MTBR and Tau MTBR have the same filament polymerization habits as the proteins isolated directly from brain tissue (13–16), post-translational modification is not required for these differences in filament morphology. We therefore considered the possibility that one or more amino acid residues in their MTBRs is(are) somehow playing a mor-
other details.

Fig. 2. SDS gel electrophoresis of MAP2 Module-A and Module-B mutants in the presence and absence of dithiothreitol. The mutant forms are indicated beneath each pair of lanes. Odd-numbered lanes contain protein incubated in the absence of dithiothreitol, and even-numbered lanes contain protein treated with dithiothreitol for 30 min prior to loading the samples for electrophoresis. See “Results” for other details.

Fig. 3. Montage of electron micrographs illustrating the SF morphology of MAP2 MTBR-modA(HGKYP) polymers. Note the absence of periodic paired helical structure. These SFs tend to undulate, but are less curved than wild-type MAP2 SFs (see inset to Fig. 1). The bar corresponds to 50 nm.

In Vitro Assembly of Alzheimer-like Filaments

RESULTS

Mutations in Module-A of the MAP2 MTBR—To assess the importance of Module-A in determining PHF morphology, we used site-directed mutagenesis to replace the Tyr, Lys, Gln, Thr, and Pro residues in MAP2 by the corresponding His, Gly, Lys, Tyr, and Pro residues in Tau. We purified this bacterially expressed mutant polypeptide, designated MTBR-modA(HGKYP), to homogeneity, as confirmed by SDS-polyacrylamide gel electrophoresis. Upon overnight dialysis in the absence of a thiol reducing agent, MTBR-modA(HGKYP) formed the disulfide cross-linked dimer (lane 1 of Fig. 3A) which is known to hasten filament self-assembly (13–15, 22). As shown in lane 2, treatment with 4 mM dithiothreitol converts the cross-linked dimer into monomer. (Other lanes in Fig. 2 document the purity and dimerization of two other MAP2 MTBR mutants described later in this report.) When incubated in the absence of polyanions, MTBR-modA(HGKYP) formed filaments slowly, usually requiring 7–10 days. In the presence of tRNA, however, the full extent of filament self-assembly required only 1–2 days. In any case, only straight filaments assembled from the MTBR-modA(HGKYP) mutant (see micrographs in Fig. 3), suggesting that Module-A is not a PHF morphology-determining region.

Mutations in Module-B of the MAP2 MTBR—We next expressed and prepared the MTBR-modB(QEKE) mutant of MAP2, in which we replaced the four MAP2 residues in Module-B (shown in Fig. 1) by those found at corresponding positions in Tau. The purity and dimerization of MTBR-modB(QEKE) are documented in lanes 3 and 4 of Fig. 2. When assembled in the presence of tRNA, the MTBR-modB(QEKE) filaments of MAP2 were predominantly of the paired-helical type (see montage in Fig. 4). These MTBR-modB(QEKE) filaments were also considerably longer (often >1 μm) than wild-type MAP2 MTBR polymers, which rarely exceed 0.3–0.4 μm (14, 15). Compared with Tau PHFs (see inset to Fig. 1), the filaments formed from the modB(QEKE) mutant have an exceptionally regular cable-like morphology. Their helical periodicity is 45–55 nm for the modB(QEKE) mutant versus 80–90 nm observed for Alzheimer PHFs and those formed in other neurodegenerative diseases.

To determine the relative importance of individual Module-B residues in promoting the formation of PHF-like structures, we prepared the four single-residue mutants: modB(Arg-to-Gln), modB(Lys-to-Glu), modB(Glu-to-Lys), and modB(Val-to-Glu). All four polymerized into filaments, and each had its own characteristic frequency of PHF formation, indicated as the number of PHF-like structures divided by the total number of assembled filaments (Fig. 5A). We also determined the relative abundance of protein present as paired helical filament structure, indicated by the total length of PHF-like structures divided by the total length of all assembled filaments (Fig. 5B). In the latter case, we assumed that SF and PHF structures have the same mass per unit length, based on image reconstruction work on Tau filaments (21).

By far, the most interesting Module-B point mutant was the modB(Val-to-Glu), whose purity and dimerization properties are shown in lanes 5 and 6 of Fig. 2. As shown in Fig. 6, this single-point MAP2 MTBR mutant preferentially assembled into PHF-like polymers. These data, which show that a single residue can have profound effects on PHF formation, underscore the idea that Module-B residues comprise the morphology-determining region in microtubule-associated proteins.

DISCUSSION

Neurofibrillar tangles are a complex aggregate comprised of straight filaments and/or paired helical filaments, with the microtubule-binding region of Tau serving as the basic building block. Although image reconstruction studies indicate that straight filaments and paired helical filaments arise from two distinct filament-packing schemes (21), surprisingly little is known about what residues in the MTBR control filament morphology. By identifying Tau residues that induce MAP2 to
form paired helical filaments, we localized a cluster of key morphology-conferring residues. We found that MAP2 mod B-(QEKE), which contains four Tau Module-B residues, formed PHF-type filaments. Moreover, introduction of individual charged residues from Tau’s Module-B into the Module-B of MAP2 likewise resulted in the formation of PHF-like structures, albeit with lower frequency. In the context of the three-repeat microtubule-binding region, the only known tauopathy mutations are located at positions 272, 337, and 342. The G272V mutation is situated in Module-A, whereas the two other, V337M and E342V, lie squarely within Module-B. Mutations at position 337 form PHF-type filaments from all six Tau isoforms (23), and the E342V mutant is known to alter the abundance of three- and four-repeat Tau in favor of the latter.

It should be mentioned that PHFs isolated from AD neurofibrillary tangles typically exhibit 80-nm helical periodicities, whereas those in Chromosome-17 tauopathies range from 90 to 120 nm. Detailed morphological studies of neuropathic filaments are limited by slight irregularities in Tau filament morphology. In fact, image reconstruction on Tau filaments has failed to reveal the boundaries of each repeating unit, denying the opportunity to establish the number of MTBRs per unit length. Electron-opaque uranyl acetate apparently does not penetrate the boundaries of each morphological unit and fails to reveal the longitudinal-repeat periodicity. In this regard, there is an obvious need to identify filament structures that are more suitable for establishing the MTBR packing scheme. Although beyond the scope of this study, the remarkably regular morphology of MTBR modB(QEKE) filaments (shown in Fig. 4) may provide important clues for refining the structure of Tau PHFs.

We have also addressed the question of whether the equivalent MAP2 residues substituted into Tau would prevent the formation of Tau PHFs. In principle, if the introduced mutations were the sole morphology-determining factors, the MAP2 mutants should have formed structures that were indistinguishable from wild-type Tau filaments. We tested this possibility by replacing the Tau residues in the Module-A and Module-B regions by those found in MAP2. We consistently observed that the Tau Module-A mutants formed fewer filaments, and those that were formed were predominantly straight filaments, like those observed with MAP2 (data not shown). On the other hand, the Tau Module-B mutants containing the corresponding MAP2 residues were largely indistinguishable from wild-type Tau filaments assembled in vitro. These unpublished findings suggest that other residues outside Module-A and/or Module-B must also play some role in promoting PHF formation.

Further work is needed to understand the significance of the persistently different locations of charged residues in both Module-A and Module-B of Tau and MAP2. In the absence of reducing agents, Tau and MAP2 form Tau-S-S-Tau and MAP2-S-S-MAP2 homodimers that bind to assembled microtubules (16) and also facilitate SF and PHF formation (13, 14). Despite numerous attempts, we have failed to identify conditions leading to the formation of Tau-S-S-MAP2 heterodimers.2 Because Module-A and Module-B regions flank the only thiol group in three-repeat MAPs, one cannot dismiss the possibility that the arrangement of charged residues may prevent Tau-MAP2 heterodimer formation.

In conclusion, while the Module-B of Tau appears to be an especially important determinant of filament morphology, our

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2 M. A. DeTure, L. Di Noto, and D. L. Purich, unpublished data.
findings do not exclude the possibility that other residues play a role. Our discovery of this PHF morphology determinant may also provide new tools for examining the factors affecting the formation and stability of neuropathic filaments.

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