In Vitro Polymerization of Embryonic MAP-2c and Fragments of the MAP-2 Microtubule Binding Region into Structures Resembling Paired Helical Filaments*

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The microtubule-associated protein Tau is widely regarded as the principal component of paired helical filaments comprising Alzheimer neurofibrillary tangles. Tau fragments containing the non-identical repeat region formed structures resembling paired helical filaments (Schweers, O., Mandelkow, M., Biernat, J., and Mandelkow, E. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8463–8467). MAP-2, the other structurally related neuronal microtubule-associated protein, has not been implicated in paired helical filament formation. We now describe the assembly of paired helical filament-like structures from MAP-2 polypeptides containing only 100 residues. A dimeric species, stabilized by an interchain disulfide, appears to be involved in the assembly reaction. We also investigated the polymerization of embryonic MAP-2c, which, except for its microtubule binding region, is structurally distinct from Tau. Full-length MAP-2c formed paired helical filament-like polymers. Polymerized MAP-2c and the microtubule binding region fragment readily bound thioflavin-S, a dye that stains paired helical filaments in the histochemical diagnosis of Alzheimer's disease. Our unprecedented finding that a small MAP-2 microtubule binding region fragment and MAP-2c can form structures resembling straight filaments or Pronase-treated paired helical filaments raises fundamental questions concerning the role of MAP-2 in the pathobiology of Alzheimer disease.

Neurofibrillary tangles (NFTs), a histological feature characteristic of Alzheimer disease (1, 2) are formed from paired helical filaments (PHFs) (3–5). Although other cytomatrix proteins are associated with NFTs, the microtubule-associated protein (MAP) known as Tau (6–9) is generally accepted as the principal component of PHFs (2, 6, 7). Tau protein and MAP-2 are highly abundant in brain tissue where they appear to stabilize microtubules (MTs) and to suppress dynamic behavior of MTs and to suppress dynamic behavior of MTs. In adults, Tau is the predominant axonal MAP, whereas MAP-2a and -2b are found in dendrites and perikaryal regions. MAP-2 cross-links microtubules to one another as well as to microfilaments, intermediate filaments, and vesicular components (9–11). A shared structural motif in these MAPs is a tri- or quartet of non-identical 31-amino acid repeats, and synthetic octadecapeptide repeat analogues can directly promote tubulin polymerization (12, 13) and/or block MAP binding to MTs (14, 15). Schweers et al. (16) recently found that conditions permitting oxidation of cysteine 322 in the 122-amino acid fetal Tau fragment K12 leads to the assembly of paired helical filament-like structures.

Evidence for the association of MAP-2 with neurofibrillary tangle formation is less convincing. While antibodies directed against MAP-2-specific epitopes do bind to NFTs in autopsy brain tissue from Alzheimer patients (17–19), no direct biochemical connection of MAP-2 to PHF formation has ever been reported. The striking conservation of primary structure within the microtubule binding regions (MTBRs) of Tau protein and MAP-2 persuaded us to investigate the polymerization properties of MAP-2 MTBR fragments analogous to the Tau fragment that forms PHFs (16). We had expected that this fragment would not polymerize, and our goal was to use such a nonpolymerizing form to identify the minimal set of site-directed mutations needed for its assembly into PHFs. We first examined a smaller 100-amino acid fragment of bovine brain MAP-2 that includes the complete three-repeat motif and can bind to MTs (dissociation constant of 8 μM). To our great surprise, this MTBR fragment readily polymerized into structures resembling PHFs. We also discovered that embryonic MAP-2c can self-assemble into PHF-like structures, indicating that other structural elements within this naturally occurring MAP-2 polypeptide do not suppress the assembly process.

EXPERIMENTAL PROCEDURES

Bacterial expression and purification of bovine MAP-2 fragments were described previously (15, 20). Each expressed protein was dialyzed against 100 volumes of 100 mM Tris-HCl (pH 6.8), with three buffer changes over 6–9 h. To obtain a full-length MAP-2c cDNA clone, reverse transcription-polymerase chain reaction was performed on rat brain poly(A)⁺ RNA (Clontech). A random hexamer was used to generate first strand DNA sequence, and the entire coding sequence was produced by polymerase chain reaction with a sequence-specific 5’ primer (5’- GCAGTCCGGATCCATATGGCTGACGAGAGGAAAGA-3’) and a sequence-specific 3’ primer (5’-GATATCAAGCTTTCACACAGGCCTGCTTAGCGA-3’). The sequence of the polymerase chain reaction product was confirmed by DNA sequencing using AmpliTaq Cycle Sequencing Kit (Perkin-Elmer Corp.). The MAP-2c coding sequence was subsequently cloned into pETh-3b vector through the 5’-NdeI site and 3’-HindIII site. Bacterial expression and purification of recombinant rat brain MAP-2c are similar to those of bovine brain MTBR with the following modifications. Expressed MAP-2c, purified as described elsewhere (15), was then dialyzed into Tris buffer and handled as described for bovine MAP-2 MTBR-123 fragment.

We employed matrix-assisted laser desorption mass spectroscopy to verify the molecular weights of each MAP-2 polypeptide (15). Protein concentration assays were also carried out as described elsewhere (20).

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The abbreviations used are: NFT, Neurofibrillary tangles; PHF, paired helical filaments; MAP, microtubule-associated protein; MT, microtubules; MTBR, microtubule binding regions.

¶D. L. Purich, A. M. Ainsztein, and R. L. Coffey, unpublished results.
For polymerization studies, samples were brought to 12–18 mg/ml using an Amicon Centriprep concentrator at room temperature, and the buffer was changed to 0.2 M Tris, 0.2 M sodium acetate (pH 6.8) by diluting with 0.6 M Tris-HCl and 1.0 M sodium acetate at the same pH. Aliquots (20 μl) were placed in sitting-bridge crystallization cells (Ham-ilton Research, Inc., Laguna Hills, CA). The bridges were kept in a sealed vapor equilibration chamber containing 1 ml of 0.6 M Tris-HCl and 1.0 M sodium acetate (pH 6.8) at room temperature for 1–4 weeks.

Samples were added to carbon-coated 400-mesh copper grids for 30 s, followed by negative staining with 1–2% uranyl acetate for 30 s. Grids were examined on a Hitachi H-7000 transmission electron microscope at 75 kV. For polymer length measurements, digitally scanned images were analyzed using Image-1 software (Universal Imaging, Inc., West Chester, PA).

Sedimentation equilibrium runs were performed in the Beckman XLA analytical ultracentrifuge (21), and data were collected at 0.003-cm increments with UV-visible absorption scans at 280 nm. The system was deemed to be at sedimentation equilibrium when no further change in scans was noted during a 24-h interval. Binding of thioflavin-S to polymers of MAP-2 or MAP-2 MTBR fragments was monitored by the change of extrinsic fluorescence of the dye using a Photon Technology International model A1010 photon-counting spectrofluorimeter. The excitation wavelength was set at 440 nm, and the emission spectra was recorded from 460 to 550 nm.

RESULTS

Polymerization of MAP-2 MTBR-123—Tau and MAP-2 possess structurally related microtubule-binding regions that closely resemble each other (Fig. 1), but their projection-arm regions have different amino acid sequences. Our 100-residue fragment, MAP-2 MTBR-123, includes the first, second, and third nonidentical repeat region, and it is 22 residues shorter than Tau K12. MTBR-123 also contains a single sulfhydryl group in the second repeat, and this is positioned in an analogous manner in the Tau K12 polypeptide. Schweers et al. (16) reported that oxidation of this thiol is involved in the polymerization process, and we therefore omitted a sulfhydryl reducing agent to permit thiol oxidation. Upon incubation for a period of 5 days or longer, the MTBR-123 fragment formed a viscous gel, suggesting that aggregation and/or polymerization of the MAP-2 fragment had occurred. Electron microscopy of this material, using uranyl acetate as a negative contrast stain for polymeric species, revealed that massive polymerization had taken place, and numerous long filamentous structures are evident (Fig. 2A). These polymers are similar in size and appearance to straight filaments and regions of Pronase-treated PHF cores isolated from autopsy brain of Alzheimer patients (6). This extensive polymerization of MAP-2 MTBR-123 is further illustrated in Fig. 2B. The histogram (Fig. 2C) depicts the...
As shown in Fig. 3A, dimerization of MAP-2 MTBR-123. Electrophoresis (18% cross-linked acrylamide containing 0.1% SDS) was carried out on samples incubated in crystallization wells for 1, 2, and 5 days (lanes 1–3). One additional day-5 sample (lane 4) was treated with 4 mM dithiothreitol (DTT) to reduce disulfides prior to electrophoresis. The values on the right indicate the mobilities of molecular weight standards; dye-labeled soybean trypsin inhibitor (27 kDa) and lysozyme (19 kDa) were from Bio-Rad. B, sedimentation equilibrium analysis of MAP-2 MTBR-123 in the presence or absence of 4 mM dithiothreitol. The illustrated scan of absorbance at 280 nm versus radial position was recorded 1 day after equilibrium had been reached.

We also observed that MAP-2 readily dimerizes (Fig. 6A), a finding that agrees with earlier electron micrograph evidence pointing to an antiparallel dimeric structure for MAP-2c (22). Indeed, at two different relative centrifugal field strengths, we observed that about 30% of MAP-2c is present as a dimer. Finally, we used fluorescence spectroscopy to demonstrate that thioflavin-S binds to polymeric MAP-2c (Fig. 6B), again pointing to the overall structural similarity of polymerized Tau and MAP-2.

DISCUSSION

While the core structure of Alzheimer PHFs clearly contains Tau protein (6), there is no compelling evidence that Tau protein is the exclusive building block of paired helical filaments.
Earlier workers (19) observed that antibodies directed against MAP-2-specific epitopes bind to NFTs in autopsy brain tissue from Alzheimer disease patients, but cross-reactivity with Tau protein could not be definitively eliminated. One monoclonal antibody to MAP-2 was especially effective in staining NFTs in areas of Alzheimer disease brain that tend to be most affected, including the perihippocampal cortex and the CA1 region of the hippocampus (17). Polyclonal antibodies raised against MAP-2 also immunocytochemically stain abnormal neurites around senile plaque (18). Our investigations now demonstrate that MAP-2 fragments as well as embryonic MAP-2c readily polymerize into structures resembling PHFs. We have uniformly obtained assembly in some 90–100 individual runs, confirming the reproducibility of the polymerization process.

The assembly of Alzheimer-like straight and paired helical filaments from full-length Tau protein has been demonstrated (23, 24). One might have expected that structural features within the full-length Tau molecule may permit PHF formation, whereas their absence in high molecular weight MAP-2ab may block intracellular formation and/or accumulation of intact MAP-2ab in PHFs. However, we now report that MAP-2c polymerizes into structures resembling straight and/or paired helical filaments. Furthermore, high molecular weight MAP-2a and MAP-2b are remarkably susceptible to limited proteolysis (13, 25) in the so-called hinge region joining the projection arm and MT binding regions (see Fig. 1). An MTBR fragment, for example, can be generated by thrombin cleavage of MAP-2ab (13), and we are investigating whether this polypeptide assembles into PHF-like structures. Trypsin and chymotrypsin cleavage sites are also present within the MAP-2 MTBR (25, 26). Any aberrant action of these and/or related proteases within neurons might generate proteolytic cleavage products capable of forming PHFs. Neurons contain high concentrations of high affinity protease inhibitors known as serpins, raising the possibility that errors in the synthesis of such inhibitors may lead to lesions in cytoskeletal organization.

Under normal conditions, Tau protein should be absent from the neuronal cell body where neurofibrillary tangles are most frequently observed to accumulate in affected neurons (1, 2). Incorrect targeting may direct Tau to the neuronal cell body, where proteolysis may generate MTBR fragments from both Tau and the normally resident MAP-2. In this regard, studies of Tau/MAP-2 copolymerization in vitro may reveal whether their combined presence promotes or inhibits the polymerization process. MAP-2 fragments may even alter the minimal concentration of Tau fragment for PHF assembly. Given the importance of the second repeat in the binding of MAP-2 to microtubules (13, 15), one would anticipate that such interactions should alter dimerization, which is a process likely to involve the non-identical repeats. For example, MAP-2 phosphorylation at sites near the cysteine thiol group suppresses MAP-2 binding to microtubules in vitro (20), and other post-translational modifications may also influence the course of PHF assembly.

NFTs and amyloid plaque bind the dyes Congo red and thioflavin-S, constituting a clinically useful marker in Alzheimer disease (27, 28). Schweers et al. (16) demonstrated that PHF-like structures assembled from the Tau MTBR-K12 fragment also bind these aromatic dyes. Given the striking conservation of sequence within the Tau K12 and MAP-2 fragments, the PHF-like structures formed in our experiments were also expected to bind these dyes. Binding of thioflavin-S to MAP-2 MTBR-123 as well as to MAP-2c was attended by a characteristic fluorescence enhancement previously reported for thiofla-
vin-S interactions with polymerized Tau K12 fragment (16). Nonetheless, more work will be required to characterize the structural motif responsible for thioflavin S and Congo red binding to polymerized Tau and MAP-2.

Lastly, one may ask why MAP-2 has not been identified biochemically as a core component of Pronase-treated neurofibrillary tangles. A definitive answer to this question will require further investigation, but there are several possibilities. First, MAP-2 may have nothing at all to do with NFT formation. Second, MAP-2 may be present in PHFs at levels below the detection limits of previously employed methods. Third, a small pool of MAP-2-enriched NFTs in one or more regions of the brain may be obscured by whole brain tissue sampling. Fourth, MAP-2 may be selectively removed during preparation and analysis of Alzheimer PHFs. Whatever the answer, the findings presented in this report should rekindle interest in exploring the potential connection of MAP-2 to the pathobiology of Alzheimer disease.

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