The Juvenile Microtubule-associated Protein MAP2c Is a Rod-like Molecule That Forms Antiparallel Dimers*

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We have developed a procedure to isolate the microtubule-associated protein 2c (MAP2c), a juvenile form of MAP2 occurring in mammalian brain. The shape, size, self-association, and antibody interactions of MAP2c were studied. Monomeric MAP2c is an elongated molecule with a length approximately 48 nm, considerably shorter than the higher molecular weight forms MAP2a or b of adult brain. Two monoclonal antibodies whose epitopes are near the N or C terminus, respectively, are located close to the opposite ends of the MAP2c rods. This places constraints on the types of internal folding of the molecule.

MAP2c self-associates into dimers and fibrous aggregates. The dimers are predominantly antiparallel and nearly in register, as judged by antibody labeling.

Microtubules contain the core protein tubulin and a variety of microtubule-associated proteins, MAPs, which coassemble with microtubules and stabilize them. The best-studied MAPs are the MAP2 and tau proteins from mammalian brain. Several cDNA sequences have been determined, including that of the high molecular weight MAP2 (1828 or 1830 amino acid residues, Lewis et al., 1988; Kindler et al., 1990) and its smaller derivative MAP2c (467 residues, Doll et al., 1990), or various isoforms of tau protein (ranging between 352 and 441 residues, Lee et al., 1988; Goedert et al., 1988; Himmler et al., 1989). These proteins share several notable features.

First, their C-terminal parts are highly homologous to one another; they contain three or four internal pseudo-repeats of about 31 residues length. This region appears to be involved in the interaction with microtubules (Aizawa et al., 1988; Joly et al., 1989; Ennulat et al., 1990), and it is preferentially phosphorylated by several kinases (Steiner et al., 1990).

Second, the isoforms within each group are generated by alternative splicing of a single gene transcript. In the case of rat MAP2b, a central region may be removed (residues 152-1514, i.e., 1363 out of 1830 residues), thus generating MAP2c with 467 residues (Garner and Matus, 1988; Doll et al., 1990).

Third, all of these proteins can occur in several states of phosphorylation (MAP2, Binder et al., 1984; MAP2c, Crandall and Fischer, 1989). Some of these affect the mobility in SDS gels, shifting the $M_r$ to a higher value and increasing the apparent number of isoforms seen on SDS gels. Apart from having different molecular weights, the different types of MAPs can be distinguished by using several monoclonal antibodies. Since the MAPs have both homologous and heterologous regions, some antibodies cross-react between the different isoforms while others do not. In this study we have used mainly three antibodies: AP18 and C both recognize all MAP2 isoforms but not tau (Binder et al., 1986; Tucker et al., 1988a). The epitope of AP18 is close to the N terminus (within the first 150 residues; Wille et al., 1992). Antibody 2-4 cross-reacts with all isoforms of MAP2 and tau; its major epitope is at the end of the last internal repeat (sequence HHVPGGG), about 75 residues from the C terminus, in a region where these MAPs are homologous (Dingus et al., 1991).

A common property of MAP2 and tau isoforms is that they remain in solution during boiling. This fact can be exploited as a first step in the purification scheme (Fellous et al., 1977).

When such heat-stable fractions were prepared from juvenile rat brain they contained a protein of $M_r$ 70,000 which was initially considered a variant of tau ("slow young tau," Coughie and Nunex, 1985). The proof that this protein was part of the MAP2 family came with peptide mapping and the cDNA sequence (Garner et al., 1988; Doll et al., 1990).

Little is known about the structure of MAP2a,b. The protein has almost no secondary structure as judged by circular dichroism (Voter and Erickson, 1982; Hernandez et al., 1986), and it is rather flexible as judged by NMR (Woody et al., 1983). It is hardly visible in negative stain electron microscopy (Zingheim et al., 1979), but in thin sections MAP2a,b form about 40-nm projections on the surface of microtubules which correspond to the projection domain (Kim et al., 1978; Vallee, 1980). Judging by metal shadowing the molecules of MAP2a,b were considered to be up to 180 nm long (Voter and Erickson, 1982), but later studies emphasized a shorter length of 90 nm (Gottlieb and Murphy, 1985). Tau shows no recognizable projections on the microtubule surface in conventional thin...
sections, but 20-nm stubs after quick-freeze deep etching. With this method, isolated tau molecules are about 56-nm long (Hirokawa et al., 1988). No data have been reported so far on the length and self-association of MAP2c; this will be described in the present paper.

MATERIALS AND METHODS

Protein Preparation—Microtubule protein from bovine (adult and juvenile) or porcine (adult only) brain was prepared by a modified temperature cycle method (Mandelkow et al., 1985) and boiled for 15 min (Fellous et al., 1977), followed by a clearing spin. The supernatant containing MAP2 and tau was then applied to a Pharmacia FPLC Mono-S column and eluted with a NaCl gradient (buffer 20 mM PIPES, pH 6.9, 0.05-1 M NaCl, 2 mM DTT, 1 mM EGTA, 1 mM MgSO4). The elution profile shows three main peaks (not shown, for details see Hagestedt et al., 1989). SDS-PAGE of peak 1 shows the usual four to six bands of tau protein in the M, range of 50,000-70,000, depending on phosphorylation (Fig. 1B, fraction 12-15). Peak 2 contains mostly MAP2a,b and a small contribution from MAP2c (Fig. 1B, fraction 25-35). MAP2c-containing fractions were pooled and concentrated by precipitation in 45% ammonium sulfate. The solubilized pellet was dialyzed against 50 mM Tris, pH 8.0, 1 M LiCl, 1 M NaSCN, 2 mM DTT, 2 mM NaN3 and then applied to a Pharmacia FPLC Superose 12 column (buffer: 50 mM Tris, pH 8.0, 0.5 M LiCl, 0.5 M NaSCN, 2 mM DTT, 2 mM NaNaN3). The elution profile shows only one main peak with an accompanying shoulder (data not shown). SDS-PAGE reveals an incomplete separation of MAP2a,b and MAP2c (Fig. 2A). MAP2a,b is mainly contained in fraction 5-11, MAP2c mainly in fraction 9-12. The incomplete separation made it necessary to re-run the MAP2c containing fractions (pooled and concentrated; Centricron 10 microconcentrator, Amicon) on the Superose 12 column. The second run led to a complete separation of MAP2a,b (Fig. 2B, fraction 5-9) and MAP2c (Fig. 2B, fraction 10-12), monitored by SDS-PAGE. MAP2c containing fractions were pooled, concentrated (Centricron 10 microconcentrator, Amicon) and dialyzed against reassembly buffer (0.1 M PIPES, pH 6.9, 1 mM EGTA, 1 mM MgSO4, 1 mM DTT).

SDS-PAGE and Immunoblotting—SDS gel electrophoresis was done with a gradient of 4-20 or 7-15%. Immunoblotting was done by transferring the proteins to Immobilon membrane (Millipore) and incubating with the monoclonal antibodies C, AP18, or 2-4 (antibody C kindly provided by Dr. A. Matus, see Tucker et al., 1988a, antibody AP18 from Dr. L. Binder, see Binder et al., 1986, antibody 2-4 from Drs. J. Dingus and R. Vallee, see Dingus et al., 1991). Nonspecific binding was blocked by 1% bovine serum albumin. The bound antibody was detected by a peroxidase conjugated secondary antibody (anti-mouse IgG).

Glycerol Spray Experiments—Spraying was done following Tyler and Branton (1980). The samples were diluted 1:10 in spraying buffer (50 mM ammonium acetate, pH 8.0, 150 mM NaCl, 1 mM MgCl2, 0.1 mM EGTA), made up to 70% glycerol, and sprayed onto freshly cleaved mica. The addition of 1-10 mM ATP promoted the formation of rod-like aggregates and fibers. The sprayed samples were vacuum dried for 2 h, shadowed with platinum/carbon (thickness about 1.5 nm, shadowing angle 4°) using a BAE 080T shadowing unit (Balzers Union), followed by 20-30 nm carbon. Finally the replicas were floated off on doubly distilled water and picked up with 600-mesh copper grids.

Antibody Labeling—Samples were made up to 10 mM ATP (to increase the number of aggregates and fibers), mixed with excess antibody, and incubated for 1-1.5 h at room temperature. Afterward the samples were diluted 1:10 in spraying buffer and treated following the normal glycerol spray procedure.

Electron Microscopy—The specimens were examined on a Philips CM12 microscope at 35,000-60,000 magnification. Electron micrographs were taken on Kodak electron image film SO-163. Magnification calibration was performed using negatively stained catalase crystals.

Data Analysis—Fields of particles were examined on enlarged prints. Clearly distinguishable filaments were measured in length and judged by their widths to be monomers (about 4.5 nm or less) or dimers (about 6.7-10.0 nm). These values refer to the apparent thickness given by the protein plus the surrounding metal layer. As a control we compared the protein samples with single- or double-stranded DNA; the MAP2c monomers were comparable in thickness and contrast to single-stranded oligonucleotides. Oligomeric aggregates or fibrous polymers (more than 6.7-nm wide) were distinguished from dimers by their much longer size (which showed no prominent value in the length distribution).
RESULTS

Preparation and Characterization of MAP2c—When MAPs are prepared from brain tissue by the boiling procedure, the main components are the high molecular weight MAP2a,b (M, about 270,000), and the intermediate tau proteins (M, about 50,000–70,000). MAP2c has an M, value around 70,000, slightly above tau. Note that the apparent M, values of most MAP proteins are much higher than the actual molecular weights derived from the sequences. The relationship of the 70-kDa protein to MAP2 was first shown using antibodies that cross-react with MAP2 but not with tau (antibody C, Tucker et al., 1988a; compare Fig. 1A, lanes 5 and 6, and note that this antibody reacts with bovine brain MAP2 but not with porcine). MAP2c is enriched in juvenile tissue, and when the boiled MAPs are fractionated on a Mono-S column, MAP2c coelutes with MAP2 rather than with the tau components (Fig. 1B, fractions 33 and 34).

One of the problems in the biochemical separation of MAP2c is that all MAP2 species coelute under most conditions of salt, pH, etc., suggesting that they interact with one another. We found that MAP2c could be separated only in rather harsh conditions (high concentrations of chaotropic salts such as LiCl and NaSCN) in solutions containing DTT. Even then one requires two successive gel filtration runs to obtain good separation (compare Fig. 2, A and B). Typical yields were 13 ng of MAP2c/Gram of adult brain, about 2,000 times less than MAP2a,b.

In this study we made use of three monoclonal antibodies. The antibodies AP18 (Binder et al., 1986) and C (Tucker et al., 1988a) react with all MAP2 variants but not with tau, whereas the antibody 2-4 reacts with all MAP2 variants and with tau. These antibodies were used for immunoblots, AP18, and 2-4 were also used in the EM studies. The epitope of AP18 is located within the first 150 residues of MAP2c (Wille et al., 1992), while the epitope of 2-4 is within 75 residues of the C terminus (Dingus et al., 1991).

Molecular Length of MAP2c—Glycerol spraying and rotary shadowing of MAP2c molecules reveals rod-like structures in the electron microscope, as illustrated in Figs. 3 and 4. Their minimum length is typically 40–50 nm. The minimum width is around 4 nm; since this value depends largely on the metal deposit the actual width of MAP2c is probably much smaller. In fact it is difficult to obtain contrasting images of MAP2c except by shadowing at very shallow angles; the comparison with images from myosin (not shown) suggests that MAP2c must be much thinner than a coiled coil a-helix. In many cases the particles appear to consist of two or more molecules. These may have a wider apparent diameter, up to 13 nm, they sometimes splay apart into two components, and they can form long fibers (Fig. 4). A length histogram of particles is shown in Fig. 5A. It has a peak around 50 nm, but no sharp upper limit. The histogram of Fig. 5B is obtained by eliminating all composite particles on the basis of their apparent width. This eliminates the tail of the distribution; in other words, the longer particles also tend to be the thicker ones.
presumably because they consist of several molecules side-by-side. The subset of single molecules has a mean length of 48 ± 7 nm. This is about half the length of MAP2a,b reported by Gottlieb and Murphy (1985).

The interpretative diagrams of Fig. 4 suggest several possibilities of joining MAP2c molecules into dimers or oligomers. Many particles appear as dimers aligned closely in register, that is they are distinctly wider than the single molecules and sometimes splay open (Fig. 4B). These dimers are somewhat longer than the monomers, about 58 nm on average, indicating a small offset of about 5 nm between the two members. In addition, dimers with a large stagger, oligomers, and fibers are observed as well (Fig. 4, C and D). The fibers appear smooth and show no axial substructure due to the constitutive monomers; however, they can be branched (Fig. 4D). Occasionally one finds faint knobs at the ends of monomers or oligomers, but this is not a reproducible feature.

**Location of Antibody-binding Sites**—The above observations show that MAP2c has a tendency to self-associate, with dimers being particularly prominent. Since a MAP-mediated interaction between microtubules may play a role in neuronal differentiation we wanted to characterize the interaction in more detail. One approach is to map the positions of antibody-binding sites on the monomers and then compare them with dimers. This requires antibodies whose affinity is high enough to survive the glycerol spray procedure. We concentrated on three antibodies, AP18, C, and 2-4; of these, C was useful only for immunoblotting, while the other two could be imaged in the electron microscope (Fig. 6).

Single MAP2c molecules labeled with 2-4 are shown in Fig. 6A, top row. The antibody-binding site is clearly near one end of the rod. Since the epitope is near the C terminus (Dingus et al., 1991), this means that the molecule is folded such that one of the physical ends coincides roughly with the C-terminal region.

Dimers of antibody-labeled MAP2c are shown at the bottom of Fig. 6A. They have a dumb-bell shape, with single antibody molecules at both ends. This appearance is very reproducible; it means that the dimers are antiparallel. The separation between the two antibody labels is somewhat larger than the monomer length, typically 55–60 nm. This suggests that the two monomers are not in exact alignment, but shifted by about 5 nm, as mentioned above for the unlabeled dimers.

Similar results are obtained with the antibody AP18 (Fig. 6B). Here, too, the antibody site is near one end of the rod. Since the epitope of AP18 is within the first 150 residues of MAP2c, this result means that the N terminus of the chain is near one end of the rod. As with antibody 2-4, labeled dimers show antibodies at opposite ends, as expected for an antiparallel alignment.

Thus far these data leave open the question of whether the two antibodies 2-4 and AP18 bind to the same or to opposite ends of the MAP2c rods. In order to settle this we performed double label experiments. Fig. 6C (left) shows MAP2c molecules (judged to be monomers on the basis of their width, see “Materials and Methods”) labeled with antibodies at opposite ends. This pattern was not observed with monoclonic MAP2c when only one antibody was used. This suggests that the epitopes of antibodies 2-4 and AP18 are in fact located at opposite ends of the MAP2c structure. We also found dimers with one or two antibodies at each end (Fig. 6C, upper row, right, and lower row); however, these contain little information about the location of the ends because the different antibodies cannot be distinguished from one another by electron microscopy. With three or more antibodies bound, their shielding reduces the amount of platinum in such a way that it is difficult to decide whether the MAP2c aggregate contains two or more molecules.

It is interesting to note that both ends of the polypeptide chain are near opposite ends of the MAP2c rod. With about 467 residues, the maximum length of the extended chain is on the order of 170–180 nm, more than three times the observed length of 50 nm. This means that several ways of folding could be envisaged which will be discussed below.

**DISCUSSION**

**Preparation of MAP2c**—This study represents a step toward understanding the molecular structure and interactions of MAP2c. This protein of 467 residues is a juvenile variant
of MAP2a,b and occurs mainly in developing or regenerating neuronal tissue (reviewed by Tucker, 1990); it differs from MAP2a,b by the lack of 1363 amino acids due to alternative splicing. The function of MAP2c is unknown so far, but it might be involved in controlling microtubule dynamics since the juvenile MAPs stabilize microtubules less efficiently than adult ones (review by Nunez, 1988). The very low abundance of MAP2c in adult tissue has been an obstacle to studying its structural features; this problem has now been partly solved with the improved method of preparation described here.

MAP2c is related to the tau proteins in that it shares a homologous C-terminal region which carries the microtubule-binding site. MAP2a,b and tau can be isolated from mammalian brain in milligram quantities, and therefore several structural and biophysical studies have been reported, such as electron microscopy (MAP2a,b: Zingsheim et al., 1979; Voter and Erickson, 1982; Gottlieb and Murphy, 1985; Tau: Hirokawa et al., 1988; Hagesiödt et al., 1989), or NMR (Woody et al., 1983). These studies point to an elongated molecule with very little regular secondary structure and a high flexibility. The main problem with MAP2c has been so far its low abundance. In adult brain its concentration is about 2,000 times less than that of MAP2a,b. We have now developed a procedure to prepare MAP2c in amounts sufficient to initiate a structural analysis.

Like MAP2a,b and tau, MAP2c is best stable, i.e. it can be boiled without losing its microtubule binding activity; unlike tau, MAP2c it is not soluble in perchloric acid. These features, along with the unusual amino acid composition (Doll et al., 1990), suggest that the function of the protein survives denaturation or that it does not depend on a particular conformation.

Shape and Conformation of MAP2c—One interesting result of this study is that both the N and C terminus of MAP2c are near the ends of the rod-like structure and that they are in fact at opposite ends. This can be interpreted in several ways (Fig. 7). The simplest and most probable view would be that the sequence is roughly collinear with the structure (Fig. 7B). However, with about 467 amino acid residues the fully extended chain of MAP2c would be around 177-nm long (0.38 nm/residue, Fig. 7A). In principle this would suffice for traversing the observed length up to three times (Fig. 7, C and D). The extended conformations are not very probable, but the examples show that the two ends of the polypeptide chain could in principle be at the same end (Fig. 7C) or at opposite ends (Fig. 7D). The double label experiments argue against the first possibility (Fig. 7C), leaving a collinear arrangement (Fig. 7B) as the most likely one, although more complicated models cannot strictly be excluded (e.g. Fig. 7D).

For the present discussion we make the assumption that the sequence is on average collinear with the rod, without specifying how the structure might be compacted from the maximum of 177 nm down to 48 nm. It is unlikely that MAP2c has sizable stretches of regular secondary structure. Computer predictions show little evidence for it, the protein has a high content of prolines (9%), and circular dichroism spectra of the related proteins tau and MAP2a,b show a very low content of β-sheet or α-helix (Voter and Erickson, 1982; Hernandez et al., 1986). Thus we do not know at present what factors determine the shape of MAP2c, i.e. why it is rod-like rather than globular. We note, however, that MAP2c has a surprising asymmetric charge distribution. The N-terminal region is predominantly negative, the C-terminal region positive (Fig. 7E). This property might be important for the dimer formation and interactions with other molecules.

As described in other reports, the epitope of antibody 2-4 is at the last internal repeat about 75 residues from the C terminus (Dingus et al., 1981), equivalent to about 15% of the polypeptide chain. If the chain folding were collinear with the structure (as in Fig. 8B) one might expect to see a short protrusion of the rod beyond the antibody label. This is not observed (Fig. 6), but this could also be due to insufficient resolution because of the large apparent size of the antibody (around 7 nm after metal shadowing). The epitope of antibody AP18 is before the MAP2c splice site, i.e. within the first 150 residues (Wille et al., 1992). As with 2-4 we do not observe a protrusion of the rod beyond the antibody label. In view of the limited resolution the observed site would also be consistent with a roughly collinear structure as in Fig. 7B.

Interactions of MAP2c—Several authors (Aizawa et al., 1988; Joly et al., 1988; Ennulat et al., 1989) have shown that the internal repeats in the C-terminal regions of MAP2 or tau isoforms are involved in microtubule binding. Thus the “assembly domain” (defined for MAP2 by Vallee, 1980) corresponds to the C-terminal half; this part carries a net positive charge which is complementary to the negative character of the C-terminal MAP binding region of tubulin. The N-terminal “projection domain” of MAP2c does not bind to microtubules and is thought to extend into the solution, away from the microtubule surface.
The possibility of a role of tau or MAP2 in microtubule bundling was suggested by transfection experiments (Kanski et al., 1989; Lewis and Cowan, 1990), but it is not clear whether this effect is a direct consequence of microtubule-MAP interactions, or an indirect result of microtubule stabilization (see Chaplin et al., 1991). In vitro, bundling can be induced by certain proteins such as glyceraldehyde-3-phosphate dehydrogenase (Somers et al., 1990) or dynamin (Ober et al., 1990), but this has not been demonstrated for the bona fide MAPs so far. Our recent studies on the interaction between microtubules, MAPs, and motor proteins point to a more subtle role: MAPs could act both as spacers (keeping microtubules at a certain distance from other objects) and/or interlocking agents which prevent motility. With regard to this latter function there is a clear difference between the full-length MAP2a,b and the smaller MAP2c (Heins et al., 1991).

An unexpected result of this study was the finding that MAP2c molecules interact not only with tubulin, but with another to form antiparallel dimers. Dimers (and other aggregates) can be distinguished from monomers on the basis of their greater apparent width (Fig. 4), however, another direct proof comes from the antibody labeling experiments (Fig. 6). The relationship between monomers, dimers, and antibody epitopes is diagrammed in Fig. 8, assuming a colinear model for MAP2c (as in Fig. 7B).

What could be the basis for dimerization? We do not know enough about the folding of the protein. Associations of α-helices (as in coiled coil proteins) or β-sheets would seem to be of minor importance, considering the low content of secondary structure mentioned above, but specific interactions based on short sequences cannot be ruled out. On the other hand, MAP2c has an asymmetric charge distribution, as mentioned above (diagrammed as bright and dark in Fig. 8). This would favor the antiparallel alignment of pairs of MAP2c molecules.

The two members of a dimer are nearly in register, but probably not exactly. Both unlabeled and antibody-labeled dimers are somewhat longer than the monomers, about 58 nm on average. This would mean that there is a shift of about 5 nm between the molecules so that the single molecules protrude for about 5 nm at each end of a dimer. These could act as ‘sticky ends’ and generate the more complex assembling forms, including the fibers.

Could MAP2c Play a Role in Microtubule Bundling?—This possibility is suggested by the antiparallel association into dimers. If the C-terminal half binds to a microtubule and the N-terminal half protrudes away from it, then the interaction between two microtubules could be mediated by an antiparallel interaction between the MAP2c molecules. One could speculate that such an interaction of microtubule arrays might be important during neuronal differentiation when MAP2c molecules are preferentially expressed.

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