Different Forms of Microtubule-associated Protein 2 Are Encoded by Separate mRNA Transcripts

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Abstract. Brain microtubule-associated protein 2 (MAP2) consists of a pair of high molecular mass (280 kD) polypeptides, MAP2a and MAP2b, and a recently identified 70-kD protein, MAP2c, which is antigenically related to these high molecular mass MAP2's. Using cDNA clones we have analyzed the expression of these three proteins at the nucleic acid level. cDNA probes selective for the high molecular mass MAP2's a and b identified only a 9-kb mRNA, whereas a probe for sequence common to all three MAP2 isoforms, a, b, and c, recognized the 9-kb transcript and additionally a 6-kb mRNA. Southern blot analysis with cDNA probes indicated that there is only one MAP2 gene from which these two distinct mRNAs are derived. The 70-kD MAP2c protein is much more abundant in neurons of developing brain than those of adult tissues. Similarly the expression of the 6-kb MAP2c-related mRNA, is much greater in neonatal than adult rat brain, indicating that the developmental expression of MAP2 is determined by transcriptional regulation from a single MAP2 gene.

Materials and Methods

A random hexanucleotide-primed cDNA library, prepared from postnatal day 5 rat brain poly A+ RNA, was constructed in bacteriophage λgt11 using the methods described in Watson and Jackson (28) and Young and Davis (29). A mouse polyclonal antiserum was raised against thrice-cycled microtubules prepared from 5-d-old rat brain by the method of Carr et al. (8). The mice were injected i.p. with 0.5 ml of a 1:1 emulsion of 1 mg/ml microtubule proteins with Freund's complete adjuvant on day 1. The injection was repeated using Freund's incomplete adjuvant on day 14, and the mice were bled 3 d later. This antiserum gave positive staining of MAP2 on Western blots of 5-d-old rat brain microtubules at 1:1,000 dilution.

The antiserum was used at 1:500 dilution to screen the λgt11 library using peroxidase-conjugated secondary antibody (Dako-Patts, Copenhagen, Denmark) to detect positive clones. These were plaque purified, grown at a density of 10<sup>4</sup> pfu/10 cm plate and overlayed with nitrocellulose filters soaked in 10 mM isopropyl-β-d-thiogalactopyranoside (Boehringer, Mannheim) to induce and absorb the fusion proteins. The filters, bearing immobilized fusion protein, were used to absorb monospecific antibodies from the polyclonal mixture by the method of Lewis et al. (14). These monospecific antibodies were used to stain Western blots, and the clones 19a and 38a, whose fusion proteins adsorbed antibodies that reacted with MAP2, were used for further study. The bridging clone 14b was isolated by rescreeing the same library with a 38a probe radioactivity labeled by oligo-labeling with random primers (3) and then testing the positives for crosshybridization with an oligo-labeled 19a probe.

Supernatant (S1) fractions were made from brain homogenates (21), and their proteins separated on SDS-polyacrylamide gels (11), blotted onto a nitrocellulose sheet and immunoperoxidase stained (Western blotted) as described by Towbin et al. (24). Escherichia coli extracts containing fusion proteins were obtained by eluting the proteins directly from a plate lysate. λgt11 clones were grown on E. coli strain Y1090 at 10<sup>4</sup> pfu/plate containing 10 mM isopropyl-β-d-thiogalactopyranoside in the top agar. After incubating overnight at 37°C, proteins were eluted for 1 h in 1 X SDS-polyacrylamide gel sample buffer (11) and boiled for 5 min before loading on a gel.

Poly A+ RNA was selected with oligo-β-D-cellulose from RNA isolated from postnatal day 5 and adult rat brain using guanidinium thiocyanate as described by Maniatis et al. (15). Western blots were prepared by separating the selected RNA on 0.8% agarose-formaldehyde gels (15) and blotted onto GeneScreen (New England Nuclear, Boston, MA) in 25 mM sodium phosphate buffer, pH 6.5. The blots were hybridized with cDNA probes at 10 ng/ml in 50% formamide, 0.2% polyvinylpyrrolidone, 0.2%...
Figure 1. Western blot analysis of monospecific antibodies selected using fusion-proteins from two non-overlapping MAP2 cDNA clones. Protein extracts from *Escherichia coli* strain Y1090 infected with bacteriophage λ-gtll, clone 19a, or clone 38a (lanes 1-3, respectively) were run on a 3-15% SDS-polyacrylamide gel, along with S1 supernatants from postnatal day 5 and adult rat brain homogenates (lanes 4 and 5, respectively) and heat stable juvenile MAPs (lane 6). A Coomassie Blue-stained gel (A) and two Western blots made using selected monospecific antibodies from clone 19a (B) and 38a (C) are shown. The positions of MAP2s a, b, and c and λ-gtll β-galactosidase fusion protein (f) are indicated by lettered arrows at the right of each panel.

BSA, 0.2% Ficoll 400, 50 mM Tris-HCl, pH 7.5, 1 M NaCl, 0.1% sodium pyrophosphate, 1% SDS, 10% dextran sulphate and 100 μg/ml denatured salmon sperm DNA at 42°C for 16 h, and washed with 2 × SSC and 1% SDS at 65°C. The cDNA probes were labeled by nick translation (15).

For Southern blotting rat liver DNA was prepared by the method of Blin and Stafford (1) and 20 μg of DNA per well was digested with restriction enzymes (Boehringer, Mannheim). DNA fragments were separated on 0.9% agarose gels and Southern blotted onto Genescreen in 25 mM sodium phosphate buffer, pH 6.5, and hybridized with 10 ng/ml radioactively-labeled probes as described for the Northern blots. The blots were washed with 0.3 M NaCl, 60 mM Tris–HCl, pH 8.0, 2 mM ethylenediaminetetraacetic acid and 1% SDS at 60°C. Radioactive cDNA probes were prepared by oligolabeling with random primers (3).

Results

MAP2 clones were isolated from a rat brain λgtll cDNA library and characterized using monospecific antibodies selected from a polyclonal anti–MAP serum. Several of the MAP2 clones isolated fell into one of two different classes, as exemplified by clones 19a and 38a. Fig. 1 shows the relationships of these two clones to the different forms of MAP2 protein. The monospecific antibodies used to establish these relationships were selected by immunoabsorption with the expressed fusion proteins of clone 19a (to give monospecific anti–19a) and clone 38a (to give monospecific anti–38a). As expected, anti–19a reacts strongly with the fusion protein from clone 19a (Fig. 1 B, lane 2). It does not react with either the β-galactosidase from λgtll (Fig. 1 B, lane 1) or with the fusion protein from clone 38a (Fig. 1 B, lane 3). Conversely, anti–38a reacts only with the fusion protein from clone 38a (Fig. 1 C, lane 3) and not with any of the proteins from λgtll or clone 19a (Fig. 1 C, lanes 1 and 2).

The two antibodies differed in their reaction with the various MAP2 forms. On western blots of microtubule proteins, anti–19a reacted only with MAP2 a and b (Fig. 1 B, lanes 4–6), whereas anti–38a reacted not only with these same two high molecular mass proteins, but also with MAP2c (Fig. 1 C, lanes 4–6). Thus clone 19a represents sequences present only in MAP2a and MAP2b, whereas clone 38a corresponds to sequences present in all three forms.

The cDNA 38a and 19a clones do not cross-hybridize (data not shown) and thus represent non-overlapping sequences. However, a further MAP2 cDNA clone, 14b, was identified that hybridized to both 38a and 19a. The existence of such a bridging clone indicates that the sequences in clones 38a and 19a represent separate domains on one continuous stretch of DNA. Fig. 2 is a restriction map of these three clones.
showing the extent of their overlap and their order, with 38a lying 5' to 14b, which is in turn 5' to 19a. Also shown in Fig. 2 are the two probes, 38a' and 19a', that were used in Southern blot experiments.

Fig. 3 shows Northern blot analysis of postnatal day 5 and adult rat brain poly-A+ RNA with radioactive probes derived from clones 19a and 38a. The 19a probe (lanes 1 and 2) revealed a single 9 kb mRNA that is expressed with equal abundance in both juvenile and adult brain. The 38a probe hybridized not only with the 9 kb mRNA but also with a 6 kb mRNA that is much more abundant in juvenile brain than adult (Fig. 3, lanes 3 and 4). The right hand panel of Fig. 3 shows that the probes 38a' and 19a', which were used in Southern blot experiments (see below), hybridize with the same mRNAs as their respective full length cDNAs.

To investigate further the relationship between MAP2c and the 6 kb mRNA, we examined poly-A+ RNA from rat glioma C6 cells, which contain abundant MAP2c but in which MAP2a or MAP2b are not detectable (Fig. 4 A). If MAP2c is encoded on the 6 kb mRNA then these cells should contain the 6 kb mRNA but not the 9 kb mRNA. Fig. 4 B shows Northern blots of poly-A+ RNA from C6 cells, developed with probes 19a and 38a. Juvenile rat brain poly-A+ RNA served as a positive control. Probing with clone 19a, which acts as a specific marker for the 9 kb mRNA, confirmed its presence in juvenile rat brain (Fig. 4 B, lane 1).

By contrast C6 cells showed no evidence of RNA cross-hybridizing with the 19a probe (Fig. 4 B, lane 2), even after ten times longer exposure (not shown). Probing with clone 38a showed that both mRNAs are present in juvenile brain but that only the 6 kb mRNA occurs in C6 cells (Fig. 4 B, lanes 3 and 4). Thus the high molecular mass MAP2's a and b are encoded by one or more 9 kb mRNAs whereas the 70 kD molecular mass MAP2c is encoded by a 6 kb mRNA. To determine whether these two mRNAs were transcribed from one or two genes, Southern blots of rat genomic DNA were prepared using probes 19a' and 38a' (Fig. 5). The 19a' probe hybridizes with only single bands. Similarly, despite the fact that the 38a' probe cross-reacts with two distinct mRNAs on Northern blots (Fig. 3, right hand panel), on Southern blots it reacted with only single bands in genomic DNA cut with several restriction enzymes (Fig. 5). If there were more than one MAP2 gene then probe 38a', which contains sequences represented on both the 6 kb and 9 kb mRNAs, should have produced two or more bands. The fact that it did not indicates that both mRNAs are derived from a single gene.

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Discussion

These results demonstrate that MAP2c is derived from a 6 kb mRNA that is distinct from the 9 kb mRNA that encodes high molecular mass MAP2. Other explanations for the occurrence of MAP2c, such as derivation from high molecular mass MAP2 by proteolysis or other posttranslational modifications are thus excluded. Posttranslational modification of the MAP2c protein itself remains possible and is in-
Indeed suggested by the presence of minor anti-MAP2-reactive bands of similar size to MAP2c whose exact pattern changes during development (Tucker, R., and A. Matus, unpublished observations). The pattern of developmental expression of the 6 kb MAP2c mRNA mirrors that of the high molecular mass MAP2 (25). Such an influence of MAP2c could contribute to the plastic nature of growing dendrites compared with the rigid form of adult dendrites in which only the high molecular mass forms of MAP2 are present.

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Both peptide mapping (4) and the data presented here indicate that MAP2c is homologous to a stretch of high molecular mass MAP2. Both high molecular mass MAP2 and MAP2c co-assemble with microtubules in vitro (reference 4, also see Fig. 1 above) and this, together with the structural homology, suggests that all three forms possess a common tubulin-binding domain, although their precise relationship remains to be established. The tubulin-binding domain of high molecular mass MAP2 has been shown to be contained in a 35-kD fragment at one end of the molecule (26), with the remainder of the polypeptide protruding from the wall of the microtubule as an extended tail (9, 27, 30). Thus in essence MAP2c appears to be a form of MAP2 that lacks ~200 kD from the tail domain of high molecular mass MAP2.

The two MAP2 mRNAs are transcribed from one gene. Southern blots of total rat liver DNA (20 Itg/well) digested with either Kpn I, Eco RI, Hind III, Pst I, or Bam HI (lanes K, E, H, P, and B, respectively). The blots were hybridized with radioactive probes derived from either the Eco RI-Pst I fragment from clone 19a (19a') or with the Eco RI-Sal I fragment of clone 38a (38a') (see Fig. 2).

Figure 5. The two MAP2 mRNAs are transcribed from one gene. Southern blot analysis revealed only one rat MAP2 gene. This does not exclude the possibility that other MAP2-related genes may exist to which our probes hybridize only weakly. Lewis et al., since both identify only a 9 kb mRNA transcribed from either the Eco RI-Pst I fragment from clone 19a (19a') or with the Eco RI-Sal I fragment of clone 38a (38a') (see Fig. 2).
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