

New forms of HMW MAP2 are preferentially expressed in the spinal cord

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Abstract The high molecular weight forms of microtubule-associated protein 2 (MAP2a and b) play a central role in the specification of dendrites. RT-PCR amplification of a portion of the N-terminal and middle MAP2b domains of rat spinal cord cDNAs allowed identification of new variants containing both exon 8 (246 bp) and a new exon, 7A (237 bp), located at the beginning of the middle MAP2b region. The brain and the spinal cord express transcripts containing exon 8, whereas exon 7A alone or exons 7A+8 were detected, whatever the developmental stage, only in the spinal cord.

Key words: Microtubule-associated protein 2; Spinal cord; New exon

1. Introduction

Microtubule-associated proteins seem to play a crucial role in determining the shape of nerve cells, the plasticity of neuronal processes and the acquisition of neuronal polarity. For instance, the specification of the dendrites during brain development probably depends [1] on the expression and transport of one of the major microtubule-associated proteins, MAP2, whereas another group, tau proteins, is targeted essentially towards the axons [2,3].

Both tau and MAP2 proteins are heterogenous and the expression of the various species is developmentally regulated. Immature and mature LMW tau forms [4] differing in the number of tubulin binding repeats (three and four, respectively) and in the presence or absence of two exons located in the N-terminal domain [5,6] are expressed successively during development. In addition, HMW tau species [7,8] are expressed in the spinal cord [9] and dorsal root ganglia [10] which contain either one or two additional exons (exons 4A and 6) whereas they are present only in trace amounts in the brain.

MAP2 proteins are also heterogenous and developmentally regulated in the brain: two HMW species [11,12] of 270 kDa (MAP2a/b) are present in the adult brain whereas MAP2b and a much smaller variant [13,14] of 70 kDa (MAP2c) are present at immature stages. MAP2a/b are distributed only in dendrites whereas MAP2c seems to be present in all the neuronal domains [15] and astroglial cells [16].

Compared to MAP2b, MAP2c has the same N-terminal region and the same C-terminal domain which contains the tubulin binding sites, but lacks the long middle sequence of

200 kDa [17,18]. Three tubulin binding homologous repeats [19] have been identified, irrespective of the developmental stage, in the brain MAP2b and MAP2c species. Another LMW variant, MAP2d, with four repeats has been recently discovered both in the brain [20] and in the neuronal cell line ND 7/23 [21] whereas HMW MAP2 forms with four repeats have been cloned so far only in ND cells [21] and in dorsal root ganglia [22]. We have recently described [23] a brain HMW MAP2 variant which differs from MAP2b in the presence of an insertion of 246 bp in the N-terminal side of the middle region.

MAP2b and c are respectively encoded by mRNAs of 9 and 6 kb [24] which are produced by alternative splicing of a primary transcript produced from a single gene [25]. The difference between MAP2a and b is unknown.

We describe in this work new HMW MAP2 species which are preferentially expressed in the spinal cord.

2. Material and methods

2.1. RNA extraction and hybridization analysis

Total RNA was extracted [26] from juvenile (6-day-old) and adult (36-day-old) rat spinal cord and brain. Poly(A)⁺ RNA was selected by oligo(dT)-cellulose chromatography (Pharmacia). Northern blot analysis was carried out as previously described [27]. Blots were washed for 2 h at 60°C in 0.2×SSC (1×SSC: 0.15 M NaCl/15 mM sodium citrate) containing 0.1% sodium dodecyl sulfate.

2.2. First strand cDNA synthesis and DNA amplification

Poly(A)⁺ RNA was reverse-transcribed from an oligo(dT) primer using a first strand synthesis kit (Stratagene). Amplification of the first-strand products was carried out in a DNA thermal cycler from Perkin Elmer-Cetus Instruments. The two oligonucleotides used as primers in the PCR experiments were a sense primer (TCACAGGG-CACCTATTGAGA) and an antisense primer (CATCTGC-CACCTTTGTCTGCT). They are respectively located at nucleotides 187–206 and 1128–1147 on the rat MAP2 sequence [28]. The numbering starts with 1 at the ATG coding for the first methionine. The two primers are indicated by arrows in Fig. 1.

2.3. Analysis of amplified DNA by Southern blot

An aliquot (5 or 10 µl) of the amplified DNA was fractionated on a 1.2% agarose gel and transferred to a nitrocellulose membrane filter as described by Southern [29]. Blots were washed twice for 1 h at 65°C in 1×SSPE (1×SSPE: 0.15 M NaCl/10 mM NaH₂PO₄/1 mM EDTA) containing 0.1% sodium dodecyl sulfate.

2.4. Cloning and sequencing of amplified DNA

The DNA fragments amplified by PCR were subcloned in pGEM-T (Promega) as recommended by the supplier. Sequencing of the different amplified products was performed by the dideoxynucleotide chain-termination method [30] using [³³P]dATP and sequenase enzyme (US Biochemical).

2.5. Probes used for the Northern and Southern blot analyses

Northern and Southern blot analyses were carried out either with a MAP2c specific cDNA probe or with two other probes corresponding respectively to insertion 7A (sense primer ATGAAGGGGCTGAG-

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GATCAA and antisense primer CTTTCTCCTGGCCTAAGTGG) or to insertion 8 (sense primer TAGAGGGTGTGATGGCTGAG and antisense primer GGCAGAGGAAGGGATTCTA). The two last probes were obtained by PCR amplification and were sequenced to verify that they had the expected sequence. The position of the different primers used is indicated in Fig. 2. All these probes were labeled using the megaprime technique (Amersham) with [α - 32 P]dCTP.

3. Results

3.1. Southern blot analysis and cloning of rat spinal cord

RT-PCR products

The rat spinal cord expresses the two mRNAs of 9 and 6 kb which encode in the brain the HMW and LMW MAP2 species, respectively. To identify more precisely the HMW MAP2 species expressed in the spinal cord, RNAs were prepared from 6- and 36-day-old rat spinal cord to generate MAP2b-specific RT-PCR products. According to the known sequence of MAP2b [28], a single product of 961 bp was expected when using the oligonucleotides described in Fig. 1 for PCR amplification. In contrast, ethidium bromide staining (results not shown) and Southern blot analysis (Fig. 2A) revealed two additional bands of varying intensity and of approx. 1200 and 1450 bp. This suggests that at least two spinal cord variants have, compared to MAP2b, longer sequences (of 240 and 490 bp) in this region. The HMW MAP2 PCR products were therefore cloned and sequenced. Compared to MAP2b the set of clones of 1207 bp had an insertion of 246 bp located directly downstream of the splice junction between MAP2c and b (Fig. 1). This insertion has previously been found in a brain HMW MAP2 PCR product [23] and is homologous to exon 8 of the recently described human MAP2 gene [31].

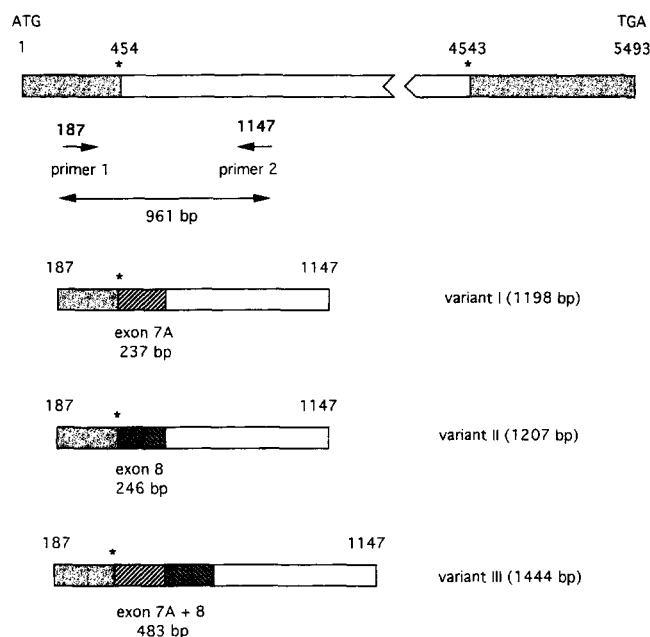


Fig. 1. Schematic representation of the rat MAP2b sequence (numbering refers to the sequence reported by Kindler et al. [28]) compared to the three new HMW MAP2 variants identified in this work. The arrows show the position of oligonucleotides 1 and 2 used as primers to amplify, by RT-PCR, a HMW MAP2 fragment. Asterisks indicate the splice junctions between MAP2c and MAP2b. The sequence of MAP2c is shown by the N- and C-terminal greyish regions. The hatched boxes represent the two new exons 7A and 8.

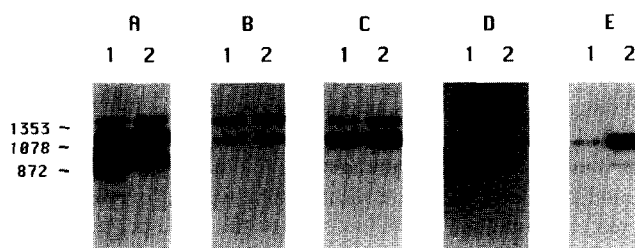


Fig. 2. Southern blot analysis of the RT-PCR products obtained with oligonucleotides 1 and 2 (see Fig. 1) and RNA extracted from rat spinal cord (A–C) or brain (D,E) at postnatal days 6 (lanes 1) and 36 (lanes 2). Hybridization was performed with a probe which detects MAP2b and MAP2c (A) and with probes specific for exon 7A (B,D) or exon 8 (C,E). The DNA markers are ϕ X174 RF DNA/HaeIII fragments.

Clones of 1444 bp were also isolated and shown to contain an insertion of 483 bp. The first 237 bp of this insertion, referred to exon 7A (Fig. 1), which were detected for the first time in this work, are followed downstream by a stretch of 246 bp identical to exon 8. The sequence of the two insertions of 237 and 246 bp is represented in Fig. 3. Finally, the clones of 961 bp had, as expected, the sequence previously reported for MAP2b [28].

The data reported above clearly suggest that the new insertion of 237 bp is encoded by a different exon of the MAP2 gene. Experiments (results not shown) performed by PCR amplifying this region with rat genomic DNA in the presence of a set of primers located at both sides of the insertion confirmed this conclusion and showed that this region is a single exon. Other experiments (results not shown) also performed with a probe specific for the insertion of 237 bp confirmed that there is a single MAP2 gene [25,31] and that the new exon which is now referred to exon 7A belongs to this gene.

3.2. Southern blot analysis of brain and spinal cord RT-PCR products with probes specific for exons 7A and 8

Southern blots of the RT-PCR products obtained with 6- and 36-day-old spinal cord RNA in the presence of primers 1 and 2 (Fig. 1) were hybridized with the probes specific for exons 7A (Fig. 2B) and 8 (Fig. 2C), respectively. Two bands of approx. 1200 and 1444 bp were detected in both cases but their relative proportion was different depending on the probe, suggesting further heterogeneity. The minimal conclusion is therefore that the spinal cord expresses not only a transcript containing both exons 7A and 8 but also two other transcripts containing each one of these two inserted sequences.

Southern blot analysis performed with rat brain PCR products and the probe specific for exon 7A revealed, at the adult stage, trace amounts of the transcripts of 1198 and 1444 bp (Fig. 2D). In contrast, the probe specific for exon 8 revealed a band of 1207 bp which increased in intensity at mature stages (Fig. 2E). A faint band of approx. 950 bp was also detected by this probe; this band probably corresponds to a clone which contained this new insertion but lacked a stretch of approx. 250 bp. In addition and since the transcript of 1444 bp revealed by the probe specific for exon 7A was not detected by the probe specific for the exon 8, one may assume that it contains another, yet unknown, MAP2 domain.

3.3. Northern blot analysis of brain and spinal cord mRNAs with probes specific for exons 7A and 8

Northern blot analysis was also performed to determine whether MAP2 mRNAs containing the insertion(s) are transcribed in rat brain and spinal cord. Fig. 4A shows that a transcript of 9 kb containing exon 7A was expressed in the spinal cord and that its concentration was apparently much lower at immature stages. In contrast, the same probe did not reveal a 9 kb band with brain mRNAs. When the probe specific for exon 8 was used, 9 kb transcripts were detected with both brain and spinal cord mRNAs (Fig. 4B), however, the highest concentration was again observed with adult spinal cord. Another transcript of 2.4 kb was also revealed by the same probe but its significance remains unknown.

4. Discussion

The data reported in this work show that several new HMW MAP2 variants are preferentially expressed in the spinal cord. These MAP2 forms differ from the species sequenced previously in the presence of two domains of approximately the same size, 237 and 246 bp.

The PCR products containing these insertions are not artefacts because there are MAP2b-specific sequences downstream and upstream and because the insertions do not alter the open reading frame of the remaining protein. They are located downstream of a known splice junction which is located between the N-terminal domain, common to MAP2b and MAP2c, and the middle region which is present only in MAP2b. The presence at the 3' end of the PCR products containing the insertion(s) of a portion of the sequence of the middle region means that the new transcripts belong to the HMW MAP2 family and are produced by alternative splicing.

Since the PCR technique can overamplify transcripts present in trace amounts, Northern blot analysis was also performed with a probe specific for the new exon identified in

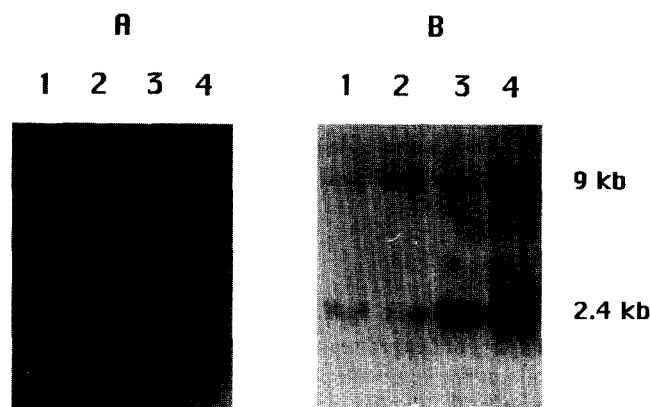


Fig. 4. Northern blot analysis of total RNA (20 µg/lane) extracted from rat brain (lanes 1,2) and spinal cord (lanes 3,4) at postnatal days 6 (lanes 1,3) and 36 (lanes 2,4). Hybridization was performed with probes specific for exon 7A (A) or exon 8 (B).

this work. Comparison of the data obtained with the two techniques suggests that the HMW MAP2 variants containing exon 7A are essentially expressed in the adult spinal cord whereas negligible amounts were detected in this region at immature stages and, whatever the stage, in the brain. Exon 8 is also preferentially expressed in the adult spinal cord although it could be detected in the brain in higher amounts than exon 7A.

Exons 7A and 8 encode 80 and 83 amino acid residues, respectively: this would increase the molecular weight of the three variants containing exon 7A or 8 alone or both exons 7A and 8 by 8.6, 8.8 and 17.4 kDa. This increase can account for the higher apparent mass of MAP2a compared to MAP2b. In addition, exons 7A and 8 are clearly expressed, like MAP2a, in higher amounts at adult stages. It has previously been suggested that MAP2a is generated from MAP2b by phosphorylation [32] but the possibility that MAP2a is produced by alternative splicing has never been disproved. However, we have recently reported that HMW MAP2 transcripts containing exon 8 are expressed in the DRG which do not express MAP2a [33].

Little is known of the functional significance of the various domains of the different MAP2 species. Early work [34] on MAP2a/b showed that a C-terminal fragment of 36 kDa, which retains polymerization activity, can be cleaved and binds to the microtubule lattice, whereas the middle and N-terminal regions, that project from the microtubule wall, determine the spacing between microtubules in the dendrites. More recently, sequence data and transfection experiments have provided information on the function of the C-terminal domains of the various MAP2, tau and MAP4 species which all contain tubulin binding repeats. For instance, experiments performed with synthetic peptides [35] have confirmed that these repeats are sufficient for microtubule binding. The polymerization activity of MAP2d, which contains four repeats, is twice that of MAP2c which has only three repeats [36]. Other data suggested [37,38] that regions of tau proteins which flank the microtubule binding domain affect the bundling of microtubules in vitro. Transfection experiments [39] within non-neuronal cells performed with different MAP2 deletion mutants also suggested that the strength of binding to microtubules not only increases with the number of repeats but also

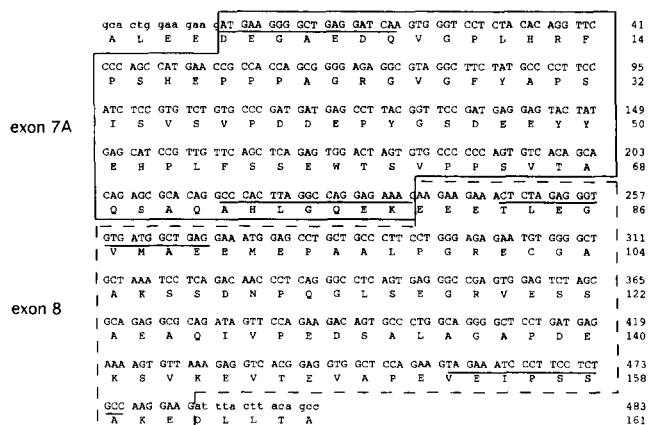


Fig. 3. Nucleotide and predicted amino acid sequences of the 483 bp insertion identified in a spinal cord HMW MAP2 variant of 1444 bp. This domain is composed of two stretches of 237 bp (exon 7A) and 246 bp (exon 8) which are boxed. Exons 7A (continuous line) and 8 (dashed line) are also separately found in other clones of approx. 1200 bp. Numbers to the right designate nucleotides (upper) and amino acids (lower). The 5' and 3' MAP2b sequences determined at both sides of the inserted region are indicated in lower-case letters. The positions of the oligonucleotides used to amplify separately each insert are underlined.

requires contiguous sequences. It cannot be excluded therefore that MAP2 domains located in the N-terminal end or the middle region may contribute to the polymerization activity and/or microtubule stabilization.

It has also been suggested that the N-terminal and middle regions of MAP2 would be responsible for the interactions between two adjacent microtubules or between microtubules and other elements of the cell. For instance, the formation of cross-bridges between microtubules and neurofilaments was demonstrated in the dendrites of motor neurons from bovine spinal cord [40]. The existence of a number of HMW MAP2 forms with projecting filamentous side arms of varying lengths might allow generation of different spacings between microtubules and other cellular elements. The Blast program suggested that the first 32 amino acid residues of exon 8 have 41% homology with the N-terminal region of MARCKS, a filamentous bovine myristoylated alanine-rich substrate of protein kinase C which is an actin-binding protein that seems to regulate actin-membrane interactions [41,42]. The same program shows that some regions of exon 7A have homologies with sequences of nestin, an intermediate filament protein, but the significance of this observation remains unknown.

Exon 8 detected within the human MAP2 gene [31] has 80% homology with the rat new exon of 246 bp that we described previously [23]. The other new exon of 237 bp (exon 7A) which has so far been identified only in this work is preferentially expressed in the spinal cord and only in trace amounts in the brain and the dorsal root ganglia [22].

In conclusion, the data reported here show that new HMW MAP2 variants containing exons which were not detected within MAP2b, are preferentially expressed in the adult spinal cord. It remains to be determined whether this species confers specific properties to the dendrites of this nervous tissue. This possibility, of course, depends on their as yet unproven dendritic localization in the spinal cord and of their co-distribution with MAP2a/b.

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