

Invited Review

Molecular genetic approaches to microtubule-associated protein function

C. González-Billault and J. Avila

Center of Molecular Biology "Severo Ochoa", Universidad Autónoma de Madrid, Madrid, Spain

Summary. Protein function *in vivo* can be studied by deleting (knock-out) the gene that encodes it, and search for the consequences. This procedure involves different technologies, including recombinant DNA procedures, cell biology methods and histological and immunocytochemical analysis.

In this work we have reviewed these procedures when they have been applied to ascertain the function of several microtubule-associated proteins. These proteins have been previously involved, through *in vitro* experiments, in having a role in the microtubule stabilization. Here, we will summarize the generation and characterization of different microtubule-associated protein knock-out mice. Special attention will be paid to MAP1B knock-out mice. Amongst the different MAPs knock-out mice these show the strongest phenotype, the most likely for being MAP1B, the MAP that is expressed earliest in neurogenesis.

Molecular genetics could be considered as a valid and useful procedure to truly establish the *in vivo* functions of a protein, although it is necessary to be aware of possible artifacts such as the generation of some kinds of RNA alternative splicing. To avoid this the best strategy to be used must consider the deletion of the exon that contains the functional domains of the protein.

Key words: Microtubule-associated proteins, Knock-out, Review

Introduction

The complexity of a cell depends on a structured organization of its components. Thus, cytoskeleton plays a key role in controlling a wide variety of cellular processes, such as cellular organelle movement, and intracellular transport, cellular motility, cell

morphological differentiation, establishment of cell polarity and chromosome segregation (Hyman and Karsenti, 1998; Baas, 1999).

Microtubules (a main cytoskeleton compound) directly participate in all of the above mentioned processes. Although they are present in many cellular types, microtubules are specially abundant in neurons, where they contribute to the development and maintenance of neural polarity (Baas, 1998; Mattson, 1999). To accomplish all of their function the dynamic properties of microtubules must be under a tight control.

Microtubule dynamics are finely regulated, through a well known process termed dynamic instability (Mitchinson and Kirschner, 1984). These dynamics, can be modified by a group of proteins that copurify with the main component of microtubules, tubulin, through cycles of assembly and disassembly, termed microtubule-associated protein (MAPs) (Weingarten et al., 1975; Hirokawa et al., 1988). These proteins could stabilize microtubules reducing the dynamic instability (Horio and Hotani, 1986). Additionally, MAPs diminished the critical concentration of tubulin required to form polymers *in vitro* (Murphy and Borisy, 1975), thus promoting the assembly of microtubules (Avila, 1990). However, the *in vivo* functions of these MAPs have not been fully established.

Nowadays, to really ascertain the function of a protein an *in vivo* approach has become an important and desirable task. For this, scientists have taken advantage of two major breakthroughs in the mouse molecular genetics. First, the development of techniques to isolate homologous recombination events in mammalian cells (Doetschman et al., 1987; Thomas and Capecchi, 1987) and second, the establishment of embryonic stem cells culture (Evans and Kaufman, 1981; Martin, 1981). These techniques have led to the manipulation of mouse genome, and the subsequent generation of transgenic and knock-out mice. These techniques have become very popular during the last decade, as can be observed by the increasing number of articles describing molecular genetic models in mice (Brandon et al., 1995)

The generation of knock out mice can be faced using mainly two different strategies. The gene targeting

Offprint requests to: Dr. Jesús Avila, Center of Molecular Biology "Severo Ochoa", Universidad Autónoma de Madrid, Campus Cantoblanco, 28049, Madrid, Spain. Fax : 34 91 397 4799. e-mail: javila@cbm.uam.es.

(Thomas and Capecchi, 1987; Wurst and Joyner, 1993) and the gene trapping (Evans et al., 1997; Zambrowicz and Friedrich, 1998) techniques.

In the gene targeting approach, a targeting vector is introduced in a site-directed manner into the genome. This vector contains an appropriate selection marker (for example, *neo^r*) flanked by sequences present in the genomic sequence of the gene to be disrupted. Thus it may allow the homologue recombination. Therefore the gene expression is interrupted at a known and chosen site.

On the other hand, in the gene trapping approach, the vector is randomly integrated into intronic or exonic regions of the genome. This fact allows the trapping of several genes in a single event (Stoykova et al., 1998). The trapping vector contains a reporter gene (for example, *lacZ* gene), besides the selection marker gene. The use of splicing acceptor sequences in the 5' of the vector allows the splicing and fusion of the construct with known proteins, that can be rapidly identified using the RACE-PCR strategy (Frohman et al., 1988). Because of the absence of promoter sequences in the trapping vector, the expression pattern of reporter gene mimics the one of the trapped gene protein. Depending on the site of integration of trapping vector, a complete or partial knock out can be created (Friedrich and Soriano, 1991; Chowdhury et al., 1997)

Once knock-out mice have been generated, a detailed phenotype analysis should be taken into account. For this purpose, morphological, histological and immunochemical procedures should be used.

Here we described the results of several independent studies designed to elucidate the microtubule-associated proteins *in vivo* functions. The results are also summarized in Table 1.

Tau protein

Tau protein is one of the most studied MAPs, since it is not only a microtubule-associated protein, but also it forms aberrant aggregates found in the brain of patients suffering Alzheimer's disease or other taupathies. It has been found to be enriched in the axonal compartment. It is composed by a family of isoforms derived by alternative splicing from a single gene (Goedert et al., 1989). Two classes of tau protein have been described

depending on the number of tubulin binding domains that it contains. Juvenile tau proteins have three binding domains while adult tau contains four tubulin binding sites (Kosik et al., 1989; Lee et al., 1990; Goedert et al., 1991). Additionally a high molecular weight tau have been described in peripheral nervous system (Georgieff et al., 1991). This protein contains an extra exon termed 4a that it is responsible for such a increase in molecular weight (Couchie et al., 1992).

Tau protein was the first microtubule-associated protein to be disrupted through molecular genetic approaches (Harada et al., 1994). The molecular strategy consisted in exon 1 (Himmler, 1989) disruption by homologue recombination (Harada et al., 1994). Mice homozygous for this mutation were indistinguishable from heterozygous and wild type littermates. Histological analysis in sections stained with haematoxylin-eosin did not show abnormalities along development. The expression of low and high (Georgieff et al., 1991) molecular tau proteins were completely abolished.

Authors analyzed the expression levels of other MAPs and cytoskeleton-relevant proteins, including, MAP1A, MAP1B, MAP2, neurofilament proteins, synapsin-1 and tubulin isoforms and they only found an increase in MAP1A levels.

Using electron microscopy Harada et al. (1994), looked for further morphological changes in tau homozygous mice. They found a reduction in the number and density of axons corresponding to parallel fibers in the cerebellum. These effects were not observed in dendrites of Purkinje cells. Quick-freeze deep-edge electron microscopy allowed authors to establish a great reduction in the number of cross-bridges between microtubules, but not with axonal membrane in cerebellum from homozygous mice (Table 2).

An intriguing aspect of this study was the fact that primary cultures of hippocampal neurons from homozygous were indistinguishable from wild type control littermates, arguing against a role of tau protein in axonal elongation and neuronal polarization. The authors explained this apparently controversial result, explaining that the absence of tau protein should be important only in small caliber axons (such as cerebellum axons), while in large caliber axons (such as those present in DRG and hippocampal neurons), the

Table 1. Comparison of MAPs knock-out strategies.

MAP	GENETIC BACKGROUND	TARGETING APPROACH	SITE OF MUTATION	TYPE OF MUTATION	LETHALITY
1B (1)	C57BL6/129	Homologous recombination	Exon 5/ aa 571	Null	Before E8
1B (2)	C57BL6/129	Homologous recombination	Exon 1/ aa 11	Hipomorphous?	Non lethal
1B (3)	CD1/129	Gene trapping	Downstream exon 2/aa 95	Hipomorphous	Perinatal
Tau	C57BL6/129	Homologous recombination	Exon 1	Null	Non lethal
2	N.D	Homologous recombination	N.D	N.D	Non lethal
4	MPI-II/?	Gene trapping	Downstream aa 139	-	Non lethal
Tau/1B	C57BL6/129	Homologous recombination	Exon 1 for both genes	Null for tau/ Non null for 1B	N.D

(1): Edelmann et al., 1996; (2): Takei et al., 1997; (3): González-Billault et al., 2000; aa: aminoacid; N.D: not described.

presence of other MAPs (such as MAP1A and MAP1B) should be compensating the absence of tau protein, as was suggested by the increase of MAP1A levels in homozygous protein extracts.

Microtubule-associated protein 1B

MAP1B (Bloom et al., 1985), is the first MAP which is specifically expressed in neurons during nervous system development (Tucker and Matus, 1988; Tucker et al., 1989). During brain development, the expression of MAP1B changes in such a way that the highest level of the protein is reached in the earliest developmental stages during neuritogenesis (Riederer et al., 1986; Schoenfeld et al., 1989; Díaz-Nido et al., 1990). From synaptogenesis on, it is down regulated until reaching the low adult levels (Schoenfeld et al., 1989; Fischer and Romano-Clarke, 1990; Riederer et al., 1990). However, through histological and immunochemical analysis it was shown that in some brain regions where neuritogenesis persists into adulthood, such as the olfactory system and photosensitive cells of the retina and/or in areas with greater neural plasticity potential, such as the hippocampus, high levels of MAP1B expression are maintained (Tucker and Matus, 1988; Viereck et al., 1989).

Edelman's knock-out

In 1996, Edelman and colleagues (1996) described for the first time a knock-out mouse lacking the microtubule-associated protein 1B. The strategy used by the authors was gene targeting through homologue recombination. The targeted site chosen was the exon 5, specifically creating a premature stop codon in the zone corresponding to the MAP1B 571 codon, in the vicinity of the microtubule-binding domain. The genetic background for these mutants was contributed by 129/J and C57BL/6J strains. In this study they could not clearly ascertain the function of the protein because of the drastic phenotype obtained. As was determined by a Southern blot analysis, homozygous mice died before

embryonic day 8 which is assumed to be the neural fold stage (Table 2). Therefore Edelman's analysis was indeed based on heterozygous animals for this mutation.

Heterozygous were smaller in size than their wild type littermates. The most severe of the abnormalities encountered in the nervous system was the size and shape of the cerebellum. Some other abnormalities although milder were found in the hippocampus and retina.

An important aspect of the analysis was the fact that authors could not detect the presence of a truncated polypeptide corresponding to the 64 kDa amino terminal domain of the protein, concluding that if this peptide was eventually synthesized it was not stable enough to be detected. Thus, the mutation should be considered as a null, and the effect of a truncated protein acting as a dominant negative should be discarded.

Takei's knock-out

Another molecular genetic approach to study MAP1B function was published by Takei and colleagues (1997). In this targeting approach the protein expression was interrupted using the homologue recombination technique, at aminoacid 11, corresponding to exon 1 in the genomic organization of *map1b* gene. The genetic background for these animals was also contributed by 129/J and C57BL/6 as in the case of Edelman's report.

Surprisingly, the results described by Takei et al. (1997) were completely different to the preceding study. In this case, heterozygous showed no apparent abnormalities when compared with their wild type littermates.

Although entire homozygous brains were slightly reduced in size, the brain architecture was not different from control samples, as was judged in nervous system sections corresponding to hippocampus, retina, olfactory bulb and spinal cord. Homozygous animals could reach adulthood and only slightly differences in nervous system of young animals were observed. These differences were described as a retardation in the myelination levels of certain tracks of the nervous

Table 2. Histological characteristics of MAPs knock-out mice.

MAP	OVERALL ANALYSIS	HIPPOCAMPUS	CORTEX	CEREBELLUM	MYELINIZATION
Tau	Mild phenotype	No abnormalities	No abnormalities	Diminished caliber of axons	Not affected
1B (1)	No phenotype because of embryonic lethality				
1B (2)	Mild phenotype	No abnormalities	No abnormalities	No abnormalities	Delayed in optic nerve and spinal cord
1B (3)	Strong phenotype	Abnormal lamination	Abnormal lamination	Abnormal lamination	Not affected
Tau/1B	Not described	Abnormal neurite extension (in primary cell cultures)	Defects in corpus callosum	Not described	Not described

Analysis of histological description of nervous system on MAPs knock-out's. (1): Edelman et al., 1996; (2): Takei et al., 1997; (3): González-Billault et al., 2000.

system of juvenile animals, mainly optic nerve and spinal cord (Table 2). However, this delayed myelination could not be found in other tracts such as trigeminal and sciatic nerves. No obvious changes in axonal diameter were detected. The authors could not detect any difference in the expression levels of other known MAPs, such as tau, MAP2 and MAP1A that could be compensating the absence of MAP1B in homozygous animals. An interesting feature of this study is the fact that when they used a MAP1B antibody to analyze the expression of the protein in the different alleles for the mutation, they found immunoreaction of a protein with an electrophoretic mobility quite similar to MAP1B in homozygous protein extracts. This fact was interpreted by authors as an immunoreaction with some MAP1B related protein or with a yet unknown alternative splicing product yielding a novel MAP1B isoform. Quantitation of this protein accounted for 5% of the total protein, according to the authors' estimations.

Gonzalez-Billault's knock-out

The third MAP1B knock-out mouse (González-Billault et al., 2000) has derived from a gene trapping study (Chowdhury et al., 1997). The genetic background for these animals was contributed by 129/J and CD1 strains.

Gonzalez-Billault et al. found that mice homozygous for the mutation, died during their first day of postnatal

life. Histological analysis showed gross alterations in the patterning of lamination of several brain structures, including cerebral cortex, olfactory bulb, hippocampus and cerebellum (Table 2). Abnormalities also included a great enlargement of cerebral ventricles, with an acute decrease in surrounding cerebral areas, as was determined in histological cryostat sections.

At the molecular level, an increase in the amount of recently described alternative transcripts (Kutschera et al., 1998) was verified in hetero- and homozygous animals. Additionally, a very little quantity of full length protein was detected in homozygous mice, indicating that the mutation should be considered as hypomorphous.

Differences in phenotype can be explained

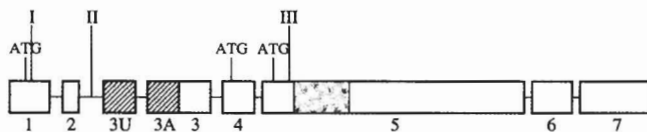
The fact that three independent studies gave rise to three different phenotypes can be explained observing the genomic structure of MAP1B gene (Figure 1 upper panel) (Kutschera et al., 1998). MAP1B gene is composed of seven coding (1-7) and two non-coding exons (3A and 3U). Besides the AUG codon, with an exon 1, two other putative AUG codons are located in exons 4 and 5, that could give rise to amino terminal truncated forms (Kutschera et al., 1998). These forms could be functional because of the presence of the tubulin-binding domain in exon 5, downstream of those putative AUG.

In the case of Edelmann's report, the targeting at aminoacid 571 should interrupt all the MAP1B isoforms expression (Figure 1, III), resulting in the generation of a null mutant, that could give account for the severity of the phenotype of homozygous animals. In Takei's report, the interruption of protein expression in the exon 1 (Figure 1, I), would interrupt the expression of the full length protein, but not the alternative splicing products, that could give account for the immunoreactivity with antibodies against MAP1B in homozygous protein extracts (Takei et al., 1997). In fact, considering that both studies were conducted over the same genetic background this explanation is very feasible. In Gonzalez-Billault's report (Figure 1, II), the presence of very low quantities of full length protein and the presence of alternative transcripts could be enough for reaching the day of birth, but insufficient to overcome MAP1B loss of function.

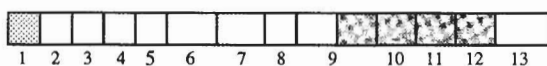
The presence of a little quantity of full length protein in homozygous animals is not an unusual fact, similar mechanisms giving rise to hypomorphic mutated alleles have already been described for other gene trapping studies (Serafini et al., 1996; Yeo et al., 1997; Voss et al., 1998). Moreover, a gene targeting approach directed to disrupt the β -actin locus also generated a hypomorphic mutation (Shawlot et al., 1998).

Another question to be addressed are the phenotypic differences between Takei's report and ours, since both could potentially be producing alternative splicing MAP1B transcripts. One explanation would come from the fact that Takei's report has not dealt with the quantitation of the alternative transcripts that could be

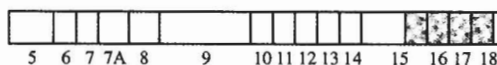
MAP1B gene



Tau gene



MAP2 gene



MAP4 protein



Fig. 1. Schematic diagram showing genomic organization of microtubule-associated proteins 1B, 2 and tau gene; and MAP4 protein. MAP1B gene: boxes, coding exons; hatched boxes, non coding exons; shaded box, microtubules binding domain; I-III, sites of mutations. Tau gene: boxes, coding exons; shaded boxes, microtubules binding domain; dotted box, site of mutation. MAP2 gene: boxes, coding exons; shaded boxes, microtubules binding domain. MAP4 protein: P, proline-rich domain; shaded boxes, microtubule binding domain. Arrow indicates the site used for gene trapping (aminoacid 139).

MAPs function: knockout approaches

overexpressed in mutant mice. A second alternative might consider a functional compensation by other yet unknown or not well characterized MAPs, such as the products of *lis1* gene (Sapir et al., 1997; Hirotsune et al., 1998) or Doublecortin (Francis et al., 1999; Gleeson et al., 1999). Finally, differences in genetic background of these animals could be related with discrepancies in phenotype.

Microtubule-associated protein 4

Microtubule-associated protein 4 derives from a single copy gene encoding a 200 kDa protein produced in most tissues (Parysek et al., 1984). Its mRNA level varies between different tissues (West et al., 1991) and it is the predominant MAP in non-neural cells (Chapin and Bulinsky, 1994). A wide variety of transcripts generated by alternative splicing (Chapin et al., 1995) and alternative polyadenylation (Code and Olmsted, 1992) have been described.

MAP 4 locus was mutated using a splice-acceptor gene trap vector (Voss et al., 1998). This trapping strategy considered the use of a vector very similar to that used by the same group in the generation of a MAP1B knock-out mice (Chowdhury et al., 1997). However, in this case the gene trap construct did not contain the viral IRES sequence (a sequence required for ribosome binding). Thus, the resulting product after gene trapping is a fusion protein composed by the 139 amino terminal aminoacids of MAP4 linked to β -galactosidase and neomycin phosphotransferase proteins. β -galactosidase activity was used to analyze the expression pattern of the construct along development. It was expressed ubiquitously as was expected for a fusion protein under the control of MAP4 promoter.

The presence of a 5.4-kb mRNA fusion transcript containing 5' MAP4 and lacZ was verified. Additionally, the absence of lacZ and MAP4 3' sequence fusion transcript was confirmed. However, the analysis of the transcripts showed that the 5.4-kb fusion transcript did not replace the normal 5.5-kb and 6.5-kb normal transcripts, since normal levels of these transcripts were found in homozygous mice. This suggests that primary RNA was processed to both the normal and the predicted chimaeric mRNA. A very likely explanation for these phenomena have been discussed by authors (Voss et al., 1998). They explained that the results obtained are a possible combination of 1.- splicing complex might ignore the SV40 polyadenylation signal, and 2.- a splicing event around the trapping vector, leading to the expression of the wild type MAP4 protein in homozygous mice

Homozygous mutant did not show any phenotypic abnormalities in this case, as was expected because of the presence of normal transcripts.

MAP2

Microtubule-associated protein 2 is a protein that is

expressed mainly in the cell body and dendrites of neurons in the central nervous system (Matus et al., 1981). It is composed by several isoforms derived from a single gene by alternative splicing (Garner and Matus, 1988; Kalcheva et al., 1995)

MAP2 knock out mice have also been generated by Hirokawa's group (Harada et al., 1998) by gene targeting techniques. Homozygous animals have been described as healthy and fertile, with an apparent normal architecture of the nervous system, and with no variations in the Purkinje dendrites. Since the above described results have been presented only as a brief communication (Harada et al., 1998), further analyses should be needed to have a more complete view of the phenotype of those mice.

MAP1B/Tau double knock-out

MAP1B/Tau double knockout mice have been generated by crossing MAP1B and tau mice (Takei et al., 1998). Double homozygous were characterized as having brain abnormalities, including defects of formation of corpus callosum. Besides, in neuron primary cultures of double mutants cells, neurite elongation was severely affected. However, since no many data have been reported, further characterization should be described to understand better these mice.

Conclusions

The conclusions are that mouse genetic procedures involving gene deletion could be used to study the function of MAPs like MAP1B, in which it was clear, from some data, that it is required for neuronal development. However, in the case of other MAPs no major phenotypical differences compared to wild type mice were observed in knock-out mice. Although some functions were already discussed for some of these MAPs using oligonucleotide antisense technology (Caceres and Kosik, 1990; Caceres et al., 1992), these functions could be performed in the mutated mice by other proteins with similar functions.

According to this, a very good alternative should be the generation of knock out mice depleting more than one of these proteins with related functions, like tau and MAP2 or the analysis of the double tau/MAP1B double mutant. This will contribute to elucidate if these complement of functions can be take place.

Additionally, it should be indicated that the recombinant DNA procedures used in some cases may result in the expression of alternative transcripts in which the function of the protein could be in part conserved, although perhaps in a decreased form.

Perspectives

Molecular genetics could be considered as a valid and useful procedure to truly establish the *in vivo* functions of a protein, although it is necessary to be

aware of possible artifacts such as the generation of some kinds of RNA alternative splicing. To avoid this the best strategy to be used must consider the deletion of the exon that contains the functional domains of the protein.

Once the mutated animals have been obtained, extensive histological and immunocytochemical approaches are of great importance for this type of studies.

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