The projection domain of MAP2b regulates microtubule protrusion and process formation in Sf9 cells

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Summary
The expression of microtubule-associated protein 2 (MAP2), developmentally regulated by alternative splicing, coincides with neurite outgrowth. MAP2 proteins contain a microtubule-binding domain (C-terminal) that promotes microtubule assembly and a poorly characterized domain, the projection domain (N-terminal), extending at the surface of microtubules. MAP2b differs from MAP2c by an additional sequence of 1372 amino acids in the projection domain. In this study, we examined the role of the projection domain in the protrusion of microtubules from the cell surface and the subsequent process formation in Sf9 cells. In this system, MAP2b has a lower capacity to induce process formation than MAP2c. To investigate the role of the projection domain in this event, we expressed truncated forms of MAP2b and MAP2c that have partial or complete deletion of their projection domain in Sf9 cells. Our results indicate that process formation is induced by the microtubule-binding domain of these MAP2 proteins and is regulated by their projection domain. Furthermore, the microtubule-binding activity of MAP2b and MAP2c truncated forms as well as the structural properties of the microtubule bundles induced by them do not seem to be the only determinants that control the protrusion of microtubules from the cell surface in Sf9 cells. Rather, our data suggest that microtubule protrusion and process formation are regulated by intramolecular interactions between the projection domain and its microtubule-binding domain in MAP2b.

Key words: MAP2, Microtubules, Process formation, Sf9 cells, Projection domain

Introduction
Neurons undergo important morphological remodeling during their differentiation and adaptive events in adult brain. Differentiating neurons become polar by elaborating two types of neuritic compartments: dendrites and axon. The cytoskeletal proteins act as intrinsic determinants in the acquisition of the shape of a neuron during its differentiation. As such, the microtubule-associated proteins are involved in the elaboration of the neuritic compartments. In particular, the suppression of the microtubule-associated protein 2 (MAP2) expression in primary neuronal culture inhibits the formation of neurites (Caceres et al., 1992). Moreover, the inhibition of MAP2 expression in ECP19 cells, which undergo neuronal differentiation in the presence of retinoic acid, inhibits the development of neurites as well as the withdrawal of these cells from the cell cycle (Dinsmore and Solomon, 1991). In adult neurons, MAP2 is enriched in dendrites (Ludin and Matus, 1993) and seems to exert a stabilizing effect on the dendritic morphology since its suppression or degradation was correlated with dendritic loss or remodeling (Faddis et al., 1997; Sharma et al., 1994). Furthermore, changes of MAP2 phosphorylation were observed during neuronal plasticity (Halpain and Greengard, 1990; Quinlan and Halpain, 1996b).

Diverse MAP2 isoforms generated by alternative splicing indicate that process formation is induced by the microtubule-binding domain of these MAP2 proteins and is regulated by their projection domain. Furthermore, the microtubule-binding activity of MAP2b and MAP2c truncated forms as well as the structural properties of the microtubule bundles induced by them do not seem to be the only determinants that control the protrusion of microtubules from the cell surface in Sf9 cells. Rather, our data suggest that microtubule protrusion and process formation are regulated by intramolecular interactions between the projection domain and its microtubule-binding domain in MAP2b.

Key words: MAP2, Microtubules, Process formation, Sf9 cells, Projection domain
Materials and Methods
Baculoviral recombinants
All MAP2 constructs contain a 6xHis tag at the N-terminus. The engineering of the MAP2-His fusion protein was performed in three steps. First, the full-length MAP2b cDNA cloned in the baculovirus expression vector pVL1392 was digested with BamHI and cloned into the sites BamHI and BgII of the vector pBacPAK His2 (Clontech, Palo Alto, CA) (MAP2b-Bac PAK His2). Second, the non-coding sequence of MAP2b was deleted to engineer the His fusion protein. This was done by adding by PCR a BamHI site in 5′ of the coding sequence of MAP2b. All PCR products were generated by using the Pfu enzyme (Stratagene, La Jolla, CA) and were cloned into the blunt end vector PstBlue (Novagen). The PCR product, cloned in the PstBlue vector, containing the BamHI site, was digested with BamHI and XhoI and cloned into the pBacPAK His2 vector (PCR1-BacPAK His2). Third, the PCR1-Bac PAK His2 was digested with NotI (this site is found in the MAP2b sequence amplified by PCR) and inserted in the MAP2b-pBacPAK His2 vector that was also digested with NotI.

Five MAP2b constructs having a deletion of nucleotides 656-1921 (MAP2b-I, 1921-3320 (MAP2b-II), 3329-4772 (MAP2b-III), 1-4772 (M) and 4772-5881 (Prob) were produced. To delete the nucleotides 656-1921 (MAP2b-1), a Scal site was inserted in 3′ of nucleotide 656 by PCR. A PCR product containing the MAP2b sequence from nucleotides 378-1921 was subcloned into the BamHI and XhoI sites of the Bluescript vector (MAP2b1-1,5471-BS). The nucleotides from 378-1921 of the MAP2b sequence were deleted by digestion of MAP2b1-1,5471-BS with KasI and Scal. Second, the PCR-C1 cloned into the XhoI site was inserted in 3′ of nucleotide 2192 by PCR. A PCR product containing the MAP2b sequence from nucleotides 378-1921-XhoI (PCR-C2) was cloned into the vector PstBlue. The insertion of PCR-C2 into MAP2b-BacPAK His2 vector was done in three steps. First, the nucleotides 1-3121 of MAP2b-His sequence were subcloned into the Bluescript vector (MAP2b1-1,5471-BS). Second, the PCR-C2 cloned into the PSTBlue vector was digested with KasI and XhoI and subcloned into the corresponding sites of MAP2b1-1,5471-BS (C2-MAP2b1-1,5471-BS). Third, the sequence of MAP2b containing the deletion from 1921 to 3329 was cut from the C2-MAP2b1-1,5471-BS by digestion with BamHI and XhoI and re-inserted in the MAP2b-BacPAK His2 vector.

To delete the nucleotides 3329 to 4472 (MAP2b-3), an XhoI site was inserted in 3′ of nucleotide 4472 by PCR. A PCR product containing the MAP2b sequence from nucleotides 3329-4472-XhoI (PCR-C3) was cloned into the blunt end PstBlue vector. The insertion of PCR-C3 into MAP2b-BacPAK His2 vector was done in three steps. First, the nucleotides 3329-5513 of MAP2b-His sequence was subcloned into the PstBlue vector (MAP2b3-3329-5513-Pst) at the SphI and XhoI sites. Second, the PCR-C3 cloned into the PstBlue vector was digested with Smal and XhoI and subcloned into the corresponding sites of MAP2b3-3329-5513-Pst (C3-MAP2b3-3329-5513-Pst). Third, the sequence of MAP2b containing the deletion from 3329 to 4772 was cut from the C3-MAP2b3-3329-5513-Pst by digestion with XhoI and SphI and re-inserted in the MAP2b-BacPAK His2 vector.

A truncated form of MAP2b corresponding to the microtubule-binding domain (Mt) was generated by deleting the nucleotides 1-4772. To do so, a BamHI site was inserted at the 4765 nucleotide by PCR. MAP2b-pBacPAK His2 was digested with BamHI and SphI. The deleted sequence was replaced by the PCR product digested with the same restriction enzymes.

A truncated form of MAP2b corresponding to the projection domain of MAP2b (Prob) was produced by deleting the nucleotides 4772-5881. First, a NotI site was inserted at the 4772 nucleotide of the MAP2b sequence by PCR. MAP2b-pBacPAK His2 was digested with NotI and XhoI. The deleted sequence was replaced by the PCR product digested with the PCR product digested with the
same restriction enzymes. To generate a truncated form of MAP2c corresponding to its projection domain, a NotI site was inserted by directed mutagenesis at the nucleotide 444 of the MAP2c sequence. MAP2c-pBacPAK His2 was digested with NotI and NcoI. The deleted sequence was replaced by the PCR product digested with the same restriction enzymes.

The transfer vector containing the different mutated forms of MAP2b and MAP2c were co-transfected with the Bsu36I-digested BacPAK6 viral DNA onto the Spodoptera frugiperda (Sf9) cells using bacfectin (Clontech, Palo Alto, CA).

**Cell culture**

The Sf9 cells were obtained from the American Type Culture Collection (ATCC # CRL 1711; Rockville, MD). Sf9 cells were grown in Grace’s medium (Gibco BRL, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (Immunocorp, Montreal, Quebec, Canada) as a monolayer at 27°C. For infection, cells were plated on glass coverslips at a density of 1×10^6 cells/60 mm dish. Cells were infected for 24 or 72 hours with viral stock at various multiplicities of infection (m.o.i.).

**Immunofluorescence**

For immunochemistry, the cells were fixed in 4% paraformaldehyde in PBS for 20 minutes. Then the cells were permeabilized with 0.2% Triton X-100 in PBS for 5 minutes. The expression of the truncated forms of MAP2b and MAP2c except MT was revealed by using a monoclonal antibody directed against MAP2 (clone HM2, dilution 1:200) purchased from Sigma (Mississauga, Ontario, Canada). The Mt mutant was revealed by the monoclonal antibody 46.1 directed against the microtubule-binding domain of MAP2 (kindly provided by V. Lee, University of Pennsylvania). To visualize the microtubule reorganization, a monoclonal antibody directed against α-tubulin (Sigma, dilution 1:500) was used. The actin reorganization was visualized with Rhodamin-phalloidin (Molecular Probes, Eugene, OR), at a dilution of 1:200. We used the following secondary antibodies (Jackson ImmunoResearch Laboratories, Bio/Cam, Mississauga, Ontario, Canada): the anti-mouse Fab fragment conjugated to rhodamine (dilution 1:500) and a donkey anti-mouse conjugated to FITC (dilution 1:500). All these antibodies were diluted in PBS plus 5% BSA. Incubation was carried out at room temperature for 1 hour. After

![Fig. 1. Baculoviral constructs of MAP2 expressed in Sf9 cells. (A) The three open boxes represent the three repeat sequences involved in the microtubule-binding activity of MAP2. The highlighted parts of the sequence correspond to the highly conserved regions. The percentage of cells with processes are indicated for each MAP2 construct. For details on the morphological analysis see Materials and Methods. (B) Sf9 cells were infected with the recombinant baculovirus containing the truncated forms of MAP2 at an m.o.i. of 5.0. At 72 hours post-infection, the cells were lysed and 30 μg of cell lysates was separated on SDS-PAGE (7.5%). The proteins were transferred to a nitrocellulose membrane and the expression of the constructs was revealed using the anti-MAP2 antibody HM2, except for the construct corresponding to the microtubule-binding domain (Mt), which was revealed with the antibody 46.1. The molecular mass of the standards are indicated on the left: myosin (209 kDa), β-galactosidase (134 kDa), bovine serum albumin (84 kDa), carbonic anhydrase (40 kDa), soybean trypsin inhibitor (32 kDa) and lysozyme (19 kDa). In lane MAP2b, MAP2b-1 and Prob, the multiple bands are caused by the protein degradation that occasionally occurs during preparation of cell lysates.
three washes in PBS, the coverslips were mounted in moviol and visualized by fluorescence microscopy.

Preparation of microtubules

Microtubules were purified from SF9 cells as previously described, with slight modifications (Vallee and Collins, 1986). Briefly, cells were collected by centrifugation at 250 \( \times \) g for 3 minutes. The cells were then suspended in the PEM buffer (0.1 M Pipes–NaOH, pH 6.6, 1 mM EGTA and 1 mM MgSO\(_4\)), to which a cocktail of protease inhibitors were added prior to use. Preparation of microtubules

Extraction of the cytoskeleton

At 48 hours post-infection, SF9 cells were washed in PBS and then suspended in the extraction buffer (80 mM Pipes pH 6.8, 0.05% IGEPAL, 1 mM MgCl\(_2\), 5 mM EGTA) to which protease inhibitors were added prior to use. Extraction was allowed to proceed for 2 minutes. Cells were then centrifuged at 250 \( \times \) g for 2 minutes. The pellet was resuspended in 80 mM Pipes pH 6.8, 1 mM MgCl\(_2\) and 5 mM EGTA and cells lysed using PBS buffer containing 1% IGEPAL and 0.1% SDS. For drug treatment, 10 \( \mu \)M colchicine was added to the cultures 2 hours before extraction. For cold treatment, cultures were incubated in an ice bath for 30 minutes before the extraction was performed.

Immunoblotting and dot blotting

The expression of the truncated forms of MAP2 in the SF9 cells was confirmed by western blot. To do so, the transfected cells were centrifugated at 250 \( \times \) g for 3 minutes. The pellets were resuspended into PBS containing protease inhibitors (5 \( \mu \)g/ml of antipain, aprotinin and leupeptin, 1 mM EDTA, 100 \( \mu \)g/ml PMSF and 7 mM DFP). An equal volume of sample buffer was added to the cell suspension before it was boiled for 5 minutes. The protein were separated on a 7.5% polyacrylamide gel and transferred on nitrocellulose membrane. The antibodies used to visualized the proteins were HM2 (Sigma) 46.1 (kindly provided by V. Lee, University of Pennsylvania) or an anti-His antibody (Santa Cruz, Santa Cruz, CA). The secondary antibodies were conjugated to HRP (Jackson Immunoresearch Laboratories, Bio/Cam, Mississauga, Ontario, Canada) and revealed by chemiluminescence (Roche, Laval, Québec, Canada). For drug treatment, 30 \( \mu \)g of total protein extract prepared from infected cells were applied to a nitrocellulose membrane using the dot blot Manifold apparatus. The membrane was air dried for 30 minutes and incubated in the primary antibody against MAP2 for 60 minutes at room temperature. The membrane was then washed and incubated in the secondary antibody conjugated to HRP (Jackson Immunoresearch Laboratories) and revealed by chemiluminescence (Roche). To quantify the protein level, the autoradiographic dots were scanned and the digitized data were quantified using the program ImageQuant (Molecular Dynamics).

Co-immunoprecipitation

The cells were washed twice in PBS and lysed in RIPA buffer (50
or the Pro AnalySIS program. Measurements were made in the proximal region of the processes. We performed 50 random measurements per process.

Quantitative morphological analysis

The morphological analysis was performed by two observers. Three sets of experiments were analyzed. The morphological phenotypes observed with the different truncated forms of MAP2 were highly reproducible from one set of experiments to another. To evaluate the number of processes per cell, 150 cells were measured for each truncated form of MAP2 in each set of experiment. To analyze the process length, 50 cells were used for each truncated form of MAP2 in each set of experiments.

Statistical analysis

The distribution of the percentage of cells with one, two or more than two processes was analyzed for each truncated form and full-length MAP2c and MAP2b in three sets of experiments. To analyze the reproducibility of the data from one set of experiments to another, a chi-square test followed by the Fisher’s Exact test was performed. Since the distribution was not statistically different from one experiment to another, the three experiments were combined for the statistical analysis. The differences among truncated and full-length MAP2c and MAP2b in the distribution of the number of processes per cell were analyzed by a chi-square test followed by the Fisher’s Exact test. The length of process was analyzed by one-way ANOVA followed by the Sheffe’s test. For electron microscopy analysis, the statistical significance of the spacing between microtubules was determined using one-way ANOVA followed by Fisher’s PLSD test. Statistical significance was accepted if $P<0.05$.

Results

The projection domain of MAP2b negatively regulates the capacity of the microtubule-binding domain to induce process formation

Our previous work showed that the expression of MAP2b and MAP2c results in the formation of cytoplasmic processes in Sf9 cells. These processes seem to be induced by the protrusion of microtubule bundles from these cells. MAP2b has a significantly lower tendency than MAP2c to induce cell processes, indicating that the MAP2b-induced microtubule bundles are less capable than those of MAP2c of protruding at the cell surface. Since MAP2b and MAP2c differ by their projection domain, the above observations suggest that this domain regulates the MAP2-induced protrusion of microtubule bundles. To examine this possibility, we have produced recombinant baculovirus that contain either the projection domain of MAP2b (Prob), the projection domain of MAP2c (Proc) or the microtubule-binding domain (Mt) that

Electron microscopy

For transmission electron microscopy, Sf9 cells were grown on glass coverslips at a density of 2.0×10^6 cells/60 mm dish. Cells were infected for 48 or 72 hours with viral stock at a multiplicity of infection (m.o.i.) of 5. The cultures were fixed in a solution containing 2% glutaraldehyde and 2 mg/ml tannic acid for 15 minutes, rinsed in a solution of 5% sucrose in 0.1 M cacodylate, postfixed for 10 minutes with 1% osmium tetroxide, dehydrated with increasing concentrations of ethanol, and embedded using EPON resin (Cedarlane Laboratories, Hornby, Ontario, Canada). After curing the resin, cells were sectioned parallel to the long axis of the processes. The spacing between microtubules was measured using either the NIH Image 1.62 program

MAP2 protein level in Sf9 cells

<table>
<thead>
<tr>
<th>Proteins</th>
<th>24 h</th>
<th>72 h</th>
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<tbody>
<tr>
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<td>20</td>
</tr>
<tr>
<td>MAP2b</td>
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<td>20</td>
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<tr>
<td>Mt</td>
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<td>18</td>
</tr>
<tr>
<td>MAP2b-1</td>
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<tr>
<td>MAP2b-3</td>
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MAP2 protein level was measured as described in Materials and Methods.

Table 1. MAP2 protein level in Sf9 cells
is common to MAP2b and MAP2c (Fig. 1A). The expression of these truncated forms was analyzed by immunoblotting in Sf9 cells. Each mutant was found to migrate at its expected molecular weight and was immunoreactive to antibodies directed against MAP2 isoforms (Fig. 1B). We first examined the ability of each of these constructs to induce process formation in Sf9 cells. As expected, the expression of the truncated form that corresponds to the projection domain of MAP2b (Prob) did not induce process formation. Similar results were obtained with the truncated form corresponding to MAP2c projection domain (Proc). By contrast, Mt promoted formation of cell processes.

The pattern of process formation of Mt in Sf9 cells was analyzed according to two parameters: number of cells with processes and number of processes per cell. To evaluate the percentage of cells having processes, at 24 and 72 hours post-infection the cells were fixed and stained with the anti-MAP2 antibody, HM2, which recognizes MAP2b and MAP2c, and the antibody 46.1 directed against an epitope located in Mt (Kosik et al., 1988). The percentage of cells positive to HM2 or 46.1 and having cell processes was determined. 44% of the Mt-expressing cells presented processes compared with 7% and 80% of MAP2b- and MAP2c-expressing cells, respectively (Fig. 1A). This indicates that the projection domain of MAP2b negatively regulates the capacity of the microtubule-binding domain to induce process formation, whereas the projection domain of MAP2c appears to increase its capacity to initiate process formation. For the cells expressing Mt and presenting processes, the number of processes per cell was examined and compared with that of MAP2b and MAP2c. As reported before, MAP2b induced the formation of one process, whereas MAP2c had the tendency to induce the formation of multiple processes (Figs 2, 3). Interestingly, in the cells expressing Mt, the number of processes per cell were significantly different from that of MAP2c- and MAP2b-expressing cells. Indeed, 39% of Mt-expressing cells had multiple processes compared with 57% and 9% of MAP2c- and MAP2b-expressing cells (Fig. 3). However, Mt-expressing cells (37%) had a slightly higher tendency than MAP2c-expressing cells (30%) to develop one process but, importantly, a lower tendency than MAP2b-expressing cells (74%). Thus, the projection domain of MAP2c seems to contribute to the production of multiple processes by Sf9 cells whereas that of MAP2b impairs it. To eliminate the possibility that the difference in the Mt pattern of process formation, compared with that of full-length MAP2b and MAP2c, was attributable to different levels of protein expression, their protein level was analyzed by dot blotting. The antibody, 46.1, which recognized an epitope located in the C-terminus of MAP2, was used to compare the protein level of MAP2b, MAP2c and Mt (Kosik et al., 1988). At 72 hours post-infection, the expression of Mt was similar to that of MAP2c and MAP2b (Table 1). This indicates that the molar expression of Mt was approximately two times higher than that of MAP2c and approximately seven times higher than that of MAP2b at this time of infection. While the molar expression of Mt was higher than that of MAP2c, it induced a lower percentage of cells with processes and a lower number of processes per cell than MAP2c. Thus, the protein level does not influence the pattern of process formation of MAP2 proteins in Sf9 cells as reported before (Leclerc et al., 1996). At 24 hours post-infection, Mt and MAP2c had a similar percentage of cells with processes (~20%) despite the fact that the molar expression of Mt is higher than that of MAP2c (Table 1). By contrast, at 48 hours post-infection, 44% of Mt-expressing cells had processes compared with 80% of cells expressing MAP2c. These results indicate that Mt is less efficient than MAP2c at inducing process outgrowth in parallel to the increase of its protein level.

**Regions of Prob that regulate the process formation by the microtubule-binding domain**

To identify which region(s) of the 1372 a.a. domain were involved in regulating the process formation by Mt, we subdivided this sequence into three portions of equal size corresponding to the region adjacent to the N-terminus common to MAP2c and MAP2b (MAP2b-1), the median region (MAP2b-2) and the region adjacent to the microtubule-binding domain (MAP2b-3) (Fig. 1A). MAP2b-1 is deleted of amino acids 147-569, 147 a.a. is the amino acid located at the splicing site of MAP2c. MAP2b-2 has a deletion from amino acids 1035 to 1519, 1519 corresponds to the splicing site of MAP2c. These truncated forms were expressed in Sf9 cells. Their expression was analyzed by western blotting and each truncated form migrated at the expected apparent molecular weight on SDS-PAGE (Fig. 1B). Their protein level was evaluated by dot blotting (Table 1). At 24 and 72 hours post-infection, MAP2b-3 presented the highest protein level. The protein level of MAP2b-
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1 was similar to that of MAP2b, whereas that of MAP2b-2 was slightly lower than that of MAP2b at 72 hours post-infection.

We performed a quantitative morphological analysis as described above to verify whether these truncated forms of MAP2b induced different patterns of process formation in Sf9 cells. As noted for MAP2b, they had the tendency to induce one process per cell (Figs 2, 3). The percentage of cells with processes induced by MAP2b-1 and MAP2b-2 was not significantly different from that of MAP2b. For the cells that expressed MAP2b-1, MAP2b-2 or MAP2b-3 and presented processes, the number of processes per cell was examined. The number of processes per cell induced by MAP2b-1, MAP2b-2 and MAP2b-3 was reminiscent of that of MAP2b. Indeed, ~74% of the MAP2b-expressing cells had one process compared with 75%, 69% and 71% of the MAP2b-1-, MAP2b-2- and MAP2b-3-expressing cells, respectively. Furthermore, the percentage of MAP2b-1-, MAP2b-2- and MAP2b-3-expressing cells having two or more than two processes was similar to that of MAP2b. Thus, in the present expression system, it seems that the deletion of the amino acids 1035 to 1519 deleted in MAP2b-3 had the most important positive effect on the formation of processes by MAP2b in Sf9 cells. This could be related to the fact that MAP2b-3 presented the highest protein level at 72 hours post-infection. Indeed, there is an increase in the number of cells with processes in parallel to the increase of MAP2 protein expression in Sf9 cells. However, the level of MAP2 proteins does not seem to be the sole determinant involved in the production of processes by these cells. For instance, at 24 hours post-infection, MAP2b-3 protein level was ten times lower than that of MAP2b at 72 hours post-infection, but the percentage of cells presenting processes (~10%) was identical to that of MAP2b at 72 hours post-infection (Table 1). This suggests that MAP2b-3 has a higher capacity than MAP2b, MAP2b-1 and MAP2b-2 to initiate process formation.

Process length induced by MAP2 truncated forms

The process length was analyzed for each truncated form of MAP2b and MAP2c (Fig. 4). We first compare the process length of cells bearing one process. MAP2c-expressing cells presented the longest process (72.7±2.2 µm) followed by MAP2b-3 (62.4±2.5 µm), MAP2b-1 (52.4±1.9 µm), Mt (47.9±2.0 µm), MAP2b-2 (41.9±1.6 µm) and MAP2b (35.8±1.4 µm). These differences in process length were statistically significant except for Mt and MAP2b-1. Thus, a partial deletion in the 1372 a.a. domain had a positive effect on process length, whereas a deletion of Proc had a negative one. However, the process length of cells expressing MAP2b-1, MAP2b-2 and MAP2b-3 seems to correlate with their level of protein expression (Table 1). Indeed, MAP2b-2-expressing cells present the lowest protein expression and the shortest processes, whereas MAP2b-3-expressing cells present the highest protein expression and the longest processes. However, the level of protein expression does not seem to be the only parameter that determines process length since MAP2b, which has an expression level that is lower than that of MAP2b, induces the formation of processes longer than those generated by MAP2b. Moreover, the process length induced by Mt, whose level of

Fig. 5. Micrographs showing the distribution of microtubules and F-actin in Sf9 cells expressing the truncated forms of MAP2. The analysis was done by confocal microscopy. Cells were fixed at 72 hours post-infection and double-stained with an anti-α-tubulin antibody (DM1A, Sigma) and rhodamine-phalloidin (Molecular Probes) to reveal F-actin. Bar, 20 µm.
expression is much higher than that of MAP2b-1 and MAP2b-2, is similar or slightly longer than the process length induced by these MAP2b constructs. From the above observations, it appears that the additional domain of 1372 a.a. in the projection domain of MAP2b not only is involved in determining the number of processes per cell but also exerts an effect on process length.

Finally, we examined the process length of cells bearing multiple processes. Since MAP2c and Mt induced the highest percentage of cells with multiple processes, the process length was analyzed only for these two constructs. The process length of MAP2c- and Mt-expressing cells bearing multiple processes was 31.5±1.4 μm and 29.6±1.4 μm, respectively. These lengths were not statistically different but were significantly shorter than the process length of MAP2c- and Mt-expressing cells that have one process.

Distribution of the microtubules and actin microfilaments in Sf9 cells expressing MAP2c and MAP2b truncated forms

According to our previous work in Sf9 cells, microtubule formation is required for process outgrowth in Sf9 cells. Thus, we examined the distribution of microtubules in cells expressing the full-length and the truncated forms of MAP2b and MAP2c. As shown previously, in MAP2c-expressing cells that have multiple processes, thin microtubule bundles radiate tangentially from the cell surface to form processes (Boucher et al., 1999). In MAP2b-expressing cells that develop one process, a thick bundle of microtubules originating in the cell body extends into the process. In MAP2b-expressing cells that have no processes, microtubules are organized in a thin bundle that forms a ring under the plasma membrane (Fig. 5).

We analyzed the distribution of microtubules in cells expressing the different truncated forms of MAP2b and MAP2c using confocal microscopy (Fig. 5). In cells expressing Mt and having one process, several thin bundles of microtubules were found in the cell body that formed a thick bundle at the hillock region of the process and extended into the process. In cells having multiple processes, the distribution of the microtubules was reminiscent of that found in MAP2c-expressing cells presenting multiple processes. In MAP2b-1, MAP2b-2 and MAP2b-3-expressing cells, a thick bundle of microtubules was found in the cell body that extended into a process at one pole of the cell body as previously described for MAP2b (Leclerc et al., 1996). In cells expressing either of these MAP2b truncated forms that did not have processes, microtubules formed a ring under the plasma membrane as reported for full-length MAP2b (Boucher et al., 1999). From these observations, it appears that the projection domain is not necessary to induce microtubule bundling since the construct Mt promoted microtubule bundling. However, the 1372 a.a. domain in the MAP2b projection domain seems to favor the formation of a thick bundle of microtubules rather than the formation of multiple thin bundles. Furthermore, the packing density of microtubules does not seem to influence their capacity to protrude from cell surface. For instance, Mt can induce the formation of one thick bundle of microtubules (Fig. 5) or of multiple thin bundles. The thick bundles seem to have a capacity to protrude from the cell surface similar to that of the thin ones. Indeed, an equal number of Mt-expressing cells develop multiple processes (37%) and one process (39%).

In cells expressing Prob, there was no formation of microtubules (Fig. 6). Interestingly, Prob expression was concentrated in the cell periphery. In these cells, F-actin formed a ring under the plasma membrane and seemed to co-localize with Prob (Fig. 6). In addition, Proc expression had the tendency to be concentrated in the cell periphery and to co-localize with F-actin (Fig. 6). F-actin nuclear staining was often noted in cells infected with any truncated form of MAP2b and MAP2c as noted in wild-type baculovirus infection (Charlton and Volkman, 1991). Finally, in Sf9 cells expressing either full-length or truncated forms of MAP2b and MAP2c and presenting processes, F-actin was found in the cell body and in the processes (Fig. 5).

Bundling of microtubules in Sf9 cells expressing MAP2c and MAP2b truncated forms

As revealed by light microscopy, MAP2c and Mt have a higher tendency to induce multiple thin microtubule bundles than MAP2b, MAP2b-1, MAP2b-2 and MAP2b-3. This suggests that the 1372 a.a. domain favors the formation of a unique thick bundle of microtubules. To better understand its role in microtubule bundling, we examined the effect of partial or complete deletion of this domain on the spacing between microtubules along the processes in Sf9 cells. This was examined in cells expressing MAP2b-1, MAP2b-2, MAP2b-3 or Mt as well as full-length MAP2b and MAP2c 72 hours after infection. Longitudinal sections were used (Fig. 7). We measured wall to wall spacing between neighboring microtubules at 50 randomly selected locations in the proximal region of the process (Table 2). Because of the very low capacity of MAP2b to induce process formation in Sf9 cells, measurements were made in the cell bodies of the round-infected cells expressing MAP2b. In these cells, microtubules form a ring under the plasma membrane (Fig. 7). To confirm that the spacing between microtubules is not different between the cell body of the round infected cells and the processes, we performed measurements in the cell bodies of the roundcells expressing MAP2b-2 or MAP2b-3. The microtubules are also organized as...
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MAP2b-induced microtubule protrusion

There was no statistically significant difference between the cell bodies and the processes for MAP2b-2 and MAP2b-3 expressing cells. Thus, the measurements in these two compartments were combined for those constructs (Table 2). However, differences were observed in the case of MAP2c-expressing cells where the average spacing between microtubules was 13.05±0.23 nm in the cell bodies compared with 16.40±0.51 nm in the processes. The average spacing between microtubules in MAP2b and MAP2c induced bundles was 53±1.90 and 16.40±0.51 nm, respectively. These values confirm previous findings (Chen et al., 1992; Leclerc et al., 1996). As for MAP2b-1, MAP2b-2 and MAP2b-3, the average spacing between microtubules is 33.40±1.11, 39.70±1.10 and 41.00±1.00 nm, respectively. Thus, the spacing between microtubules is significantly different between MAP2b-1 and MAP2b-2 and between MAP2b-1 and MAP2b-3. These results indicate that equal size deletions in the 1372 a.a. domain of MAP2b give different microtubule spacing. Moreover, MAP2b-1, which deleted sequence was slightly shorter than that of MAP2b-2 and MAP2b-3, gave a narrower microtubule spacing than these constructs. This suggests that the length of the projection domain of MAP2, although important, is not the sole determinant of the spacing between microtubules. As for Mt-induced processes, the microtubules were so tightly packed that it was impossible to make measurements (Fig. 7).

To verify whether the expression level of the protein affects the spacing between microtubules, measurements were made 48 hours post-infection and no differences were observed (data not shown).

The above observations suggest that the structural properties of microtubule bundling are not the sole parameters that regulate microtubule protrusion from Sf9 cells. Indeed, MAP2b-1, which gives rise to a narrower spacing than MAP2b, MAP2b-2 and MAP2b-3, does not induce higher microtubule protrusion than that induced by these proteins. Furthermore, MAP2b-3 induces a microtubule spacing similar to that of MAP2b-2 but has a

Table 2. Spacing between microtubules in the processes induced by truncated and full-length forms of MAP2

<table>
<thead>
<tr>
<th>Construct</th>
<th>MAP2b</th>
<th>MAP2c</th>
<th>MAP2b-1</th>
<th>MAP2b-2</th>
<th>MAP2b-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>18</td>
<td>15</td>
<td>21</td>
<td>27</td>
</tr>
<tr>
<td>Average (nm)</td>
<td>53.00</td>
<td>16.40</td>
<td>33.40</td>
<td>39.70</td>
<td>41.00</td>
</tr>
<tr>
<td>s.e.m.</td>
<td>1.90</td>
<td>0.51</td>
<td>1.11</td>
<td>1.10</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Cells were fixed 72 hours post-infection and processed for electron microscopy as described in Materials and Methods. We performed 50 measurements per process, n being the number of processes. The statistical significance of the spacing between microtubules was determined by using a one-way ANOVA, followed by a Fisher’s PLSD test. Statistical significance was accepted if P<0.05.
Interestingly, MAP2b-2, which has the lowest protein level, and MAP2b-3 was bound to microtubules, respectively. Preparation, whereas 9%, 50% and 40% of MAP2b-1, MAP2b-2, and MAP2b-3, respectively, were found in the microtubule positive effect on the microtubule-binding affinity of MAP2b revealed with an anti-tubulin monoclonal antibody. Transfer on a nitrocellulose membrane. The membrane was visualized with a 46.1 antibody. (B) To verify the level of tubulin, proteins were transferred on a nitrocellulose membrane for visualization. Moreover, Mt was revealed by dot blotting using the tubulin monoclonal antibody.

The microtubule-binding properties of MAP2c and MAP2b truncated forms

The microtubule-binding affinity of MAP2b and MAP2c truncated forms was evaluated by quantifying the amount of each truncated form in a preparation of microtubules by dot-blot. The microtubules were prepared as described previously (Vallee and Collins, 1986). As illustrated in Fig. 8, similar amounts of tubulin were found in the microtubule preparations from Sf9 cells expressing either the truncated forms of MAP2 or full-length MAP2b and MAP2c. The amount of MAP2b, MAP2c, and MAP2b truncated forms in the microtubule preparation is presented as a percentage of the total amount of protein in 4x10^6 Sf9 cells. All the deletion in the 1372 a.a. domain had a positive effect on the microtubule-binding affinity of MAP2b (Fig. 8). 5% of MAP2b was found in the microtubule preparation, whereas 9%, 50%, and 40% of MAP2b-1, MAP2b-2, and MAP2b-3 was bound to microtubules, respectively. Interestingly, MAP2b-2, which has the lowest protein level, presented the highest microtubule-binding affinity. Moreover, the microtubule-binding affinity does not seem to influence the capacity of microtubules to protrude from Sf9 cells since MAP2b-2 induced a lower percentage of cells with microtubule protrusion than MAP2b-3. Moreover, MAP2c and Mt, which showed a significantly higher percentage of cells with microtubule protrusion than MAP2b-2 and MAP2b-3, presented an equal or lower percentage of protein bound to microtubules than these MAP2b truncated forms. Indeed, 34% and 39% of MAP2c and Mt was bound to microtubules compared with 50% and 40% for MAP2b-2 and MAP2b-3.

The amount of polymerized tubulin induced by the expression of MAP2c and MAP2b truncated forms was also evaluated by extracting the cytoskeleton 48 hours following infection, as described in Materials and Methods. Similar amounts of tubulin were found in the extracted cytoskeletal pellets indicating that differences in the binding affinities of MAP2c and MAP2b truncated forms to microtubules did not affect the amount of polymerized tubulin. Thus, the amount of polymerized tubulin is not a limiting factor in microtubule protrusion from the cell surface in Sf9 cells. For instance, MAP2b-3, which has a higher protrusion activity than MAP2b, MAP2b-1, and MAP2b-2, induces a similar amount of polymerized tubulin to these proteins. Furthermore, we examined the effect of cold treatment (30 minutes) on the stability of the polymerized microtubules in the cells expressing MAP2c and MAP2b truncated forms. The amounts of tubulin remaining in the extracted cytoskeletal pellet following cold treatment, compared with control levels are the following: MAP2b, 30%; MAP2c, 30%; MAP2b-1, 35%; MAP2b-2, 28%; MAP2b-3, 25%; and Mt, 26%. These results indicate that cold treatment destabilized the microtubule polymers induced by MAP2c and MAP2b truncated forms as well as those induced by full-length MAP2b and MAP2c, to the same extent. Finally, we examined the effect of treatment with the microtubule depolymerizing agent colchicine (2 hours) on the stability of the microtubule polymers induced by MAP2c and MAP2b truncated forms. The amounts of tubulin remaining in the extracted cytoskeletal pellet following treatment with colchicine, compared with control levels, are the following: MAP2b, 34%; MAP2c, 12%; MAP2b-1, 17%; MAP2b-2, 33%; MAP2b-3, 28%; and Mt, 25%. Thus, treatment with colchicine destabilized the microtubule polymers induced by MAP2c and MAP2b truncated forms as well as those induced by full-length MAP2c and MAP2b, to different extents. After 2 hours of treatment with colchicine, the amount of polymerized tubulin in the extracted cytoskeletal pellet was higher for MAP2b than for MAP2c indicating that MAP2b might confer more resistance to drug treatment. Furthermore, partial deletion in the 1372 a.a. domain of MAP2b seems to render microtubule polymers less resistant to drug treatment. This is particularly evident for MAP2b-1. Therefore, the 1372 a.a. domain might play a role in conferring drug resistance to polymerized microtubules. Moreover, MAP2c seems to induce the formation of microtubule polymers more sensitive to colchicine treatment than Mt. This indicates that the projection domain of MAP2c confers drug sensitivity to polymerized microtubules. Thus, the microtubule resistance to colchicine seems to be induced mainly by Mt, but Proc decreases the resistance whereas the 1372 a.a. domain enhances it.

As reported above for the structural properties of microtubules, one can conclude from the present observations...
that the microtubule-binding affinity and the polymerizing activity of MAP2c and MAP2b truncated forms are not the only factors that control microtubule protrusion and process formation in Sf9 cells.

Interactions between the projection domain and the microtubule-binding domain

To explore the mechanism by which Prob regulates microtubule protrusion and process formation in Sf9 cells, we first verified whether Prob has to be attached to Mt to impair the protrusion of microtubule bundles. Thus, Sf9 cells were co-infected with Prob and Mt recombinant baculovirus. A quantitative morphological analysis was performed as described in the previous section. To identify the cells that co-expressed Prob and Mt, the cells were double stained with a polyclonal anti-MAP2 antibody (kindly provided by Richard Vallee, University of Massachusetts Medical School, Worcester, MA), which recognizes an epitope contained in the projection domain of MAP2b, and the antibody 46.1, which recognizes an epitope found in Mt as described above. For the quantitative morphological analysis, only the cells presenting a high protein level of Prob and Mt were selected (Fig. 9). The percentage of Prob/Mt co-infected cells presenting processes was significantly lower (30%) than that of Mt-infected cells (44%) (Fig. 10). Moreover, the number of processes per cell was significantly lower in Prob/Mt-expressing cells than in Mt-expressing cells (Fig. 10). In Mt-infected cells, 37% and 39% of the cells had one and multiple processes, respectively, compared with 51% and 28% of co-infected cells. From this set of experiments, it appears that Prob does not have to be attached to Mt to impair its capacity to induce process formation in Sf9 cells. Interestingly, co-expression of Proc with Mt did not result in the pattern of process formation of full-length MAP2c but rather gave rise to a pattern resembling that of Mt: 38% of cells co-expressing Proc and Mt had processes compared with 44% of Mt-expressing cells (Fig. 10).

The co-infection experiments demonstrated that the projection domain of MAP2b impairs process formation activity of Mt. This could be done by intramolecular interactions between these two domains, which would result in masking functional domain(s) involved in process formation. Another possibility is that Prob could compete with Mt for a common element that when bound to Mt allows microtubule protrusion and process formation and, when bound to Prob, blocks them. However, previous studies on MAP2 structure have suggested that MAP2b could adopt different conformations, including the formation of hairpin structures (Wille et al., 1992a; Wille et al., 1992b). This indicates that Prob and Mt would interact to give rise to such a conformation. Moreover, these electron microscopic studies demonstrated that MAP2b can form anti-parallel dimers reinforcing the possibility that Prob and Mt can interact. To verify this possibility we performed a co-immunoprecipitation experiment (Fig. 11). We used the anti-MAP2 antibody, AP20, which recognizes an epitope located in the additional sequence of 1372 a.a. in the MAP2b projection domain, or the antibody HM2 to immunoprecipitate Prob. The membrane was revealed with AP20 or HM2 to show that Prob was immunoprecipitated. Then, the same membrane was probed with the antibody, 46.1, which recognizes the microtubule-binding domain of MAP2 and prefers the formation of a single thick bundle of microtubules. Most notably, it regulates the capacity of this microtubule bundle to protrude from cells. This could be through its interaction with Mt.

Discussion

Even though the expression of MAP2b and MAP2c has been correlated with the differentiation of neurites (Ludin and Matus,
The microtubule-binding domain and the proline-rich region of MAP2 proteins are involved in microtubule protrusion and process formation

Our present results show that the expression of the construct Mt, which contains the microtubule-binding domain and the adjacent proline-rich region, promotes microtubule bundling, microtubule protrusion and process formation in Sf9 cells. Indeed, it was previously shown that the proline-rich region and the microtubule-binding domain were sufficient to induce microtubule bundling in non-neuronal cells (Ferralli et al., 1994; Umeyama et al., 1993). The microtubule-binding domain contains the three repeated sequences of 18 a.a. responsible for microtubule assembly that share sequence homology with MAP4 and tau (Lewis et al., 1988). Furthermore, MAP2, MAP4 and tau present sequence homology in the proline-rich region adjacent to the microtubule-binding domain (Chapin and Bulinski, 1991; Ferralli et al., 1994; West et al., 1991). The homology is found in the 25-30 a.a. adjacent to the first repeat. Two residues, Lys_{215} and Arg_{221}, are highly conserved. These amino acids are known to enhance the microtubule-binding activity of the microtubule-binding domain of tau (Goode et al., 1997). The sequence homology indicates that this function is conserved between MAP2, MAP4 and tau. Furthermore, previous studies demonstrated that the proline-rich region adjacent to the microtubule-binding domain in tau interacts with the src-family of non-receptor tyrosine kinases such as fyn through the SH3 domains of these kinases (Lee et al., 1998). Interestingly, the binding of tau to fyn alters cell morphology, which is associated with a reorganization of the microtubules. A similar binding sequence to SH3 domains is found in MAP2 isoforms from amino acids 286 to 294. However, this region does not seem to be involved in the binding of the SH3 domains of Src and Grb2 to MAP2c (Lim and Halpain, 2000). This interaction is rather mediated by the 300-400 a.a. region located in the microtubule-binding domain. Moreover, Src and Grb2 interact preferentially with non-microtubule-associated MAP2c (Lim and Halpain, 2000). Nonetheless, these data indicate that, as noted for tau, the proline-rich region in MAP2 proteins could influence the microtubule bundling and protrusion from cells by its binding to signaling proteins.

Effects of the additional domain of 1372 a.a. in the projection domain of MAP2b on microtubule protrusion and process formation

Here, we show that a partial or complete deletion of the 1372 a.a. domain has a strong positive effect on microtubule protrusion and process formation in Sf9 cells. However, Proc, the projection domain of MAP2c, seems to enhance these events since MAP2c presents the highest percentage of cells with processes. This enhancing effect of Proc on MAP2c ability to induce process formation was also reported in human hepatoma cell line PLC (Ferralli et al., 1994). Previous studies have
suggested that the capacity of MAP2c constructs to support process formation was related to their strength of binding to microtubules (Ferralli et al., 1994). However, in Sf9 cells this correlation does not seem to exist. Indeed, Mt has a slightly higher binding affinity than MAP2c but presents a lower capacity to induce process formation in Sf9 cells. This difference might be explained by the fact that, in previous studies, cortical actin had to be depolymerized by cytochalasin D to induce process formation, whereas in Sf9 cells process formation occurs spontaneously (Ferralli et al., 1994; Leclerc et al., 1996; Leclerc et al., 1993). Therefore, in Sf9 cells microtubule protrusion and process formation might require additional cellular elements that influence MAP2 protein activity. Moreover, Proc seems to decrease microtubule stability as revealed by the lower percentage of microtubules resistant to colchicine treatment in MAP2c-expressing cells compared with Mt-expressing cells. Our results correlate with previous studies showing that MAP2c does not confer colchicine resistance to microtubules (Caceres et al., 1992; Olmsted et al., 1989; Takemura et al., 1995). However, some studies reported that MAP2c can induce drug resistance to microtubules (Ferhat et al., 1996; Takemura et al., 1992). These studies used immunocytochemistry to evaluate the amount of polymerized tubulin whereas, in our study, we used a biochemical approach. Furthermore, a different cellular system was used in these studies. These experimental differences might explain the discrepancy between the results. In neurons, the suppression of MAP2 protein expression blocks the induction of labile or tyrosinated microtubules but does not affect the population of stable or acetylated microtubules resistant to colchicine (Caceres et al., 1992). Moreover, it was shown that, in the presence of MAP2c, microtubules display dynamic instability (Kaech et al., 1996). The high capacity of MAP2c to induce microtubule protrusion and process formation might be due to the induction of labile microtubules that are mainly located in the growth region in neurons.

Our present results indicate that the additional domain of 1372 a.a in the MAP2b projection domain decreases the positive effects of Proc on process formation in Sf9 cells. This could be explained by the fact that it significantly decreases the microtubule-binding affinity of MAP2b. However, MAP2b-2 and MAP2b-3, which have a higher or similar binding affinity to microtubules than MAP2c, present a lower percentage of cells with processes than MAP2c. Thus, the microtubule-binding activity does not seem to be the sole factor involved in process formation by MAP2b in Sf9 cells. Interestingly, the 1372 a.a. domain seems to favor the formation of a unique thick bundle of microtubules resulting in the formation of a unique process, whereas MAP2c induces the formation of multiple thin bundles that give rise to the formation of multiple processes. The induction of multiple thin bundles of microtubules was reported in other non-neuronal cell lines (Ferhat et al., 1996). In cells expressing full-length or MAP2b truncated forms that do not have processes, the thick bundle of microtubules forms a ring under the plasma membrane. Since microtubules have to penetrate the actin network to protrude from cell surface, the thickness of the microtubule bundles might be a limiting factor (Tanaka and Sabry, 1995). However, this does not seem to be the case in Sf9 cells since MAP2b-3, which induces a thick bundle of microtubules, can induce three-times as much process formation as MAP2b. Futhermore, the 1372 a.a. domain seems to enhance the resistance of microtubules to colchicine, resistance that is mainly induced by Mt expression. This could contribute to lower microtubule dynamics and thereby their capacity to protrude from cells. The distinct organization of microtubule bundles by MAP2c and MAP2b might reflect their distinct role in the elaboration of the dendritic arborization. MAP2c, which leads to the formation of thin and labile microtubule bundles that could easily penetrate the actin network of the growth cone, would be involved in the initial stage of dendritic outgrowth (Tanaka and Sabry, 1995). By contrast, MAP2b would be involved in the production of thicker bundles to increase the diameter of dendrites and stabilize microtubules to consolidate the newly formed dendritic branches (Hillman, 1988).

Mechanisms regulating the effects of the additional domain of 1372 a.a in the projection domain of MAP2b on microtubule protrusion

Microtubule protrusion and process formation induced by MAP2 proteins depend on the actin cytoskeleton, as previously shown (Boucher et al., 1999; Edson et al., 1993). Thus, the distinct effect of MAP2b and MAP2c on microtubule protrusion might be related to their distinct effect on actin cytoskeleton. Indeed, MAP2b and MAP2c organize differentially F-actin in vitro. MAP2c is able to induce the formation of an isotropic gel of F-actin, whereas MAP2b induces the formation of F-actin bundles (Cunningham et al., 1997). To bundle or croslink F-actin, a protein has to contain two actin-binding domains or to
have the capacity to dimerize (Puius et al., 1998). In either case, the 1372 a.a. domain, by allowing different conformational states of MAP2b, might modify the structural relation between the actin-binding domains and thereby the organization of F-actin by MAP2b. An actin-binding domain was identified in one of the repeats of the microtubule-binding domain (Correas et al., 1990). Furthermore, as revealed in the present study, Proc and Prob co-localize with F-actin in Sf9 cells indicating that an F-actin-binding site could be located in the region common to MAP2c and MAP2b. This needs to be confirmed by in vitro studies. Finally, since simultaneous changes in microtubule and actin organization are observed in process outgrowth, the 1372 a.a. domain might compromise the molecular link between these cytoskeletal elements and thereby impair process formation (Tanaka and Sabry, 1995). The 1372 a.a. domain also influences the length of the processes since a partial deletion in this domain increases process length in Sf9 cells. This could occur through its effect on actin organization. Indeed, depolymerization of F-actin by cytochalasin increased the rate of process outgrowth in Sf9 cells (Knowles et al., 1994). Thus, the organization of F-actin induced by these MAP2b truncated forms could favor a higher rate of process elongation.

The effect of the domain of 1372 a.a. on process formation can be also mediated through its binding to signaling proteins. This domain contains a binding site for calmodulin, which is known to decrease the actin-binding activity of MAP2b (Kindler et al., 1990; Kotani et al., 1985). This calmodulin-binding domain was deleted in MAP2b-3, the MAP2b truncated form that gave rise to the highest number of cells with processes. Moreover, the domain of 1372 a.a. also contains a high affinity phosphatidylinositol-binding site (Burns and Surridge, 1995; Surridge and Burns, 1994). A recent study demonstrated that MAP2c process outgrowth activity can be inhibited by the co-expression of a subtype of metabotropic glutamate receptors, mGluR1, in Sf9 cells (Huang and Hampson, 2000). mGluR1 stimulates phosphoinositide (PI) hydrolysis (Pickering et al., 1993). Treatment of the cells with a phospholipase C inhibitor reversed the inhibitory effect of mGluR1 suggesting that the PI pathway was involved in the suppression of MAP2c-mediated process formation in Sf9 cells (Huang and Hampson, 2000). It was proposed that binding of PI to MAP2c reduced its binding to tubulin and consequently its microtubule assembly activity (Yamauchi and Purich, 1987). In Sf9 cells, MAP2b promotes microtubule assembly (Leclerc et al., 1996). Therefore, if binding of PI to MAP2b is responsible for its low capacity to induce process formation, it regulates a function of MAP2b other than that of microtubule assembly. PI is also known to decrease the binding of MAP2 to actin (Yamauchi and Purich, 1993). Since MAP2b contains a high affinity PI-binding site, its actin-binding activity might be lower than that of MAP2c.

By co-immunoprecipitation, we showed that intermolecular interactions occur between Prob and Mt in Sf9 cells. Previous studies highlighted the possibility of such interactions by demonstrating that MAP2b can form antiparallel dimers that are nearly in complete overlap (Wille et al., 1992a; Wille et al., 1992b). However, another study reported that autonomous dimerization of MAP2c did not occur in human hepatoma cell line PLC or Hela cells (Burgin et al., 1994). It was reported that the ERM protein, ezrin, forms oligomers and that the formation of oligomers depends on its state of phosphorylation (Gautreau et al., 2000). Similarly, the interaction between MAP2 proteins might depend on their state of phosphorylation, which could vary from one cell type to another. Our present data indicate that, in Sf9 cells, interactions seem to exist between the projection domain and the microtubule-binding domain of MAP2b, suggesting that MAP2 proteins could form antiparallel dimers in Sf9 cells. Moreover, these interactions might be responsible for the negative effect that Prob exerts on process formation by Mt. The inhibitory effect of Prob is most likely mediated by the additional domain of 1372 a.a. since we could not co-immunoprecipitate Proc and Mt in Sf9 cells. However, this does not exclude the possibility that Proc interacts with Mt. Indeed, the effect of Prob on process outgrowth could occur through its interaction with Mt and/or by modulating the interaction of Proc with Mt. Furthermore, our data does not indicate whether the interactions between Prob and Mt are direct or indirect. For example, the interactions between these two domains could be mediated by signaling proteins involved in neurite outgrowth and neuronal plasticity.

The length of the projection domain of MAP2b is not the sole determinant of the spacing between microtubules

One known function of the projection domain is to set the spacing between microtubules. Previous studies suggested that the primary sequence of this domain is one primary determinant of the spacing between microtubules (Chen et al., 1992; Leclerc et al., 1996). Furthermore, the loss of MAP2 and MAP1B in MAP2/MAP1B knock-out mice results in a decreased microtubule spacing in axons and dendrites (Teng et al., 2001). In this study, we show that the deletion of equal portions of the projection domain of MAP2b gives different microtubule spacing. Moreover, MAP2b-1, which was deleted of a slightly shorter sequence than MAP2b-2 and MAP2b-3, induces a narrower spacing between microtubules than these proteins. This indicates that the primary sequence of the projection domain of MAP2, although important, is not the sole determinant of the spacing between microtubules. One possibility is that the deleted portions in the 1372 a.a. domain contain different phosphorylation sites. Previous studies suggested that the phosphorylation of the projection domain of MAP2 causes it to expand due to an increase in intramolecular repulsion. This in turn could cause the distance between adjacent microtubules to increase (Mukhopadhyay and Hoh, 2001). Thus, the phosphorylation state of MAP2b might also be a key determinant of the spacing between microtubules. This has previously been shown for neurofilament proteins whose phosphorylation state regulates the spacing between them by regulating their structural features (Glicksman et al., 1987; Myers et al., 1987). It is also well known that the phosphorylation of tau protein increases its rigidity (Hagedstedt et al., 1989). Thus, the phosphorylation state of MAP2 might regulate its flexibility, determining the spacing between microtubules.

Another possibility is that those deleted portions are involved in different structural configuration regulating the length of the protein. Previous atomic force microscopy studies suggested that the projection domain of MAP2b could arbor different structural conformations due to the existence of repulsive intramolecular forces (Mukhopadhyay and Hoh, 2001). In this study, we demonstrate by co-immunoprecipitation the interaction of the projection domain of MAP2b with its microtubule-binding
domain. Thus, the deletion of different portions in the 1372 a.a. domain, although of equal length, might have affected differently the conformation of the projection domain.

Structural conformation of operative and inoperative MAP2b

Several studies, using different approaches, highlighted the possibility that Prob can exist in different structural configurations. First, this domain was shown to be flexible (Woody et al., 1983). Computer-generated secondary structure predictions suggest that the projection domain of MAP2b has a very important stretch of helices separated by short turns (Kindler et al., 1990). This secondary structure could contribute to its flexibility. Interestingly, tau’s flexibility decreases considerably when it binds to microtubules but not that of MAP2b (Woody et al., 1983). Phosphorylation also diminishes tau’s flexibility (Hagestedt et al., 1989). Such data do not exist for MAP2b. Given that the projection domain of MAP2b contains several sites of phosphorylation, it is possible that its phosphorylation has also some effects on MAP2’s flexibility. Variation of phosphorylation of MAP2b could allow a higher or lower degree of extension, which could result in masking or unmasking sites involved in process formation. Second, several studies point out that the length of the projection domain can vary. Votier and Erickson demonstrated by rotary shadowing that the length of MAP2b varies by folding back (Votier and Erickson, 1982). Moreover, it was shown that the length of the microtubule-binding domain is half of the length of the total protein, despite the fact that it contains only one-sixth of the mass (Wille et al., 1992a). Third, there is evidence from electron microscopy studies that MAP2b can form hairpin structures (Wille et al., 1992a). This indicates that Prob could fold back on the microtubule-binding domain in full-length MAP2b and thus interactions between these two domains could contribute to the low capacity of MAP2b to induce process formation in Sf9 cells. The detection of an interaction between Prob and Mt in cells that co-express these two domains indicates that such a situation might exist in Sf9 cells. However, the fact co-expression of Prob and Mt does not completely reconstitute the low capacity of MAP2b to induce process formation might indicate that in these conditions the interactions between these two domains do not fully match.

The folding back of the projection domain could mask binding sites to the cytoskeletal or signaling proteins located in the microtubule-binding domain and in the proline-rich region. This situation was reported for ezrin, a member of the ERM family of proteins that links the actin cytoskeleton to the membrane. It was shown that ezrin exists in a dormant form in which its actin-binding site located in the C-terminus is masked by the N-terminus that is folded back (Gary and Bretscher, 1995). Moreover, the folding back of the N-terminus of ezrin masks its binding site for EBP50, the ezrin-radixin-moesin-binding phosphoprotein 50 (Reezek and Bretscher, 1998). These conformational changes of ezrin are controlled by intramolecular interactions. Similarly, MAP2b function could be regulated by intramolecular interactions as suggested by our data.

The truncated form, MAP2b-3, which has a deletion from 1035 to 1519, had the highest capacity to induce process formation in Sf9 cells. This region comprises a proline-rich region extending from 1370 to 1650 a.a., which includes the splicing site of MAP2c (1519 a.a.) (Kindler et al., 1990). The computer programs predict that there are several secondary structures, 19 helices separated by very short turns, in the last two-thirds of the projection domain adjacent to the MAP2c splicing site. Therefore, this region could serve as an hinge that would determine the position of projection domain. In the truncated form, MAP2b-3, one part of the hinge (1370 to 1519 a.a.) was removed. This could compromise the folding back of the projection domain on the microtubule-binding domain. Consequently, it would have reduced the possibility of intramolecular interactions between the projection domain and the microtubule-binding domain and thereby increase process formation by this MAP2b truncated forms.

None of the deletions performed in the 1372 a.a. could induce the formation of multiple processes such as MAP2c. In a previous study, we reported that the expression of a truncated form of MAP2b, that has a deletion from amino acid 228 to 1621 induces half as many cells with multiple processes than MAP2c (Leclerc et al., 1993). Therefore, the formation of multiple processes would not depend solely on the unfolding of the projection domain but it appears that the junctional sequence at the splicing site of MAP2c plays a role in this event.

Our data demonstrate that the projection domain of MAP2b regulates the capacity of the microtubule-binding domain to induce microtubule protrusion and process formation. As suggested by our present data, this regulation could happen through intramolecular interactions between the projection domain and the microtubule-binding domain, most probably involving the 1372 a.a. domain present in MAP2b. Our data suggest that the projection domain would allow MAP2b to exist in an operative form that is able to induce microtubule protrusion and process formation and in an inoperative form that would not induce microtubule protrusion and process formation. In the latter form, the projection domain would be folded back on the microtubule-binding domain, reducing the capacity of the microtubule-binding domain to induce process formation. In the inoperative form, MAP2b would stabilize the cytoskeleton to maintain the dendritic shape whereas, in the operative form, it would promote process formation and remodeling of dendrites.

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