Development 116, 1151-1161 (1992)
Printed in Great Britain © The Company of Biologists Limited 1992

Reorganisation of the microtubular cytoskeleton by embryonic microtubule-associated protein 2 (MAP2c)

BRIGITTE WEISSHAAR, THIERRY DOLL and ANDREW MATUS

Friedrich Miescher Institute, PO Box 2543, 4002 Basel, Switzerland

Summary

Microtubule-associated protein 2c (MAP2c) is one of a set of embryonic MAP forms that are expressed during neuronal differentiation in the developing nervous system. We have investigated its mode of action by expressing recombinant protein in non-neuronal cell lines using cell cDNA transfection techniques. At every level of expression, all the MAP2c was bound to cellular microtubules. At low MAP2c levels, the microtubules retained their normal arrangement, radiating from the centrosomal microtubule-organising centre (MTOC) but at higher levels an increasing proportion of microtubules occurred independently of the MTOC. In most cells, radially oriented microtubules still attached to the MTOC co-existed with detached microtubules, suggesting that the primary effect of MAP2 is to increase the probability that tubulin polymerisation will occur independently of the MTOC. The MTOC-independent microtubules formed bundles whose distribution depended on their length in relation to the diameter of the transfected cell. Short bundles were attached to the cell cortex at one end and followed a straight course through the cytoplasm, whereas longer bundles followed a curved path around the periphery of the cell. By comparing these patterns to those produced by two chemical agents that stabilise microtubules, taxol and dimethyl sulphoxide, we conclude that effects of MAP2c arise from two sources. It stabilises microtubules without providing assembly initiation sites and as a result produces relatively few, long microtubule bundles. These bend only when they encounter the restraining influence of the cortical cytoskeleton of the cell, indicating that MAP2c also imparts stiffness to them. By conferring these properties of stability and stiffness to neuronal microtubules MAP2c contributes to supporting the structure of developing neurites.

Key words: microtubules, cytoskeleton, microtubule-associated proteins, differentiation, neurons, transfection.

Introduction

In the cytoplasm of axons and dendrites, microtubules are organised as longitudinal bundles connected by fine filaments (Peters et al., 1976; Heuser and Kirschner, 1980; Hirokawa, 1982) and it has long been presumed that this arrangement is a significant factor in determining the shape of neuronal processes. This idea is supported by the effects of tubulin depolymerising drugs such as colchicine, which cause neuroblastoma cells to retract their processes (Seeds et al., 1970; Yamada et al., 1970) and both axons and dendrites to extend lateral filopodia and lose their cylindrical outline (Bray et al., 1978; Matus et al., 1986). This suggests that, rather than passively following the course of the processes, the microtubule fascicles that they contain make an important contribution to their morphology. This function is particularly important during neuronal development when the transition from motile growth cone to established process is marked by the formation of stabilised bundles of microtubules (Forscher and Smith, 1988; Tanaka and Kirschner, 1991). It has become increasingly clear that several prominent brain microtubule-associated proteins (MAPs) play a significant role in neuronal morphogenesis through their influence on the microtubular cytoskeleton. These proteins promote tubulin polymerisation and stabilise microtubules (Murphy and Borisy, 1975; Weingarten et al., 1975; Sloboda and Rosenbaum, 1979) and occur in alternative 'embryonic' and 'adult' forms whose differential expression during brain development correlates with the maturation of neuronal circuitry (Burgoyne, 1986; Nunez, 1986; Matus, 1988). They are persistently expressed in areas of the brain where growth of neuronal processes continues in the adult or where there is evidence of connective plasticity (Viereck et al., 1989; Schoenfeld et al., 1989; Viereck and Matus, 1990).

Despite extensive evidence of a correlation between the expression of MAPs and neuronal development and plasticity, little is known of the molecular mechanisms involved in their function. One aspect that has excited considerable debate is the nature of their role in the fasciculation of microtubules in neuronal processes. Electron microscopy has shown that MAPs decorate tubulin polymers with fine filamentous side arms corresponding to a so-called projection domain of the MAP molecules (Vallee and Borisy, 1977; Kim et al., 1979; Brown and Berlin, 1985; Friden et al., 1988). In appearance these filaments resemble those that

connect microtubules in axons and dendrites and immunoelectron microscopy has indicated that several of the MAPs are associated with these cross-linking filaments in neuronal processes (Hirokawa et al., 1988a,b; Sato-Yoshitake et al., 1989). These observations have been interpreted in two very different ways. According to the first, the projection domains of the MAPs act as mechanical struts whose function is to maintain regular spacing between the microtubules in neuronal processes. This interpretation is based in part on the observation that polymers of pure tubulin or microtubules from which the filamentous MAP projection domains have been removed by proteolysis pack together closely when pelleted whereas microtubules bearing intact MAPs are separated by a regular space spanned by the filamentous MAP side arms (Kim et al., 1979; Brown and Berlin, 1985; Friden et al., 1988; Hirokawa et al., 1988b). The other major argument for the MAPs having a passive spacer function is that MAP-containing microtubules do not spontaneously form bundles when reassembled in vitro and in concentrated suspensions their physical properties, such as viscosity, do not differ from those of pure tubulin polymers or microtubules from which the MAP side arms have been digested. This suggests that, if there is any MAP-mediated interaction between microtubules, it must be very weak (Sato et al., 1988; Friden et al., 1988).

The other interpretation, that the MAPs actively cross-link neuronal microtubules, has been inferred from recent experiments in which it was shown that, when recombinant MAP2 or tau are expressed in non-neuronal cells by transfection, they induce the formation of microtubule bundles (Lewis et al., 1989; Kanai et al., 1989). However, when microtubules in non-neuronal cells are stabilized by chemical agents, they also form bundles connected by fine filaments, even though such cells do not contain MAP2 or tau (Sandoval et al., 1977; Schiff and Horwitz, 1980). Because of this, it has been argued that microtubule bundling by MAP2 and tau results from their stabilization of normally dynamically unstable cellular microtubules and that the cross-bridges are formed by other molecules that exist in all cells (Chapin et al., 1991).

These issues raise important questions about MAP function in neuronal processes. Firstly, what is the nature of MAP-containing microtubule bundles? Are they regular arrays whose structure is determined by specific ligand-like interactions between MAP molecules, as proposed by the active cross-linking hypothesis? Or are they less ordered structures produced by weaker, non-specific interactions between stabilised microtubules and their accessory components, as is implied by physical measurements (Friden et al., 1988; Sato et al., 1988)? Secondly, what is the significance of the bundling of microtubules seen in MAP transfected non-neuronal cells? Does it represent a primary event that determines the arrangement of microtubules in neuronal processes, or is it secondary to some prior effects of the MAPs acting on individual microtubules, such as stabilisation? We have approached these questions by characterising in detail the rearrangement of microtubules in non-neuronal cells induced by their transfection with MAP2. We were particularly interested in the low molecular mass form of MAP2, MAP2c, whose expression in both the embryonic and adult brain correlates with active growth of neuronal processes (Tucker et al., 1988b; Garner and Matus, 1988; Viereck et al., 1988, 1989). Compared to the adult high molecular mass forms of MAP2, MAP2c lacks more than 1300 amino acids from a central domain (Papandrikopoulou et al., 1989; Doll et al., 1990; Kindler et al., 1991) and whereas the high molecular mass forms of MAP2 are selectively associated with dendrites (Bernhardt and Matus, 1984; De Camilli et al., 1984; Burgoyne and Cumming, 1984) MAP2c is also present in axons (Tucker et al., 1988a,b). For these reasons, it was of interest to determine whether it would induce bundling of microtubules as had previously been shown for high molecular mass MAP2 (Lewis et al., 1989).

To distinguish specific effects of MAP2 from those of stabilisation, we compared it to two chemical stabilisers of microtubules, dimethyl sulphoxide and the plant alkaloid taxol. We find that MAP2 induces a characteristic phenotype that results from the formation of long, stiff microtubule bundles whose arrangement within the cell appears to be determined by their interaction with the cortical cytoskeleton.

Materials and methods

Cell culture and transfection

The full-length MAP2c coding sequence (Doll et al., 1990) was assembled in the Bluescript vector (Stratagene, Heidelberg) then excised by digestion with *Eco*RI and ligated into the eukaryotic expression vector pECE (Pharmacia, Dübendorf). The full coding sequence of high molecular mass adult MAP2 was similarly assembled in Bluescript from separate cDNA clones isolated from a randomly primed rat brain cDNA library (Garner et al., 1988a) and subcloned into the pECE vector. Cell lines were cultured under standard conditions and transfected using the calcium phosphate method (Chen and Okayama, 1987). Taxol treatment was performed by adding taxol as a 5 mM stock solution in dimethyl sulphoxide to final concentrations of between 0.1 and 40 mM. After incubation for various lengths of time, the cells were washed twice in PBS (0.05 M sodium phosphate; 0.15 M sodium chloride) and fixed.

Fixation and staining

To ensure reliable localisation of MAP2 and the cytoplasmic distribution of microtubules, cells were fixed by methods involving rapid freezing as the initial step. In its most rigorous form, this consisted of plunging coverslips with the growing cells into a 3:1 vol/vol mixture of liquid propane and ethane maintained at liquid nitrogen temperature followed by freeze-substitution fixation, performed by transferring the coverslips to 2% glutaraldehyde in methanol at −70°C and allowing them to warm up to −20°C (Bridgman et al., 1990). Because of the rapid freezing and fixation in the frozen state, this method prevents extraction of soluble MAP2, which was important in assessing the correlation between cellular MAP2 levels and microtubule rearrangement. Less rigorous forms of fixation proved equally capable of consistently localising microtubule-bound MAP2 and preserving the same features of microtubule organisation and, for routine observation, cells were frozen and fixed for 3 minutes in methanol cooled to −70°C in a methanol-dry ice bath. For subsequent staining, the cells were incubated in PBS containing 2% BSA for 20 minutes to block non-specific binding of antibodies and then incubated with monoclonal antibody that recognizes all MAP2 forms (anti-MAP2 clone C, Sigma Immunochemicals, Deisenhofen, Ger-

many). The second antibody was rhodamine-conjugated goat anti-mouse IgG (Jackson Labs., Milan Analytica, La Roche). For double-label immunofluorescence, cells were labeled with a rabbit polyclonal anti-tubulin antibody (a gift from Dr Chloe Bulinski, Columbia Medical School, New York) and monoclonal C antibody. Second antibody for the polyclonal rabbit antibody was fluorescein-conjugated goat anti-rabbit (Jackson Labs, Milan Analytica, La Roche). The measurements of relative intensity of fluorescence measurements were made using Image1/AT software (Universal Imaging, West Chester, Pennsylvania). Each cell was outlined by cursor and the fluorescent emission was integrated over the entire area.

For whole-mount electron microscopy, cells were grown on carbon/formvar-coated gold coverslips. Transfected cells were identified by immunofluorescence staining with anti-MAP2 and their position determined in relation to orientation marks built into the finder grids. The cells were fixed and prepared for viewing by negative staining with 1% uranyl formate as described by Small (1988).

Results

The embryonic low molecular mass MAP2 form (MAP2c) used in these experiments had identical effects to those previously reported for the adult high molecular mass form (Lewis et al., 1989). When transfections were performed in parallel using cDNAs encoding both forms, there was no detectable difference in their effects. In both cases, all the expressed protein was bound to microtubules and their influence on the arrangement of microtubules (described in detail below for MAP2c) was indistinguishable. All the experiments were repeated using various non-neuronal cell lines, including the rodent lines 3T3, L-6 and RAT-1, and the human lines, HeLa, 293 and PLC, all of which gave essentially the same results. Results are presented for PLC cells, which have the advantage of being large so that individual microtubules are well resolved by immunofluorescence microscopy. Fig. 1 shows a typical experiment using PLC cells. The transfected cells were recognisable by their positive staining with anti-MAP2 (Fig. 1A), whereas untransfected cells were visible only in the anti-tubulin-stained image (Fig. 1B). In all transfected cells, the expressed MAP2 was completely bound to tubulin polymers (Fig. 2) and this was the case whether or not the microtubules were bundled.

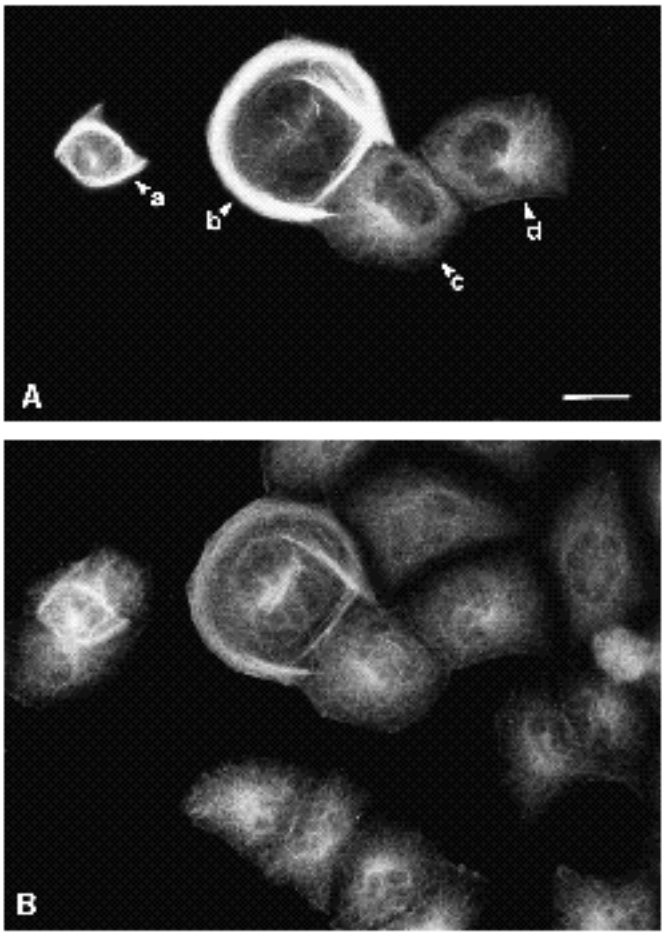

Fig. 1. Microtubule reorientation and bundling in MAP2c transfected PLC cells. A transfected culture was double stained for MAP2 (A*)* and tubulin (B*)*. Cells expressing high MAP2 levels (labeled a, b) show bundled microtubules while in cells with low MAP2 levels (labeled c, d) microtubule distribution is the same as in surrounding untransfected cells. Note the wide variation in cell size of the PLC line. Scale bar, 20 μm.

Changes in microtubule distribution in MAP2-transfected cells

All transfected cultures contained some cells in which the expressed MAP2 had not affected the distribution of microtubules, which remained in their typical organisation radiating from a microtubule-organising centre (MTOC) located at the centrosome (Fig. 1A, cells c and d). In other cells, the expression of MAP2 had induced bundling of the microtubules to various degrees (Fig. 1A, cells a and b). Cells where the microtubules were bundled stained noticeably brighter for MAP2 than those in which microtubules were not bundled (compare cells a and b in Fig. 1A with cells c and d). This suggested that the degree of bundling might depend upon the level of MAP2 expression and we therefore compared the average levels of anti-MAP2 immunofluorescence measured by microphotometry in 50 cells showing a high degree of bundling and 50 cells showing no bundling. These measurements were repeated in three separate transfection experiments. To avoid the extraction of MAP2 during the processing of the cells for immunohistochemistry, these measurements were made on cells that had been rapidly frozen in propane/ethane at liquid nitrogen temperature and then fixed with glutaraldehyde by freeze substitution (see Materials and methods). Although it is not possible to derive absolute values by this method, the results support a correlation between the level of MAP2 expressed in the cell and the degree of microtubule bundling (Fig. 3).

Formation of marginal band-like arrays

The expression of high levels of MAP2 was frequently associated with the appearance of rings of microtubules running around the periphery of the cell (Figs 1A, 4A). This was in turn associated with a change in cell shape and cytoplasmic organization, in which the outline of the cell became a rotund reflection of the bundled microtubules and

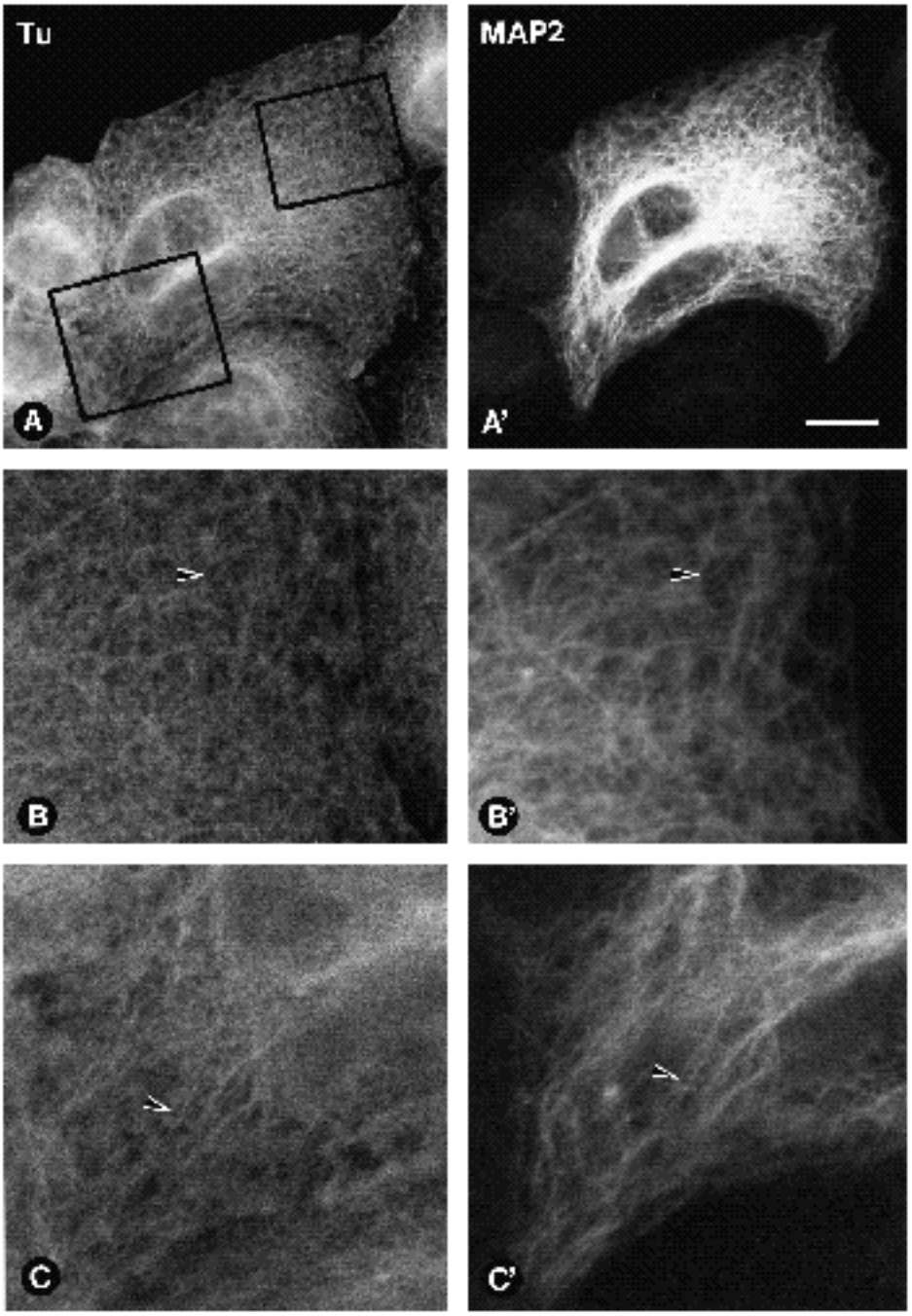

Fig. 2. Co-localization of MAP2 and polymerized tubulin in transfected cells. MAP2 transfected PLC cell culture double-stained for tubulin (A-C) and MAP2 (A′-C′). Boxes in A outline the fields shown at higher magnification in B and C. Arrowheads in B,B′ and C,C′ indicate individual microtubules stained with both anti-tubulin and anti-MAP2. Scale bar, 18 μm.

the particulate contents of the cells were excluded from the periphery (Fig. 4B). In most cells showing this phenotype, the peripheral bundles of microtubules co-existed with microtubules that still originated from the centrosomal microtubule-organising centre (MTOC) and ran radially through the cytoplasm (Figs 4A, 5A). These MTOC-attached, radially oriented microtubules did not form bundles (Figs 5, 6), even though they were decorated with MAP2 along their length (Fig. 5B,C), suggesting that detachment from the MTOC is the primary event to which both bundling and reorientation are secondary. In cells viewed at low magnification, the peripheral bundled microtubules appeared to stain more strongly for MAP2 than those originating from the MTOC (Fig. 1A), suggesting that microtubule re-orientation might depend on binding of a critical amount of MAP2 to each individual microtubule. However, at higher magnification individual neighbouring microtubules could be resolved in both orientations, i.e. either emanating from the MTOC or crossing the cytoplasm independent of the MTOC, and both were stained at similar intensity by anti-MAP2 (arrowheads at points 1 and 2 in Fig. 5B).

Although most of the reoriented microtubules at the cell periphery were part of a bundle, in some cells single microtubules running circumferentially at the cell periphery could be traced over relatively long distances (Fig. 5C, the area

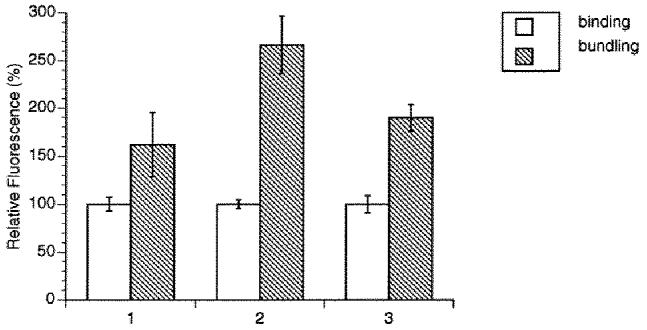

Fig. 3. Microtubule bundling is a function of MAP2 concentration. Anti-MAP2 immunofluorescence was measured (see Materials and methods) in 50 cells with bundled microtubules and 50 cells where microtubules were not bundled in each of three separately transfected cultures (numbered 1-3 beneath). The histogram shows the mean relative intensity ± s. e. m. for each experiment.

marked by an asterisk, Fig. 6A, arrowhead at the left). However, even in transfected cells with relatively few microtubules at the periphery (such as that shown in Fig. 5C) the high density of microtubules made it impossible to trace reoriented microtubules as single filament along their entire length. Thus, it remains possible that the peripheral reorientation shown by single MAP2-containing microtubules depends upon an association with a microtubule bundle at some point along their trajectory. Electron microscopy confirmed the arrangement of microtubules in transfected cells seen by immunohistochemistry (Fig. 6). At the periphery of transfected cells, microtubules were present running both circumferentially and radially (Fig. 6A, arrowheads at the right) and both in tight bundles and as single filaments. Neighbouring microtubule bundles were often fused along part of their length but separate elsewhere, and similarly microtubules that were part of a bundle frequently branched off as individual filaments (Fig. 6B).

Association of peripheral microtubules with the cell surface

The peripheral microtubule bundles in MAP2-transfected cells commonly followed a curved path under the plasma membrane. The most obvious explanation for this arrangement is that these microtubules are attached to the cell surface, either to some component of the cell membrane or of the cortical cytoskeleton. However, another important factor in determining the distribution of microtubule bundles inside MAP2-transfected cells was their length in relation to the cell diameter. We consistently found that where microtubules were short in relation to the cell diameter, their curvature was less extreme. This is illustrated in Fig. 7 where each panel shows a cell, labelled '1', that contains longer microtubule bundles, which are strongly curved, and another cell, labelled '2', containing shorter microtubule bundles that are less curved.

Transfected cultures also contained cells with short MAP2-induced bundles that were not curved around the cell periphery but were straight and ran across the interior of the cell (Fig. 8). The cell shown in Fig. 8A is particularly interesting in that it shows both features, containing long microtubules, which run circumferentially around the periphery, and short straight microtubules running across the cytoplasm. In order to be certain that these short microtubule bundles were situated in the cytoplasm and were not attached to the upper or lower surface of the cell, we examined optical sections through the depth of cells with short microtubules using confocal microscopy (Fig. 9). In all of these cells, viewed by both conventional and confocal microscopy, it was evident that these short microtubule bundles were attached at one end to the interior surface of the cell, from which they radiated out into the cell cytoplasm with no obvious preferred orientation (Figs 8, 9). Such focal attachments might also exist for longer microtubule bundles but they could not be detected because they are against the cell membrane for their entire length.

The effects of microtubule stabilising agents

To determine to what extent these effects of MAP2 arise from its capacity to stabilise microtubules, we examined cells treated with two microtubule-stabilising agents, taxol and dimethyl sulphoxide (DMSO). Both of these agents induce bundling of microtubules and cause changes in their distribution within the cell (Fig. 10). The effects of these agents differed significantly from one another and from those produced by MAP2. As previously described (Schiff and Horwitz, 1980; De Brabander et al., 1981) taxol produced bundles that were much shorter and more numerous than those produced by MAP2 (Fig. 10 A,B). In addition, taxol-induced bundles were always straight and they showed no preferred orientation within the cytoplasm or in

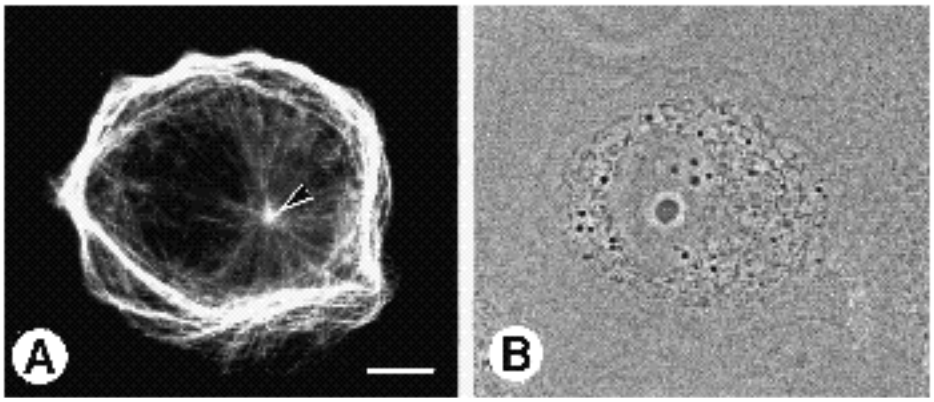

Fig. 4. Cell shape and MAP2-induced microtubule bundles. (A) MAP2-decorated microtubules run as long strands around the cell periphery and define a zone that excludes other cytoplasmic components (shown in the phase-constrast image B). The bundled microtubules at the periphery co-exist with microtubules orginating from the centrosomal microtubule organising centre (MTOC; arrowhead). Scale bar, 20 μm.

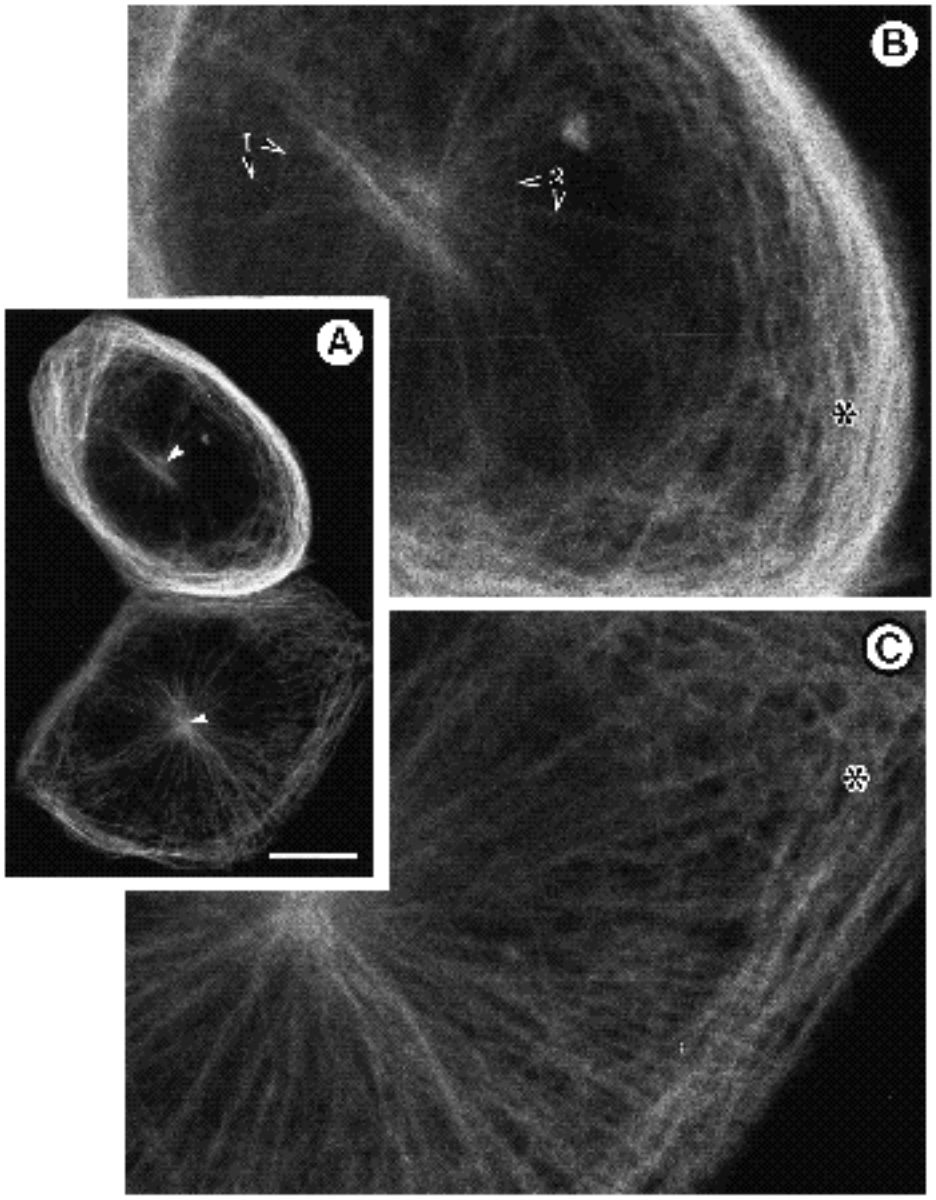

Fig. 5. Two transfected cells expressing different levels of MAP2 (A) have both MTOC-dependent (arrowheads) and MTOC-independent microtubules. Both types of microtubules stain with anti-MAP2 at a similar intensity (arrowheads 1 and 2 in B). Peripheral curved microtubules exist both singly (region of asterisk in B) and as narrow separate bundles (region of asterisk in C). Scale bars: A, 10 μm; B, C, 30 μm.

relation to the cell surface. DMSO, which also stabilises tubulin polymers in vitro (Himes et al., 1976; Algaier and Himes, 1988), also induced microtubule bundles (Fig. 10C). Like those produced by MAP2 these were long and thin, but unlike the MAP2-induced bundles, the DMSO bundles were not attached to the cell membrane but were situated close to the nucleus where they maintained an association with the MTOC at one point (arrowheads in Fig. 10C) while the rest of each bundle followed a curved path in the cytoplasm.

Discussion

These transfection experiments demonstrate three characteristic changes in the microtubular cytoskeleton that occur progressively with increasing cellular concentration of MAP2c: (i) microtubules are formed independently of the microtubule-organising centre, (ii) they become bundled and (iii) their orientation and position in the cell changes from a radial to a circumferential pattern. These same alterations were produced by both the adult high molecular mass form of MAP2 and the embryonic low molecular mass form, MAP2c, without any distinguishable difference between them. This suggests that the function of the large central domain of the high molecular mass MAP2 form, which it bears in addition to the sequences present in the low molecular mass form (Papandrikopoulou et al., 1989), is specific to its function in neuronal cells. The most obvious candidate for such a function is in determining the specific association of high molecular mass MAP2 and its mRNA with dendrites rather than axons (Garner et al., 1988b).

Formation of MTOC-independent microtubules by MAP2

In mammalian cells grown in culture microtubules charac-

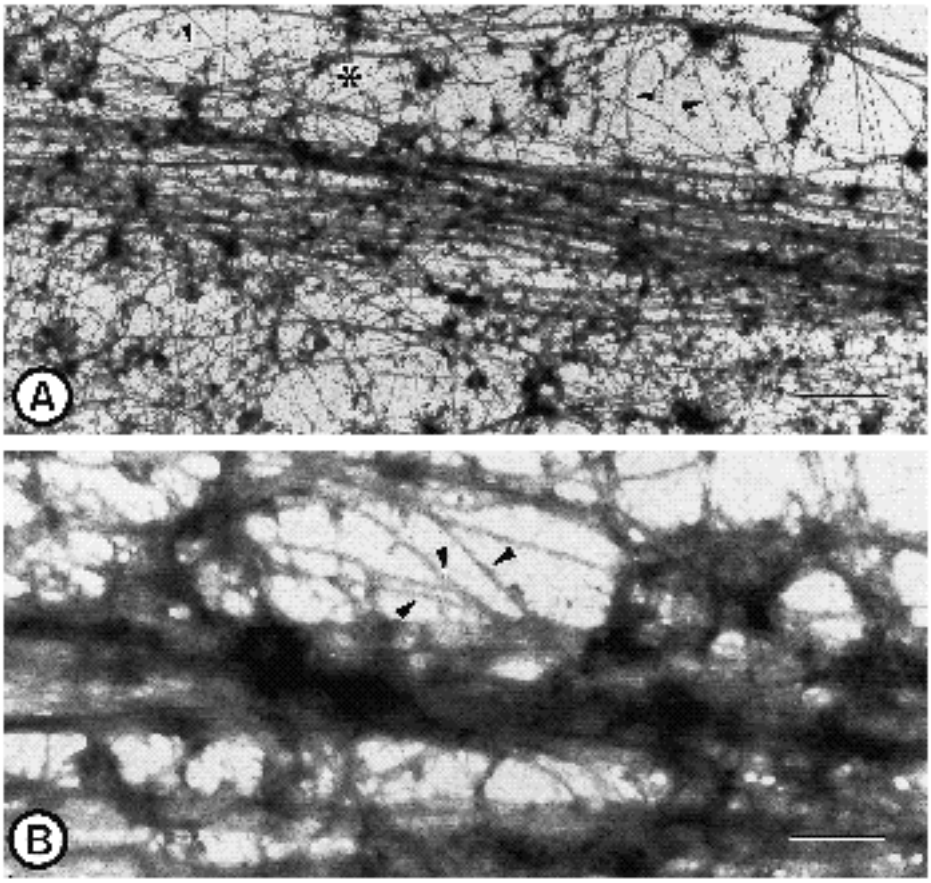

Fig. 6. Whole-mount electron microscopy of MAP2-transfected cells, negatively stained with uranyl formate. Arrowheads in A point to single microtubules in both radial (right) and circumferential (left) orientations. Arrowheads in B indicate single microtubules emerging from bundles. Scale bars: A, 0.17 μm; B, 0.38 μm.

teristically arise from a centrally placed microtubule-organising centre located at the centrosome (see Bornens, 1991 for a recent review). However, centrosome-independent microtubules have been described in the epithelial cell line MDCK (Bre et al., 1987). Both MAP2c and taxol can break the dominance of the centrosome over microtubule organization, but beyond this fundamental similarity their influence differs significantly. The effects of taxol are characterized by the appearance of many short microtubule bundles scattered throughout the cytoplasm (Schiff and Horwitz, 1980; De Brabander et al., 1981) and Fig. 10A. At the same time, all the pre-existing centrosomal microtubules disappear, suggesting that the effects of taxol do not derive from its stabilising effect on existing microtubules but from its stimulatory effect on the initiation of assembly (De Brabander et al., 1981). This interpretation is supported by the effects of taxol in vitro, where it is a powerful initiator of tubulin polymerization (Kumar, 1981; Hamel et al., 1981; Carlier and Pantaloni, 1983). Like taxol, MAP2 lowers the critical concentration for tubulin polymerization (Sloboda et al., 1975; Murphy and Borisy, 1975) but its effect on the initiation of polymerization is weaker than that of taxol (Kumar, 1981; Hamel et al., 1981). This is probably the reason that cells expressing lower concen-

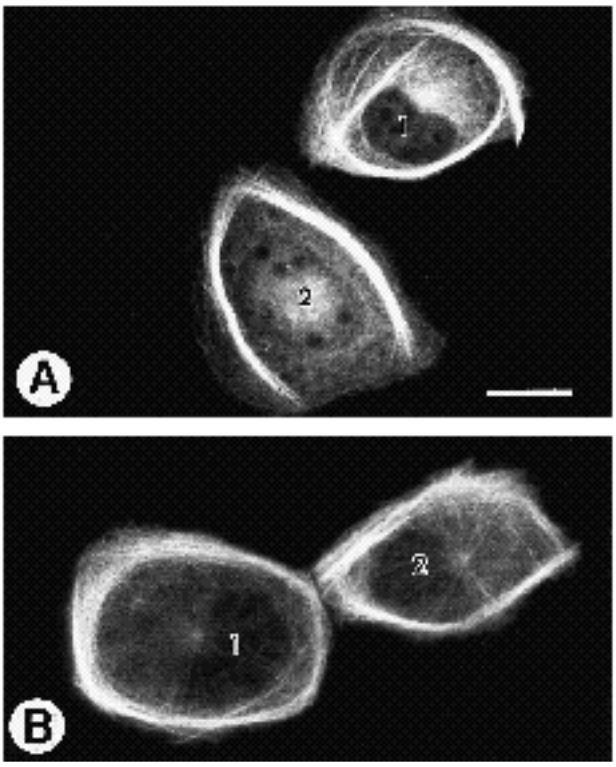

Fig. 7. The degree of curvature of MAP-induced microtubule bundles depends on their length in relation to cell diameter. Each panel shows transfected cells stained with antibodies against MAP2 in the cells labelled '1' have long microtubule bundles that bend acutely around the cell perimeter whereas the cells labelled '2' have microtubule bundles that are shorter in relation to the cell diameter that bend less. Scale bar, 15 μm.

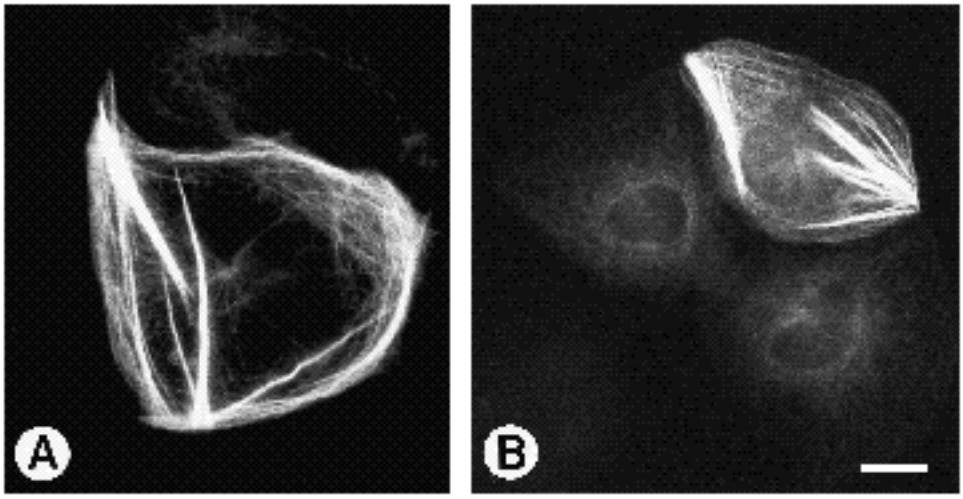

Fig. 8. Short MAP-induced microtubule bundles follow a straight path through the cytoplasm. Note particularly in A that short bundles are straight whereas long bundes are curved against the cell periphery. These short microtubule bundles are all attached at one end to a point on the inner surface of the cell. Scale bar, 15 μm.

trations of MAP2c still contain microtubules that are attached to the MTOC despite being decorated with MAP2c along their length. It has also been reported that tau, a microtubule-associated protein with a tubulin-binding domain similar to MAP2 (Lewis et al., 1988), also influences the initiation of centrosomal microtubules (Bre and Karsenti, 1990).

The difference between MAP2 and taxol in their potency as initiators of microtubule assembly can also explain the marked difference in the number of microtubule bundles that they produce. Taxol, which is very potent, produces large numbers of short bundles because it initiates polymerization at many sites in the cytoplasm. Both MAP2c and DMSO, which are relatively weaker, produce longer bundles because there are fewer initiation sites available onto which the cellular tubulin can polymerize. This has significant consequences for the arrangement of microtubules in the cell because, as our results indicate, one of the important factors determining the ultimate conformation and location of the MTOC-independent microtubule bundles produced by MAP2c is their length.

Relocation and reorientation of microtubules by MAP2

Most MAP2c-transfected cells contain both bundled microtubules that are independent of the MTOC and, in addition, single microtubules arising from the MTOC. With increasing levels of MAP2 expression an increasing proportion of the cellular microtubules becomes independent of the MTOC suggesting that the effect of MAP2c is to increase the probability that microtubule assembly will be initiated elsewhere in the cell. Our observations of short microtubules show that this occurs at sites located on the inner surface of the plasma membrane. A similar phenomenon, of microtubules attaching to the plasma membrane after the loss of centrosomal MTOCs, has been observed in *Drosophila* development and in this case the initiating sites were hemidesmosomes (Mogensen et al., 1989). MAP2 has been shown to bind to actin filaments in vitro (Griffith and Pollard, 1982; Sattilaro, 1986) so another possibility is that MAP2 might mediate an interaction between microtubules and actin filaments of the cortical cytoskeleton. However, the microtubule bundles in transfected cells are decorated with MAP2c along their length so it is not immediately

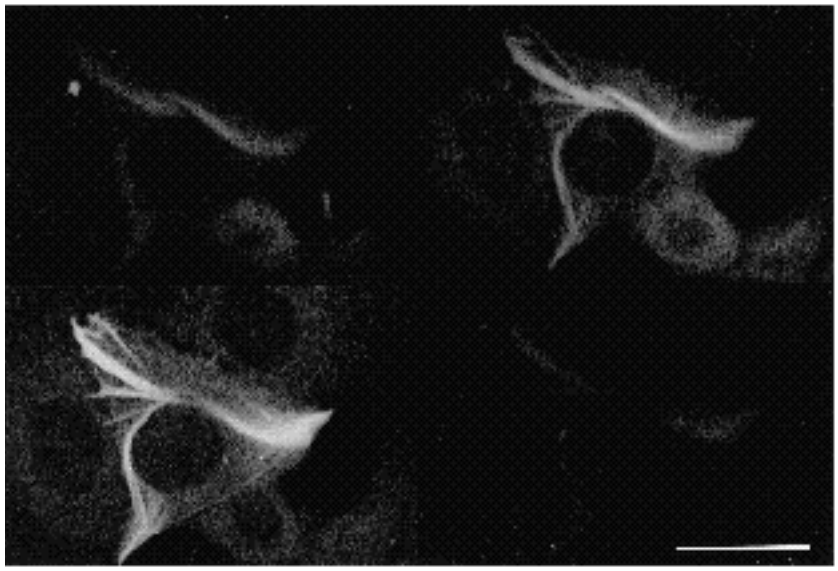

Fig. 9. Optical sections taken by confocal microscopy of anti-MAP2 stained microtubule bundles in MAP2c-transfected cells. The depth of section progresses from the bottom of the cell (top left image) to the top (image at bottom right) in steps of 2.3 μm. Scale bar, 30 μm.

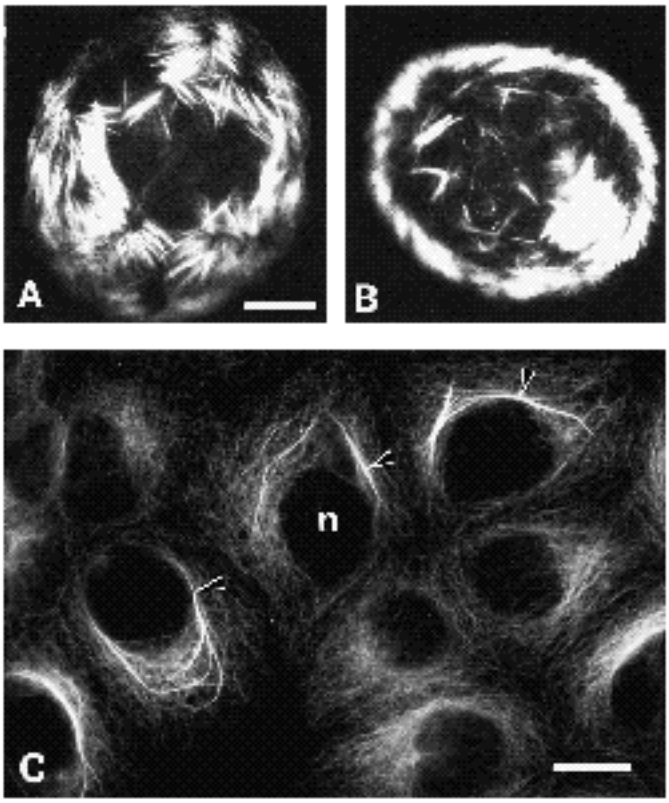

Fig. 10. The effects of microtubule stabilising agents on cellular microtubules of PLC cells. In A and B cells were treated with taxol at 0.1 μg/ml and 5 μg/ml respectively. In C cells were treated with 128 μM DMSO. Scale bars; A and B, 10 μm; C, 20 μm.

obvious how MAP2c could attach short bundles only at one end.

Microtubule bundles that are shorter than the cell diameter, whether they are produced by MAP2c or taxol, are straight, and they show no preferred orientation in the cytoplasm. However, most microtubule bundles produced by MAP2c are greater than the cell diameter, and in this case they follow a curved path around the cell perimeter. The explanation that we favour for this phenomenon is that both the curvature and the peripheral location of MAP2c-containing microtubules are the consequence of spatial constraints imposed on them by the cortical cytoskeleton. The mechanism that we envisage is as follows. Untransfected non-neuronal cells contain dynamically unstable microtubules (Schulze and Kirschner, 1986; Cassimeris et al., 1988) whose polymerization is seeded from the juxtanuclear MTOC and this determines their radial orientation in the cytoplasm. This is also the situation in transfected cells expressing levels of MAP2c too low to induce the formation of microtubules independently of the centrosomal MTOC. At higher levels of MAP2c expression, tubulin polymerisation is initiated independently of the MTOC and the resulting microtubules form bundles that are anchored to the cell surface at one end. Where these bundles are shorter than the cell diameter they remain straight but as they grow longer and encounter the cell envelope they are increasingly subjected to compressive forces that cause them to bend and adopt the most spatially economical configuration, around the edge of the cell. The proportion of MTOC-independent microtubules in a cell depends on the level of MAP2c expressed, and where this is very high long microtubule bundles are produced that form rings around the cell periphery. These effects could in principle operate as well on long single microtubules as they do on microtubule bundles. However, the density of microtubules in the periphery of MAP2c-transfected cells suggests that all of them are influenced by association with bundles at some point along their length, and it is therefore impossible to determine whether the effects that we observe can occur for completely separate individual microtubules.

This scheme implies that the MAP2c-induced microtubule bundles possess a certain mechanical stiffness and this is supported by several of our results. Both MAP2c and DMSO induce long microtubule bundles but whereas the MAP2c bundles are straight unless they encounter the resistance of the cell cortex, the DMSO bundles bend in the cytoplasm even though they do not touch the cell surface. When the MAP2c-induced bundles form peripheral rings, they distort the shape of the cell, causing the surface membrane to distend. The effect is reminiscent of that produced by the flexible stays of an igloo tent that cannot penetrate the enveloping material but while bending under the influence of its tension push it outwards by virtue of their stiffness. The most likely source of the restraining influence at the cell surface that induces the bending of microtubules is the cortical cytoskeleton, which maintains an isotropic tension that resists deformation and supports the pliable plasma membrane (Bray and White, 1988). The rigid network of actin filaments that it contains is more resistant to deformation than are microtubules (Janmey et al., 1991), which prevents the microtubules from accommodating their full length by distorting the cell surface. By contrast, the lipid bilayer itself offers little resistance to microtubule extension and microtubules assembled in liposomes composed of plasma membrane lipids are straight, not bent, and they push out protrusions from the liposomal membrane (Hotani et al., 1992).

Bundling of microtubules

Although the formation of microtubule bundles is the most striking effect of MAP2c, it is not clear what role it plays in their formation. One hypothesis proposed for both MAP2 and tau (Hirokawa et al., 1988b; Lewis et al., 1989; Kanai et al., 1989), is that these molecules actively cross-link tubulin polymers, presumably by a ligand-like dimerisation mediated through some site distinct from the tubulin-binding domain. However, microtubules in non-neuronal cells are also bundled by chemical agents that stabilise microtubules, such as taxol and DMSO, as we show here. Such observations have led to the proposal that the MAPs are not cross-linkers but act merely by stabilising microtubules, which are subsequently cross-linked by other molecules present in all cells (Chapin et al., 1991). However, comparing MAP2c-induced bundles to those produce by DMSO suggests a further important contribution of MAP2, namely that it imparts stiffness to the microtubule bundles in which it is present. How this arises remains to be determined, but

the presence of three contiguous repeats of the tubulin-binding motif in the MAP2 and MAP2c molecules(Lewis et al., 1989; Papandrikopoulou et al., 1989) could play a role by binding to tubulin subunits present in neighbouring protofilaments of the microtubule wall and thus limiting their movement relative to one another.

Significance of MAP2c-induced rearrangement for neuronal morphogenesis

The effects of MAP2c on cellular microtubules found in this study have implications for the organization of the cytoskeleton in neurons. Developing neurons express extremely high levels of MAP2c, comparable in immunofluorescence staining intensity to those in the most highly expressing transfected cells in the present study. Like MAP2c-transfected cells, neurons lack a centrosomal MTOC, instead microtubules arise at various sites in the cytoplasm with no distinct initiating organelle (Sharp et al., 1982; Okabe and Hirokawa, 1988; McNiven and Porter, 1988). The microtubules in neurons form bundles that are linked by fine filaments and these interactions appear to be of low affinity because microtubules part readily at the branch points of dendrites and at sites where transported particles, such as the various types of neuronal cytoplasmic vesicles, pass between them (Peters et al., 1976).

In developing neurons, which express MAP2c naturally, the microtubules are essential structural element that support axons and dendrites. Of the properties necessary for this function, our results suggest that MAP2 contributes stabilisation, which it turn enables the formation of bundles, and stiffness.

We thank Dr M. Terasaki for advice with freeze substitution fixation, and Drs U. Aebi and M. Dürrenberger for advice and assistance with the whole-mount negatively stained electron microscopy preparations.

References

Algaier, J. and Himes, R. H. (1988). The effects of dimethyl sulfoxide on the kinetics of tubulin assembly. *Biochim. Biophys. Acta.* **954**, 235-243.

Bernhardt, R. and Matus, A. (1984). Light and electron microscopic studies of the distribution of microtubule-associated protein 2 in rat brain: a difference between dendritic and axonal cytoskeletons. *J. Comp. Neurol.* **226**, 203-221.

Bornens, M. (1991). Structure and function of isolated centrosomes. In *The centrosome* (ed. V. Kalnins). New York: Academic Press.

Bray, D., Thomas, C. and Shaw, G. (1978). Growth cone formation in cultures of sensory neurons. *Proc. Natl. Acad. Sci. USA* **75**, 5226-5229.

Bray, D. and White, J. G. (1988). Cortical flow in animal cells. *Science* **239**, 883-888.

Bre, M. H., Kreis, T. E. and Karsenti, E. (1987). Control of microtubule nucleation and stability in Madin-Darby canine kidney cells: the occurrence of noncentrosomal, stable detyrosinated microtubules. *J. Cell Biol.* **105**, 1283-1296.

Bre, M. H. and Karsenti, E. (1990). Effects of brain microtubule-associated proteins on microtubule dynamics and the nucleating activity of centrosomes. *Cell Motil. Cytoskel.* **15**, 88-98.

Bridgman, P. C., Kachar, B. and Reese, T. S. (1990). The structure of cytoplasm in directly frozen cultured cells. II. Cytoplasmic domains associated with organelle movements. *J. Cell Biol.* **102**, 1510-1521.

Brown, P. A. and Berlin, R. D. (1985). Packing volume of sedimented microtubules: regulation and potential relationship to an intracellular matrix. *J. Cell Biol.* **101**, 1492-1500.

Burgoyne, R. D. (1986). Microtubule proteins in neuronal differentiation. *Comp. Biochem. Physiol. (B.)***83**, 1-8.

Burgoyne, R. D. and Cumming, R. (1984). Ontogeny of microtubule-associated protein 2 in rat cerebellum: differential expression of the doublet polypeptides. *Neurosci.* **11**, 156-167.

Carlier, M.-F. and Pantaloni, D. (1983). Taxol effect on tubulin polymerization and associated guanine 5′-triphosphate hydrolysis. *Biochemistry* **22**, 4814-4822.

Cassimeris, L., Pryer, N. K. and Salmon, E. D. (1988). Real-time observation of microtubule dynamic instability in living cells. *J. Cell Biol.* **107**, 2223-2231.

Chapin, S. J., Bulinski, J. C. and Gundersen, G. G. (1991). Microtubule bundling in cells. *Nature* **349**, 24.

Chen, C. and Okayama, H. (1987). High efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell Biol.* **7**, 2745-2752.

De Brabander, M., Geuens, G., Nuydens, R., Willebrords, R. and De Mey, J. (1981). Taxol induces the assembly of free microtubules in living cells and blocks the organizing capacity of the centrosomes and kinetochores. *Proc. Natl. Acad. Sci. USA* **78**, 5608-5612.

De Camilli, P., Miller, P. E., Navone, F., Theurkauf, W. E. and Vallee, R. B. (1984). Distribution of microtubule-associated protein 2 in the nervous system of the rat studied by immunofluorescence. *Neurosci.* **11**, 817-846.

Doll, T., Papandrikopoulou, A. and Matus, A. (1990). Nucleotide and amino acid sequences of embryonic rat MAP2c. *Nucl. Acids Res.* **18**, 361.

Forscher, P. and Smith, S. J. (1988). Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. *J. Cell Biol.* **107**, 1505-1516.

Friden, B., Nordh, J., Wallin, M., Deinum, J. and Norden, B. (1988). Effects of proteolysis of the extending parts of high-molecular-weight microtubule-associated proteins on interactions between microtubules. *Biochim. Biophys. Acta* **955**, 135-142.

Garner, C. C., Farmer, L. and Matus, A. (1988a). Single-shot cloning of multiple cDNAs coding for a set of related microtubule-associated proteins. *Gene* **71**, 483-490.

Garner, C. C., Tucker, R. P. and Matus, A. (1988b). Selective localization of messenger RNA for cytoskeletal protein MAP2 in dendrites. *Nature* **336**, 674-677.

Garner, C. C. and Matus, A. (1988). Different forms of microtubule-associated protein 2 are encoded by separate mRNA transcripts. *J. Cell Biol.* **106**, 779-783.

Griffith, L. M. and Pollard, T. D. (1982). The interaction of actin filaments with microtubules and microtubule-associated proteins. *J. Biol. Chem.* **257**, 9143-9151.

Hamel, E., del Campo, A., Lowe, M. C. and Lin, C. M. (1981). Interactions of taxol, microtubule-associated proteins, and guanine nucleotides in tubulin polymerization. *J. Biol. Chem.* **256**, 11887-11894.

Heuser, J. E. and Kirschner, M. (1980). Filament organization revealed in platinum replicas of freeze-dried cytoskeletons. *J. Cell Biol.* **86**, 212-234.

Himes, R. H., Burton, P. R., Kersey, R. N. and Pierson, G. B. (1976). Brain tubulin polymerization in the absence of 'microtubule-associated proteins'. *Proc. Natl. Acad. Sci. USA* **73**, 4397-4399.

Hirokawa, N. (1982). Cross-linker system between neurofilaments, microtubules and membranous organelles in frog axons revealed by the quick-freeze, deep-etch method. *J. Cell Biol.* **94**, 129-142.

Hirokawa, N., Hisanaga, S. I. and Shiomura, Y. (1988a). MAP2 is a component of crossbridges between microtubules and neurofilaments in the neuronal cytoskeleton: quick-freeze, deep-etch immunoelectron microscopy and reconstitution studies. *J. Neurosci.* **8**, 2769-2779.

Hirokawa, N., Shiomura, Y. and Okabe, S. (1988b). Tau proteins: The molecular structure and mode of binding to microtubules. *J. Cell Biol.* **107**, 1449-1459.

Hotani, H., Lahoz-Beltra, R., Combs, B., Hameroff, S. and Rasmussen, S. (1992). Microtubule dynamics, liposomes and artificial cells: In vitro observation and cellular automata simulation of microtubule assembly/disassembly and membrane morphogenesis. *Nanobiol.* **1**, 61-74.

Janmey, P. A., Euteneur, U., Traub, P. and Schliwa, M. (1991). Viscoelastic properties of vimentin compared with other filamentous biopolymer networks. *J. Cell Biol.* **113**, 155-160.

Kanai, Y., Takemura, R., Oshima, T., Mori, H., Ihara, Y., Yanagisawa, M., Masaki, T. and Hirokawa, N. (1989). Expression of multiple tau isoforms and microtubule bundle formation in fibroblasts transfected with a single tau cDNA. *J. Cell Biol.* **109**, 1173-1184.

Kim, H., Binder, L. I. and Rosenbaum, J. L. (1979). The periodic

association of map2 with brain microtubules in vitro. *J. Cell Biol.* **80**, 266-276.

Kindler, S., Schulz, B., Goedert, M. and Garner, C. C. (1991). Molecular structure of microtubule-associated protein 2b and 2c from rat brain. *J. Biol. Chem.* **265**, 19679-19684.

Kumar, N. (1981). Taxol-induced polymerization of purified tubulin. *J. Biol. Chem.* **256**, 10435-10441.

Lewis, S. A., Wang, D. and Cowan, N. J. (1988). Microtubule-associated protein MAP-2 shares a microtubule binding motif with tau protein. *Science* **242**, 936-939.

Lewis, S. A., Ivanov, I. E., Lee, G. H. and Cowan, N. J. (1989). Organization of microtubules in dendrites and axons is determined by a short hydrophobic zipper in microtubule-associated tau. *Nature* **342**, 498-505.

Matus, A., Bernhardt, R., Bodmer, R. and Alaimo, D. (1986). Microtubule-associated protein 2 and tubulin are differently distributed in the dendrites of developing neurons. *Neurosci.* **17**, 371-389.

Matus, A. (1988). Microtubule-associated proteins: their potential role in determining neuronal morphology. *Ann. Rev. Neurosci.* **11**, 29-44.

McNiven, M. A. and Porter, K. R. (1988). Organization of microtubules in centrosome-free cytoplasm. *J. Cell Biol.* **106**, 1593-1605.

Mogensen, M. M., Tucker, J. B. and Stebbings, H. (1989). Microtubule polarities indicate that nucleation and capture of microtubules occurs at cell surfaces in Drosophila. *J. Cell Biol.* **108**, 1445-1452.

Murphy, D. B. and Borisy, G. G. (1975). Association of high-molecular-weight proteins with microtubules and their role in microtubule assembly in vitro. *Proc. Natl. Acad. Sci. USA* **72**, 2696-2700.

Nunez, J. (1986). Differential expression of microtubule components during brain development. *Dev. Neurosci.* **8**, 125-141.

Okabe, S. and Hirokawa, N. (1988). Microtubule dynamics in nerve cells: analysis using microinjection of biotinylated tubulin into PC12 cells. *J. Cell Biol.* **107**, 651-664.

Papandrikopoulou, A., Doll, T., Tucker, R. P., Garner, C. C. and Matus, A. (1989). Embryonic MAP2 lacks the cross-linking sidearm sequences and dendritic targeting signal of adult MAP2. *Nature* **340**, 650-652.

Peters, A., Palay, S. L. and Webster, H. deF. (1976) *The Fine Structure of the Nervous System,* Philadelphia, PA: W. B. Saunders.

Sandoval, I. V., Macdonald, E., Jameson, J. L. and Cuatrecasas, P. (1977). Role of nucleotides in tubulin polymerization: effect of guanylyl 5′-methylenediphosphonate. *Proc. Natl. Acad. Sci. USA* **74**, 4881-4885.

Sato, M., Schwartz, W. H., Selden, S. C. and Pollard, T. D. (1988). Mechanical properties of brain tubulin and microtubules. *J. Cell Biol.* **106**, 1205-1211.

Sato-Yoshitake, R., Shiomura, Y., Miyasaka, H. and Hirokawa, N. (1989). Microtubule-associated protein 1B: molecular structure, localization, and phosphorylation-dependent expression in developing neurons. *Neuron* **3**, 229-238.

Sattilaro, R. F. (1986). Interaction of microtubule-associated protein 2 with actin filaments. *Biochemistry* **25**, 2003-2009.

Schiff, P. B. and Horwitz, S. B. (1980). Taxol stabilizes microtubules in mouse fibroblast cells. *Proc. Natl. Acad. Sci. USA* **77**, 1561-1565.

Schoenfeld, T. A., McKerracher, L., Obar, R. and Vallee, R. B. (1989). MAP 1A and MAP 1B are structurally related microtubule associated proteins with distinct developmental patterns in the CNS. *J. Neurosci.* **9**, 1712-1730.

Schulze, E. and Kirschner, M. (1986). Microtubule dynamics in interphase cells. *J. Cell Biol.* **102**, 1020-1031.

Seeds, N. W., Gilman, A., Amano, T. and Nirenberg, M. W. (1970). Regulation of axon formation by clonal lines of a neural tumor. *Proc. Natl. Acad. Sci. USA* **66**, 160-167.

Sharp, G. A., Weber, K. and Osborn, M. (1982). Centriole number and process formation in established neuroblastoma and primary dorsal root ganglion neurons. *Eur. J. Cell Biol.* **29**, 97-103.

Sloboda, R. D., Rudolph, S. A., Rosenbaum, J. L. and Greengard, P. (1975). Cyclic AMP-dependent endogenous phosphorylation of a microtubule-associated protein. *Proc. Natl. Acad. Sci. USA* **72**, 177-181.

Sloboda, R. D. and Rosenbaum, J. L. (1979). Decoration and stabilization of intact, smooth-walled microtubules with microtubule-associated proteins. *Biochemistry* **18**, 48-55.

Small, J. V. (1988). The actin cytoskeleton. *Electron Microsc. Rev.* **1**, 155-174.

Tanaka, E. M. and Kirschner, M. (1991). Microtubule behaviour in the growth cones of living cells during axon elongation. *J. Cell Biol.* **115**, 345-363.

Tucker, R. P., Binder, L. I. and Matus, A. I. (1988a). Neuronal microtubule-associated proteins in the embryonic avian spinal cord. *J. Comp. Neurol.* **271**, 44-55.

Tucker, R. P., Binder, L. I., Viereck, C., Hemmings, B. A. and Matus, A. (1988b). The sequential appearance of low- and high-molecular weight forms of MAP2 in the developing cerebellum. *J. Neurosci.* **12**, 4503-4512.

Vallee, R. B. and Borisy, G. G. (1977). Removal of the projections from cytoplasmic microtubules in vitro by digestion with trypsin. *J. Biol. Chem.* **252**, 377-382.

Viereck, C., Tucker, R. P., Binder, L. I. and Matus, A. (1988). Phylogenetic conservation of brain microtubule-associated proteins MAP2 and tau. *Neurosci.* **26**, 893-904.

Viereck, C., Tucker, R. P. and Matus, A. (1989). The adult rat olfactory system expresses microtubule-associated proteins found in the developing brain. *J. Neurosci.* **9**, 3547-3557.

Viereck, C. and Matus, A. (1990). The expression of phosphorylated and non-phosphorylated forms of MAP5 in the amphibian CNS. *Brain Res.* **508**, 257-264.

Weingarten, M., Lockwood, A., Hwo, S.-Y. and Kirschner, M. (1975). A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. USA* **72**, 1858-1862.

Yamada, K. M., Spooner, B. S. and Wessels, N. K. (1970). Axon growth: roles of microfilaments and microtubules. *Proc. Natl. Acad. Sci. USA* **66**, 1206-1212.

(Accepted 10 September 1992)