

## Actin depolymerisation induces process formation on MAP2-transfected non-neuronal cells

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### SUMMARY

We have previously shown that microtubules in non-neuronal cells form long, stable bundles after transfection with the embryonic neuronal microtubule-associated protein MAP2c. In this study, we found that treating MAP2c-transfected cells with the actin depolymerising drug cytochalasin B led to the outgrowth of microtubule-containing processes from the cell surface. This effect was specific to MAP2c and did not occur in untransfected cells whose microtubules had been stabilised by treatment with taxol. The outgrowth and retraction of these processes during repeated cycles of cytochalasin addition and removal was followed by video time-lapse microscopy and was suggestive of a physical interaction between compressive forces exerted by the MAP2c-stabilised microtubule bundles and tensile forces originating in the cortical actin network. We suggest that MAP2c confers three properties on cellular microtubules that are essential for process out-

growth: stability, bundling and stiffness. The latter probably arises from the linking together of neighbouring tubulin subunits by three closely spaced tubulin-binding motifs in the MAP2 molecule that limits their motion relative to one another and thus reduces the flexibility of the polymer. Similar multimeric tubulin-binding domains in other proteins of the MAP2 class, including tau in axons and MAP4 in glial cells, may play the same role in the development and support of asymmetric cell morphology. Axial bundles of microtubules are found in growing neurites but not in growth cones, suggesting that the regulated expression of these MAP-induced properties makes an important contribution to the establishment of a stable process behind the advancing growth cone.

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

### INTRODUCTION

The outgrowth of neuronal processes involves the interaction of two major components of the neuronal cytoskeleton, microtubules and actin filaments. These produce counteractive forces, tension generated by actin filaments of the cortical cytoskeleton (Bray et al., 1988; Zheng et al., 1991) and compression produced by microtubule bundles in the cytoplasm (Letourneau et al., 1987; Denneril et al., 1989). The interplay between these forces has been illustrated by the effects of drugs that alter the behaviour of the two filament systems. When neurons growing processes in culture are treated with tubulin depolymerising drugs, such as colchicine, the neurites retract (Bray et al., 1978; Matus et al., 1986; Seeds et al., 1970; Yamada et al., 1970), but this retraction itself is inhibited by the actin depolymerising drug cytochalasin B (Solomon and Magendanz, 1981; Joshi et al., 1985). When primary neurons are treated with cytochalasin alone, neurite growth continues despite the absence of filamentous actin and an organised growth cone, indicating that the 'push' provided by bundled microtubules is sufficient for the establishment of a stable neuronal process

(Letourneau et al., 1987). To be capable of supporting processes in this way, the neuronal microtubules need to have several special properties. They must be more stable than the microtubules of non-neuronal cells, which assemble and disassemble rapidly in a state of dynamic equilibrium (Schultze and Kirschner, 1986; Sammak and Borisy, 1988; Cassimeris et al., 1988). They must all be oriented along the longitudinal axis of the process, unlike the microtubules of non-neuronal cells, which follow various paths through the cytoplasm. Finally, they must be sufficiently stiff to support the elongate processes as they are formed.

These special properties of neuronal microtubules are thought to depend on a set of structural microtubule-associated proteins (MAPs) that are expressed at high levels in neuronal processes (Nunez, 1986; Matus, 1988). Recent experiments have shown that, when two of these proteins, MAP2 and tau, are expressed at sufficient levels in non-neuronal cells, they stabilise microtubules and induce their bundling. This is often also accompanied by striking changes in their distribution within the cytoplasm (Lewis et al., 1989; Weisshaar et al., 1992; Kanai et al., 1989; Lee and Rook, 1992). In a recent study, we found that the

arrangement of the MAP2-induced microtubule bundles inside transfected cells depends upon their length (Weisshaar et al., 1992). If they were shorter than the cell diameter they were straight and projected across the inner portion of the cytoplasm. If, however, the microtubules were longer than the cell diameter, they were bent, forming peripheral rings around the edge of the cell. The differing conformations of the long and short MAP2-induced microtubule bundles suggest that they possess an inherent rigidity, bending only when they encounter a restraining influence situated at the cell surface. The most likely source of this restraint lies in the actin filaments of the cortical cytoskeleton, which impart tensile strength to the plasma membrane (Petersen et al., 1982; Usi and Yoneda, 1982; Bray et al., 1988) and which are more resistant to mechanical deformation than are microtubules (Janmey et al., 1991). This interpretation is supported by the observation that microtubules repolymerised from pure tubulin inside liposomes can push out protrusions of the unsupported phospholipid bilayer (Hotani et al., 1992). On this basis, we proposed a model in which the cortical actin network acts as physical barrier, preventing the extension of the MAP2-induced microtubule bundles beyond the cell periphery (Weisshaar et al., 1992). If such a mechanism does exist, then it might be expected that, if the cortical actin filaments were removed, then the MAP2-induced microtubule bundles would be able to straighten, rather as springs unwind when removed from a clock, and push out process-like protrusions from the cell surface. These considerations prompted the experiments reported here, in which MAP2-transfected non-neuronal cells were treated with the drug cytochalasin B, which depolymerises actin filaments. We show that, as predicted, this leads to the appearance of microtubule-filled processes on cells expressing MAP2 but not on cells that lack MAP2. The nature of these processes and the dynamics of their extension and retraction is discussed in relation to the contribution of MAP2 to the structure of neuronal processes.

## MATERIAL AND METHODS

### Cell culture and transfection

Cell lines were cultured under standard conditions. The human hepatoma cell line PLC, for which results are presented, was cultured in Dulbecco medium with 10% fetal calf serum. Cells were transfected using the calcium phosphate method (Chen and Okayama, 1987) with cDNA encoding rat MAP2c or MAP2b cloned into the eukaryotic expression vector pECE (Pharmacia) as previously described (Weisshaar et al., 1992). 20 µM cytochalasin B (Sigma) and 10 µM taxol (a gift from Dr M Suffness, NCI) were added to cells 48 hours after transfection. Cells were fixed in rapidly stirred methanol maintained at -70 °C in a methanol-dry ice bath and were immunofluorescence stained with mouse monoclonal antibodies against MAP2 and rabbit polyclonal antibodies against tubulin (a gift from Dr Chloe Bulinski) as previously described (Weisshaar et al., 1992). In video time-lapse experiments, the cells were fixed while still under video observation with 5 mM ethylene glycol bis-succinimidylsuccinate (EGS, Pierce, Cologne, BRD) containing 0.15% Triton X-100. They were subsequently stained with mouse anti-MAP2 followed by fluorescein conjugated goat anti-mouse secondary antibody to

visualise transfected MAP2 and simultaneously with rhodamine-phalloidin to visualise actin filaments (Molecular Probes, Eugene, Oregon, USA).

Whole-mount electron microscopy specimens were prepared from cells grown and transfected on carbon/formvar-coated gold 'finder' grids with built-in orientation marks. Cells were fixed and negatively stained with 1% uranyl formate as described by Small (Small, 1988). Transfected cells were identified after fixation by immunofluorescence staining with anti-MAP2 and their position noted so that they could be located for viewing.

### Video analysis

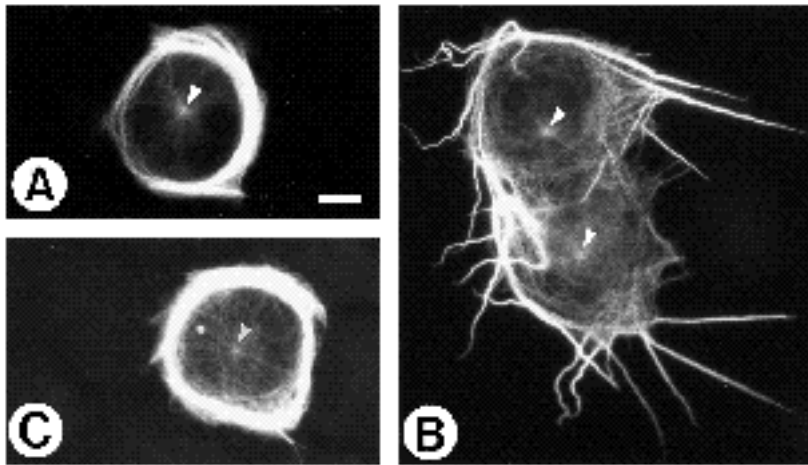
For video analysis of living cultures, transfected cells expressing high levels of MAP2 were identified by their ability to extend processes in response to cytochalasin and this was confirmed after fixation by staining with anti-MAP2. When cytochalasin was withdrawn the processes resorbed, and time-lapse observations of the identified cell were made during a second round, and in some cases a third round, of process extension after readdition of cytochalasin. Cells were observed in phase contrast using a 25× Zeiss objective (NA 0.6) and images recorded at 10 second intervals using a Hamamatsu C2400 camera and a Panasonic TQ-2028F Optical Magnetic Disc Recorder. Images composed of 16 frames were captured and averaged using Universal Imaging 'Image 1' software. Process extension and retraction rates were determined using the 'Image 1' software by measuring process lengths at various time points on images recorded on optical disc during videotaping experiments.

## RESULTS

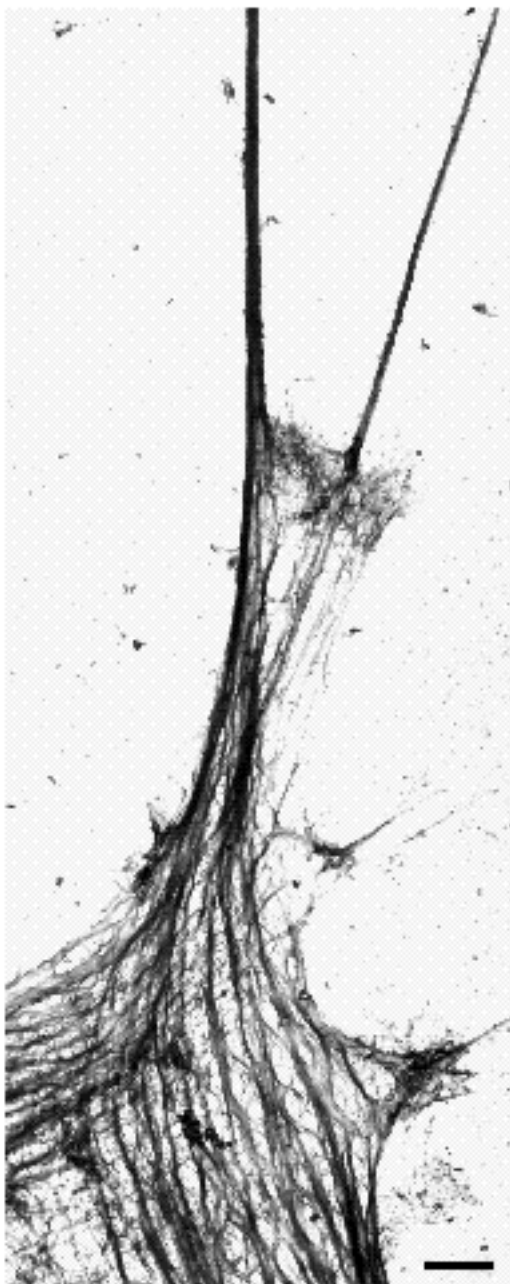
### Process formation by cells expressing MAP2

The human hepatoma line PLC, which is particularly favourable for visualising the cellular organisation of microtubules (Weisshaar et al., 1992), was transfected with the embryonic low molecular weight form of MAP2, MAP2c (Garner and Matus, 1988). All the experiments were also performed with the adult high molecular weight form, MAP2b (Papandrikopoulou et al., 1989; Kindler et al., 1991), which gave results indistinguishable from those reported here for MAP2c. As previously reported, at high levels of MAP2 expression cellular microtubules were bundled and showed a characteristic rearrangement into peripheral rings under the plasma membrane (Fig. 1A, see also Weisshaar et al., 1992). When transfected cultures were additionally treated with cytochalasin, cells expressing high levels of MAP2 underwent a striking change in shape, involving the appearance of thin processes containing bundles of microtubules (Figs 1B, 2). When cytochalasin was removed, both cell shape and microtubule organization returned to the previous pattern (Fig. 1C). Whole-mount electron microscopy showed that the cytochalasin-induced processes were formed by the funnelling together of many separate MAP2-induced microtubule bundles in the cell body to form a single compact bundle in the process (Fig. 2).

In all cultures, only cells expressing high levels of MAP2 formed processes when treated with cytochalasin. Cells in the same cultures expressing lower levels of MAP2, in which the microtubules were not bundled and were still attached to the microtubule-organising centre (Weisshaar et al., 1992), did not form processes (not shown). MAP2-



**Fig. 1.** MAP2c induces microtubule rearrangement and process formation on non-neuronal cells. Immunofluorescence micrographs of human hepatoma PLC cells expressing transfected MAP2c, stained with anti-MAP2. (A) Untreated cells; (B) Cells treated with 20  $\mu\text{m}$  cytochalasin for 30 minutes; (C) Cells 2 hours after removal of cytochalasin. In control cells (A), some MAP2-labelled microtubules still originate from the microtubule-organising centre (MTOC; arrowhead), but most are reorganised into long bundles around the cell periphery. After cytochalasin treatment (B), cells extend processes with MAP2-containing microtubule bundles. When the cytochalasin is removed (C), the processes are resorbed and the microtubules revert to the control pattern. Bar, 10  $\mu\text{m}$ .



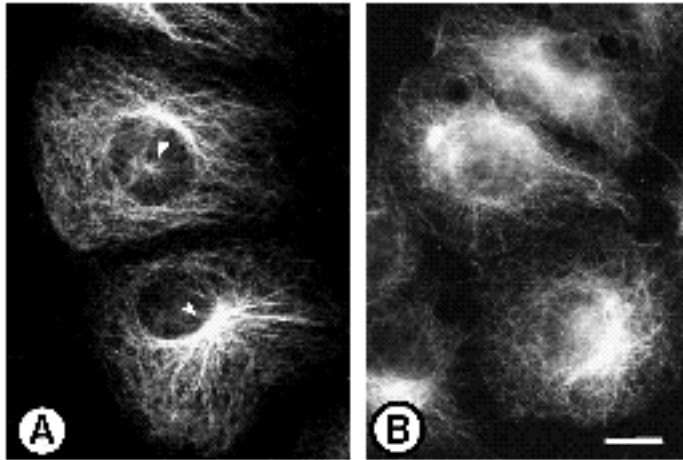
transfected cells commonly contain both bundled and single microtubules and upon cytochalasin treatment single microtubules remained in the cell body even though microtubule-containing processes were formed (this is visible in the cells in Fig. 1B). Thus process formation is linked to the rearrangement of microtubules induced by high MAP2 concentrations in the cell. This dependence was further illustrated by experiments in which untransfected cells were treated with cytochalasin (Fig. 3). Anti-tubulin staining showed that these cells contain microtubules in the pattern, common for non-neuronal cultured cells, of individual filaments emerging from a centrosomal MTOC (Fig. 3A). When treated with cytochalasin, the microtubules of these cells showed some change in appearance but none of the changes induced by MAP2, such as bundling and relocation to the periphery, were observed and these untransfected cells did not form processes (Fig. 3B).

To see whether microtubule stabilisation and bundling is sufficient for cytochalasin-induced process formation to occur, or whether additional properties conferred on microtubules by MAP2 are required, we treated untransfected cells with taxol, a drug that stabilises cellular microtubules and causes them to form short, randomly oriented bundles in the cytoplasm (Schiff and Horwitz, 1980; De Brabander et al., 1981) and Fig. 4A). When these taxol-treated cells were additionally treated with cytochalasin, they did not form processes (Fig. 4B), indicating that additional properties contributed by MAP2 are necessary for process outgrowth.

#### Depolymerisation of actin

To study the effects of cytochalasin on the organisation of actin in MAP2-transfected cells, cultures were observed by time-lapse video microscopy and fixed at maximum process extension. Fig. 5 shows such a culture that was stained for

**Fig. 2.** Cytochalasin B-induced processes on a MAP2-transfected cell containing tightly bundled microtubules. PLC cells were grown on gold electron microscope grids, transfected with MAP2, treated with cytochalasin and prepared for whole-mount electron microscopy. Loosely bundled microtubules in the cell body (bottom) progressively gather to form tight cores inside narrow elongate processes of regular diameter. Bar, 0.5  $\mu\text{m}$ .



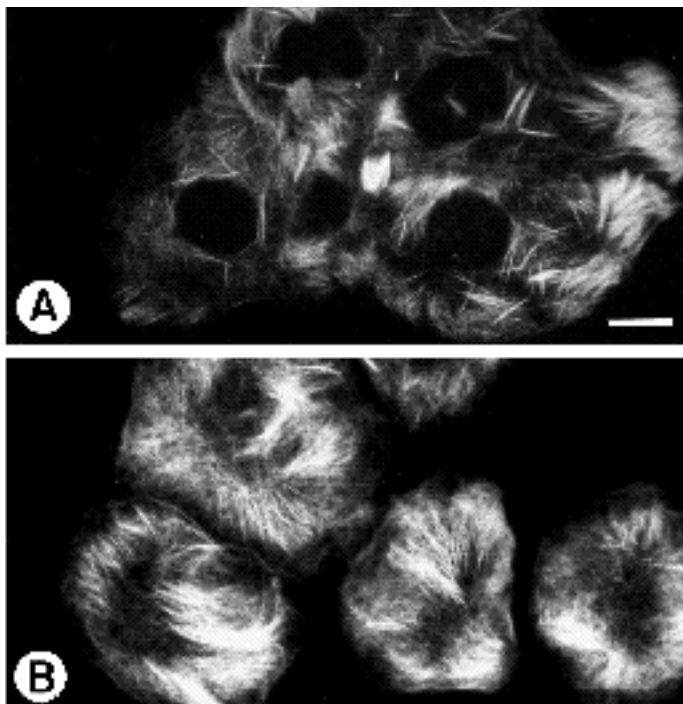
**Fig. 3.** Untransfected PLC cells do not form processes in cytochalasin. Cultures of untreated cells (A) or cells treated with cytochalasin (B) were immunofluorescently stained with monoclonal anti-tubulin antibody. Although there are difference in the appearance of these cells, in neither case do the microtubules show the bundling or spatial rearrangement characteristic of MAP2 transfected cells, and no processes are formed in the presence of cytochalasin. Arrowheads mark the microtubule-organising centre (MTOC). Bar, 10  $\mu$ m.

MAP2 by immunofluorescence (Fig. 5B) and for filamentous actin with rhodamine-labelled phalloidin (Fig. 5C). Arrowheads in each frame of the figure indicate the location of processes. As shown previously, these processes contained bundles of microtubules decorated with MAP2 but, as expected following cytochalasin treatment, they did not contain actin filaments (Fig. 5C). Instead rhodamine-phalloidin staining in these cells appeared as a dense cloud in the cell body and discrete patches at the periphery of the perikaryon. Untransfected cells in the same cultures were differently affected. In many cells, the actin filaments of stress fibres were still visible after cytochalasin treatment. This is consistent with previous studies in which stress fibres have been found to be more resistant to cytochalasin B depolymerisation than cortical actin filaments (see Letourneau et al., 1987). In other cells, actin labelling appeared in patches that were especially marked at the cell periphery. In the context of this study, the significant result

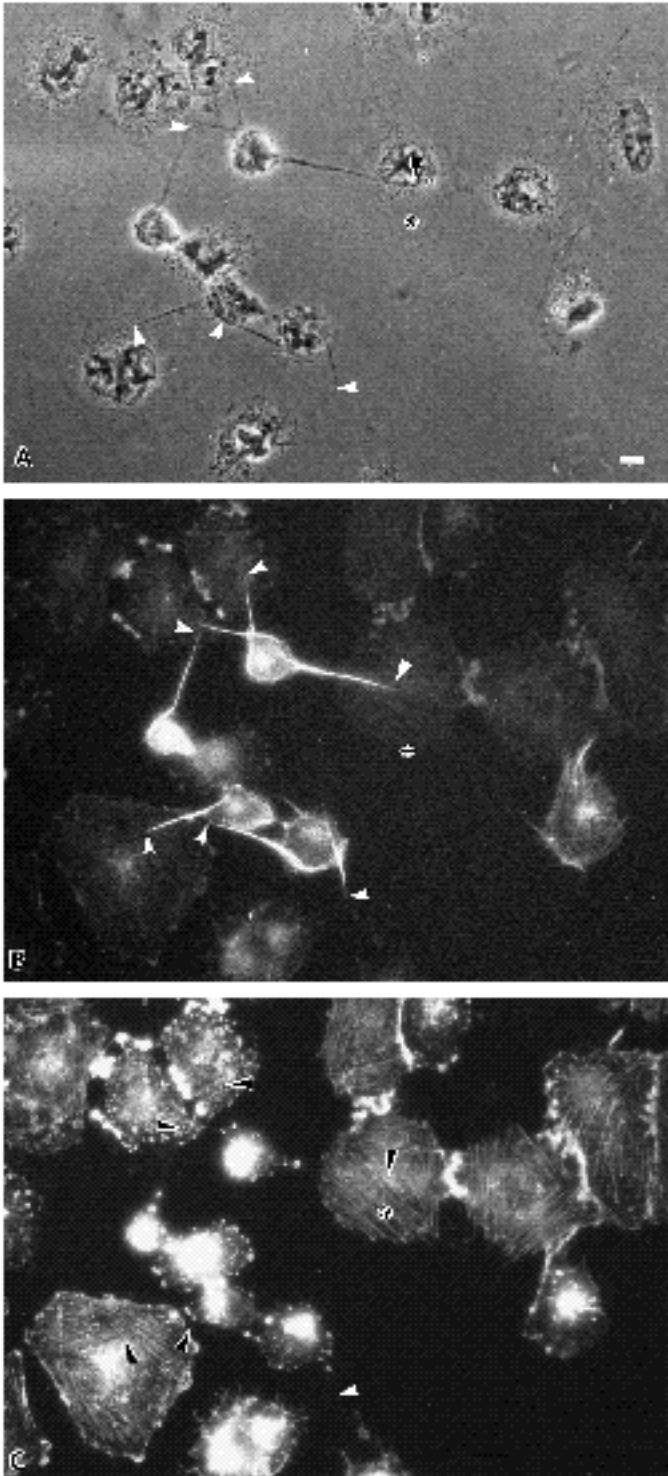
of these experiments is that process formation occurred in the absence of cortical actin filaments.

#### Video time-lapse observations

To determine how these processes are formed, MAP2-transfected cells were observed by time-lapse video microscopy during cytochalasin treatment. Typically, a candidate transfected cell could be recognised by its rotund outline (Fig. 6A). Our previous results suggest that this characteristic shape results from MAP2-induced microtubule bundles pushing out against the restraining envelope of the cortical actin filaments (Weisshaar et al., 1992). Upon addition of cytochalasin, straight processes emerged rapidly from the cell body at a rate of 2.3  $\mu$ m/min, their outgrowth being complete by 15 to 20 minutes (Fig. 6B-F). After this time the processes did not grow further but remained stable during further video recording until the cytochalasin was removed. The average rate of growth of such processes was



**Fig. 4.** Taxol-treated PLC cells do not form processes in cytochalasin. PLC cells were treated with 20  $\mu$ M taxol for 12 hours to stabilise microtubules, then fixed and stained with anti-tubulin as in Fig. 2. Taxol shows its typical effect of reorganising the microtubules into short bundles. A shows the results of taxol alone whereas in B the cells were treated additionally with 20  $\mu$ M cytochalasin for 30 minutes. No processes were formed. Bar, 10  $\mu$ m.



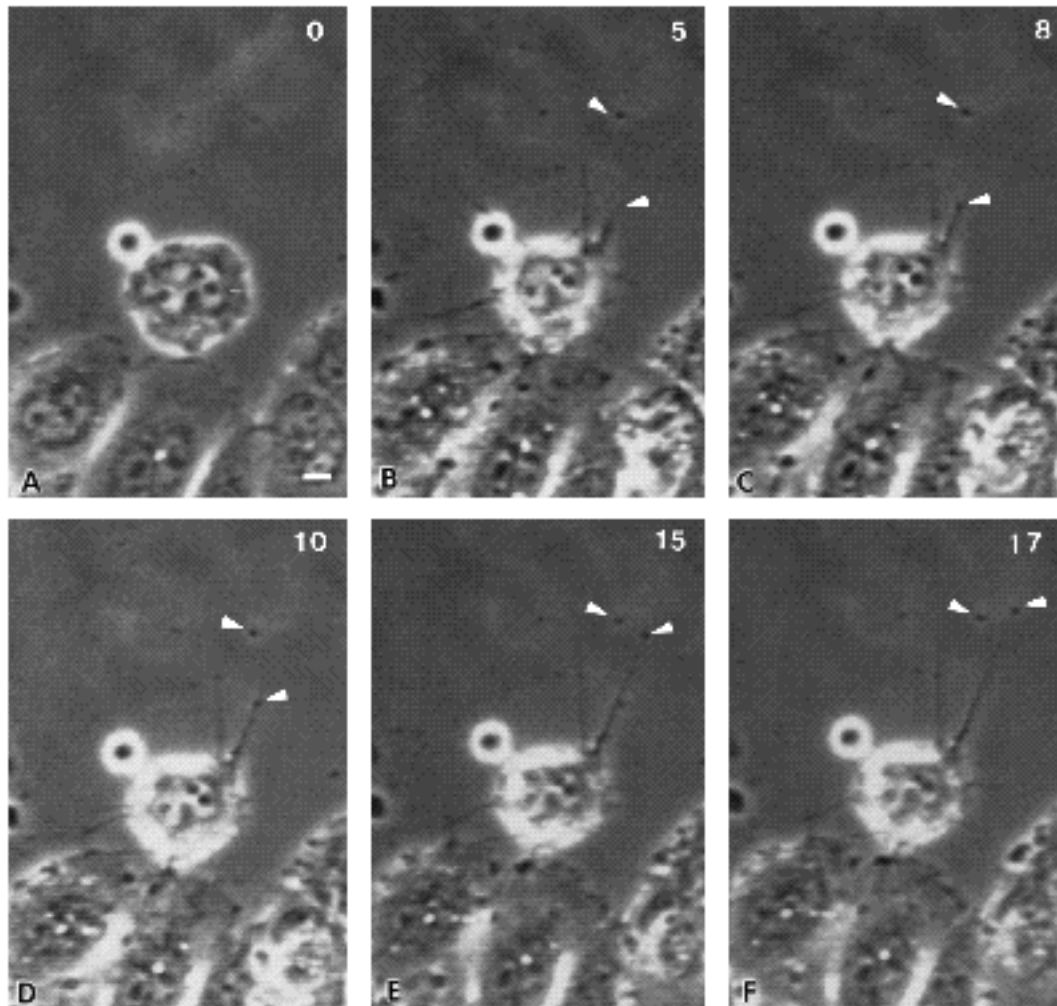
**Fig. 5.** Formation of MAP2-induced processes correlates with the disruption of cortical actin filaments. Three images of the same MAP2c-transfected cytochalasin-treated cell culture. (A) Phase-contrast video frame taken after fixation. (B) Anti-MAP2 image showing that processes are formed only by cells expressing MAP2c. (C) The same culture stained with rhodamine-phalloidin to reveal actin filaments. Arrowheads indicate the ends of processes. Note the lack of organised actin filaments in processes of transfected cells. Asterisks refer to a cell that still contains stress fibres. Bars in A,B and C, 10  $\mu$ m.

2.9  $\mu$ m/min. This is within the range of microtubule growth rates observed in non-neuronal cells (Sammak and Borisy, 1988; Schulze and Kirschner, 1988) and in neuronal growth cones (Tanaka and Kirschner, 1991) so that MAP2-induced process outgrowth could result from the assembly of new microtubules. Sliding of microtubules relative to one another also constitutes a possible mechanism for the establishment of the microtubular cores of these processes. However, the time-lapse recordings are more suggestive of outgrowth resulting from pre-existing MAP2-induced microtubule bundles pushing out protrusions from the cell surface when they are released from the constraining influence of the cortical actin network. This latter scheme is favoured by other time-lapse recordings in which thick microtubule bundles visible by phase-contrast microscopy could be seen pushing out of the cell following the application of cytochalasin (e.g. the example shown in Fig. 9A).

Time-lapse observations of the retraction of processes after cytochalasin removal also suggested that process outgrowth involves compressive forces. Fig. 7 shows the retraction of a process following the removal of cytochalasin. Retraction was marked by the process bending (Fig. 7D,E) and being pulled sideways (Fig. 7F,G), suggesting that its core was being recompressed into the cell cytoplasm. Another example is shown in Fig. 8, where a round MAP2-transfected cell produced a set of tangential processes after cytochalasin treatment (Fig. 8A). After withdrawal of cytochalasin, the processes bent upwards and backwards toward the cell body as the actin filaments reformed (Fig. 8B-D) and were slowly drawn into a circumferential alignment inside the cell (Fig. 8F). The entire procedure is suggestive of the microtubule core of each process being recompressed back into the cell body under the growing tensile force of the reforming actin filaments.

In the example shown in Fig. 7 the shortening of the process began within 30 seconds of cytochalasin removal and proceeded at a rate of 11.6  $\mu$ m/minute. We collected dynamic histories of extension and retraction for processes on 26 different cells by making measurements from video frames of complete time-lapse recordings, the consolidated data from which is presented in Table 1. This confirms that the resorption of processes into the cell body after removal of cytochalasin was faster than their original outgrowth when cytochalasin was added.

We followed many cells through several cycles of process outgrowth and retraction by the repeated addition and removal of cytochalasin (Fig. 9) and found that processes tended to reappear at the same place on the cell periphery (for example, the process labelled '1' in Fig. 9). This suggests that stable microtubules can be alternately released and restrained by the cortical cytoskeleton during successive cycles of drug treatment. However, the relationship of individual bundles at a particular site also tends to change so that the processes on the right-hand side of the cell in Fig. 9 show some evidence of amalgamation and splitting during the 2nd and 3rd cycles (Fig. 9C,E). This suggests that the microtubules that support them are not tightly cross-linked but are only loosely associated as labile bundles. Consistent with this interpretation new processes can also arise, like the one labelled '2' at the top of the transfected cell in Fig. 9.

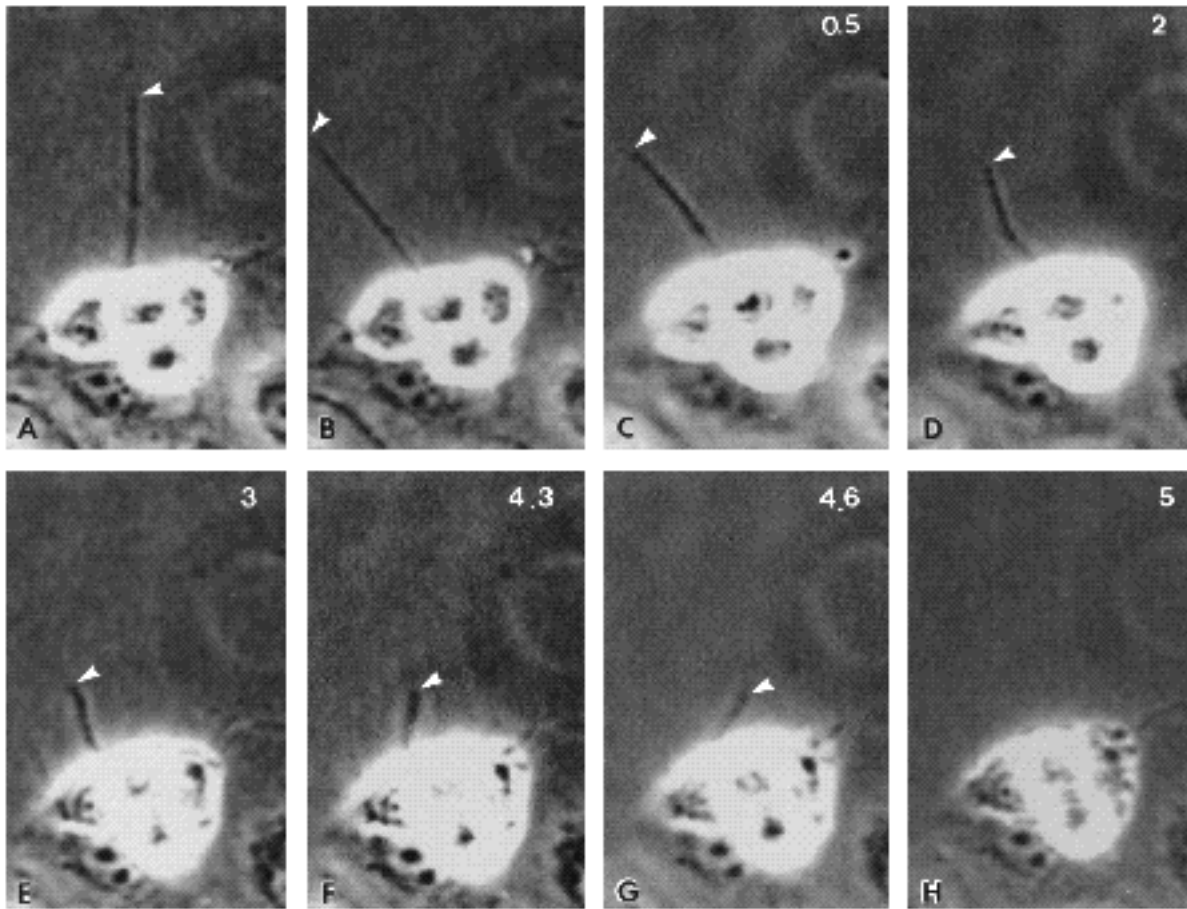


**Fig. 6.** Time course of process outgrowth from a cytochalasin-treated MAP2c-transfected cell. These frames were taken from a continuous video recording at the indicated times (in minutes). A MAP2c-transfected cell of typically rotund morphology extended several processes over a time course of 17 minutes (B-F) whereas neighbouring untransfected cells retained their original shape. The end of one extending process is marked in successive frames by the right-hand arrowheads. The left-hand arrowheads indicate a stationary particle attached to the substratum. Expression of MAP2 by process-extending cells was confirmed by immunofluorescence staining with anti-MAP2 at the end of the experiments. Bar, 10  $\mu$ m.

## DISCUSSION

In a previous study, we found that when non-neuronal cells were transfected with MAP2, the microtubules lost their normal radial pattern and instead formed long bundles that were bent around the periphery of the cell. We suspected that this arrangement resulted from a physical interaction between compressive forces exerted by the MAP2-containing microtubule bundles and restraining tensile forces originating in the cortical actin cytoskeleton (Weisshaar et al., 1992). The results of the present study confirm that interpretation by showing that, when the cortical actin filaments are depolymerised, the microtubule bundles straighten and push out processes from the cells. Process formation following treatment with cytochalasin has previously been demonstrated in avian erythrocytes (Winkler and Solomon, 1991) and in astroglial cells (Baorto et al., 1992), and it is interesting that both these cells contain microtubule-asso-

ciated proteins of the MAP2 class; MAP4 (also known as MAP3) in astroglia (Parysek et al., 1984; Huber et al., 1985) and tau in the erythrocytes (Stetzkowski-Marden et al., 1991; Lichtenberg Kraag and Mandelkow, 1990). This raises the possibility that all microtubule-associated proteins of this class may be able to confer properties on microtubules that enable them to support cellular processes. This conclusion is also supported by transfection experiments using the insect ovarian cell line SF9. These cells are interesting in that they form processes spontaneously, without any requirement for cytochalasin treatment, when transfected with either tau (Knops et al., 1991) or MAP2 (Chen et al., 1992). This might indicate that the cortical cytoskeletons of SF9 cells offers less resistance to process formation than do those of most non-neuronal cells. Alternatively the much higher MAP expression levels achieved by the baculovirus system (see below) may increase the compressive forces exerted by cellular microtubules to a level where they



**Fig. 7.** Retraction of processes by a MAP2c-transfected cell after removal of cytochalasin. A and B show the process at maximum extension in the presence of cytochalasin. Frames C to H show successive times during retraction from a continuous series of video recordings. Times after wash-out are indicated in minutes. Bending of the process during retraction (D-G) suggests that it is 'pulled' into the cell body by the re-forming cortical actin network. Note the faster time course of retraction as opposed to extension. Compare this series with that in Fig. 5. See text for further discussion. Bar, 10  $\mu$ m.

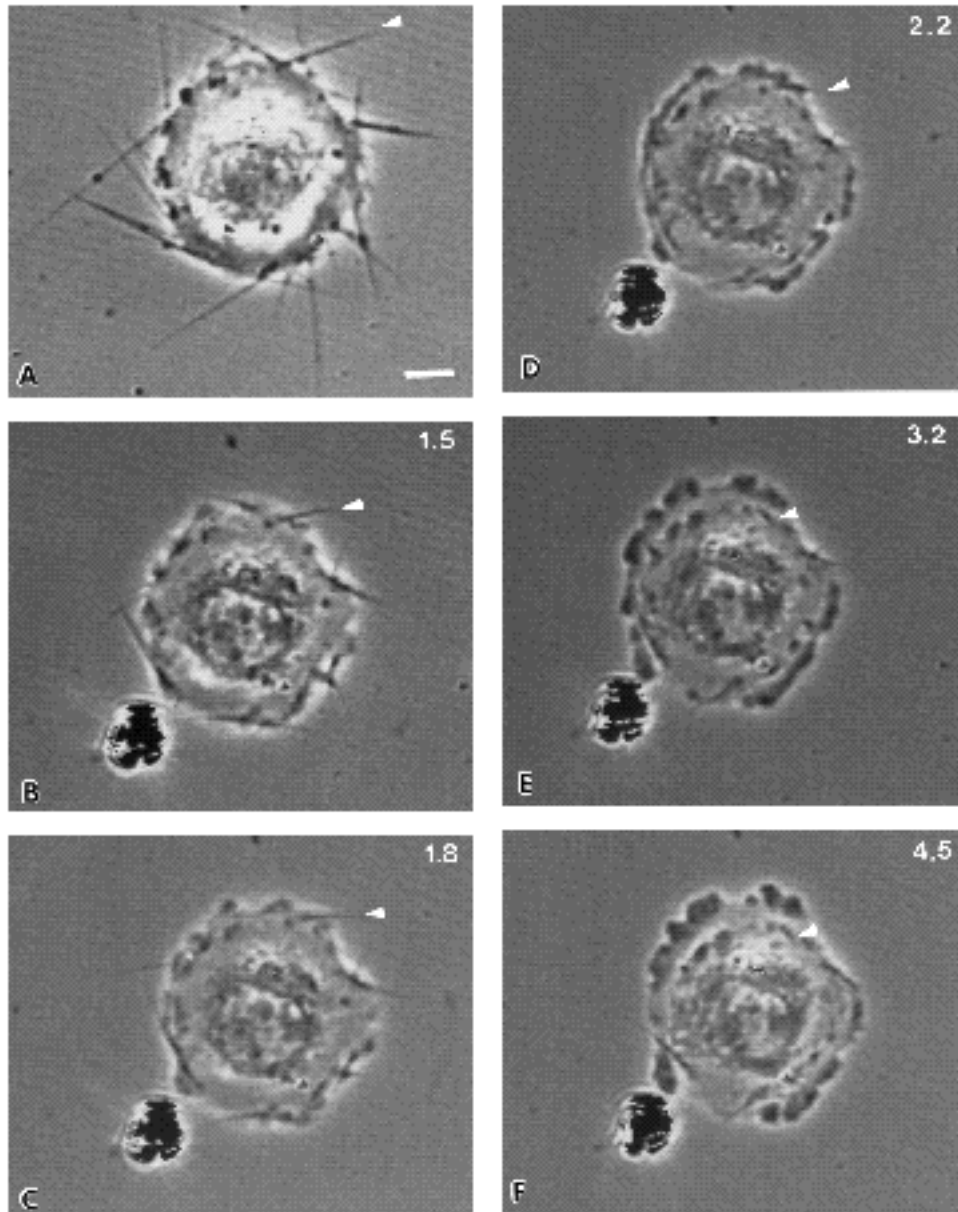
can overcome the tensile forces of the cortical actin network.

The processes induced on MAP2-transfected non-neuronal cells by cytochalasin bear an interesting resemblance to the processes of neuronal cells grown in the presence of the same drug (Marsh and Letourneau, 1984). Both lack growth cones, neither are attached to the substratum and neither shows the extensive branching that is characteristic of normal neurites. The MAP2-induced processes are short compared to cytochalasin-treated neurites. However, like tau (Knops et al., 1991), MAP2 induces long processes on SF9 cells (Chen et al., 1992), suggesting that the shortness of the processes in our experiments is not because of any limitation inherent to MAP2 itself. The difference between SF9 and other non-neuronal cells probably results from the greater expression achieved with the baculovirus system, which in the case of tau produces 10-fold more protein in SF9 cells than do conventional transfection techniques in mammalian cell lines (Lee and Rook, 1992). This explanation is consistent with the very high levels of MAP2 and tau expression in neuronal processes in the developing nervous system. Another factor that may limit process growth is the amount of tubulin available. In order to grow long

neurites, developing neurons synthesise much larger quantities of tubulin than do cells of other tissues. Thus, in order to grow processes of neuritic dimensions, MAP2-transfected cells would have to increase their synthetic capacity to neuronal levels. However, the various non-neuronal cell lines that we have examined show only modest increases in tubulin levels when transfected with MAP2 (Weisshaar et al., 1992).

#### MAP2 and the generation of microtubule 'push' in process outgrowth

In neurons, the extension of processes after cytochalasin treatment has been attributed to compressive forces exerted by microtubules (Letourneau et al., 1987; Marsh and Letourneau, 1984). On the basis of their studies, Letourneau and colleagues concluded that microtubule 'push' is sufficient for neurite elongation and growth cone 'pull' is not required (Letourneau et al., 1987). They also proposed that the same properties that are responsible for the stability, extension and cylindrical form of normal neuronal processes also underlay the unbranched, unattached neurites produced by neurons growing in the presence of cytochalasin. Our results support this conclusion and in addition



**Fig. 8.** Processes are rapidly pulled into the cell body upon removal of cytochalasin. Video sequence during one cycle of cytochalasin B removal. Times indicated are minutes after removal of cytochalasin by washing the culture with fresh medium. Processes (arrowhead) seems to be retracted by being pulled and bent back. Bar, 10  $\mu$ m.

indicate that these same three characteristic properties of neurites can be produced by a single microtubule-associated protein that is expressed during neuronal development, MAP2c.

What properties does MAP2 confer on the microtubules

**Table 1. Dynamics of process formation**

|                                     | Elongation           | Retraction         |
|-------------------------------------|----------------------|--------------------|
| Mean rate ( $\mu$ m/minute)*        | 2.9 $\pm$ 0.8 (32)   | 8.3 $\pm$ 3.9 (36) |
| Mean length ( $\mu$ m) <sup>†</sup> | 23.3 $\pm$ 11.8 (76) | NA                 |
| Range of lengths                    | 8-62 (76)            | NA                 |

Numbers in parentheses are the number of events measured (*n*).

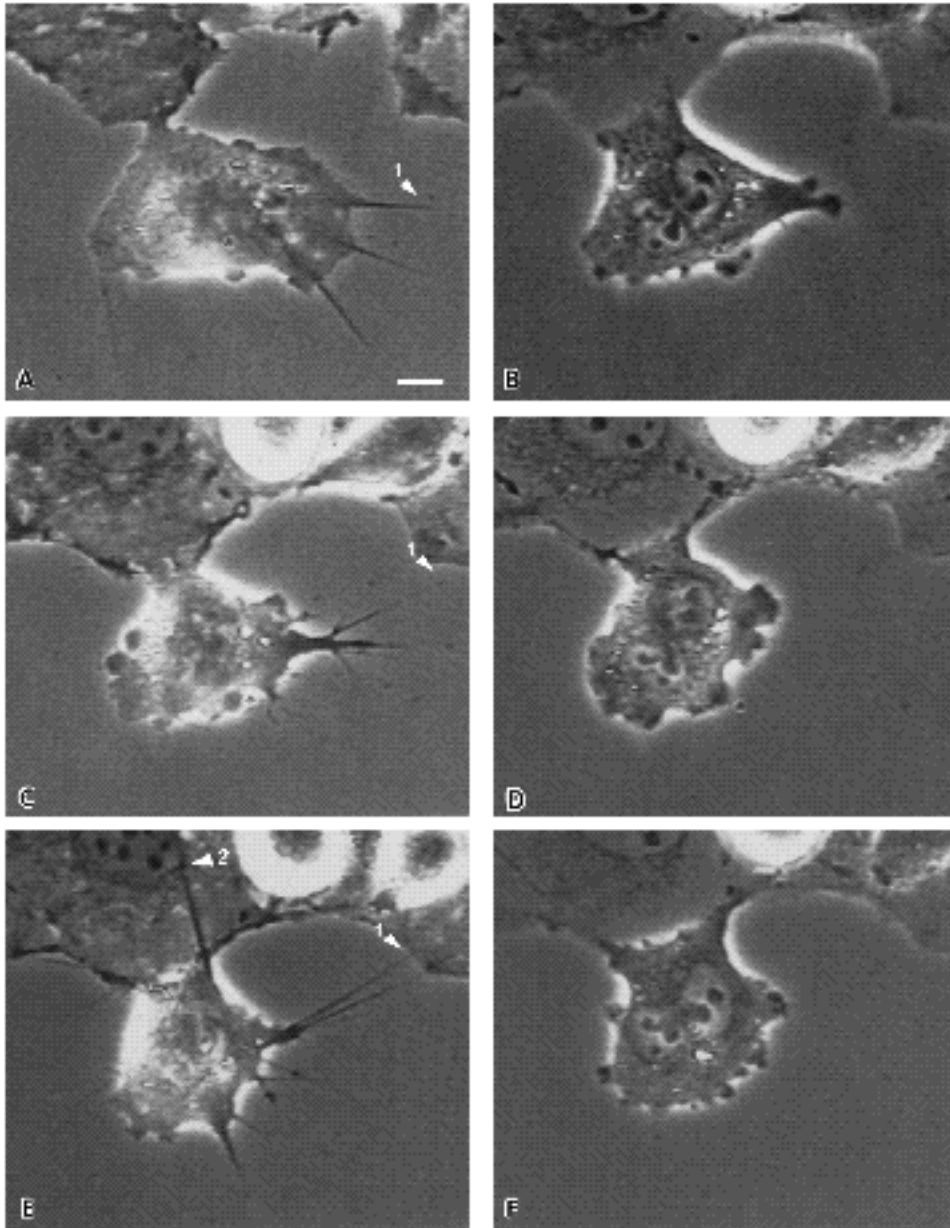
\*Rate, mean rate of elongation or retraction  $\pm$  s.d. (standard deviation of the mean).

<sup>†</sup>Length, length of processes at maximum elongation.

Total number of cells observed was 26.

of neurons and transfected non-neuronal cells that enable them to support process formation? The well-documented stabilising effect of these proteins on cellular microtubules is obviously of importance. Non-neuronal cells lack these MAPs, and the repeated excursions of growth and shrinkage that their microtubules undergo during dynamic equilibrium (Schultze and Kirschner, 1986; Cassimeris et al., 1988; Sammak and Borisy, 1988) are clearly incompatible with the formation of a stable process (Matus, 1988). However, as our results with taxol show, stabilisation alone is not sufficient for process formation to occur. Another consequence of MAP expression at high levels is that microtubules are formed independently of the centrosomal microtubule-organising centre (Weisshaar et al., 1992). Microtubules in neurons are also independent of a recognisable centrosomal organiser (Sharp et al., 1982; McNiven and Porter, 1988), which is important in facilitating their arrangement along the length of very long axons and den-



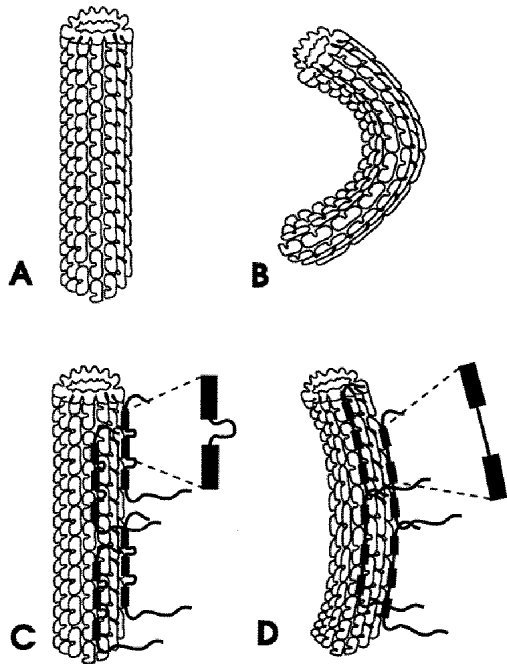


**Fig. 9.** MAP2-transfected cells repeatedly extend and retract processes during cycles of addition and removal of cytochalasin. Video microscopy images during one 4 hour experiment in which cytochalasin was added three times (cycle 1 (A,B), cycle 2 (C,D), cycle 3 (E,F)). Maximum process extension occurred in each case within 15 minutes (A,C,E). Retraction occurred within 5 minutes (B,D,F). Although processes appear at the same location on the cell during each cycle (A,C,E, arrowhead 1) processes may also appear in new locations (E, arrowhead 2) and may be of different length and diameter. Bar, 10  $\mu$ m.

drites (Okabe and Hirokawa, 1988; Baas and Black, 1990). In MAP2-transfected cells, the MTOC-independent microtubules form bundles but this does not appear to depend directly on MAP expression because microtubule bundling is also induced by chemical agents that stabilise microtubules, such as taxol and DMSO (Chapin et al., 1991; Weisshaar et al., 1992). A more significant feature of the microtubule bundles produced by MAP2 is that they are often longer than the diameter of the cell, and it is when this happens that they adopt a curved conformation under the cell surface (Weisshaar et al., 1992). The length of MAP2-containing microtubules is an important factor in their ability to support processes because, as our results show, short microtubule bundles, like those induced by taxol, do not support process formation in cytochalasin-treated non-neuronal cells.

The ability to support processes also depends on an addi-

tional property that MAP2 confers on microtubules, namely that it makes them stiff. The microtubules of non-neuronal cells that lack MAP2 or tau are very flexible, bending readily in the cytoplasm (Cassimeris et al., 1988) and even when stabilised by DMSO they follow a wavy course in the cytoplasm (Weisshaar et al., 1992). By contrast, the microtubules of MAP2-transfected cells have considerable intrinsic rigidity, which shows itself in two ways. Firstly, MAP2-microtubule bundles that are shorter than the diameter of transfected cells have a needle-like straightness. Secondly, the bending of longer bundles when they encounter the cell cortex causes normally polygonal non-neuronal cells to adopt a bulbous shape (Weisshaar et al., 1992), suggesting the interaction of counteractive forces exerted by the semi-rigid rods of the MAP2-reinforced microtubules and the tensile envelope of the actin filament network. It is probably this stiffness that MAP2 imparts to the micro-



**Fig. 10.** The multimeric binding domain of MAP2 increases the rigidity of microtubules. This diagram represents the response of microtubules when they encounter resistance. Without bound MAP2 the microtubule can bend readily (A,B). However, when the multimeric binding domain of MAP2 is applied to the surface (C,D), flexion of the microtubule is limited because neighbouring tubulin subunits are linked by the short amino acid sequences that connect the three 18 amino acid tubulin-binding moieties (black boxes). The length of this connection sets an upper limit on the movement of the subunits relative to one another (expanded views in C and D). For the sake of clarity only four MAP2c molecules are represented; in reassembled microtubules many more MAP2 molecules are applied to the microtubule surface (Amos, 1977).

tubules that drives the emergence of processes when the actin filaments are dissolved, and it seems probable that this same MAP2-induced rigidity makes a significant contribution to the ability of neuronal microtubules to support neurites.

The origin of the rigidifying effect of MAP2 on microtubules is suggested by its molecular structure. The MAP2 tubulin-binding domain is situated near the carboxyl terminus of the molecule and consists of 3 repeats of an 18 amino acid motif separated by flexible linkers of 13 amino acids (Lewis et al., 1988). This region of MAP2 is highly homologous to the carboxy-terminal region of tau, which contains similarly spaced tubulin-binding domains (Lee et al., 1988). Recent experiments with tau indicate that each individual tubulin-binding motif in a single MAP molecule can bind to a separate neighbouring tubulin subunit in the microtubule lattice, effectively linking them to one another (Butner and Kirschner, 1991) and the similar spacing of the tubulin-binding motifs in MAP2 (Lewis et al., 1988) suggest that they have the same function. This subunit cross-linking would impose limitations on the motion of tubulin subunits relative to one another that would not exist in microtubules that lack these MAPs (Fig. 10), and we

hypothesise that this has the effect of lowering their flexibility and raising the compressive force that they are capable of exerting.

### Relationship of MAP2-induced process outgrowth to neurite formation

Stable processes can be induced on neurons by physically 'tugging' on the surface membrane with a micropipette (Bray, 1984), suggesting that the tensile forces generated by actin filaments in the cortical cytoskeleton also operate in neurons. Under normal circumstances, this potentially restrictive influence on neurite elongation is overcome at the growth cone, where actin derived from the cortical cytoskeleton (Schnapp and Reese, 1982; Hirokawa, 1982; Letourneau and Ressler, 1983) is specially organised to produce motile surface properties (Bray and Hollenbeck, 1988; Gordon Weeks, 1988; Smith, 1988). The observation that growth cones adhere to the substratum and pull on it (Harrison, 1914; Letourneau, 1975) contributed to the idea that they promote neurites outgrowth by tugging on the nascent process. However, recent studies have tended to emphasise the steering function of the growth cone (Cypher and Letourneau, 1992) and suggest that the primary function of its adhesion is to provide expansion in a preferred direction where microtubules are assembled and bundled, so that they can form the axial element necessary for neurite consolidation and further extension (Tanaka and Kirschner, 1991). Growth cones contain a surplus of unpolymerised yet assembly competent tubulin (Gordon Weeks et al., 1989), and the microtubules they contain are very labile (Bamburg et al., 1986; Lim et al., 1989) in contrast to the stable microtubule bundles that are present in the newly formed process behind them. When microtubules are formed in growth cones, either during normal growth (Tanaka and Kirschner, 1991) or in the presence of taxol (Gordon Weeks et al., 1989), they do not form bundles or cause protrusions of the growth cone membrane and they are very flexible. Thus increased stability and rigidity of microtubules, together with bundling, is closely correlated with the transition from growth cone to established neurite. Our data suggest that, by conferring these properties on microtubules, proteins of the MAP2 class play a crucial role in laying down the axial skeleton required for the formation of stable neurites. Changes in the activity of these molecules, via their developmentally regulated expression, or through changes in phosphorylation that regulate their binding to tubulin (Matus, 1991), are thus likely to be of major significance in regulating neurite growth.

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