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Bundling of Microtubules in Transfected Cells Does Not Involve an Autonomous Dimerization Site on the MAP2 Molecule

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We have searched for putative dimerization sites in microtubule-associated protein 2 (MAP2) that may be involved in the bundling of microtubules. An overlapping series of fragments of the embryonic form MAP2c were created and immunologically "tagged" with an 11 amino acid sequence from human c-myc. Nonneuronal cells were transfected simultaneously with one of these myc-tagged fragments and with full-length native MAP2c. Immunolabeling with site-specific antibodies allowed the two transgene products to be located independently within the cytoplasm of a single double-transfected cell. All transfected cells contained bundled microtubules to which the full-length native MAP2 was bound. The distribution of the tagged MAP2 fragment relative to these MAP2-induced bundles was determined by the anti-myc staining. None of the fragments tested, representing all of the MAP2c sequence in overlapping pieces, were associated with MAP2-induced microtubule bundles. These results suggest that MAP2-induced bundle formation in cells does not involve an autonomous dimerization site within the MAP2 sequence.

INTRODUCTION

Two sequence-related neuronal microtubule-associated proteins (MAP), MAP2 and tau, can both induce the formation of microtubule bundles when they are expressed in nonneuronal cells by transfection (Kanai *et al.*, 1989; Lewis *et al.*, 1989; Lee and Rook, 1992; Weisshaar *et al.*, 1992; Umeyama *et al.*, 1993). This has been taken as indicating that the high levels of these two proteins in neuronal processes is associated with their involvement in the characteristic arrangement of neuronal microtubules in fascicles (Peters *et al.*, 1976). However, the nature of the contribution that MAP2 and tau make to microtubule bundling is less clear. According to one interpretation, bundling of microtubules by MAP2 and tau involves their forming dimers in the cytoplasm and thus actively cross-linking the adjacent microtubules to which they are bound (Kanai *et al.*, 1989; Lewis *et al.*, 1989). The alternative view is based on the observation that microtubules in nonneuronal cells that do not express MAP2 or tau are also bundled by a variety of chemically dissimilar stabilising agents, including taxol, GTP analogues, and dimethyl sulfoxide (DMSO) (Sandoval *et al.*, 1977; Schiff and Horwitz, 1980; Weisshaar *et al.*, 1992). From this it has been argued that bundling by MAPs reflects their property of stabilizing microtubules, which are then bundled by other ubiquitous cellular proteins (Chapin *et al.*, 1991) or because they have an intrinsic affinity for one another (Lee and Brandt, 1992) or because they are brought together by physical interactions within the cell (Matus, 1991; Weisshaar *et al.*, 1992). The available biochemical evidence is inconclusive. If the MAPs actively cross-link microtubules then differences in physical properties, such as viscosity, would be expected to exist between suspensions of microtubules containing MAPs and pure tubulin polymers. However, no significant differences have been found (Friden *et al.*, 1988; Sato *et al.*, 1988). Evidence regarding the state of purified MAP2 in solution is also conflicting; originally it was reported as being monomeric (Hernandez *et al.*, 1986), but recently antiparallel dimers of MAP2 have been found to exist (Wille *et al.*, 1992).

The dimerization hypothesis of microtubule bundling by MAP2 and tau implies the existence of an interaction site on these molecules that is independent of the tubulin-binding domain. Indeed, the identification of a

unique area required for bundling has been proposed as a test of the validity of MAPs as proteins that directly cross-link microtubules (Lee and Brandt, 1992). We have now devised a means of searching for such putative dimerization sites in living cells, using double transfection with immunologically-tagged recombinant DNA constructs. However, the results of this search, covering the entire MAP2c molecule in overlapping fragments, has failed to reveal any evidence for the involvement of an interaction between MAP2 molecules in the formation of microtubule bundles.

MATERIALS AND METHODS

The MAP2c coding sequence (Doll *et al.*, 1990) was cloned into a eukaryotic vector containing the chicken β-actin promoter (Fregien and Davidson, 1986) using standard techniques (Maniatis *et al.*, 1982). Myc-tagged fragments of MAP2c were prepared by three successive polymerase chain reactions using Taq DNA polymerase (Boehringer-Mannheim, Rotkreuz, Germany) according to the maker's instructions. In each reaction primers were constructed to add part of the required additional sequence, which included the 33 basepairs needed to encode the myc-tag sequence (Munro and Pelham, 1987), a Kozak consensus sequence, and restriction sites to enable subcloning into the β-actin vector. The fidelity of the final products were checked by sequencing using Sequenase (USB, Lucernachem, Lucerne, Switzerland) according to the maker's instructions. Correct expression of the clones was verified by staining transfected cells with antibodies directed against epitopes situated at or near either end of the cloned sequence. Polyclonal antibodies were raised to synthetic oligopeptides coupled to keyhole limpet haemocyanin (KLH) with glutaraldehyde (Harlow and Lane, 1988). The specificity of each antibody was confirm by Western blot analysis (Towbin *et al.*, 1979). Chickens were injected subcutaneously on two occasions separated by 10 d with a peptide corresponding to the initial 14 amino acids of MAP2c coupled to KLH. IgY was isolated from egg yolks by affinity chromatography using the peptide immunogen coupled to affi-gel 10 (Bio-Rad, Glattbrugg, Switzerland) according to the maker's instructions. The dilution of antibodies used for immunohistochemistry was determined for each purified batch by serial dilution assay. Rabbits were immunized with a similar KLH-coupled synthetic peptide corresponding to the carboxy-terminal 14 amino acids of MAP2c. The immune serum was used without further purification at a dilution of 1:900. Myc-tagged constructs were identified using monoclonal antibodies derived from the GE10 hybridoma cell line (ATCC, Porton Down, UK). Second antibodies labeled with either fluorescein or Texas Red were obtained from Jackson Laboratories (Milan Analytica, La Roche, Switzerland) and were used at 1:100 dilution.

Cells of the human hepatoma cell line PLC (ATCC) or HeLa cells were grown in 24-well culture dishes containing 12-mm glass coverslips at 10^4 cells per well. They were transfected with a total of 2 μg of plasmid DNA either individually or in pairs at the ratios described in the text using the calcium phosphate precipitate method (Chen and Okayama, 1987). Cells were fixed with 1% glutaraldehyde for 5 min and stained as previously described (Weisshaar *et al.*, 1992).

RESULTS

Strategy of the Cross-linkage Test

The experimental system is based on the following considerations. First, nonneuronal cells transfected with the MAP2c isoform of MAP2 display prominent microtubule bundles (Weisshaar *et al.*, 1992; Umeyama *et al.*, 1993), showing that the bundling activity is contained within the MAP2c sequence and that any other molecular components necessary for bundling are present in the cells. Second, microtubule binding by MAP2c requires that the entire carboxy-terminal tubulin-binding domain remains intact (see below). Thus MAP2c can be divided into recombinant "halves", such as those shown in Figure 1, each of which lacks the ability to bind to microtubules by itself. Third, if MAP2c contains a cross-linking site, it must necessarily be located outside of the tubulin-binding domain, which is unavailable for cross-linking because it is bound to the wall of the microtubule. Fourth, if such a cross-linking site exists, then a recombinant MAP2c half that cannot bind to microtubules by itself should nevertheless be able to associate with full-length MAP2c that is bound to microtubule bundles.

The experiments consisted of simultaneously transfecting full-length MAP2c and a partial MAP2c construct into nonneuronal cells and asking whether the partial construct remains distributed throughout the cytoplasm, as it does if transfected alone, or whether it now colocalizes with the bundled microtubules. The test also requires a means of distinguishing between full-length MAP2c and the partial MAP2c construct in a single cell. To do this we have raised separate antisera

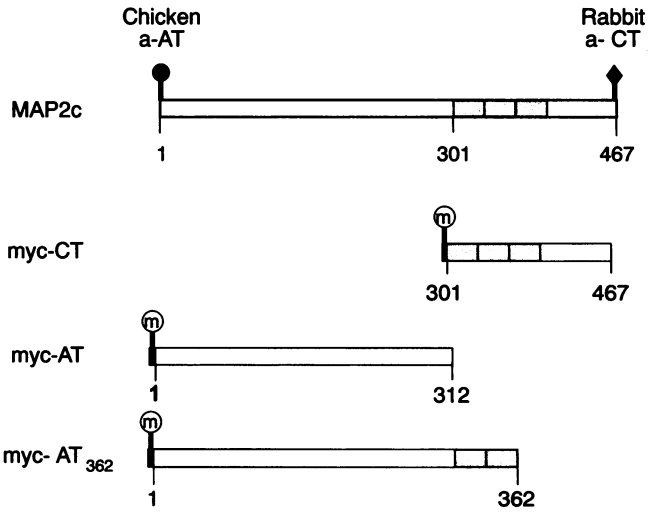

Figure 1. The amino acid sequence structure of the cDNA constructs used in the experiments. The top line shows the full length native MAP2c molecule with the three tandem repeats of the tubulin-binding domain indicated by shading. The epitopes for the amino-terminal and carboxy-terminal antibodies are indicated respectively by the round and diamond-shaped flags. The other three lines show the three overlapping partial fragments used, each of which contains an 11 amino acid tag from the human c-myc sequence (flagged by the round marker with the letter "m") at the amino-terminus. One tagged clone from the MAP2c carboxy-terminus (myc-CT) and two from the amino-terminus (myc-AT and myc-AT_{362}) are shown.

against the two ends of MAP2c, one in chicken against the first 14 amino acids (a-AT in Figure 1), the other against the last 14 amino acids (a-CT in Figure 1). To visualize separately the partial MAP2c constructs, each was provided with an amino-terminal epitope tag consisting of an 11 amino acid sequence taken from the human c-myc coding sequence (Figure 1). The test procedure is shown diagramatically in Figure 2. The nonneuronal cell line is simultaneously transfected with full-length MAP2c and a myc-tagged partial construct. The transfected culture is double-stained by immunofluorescence with mouse monoclonal antibodies against the c-myc epitope and polyclonal antibodies against whichever terminus of MAP2c is not present in the partial construct. Cells expressing both transgene products can be identified because they are stained with fluorophores for both antibodies. The anti-myc tag immunofluorescence reveals whether the partial construct is localized with the microtubules or spread throughout the cytoplasm (Figure 2).

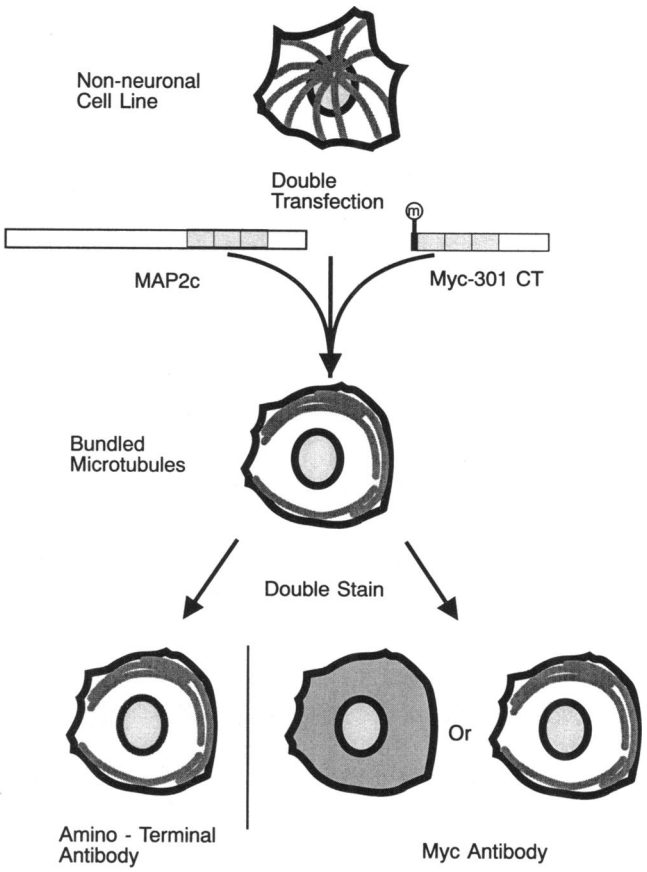

Figure 2. The strategy of the dimerization-site search experiment. Nonneuronal cells were cotransfected with full length MAP2c and one of the myc-tagged constructs shown in Figure 1. A proportion, typically 5% of the cells in any one experiment, expressed both transgene products and showed microtubule bundling induced by the full length MAP2 protein. Staining with chicken or rabbit antibody against either the amino- or carboxy-terminus of MAP2c revealed the location of the native molecule (invariably on the microtubule bundles) independently of the myc-tagged fragment (left cell, bottom line). Simultaneous staining with mouse monoclonal anti-myc antibodies and a different fluophore revealed the location of the tagged fragment, which could in principle be either spread throughout the cytoplasm (middle cell, bottom line), or associated with the microtubule bundles (right cell, bottom line).

Distribution of Partial MAP2 Constructs in Double-transfected Cells

Most experiments were conducted using the human hepatoma line PLC, whose large size enables the distribution of microtubules to be resolved clearly (Weisshaar *et al.*, 1992). Experiments in HeLa cells also gave the same results. All combinations of full-length MAP2c with each of the three partial constructs shown in Figure 1 yielded cells in which both transfected products were expressed. Figure 3 shows doubly transfected cells from experiments using the myc-AT partial construct. In all cells expressing full-length MAP2c microtubules were arranged in bundles with the typical peripheral location previously described (Weisshaar *et al.*, 1992) (Figure 3, left). In the same cells the amino-terminal construct was spread throughout cytoplasm and showed no colocalization with the MAP2c-induced bundles, not even partially (Figure 3, right). Figure 4 shows the same experiment using the myc-tagged carboxy-terminal construct (myc-CT in Figure 1). In this case also, MAP2c-induced microtubule bundles can be readily identified (left) and the coexpressed carboxy-terminal construct shows no hint of association with them (right).

We considered the possibility that the ratio of full-length and partial MAP2 construct products in the cell might influence their ability to interact. For this reason we performed double transfections at ratios of full-length cDNA to partial construct cDNA of 9:1, 5:1, 1:1, 1:5, and 1:9. Some of these results for the myc-AT construct are shown in Figure 5. Note that the preparations were photographed using automatic exposure to visualize all the immunofluorescence-labeled protein products in the cells. At the extreme ratios of 9:1 and 1:9, staining of the product from the more highly concentrated cDNA was much brighter than the less concentrated cDNA. No matter which partner was expressed at the higher level, the amino terminal construct never showed any hint of binding to the MAP2c-induced microtubule bundles. Similar double-transfection experiments were done with varying ratios of MAP2c and the carboxy-terminal construct (myc-CT) and confirmed that myc-CT did not associate with MAP2-induced microtubule bundles over a wide range of relative concentrations in the cell.

Figure 6 shows experiments with a construct, myc-AT_{362} (Figure 1), that overlaps the start of the repeat region of the MAP2 tubulin-binding domain, including two of the 31 amino acid tandem repeats. Over a wide

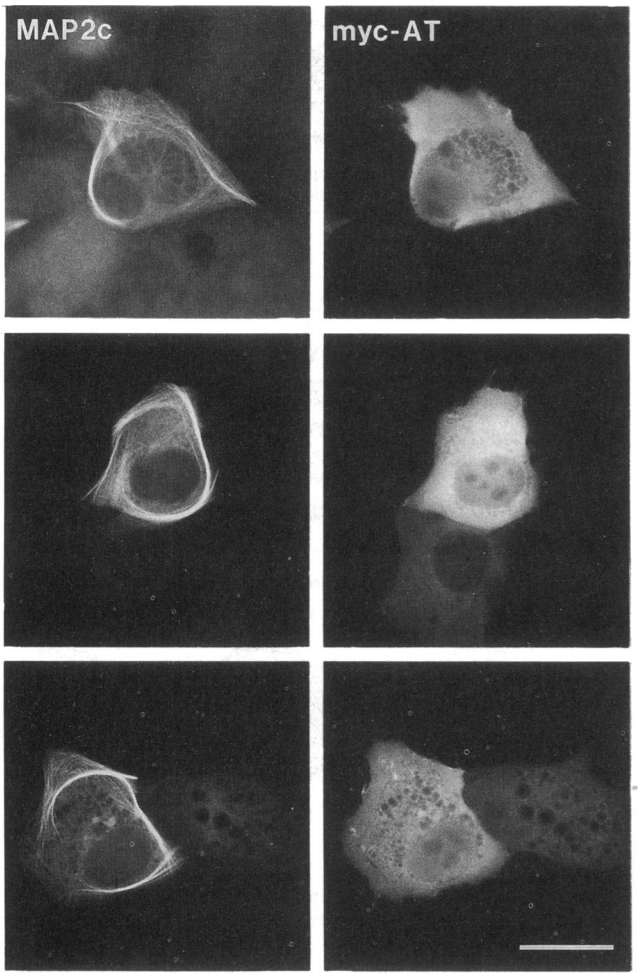

Figure 3. Colocalization of full length native MAP2c (left, stained with rabbit anti-CT) and a myc-tagged amino-terminal fragment of MAP2c (myc-AT, right, stained with mouse anti-myc). Magnification, × 400.

range of concentrations this construct also did not associate with MAP2c-induced microtubule bundles. This indicates that the failure of the AT and CT constructs to associate with MAP2c-containing bundles was not because a cross-linking sequence situated around residues 301 to 312 had been transected in the first two constructs.

DISCUSSION

These experiments show that divided halves of MAP2c do not associate with microtubule bundles containing full length MAP2c, even though in combination they span the entire MAP2c sequence. This is not an artifact of the epitope-tagging procedure because full length MAP2c bearing the same amino-terminal 11 amino acid c-myc tag sequence as the myc-AT and myc-AT_{362} constructs binds to and bundles microtubules in nonneuronal cells in a manner indistinguishable from unmodified native MAP2c (Cravchik and Matus, 1993). The region of MAP2 containing the 18 amino acid repeat motif has been shown to be essential for binding to microtubules (Lewis *et al.*, 1989). Our results show, however, that it alone is insufficient for microtubule binding because the CT construct contains all three repeats, together the rest of the carboxy-terminal sequence of the protein, and it does not bind to microtubules in our experiments. Thus sequence upstream of the repeats is required for tubulin-binding competence, and in this respect MAP2 resembles the other two members of its gene family, tau and MAP4, both of which have been shown to require sequence upstream of the repeat domain for tubulin binding (Aizawa *et al.*, 1991; Lee and Rook, 1992).

The fact that microtubules are bundled in the cells transfected with full-length MAP2c demonstrates that

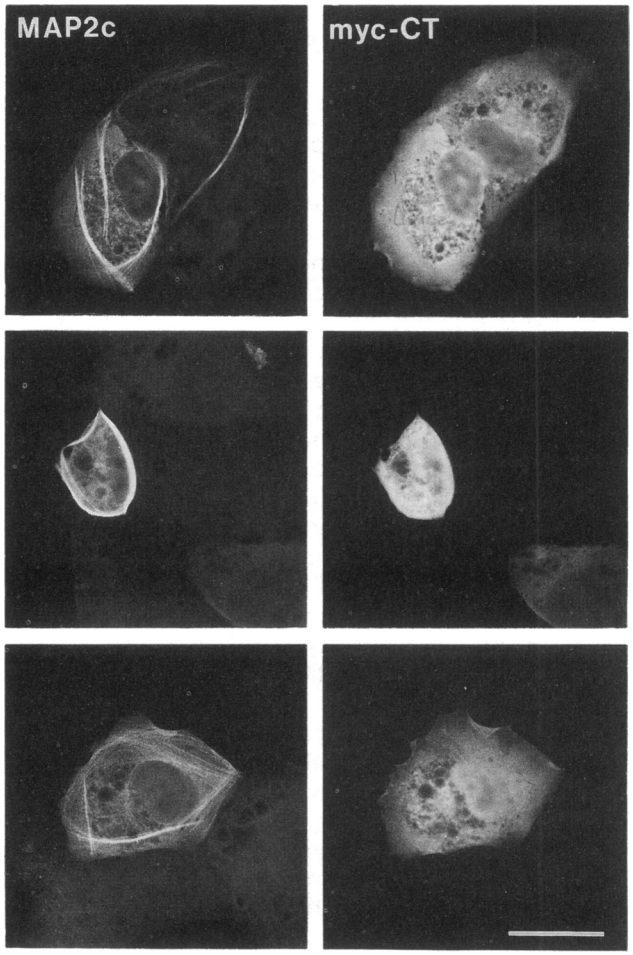

Figure 4. Colocalization of full length native MAP2c (left, stained with chicken anti-AT) and a myc-tagged carboxy-terminal fragment of MAP2c (myc-CT, right, stained with mouse anti-myc). Magnification, × 400.

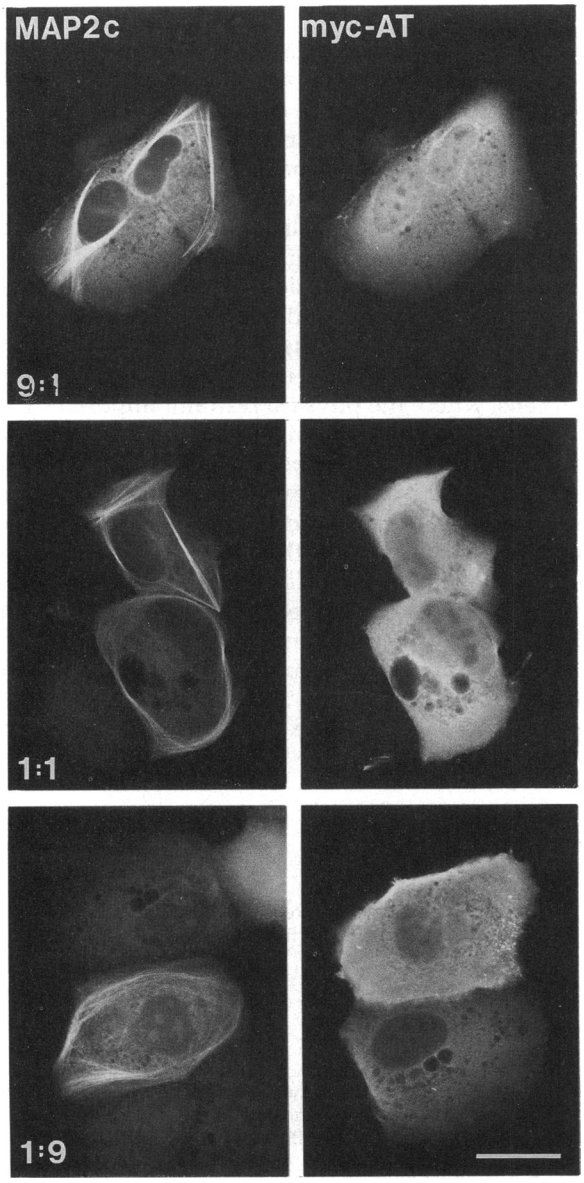

Figure 5. Colocalization of full length native MAP2c (left) and myc-tagged amino-terminal fragment of MAP2c (myc-AT, right) in cultures treated with various ratios of MAP2c to myc-AT cDNA as indicated on the left. Note that the micrographs were made with automatic exposure to compensate for the weakness of fluorescence produced by the less concentrated cDNAs to make their products visible. Other details as in Figure 3.

all the components necessary for bundling are present in the cell and furthermore that they are in the form necessary for bundling to occur. For example, if bundling requires that sequences in MAP2c be phosphorylated, then the existence of MAP2c-containing bundles shows that the appropriate kinases are active in the transfected cells. Similarly, if a third party bridging molecule is needed to mediate interaction between MAP2c molecules then this too is evidently present and active in the cells because MAP2c-containing microtubule bundles are indeed formed. In fact the presence of MAP2c-containing bundles indicates that if bundle formation requires dimerization of MAP2 molecules, either directly or indirectly, then dimerization must have occurred in these cells. The fact that none of the three partial MAP2c clones associated with the MAP2c-containing bundles at any MAP2c to partial construct ratio, even though the entire MAP2c sequence was covered

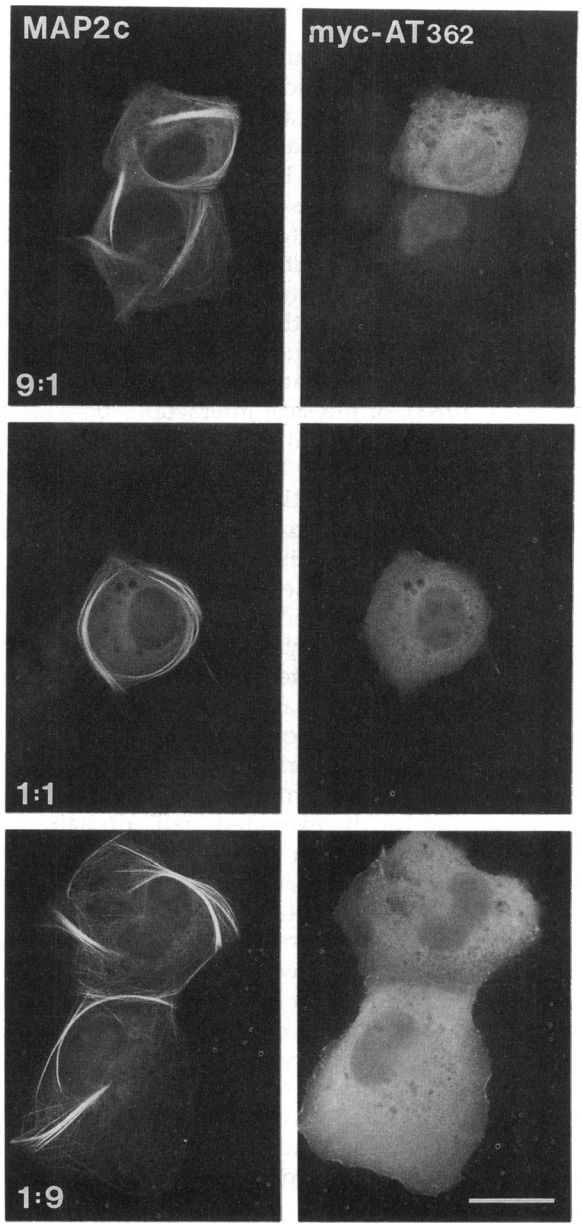

Figure 6. Colocalization of full length native MAP2c (left) and a myc-tagged amino-terminal fragment of MAP2c that includes two repeats of the tubulin-binding domain (myc-AT_{362}, right) in cultures treated with various ratios of MAP2c to myc-AT_{362} cDNA as indicated on the left. Other details as in Figures 3 and 5.

in overlapping partial constructs, leads us to conclude that no such dimerization is needed for bundle formation.

How can these findings be reconciled with the existing evidence for MAP2 dimerization? One possibility is that tubulin polymers have an intrinsic tendency to interact that is not seen in nonneuronal cells because of the conditions of dynamic instability that prevail in the absence of significant levels of stabilizing MAPs (Schulze and Kirschner, 1987; Cassimeris *et al.*, 1988; Sammak and Borisy, 1988). If this were the case, then no dimerizing function of MAP2 would be necessary and its promotion of bundling would reflect its stabilizing effect on microtubules. The induction of microtubule bundles by microtubule stabilizing agent as chemically different as taxol, GTP analogues, and DMSO (Sandoval *et al.*, 1977; Schiff and Horwitz, 1980; Weisshaar *et al.*, 1992) in cells that do not express MAP2 or tau also points to bundling resulting from interactions between stabilized tubulin polymers. Contributions from other factors, such as changes in the surface properties of tubulin polymers when MAPs are bound to them (Lee and Brandt, 1992), cross-linking by ubiquitous cellular proteins such as glyceraldehyde 3-phosphate dehydrogenase (Kumagai and Sakai, 1983), or physical interactions between microtubules and the cortical cytoskeleton (Weisshaar *et al.*, 1992; Edson *et al.*, 1993), could also be involved.

Investigations of interactions between MAP2- or tau-containing microtubules in vitro have reached various conclusions. MAP-containing microtubules do not spontaneously form bundles in vitro, but arrays of parallel microtubules separated by fine filaments are found when MAP-tubulin copolymers were pelleted by centrifugation. Because pelleted microtubules containing pure tubulin or proteolytically cleaved MAPs also form parallel arrays but with no gap between adjacent microtubules (Brown and Berlin, 1985; Kim *et al.*, 1979), this has been most frequently interpreted in terms of a spacer function for the extended "tail" domain of the MAP molecules, although similar findings have been interpreted in terms of a cross-linker function (Hirokawa *et al.*, 1988). In one recent study it was found that in processes formed on baculovirus-transfected cells, high molecular weight MAP2, with its long amino-terminal side-arm, produced larger spacing between microtubules than MAP2c (Chen *et al.*, 1992), whose shorter side-arm is suggestive of a variable spacer function (Papandrikopoulou *et al.*, 1989), which suggests that the determination of intermicrotubule spacing is a significant aspect of MAP2 function. This influence of the length of the amino-terminal portion of MAP2 on microtubule spacing cannot readily be reconciled with previous experiments suggesting that sequences near the carboxyl terminus were essential for cross-linking (Lewis *et al.*, 1989), because the carboxy-terminal sequence is the same in both the long and the short forms of MAP2 (Papandrikopoulou *et al.*, 1989; Doll *et al.*, 1990; Kindler *et al.*, 1991). In bundling microtubules in vitro, fragments of tau and MAP4 (Aizawa *et al.*, 1991; Lee and Brandt, 1992) or MAP2 (Murphy, personal communication) appear to perform as effectively or better than the intact native molecule. This lends further support to the contention that it is the "capping" of the MAP-binding domain of tubulin, together with the accompanying polymer stabilization, that is responsible for the microtubule bundling activity of these molecules. A corollary to this conclusion is that MAP-stabilized microtubules may form bundles as a result of short range weak interactions when bought into physical proximity. Such conditions can occur inside transfected nonneuronal cells, where the physical restraint imposed by the cortical cytoskeleton leads MAP-stabilized microtubules to accumulate in the cell periphery (Weisshaar *et al.*, 1992). These same conditions are the norm inside neuronal processes, where microtubules are many times more densely packed than they are in the cytoplasm of other cells types (Peters *et al.*, 1976). Finally, it is worth considering the potential benefit, in terms of neuronal cell function, of having microtubules that are only weakly interactive and would thus present no barrier to the constant and highly active particle traffic along the processes that is borne by the microtubule-based motor-molecule systems.

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