Cellular Regulation of Microtubule Organization

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Microtubules are constituents of axonemes, mitotic spindles, and elaborate arrays in interphase cells, and, with intermediate filaments and microfilaments, are among the most prevalent structures visualized in the cytomatrix (22, 44). With the exception of the A microtubule of cilia and flagella, the lattice geometry of microtubules is highly conserved. However, each of the major subunits of microtubules, α- and β-tubulin, shows heterogeneity. The number of α- and β-tubulin subspecies differs among tissues and organisms, and a number of types of analysis are used to examine how these tubulin variants are related to specific cell functions (1, 9–11, 33, 40). Investigations of the number and complexity of genes coding for these polypeptides have also been initiated (see reference 13 for review). However, the mechanisms that regulate the posttranslational compartmentalization of subunits, the spatial and temporal assembly of subunits into microtubules, and the integration of microtubules in various cellular events are still largely unknown.

There are many levels at which the formation and organization of microtubules might be determined. A postulate originating from early analyses of mitotic spindle formation (32) was that a pool of subunits existed in equilibrium with formed microtubules; increases in the subunit concentration could therefore result in a net increase in polymer. With few exceptions, however, a rapid increase in the total tubulin pool does not appear to occur before the elaboration of more extensive microtubule arrays. For example, our studies (42, 50) have demonstrated that mouse neuroblastoma cells possessing microtubule-filled neurites contain four to five times more tubulin polymer than rounded, nondifferentiated cells, but the total tubulin content of these two cell types is the same. On the basis of volume calculations, the equilibrium concentration of subunits in the nondifferentiated cells is at least twice that in differentiated cells. Data such as this indicate that a simple equilibrium between subunit and polymer cannot account for the changes in microtubule formation coordinated with certain cellular events. In addition, recent findings show that an increase in the subunit concentration in cells, brought about either by drug treatment (15) or injection of tubulin (16), results in a depression of tubulin synthesis and the loss of tubulin mRNA. These data suggest that cells autoregulate the total tubulin pool and that this may be effected by "monitoring" of the monomer concentration (14).

Although the monomer concentration in cells can be quantified, it is not known whether all subunits measured are equivalent in their potential to be incorporated into microtubules. For example, it is possible that subtle changes in subunits promote or prevent formation of stable nucleation centers or microtubule elongation. Expression of critical amounts of certain of the variants might trigger changes in distribution. Posttranslational modifications, such as occupancy of the guanine nucleotide binding sites, GTPase activity, or phosphorylation, could change the conformation of the subunits such that integration into or stabilization of the lattice is affected. Recent findings (27) indicate that there is a correlation between the phosphorylation of a β-tubulin variant in neuroblastoma cells and its incorporation into polymer. Whether this or other types of modification of tubulin is significant in the general regulation of polymer formation remains to be determined.

Proteins that interact with subunits or polymers could also affect the distribution of the tubulin pool. Proteins favoring microtubule formation might (*a*) stabilize or concentrate subunits in a defined region such that the critical concentration for assembly is exceeded; (*b*) function as templates for the initiation of nuclei from which elongation occurs; or (*c*) add to microtubules during assembly and stabilize the polymer. A number of studies have suggested that a class of proteins, termed microtubule-associated proteins, favor the formation of microtubules in vitro by lowering the critical concentration for assembly and stabilizing microtubules (17, 34, 39, 45, 46); the properties of some of these proteins will be discussed in this paper and elsewhere in this volume (49). Conversely, proteins might exist that depress polymer amount. Such proteins might bind to the ends of microtubules, preventing further elongation, or exist complexed with subunits such that initiation or elongation of microtubules could not occur. These types of molecules have been found to affect the organization of actin (36, 47, 53), but similar molecules that might be involved in the regulation of microtubules have not yet been identified. Tubulin subunits have sites for the binding of antimitotic drugs, such as colchicine, and it is conceivable that cellular molecules analogous to these substances exist.

Cells regulate not only the extent of polymer formation but also the strict geometric organization of microtubules within the cytoplasm. A number of studies have shown that microtubules possess an intrinsic polarity (23–25, 29, 30, 48), and

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others have demonstrated that subunits add preferentially to one end of the microtubule and are lost from the other end (2, 36, 37). Thus, once a microtubule is initiated, elongation in a given direction is dictated. A higher level of organization may be imposed by microtubule organizing centers (MTOC);[1] these may be sites from which microtubules initiate or regions with which microtubules interact (6). The two most prominent organizing centers in cultured mammalian cells are the centrosome and the kinetochore. However, little is known about the mechanisms by which tubulin becomes associated with these regions and how the capacity to organize microtubules is mediated. Other MTOC of less defined structure may also exist. For example, in cells treated with the microtubule-stabilizing drug, taxol, microtubule bundles end abruptly and center around regions that are structurally amorphous (19, 31). Whether these regions are analogous to centers that normally exist in cells for the initiation or organization of microtubules is unknown. A tertiary level of organization is postulated to arise from the interaction of microtubules with other organelles or with elements of the cytomatrix. Although the biochemistry of these interactions is not yet defined, it is this level of organization that is likely to result in the regular pattern of interphase or mitotic microtubules visualized in all cell types. The following discussion focuses on some of our efforts to identify some of the molecules involved in both the spatial and temporal establishment of this microtubule array.

Microtubule-associated Proteins in Neuroblastoma Cells and Mouse Tissues

Studies (17, 34, 39, 45, 46) on microtubule assembly in brain extracts have defined a group of proteins that coassemble with microtubules in constant stoichiometry and favor the assembly of tubulin into polymer in vitro. These proteins have been called microtubule-associated proteins (MAP). To investigate whether proteins having a role in the organization of microtubules during neurite elaboration might be identified, we have undertaken an examination of MAP in neuroblastoma cells. Using D_2O to favor microtubule formation, we identified proteins copurifying with tubulin from extracts of differentiated neuroblastoma cells (43). A number of proteins coassembled with microtubules through multiple cycles of assembly, but only one, with a molecular weight of 215,000, copurified stoichiometrically and quantitatively with polymerized tubulin. This protein was not purified if microtubule assembly was inhibited and therefore had characteristics typical of MAP.

No polymerization of microtubules was obtained from extracts of neuroblastoma cells that did not possess neurites. Two-dimensional gel analyses of stained or isotopically labeled material indicated that the 215-kdalton MAP was not detectable in rounded cells, and if present, it was in amounts at least 10 times lower relative to tubulin than in the neurite-bearing cells (43). As shown in Fig. 1, the synthesis of the 215-kdalton MAP increases when rounded cells are plated onto a substrate, whereas synthesis in replated, neurite-bearing cells is constant. These data suggest that there is a correlation between the synthesis of this protein and the shift in the total tubulin pool toward polymer formation. An analogous result has been observed in rat pheochromacytoma cells (PC12). These cells elaborate neurites when incubated with nerve growth factor, and both the synthesis and phosphorylation of MAP 1 increase during this response (28). All of these data suggest that increases in the amount and/or modification of MAP parallel cellular events in which microtubule organization is increased. As discussed below, at least a portion of these MAP decorate the surface of microtubules in situ. In this capacity, the MAP may serve to stabilize formed microtubules as they elongate and/or mediate the interaction of microtubules with other elements in the cytoplasm.

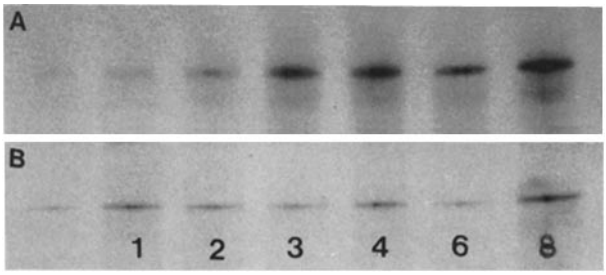

FIGURE 1 Synthesis of 215-kdalton MAP in neuroblastoma cells. Cells that had been growing either in suspension (*A*) or monolayer (*B*) culture were plated onto substrates and pulsed with [^{35}S]methionine (100 μCi/ml) for 2 h at 0, 1, 2, 3, 4, 6, and 8 h after plating. The figure is an autoradiogram of material immunoprecipitated with the 215-kdalton MAP antiserum from equal number of cells. The total incorporation of amino acid into TCA precipitable counts was the same for all points. The synthesis of the 215-kdalton protein is relatively constant in replated, differentiating cells but increases with time of plating on a substrate and neurite elaboration in cells originally grown in suspension.

To examine the distribution of the 215-kdalton protein in cells and tissues, immunologic approaches have been employed. As shown in Fig. 2, antisera raised to the 215-kdalton protein labels microtubules formed in vitro and microtubule arrays in vivo. These data demonstrate that this protein, defined as a MAP in vitro, also exists in association with microtubules in normally growing cells. Whether the distribution of this protein is only on microtubules still must be established. For example, taxol-treated neuroblastoma cells stained with the 215-kdalton antiserum show labeling both in microtubule bundles and in discrete perinuclear spots with which microtubules are associated.[2] Antiserum to high molecular weight MAP from brain has been shown to localize to regions where dendrite elaboration is occurring (3), and MAP 2 to redistribute with intermediate filaments when microtubules are depolymerized by drug treatment (4). While MAP may exist predominantly on the surface of microtubules, these other patterns suggest that dynamic alterations in the distribution or function of these proteins occur during morphogenetic or physiologic changes in cells.

A number of MAP have been identified in cultured cells that have molecular weights ranging from 200,000 to 220,000. Cycles of assembly have defined a 210-kdalton MAP in HeLa cells (7, 8, 51, 52), and selective extraction techniques have shown that 220-kdalton MAP exist in a number of rodent cells (21). Antibodies raised to the 210-kdalton MAP were found to react only with cells of primate origin (8). Conversely, the antisera that we raised to the mouse 215-kdalton protein did not react with the 210-kdalton HeLa MAP.[2] Moreover,

[1] *Abbreviations used in this paper:* MAP, microtubule-associated proteins; MTOC, microtubule-organizing centers.

[2] Olmsted, J. B., 1982, *J. Cell Biol.* 95(2 Pt. 2):345a, and unpublished results.

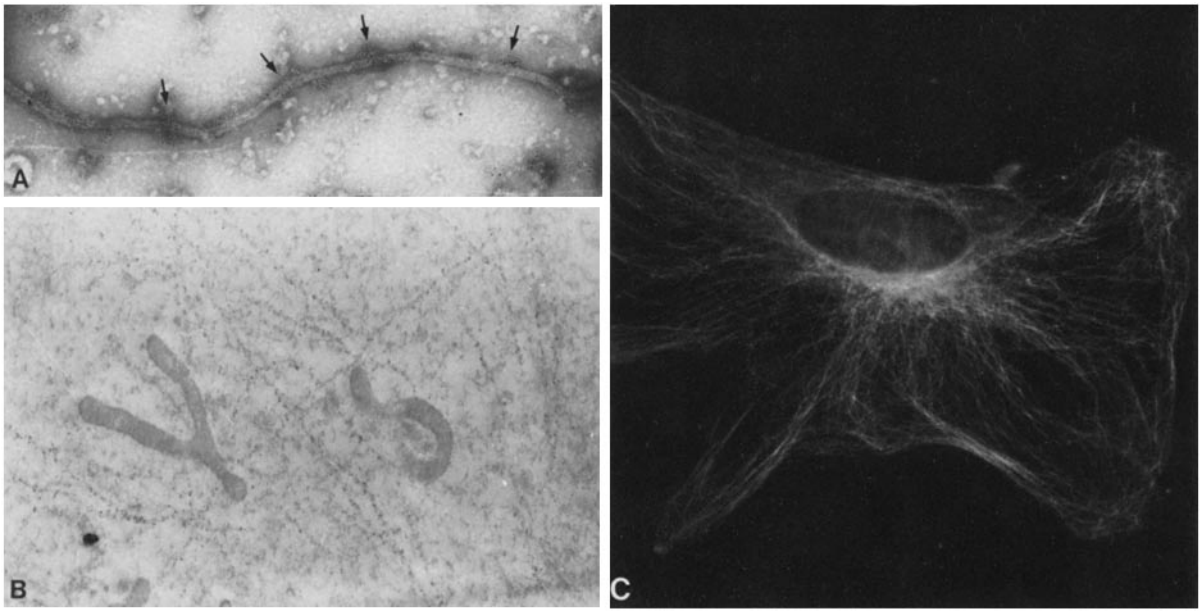

FIGURE 2 Immunolabeling of 215-kdalton protein. (*A*) Immunogold labeling (1) with 215-kdalton antiserum of microtubules formed in extracts of mouse 3T3 cells. Arrows point to region of 5-nm gold decoration. × 40,000. (*B*) Immunogold labeling (20) of microtubules in 3T3 cells incubated with 215-kdalton antiserum. Microtubules are extensively decorated. × 20,000. (*C*) Immunofluorescent labeling (1) of 3T3 cells with antibodies affinity purified (41) from the 215-kdalton complex on blots. × 700.

the antiserum showed no reaction with other rodent cells. These data indicate that the 200- to 220-kdalton MAP have homologous functions but are immunologically, and perhaps evolutionarily, diverse.

The sensitivity of immunoblotting in detecting minor reactive species has recently made it possible to identify complexity in the 215-kdalton MAP. As shown in Fig. 3, material at 215 kdaltons is resolved into several bands, and all of these species coassemble with microtubules in vitro. However, stained gels of cycled microtubules show only a single band at 215 kdaltons (43); this single band corresponds to the uppermost band seen by immunoblotting. The lower two peptides are rarely detectable on gels, suggesting they are minor constituents of the MAP complex. Affinity purification of antibodies (41) from each of the bands has shown that all of the bands are antigenically related.[3] Proteolysis inhibitors have little effect on the appearance of these proteins, suggesting that this complexity is not generated in vitro. In addition to coassembling with microtubules, all bands of the triplet are stable to boiling, indicating that they share molecular properties. At the simplest level, this complexity might arise either from the existence of a family of genes, one of which is more actively expressed, or from the postranslational modification or proteolytic processing of a single polypeptide. How these polypeptides are regulated and distributed in cells and whether the minor polypeptides have functions distinct from the major one is unknown.

The distribution of the 215-kdalton MAP in tissues isolated directly from mouse has also been examined.[3] The immunoreactive species show not only the same complexity as in neuroblastoma cells but also variations among tissue types. Immunoblotting has demonstrated that the 215-kdalton MAP antiserum reacts with a triplet of polypeptides in extracts from

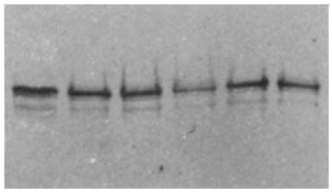

FIGURE 3 Immunoblot of microtubule fractions stained with 215-kdalton MAP antisera. Lanes left to right: X, C_1S, C_2S, C_3S, H_1P, H_2P obtained by D_2O-driven assembly of microtubules in neuroblastoma extracts (43). The 215-kdalton serum reacts with a number of bands, all of which cycle with microtubules and all of which are immunologically related (see text).

adult brain, heart, liver, and lung (Fig. 4*A*). In contrast, kidney, spleen, and stomach contain only a single, lower molecular weight band (band 4) reacting with the antiserum. As demonstrated with antibodies affinity purified from blots, band 4 is immunologically related to the triplet and also is present in all tissues positive for the triplet. Skeletal muscle, sperm, and peripheral blood contain no reactive polypeptides. When microtubules are prepared from any of these tissues, the 215-kdalton triplet associates with microtubule fractions, whereas band 4 does not. These data indicate that some tissues contain no 215-kdalton homologues, that some tissues contain 215-kdalton MAP similar to those seen in cultured cells, and that other tissues contain a protein that does not associate with microtubules in vitro but that is immunologically related to the 215-kdalton MAP. The biological significance of the distribution of different polypeptides antigenically related to the 215-kdalton MAP from mouse neuroblastoma is still unknown. However, on the basis of the data that demonstrate that band 4 does not associate with microtubules in vitro, one can speculate that the polypeptides antigenically similar to the 215-kdalton MAP have functions that are not related to the binding of these proteins to the surface of microtubules in vivo.

[3] Parysek, L. M., C. F. Asnes, and J. B. Olmsted. MAP 3: characterization, occurrence in mouse tissues, and changes in complexity during brain development. Submitted for publication.

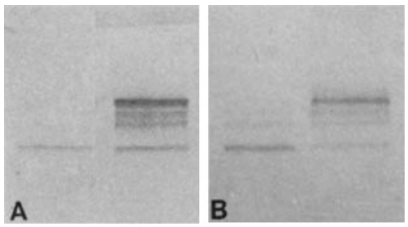

FIGURE 4 Immunoblots of mouse tissues with 215-kdalton antiserum. (*A*) Immunoblots of extracts prepared from kidney (*left*) and lung (*right*). Kidney contains only band 4, whereas lung contains the 215-kdalton triplet and band 4. (*B*) Immunoblots of extracts prepared from 17-d fetal liver (*left*) and adult liver (*right*). Fetal liver contains band 4 predominantly and shows faint reaction with the 215-kdalton triplet. Adult liver contains band 1 predominantly and lesser amounts of bands 2, 3, and 4 (see text).

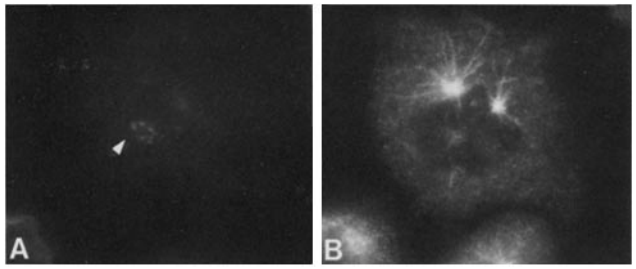

FIGURE 5 Lysed cell models incubated with anticentromere sera. Double immunofluorescence of PtK_1 cells stained for centromere antibodies (*A*) and tubulin antibodies (*B*). Note the formation of microtubules at the spindle poles and the absence of microtubule formation in the region of centromeres (arrowhead in *A*). See text and reference 18 for experimental details. × 990.

An analysis of tissues taken from mice at various stages of development has demonstrated that the complexity and occurrence of the 215-kdalton MAP triplet changes with age.[3] Extracts derived from brain show that 13-d fetal mice contain only the major 215-kdalton species, whereas 17-d fetal mice have both bands 1 and 2, and newborn mice contain all three bands. As mice reach adulthood (6 weeks), the amount of band 3 diminishes. As shown in Fig. 4*B*, changes in the distribution of the material reacting with 215-kdalton MAP sera are also seen in liver. In 17-d fetal liver, band 4 predominates, whereas adult liver contains the triplet and lesser amounts of band 4. The variety of changes occurring both within tissues and between tissues suggests that the polypeptides reactive with the 215-kdalton antiserum have various tissue-specific functions during development.

Composition of the Kinetochore, an MTOC

The centrosomes of interphase and mitotic cells and the kinetochore of mitotic cells are cellular components defined as MTOC. Although the morphology of the kinetochore of mammalian cells is well defined, little is known about the composition of this structure. The discovery that patients with the CREST variant of scleroderma have antibodies reacting with the centromere (26, 38) has provided a convenient probe for the analysis of this chromosomal region. Immunoelectron microscopy has shown that these sera react with the inner and outer plates of the kinetochore in metaphase chromosomes and with amorphous material in interphase nuclei; the interphase "prekinetochores" double in late G_2 (5). These studies demonstrated that the antisera reacted with discrete entities in both interphase and mitotic cells, even though this region undergoes a structural transition at the onset and termination of mitosis.

To resolve some of the components in interphase prekinetochores and mitotic kinetochores, we undertook identification of the antigens reacting with the anticentromere sera (18). Immunoprecipitates obtained from both chromosomes and interphase nuclei showed that the antisera reacted with proteins of 34, 23, 20, and 14 kdaltons. Preabsorption of the antiserum with any of these proteins resulted in the loss of centromere staining and indicated that all of these antigens were localized at the centromere. We also prepared matrices (12) from both mitotic and interphase cells from which the bulk of the DNA, RNA, and nuclear proteins had been removed. Immunofluorescent staining of these preparations revealed patterns that appeared identical to those of intact cells. Immunoprecipitates from matrices were similar to those obtained from intact cells, except the amount of 14-kdalton protein was greatly reduced. Although the identity and function of the nuclear matrix in vivo is still debated, these data indicate that the 20- and 23-kdalton antigens are tightly complexed with this preparation in vitro. Although the loss of the 14-kdalton protein from matrices after nuclease treatments suggests that this protein is complexed with nucleic acid, the role of any of these proteins in the transition of interphase prekinetochores to become the trilaminar kinetochore active in microtubule organization remains to be determined.

To establish whether the anticentromere sera might also be useful in understanding the mechanism by which microtubules interact with the kinetochore, the effect of the antisera on microtubule organization in lysed cell models was examined (18). In these preparations, cells are gently permeabilized, and endogenous tubulin is removed by dilution. Under the appropriate conditions, exogenous tubulin forms into microtubules only in association with cellular organizing centers. When either normal or anticentromere serum was added to the lysed cells before the addition of exogenous tubulin, microtubules formed at the centrosomes of interphase and mitotic cells. In mitotic cells, microtubules were associated with centromeres in samples that had been incubated with normal serum but did not form in lysed cells preincubated with anticentromere sera (Fig. 5). These data suggest that the centromere antisera bind at or near a chromosomal site important for the organization of microtubules. In addition, because the anticentromere serum did not interfere with the formation of microtubules at the centrosome, it is likely that the compositions of the two mitotic organizing sites are at least partially different. By using this serum in microinjection experiments, it should be possible to establish whether microtubule formation is initiated before interaction with the kinetochore or whether they form at the site and grow with a polarity opposite to that determined from in vitro studies (for discussion of this problem, see reference 25).

The studies described above indicate that the microtubule organization can be regulated at multiple levels and that the complexity of this regulation in cells and tissues is just starting to be understood. Whereas the existence of the specific polypeptides that associate with microtubules assembled in vitro has been discerned, the location of molecules that affect the overall organization of microtubules remains unknown. Polysomes synthesizing tubulin, enzymes that modify tubulin or MAP, proteins involved in nucleation or stabilization of

microtubules, and elements linking microtubules to other cellular organelles may all comprise portions of the cytomatrix. How this array of macromolecules is coordinated and localized for particular cellular functions awaits further investigation.

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