Generation of Antisera That Discriminate among Mammalian α-Tubulins: Introduction of Specialized Isotypes into Cultured Cells Results in Their Coassembly without Disruption of Normal Microtubule Function

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Abstract. To assay the functional significance of the multiple but closely related α-tubulin polypeptides that are expressed in mammalian cells, we generated three specific immune sera, each of which uniquely recognizes a distinct α-tubulin isotype. All three isotypes are expressed in a tissue-restricted manner: one (Ma3/7) only in mature testis, one (Ma4) mainly in muscle and brain, and the third (Ma6) in several tissues at a very low level. A fourth specific antiserum was also generated that distinguishes between the tyrosinated and nontyrosinated form of a single α-tubulin isotype. Because individual tubulin isotypes cannot be purified biochemically, these sera were raised using cloned fusion proteins purified from host Escherichia coli cells. To suppress the immune response to shared epitopes, animals were first rendered tolerant to fusion proteins encoding all but one of the known mammalian α-tubulin isotypes. Subsequent challenge with the remaining fusion protein then resulted in the elicitation of an immune response to unique epitopes. Three criteria were used to establish the specificity of the resulting sera: (a) their ability to discriminate among cloned fusion proteins representing all the known mammalian α-tubulin isotypes; (b) their ability to uniquely detect α-tubulin in whole extracts of tissues; and (c) their capacity to stain microtubules in fixed preparations of cells transfected with sequences encoding the corresponding isotype. The transfection experiments served to demonstrate (a) the coassembly of Ma3/7, Ma4, and Ma6 into both interphase and spindle microtubules in HeLa cells and NIH 3T3 cells, and (b) that the Ma4 isotype, which is unique among mammalian α-tubulins in that it lacks an encoded carboxy-terminal tyrosine residue, behaves like other α-tubulin isotypes with respect to the cycle of tyrosination/detyrosination that occurs in most cultured cells.

Microtubules mediate a broad range of both essential and specialized biological functions in the cells of higher eukaryotes. These functions include, for example, the segregation of chromosomes during meiosis and mitosis; the maintenance of cell shape via dynamic modulation of cytoplasmic microtubules; cell motility, as an integral part of cilia and flagella; and intracellular transport, involving the bidirectional shunting of organelles along axons. Several possible factors could contribute to this functional diversity. First, the two major soluble microtubule proteins, the α- and β-tubulins, are each encoded by multigene families (Cleveland et al., 1980; Lee et al., 1983). In mammalian species, these multigene families encode about six α- and β-tubulin polypeptides, termed isotypes, that are distinct from one another by virtue of one or more amino acid substitutions in the polypeptide chain (Hall et al., 1983; Lewis et al., 1985b; Villasante et al., 1986; Wang et al., 1986; Sullivan and Cleveland, 1986). In principle, therefore, genetically encoded differences between tubulin isotypes could contribute to microtubule function. Second, in addition to the α- and β-tubulins, microtubules possess accessory proteins (microtubule-associated proteins) whose number and composition vary among functionally distinct kinds of microtubule (e.g., Huber and Matus, 1984; Bloom et al., 1984; Parysek et al., 1984; Binder et al., 1985). Third, there are populations of microtubules in which the tubulin proteins themselves undergo specific posttranslational modification (e.g., L'Hernault and Rosenbaum, 1983; Barra et al., 1974; Raybin and Flavin, 1977; Argarana et al., 1978). However, the extent to which these (and most likely other) determining factors contribute to the diversity of microtubule function remains essentially unknown.

An intriguing paradox exists with regard to the functional significance of the expression of multiple α- and β-tubulin isotypes. Our analysis of these isotypes in mouse (Lewis et al., 1985b; Villasante et al., 1986; Wang et al., 1986) and man (Hall et al., 1983; Cowan et al., 1983; Lee et al., 1983; Lee et al., 1984; Lewis et al., 1985a) taken together with data...
from other mammalian species (Elliot et al., 1986; Farmer et al., 1984) shows that, in general, their characteristic sequences (in particular, a heterogeneous region spanning the 15 carboxy-terminal amino acids) as well as their patterns of expression have been rigidly conserved since the mammalian radiation. A single major exception to this interspecies conservation rule, namely the 10% sequence divergence of a hematopoietic-specific β-tubulin isotype since the mammalian radiation (Cowin et al., 1987), merely serves to underline the extent of the selective pressure operating on the other isotypes. An obvious possible explanation for the interspecies conservation of isotype sequences would be that different tubulin isotypes themselves contribute to the functional distinctions among microtubules by a mechanism involving either segregation or selective enrichment. However, neither genetic experiments on lower eukaryotic species nor the development of antisera that discriminate among several vertebrate β-tubulin isotypes have provided evidence to support this idea. For example, in Drosophila, studies on mutations in a single β-tubulin gene have shown that this isotype contributes to all the types of microtubule involved in spermatogenesis in that organism (Kemphues et al., 1982; Fuller et al., 1987). In yeast, although the two α-tubulin genes in Saccharomyces cerevisiae are 10% divergent (Schatz et al., 1986a), each isotype on its own can support all the microtubule functions involved in yeast cell growth and division (Schatz et al., 1986b) while one of the two α-tubulins in the yeast Schizosaccharomyces pombe is nonessential (Adachi et al., 1986). In Aspergillus nidulans the β-tubulin gene expressed in vegetative cells can substitute for that expressed in conidiation (May et al., 1985; Weatherbee et al., 1986). Finally, experiments with β-tubulin isotype-specific antisera have shown that at least in cultured mammalian cells, microtubules are mixed copolymers of all expressed α-tubulin isotypes (Bond et al., 1986; Lewis et al., 1987; Lopata and Cleveland, 1987).

The patterns of expression of β-tubulin isotypes are not paralleled by corresponding patterns among α-tubulin isotypes; that is, α- and β-tubulin isotypes are not expressed in pairs. This lack of correspondence, plus the fact that α-tubulin is capable of undergoing two unique kinds of posttranslational modification (i.e., acetylation of lysine residues [e.g., L'Herault and Rosenbaum, 1985a] and cycles of carboxy-terminal tyrosination and detyrosination [e.g., Gundersen et al., 1987]), prompted us to examine whether mammalian α-tubulin isotypes, like their β-tubulin counterparts, freely assemble into functionally distinct microtubules. Here we report the generation of antisera that discriminate among several distinct mammalian α-tubulin isotypes, and their use to analyze microtubules formed after their introduction into tissue culture cells by transfection.

Materials and Methods

Engineering of Constructs Designed to Express α-Tubulin Isotypes

To express α-tubulin isotypes as cloned fusion proteins in Escherichia coli, 3' Eco RI fragments from cDNAs encoding Ma1, Ma7, Ma4, and Ma6 (Villasante et al., 1986) were cloned into the Eco RI site of the inducible expression vector pATHII (generously provided by T. Koerner). The fusion proteins expressed by these constructs are diagrammed in Fig. 1 A. Two of these constructs were altered by site-directed mutagenesis and are shown in Fig. 1 B. A mismatched antisense oligonucleotide 5'AGGCTCCAG- TACCCTAGTACTC TTCCTCCCT 3' was synthesized and used by the procedure of Kunkel (1985) to add a tyrosine codon to the carboxy terminus of the trpE/Ma4 fusion protein, while the antisense oligonucleotide 5'CCGC- ATGCCTACA TACTC TTCCTCCTCCT 3' was used to change the encoded carboxy-terminal tyrosine residue of the trpE/Ma3/7 construct to a stop codon. (Mismatched residues are underlined in the oligonucleotide sequences.)

The fusion protein sequences were altered through the cloning of a 250-bp Sph I-Bam HI fragment from each construct into MI3, and checked by dideoxy sequencing (Sanger et al., 1980).

To express α-tubulin isotypes in cultured cells, full-length cDNAs encoding Ma3/7, Ma4/5, and Ma6 (Villasante et al., 1986) were inserted into the eukaryotic expression vector pSV-dhfr (Mulligan and Berg, 1981), replacing the dhfr-encoding sequences. Hybrid Ma3/7-Ma6 constructs were made by substituting either the 5' or 3' Eco RI fragment of the Ma6 cDNA in the pSV expression plasmid with the homologous fragment from the Ma7 cDNA. In all these constructs, tubulin cDNA sequences are flanked by SV-40 tissues promoter and terminator sequences. Transfection was by the method of Wigler et al. (1979).

Generation of α-Tubulin Isotype-Specific Sera

Five of the six cloned fusion proteins described above were used either as tolerogens or as immunogens to raise isotype-specific antisera in rabbits and guinea pigs. The method was as described in Lewis et al. (1987) with two modifications: (a) the cyclophosphamide dose used for guinea pigs was 75 mg/kg on 3 successive days, and (b) the animals received two (rather than one) injections of immunogen in Freund's complete adjuvant with a 2-wk interval before the final boost.

Affinity purification of the sera was accomplished by absorption onto and elution from nitrocellulose strips cut from Western blots of SDS gels heavily overloaded with whole extracts of E. coli expressing the appropriate trpE/α-tubulin isotype fusion protein. Briefly, the procedure was as follows. Strips were cut into small squares and blocked for 0.5 h in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 3% BSA and 0.04% Na azide. Approximately 1 ml of whole serum was incubated
Specificity of antisera to mammalian α-tubulin isotypes. Cloned sequences encoding the carboxy-terminal portions of Mal/2, Ma3/7, Ma4, or Ma6 were fused to sequences encoding E. coli trp E (see Fig. 1) and expressed in host E. coli cells. The resulting fusion proteins were purified and used to generate affinity-purified isotype-specific antisera (see Materials and Methods). The specificity of each of these antisera was tested against whole extracts of bacteria expressing all four fusion proteins (A) and against whole extracts of mouse tissues (B).

Results

Generation of Specific Antisera to Three Mammalian α-Tubulin Isotypes

Five mouse α-tubulin isotypes have been described, each distinguished by the presence of unique amino acid substitutions within the coding region (Villasante et al., 1986). Three of these isotypes (Mal, Ma2, and Ma6) are widely expressed; Mal and Ma2 differ by only a single amino acid substitution. Ma3/7 (encoded by two mRNAs, Ma3 and Ma7) is expressed exclusively in testis, while Ma4 is expressed predominantly in muscle and brain and is unique among mammalian α-tubulins in that it lacks an encoded carboxy-terminal tyrosine residue.

To investigate the biological significance of the mammalian α-tubulin multigene family, we decided to raise isotype-specific sera. The specificity of the resulting sera was established in two ways. First, to investigate potential cross reactivity among different isotypes, affinity-purified sera were used on Western blots of whole extracts of bacterial cells expressing fusion proteins corresponding to all the α-tubulin isotypes. The data are shown in Fig. 2A. The antibody raised against Mal/2 is not specific, as it cross reacts with three out of four of the cloned fusion proteins. In con-
An Antiserum That Discriminates between the Tyrosinated and Nontyrosinated Form of a Single α-Tubulin Isotype (Ma4)

An interesting feature of the biochemistry of α-tubulins is a posttranslational modification in which a carboxy-terminal tyrosine residue is either added to the heterodimer, or removed from the assembled microtubule (Thompson et al., 1979; Kumar and Flavin, 1981; Gundersen et al., 1987). To establish the specificity of our isotype-specific sera with respect to the presence or absence of the carboxy-terminal tyrosine, we engineered two fusion proteins by site-directed mutagenesis (Fig. 1 B). First, we wanted to know whether the Ma4 antibody would recognize any detyrosinated α-tubulin isotype or whether it was truly specific for Ma4. This question arises because Ma4 is the only mammalian α-tubulin isotype (and therefore the only one of our cloned fusion proteins) that is translated without a carboxy-terminal tyrosine residue. Therefore, using Ma3/7 as representative of the α-tubulin isotype (and therefore the only one of our cloned fusion proteins) ending in tyrosine, we expressed an altered Ma3/7 fusion protein in which the tyrosine codon (TAC) was changed to a termination codon (TAG). Second, we engineered a fusion protein in which a tyrosine codon was inserted between the penultimate codon and the termination codon of Ma4. Western blots of whole bacterial extracts expressing altered and unaltered fusion proteins are shown in Fig. 3. The data show that the Ma3/7-specific antibody recognizes both tyrosinated (Ma3/7) and nontyrosinated (Ma3/7-Y) forms of this isotype, but neither form of Ma4 (Ma4 or Ma4+Y). On the other hand, the Ma4-specific antibody recognizes preponderantly the nontyrosinated form of this isotype on Western blots (Fig. 3 A) and exclusively this form in fixed microtubules (see below). However, it does not detect either form of Ma3/7 and is therefore truly specific. Because of the bias of the Ma4 antibody, we raised an additional antiserum against the engineered fusion protein Ma4+Y (Fig. 1 B). The specificity of this serum with respect to all six cloned fusion proteins is demonstrated in Fig. 3 B; there is dominant recognition of Ma4+Y. Although the serum raised against Ma4 detects tubulin in extracts from brain and spleen, the antibody raised against Ma4+Y is negative on Western blots from these tissues (data not shown) suggesting that in these tissues the Ma4 isotype is predominantly present in its nontyrosinated form. This implies one of two things: either the α-tubulin tyrosine ligase activity is not present in the particular cells in which Ma4 is expressed or, more likely, Ma4 exists mainly in stable detyrosinated microtubules in these instances.
Expression of Ma3/7, Ma4, and Ma6 in HeLa and NIH 3T3 Cells

The specific sera that we raised recognize three distinct iso-types: one (Ma3/7) is expressed exclusively in testis; a second (Ma4) is expressed most abundantly in muscle and brain; and a third (Ma6) is expressed in several tissues, though at a very low level (Villasante et al., 1986). To examine whether these three isotypes assemble into microtubules or affect microtubule function outside the context of their normal pattern or level of expression, the cDNAs encoding Ma3/7, Ma4, and Ma6 were each cloned into a vector designed to express these sequences upon transfection into cultured cells via transcription from the SV-40 T-antigen promoter (see Materials and Methods). In each case, the transfected culture was analyzed by double-label immunofluorescence using an isotype-specific antibody (to detect the expression of transfected sequences) and a general tubulin antibody (to detect all microtubules irrespective of their content of tubulin isotypes). The data show that expression of Ma3/7 and Ma6 in transfected cells results in the incorporation of these isotypes into all interphase microtubules of either HeLa cells (Fig. 4, a, b, e, and f) or NIH 3T3 cells (Fig. 4, c, d, g, and h), since labeling with the specific and general tubulin antisera is in all cases coincident. Furthermore, these experiments provide additional evidence concerning the specificity of the Ma3/7 antisera, since untransfected cells are not detected. The presence of weakly labeled untransfected cells in the case of Ma6 is due to an extremely low level of endogenous Ma6 expression in HeLa and NIH 3T3 cells, and not to cross-reactivity with other α-tubulin isotypes.

Parallel transfection experiments performed using a cDNA sequence encoding Ma4 cloned into the SV-40 expression vector are shown in Figs. 5 and 6. When transfected cells were analyzed using the Ma4+Y antisera, this form of the Ma4 isotype was found to be assembled into all interphase microtubules in a manner indistinguishable from that detected by a general tubulin antibody (Fig. 5, a–d). In contrast, identical transfection experiments analyzed using the Ma4 antisera showed the presence of this (untyrosinated) isotype in a distinct subset of interphase microtubules that have a prominently perinuclear localization and tend to be curved (Fig. 6, a–d). However, in some transfected cells, the nontyrosinated form of this isotype occurs in all interphase microtubules in the cell (Fig. 6, e and f). Such cells appeared completely normal and had divided, as evidenced by their appearance in pairs on the coverslip (Fig. 6 e). As with the serum specific for Ma3/7 (Fig. 4, a and c), the specificity of the Ma4 and Ma4+Y antisera is confirmed by the presence of surrounding untransfected cells which are essentially unlabeled (Figs. 5 and 6). While the sera raised against the fusion proteins Ma4 and Ma4+Y show slight cross-reactivity on Western blots, little, if any, is apparent on preparations of fixed microtubules (see Fig. 6 and accompanying paper). The detection of two subsets of interphase microtubules by the antisera specific for Ma4 and Ma4+Y closely parallels the observations on tubulin tyrosination made by Gundersen et al. (1984, 1987). The implications of these data are discussed below.

Presence of Ma3/7, Ma4, and Ma6 in Spindle Microtubules

The capacity of Ma3/7, Ma4, and Ma6 to assemble into spindle microtubules was determined in cytospin preparations of unattached (i.e., mitotic) transfected HeLa cells. As with interphase cells, the selected mitotic populations were double labeled with isotype-specific and general tubulin antisera. In the case of Ma3/7 and Ma6, the data show no significant difference in microtubule labeling, irrespective of whether the isotype-specific or general tubulin antibody was used (Fig. 7). However, in the case of the Ma4 isotype, only the serum raised against the tyrosinated form of this polypeptide detected spindle microtubules, and these were (like Ma3/7 and Ma6) coincident with the microtubules detected by a general tubulin antibody. The serum that recognized only the nontyrosinated form of Ma4 failed to detect any spindle microtubules in the same transfection experiment, even though this is the form of the protein being translated in the transfected cells. Thus, the isotypes represented by Ma3/7, Ma4, and Ma6 competently assemble into all interphase and spindle microtubules upon transfection into tissue culture cells, while the detyrosinated form of Ma4 is usually found only in a limited subset of interphase microtubules and not at all in spindle microtubules.

Coassembly of Chimeric α-Tubulin Isotypes

The principal differences that distinguish one α-tubulin isotype from another lie within the carboxy-terminal 15 amino acids. Nevertheless, other characteristic differences also exist scattered throughout the polypeptide chain. Both the carboxy-terminal and internal sequences that distinguish each isotype are rigidly conserved among mammalian species. To assess the influence that different regions of individual α-tubulin isotypes might have with respect to each other vis-a-vis coassembly and/or phenotype, we spliced together different segments of cloned cDNAs so as to generate chimeric α-tubulin molecules. Two chimeric constructs were assembled: one consisted of NH2-terminal sequences corresponding to Ma6 coupled to COOH-terminal sequences corresponding to Ma3; and a second, complementary construct consisting of NH2-terminal Ma3 sequences coupled to Ma6 COOH-terminal sequences. In each case, the break-point was an Eco RI site at amino acid 254. The chimeric constructs were inserted into the SV40 expression vector and tested for their ability to direct the synthesis of assembly-competent α-tubulin after transfection into HeLa cells. In each experiment, the assembly of chimeric isotypes was monitored using the α-tubulin isotype-specific antibody corresponding to the carboxy-terminal segment; i.e., the exact segment originally used for the elicitation of each isotype-specific immune response (see Fig. 1). The result of this experiment showed that conjoining of NH2-terminal and COOH-terminal regions belonging to different α-tubulin isotypes has no apparent influence on microtubule assembly (Fig. 8). Similar results have been obtained in experiments involving chimeric β-tubulins (Bond et al., 1986; Fridovich-Keil et al., 1987).

Discussion

We describe here the generation of immune sera that are capable of discriminating among three of the five known mammalian α-tubulin isotypes, as well as sera that distinguish the tyrosinated from the nontyrosinated form of a single α-tubulin isotype. To generate these sera, we used cloned
fusion proteins expressed in bacterial cells consisting of the first 35,000 D of the *E. coli* trp E protein fused to the carboxy-terminal region of the various α-tubulin isotypes (Fig. 1). These fusion proteins therefore include the carboxy-terminal α-tubulin region containing the characteristic and divergent sequences that distinguish most of the isotypes. To avoid eliciting an immune response to common (i.e., shared) epitopes, including those present on the trp E portion of each fusion protein, animals were first rendered tolerant to all but one of the fusion proteins. Thus, upon challenge with the remaining fusion protein, only epitopes that are unique can be recognized. This method of raising antisera to discriminate among related molecules that contain one or more epitopes in common has been previously used to increase the probability of obtaining mAbs specific to unique epitopes (Matthew and Patterson, 1983), as well as for the generation of polyclonal antisera that specifically recognize distinct but highly homologous mammalian β-tubulin isotypes (Lewis et al., 1987). The method offers certain advantages over the use of synthetic peptide haptens: potential problems relating to peptide purification and solubility are avoided, and it is not necessary to attempt the selection of the optimal peptide(s).

**Figure 4.** Expression of Ma3/7 or Ma6 in transfected tissue culture cells results in their coassembly into interphase microtubules without disruption of function. Cloned cDNA sequences encoding Ma3/7 or Ma6 were inserted into the eukaryotic expression vector pSVdhfr (see Materials and Methods) and introduced into either HeLa cells (*a, b, e, and f*) or NIH 3T3 cells (*c, d, g, and h*) by calcium phosphate precipitation. Transfected cells expressing Ma3/7 or Ma6 were examined in fixed, detergent-extracted preparations by double-label indirect immunofluorescence using antisera specific for Ma3/7 (*shown in a and c*) or Ma6 (*shown in e and g*) and a β-tubulin-specific antibody that detects all microtubules (*shown in b, d, f, and h*). Controls in which both second antibodies were used only with one or the other first antibody showed both second antibodies to be completely specific (data not shown). Note the coincident labeling of microtubules with isotype-specific and general antitubulin antisera (*compare a with b, c with d, e with f, and g with h*) and the presence of surrounding (untransfected) cells in *a* and *c* that do not express Ma3 (*arrows*) or similar surrounding untransfected cells in *e* and *g* show very weak fluorescence because of a low level of endogenous expression of Ma6 in HeLa and NIH 3T3 cells. Bar, 10 μm.
Figure 6. Distribution of the untyrosinated form of Ma4 in transfected tissue culture cells. A transfection experiment identical to that described in Fig. 5 was analyzed using the antibody specific for the untyrosinated form of Ma4 (a, c, and e). (a and b) HeLa cells; (c-f) NIH 3T3 cells. b, d, and f show the same fields as a, c, and e, respectively, detected with a general tubulin antibody. Note the presence of unlabeled untransfected cells in a, c, and e (arrows). Bar, 10 µm.

This could be particularly important in situations where multiple but randomly scattered amino acid substitutions distinguish various members of an otherwise highly homologous family of proteins.

Three criteria were used to establish the specificity of the anti-α-tubulin antisera. First, each antibody was tested in Western blot experiments for its ability to discriminate among cloned fusion proteins representing each of the known mammalian α-tubulin isotypes. Second, the sera were tested in Western blot experiments of whole extracts of mouse tissues to assess their specificity for epitopes unique to α-tubulin. Finally, the ability of each antibody to discriminate among different α-tubulin isotypes in fixed microtubules was ascertained in transfection experiments in which cells expressing specific transfected α-tubulin isotypes showed strongly fluorescent microtubules, whereas surrounding untransfected cells remained negative. Using these criteria, we generated three α-tubulin isotype–specific sera that uniquely recognize
Ma3/7, Ma4, or Ma6. In addition, two sera that distinguish between the tyrosinated and detyrosinated form of a single α-tubulin isotype (Ma4) were generated. Although the two sera cross react slightly with their respective antigens on Western blots (Fig. 3 B), they are clearly discriminatory in immunofluorescence experiments on fixed microtubules (Fig. 6 and accompanying paper).

Of the three α-tubulin isotypes for which we raised specific antisera, two are expressed in tissues that undergo distinct developmental changes involving the formation and/or reorganization of microtubule structures (Villasante et al., 1986). Ma3/7 is expressed exclusively in testis, where specialized microtubule structures such as the flagellum and manchette are associated with spermatogenesis, while Ma4 is abundantly expressed in muscle and brain, in which great asymmetries are produced in cell morphology during development. On the other hand, Ma6 is expressed in many tissues but at a very low level. The existence of distinct α-tubulin isotypes in tissues containing specialized microtubule structures might be explained in terms of structural requirements conferred by distinct amino acid differences in the polypeptide chain. Such differences could, for example, be important for interaction with specific microtubule-associated proteins. Though this may indeed be the case, our data show that Ma3/7, Ma4, and Ma6 are capable of assembly into interphase and spindle microtubules in cell types (Ma4, Ma3/7) or at levels (Ma6) at which they are not normally expressed. Thus, in common with mammalian β-tubulin isotypes (Lewis et al., 1987; Lopata and Cleveland, 1987), there is no evidence for segregation of these isotypes among functionally different microtubules.

In addition to the encoded sequence heterogeneity that exists among tubulin isotypes, both α- and β-tubulin polypeptides can undergo posttranslational modification. In the case...
Figure 8. Assembly of chimeric α-tubulin isotypes into interphase microtubules. Two chimeric isotypes, one consisting of NH2-terminal sequences derived from Mt6 coupled to COOH-terminal sequences corresponding to Mt3 and a second consisting of NH2-terminal Mt3 sequences coupled to Mt6 COOH-terminal sequences were constructed. Each was inserted into the pSVdhfr expression vector (Mulligan and Berg, 1981) and introduced into HeLa cells or NIH 3T3 cells by calcium phosphate precipitation (Wigler et al., 1979). Transfected cells were detected by indirect double-label immunofluorescence using the α-tubulin isotype-specific antibody corresponding to the isotype encoded by the carboxy-terminal segment, plus a β-tubulin-specific antibody that detects all microtubules. (a and b) HeLa cells; (c and d) NIH 3T3 cells. (a and c) Cells transfected with the Mt6/Mt3 chimeric isotype detected with the Mt3/β-specific antibody (note the presence of surrounding untransfected cells [arrows]); (b and d) the same field shown in a and c, respectively, detected with a general tubulin antibody. Microtubules detected with either antibody appear coincident. Coincident microtubules were also observed in spindles in the same transfected cultures, and identical results were obtained using the Mt3/Mt6 chimeric isotype (data not shown).

of α-tubulins, these modifications include the acetylation of lysine (at position 40 [LeDizet and Piperno, 1986]), and the removal or addition of the carboxy-terminal tyrosine residue. The cycle of α-tubulin tyrosination and detyrosination has been well studied in vivo and in vitro. Tubulin tyrosine ligase rapidly tyrosinates the tubulin heterodimer (Raybin and Flavin, 1977; Gundersen et al., 1987), while tubulin tyrosine carboxypeptidase detyrosinates assembled microtubules slowly compared to the half-life of a microtubule in a cultured cell (Kumar and Flavin, 1981; Gundersen et al., 1987). Antibodies that distinguish between tyrosinated and detyrosinated microtubules have been prepared by Gundersen et al. (1984) and used to show that in cultured cells a small subset of microtubules is enriched in nontyrosinated α-tubulin. These microtubules are distinguished from the bulk of the cell's interphase microtubules by their wavy morphology and the fact that they are not growing (Gundersen et al., 1987). Schulze and Kirschner (1987) have shown that such wavy microtubules are 3–5 times more stable than average. However, several lines of evidence suggest that the detyrosination of these microtubules is a consequence, rather than a cause of their enhanced stability: tyrosinated and detyrosinated tubulin behave in a dynamically similar way in vitro (Raybin and Flavin, 1977; Kumar and Flavin, 1982); the drug-induced stabilization of microtubules in vivo results in their increased detyrosination (Gundersen et al., 1987); and spindle microtubules, which turn over more rapidly than interphase microtubules, are extremely...
poor in detyrosinated a-tubulin (Gundersen and Bulinski, 1986).

Thus, these more stable microtubules probably become detyrosinated because the tyrosine-carboxypeptidase has more time to act on them. The function of this detyrosination remains a mystery. What role it may play in differentiated cells is also unclear, although tubulin tyrosine ligase has been shown to be enriched in developing brain and muscle (Deanin et al., 1977), and in neurons, axonal microtubules have been shown to be detyrosinated compared to dendritic microtubules (Cambray-Deakin and Burgoyne, 1987). Acetylation of a-tubulin is similar to detyrosination in many respects: it is reversible (L'Hernault and Rosenbaum, 1985b), nonessential for cell growth and division (Piperno et al., 1987), and acts mainly on more stable microtubules, such as the wavy interphase microtubules of some cultured cells (Piperno et al., 1987), axonal microtubules (Cambray-Deakin and Burgoyne, 1987), and microtubules of cilia and flagella (LeDizet and Piperno, 1986; L'Hernault and Rosenbaum, 1983).

The work described above on a-tubulin tyrosination was performed using antisera directed against two short peptides derived from a single carboxy-terminal a-tubulin sequence and differing only by the presence or absence of the tyrosine residue. The sequence of these peptide hapten, GEEEGEE(Y), corresponds to Mal/2 (Fig. 1). Both Mal1 and Mal2 are widely expressed, and are particularly abundant in cultured cells, where Mal3/7, Mal4, and Mal6 are expressed at very low levels or not at all. The a-tubulin isotype Mal4 differs from Mal1, Mal2, and other mammalian a-tubulins in that it lacks an encoded carboxy-terminal tyrosine residue. We were therefore particularly interested in investigating how this isotype behaves in the cycle of detyrosination/tyrosination. Here we describe two sera raised against this isotype: one that recognizes only the detyrosinated form of Mal4 in fixed microtubules and another that recognizes only or predominantly the tyrosinated form (Mal4+Y). We have used these sera to examine the behavior of this isotype when expressed (by transfection) in cultured cells, in particular, in regard to how it fits into the cycle of tubulin detyrosination/tyrosination. Several conclusions can be drawn. Mal4 is a substrate for tubulin tyrosine ligase. When it is expressed in (transfected) cultured cells at low or moderate levels, it is found predominantly in tyrosinated form in all microtubules (Fig. 5), and in its detyrosinated form in only a subset of wavy microtubules (Fig. 6, a-d). Thus it behaves in the cycle of tyrosination/detyrosination like the bulk of a-tubulin, as described by Gundersen et al. (1984, 1987). However, in any transient transfection experiment, the level of expression varies immensely from one cell to another, due to variation in the number of copies of transfected DNA molecules that are taken up. Thus, when we transfected an Mal4-expressing construct into NIH 3T3 cells, in some cases the Mal4 isotype was incorporated into all the cells' microtubules in its tyrosinated form; i.e., without prior posttranslational tyrosination (Fig. 6, e and f). This presumably occurs because there is not enough tyrosine ligase in the cell to handle all of the nontyrosinated a-tubulin present due to the overexpression of Mal4. The overabundance of untyrosinated tubulin has no apparent effect on cell morphology or division (since overexpressing cells [like all transiently transfected cells] are usually found in groups of two or four on the cover-slip, showing that they have divided since transfection). This experiment reinforces the conclusions of others (Webster et al., 1987; Kumar and Flavin, 1982) that nontyrosinated a-tubulin is dynamically similar to tyrosinated a-tubulin, and therefore that the enhanced stability of naturally occurring detyrosinated microtubules is probably a cause rather than an effect of detyrosination.

These data, taken together with numerous other studies that have sought to address issues relating to the expression of multiple a- and b-tubulin genes both in lower and higher eukaryotes, all point to an apparent functional interchangeability within each family of isotypes. This presents us with a curious paradox, at least in regard to the mammalian tubulin multigene families: on the one hand, the isotypes appear functionally interchangeable but on the other, there has been rigid interspecies conservation of the amino acid sequences that distinguish one isotype from another, at least since the mammalian radiation (about 100 million years ago). One possible resolution of this paradox is that specific isotype sequences may indeed be required for interaction with microtubule-associated proteins. In that event, the ability of all isotypes to coassemble into functionally distinct microtubules could be regarded as adventitious: only the expression of certain isotype sequences (rather than their segregation) in concert with specific microtubule-associated proteins may be required for the proper functioning of specific kinds of microtubules. In the accompanying paper, these ideas are explored further by using our a- and b-tubulin isotype-specific sera to study tubulin isotype expression and use in cells and tissues in which reorganization and assembly of functionally different kinds of microtubule form an integral part of the differentiation process.

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References

Adachi, Y., T. Toda, O. Niwa, and M. Yanagida. 1986. Differential expression of essential and non-essential a-tubulin genes in Schizosaccharomyces pombe. Mol. Cell Biol. 6:2168-2178.

Argarana, C. E., H. S. Barra, and R. Caputto. 1978. Release of [14C]tyrosine from tubulin-[14C]-tyrosine by brain extract separation of a carboxypeptidase from tubulin tyrosine ligase. Mol. Cell. Biochem. 19:17–22.

Barra, H. S., C. A. Arce, J. A. Rodriguez, and R. Caputto. 1974. Some common properties of the protein that incorporates tyrosine as a single unit into microtubule proteins. Biochim. Biophys. Res. Commun. 60:1384–1390.

Binder, L. I., A. Frankfarter, and L. I. Rebbun. 1985. The distribution of tau in the mammalian central nervous system. J. Cell Biol. 101:1371–1378.

Bloom, G. S., F. D. Luca, and R. B. Vallee. 1984. Widespread distribution of the major component of MAP1 (microtubule-associated protein 1) in the central nervous system. J. Cell Biol. 98:331–340.

Bond, J. F., L. Fridovich-Keil, L. Pillus, R. C. Mulligan, and F. Solomon. 1986. A chicken-yeast chimeric β-tubulin protein is incorporated into mouse microtubules in vivo. Cell. 44:461–468.

Cambray-Deakin, M. A., and R. D. Burgoyne. 1987. Posttranslational modification of α-tubulin: acetylated and detyrosinated forms in axons of rat cerebral. J. Cell Biol. 104:1569–1574.

Cowan, N. J., S. A. Lewis, S. Sarkar, and W. Gu. 1987. Functional versatility of mammalian β-tubulin isotypes. In The Cytoskeleton in Cell Differentiation.
Lewis, S. A., W. Gu, and N. J. Cowan. 1987. Tyrosyl tubulin ligase activity in brain, skeletal muscle and liver of the developing chick. Dev. Biol. 105:230-233.

Elliot, E. M., G. Henderson, F. Sarrangi, and V. Ling. 1986. Complete sequence of three α-tubulin cDNAs in Chinese hamster ovary cells: each encodes a distinct isoprotein. Mol. Cell. Biol. 6:906-913.

Farmer, S. R., J. F. Bond, G. S. Robinson, D. Mbandgolo, M. J. Fenton, and E. H. Berkowitz. 1984. Differential expression of the rat β-tubulin multigene family. In Molecular Biology of the Cytoskeleton. G. G. Borisy, D. W. Cleveland, and D. B. Murphy, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 333-342.

Fridovich-Keil, J. L., J. F. Bond, and F. Solomon. 1987. Domains of β-tubulin essential for conserved functions in vivo. Mol. Cell. Biol. 7:3792-3798.

Fuller, M. T., J. H. Cautlon, J. A. Hutchens, T. C. Kaufman, and E. C. Raff. 1987. Genetic analysis of microtubule structure: a β-tubulin mutation causes the formation of aberrant microtubules in vivo and in vitro. J. Cell Biol. 104:385-394.

Gundersen, G. G., and J. C. Bulinski. 1986. Distribution of tyrosinated and nontyrosinated α-tubulin during mitosis. J. Cell Biol. 102:1118-1126.

Gundersen, G. G., M. H. Kalnoksi, and J. C. Bulinski. 1984. Distinct populations of microtubules: tyrosinated and nontyrosinated alpha tubulin are distributed differently in vivo. Cell. 38:779-789.

Kumar, N., and M. Flavin. 1981. Preferential action of a brain detyrosinolating carboxypeptidase on polymerized tubulin. J. Biol. Chem. 256:7678-7686.

Kumar, N., and M. Flavin. 1982. Modulation of some parameters of assembly of microtubules in vitro by tyrosination of tubulin. Eur. J. Biochem. 128:215-222.

Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA. 82:488-492.

LeDizet, M., and G. Piperno. 1986. Cytoplasmic microtubules containing acetylated α-tubulin in Chlamydomonas reinhardtii, spatial arrangement, and properties. J. Cell Biol. 103:13-22.

Lee, M. G.-S., S. A. Lewis, C. D. Wilde, and N. J. Cowan. 1983. Evolutionary history of a multigene family: an expressed human β-tubulin gene and three processed pseudogenes. Cell. 33:477-487.

Lee, M. G.-S., C. Loomis, and N. J. Cowan. 1984. Sequence of an expressed human β-tubulin gene containing ten Alu family members. Nucleic Acids Res. 12:5823-5838.

Lewis, S. A., M. L. Gilmartin, J. L. Hall, and N. J. Cowan. 1985a. The amino acid sequences of the human β-tubulin isotypes. J. Mol. Biol. 182:11-20.

Lewis, S. A., W. Gu, and N. J. Cowan. 1987. Free intermingling of mammalian β-tubulin isotypes among functionally distinct microtubules. Cell. 49:59-548.

Lewis, S. A., M. G-S. Lee, and N. J. Cowan. 1985b. Five mouse tubulin isotypes and their regulated expression during development. J. Cell Biol. 101:852-861.