Copolymerization of Two Distinct Tubulin Isotypes During Microtubule Assembly In Vitro

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Abstract. Cells contain multiple tubulin isotypes that are the products of different genes and posttranslational modifications. It has been proposed that tubulin isotypes become segregated into different classes of microtubules each adapted to specific activities and functions. To determine if mixtures of tubulin isotypes segregate into different classes of polymers in vitro, we used immunoelectron microscopy to examine the composition of microtubule copolymers that assembled from mixtures of purified tubulin subunits from chicken brain and erythrocytes, each of which has been shown to exhibit distinct assembly properties in vitro. We observed that (*a*) the two isotypes coassemble rapidly and efficiently despite the fact that each isotype exhibits its own unique biochemical and assembly properties; (*b*) at low monomer concentrations the ratio of tubulin isotypes changes along the lengths of elongating copolymers resulting in gradients in immuno-gold labeling; (*c*) two distinct classes of copolymers each containing a distinct ratio of isotypes assemble simultaneously in the same subunit mixture; and (*d*) subunits and polymers of different isotypes associate nearly equally well with each other, there being only a slight bias favoring interactions among subunits and polymers of the same isotype. The observations agree with previous studies on the homogeneous distribution of multiple isotypes within cells and suggest that if segregation of isotypes does occur in vivo, it is most likely directed by cell-specific microtubule-associated proteins (MAPs) or specialized intracellular conditions.

MICROTUBULES are associated with many cellular activities, including changes in cell shape, intracellular transport, mitosis, and flagellar motility, and it has been proposed that cells and organisms use various tubulin isotypes to produce microtubules with different functional properties (9, 34). In higher eukaryotes, tubulin is encoded by multiple tubulin genes each with uniquely conserved primary sequences, which in turn may be subject to different posttranslational modifications that affect their distribution and stability. In addition, the carboxy terminus of tubulin, especially that of beta tubulin, is thought to influence microtubule polymerization and the binding of microtubule-associated proteins (MAPs)[1] (32). However, it is the conservation of distinct isotype classes and their corresponding patterns of expression in different vertebrate species that suggests that multiple tubulin isotypes are required for normal microtubule function (4, 35, 37). It is therefore of particular interest to determine if different classes of microtubules each composed of different isotypes assemble in cells to perform various tasks.

To examine these questions investigators have transfected mammalian tissue culture cells with vertebrate tubulin cDNAs (2, 7, 13, 15). Although immunofluorescence microscopy revealed that the introduced tubulins were capable of copolymerizing with the endogenous cellular tubulins, in some cases they did so with reduced efficiency (7, 13). In a recent study of beta tubulin function in *Drosophila*, Fuller and co-workers (8) demonstrated that a mutation in the testis-specific beta tubulin gene caused male sterility and produced abnormal microtubules but noted that the various classes of spermatocyte microtubules were affected to different degrees. Taken together, these observations suggest that in the cell not all isotypes are equivalent with respect to assembly and function.

In comparing the assembly properties of tubulin isotypes isolated from chicken erythrocytes and chicken brain, we observed large differences in charge and solubility, oligomer stability, and the rates of polymer nucleation and elongation for the two tubulin types (19–22). These differences are most likely because of the highly divergent amino acid sequence of erythrocyte beta tubulin (23). Since brain and erythrocyte tubulins differ significantly in their assembly and biochemical properties (28, 36) and presumably also in their function, we sought to determine if these isotypes produced different classes of microtubules when mixed together in vitro.

In this paper, we show by immunoelectron microscopy that both isotypes coassemble efficiently under several different conditions in vitro. Nevertheless, in most of the cases examined, the copolymers exhibited nonuniform patterns of labeling (gradients) indicating that partial segregation had oc-

1. *Abbreviation used in this paper*: MAP, microtubule-associated protein.

curred. The factors that influence this segregation appear to be complex and may include differences in the stabilities of microtubules and tubulin oligomers as well as other factors.

Materials and Methods

Preparation of Chicken Brain and Erythrocyte Tubulin

Chicken brain tubulin was isolated by the method of Dentler et al. (5). Chicken erythrocyte tubulin was prepared from chicken blood by the method of Murphy and Wallis (20). Unless stated otherwise, microtubule assembly buffer for both preparations was 0.1 M Na-Pipes at pH 6.94 containing 1 mM $MgCl_2$, 1 mM GTP and 5% glycerol.

Tubulin was purified free of MAPs by ion exchange chromatography on phosphocellulose (P-11; Whatman, Inc., Clifton, NJ), and purified fractions were immediately supplemented with $MgCl_2$ and GTP to final concentrations of 1 mM before freezing and storage (38). In most cases, aliquots of purified tubulin were passed through an additional cycle of microtubule assembly and disassembly before use to remove inactive subunits.

Microtubule seeds were prepared by shearing polymers assembled from tubulin 10 times with a 27-gauge needle and allowing the preparation to recover for 10 min before use. This treatment produced 1–2-μm-long microtubules that were used as seeds in copolymer elongation experiments.

Procedures for Immunoelectron Microscopy

Copolymer formation was initiated by adding preformed brain or erythrocyte microtubule seeds to preparations of subunits containing a mixture of subunits of both tubulin isotypes. At various times after the initiation of elongation, aliquots of incubation mixtures were diluted to 0.1–0.2 mg/ml in assembly buffer containing 1% glutaraldehyde. EM grids containing fixed polymers were labeled with a rabbit antiserum specific for the beta subunit of chicken erythrocyte tubulin and stained with protein A-gold as described by Rothwell et al. (27, 28). Grids were examined with an electron microscope (10A; Carl Zeiss, Inc., Thornwood, NY) to determine the number of gold particles occurring along 0.3-μm segments of the polymers. In practice, we counted all of the particles within one particle diameter (10 nm) of polymer surface visible on the binocular viewing screen of the microscope at a magnification of 80,000. This distance (0.3 μm) was arbitrarily designated 1 U of length. Because the maximum amount of label varied slightly between experiments, to compare the composition of copolymers in different experiments, it was necessary to normalize the curve with respect to the maximum density of labeling observed on erythrocyte microtubules for each experiment.

The sensitivity of the antibody labeling response (Fig. 1) in copolymers containing different ratios of brain and erythrocyte tubulin isotypes was similar to that described in an earlier report by Rothwell et al. (28). The density of immuno-gold label increased linearly up to 40–50% erythrocyte tubulin, beyond which the curve rapidly reached a plateau (not shown). For most of the work described here, copolymers contained 10–20% erythrocyte tubulin, which was midway in the range of concentrations giving a linear staining response. The density of immuno-gold label was uniform as indicated by direct visual inspection (e.g., references 27 and 28) and by the low standard deviations of the samples. Differences as little as 2% in erythrocyte tubulin content could be distinguished in copolymers containing from 2 to 40–50% erythrocyte tubulin.

The background of gold particles was determined to result from tubulin subunits in the preparation and its density varied depending on the concentration of fixed microtubules applied to the grid. We used a dilution resulting in the attachment of 10–30 microtubules/grid square. Since the density of background label was uniform for any given specimen, and since gradients were observed regardless of the antibody concentration or the microtubule density, no attempt was made to subtract background from the label on the microtubules. The unit of microtubule length used in this study (0.3 μm) was determined empirically to be the minimum length required to average out local patchiness in labeling. The mean length of copolymer segments was 3–5 μm or ~10–20 length units. It was usually possible to distinguish the boundaries between seeds and copolymers to within 0.03 μm, or 1/10th of a length unit.

Protein Assay and Biochemical Materials

Protein concentrations were determined by the Bradford protein assay (3) using BSA as a standard. Pipes was obtained from Calbiochem-Behring Corp. (San Diego, CA). Other chemicals and nucleotides were obtained from Sigma Chemical Co. (St. Louis, MO).

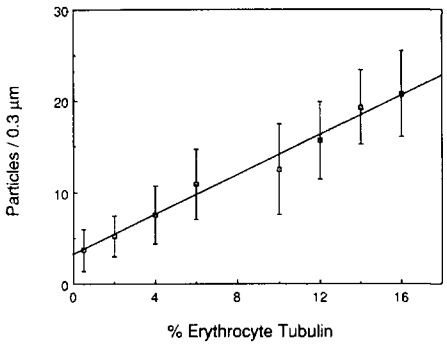


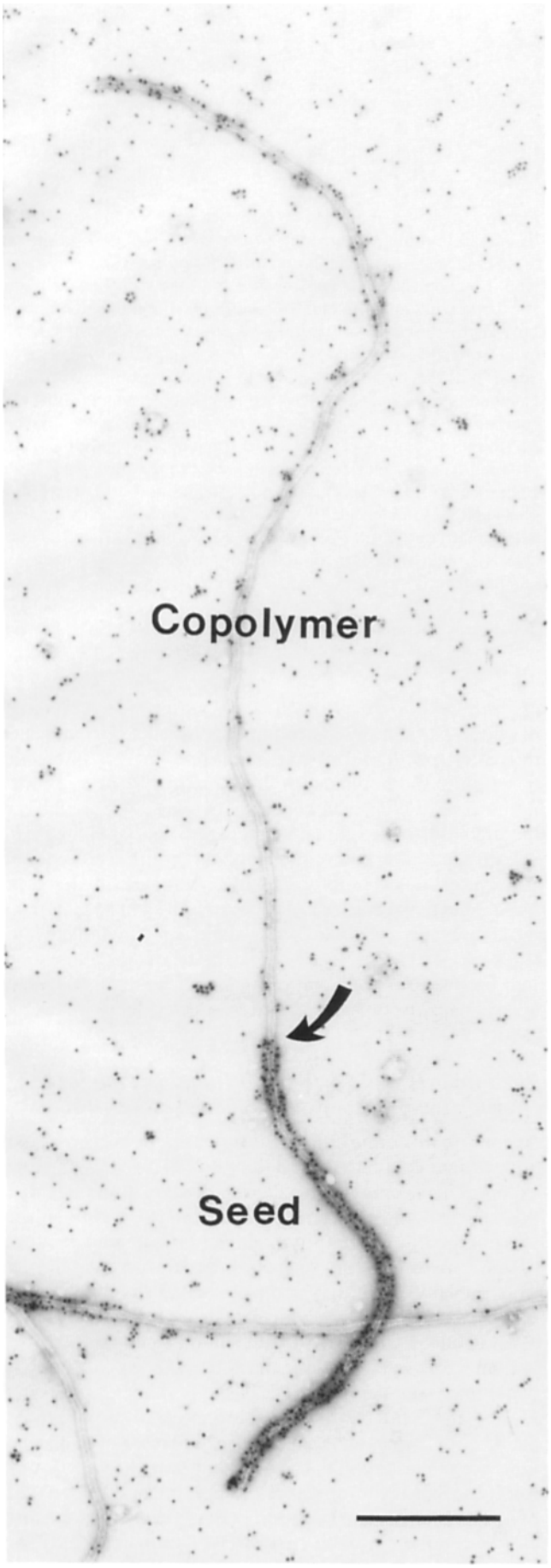
Figure 1. Density of immuno-gold label versus the percent erythrocyte tubulin contained in copolymers. Copolymers obtained by self-assembly from mixtures containing different ratios of brain and erythrocyte tubulin at 2 mg/ml were fixed and prepared for immuno-EM using antibody to erythrocyte beta tubulin. The sample size (n) was 50 microtubules/data point. The probability that the differences in the means and standard deviations of adjacent samples are because of random sampling error is <0.1% as determined by t test.

Results

Immunoelectron Microscopy of Copolymers Composed of Different Tubulin Isotypes

To determine if isotype sorting occurred in vitro, we examined copolymers assembled from mixtures of brain and erythrocyte tubulin by immuno-EM, using a rabbit polyclonal antibody to erythrocyte beta tubulin (27, 28). We previously demonstrated that brain microtubules, erythrocyte microtubules, and copolymers containing a mixture of both isotypes could easily be distinguished from each other by this method (28). When self-assembled rapidly at subunit concentrations >2 mg/ml, copolymers contained an equal distribution of isotypes (27, 28). What is remarkable is the fact that the isotypes copolymerized so efficiently with each other despite the large differences in their biochemical and assembly properties. Nevertheless, if polymer formation was initiated at or close to steady state conditions as described below, gradients in antibody labeling appeared indicating that partial segregation of the isotypes had occurred.

We first examined the composition of copolymers that formed by elongation after mixing samples of preassembled brain and erythrocyte microtubules that were prepared as described in detail in a previous report on microtubule annealing (30). In every case examined, the labeling increased progressively towards the free growing ends of the copolymers, indicating that the proportion of erythrocyte tubulin increased as the copolymers grew longer. An example of such a gradient in antibody labeling on an elongated copolymer is shown in Fig. 2. As seen in this example and described subsequently, the changes in composition were not always uniform but sometimes occurred in steps, some stretches exhibiting only slight changes in isotype composition. In this example, the region of major change occurs at the free distal tip of the copolymer, but this was not always the case, since regions of rapid change in composition sometimes occurred



adjacent to the seed itself. In yet other cases, such as those shown graphically in Fig. 3, the change in composition was evident along the lengths of the polymers. However, in all cases, the proportion of erythrocyte tubulin increased towards the free growing ends of the copolymers. Analysis of the composition of copolymers from such an experiment is presented in Table I where the mean density of immuno-gold label (x) at each of the ends of the copolymers (mean length = 2.5 μm) is designated a or b. Four configurations or arrangements of copolymers were observed. For the case of copolymers (C) elongated from the ends of both erythrocyte and brain microtubule fragments (X), the ends of copolymers attached to fragments (a) contained less erythrocyte tubulin than the ends exposed to solution (b). Gradients occurred regardless of the type (brain or erythrocyte) of microtubule fragment that served as the nucleus for copolymer elongation. Student's independent t test indicated that the difference in labeling at the two ends was highly significant (t value = 8.25 and probability <0.001).

The Special Case of Segregation during Microtubule Annealing

In addition to the simple case for elongated copolymers, there was also the more complex case where copolymers were "trapped" within annealed microtubules by endwise joining of preexisting polymers. Four representative examples of gradients in copolymers in annealed microtubules are shown in Fig. 3. Three annealed configurations involving copolymers were observed. The first of these, copolymers (C) flanked by brain (B) polymer on one end and by erythrocyte (E) microtubule on the other (configuration B-C-E in Table I) exhibited obvious gradients in labeling, with the difference in composition at opposite ends a and b being highly significant as determined by t test (t value = 8.89, probability <0.001).

We present this more complex case because it demonstrates the possible preferential association of subunits and polymers of homologous isotypes, another behavior that could cause segregation. The phenomenon is exemplified by the strict polarity of orientation of copolymers in the configuration B-C-E. In all 37 cases comprising this sample, the a end flanking brain microtubule fragments exhibited a lower density of labeling than the b end facing erythrocyte microtubules. In contrast, copolymers included between tubule fragments of the same type (configurations B-C-B and E-C-E in Table I) did not contain gradients in labeling. This was determined by direct visual inspection of the label along such copolymers (no gradients were observed) and by the fact that the standard deviations of the E-C-E and B-C-B classes that

Figure 2. Immuno-EM of an elongated copolymer. Erythrocyte and brain microtubule seeds were polymerized separately at 6 mg/ml and were mixed at a 1:3 ratio, respectively, after reaching steady state as determined by spectrophotometry. The sample was processed for immunoelectron microscopy 60 min after the addition of seeds. The junction between the erythrocyte tubulin seed and elongated copolymer is identified by an arrow. Copolymers contained a relatively greater proportion of erythrocyte tubulin at their free distal ends, resulting in a gradient of immuno-gold labeling along their lengths. Bar, 0.5 μm.

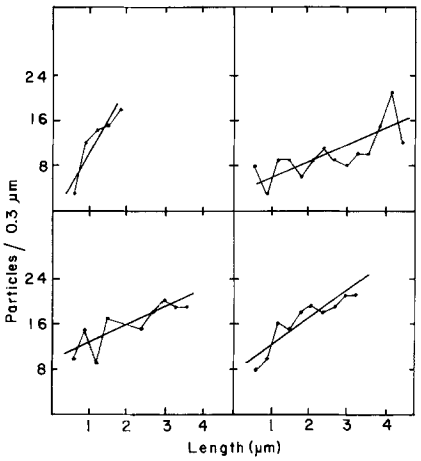


Figure 3. Examples of gradients in the ratio of tubulin isotypes in elongated copolymers. Four representative samples of gradients in copolymers in annealed microtubules are shown. Purified brain and erythrocyte tubulins were precycled, polymerized separately at 6.5 mg/ml, and combined to give a mixture containing 25% erythrocyte tubulin polymers. The copolymers shown here are from samples taken at 30 and 60 min after mixing. The gradients extend over ~4 μm of copolymer length and correspond to overall changes in erythrocyte tubulin composition ranging from 16–22%. The rate of change in composition for the copolymers shown here ranges from 4–11%/μm. Best straight line fit through the data was determined by the least-squares regression method.

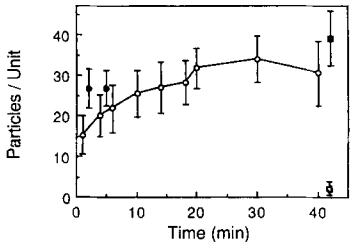


Figure 4. Copolymer elongation from brain microtubule fragments. Microtubule protein containing MAPs (H_2P) was used to prepare brain microtubule fragments and mixtures of brain and erythrocyte tubulin subunits. Brain microtubule fragments (3.6 mg/ml) were stabilized with an equimolar amount of taxol and pelleted through 20% sucrose cushions containing 10 μm taxol. Resuspended polymers were sheared 10 times with a 26 g syringe needle and an aliquot (26 μl, 3.6 mg/ml) was added to 1 ml of an equimolar mixture of subunits of brain and erythrocyte microtubule protein (final concentration, 0.94 mg/ml) to initiate the elongation of copolymers, and the mean density of label at the free ends of copolymers (1 U = 0.3 μm) was determined. In this experiment assembly buffer contained GTP regenerating enzymes, including 4 mM acetyl phosphate and 1 U/ml acetate kinase (17). The symbols designate elongated copolymer (○), self-assembled copolymers (●), and erythrocyte (■), and brain (□) microtubule fragments. The increase in labeling from 15 to 30 particles/U corresponds to an increase in the content of erythrocyte tubulin from 19 to 46%, or a net change of 27% over a 20-min period of time. Error bars indicate SD; the sample size (n) was 50 microtubules/data point.

did not exhibit gradients were less than those of the X-C and B-C-E classes that contained gradients. Thus, if gradients exist in copolymers located between seeds of the same type, they are difficult to detect by both visual and quantitative methods.

Although we do not know the details of copolymer formation during microtubule annealing, the patterns of labeling of copolymers in the annealing experiments also indicate a degree of specificity, albeit a slight one, for the association of subunits and polymers of the same isotype. For example in the case of elongated copolymers (class X-C in Table I), the ratio of isotypes contained in regions of copolymer adjacent to seeds was different depending on the seed type. For copolymers adjacent to brain and erythrocyte tubulin seeds, these densities were 10.3 ± 2.5 (n = 18) and 11.2 ± 3.6 (n = 47), respectively, which was statistically significant ($p < 0.002$) as determined by t test. The issue of specific recognition between subunits and polymers of homologous versus heterologous isotypes is treated in greater detail in the final section of Results.

*Table I. Changes in Tubulin Composition along the Lengths of Microtubule Copolymers**

	Microtubule configuration§							
	X-C (elongation)		B-C-E (annealing)		E-C-E (annealing)		B-C-B (annealing)	
Statistic‡	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
x	11.2	16.3	9.1	15.3	11.6	12.1	10.3	10.0
SD	3.3	3.8	2.6	3.3	3.3	3.3	2.1	2.4
n	67	67	37	37	33	33	7	7
t	8.25		8.89		0.56		0.24	
p	<0.001		<0.001		0.577		0.814	

* The density of immuno-gold labeling was determined for 1 U (0.3 μm) at each end (*a* and *b*) of copolymer segments. The mean and standard deviation of labeling were determined for each end and examined by t test to determine the significance of differences in the values at the two ends.

‡ The statistics indicate the mean (x), SD, sample size (n), t statistic (t), and probability (p).

§ Four microtubule configurations are indicated: (*a*) copolymer elongated from either brain or erythrocyte microtubule seed (X-C) where the end of copolymer adjacent to the seed is designated (*a*) and the distal free end of copolymer is designated (*b*); (*b*) annealed copolymers situated between brain and erythrocyte tubulin domains (B-C-E) where the ends of copolymers adjacent to *B* and *E* seeds as designated *a* and *b*, respectively; (*c*) copolymers situated between two erythrocyte tubulin domains (E-C-E) where *a* and *b* indicate the first and second ends encountered during the measuring procedure; and (*d*) copolymers situated between two brain tubulin domains (B-C-B) where *a* and *b* indicate the first and second ends encountered during the measuring procedure.

The Proportion of Erythrocyte Tubulin at the Free Growing Ends of Copolymers Increases with Time

To confirm that brain and erythrocyte isotypes became incorporated into copolymers at different rates, we designed new experiments to examine the density of label at the free distal tips of copolymers with increasing elongation time in mixtures of tubulin subunits at moderate protein concentrations (0.9–1.3 mg/ml). Brain and erythrocyte microtubule seeds were prepared separately and were added simultaneously to preparations containing approximately equimolar amounts of brain and erythrocyte tubulin subunits and the density of immuno-gold label on the terminal 0.3 μm of growing copolymer was determined at various times. As shown in Figs. 4 and 5 the density of label increased rapidly during the first 10–20 min and then reached a plateau. The observations are consistent with the idea that brain and erythrocyte isotypes were incorporated at different rates during elongation. The increase in erythrocyte tubulin content at the distal ends of copolymers during growth was estimated to be 27 and

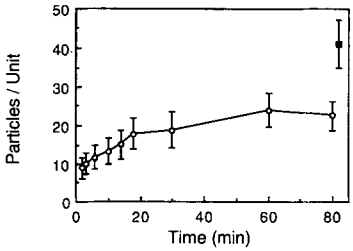


Figure 5. Copolymer elongation from erythrocyte microtubule fragments. Samples of purified brain and erythrocyte tubulin were passed through a cycle of assembly and disassembly before use. Erythrocyte microtubule fragments (50 μl, 5.6 mg/ml) were sheared 10 times with a 26 g syringe needle and added to 600 μl brain tubulin subunits (1.3 mg/ml) to initiate copolymer elongation. Erythrocyte tubulin was estimated to comprise 6% of the subunits and 26% of the total protein in the sample. Taxol and regenerating enzymes were not included. The mean density of label at the free ends of copolymers (1 U = 0.3 μm) was determined. The symbols indicate copolymers (○) and erythrocyte tubulin fragments (■). Brain microtubule fragments were not included as a reference. The observed increase in labeling from 10 to 20 particles/U corresponds to a change in erythrocyte tubulin content from 11 to 27%, or a net change of 17% over a 30-min period of time. Error bars indicate SD; the sample size (n) was 50 microtubules/data point.

17%, respectively. The fact that the density of label persisted for several hours indicates that dynamic processes such as dynamic instability and treadmilling did not rapidly change the ratio of isotypes. This is not too surprising since in previous studies we showed that erythrocyte microtubules are less dynamic than brain microtubules (reference 27, and Figs. 6 *B* and 8 in reference 30). Thus, the presence of erythrocyte tubulin at the tips of elongating copolymers could account for their relative stability.

Simultaneous Formation of Two Classes of Copolymers with Different Ratios of Tubulin Isotypes

In addition to the presence of gradients in elongating copolymers, we observed a second class of self-assembled copolymers (Fig. 4, *filled circles*) that contained a different ratio of tubulin isotypes. Self-assembled copolymers never comprised more than a few percent of the microtubules under these conditions because elongating copolymers rapidly reduced the tubulin subunit concentration and depressed spontaneous microtubule nucleation. In addition, we could only examine copolymers that self-assembled during the first few minutes of polymerization, because it was difficult to distinguish self-assembled copolymers longer than 5–10 μm in length. However, sufficient numbers were found at early time points to allow comparisons of their labeling and composition. Self-assembled copolymers, identified by the lack of discrete brain and erythrocyte tubule fragments at their ends, contained ~10% more erythrocyte tubulin than did copolymers elongated from fragments in the same preparation (Fig. 5). Unlike elongated copolymers that contained gradients, self-assembled copolymers were uniformly labeled along their lengths, and in this respect resembled copolymers produced by self-assembly at high subunit concentration. Although complete segregation of subunit isotypes was not observed, these observations provide limited but definitive evidence for the simultaneous segregation of tubulin isotypes into two distinct classes of microtubules (see Discussion).

The Increased Stability of Erythrocyte Tubulin Oligomers May Account for the Change in Ratio of Isotypes during Polymerization

Erythrocyte tubulin oligomers are relatively more stable than those composed of brain tubulin. Upon warming to 37°C, erythrocyte oligomers dissociate slowly, and since oligomers comprise the bulk of the tubulin in depolymerized samples, the rates of microtubule nucleation and elongation are noticeably retarded relative to those of brain tubulin under the same conditions (21, 22, 33). It seems reasonable that the relatively slow rate of dissociation of erythrocyte tubulin oligomers could also be responsible for the gradients observed in copolymers assembled in mixtures of brain and erythrocyte tubulin. To examine this possibility, we determined the composition of oligomers present in mixtures of brain and erythrocyte tubulin at 5°C (Table II). In 8 out of 9 cases, the proportion of erythrocyte tubulin in oligomer pellets was greater than that in the corresponding mixtures, the average difference being 11%. The results show that under depolymerizing conditions, preparations containing equal amounts of brain and erythrocyte tubulin contain a higher proportion of erythrocyte tubulin oligomers and that these do not exchange rapidly with brain tubulin subunits. The data provide indirect support for the idea that differences in oligomer stability could contribute to the observed patterns of segregation by changing the ratio of erythrocyte and brain tubulin subunits during microtubule assembly (see Discussion).

The Role of Template Recognition in the Segregation of Isotypes

Finally, we observed that subunits of one isotype did not associate equally well with the ends of microtubules composed of the other isotype. To determine if the kind of microtubule seed influenced the composition of copolymers, erythrocyte, and brain microtubule seeds were prepared separately and added together to mixtures of erythrocyte and brain tubulin

Table II. Sedimentation of Erythrocyte Tubulin Oligomers in Brain and Erythrocyte Tubulin Mixtures at 5°C

Experiment	E Tubulin in Mixture (%)	E Tubulin in Pellet (%)
1	55.0	65.2
2	48.2	53.8
3	60.2	70.2
	55.8	50.8
	56.8	65.6
4	57.5	61.0
	54.9	63.2
	57.8	61.4
	56.4	65.0
Mean ± SD	55.8 ± 3.3	61.6 ± 6.1

Samples of erythrocyte and brain microtubule protein were passed through a cycle of assembly and disassembly to remove inactive subunits. Mixtures (1–3 mg/ml) containing equal amounts of isotypes were sedimented either at low speed (37,000 *g* for 20 min, samples 1 and 2) or at high speed (200,000 *g* for 90 min, samples 3 and 4) to pellet oligomers. The percent erthrocyte tubulin contained in mixtures and pellets was determined by SDS-PAGE at pH 8.8 and quantitative densitometry. Erythrocyte tubulin exhibits a unique electrophoretic mobility at this pH. Oligomer pellets were determined to contain 10% more erythrocyte tubulin (61.6%) than the respective starting mixture (55.8%). This difference was determined to be significant as determined by t test ($P < 0.02$ in a standard 2-tailed test of the two aggregate samples).

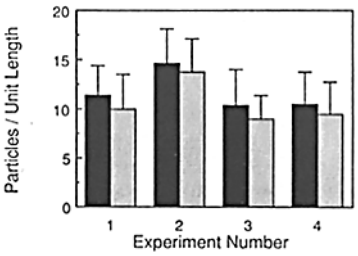


Figure 6. Compositional analysis of copolymers elongated from brain and erythrocyte microtubule seeds indicates that subunits associate preferentially with polymers of the same isotype. The bar graph shows the mean content of erythrocyte tubulin in populations of copolymers elongated from erythrocyte (*solid bar*) and brain (*stipple bar*) microtubule seeds for four separate experiments. The proximal segments of copolymers (3 U/microtubule = 0.9 μm) immediately adjacent to the seeds were examined and scored (50 microtubules for each seed type or 300 measurements/experiment). Experiments 1–3 are elongation experiments in which 25 μl of each seed type (6–12 mg/ml) was added to a mixture of subunits (each type at 0.5 mg/ml, 1.0 mg/ml total subunit concentration) and aliquots examined at 1–2 min after the initiation of elongation. The data in experiment 4 are from a steady-state experiment in which equal amounts of seeds (6–8 mg/ml) were combined and sampled within 1 min after mixing. The data show that the ratio of isotypes in copolymers elongated from brain and erythrocyte microtubule seeds is not the same. Error bars indicate SD.

subunits to initiate the elongation of copolymers, and the density of immuno-gold label was determined on the proximal 0.9 μm (3 U) of copolymer adjacent to the seeds. Although the differences were small, the data from four experiments showed that erythrocyte and brain microtubule seeds initiated the formation of copolymers whose proximal segments contained a greater amount of their respective tubulin isotype (Fig. 6). The differences in composition (2–5% differences in erythrocyte tubulin content) were slight but were significant ($p \leq 0.02$) as determined by t test for the aggregate sample of 1,200 U. In similar experiments, however, no difference in the rate of copolymer growth from the two different types of seeds was observed, suggesting that the slight differences in composition did not have functional significance as judged by the rate of copolymer elongation. Thus, the type of microtubule seed appears to play a relatively minor role in determining the composition of copolymers that elongate from its ends.

Discussion

Biochemically Distinct Tubulin Isotypes Copolymerize but Exhibit a Limited Degree of Segregation

We have observed that different isotypes of tubulin can copolymerize efficiently even though they exhibit considerable differences in their biochemical and assembly properties. The brain and erythrocyte isotypes have a far greater tendency to copolymerize than to segregate into distinct classes of polymers. Nevertheless, at low subunit concentrations, the isotypes exhibit a limited degree of segregation: (*a*) the proportion of erythrocyte tubulin changes in a regular manner along the lengths of elongating copolymers at a rate of 5–10%/μm; (*b*) two distinct classes of copolymers can assemble simultaneously in the same preparation, with copolymers formed by self-assembly containing twice as much erythrocyte tubulin as copolymers elongated from microtubule fragments; and (*c*) subunits of one isotype associate about equally as well with polymers of a different isotype as they do with polymers of the same isotype. Thus, the generation of copolymers with different ratios of isotypes appears to be the result of two distinct processes, which we described in an earlier theoretical paper on isotype sorting, if: unequal rates of incorporation of the two subunit types into elongating copolymer ends, and to a much more limited extent, a process of template recognition based on differences in the affinities between homologous and heterologous subunits.

Gradients Form Because Isotypes Polymerize at Different Rates

Gradients in copolymer composition are caused by unequal rates of incorporation of the two kinds of subunits into polymers. This net rate is determined by the concentrations of subunits and polymer ends and the rate constants for subunit association and dissociation at polymer ends. In practice a difference between the isotypes in one or several of these parameters could generate the observed gradients, but at present too little is known about the process to propose a specific mechanism. However, we do know that polymers and oligomers of erythrocyte tubulin are more stable than those of brain tubulin.

It is useful to consider the consequences of differences in stability. Erythrocyte microtubules have been observed to be 2–3 times more stable than brain microtubules in samples containing a mixture of polymers (Fig. 8 *B* in reference 30). The possibility that oligomers may contribute to the formation of gradients was also of particular interest to us. Depending on the solution conditions, oligomers are known to be relatively stable structures (33), and our previous work had shown that the presence of stable oligomers of erythrocyte tubulin markedly reduced the rates of both microtubule nucleation and growth by limiting the rate at which dimers were made available for assembly (21, 22). The present work shows that our tubulin mixtures contained a higher proportion of erythrocyte tubulin oligomers than would be expected if the oligomers of both isotypes had the same stability, and that erythrocyte tubulin oligomers did not exchange rapidly with brain tubulin subunits. We speculate that as the concentration of brain tubulin becomes depleted, the proportion of erythrocyte tubulin subunits gradually increases, resulting in the observed gradients. While our work does not prove that differential stability of erythrocyte polymers and/or oligomers causes the gradients, such a mechanism is plausible and is consistent with our observations in these and previous studies. Other processes could also affect the segregation of isotypes during polymerization. For example, the lower critical concentration of erythrocyte tubulin could cause the ratio of isotypes to gradually change with time. Alternatively, there could be time-dependent conformational changes in the tubulin, different rates of denaturation of one isotype relative to the other, or other processes that cause unequal rates of incorporation of the isotypes into polymers.

The mechanism for the simultaneous generation of two distinct classes of heteropolymers has not been examined in any detail. It is possible that self-assembled copolymers enriched in erythrocyte tubulin arise from dense aggregates of erythrocyte tubulin oligomers present in the initial subunit

mixture. Electron micrographs published by us previously (20, 22) reveal oligomer-rich aggregates consisting of extensive rings and spirals, which appear to act as sites of microtubule nucleation. Polymers that arise from these structures by self-assembly might be expected to be enriched in erythrocyte tubulin compared to copolymers elongating from seeds in the same mixture. Another possibility is that there was no simultaneous segregation into two classes of polymers, and that the copolymer segments formed by elongation from seeds or by self-assembly were identical in composition but differed in their protofilament number or alignment. It is possible that this could give rise to differential antibody binding, which would appear like a different stoichiometry of immunogold binding. Although we cannot formally rule out this possibility, we can say that we have so far not observed differences in antibody labeling of erythrocyte microtubules that are formed by elongation versus self-assembly, and that polymer types prepared by both methods contain thirteen protofilaments.

Finally, in agreement with the observation that different tubulin isotypes copolymerize freely, subunits of one isotype appear to associate nearly as well with polymers of different isotypes as they do with polymers of their own type. This is a surprising result, given the biochemical differences between the two isotypes and contrasts with the result obtained by Oosawa (24) and Kuroda (14) who observed template recognition effects of large magnitude during the seeded assembly of flagellins obtained from several species of bacteria.

Subunit Sorting In Vivo

Recently it has been shown that tubulin isotypes segregate to a limited extent in differentiating cells. Significant differences in the ratios of endogenous tubulin isotypes (gene products) contained in polymer and monomer fractions have been reported to occur in chicken erythrocytes (13) and in differentiating PC12 cells (1, 12). In the case of PC12 cells, subsets of microtubules enriched in certain isotypes can be distinguished by electron microscopy (12). The functional significance of having biochemically distinct classes of microtubules in the same cell may be to provide polymers with different degrees of stability or different affinities for MAPs and other components, which in turn are required for unique programs for cell division and differentiation. Although little is known about the segregation of isotypes for other cytoskeletal proteins, there have been reports of the spatial segregation of cytoplasmic myosins in myoblasts (6), of myosins I and II in *Acanthameba* (10), and of actin isotypes in muscle (16, 25, 26, 31) and vascular pericytes (11). Although the extent to which these examples represent true sorting has not been determined, the observations are consistent with what one might expect to see if sorting had occurred.

Since we observed sorting in vitro shortly after minor perturbation of monomer–polymer equilibria, one might expect sorting in the cell to take place during periods of rapid rearrangement of the cytoskeleton such as during mitosis, since at this time factors with high affinities for certain isotypes (certain MAPs for example) could selectively promote their incorporation into polymers. However, the ability of MAPs to promote sorting has not yet been observed by us or reported by others. Nevertheless, it is possible that other MAPs, microtubule-organizing centers, different ionic conditions, or a unique combination of isotypes might affect isotype usage in polymer formation. The present study suggests that interactions among the tubulin subunits themselves may be important in this process. Unfortunately, it is difficult to say if or how tubulin sorting occurs in cells. Sorting occurs in vitro under conditions of perturbation (shearing polymers, adding polymers to subunits). One might therefore expect to observe sorting in cells during periods of flux and change in the polymer/monomer ratio such as would occur during synthesis of a new isotype in differentiation or at times of remodeling of the microtubule cytoskeleton such as during mitosis. Further, one might predict the presence of factors such as MAPs or putative monomer-binding proteins that bind with different affinities to different tubulin isotypes. Further work will be required to determine if and to what extent tubulin isotype sorting occurs in vivo and to establish its significance for cell function.

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