Mini-Review

The Multitubulin Hypothesis Revisited: What Have We Learned?

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In the 16 years since α and β tubulin were first determined to be the principal subunits of eukaryotic microtubules, it has become abundantly clear that these polymers contribute at least two essential functions to all higher eukaryotes. First, as the primary structural component of the mitotic spindle, microtubules are responsible for the orderly segregation of replicated chromosomes into daughter cells at cell division. Second, in concert with actin filaments and intermediate filaments, cytoskeletal microtubules play a major role in establishing and maintaining the dynamic spatial organization of the cytoplasm (reviewed by Kirschner and Mitchison [11]). In addition to such ubiquitous uses, microtubules also function in specialized roles, comprising the major component of meiotic spindles and eukaryotic cilia and flagella, in establishment of the highly asymmetric morphology of neurons and serving as a substrate for the transport of vesicles and organelles within the cytoplasm.

Two Hypotheses for the Function of Tubulin Gene Families

Analysis of the molecular genetics of tubulin quickly lead to the discovery that in most eukaryotes small multigene families encoding both α and β polypeptides lay beneath this heterogeneity of tubulin use (reviewed in reference 4). While the data from unicellular eukaryotes have now demonstrated that single gene products can be sufficient for construction of all essential microtubules (17, 36), results from higher species clearly suggest that multiple tubulin gene sequences are required. But to what functional end? Two hypotheses are readily evident. First, to many the most attractive possibility was that individual tubulin genes might encode functionally divergent polypeptides which could confer some unique property to the final microtubule polymer. (Indeed, this idea was initially proposed by Fulton and Simpson [7] in advance of definitive proof of the existence of multiple genes.) The alternative hypothesis, most cogently advanced by Raff (19), was that multiple polypeptides are themselves functionally equivalent but represent the products of duplicated genes which have evolved to possess different regulatory sequences for activation of transcription during alternative programs of differentiation. So where does the issue stand at present?

α and β Tubulins in Higher Eukaryotes Are Each Encoded by Approximately Six Functional Genes

The accumulated data for vertebrate tubulin genes, derived principally from Cowan's laboratory and from my own, have established that approximately six functional α tubulin genes and a corresponding number of β tubulin genes are expressed in specific, but often complicated programs during differentiation. Cowan's comprehensive examinations of cDNA sequences encoding mouse α tubulins (33) and β tubulins (14, 34) have identified six functional α genes that encode five distinct α tubulin isotypes and five β genes each encoding a unique primary sequence, including one (used in construction of marginal band microtubules in mammalian platelets [N. Cowan, personal communication]) which is nearly as divergent from other β tubulins as β tubulins are from α tubulins (34). Similarly, our examination of the chicken genome has established that seven genes encode functional β tubulins. Complete sequence analysis of six of these has revealed that they encode five distinct isotypes (27–29) including one highly divergent isotype which assembles into the marginal band of circumferential microtubules in hematopoietic cells (15; Murphy, D. B., and D. W. Cleveland, unpublished observations).

Identification of Conserved Isotypic Classes of Vertebrate β Tubulins

Comparison of all of the available vertebrate β tubulin sequences unambiguously reveals the existence of a highly conserved polypeptide framework in which individual sequences typically diverge from each other both within and between species in 2–8% of ~450 residue positions (excluding the more divergent marginal band β tubulins). However, the carboxy-terminal ~15 residues constitute a major variable region domain for β tubulin, and, to a lesser extent, for α tubulin as well (18, 33). In this short region, substitutions, deletions, and terminal addition of amino acids are found in >75% of the residue positions.

What has emerged from an otherwise confusing array of variable region sequences (including not only the mouse and chicken sequences but also three human β tubulins determined by Cowan and co-workers [9, 13] and one determined by Sullivan and Cleveland [26], one porcine sequence determined by Krauhs et al. [12], and three fragmentary rat sequences from Farmer et al. [6]) is the identification of four distinct, evolutionarily conserved isotypes of β tubulins that are distinguished primarily by their characteristic carboxyl-terminal variable region sequence (26; see also Table I). In addition, in every case where a mammalian interspecies comparison can be made, all amino acid substitutions that distinguish one isotype from another are absolutely con-

served (34). Furthermore, such as it is known, the program of expression in different cell and tissue types for each isotypic class is similar in different species.

Are Individual Tubulin Isotypes Functionally Distinguishable?

The stringent interspecies conservation of β tubulin isotypes implies that each isotypic sequence has been positively selected during evolution. This in turn supports the hypothesis of a functional role for the structural differentiation of β tubulin isotypes. Indeed, direct evidence that β tubulin isotypes are biochemically distinguished within cells has been presented by Gard and Kirschner (8) and Edde et al. (5), who have documented the differentiation-dependent phosphorylation of a distinct isoform of β tubulin in neuroblastoma cells. Clearly, the presence of cellular mechanisms that use only a specific isotype mandates that such isotypes are in fact used for unique roles in cells. In addition, in vitro experiments have demonstrated that proteolytic removal of the carboxy-terminal 15–20 amino acids yields subunits still able to assemble, but with altered properties (3, 24). When coupled with evidence that the carboxy-terminal domain may be directly involved in binding some microtubule-associated proteins (23), these results further suggest that tubulin assembly characteristics could be modulated in vivo by preferential binding of individual microtubule-associated proteins to particular isotypes.

Genetic Approaches Do Not Support Functional Specialization of Individual Tubulin Isotypes

On the other hand, arguments which rely on sequence conservation alone are clearly insufficient in and of themselves to settle the question of the purpose of multigene families. As Raff has correctly noted (19), retention of variable region sequence may simply reflect the slow ticking of the evolutionary clock of sequence drift after the time of gene duplication. In addition, in those organisms amenable to genetic approaches, evidence has clearly suggested functional equivalence of multiple polypeptides. For example, the identification of a single *Drosophila* gene that encodes the overwhelming majority of β tubulin polypeptides in developing spermatocytes coupled with the isolation of recessive mutations in that locus which disrupt function of meiotic, axonemal, and cytoplasmic microtubules allowed Kemphues et al. (10) to conclude that this β tubulin subunit was multifunctional; i.e., it was used to assemble all classes of spermatocyte microtubules. Obviously, such findings dispel any notion of one gene product for each microtubule class. However, these data do not exclude the possibility of unique functional properties conferred to the testis-specific isotype as a result of primary sequence.

Additional genetic experiments in the fission yeast *Schizosaccharomyces pombe* have shown that this organism contains two α tubulin genes whose protein products are identical only in ~80% of residue positions (31). Through gene disruption experiments, one of the two genes has been shown to be essential, whereas the second is dispensable (1). Mutations in the essential gene, however, may be complemented by increased expression of the nonessential gene. An analogous situation has been found for the budding yeast *Saccharomyces cerevisiae* (21, 22). Although the limited diversity of microtubule function in yeast limits wide generalizations, these results (particularly when coupled with the presence of only a single β tubulin gene) argue against restriction of individual gene products to subclasses of microtubules.

Similarly, analysis of the two β tubulin genes in Aspergillus has produced the startling finding that rather than inhibiting conidiation (sporulation) as expected, mutants which fail to produce the β tubulin normally expressed only during conidiation have no phenotype at any point in the life cycle (35). Although the retention in the genome of this functional gene argues clearly that some growth condition(s) exist in which the gene product confers some selective advantage, the direct data at hand demonstrate that this gene does not provide a specialized polypeptide of essential function.

In addition, the present tubulin gene sequence data also demonstrate that in some cases gene multiplicity is used for expression of a single isotype in multiple differentiated cell types. For example, as shown in Table I, two mammalian genes encode the class IV β tubulin isotype. Similarly, recombination between two chicken genes has yielded two pathways for expression of a single isotype (28).

Other Evidence for Versatility in Tubulin Isotype Function

Two final experiments provide a different perspective on the properties of tubulin isotype expression. First, brain tubulin can substitute for the highly divergent hematopoetic β tubulin in the in vitro reassembly of the marginal band microtubules in chicken erythrocytes (30). Since the two tubulins differ in their assembly properties (16, 20), there was a distinct possibility that this substitution would fail. On the other hand, the significance of this experiment is unclear because the assay might not be sufficiently sensitive to detect the

Table I. Proposed Carboxy-terminal Isotype-defining Consensus Sequences for Four Conserved β Tubulin Isotypes

Isotype Class	Species from which isotype identified	Pattern of expression	COOH-Terminal variable sequence
I	Human (9), rat (6), mouse (14)	Major constitutive	E E E E D F G E E A E E E A
II	Chick (32), pig (12), rat (6), mouse (14)	Major neuronal	D E Q G E F E E E G E E D E A
III	Chick (25), human (26)	Minor neuronal	E E E G E M Y E D D E E E S E S Q G P K
IVa	Human (9), rat (6), mouse (14)	Brain specific	E E G E F E E E A E E E V A
IVb	Human (13), mouse (26, 34)	Constitutive	E E E G E F E E E A E E E V A

Sequences are aligned to maintain a constant position of the single aromatic residue. (Adapted from reference 26.)

functionally important differences and the assembly of the brain tubulin might be promoted by residual erythrocyte tubulin that is left in the extracted ghosts (Murphy, D. B., personal communication).

Second, when a chimeric β tubulin composed of a yeast carboxy-terminal variable region sequence linked to a chicken amino terminus is expressed in mouse fibroblasts, the novel tubulin can incorporate into all classes of microtubules that can be identified by light microscopy (2). Unfortunately, such data are silent as to the question of the possible specialized function of tubulin isotypes. If the isotype-defining carboxy-terminal domain does confer a unique functional property in its normal mammalian context, what then could one expect the mammalian machinery to do with the yeast-variable region sequence other than to use it indiscriminately?

What Can We Now Conclude about Tubulin Gene Families and Isotypes?

Consideration of the collective findings reveals that both hypotheses for the function of tubulin multigene families in higher eukaryotes are correct in some instances. Some genes use alternative transcriptional activator sequences to encode functionally equivalent polypeptides. Others encode divergent polypeptides that are likely to be functionally distinguishable. In view of the failure thus far to detect such properties unambiguously in most in vitro assays or in gene transfection experiments, it seems likely (at least to this observer) that such functional distinctions are probably subtle and manifest only in conjunctions with other specific microtubule components.

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