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Tubulin Sorting during Dimerization In Vivo

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We demonstrate sorting of β-tubulins during dimerization in the *Drosophila* male germ line. Different β-tubulin isoforms exhibit distinct affinities for α-tubulin during dimerization. Our data suggest that differences in dimerization properties are important in determining isoform-specific microtubule functions. The differential use of β-tubulin during dimerization reveals structural parameters of the tubulin heterodimer not discernible in the resolved three-dimensional structure. We show that the variable β-tubulin carboxyl terminus, a surface feature in the heterodimer and in microtubules, and which is disordered in the crystallographic structure, is of key importance in forming a stable α-β heterodimer. If the availability of α-tubulin is limiting, α-β dimers preferentially incorporate intact β-tubulins rather than a β-tubulin missing the carboxyl terminus (β2ΔC). When α-tubulin is not limiting, β2ΔC forms stable α-β heterodimers. Once dimers are formed, no further sorting occurs during microtubule assembly: α-β2ΔC dimers are incorporated into axonemes in proportion to their contribution to the total dimer pool. Co-incorporation of β2ΔC and wild-type β2-tubulin results in nonmotile axonemes because of a disruption of the periodicity of nontubulin axonemal elements. Our data show that the β-tubulin carboxyl terminus has two distinct roles: 1) forming the α-β heterodimer, important for all microtubules and 2) providing contacts for nontubulin components required for specific microtubule structures, such as axonemes.

INTRODUCTION

Microtubules support the assembly of cytoskeletal structures of diverse morphology and function. The basic building blocks of all microtubules are heterodimers composed of α- and β-tubulins encoded by multiple gene families. Studies of *Drosophila* development have demonstrated functional specialization of microtubules assembled from differentially expressed α- and β-tubulin isoforms (Hoyle and Raff, 1990; Matthews *et al.*, 1993; Hoyle *et al.*, 1995, 2000; Hutchens *et al.*, 1997; Dettman *et al.*, 2001). We have used *Drosophila* spermatogenesis as a model system to compare the intrinsic capacity of different α- and β-tubulins to assemble into different microtubules. In the *Drosophila* male germ line, a single α-β heterodimer species composed of the ubiquitous α84B-tubulin and the testis-specific β2-tubulin, supports all postmitotic microtubule functions, including assembly of the sperm tail flagellum, *Drosophila*'s only motile axoneme (Kemphues *et al.*, 1979, 1980, 1982, 1983; Matthews *et al.*, 1989). We have discovered that the constituent tubulins can determine several levels of microtubule organization, including microtubule protofilament number, morphology of specific sets of microtubules, and overall organization of axoneme superstructure (Fackenthal *et al.*, 1993; Hoyle *et al.*, 1995; Hutchens *et al.*, 1997; Raff *et al.*, 1997, 2000). These studies demonstrated that different α-β dimers contribute to differences in microtubule architecture and function. Here we ask a more basic question: Does the process of α-β dimerization discriminate between tubulin isotypes? To address this we have looked at the process of tubulin dimerization in vivo in the *Drosophila* male germ line.

Biochemical studies by Cowan, Lewis, and their colleagues identified components of the molecular machinery that guides formation of α-β-tubulin heterodimers (Gao *et al.*, 1992, 1994; Tian *et al.*, 1996, 1997, 1999). After initial in vivo folding of nascent (or in vitro, denatured) tubulin subunits via TriC chaperonin, final folding and dimerization occur in a "dimerization machine," a supermolecular complex comprising α- and β-tubulin and at least five tubulin-specific chaperones. Because the heterodimer is the stable form of native tubulin, biochemical preparations always contain equimolar amounts of α- and β-tubulin. With the *Drosophila* male germ line we are able to determine the consequences for tubulin dimerization in vivo when synthesis of α- and β-tubulin is not equimolar (Hoyle *et al.*, 1995; Hutchens *et al.*, 1997).

In this study, we examined the role of the β-tubulin carboxyl terminus in the dimerization process. The isotype-

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defining carboxyl terminus of *Drosophila* β2-tubulin is required for axonemes but is not essential for the assembly of functional cytoplasmic microtubules, including spindles (Fackenthal *et al.*, 1993; Hoyle *et al.*, 1995; Nielsen *et al.*, 2001). The α- and β-carboxyl termini are not resolved in the electron crystallographic structure of the α-β tubulin dimer. Both termini are surface features of the α-β dimer and lie on the outside of the microtubule wall (Wolf *et al.*, 1996; Nogales *et al.*, 1998, 1999). The resolved intradimer contacts between α- and β-tubulin thus support the prediction that the C termini are dispensable for the heterodimer. We tested this hypothesis for the β-tubulin subunit by comparing the ability of β2ΔC, a carboxyl terminus-truncated form of β2-tubulin (Fackenthal *et al.*, 1993), to form dimers when it was the sole β-tubulin in the male germ cells or when different full-length β-tubulins were also present. Our data show that the β-tubulin carboxyl terminus plays an important role in generating stable α-β dimers and that sorting between different β-tubulins takes place during dimerization. Competition between β2ΔC and full-length β-tubulins revealed that different β-tubulin isoforms have distinct capacities for forming dimers with α-tubulin. In vivo, many β-tubulins may be coexpressed in the same cell. We propose that isotype-specific differences in the dimerization properties of different β-tubulins may play a key role in defining the cellular isoform content and, hence, in determining cell-type specific functions of the microtubule cytoskeleton.

Because we observed that tubulins were sorted during dimerization, we wondered whether further sorting would occur during microtubule assembly. We found no evidence for this. Once formed, β2ΔC-containing dimers were incorporated into axonemes at the same ratio as they were present in the total dimer pool. As we show here, these β2ΔC-containing axonemes are structurally and functionally compromised, revealing that the periodicity of the interactions of the β-tubulin carboxyl termini with nontubulin components is an essential feature of axoneme architecture.

MATERIALS AND METHODS

Drosophila *Stocks*

Stocks were maintained at 25°C to avoid temperature-induced effects on male fertility. The β2-tubulin null allele, $\beta2^{null}$, was described by Fackenthal *et al.* (1993). The deficiency chromosome that deletes the α84B-tubulin gene, *Df(3R)Scx*^{4}, was described by Hazelrigg and Kaufman (1983); for clarity, we have herein designated this as *Df(α84B)*. Other *Drosophila* stocks are described in FlyBase (1999).

Gene Constructs

The transgenic construct p[β2ΔC] consists of the β2 gene with stop codons engineered at amino acid positions 432 and 433, resulting in the deletion of the 15 carboxyl-terminal residues (construct *B2t.432*, Fackenthal *et al.*, 1993). p[β2ΔC] supports expression of β2ΔC-tubulin in the postmitotic male germ line at a level equivalent to the endogenous β2-tubulin. Pulse-chase experiments have shown that the intrinsic stability of β2ΔC is only slightly less than that of β2 (Fackenthal *et al.*, 1993; note that the pulse-chase experiments were performed under conditions where α-tubulin was not limiting; see DISCUSSION and Figure 2.).

As described previously, the transgenic constructs p[β1], p[β3], and p[α85E] were constructed with the use of a "testis vector" containing 2.1 kb of β2 5′ and 1.5 kb of β2 3′ sequences flanking the indicated heterologous coding sequence. These β2 regulatory sequences control expression of the inserted coding sequence at the same level and tissue specificity as the endogenous β2-tubulin (Hoyle *et al.*, 1995; Hutchens *et al.*, 1997; Raff *et al.*, 2000). The transgenic construct p[α84B] consists of the wild-type α84B-coding sequence and sufficient flanking genomic sequence to direct fully wild-type expression of α84B in the male germ line (Matthews *et al.*, 1993; Hutchens *et al.*, 1997).

Analysis of Drosophila *Testis Tubulins*

In the *Drosophila* testis, the ubiquitous β-tubulin, β1, is the only β-tubulin expressed in the early, mitotic stages of spermatogenesis (Bialojan *et al.*, 1984; Kaltschmidt *et al.*, 1991). Before meiosis, β1 is replaced by the testis-specific isoform, β2-tubulin. β2 is then required for all microtubules, including meiotic spindles, all cytoplasmic microtubules, and the motile sperm tail axoneme (Kemphues *et al.*, 1979, 1980, 1982, 1983). Both β1 and β2 dimerize with α84B-tubulin, the only α-tubulin expressed in the *Drosophila* male germ line (Matthews *et al.*, 1989). After the onset of spermatogenesis in late larval development, the testes become filled with developing spermatids. In the work presented here, all testes are from 1-day-old adults, in which total testis proteins primarily represent postmitotic stages. The major tubulins in adult testes are thus postmitotically expressed species, which consist of the endogenous isoforms α84B and β2, or heterologous tubulins expressed from transgenic constructs controlled by β2 regulatory elements.

Samples for two-dimensional gel analysis and immunoblotting were prepared from 1-day-old males as described by Hoyle and Raff (1990). Four testes labeled for 1 h with [^{35}S]methionine plus six unlabeled testes were used for each gel sample. Isoelectric focusing gradients were established with the use of a 2:1 ratio of wide range (pH 3.0–10) to narrow range (pH 4.0–6.0) ampholytes (Fluka, Buchs, Switzerland). Antibodies used were a commercial anti-β antibody (N357, Amersham Pharmacia Biotech, Piscataway, NJ) and anti-α antibody (N356, Amersham Pharmacia Biotech); as previously documented, these antisera react with endogenous *Drosophila* testis tubulins and all experimental β-tubulins used in this study (Hoyle *et al.*, 1995; Hutchens *et al.*, 1997; Raff *et al.*, 2000). Primary antibodies were detected with the use of a horseradish peroxidase-conjugated goat-anti-mouse secondary antibody (Jackson ImmunoResearch, West Grove, PA) and detected with the use of 4-chloro-1-napthol and hydrogen peroxide.

In each experiment, the [^{35}S]methionine signal for each tubulin species provided a direct measure of the levels of synthesis during 1 h of labeling. Antibody-staining signals on the same blot provided a direct measure of the amount of each tubulin species that accumulated in the stable tubulin pool during spermatid maturation, a process that takes 5 days (Lindsley and Tokuyasu, 1980). Comparison between [^{35}S]methionine signal and antibody signal thus revealed the relative extent of stable dimers formed by each tubulin species present. Different sites of insertion were tested for each gene construct. Multiple blots were run for each gene combination; all gave the described phenotypes.

Determination of Male Fertility

Virgin males were collected and held away from females for 5 days. Sperm production was assayed by dissecting the reproductive tract and scoring for the presence of motile sperm in the seminal vesicles by light microscopy as described previously (Hoyle and Raff, 1990; Hoyle *et al.*, 1995; Hutchens *et al.*, 1997).

Electron Microscopy

Testes from 1-day-old males were fixed overnight in 2.5% gluteraldehyde and 0.1 M cacodylate, stained with 2% osmium tetroxide and 0.5% uranyl acetate, dehydrated, and imbedded in DER resin (Electron Microscopy Sciences, Fort Washington, PA). Sectioning and transmission electron microscopy were done by standard methods.

RESULTS

The β-Tubulin Carboxyl Terminus Participates in Formation of Stable α-β Heterodimers

We have used the *Drosophila* postmitotic male germ cells as an experimental system that allows us to assay tubulin dimerization under conditions where endogenous or heterologous tubulins can be expressed at specific levels. The stable form of soluble tubulin is the α-β heterodimer. In *Drosophila*, free, monomeric α- or β-tubulin is degraded immediately after synthesis (Kemphues *et al.*, 1982; Hoyle *et al.*, 1995; Hutchens *et al.*, 1997). We have previously shown that synthesis of both α- and β-tubulin is directly proportional to gene dose in the postmitotic male germ cells (Hoyle and Raff, 1990; Hoyle *et al.*, 1995). We have shown that this holds true for the endogenous germ line tubulins, β2 and α84B, as well as for β2ΔC and transgenic genes driven by β2 regulatory sequences (see MATERIALS AND METHODS; Hoyle and Raff, 1990; Fackenthal *et al.*, 1993; Hoyle *et al.*, 1995; Hutchens *et al.*, 1997; Raff *et al.*, 2000). In wild-type testes, the quantity of α matches the quantity of β, but the one "synthesis unit" per gene still holds true under experimental conditions in which the gene dose of α does not equal the gene dose of β (Hutchens *et al.*, 1997). Thus, as illustrated in Figure 1, we can examine the formation of stable tubulin dimers in vivo under conditions in which α- and β-tubulin synthesis is not balanced.

We tested the role of the β-tubulin C terminus in dimerization in experiments in which intact β-tubulins competed for dimerization with α-tubulin with β2ΔC, a truncated β2-tubulin missing only the final 15 amino acids that constitute the variable C-terminal isotype-defining domain (Fackenthal *et al.*, 1993). Figure 2 and Table 1 summarize experiments in which we determined the ability of β2ΔC to form stable dimers under conditions in which the availability of total α- and β-tubulins was varied. We found that, when β2ΔC was coexpressed with any full-length β-tubulin under conditions in which α-tubulin was limiting (i.e., the total α-tubulin pool was less than the total β-tubulin pool), the β2ΔC component of the β-tubulin pool failed to dimerize, and β2ΔC was degraded. Conversely, when α-tubulin was not limiting, β2ΔC formed stable heterodimers. We have found that stable α-β2ΔC heterodimers are incorporated into axonemes. This results in male sterility if β2ΔC makes up more than one-third of the stable dimer pool (discussed below). We therefore used male sterility as a second assay for the formation of stable α-β2ΔC dimers (Table 1).

Figure 2A shows that, when β2ΔC was the only β-tubulin expressed in the postmitotic germ cells, β2ΔC formed stable dimers with α84B-tubulin. Under these conditions, β2ΔC is itself stable (Table 1, lines 1and 2) and rescues the instability of α84B, which is otherwise unstable in the absence of endogenous β2 (as shown in Figure 1B). β2ΔC-containing heterodimers can be assembled into microtubule arrays that exhibit at least partial function, including meiotic spindles and the cytoplasmic microtubules that mediate mitochondrial elongation. However, β2ΔC cannot assemble axonemes (Figure 3; Fackenthal *et al.*, 1993). Here we report experiments in which coexpression of β2ΔC and intact β-tubulins reveals a new role for the C terminus in stabilization of heterodimers. Our data thus show that, although dimerization and microtubule assembly per se are not dependent on the β-tubulin C terminus, it nonetheless plays a crucial role in stability of the heterodimer and control of the specificity of microtubule assembly in vivo.

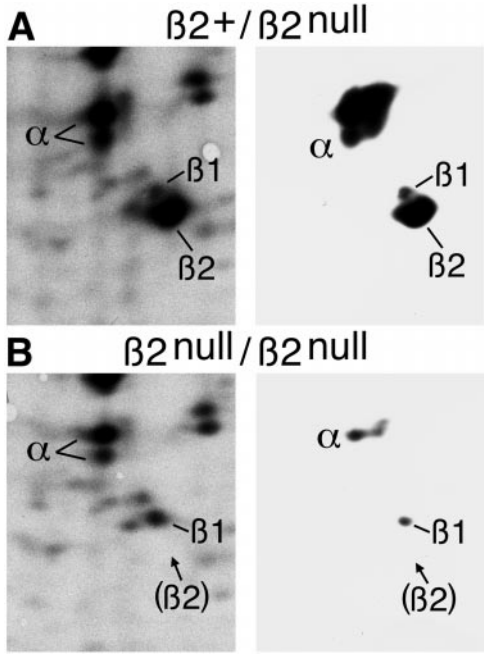

Figure 1. Monomeric tubulins are unstable in the *Drosophila* male germ line. Total testis proteins from 1-day-old males were labeled with [^{35}S]methionine, separated by two-dimensional gel electrophoresis, blotted, and immunostained as described in MATERIALS AND METHODS. Left, autoradiograms showing labeled proteins. Right, the same blots stained with anti–α-tubulin and anti–β-tubulin antibodies, showing stable testis tubulins. The α signal is from α84B-tubulin (Matthews *et al.*, 1989; Hutchens *et al.*, 1997). (A) Testes from males with one functional copy of the wild-type β2-tubulin gene in a wild-type α84B background (genes present: $\beta2^{+}/\beta2^{null}$; $\alpha84B^{+}/\alpha84B^{+}$). The stable form of soluble tubulin is the α-β heterodimer: the amount of stable α84B corresponds to the total amount of stable β-tubulin, primarily β2-tubulin for this genotype. (B) Testes from β2-null males (genes present: $\beta2^{null}/\beta2^{null}$; $\alpha84B^{+}/\alpha84B^{+}$). These males are sterile: each gonial cell produces 16 spermatocytes via four rounds of mitosis, but then spermatogenesis fails. β2-Tubulin is not synthesized (arrows denote the normal position of β2); the mature spermatocytes do not assemble meiotic spindles, and spermatids do not assemble axonemes or any other postmitotic microtubules. α84B is synthesized in the postmitotic cells (left), but in the absence of any β2, there can be no formation of α-β dimers and almost all of the newly synthesized α84B is rapidly degraded and fails to accumulate (right). The small amount of antibody-stained α84B represents α84B-β1 dimers in premeiotic cells.

Figure 2B shows that β2ΔC was not stable if full-length β2 was also present at wild-type levels and α-tubulin was therefore limiting (Table 1, line 4). These males have two gene doses of α-tubulin and three total gene doses of β-tubulin. β2ΔC synthesis was the same with or without synthesis of endogenous β2 (in Figure 2, compare B and A), but when α-tubulin is limiting, endogenous full-length β2 and β2ΔC must compete to form α-β heterodimers. β2ΔC is outcompeted, fails to dimerize, and is degraded. The extent to which β2ΔC tubulin is excluded from the dimer pool when α-tubulin is limiting is illustrated by comparing B and C in Figure 2. Figure 2B shows testis tubulins in males with two copies of both α84B and full-length β2, plus a single copy of β2ΔC. Only a trace amount of stable β2ΔC was accumulated (i.e., dimerized with α-tubulin). Figure 2C shows

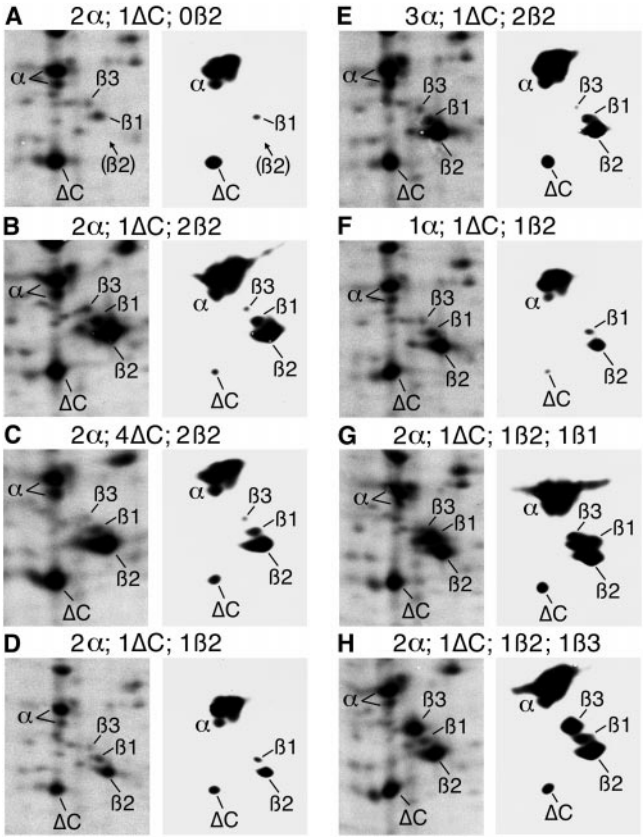

Figure 2. β2ΔC-tubulin is stable only when α-tubulin is not limiting. Two-dimensional gels of total testis proteins from 1-day-old males, prepared as described in MATERIALS AND METHODS. Left, autoradiograms showing [^{35}S]methionine-labeled proteins. Right, the same blots stained with anti–α-tubulin and anti-β-tubulin antibodies, showing stable testis tubulins. (A) Testis proteins from sterile males with two gene doses of α84B (wild type at the α84B locus), one copy of β2ΔC, and no β2-tubulin (genes present: p[β2ΔC]; $\beta2^{null}/\beta2^{null}$; $\alpha84B^{+}/\alpha84B^{+}$). β2ΔC is the only β-tubulin synthesized in the postmitotic cells and is stable (comparable to the stability of β2 itself shown in Figure 1A). Arrow denotes the normal position of β2. (B) Testis proteins from fertile males that are wild type at the α84B and β2 loci and also carrying one copy of p[β2ΔC] (genes present: p[β2ΔC]; $\beta2^{+}/\beta2^{+}$; $\alpha84B^{+}/\alpha84B^{+}$). There are two gene doses of α-tubulin and three gene doses of total β-tubulin. β2ΔC is synthesized but is unstable; stable tubulin in the postmitotic cells consists primarily of α84B-β2 dimers. β1 staining reflects the contribution of premeiotic germ cells to total testis tubulins. The amount of total protein on this gel is large enough to also allow detection of the endogenous β3-tubulin present in the somatic cyst cells (Kimble *et al.*, 1989; Hoyle and Raff, 1990). (C) Testis proteins from fertile males that are wild type at the α84B and β2 loci but also carrying four copies of p[β2ΔC]. There are two gene doses of α-tubulin and six total gene doses of β-tubulin. There is more stable β2ΔC than in the genotype shown in Figure 2B, but the amount of stable β2ΔC is less than β2 and much less than when β2ΔC is the only β-tubulin available in the postmitotic cells (compare with Figure 2A). (D) Testis proteins from sterile males carrying one copy each of p[β2ΔC] and β2 and wild type at the α84B locus (genes present: p[β2ΔC]; $\beta2^{+}/\beta2^{null}$; $\alpha84B^{+}/\alpha84B^{+}$). There are two total gene doses of β-tubulin and two gene doses of α-tubulin. β2ΔC is stable, accumulating to an amount only slightly less than endogenous β2. (E) Testis proteins from sterile males carrying one copy of p[α84B], one copy of p[β2ΔC], and wild type at the endogenous α84B and β2 loci (genes present: p[β2ΔC]; p[α84B]; $\beta2^{+}/\beta2^{+}$; $\alpha84B^{+}/\alpha84B^{+}$). There are three total gene doses of α-tubulin and three total gene doses of β-tubulin. β2ΔC is stable. (F) Testis proteins from sterile males with one gene dose each of β2ΔC, α84B and β2 (genes present: p[β2ΔC]; $\beta2^{+}/\beta2^{null}$; $\alpha84B^{+}$/Df[α84B]). There are two gene doses of total β-tubulin but only one gene dose of α-tubulin. The rates of β2ΔC and endogenous β2 synthesis are comparable (left). However, β2ΔC is unstable (right). (G) Testis proteins from fertile males carrying two gene doses of α84B and one gene dose each of β2, β2ΔC, and transgenic β1 (genes present: p[β2ΔC]; p[β1]; $\beta2^{+}/\beta2^{null}$; $\alpha84B^{+}/\alpha84B^{+}$). There are three gene doses of total β-tubulin and only two gene doses of α-tubulin. The stability of β2ΔC is reduced but to a lesser degree than when β2 is the sole competing full-length β-tubulin (compare B and C). (H) Testis proteins from males carrying two gene doses of α84B and one gene dose each of β2, β2ΔC and transgenic β3 (genes present: p[β2ΔC]; p[β3]; $\beta2^{+}/\beta2^{null}$; $\alpha84B^{+}/\alpha84B^{+}$). There are three gene doses of total β-tubulin and only two gene doses of α-tubulin. The stability of β2ΔC is reduced, comparable to the genotype in G. (Note that males with equal gene doses of p[β3]and β2 are sterile with or without β2ΔC; see Table 1, lines 18 and 19.)

testis tubulins in males with two copies of both α84B and β2, but with four copies of β2ΔC (Table 1, line 6). The amount of stable β2ΔC is increased but is still much less than the amount of stable β2. Thus, intact β2 can outcompete β2ΔC for dimerization even when synthesis of β2ΔC exceeds β2.

The instability of β2ΔC can be "rescued" by reducing the endogenous β2 gene dosage so that α-tubulin is no longer limiting. Figure 2D shows synthesis and accumulation of testis tubulins in males that are wild-type for α84B and have one copy each of β2ΔC and the endogenous β2 gene. There are two gene doses of α-tubulin and two total gene doses of β-tubulin (Table 1, line 8). Figure 2D, right, shows that the amount of accumulated β2ΔC is only slightly less than the amount of β2. Thus, if α-tubulin is not limiting, β2ΔC can form stable dimers even in the presence of β2.

A second means of stabilizing β2ΔC is to increase the level of α84B-tubulin. Figure 2E shows synthesis and accumulation of β2ΔC in males that are wild-type at the α84B and β2 loci and also carry one copy of β2ΔC, plus an additional transgenic copy of the α84B gene (Matthews *et al.*, 1993; Hutchens *et al.*, 1997). This results in three total gene doses of α-tubulin and three total gene doses of β-tubulin (Table 1, line 9). Once again, as in Figure 2D, α84B is not limiting, and β2ΔC forms stable dimers. In a separate experiment, we used a different α-tubulin isoform to increase the total α-tubulin pool (Table 1, lines 11 and 12). α85E-tubulin is a developmentally regulated isoform that is not expressed in the wild-type male germ line (Matthews *et al.*, 1990). We have previously shown that the transgenic construct p[α85E] expresses α85E in the postmitotic male germ line at a level equivalent to the endogenous α84B (Hutchens *et al.*, 1997). Increasing the level of α-tubulin in the testes by expressing one copy of p[α85E] in males together with one copy of p[β2ΔC] and two copies each of α84B and β2 also resulted in accumulation of stable β2ΔC, similar to when three doses of α84B were present. α85E is not a normal partner for β2 in wild-type males; nevertheless, α85E can rescue the instability of β2ΔC about as well as can α84B.

A reciprocal experiment showed that reducing the level of endogenous α-tubulin destabilizes β2ΔC. Figure 2F shows synthesis and accumulation of testis tubulins in males with

Table 1. The β-tubulin carboxyl terminus functions in forming stable α, β-tubulin heterodimers

Expt.	Tubulin genes present[a]	Total α-tubulin gene dose	Total C+ β-tubulin gene dose[b]	β2ΔC gene dose	Excess α-tubulin gene dose[c]	Stability of β2ΔC[d]	Male fertility phenotype[e]	Fraction of males with sperm	Amount of sperm[f]
I. β2ΔC is stable only when α-tubulin is not limiting									
1.	2 α84B; 0 β2; 1 p[β2ΔC]	2	0	1	2	Stable	Sterile (no axonemes)		
2.	2 α84B; 0 β2; 2 p[β2ΔC]	2	0	2	2	Stable	Sterile (no axonemes)		
3.	2 α84B; 2 β2 (Wild type)	2	2	0			Fertile	23/23	+++++
4.	2 α84B; 2 β2; 1 p[β2ΔC]	2	2	1	0	Unstable	Fertile	75/75	++++
5.	2 α84B; 2 β2; 2 p[β2ΔC]	2	2	2	0	Reduced	Fertile	12/12	++++
6.	2 α84B; 2 β2; 4 p[β2ΔC]	2	2	4	0	Reduced	Fertile, RF	25/34	++
7.	2 α84B; 1 β2	2	1	0			Fertile, RF	73/90	+++
8.	2 α84B; 1 β2; 1 p[β2ΔC]	2	1	1	1	Stable	Sterile	0/126	−
9.	2 α84B; 1 p[α84B]; 2 β2; 1 p[β2ΔC]	3	2	1	1	Stable	Sterile	0/77	−
10.	2 α84B; 1 p[α84B]; 2 β2	3	2	0			Fertile	76/77	++++
11.	2 α84B; 1 p[α85E]; 2 β2	3	2	0			Fertile	28/23	+++++
12.	2 α84B; 1 p[α85E]; 2 β2; 1 p[β2ΔC]	3	2	1	1	Reduced	Sterile	0/54	−
13.	1 α84B; 2 β2	1	2	0			Fertile, RF	39/40	+++
14.	1 α84B; 1 β2; 1 p[β2ΔC]	1	1	1	0	Unstable	Fertile, RF	31/31	+++
II. Heterologous β-tubulins are preferentially dimerized in competition with β2ΔC when α-tubulin is limiting									
15.	2 α84B; 2 β2; 1 p[β1]	2	3	0			Fertile	40/40	+++++
16.	2 α84B; 1 β2; 1 p[β1]	2	2	0			Fertile	113/113	+++++
17.	2 α84B; 1 β2; 1 p[β1]; 1 p[β2ΔC]	2	2	1	0	Reduced	Fertile, RF	75/82	+++
18.	2 α84B; 1 β2; 1 p[β3][g]	2	2	0			Sterile (abnormal axonemes)		
19.	2 α84B; 1 β2; 1 p[β3]; 1 p[β2ΔC]	2	2	1	0	Reduced	Sterile (abnormal axonemes)		

[a] Tubulin genes expressed in the postmitotic male germ cells. Transgenic tubulin genes presented are expressed in the male germ line at a level equivalent to that of the endogenous germ line tubulin genes (see MATERIALS AND METHODS).

[b] Total C+ β-tubulin gene dose equals the total number of genes for full-length β-tubulins (i.e., containing an intact C terminus).

[c] Excess α-tubulin gene dose equals the total α-tubulin gene dose minus the total C+ β-tubulin gene dose. (Excess α-tubulin gene dose represents the number of genes for α-tubulin that exceeds the number of genes for full-length β-tubulins and thus represents α-tubulin available for dimerization with β2ΔC.)

[d] The amount of β2ΔC accumulated in the testis tubulin pool when β2 was absent is approximately equal to the normal amount of β2 (scored as stable). When other full-length β-tubulins were present, stable β2ΔC was diminished relative to other β-tubulins in the same experiment (scored as reduced stability) or barely detectable (scored as unstable). See Figure 2.

[e] Males scored as "Fertile" exhibited fertility and motile sperm production similar to that of wild-type males. Males scored as "Fertile, RF" produced progeny but exhibited reduced fecundity and a reduced amount of motile sperm relative to wild type. Sterile males produced no motile sperm.

[f] Scoring in sperm counts: +++++, large sperm mass equivalent to wild type; ++++, large sperm mass, slightly less than wild type; +++, medium sperm mass, significantly less than wild type; ++ small amount of sperm.

[g] When β3 is >30% of the total germ line β-tubulin pool, axoneme morphology is abnormal, axonemes are not functional and males are sterile (Hoyle and Raff, 1990).

one copy of the endogenous β2 gene, one copy of β2ΔC, and only one copy of α84B-tubulin. In this genotype, α-tubulin is once again limiting, β2 outcompetes β2ΔC to form α-β dimers, and β2ΔC is degraded (Table 1, line 14).

Different β-Tubulin Isoforms Exhibit Differing Potential for Dimerization

The ability of full-length β2-tubulin to outcompete β2ΔC for dimerization with α84B revealed that the β2 carboxyl terminus is important in forming stable α-β dimers. We next wished to ask whether these results reflect a general role of the β-tubulin carboxyl terminus in dimerization. We therefore tested the ability of two other *Drosophila* β-tubulin isoforms, β1- and β3-tubulin, to compete with β2ΔC for dimerization in the male germ line. Neither β1 nor β3 is normally expressed in the postmitotic male germ line; however, both are partners with α84B elsewhere (Kimble *et al.*, 1989; Matthews *et al.*, 1989; Dettman *et al.*, 1996, 2001). We made use of the transgenic constructs p[β1] and p[β3] to express β1 or β3 in the postmitotic male germ cells at a level equivalent to that of endogenous β2 (see MATERIALS AND METHODS; Hoyle *et al.*, 1995; Raff *et al.*, 2000).

Figure 2, G and H, show that β1 and β3 outcompete β2ΔC for dimerization with α84B. In the testes of the males in these experiments, two copies of full-length β-tu-

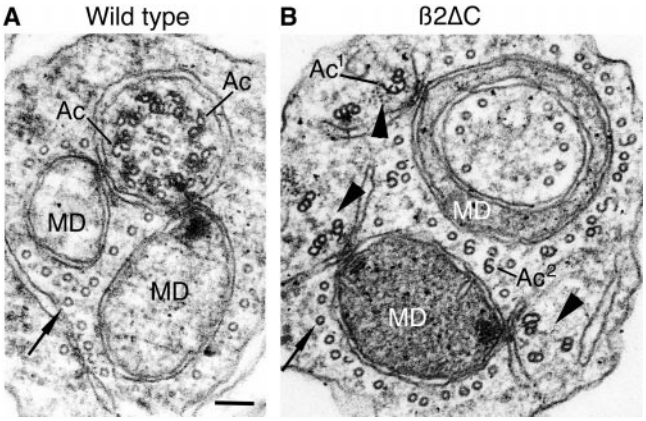

Figure 3. Microtubule assembly capacity of β2ΔC. Cross-sections of intermediate stage elongating spermatids. (A) Spermatid from a wild-type fertile male showing the normal architecture of an intermediate stage axoneme. The canonical nine doublet microtubules plus two central pair microtubules have been assembled, and the nine accessory microtubules (Ac) associated with the B tubule of each doublet are nearly completed. Inner and outer dynein arms are present on the A tubules of each doublet. Radial spokes are present between the doublets and the central pair complex (see Figure 5A for more detail). Arrow indicates cytoplasmic microtubules that at this stage surround the two mitochondrial derivatives (MD), in which typical electron-dense material has just begun to accumulate. (B) Spermatid from a sterile male in which β2ΔC is the only β-tubulin in the postmitotic germ cells (male with one copy of p[β2ΔC] in a β2-null background; see Figure 2A). Mitochondria-associated cytoplasmic microtubules are present (arrow), but axonemes are not formed. Clusters of axonemal microtubules (arrowheads) are dispersed in the cytoplasm. These clusters include doublets with multiple nascent accessory tubules (Ac^1). (β2ΔC does not support completion of closed accessory microtubules [Raff *et al.*, 2000].) There is no higher-order axonemal organization, and there are no dynein arms, radial spokes, or other nontubulin axoneme components normally associated with doublet microtubules. Microtubules with nascent accessory tubules can also be seen associated with the mitochondrial derivatives (Ac^2). Such misplaced doublet-like microtubules are never seen in wild-type spermatids. Scale bar, 100 nm for A and B.

bulin and one copy of β2ΔC were expressed in the presence of two copies of α84B. Total full-length β-tubulin thus consists of one gene dose of β2 plus one gene dose of either β1 or β3. In Figure 2, B, G, and H, the relative affinity of each full-length β-tubulin for dimerization with α84B is judged by the degree to which β2ΔC is excluded from the dimer pool and subsequently degraded. Figure 2B shows that β2ΔC is almost completely degraded in the presence of two copies each of α84B and β2. In Figure 2, comparison of B with G or H shows that, when one gene dose of β2 is replaced by one gene dose of either β1 (Table 1, line 17) or β3 (Table 1, line 19), β2ΔC is still degraded but to a lesser extent than when β2 is the sole competitor. Thus, in the context of the male germ line, any β-tubulin may act to provide α-β dimer stability, but the endogenous β2 works best. The amount of stable β2ΔC is about the same in Figure 2, G and H, indicating that β1 and β3 are approximately equal in their ability to compete with β2ΔC for dimerization with α84B.

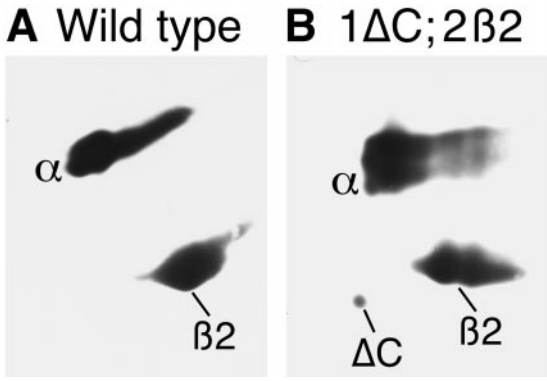

Figure 4. α84B-β2ΔC dimers are incorporated into axonemes at the same ratio at which they are present in the total testis tubulin pool. Mature motile sperm were isolated from the seminal vesicles of 14 1-week-old virgin males; sperm proteins were separated by two-dimensional gel electrophoresis, blotted, and immunostained as described in MATERIALS AND METHODS. (A) Tubulins from wild-type sperm. Both α84B and β2 undergo posttranslational modification (Piperno and Fuller, 1985; Eddé *et al.*, 1990; Hutchens *et al.*, 1997). (B) Tubulins in sperm from fertile males carrying one copy of p[β2ΔC] but otherwise wild-type at the α84B and β2 loci. Total testis tubulins from males of this genotype are shown in Figure 2B. Comparison of the β2ΔC signals with that in Figure 2B shows the same relative amount of stable β2ΔC-tubulin.

Sorting between Tubulins Occurs Only during Dimerization, Not during Microtubule Assembly

Instability of β2ΔC appears to result from competition with β2 during passage through the tubulin-specific chaperone supercomplex or "dimer-making machine." An alternative explanation is that some or all of the instability of β2ΔC results from preferential use of α84B-β2 over α84B-β2ΔC heterodimers in the assembly of axonemes and concomitant degradation of the unused α84B-β2ΔC dimers. To distinguish between these two models, we compared the relative amounts of β2ΔC in testes and in the seminal vesicles from males of the same fertile β2ΔC-expressing genotype. The testes contain only immature spermatids; mature sperm exit the testes and are stored in the seminal vesicle. In the testes there exists a soluble tubulin heterodimer pool used to assemble several transient microtubule arrays including the meiotic spindle and two classes of cytoplasmic microtubules, as well as the axoneme microtubules. The soluble tubulin, together with the rest of the cytoplasm, is lost during spermatid individualization just before the mature sperm enter the seminal vesicle. In the seminal vesicle, 100% of both α- and β-tubulin is present in the form of stable axoneme microtubules. If α84B-β2 dimers are preferentially incorporated into microtubules and α84B-β2ΔC dimers are excluded and eventually degraded, then there should be no β2ΔC found in sperm. This is not the case. Figure 2B shows the relative amounts of stable α84B, β2, and β2ΔC in total tubulins from testes of fertile males with one copy of β2ΔC in an otherwise wild-type background. Figure 4A shows the tubulins found in mature sperm isolated from the seminal vesicles of wild-type males. Figure 4B shows the amount of β2ΔC in mature sperm isolated from seminal vesicles of males of the same genotype as in Figure 2B. Comparison of

Figure 2B with Figure 4B shows that the amount of β2ΔC relative to β2 is about the same in total testis tubulins and in tubulins assembled into axonemes of mature motile sperm.

We therefore conclude that once made α84B-β2ΔC dimers are not preferentially excluded from microtubules and that incorporation of α84B-β2ΔC into axonemes directly reflects the proportion of α84B-β2ΔC dimer in the soluble α-β dimer pool. This result indicates that competition between β2ΔC and full-length β-tubulin occurs only in the dimer-making machine; the axoneme-making machinery is less picky. This is in agreement with studies showing that ectopic β1 and β3 are incorporated into axonemes at the same gene dose at which they are expressed (Hoyle and Raff, 1990; Hoyle *et al.*, 1995; Raff *et al.*, 2000). Moreover, ectopic β1 is uniformly distributed along the length of the axoneme; there is no preferential incorporation of endogenous β2 (Nielsen *et al.*, 2001). In the case of ectopic β3 expression, α84B-β3 dimers by themselves fail to support axoneme assembly and co-incorporation with endogenous β2 results in dominant male sterility (Hoyle and Raff, 1990), just as is the case with β2ΔC.

β2ΔC Disrupts the Periodicity of Organization of Nontubulin Components of the Axoneme

As discussed above, when it is the only β-tubulin in the post-mitotic germ cells, β2ΔC can support assembly and partial function of meiotic spindles and some classes of cytoplasmic microtubules (Fackenthal *et al.*, 1993). However, β2ΔC alone cannot support the testis-specific functions that are unique to the β2 isoform (Fackenthal *et al.*, 1993). β2ΔC can support assembly of doublet microtubules, but it cannot support axoneme morphogenesis (Figure 3), nor can it support microtubule-mediated shaping of the sperm nuclei.

Analysis of genotypes in which β2ΔC is coexpressed with intact β2 allowed us to discern some of the specific roles of the β2-specific C terminus in axonemes. When β2ΔC is co-incorporated with β2, meiotic spindles and cytoplasmic microtubules are fully functional, and intact full-length axonemes are assembled. A small amount of β2ΔC is compatible with axoneme motility. Figure 2C shows that, in testes of males with four gene doses of β2ΔC in an otherwise wild-type background, some β2ΔC accumulates in the total stable β-tubulin pool. These males are fertile but of reduced fecundity relative to wild-type (Table 1, line 6). However, when α-tubulin is not limiting and β2ΔC-containing dimers constitute a third or more of the total stable β-tubulin pool, males are invariably sterile (e.g., Table 1, lines 8, 9, and 12). This sterility provides another indicator that there is no sorting of dimers during microtubule assembly and that β2ΔC is being built into axonemes.

In sterile males in which β2ΔC makes up 30% of the stable β-tubulin pool, axonemes are assembled and mature individualized sperm are formed, but the axonemes are not motile and sperm do not enter the seminal vesicles. This seems paradoxical: β2ΔC is missing the carboxyl terminus, and yet β2ΔC-mediated sterility is a dominant phenotype. At the light microscope level, the sperm look normal (albeit motionless) and reach the wild-type length of ~1.8 mm. Viewed in cross-section by electron microscopy, the β2ΔC-containing axonemes are indistinguishable from wild-type axonemes at all stages. Figure 5 compares the ultrastructure of wild-type axonemes with nonmotile axonemes from sterile males carrying three copies of α84B, one copy of β2ΔC, and two copies of β2 (the genotype shown in Figure 2E; Table 1, line 9). Figure 5A shows a cross-section of a mature wild-type 9 + 2 axoneme. The cross-section of a nonmotile β2ΔC-containing axoneme in Figure 5B has all of the wild-type axonemal structures, including doublet microtubules, inner and outer dynein arms, radial spokes, and the central pair complex.

However, the sterility can be explained by defects seen in longitudinal sections of axonemes. Figure 5, C and E, shows longitudinal sections of wild-type axonemes with the regular array of radial spokes and an element repeated at 15-nm intervals within the central pair complex. Both of these regular axial arrays are disrupted in the β2ΔC-containing axonemes shown in Figure 5, D and F. Radial spokes are present, but their spacing is uneven and some spokes appear to be out of the plane of section. The spoke heads appear irregular in shape and many do not appear to contact the central pair complex. The 15-nm repeat element is lost in patches along the β2ΔC-containing central pair complexes. All axonemal microtubules are present and appear fully wild-type in cross-section. Thus, it appears that nontubulin components of the axoneme have failed to form all of the correct associations with microtubules containing β2ΔC.

When β2ΔC is increased from 30 to 50% of the stable β-tubulin pool, defects become readily apparent in axonemes examined in cross-section. These defects include severe examples of the types of radial spoke defects and central pair complex defects that can be detected only in longitudinal section when β2ΔC is just 30% of the stable β-tubulin pool. Figure 6 shows the ultrastructure of nonmotile axonemes from sterile males carrying two copies of α84B, one copy of β2ΔC, and one copy of β2 (the genotype shown in Figure 2D; Table 1, line 8). The axoneme shown in Figure 6A is missing two radial spoke heads. In addition, three intact spokes are not in contact with the central pair complex. The axoneme in Figure 6B is missing a spoke head as well as the entire central pair complex, including the central pair microtubules. Although many axonemes are defective, there is no clear ordering or pattern among the defects. We observed normal central pair complexes associated with defective spokes, normal spokes in axonemes with defective central pair complexes, and normal spokes and central pair complexes associated with defective outer doublet complexes.

DISCUSSION

To determine the functional roles of the β-tubulin carboxyl terminus, we expressed a carboxyl-truncated β-tubulin in the *Drosophila* male germ line under conditions where synthesis of α- and β-tubulins are not equimolar. Our data show that the β-tubulin C terminus is important in generating stable α-β dimers in vivo. A variety of full-length α- and β-tubulins are stable when expressed ectopically in the wild-type male germ line at nonequimolar ratios of α- and β-tubulin (Hoyle and Raff, 1990; Hoyle *et al.*, 1995; Hutchens *et al.*, 1997; Raff *et al.*, 1997, 2000). We have found that the carboxyl terminus-truncated β-tubulin, β2ΔC, forms stable dimers only when α-tubulin is not limiting, i.e., when the total gene dose of intact β-tubulin plus β2ΔC is less than or equal to the total α-tubulin gene dose. If the total β-tubulin gene dose exceeds the total α-tubulin gene dose, intact β-tubulins are preferentially dimerized. In such conditions, β2ΔC fails to dimerize and is degraded. This contrasts with results of in vitro experiments using rabbit reticulocyte extracts to express a murine β-tubulin missing the last 12 carboxyl residues (Fontalba *et al.*, 1995). In the in vitro system, the newly synthesized truncated β-tubulin was not able

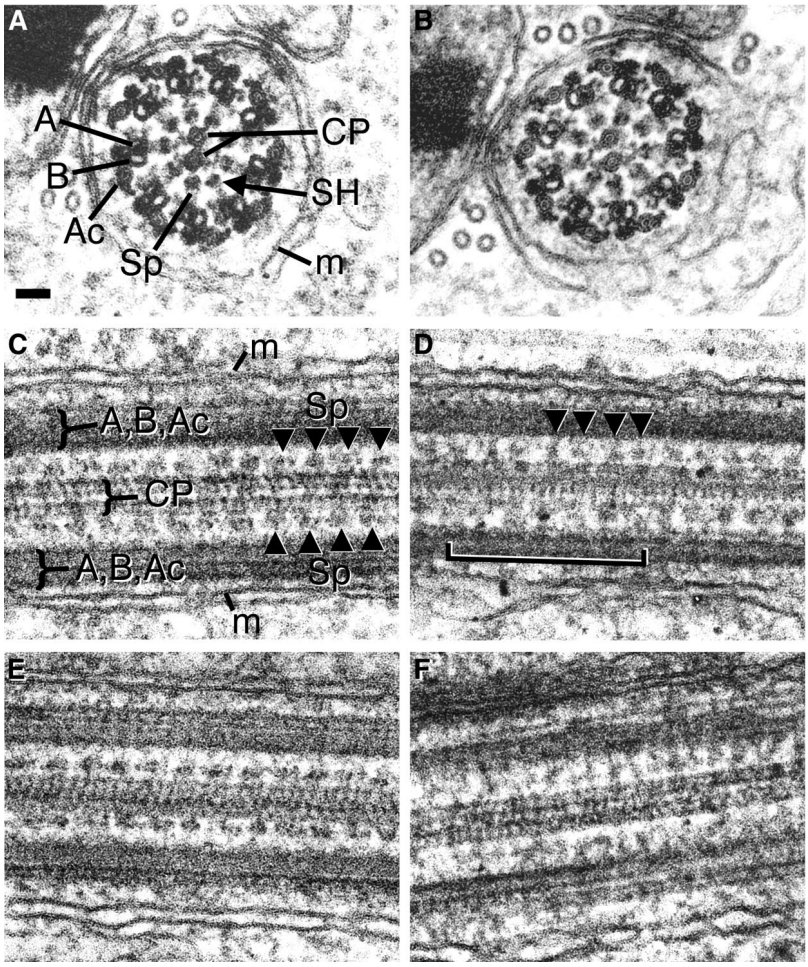

Figure 5. β2ΔC causes dominant defects in the axial organization of the axoneme. Axonemes in nearly mature spermatids before individualization. Cross-sections (A and B) and longitudinal sections (C–F) of axonemes from wild-type males (A, C, and E) and sterile males with three copies of α84B, one copy of β2ΔC, and two copies of β2 (B, D, and F; this is the same genotype as in Figure 2E and Table 1, line 9). (A) Wild-type axoneme showing the major components including the A and B tubules of the doublets, the accessory microtubules (Ac) associated with each B tubule, the central pair complex (CP), and the radial spokes (Sp) and spoke heads (SH). Other components include the inner and outer dynein arms associated with each A tubule, the membrane surrounding the axoneme (m), and the luminal filaments within the accessory and central pair tubules (which appear in cross-section as a "dot" or filling in the microtubule lumen). (B) β2ΔC-containing axoneme. In cross-section, axonemes from males of this genotype appear wild type. (C and E) Longitudinal sections through wild-type axonemes at the plane of the central pair complex. The regular spacing of the radial spokes (arrowheads) can be seen along the entire length of each section. Within the central pair complex there is a "beads-on-a-string" element with a periodicity of ~15 nm. (D) Longitudinal section through a β2ΔC-containing axoneme showing mild disorganization. Some radial spokes have a wild-type spacing (arrowheads) and some are irregularly spaced and misshapen (bracket). In addition, the beads-on-a-string element is not present along most of the top edge of the central pair complex, and there is a gap in the middle along the bottom edge (compare with A). (F) Longitudinal section through a region of a second β2ΔC-containing axoneme that is more disorganized than the axoneme in D. Very few wild-type radial spokes are present; most spokes are misshapen and irregularly spaced. There are gaps where spokes are either above or below the plane of section or are missing entirely. The beads-on-a-string element of the central pair complex is largely disrupted. Scale bar, 50 nm, for all panels.

to form α-β heterodimers and was released from the chaperone complex, undegraded, as a monomer.

In this study, we have found that different β-tubulin isoforms possess different affinities for dimerization with α-tubulin. Our data demonstrate that the carboxyl terminus is key in establishing stable heterodimers. However, just as sequence differences between different β-tubulin isoforms in regions other than the C terminus are important in determining isoform-specific function in microtubule assembly, internal sequence differences may also contribute to differential dimerization properties.

Our data support the interpretation that differences in dimerization properties are important in determining isoform-specific microtubule functions. Thus, we found that β2 was better at competing with β2ΔC than either of the other two full-length β-tubulin isoforms we tested. The finding that endogenous β2 has greater affinity than β1 for α84B suggests a developmental role for β-tubulin sorting during dimerization. β1 is expressed in primary spermatocytes and is rapidly replaced by β2 before meiosis (Kemphues *et al.*, 1982). Tian *et al.* (1999) have shown that heterodimeric tubulins can exchange in vitro by recycling through the dimer-making machine. At the onset of β2 synthesis the β1 gene is no longer expressed; however, each spermatocyte still contains β1 protein. One of several possible mechanisms by which β2 could replace this residual β1 would be for β2 to outcompete β1 for dimerization during recycling.

The equal affinities of β1 and β3 for α84B are also consistent with the developmental coexpression of these two isoforms in several cell types during development (Kimble *et al.*, 1989, 1990; Dettman *et al.*, 1996, 2001; Hoyle *et al.*, 2000). We have previously shown that β1 and β3 are coassembled into the same microtubules in vivo (Hoyle *et al.*, 2000). This would not be possible if either β1 or β3 could strongly outcompete the other for dimerization with α-tubulin.

The β2-specific carboxyl terminus is essential for assembly of the sperm tail flagella, the only motile axoneme in *Drosophila*. The 15 carboxyl residues missing from β2ΔC include the axoneme motif present in all β-tubulins incorporated into motile axonemes (Raff *et al.*, 1997), as well as sites of posttranslational modification known to be important for axoneme motility (Xia *et al.*, 2000). The axoneme motif specifies the central pair microtubules (Nielsen *et al.*, 2001). Here, we have found that even when full-length β2 makes up the majority of the β-tubulin present, incorporation of stable β2ΔC into axoneme microtubules disrupts the organization of the central pair complex and the radial spokes. Studies of paralyzed flagella mutants of *Chlamydomonas* have shown these two axonemal structures to be involved in regulating axoneme motility (reviewed by Smith and Lefebvre, 1997; Porter and Sale, 2000). A nontubulin component found in the central pair complex of a wide range of species is the central pair projection (Witman *et al.*, 1978). Transient contact between the radial spokes and the

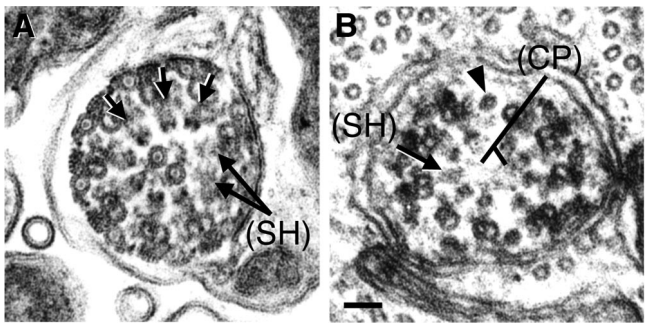

Figure 6. Axoneme defects increase with increased levels of stable β2ΔC. Cross-sections of axonemes containing equal amounts of β2ΔC and β2-tubulin show defects in the central pair complex and radial spokes. Axonemes in A and B are from sterile males with two copies of α84B, one copy of β2ΔC, and one copy of β2 (this is the same genotype as in Figure 2D and Table 1, line 8). (A) An axoneme missing two spoke heads (SH) normally found adjacent to the central pair complex. In addition, three intact spokes are detached from the central pair complex (arrows). (B) An axoneme missing the central pair complex (CP) as well as a single spoke head (arrow). One accessory microtubule lacks the electron-dense material that would normally connect it to the adjacent doublet (arrowhead). Scale bar, 50 nm, for both panels.

central pair projections is thought to play an important role in the regulation of dynein and the generation of the complex flagellar waveform (Smith and Lefebvre, 1997).

The identity of the repeated element found in the *Drosophila* central pair complex is not known. However, its disruption by β2ΔC, as well as the disruption of the radial spokes, implies that both components are in contact with the carboxyl terminus of β-tubulin in wild-type axonemes, either directly or indirectly through other protein-protein interactions. In our experiments, all nontubulin components present are wild type. When the radial spokes or central pair components are themselves mutant, as is the case with *Chlamydomonas* paralyzed flagellar mutants, the mutations are recessive. In contrast, when β2 subunits lacking the carboxyl terminus are incorporated at random into axonemes, the consequence is a dominant disruption of axoneme motility. It appears that for each α84B-β2ΔC dimer incorporated into an axoneme microtubule, there is a stoichiometric loss of interaction with nontubulin components of the axoneme. When 30% of the β-tubulin lacks the carboxyl terminus, the loss of organization becomes too great to permit axoneme function, and sperm become nonmotile. The higher the percentage of truncated β-tubulin incorporated into the axoneme, the more severe are the defects. Axonemes from males in which β2ΔC is 50% of total β-tubulin exhibit the same kind of defects that occurred in sterile males with 30% β2ΔC but to a much more marked degree, readily apparent in axoneme cross-sections.

We have discovered that the β2-tubulin carboxyl terminus plays two distinct roles. In its first role, the acidic carboxyl terminus is important for producing a stable α-β heterodimer. The generalized nature of this requirement is reflected by the fact that any acidic carboxyl terminus seems to work; β1- and β3-tubulin both form stable α-β dimers in the male germ line. Thus, although the β-tubulin carboxyl terminus is the hypervariable, isotype-defining region of the molecule (Sullivan and Cleveland, 1986), the wrong carboxyl terminus seems to be better than no carboxyl terminus. In a distinct second role, the carboxyl terminus is involved in interactions between intact microtubules and nontubulin components required to generate the architecture of specific microtubule-based structures. β2ΔC by itself fails to support any axonemal organization (Fackenthal *et al.*, 1993; Nielsen *et al.*, 2001). However, not just any carboxyl terminus can support this function: other full-length *Drosophila* β-tubulin isoforms cannot replace β2 for axoneme function (Hoyle and Raff, 1990; Hoyle *et al.*, 1995; Raff *et al.*, 2000). In contrast, the carboxyl terminus is not required for generic microtubule functions: β2ΔC can support assembly of functional meiotic spindles as well as the cytoplasmic microtubules associated with elongation of the mitochondrial derivative (Fackenthal *et al.*, 1993; Figure 3B). It should be noted that these generic functions can also be fully or partially supplied by other *Drosophila* isoforms (Hoyle and Raff, 1990; Hoyle *et al.*, 1995; Raff *et al.*, 2000).

Our data demonstrate that competition between β2ΔC and intact β-tubulins takes place during dimerization and not during subsequent microtubule assembly. However, competition between full-length β-tubulin and β2ΔC could occur at several steps in the dimerization process. Our work does not distinguish between competition for binding with cytosolic chaperonin, competition for cofactors of the dimer-making machine, or direct competition between β2 and β2ΔC for α84B. However, the resolved three-dimensional structure of the α-β dimer allows for the possibility that the unresolved β-tubulin carboxyl residues are in contact with the α-tubulin moiety of the same α-β dimer (Nogales *et al.*, 1998). Direct competition between β-tubulins for binding to α is consistent with our finding that different β-tubulin isoforms exhibit differential ability to form α-β dimers. This model suggests the possibility that α-tubulin residues contacted by the β-tubulin carboxyl terminus play a direct role in stabilizing the α-β dimer. The resolved structure predicts these α-tubulin residues to be non-carboxyl-terminal residues. As is the case with β-tubulin, the α-tubulin carboxyl terminus is also unresolved in both the structures of the heterodimer and the microtubule (Nogales *et al.*, 1998, 1999). The 11 unresolved α residues are in position to contact β-tubulin in another heterodimer, either in the same protofilament or perhaps in an adjacent protofilament (Nogales *et al.*, 1998). This leads to the prediction that the α-tubulin carboxyl terminus will be of relatively little importance in forming or stabilizing intrasubunit associations in the dimer but may be involved in interdimer associations in the protofilament substructure of microtubules.

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