Published in final edited form as:

Cytoskeleton (Hoboken). 2011 March ; 68(3): 175–187. doi:10.1002/cm.20503.

Megakaryocyte Lineage Specific Class VI β -Tubulin Suppresses Microtubule Dynamics, Fragments Microtubules, and Blocks Cell Division

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Abstract

Class VI β -tubulin ($\beta 6$) is the most divergent tubulin produced in mammals and is found only in platelets and mature megakaryocytes. To determine how this unique tubulin isotype affects microtubule assembly and organization, we expressed the cDNA in tissue culture cells under the control of a tetracycline regulated promoter. The $\beta 6$ coassembled with other endogenous β -tubulin isotypes into a normal microtubule array; but once the cells entered mitosis it caused extensive fragmentation of the microtubules, disrupted the formation of the spindle apparatus, and allowed entry into G1 phase without cytokinesis to produce large multinucleated cells. The microtubule fragments persisted into subsequent cell cycles and accumulated around the membrane in a marginal band-like appearance. The persistence of the fragments could be traced to a pronounced suppression of microtubule dynamic instability. Impairment of centrosomal nucleation also contributed to the loss of a normal microtubule cytoskeleton. Incorporation of $\beta 6$ allowed microtubules to resist the effects of colcemid and maytansine, but not vinblastine or paclitaxel; however, cellular resistance to colcemid or maytansine did not occur because expression of $\beta 6$ prevented cell division. The results indicate that many of the morphological features of megakaryocyte differentiation can be recapitulated in non-hematopoietic cells by $\beta 6$ expression and they provide a mechanistic basis for understanding these changes.

Keywords

$\beta 6$ -tubulin; dynamic instability; paclitaxel; marginal band; platelet formation; multinucleation

Introduction

Microtubules are essential cytoskeletal organelles involved in maintaining cell shape, organizing the Golgi apparatus and endoplasmic reticulum, transporting vesicles, and forming the mitotic spindle apparatus. They assemble from heterodimers of α - and β -tubulin that interact longitudinally to form protofilaments and laterally to form hollow tubes. In vertebrates, α - and β -tubulin are each encoded by 6-7 member multigene families that produce highly homologous proteins [Sullivan, 1988]. The β -tubulins are less well conserved than α -tubulins and differ at a number of internal residues in addition to their diverse C-terminal 15 amino acids. These distinctive C-terminal tails are highly conserved across vertebrate species and have been used to define specific isotypes of the protein [Lopata and Cleveland, 1987]. Mammals express 7 β -tubulin isotypes abbreviated here as

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β 1, β 2, β 3, β 4a, β 4b, β 5, and β 6. These isotypes can be categorized into two groups: a relatively conserved group (β 1, β 2, β 4a, β 4b) whose members share more than 96% identity in their amino acid sequences, and a more divergent group (β 3, β 5, β 6) whose sequences can differ by as much as 20%. In addition to sequence differences, β -tubulins differ in their tissue distributions [Ludueno, 1998; Sullivan, 1988]. Some, like β 1, β 4b, and β 5, are found in most tissues; whereas expression of the others is tissue restricted (e.g. β 3 and β 4a in neurons). Because of their distinctive yet conserved C-terminal tails and their nonuniform tissue distributions, it has long been suspected that β -tubulin isotypes may allow microtubules to serve different functions [Fulton and Simpson, 1976].

The most divergent β -tubulin isotype is β 6 which differs from the widely expressed β 1 in about 20% of its amino acids. The expression of β 6-tubulin is restricted to hematopoietic tissues: megakaryocytes and platelets in mammals [Wang et al., 1986], but also erythrocytes and thrombocytes in avian species [Murphy et al., 1987; Murphy et al., 1986]. The expression of β 6 is induced during megakaryocyte differentiation and is concurrent with the production of platelets whose discoid shape is maintained by a circumferential ring of microtubules called the marginal band [Lecine et al., 2000]. Despite its unusual tissue distribution and localization to the platelet membrane, early transfections of β 6 cDNA into tissue culture cells indicated that the protein coassembled with other existing isotypes and had no obvious effects on microtubule organization [Joshi et al., 1987; Lewis et al., 1987]. As part of ongoing efforts in our laboratory to define functional differences among the various β -tubulin isotypes, we re-examined the effects of tetracycline-regulated β 6 expression in Chinese hamster ovary (CHO) cells. Microtubules in CHO cells are composed of 70% β 1, 25% β 4b, and 5% β 5 [Ahmad et al., 1991; Sawada and Cabral, 1989]. As is the case with most cultured cell lines, there is no evidence for the production of β 6. Here we demonstrate that ectopic expression of β 6 has profound effects on microtubule assembly and organization that result in a loss of cell division.

Materials and Methods

Plasmids and Antibodies

Human β 6 (GenBankTM accession no. BC033679; ATCC no. MGC-44928) and CHO β 1 (GenBankTM accession no. U08342) cDNAs were cloned into a tetracycline-regulated expression vector pTOPneo [Gonzalez-Garay et al., 1999]. CHO β 1 was fused to a hemagglutinin antigen (HA) epitope at its C-terminal end to allow its detection amid the other cellular tubulin proteins. The plasmids were sequenced to ensure that no mutations were introduced during molecular engineering. Expression vectors for EGFP-MAP4 [Olson et al., 1995] (Dr. Joanna Olmsted, University of Rochester) and EB1-GFP [Piehl and Cassimeris, 2003] (Addgene, Cambridge, MA) were also used.

Antibodies used in these studies included mouse monoclonal antibodies DM1A (Sigma) against α -tubulin, 18D6 that recognizes the N-terminal end of all β -tubulin isotypes [Theodorakis and Cleveland, 1992], 6-11B-1 against acetylated α -tubulin (GeneTex, Irvine, CA), C4 against actin (Chemicon, Temecula, CA), GTU-88 against γ -tubulin (Sigma), and D8 against a β 6 C-terminal peptide corresponding to the human sequence (Dr. Anthony Frankfurter, University of Virginia). A rabbit antibody against α -tubulin (Dr. Jeanette Bulinski, Columbia University) was also used in some experiments. Secondary goat antimouse and antirabbit IgGs tagged with Alexa-488, Alexa-594 and Alexa-647 were from Invitrogen.

Transfection and Selection of Stable Cell Lines

CHO/tTA cells, that express a tetracycline-regulated transactivator [Gonzalez-Garay et al., 1999], were maintained in alpha modification of minimum essential medium (α MEM; Sigma-Aldrich Co., St. Louis, MO), supplemented with 5% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA), 50 U/ml penicillin, and 50 μ g/ml streptomycin (Invitrogen Life Technologies, Carlsbad, CA). Transfections were carried out using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. Cells were trypsinized and seeded in α MEM with 1 μ g/ml tetracycline (Sigma-Aldrich) and 2 mg/ml G418 (Invitrogen) for the selection of stable cell lines. After about 7 d, individual G418-resistant colonies were isolated and maintained in α MEM containing G418 and tetracycline to repress the expression of the ectopic protein until analysis.

Electrophoresis and Quantification of Tubulin

Cells grown in 24-well dishes were lysed in 1% SDS. The proteins were precipitated with 5 volumes of acetone, dissolved in SDS sample buffer (0.0625 Tris-HCl, pH 6.8, 2.5% SDS, 5% 2-mercaptoethanol, 10% glycerol), resolved on polyacrylamide minigels (Bio-Rad, Hercules, CA) and transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Tubulin was probed on these western blots with 1:2,000 dilutions of 18D6 to detect all isoforms of the protein, or 6-11B-1 specific for acetylated α -tubulin. Antibody C4 to actin was included at a 1:40,000 dilution to act as a loading control. Reacting bands were visualized by reaction with a 1:2,000 dilution of Alexa 647-conjugated goat antimouse IgG (Invitrogen). Bands were imaged and quantified by capturing fluorescence emission with a STORM 860 scanner (Molecular Dynamics Inc., Sunnyvale, CA). Relative levels of acetylated tubulin between cell lines was determined by comparing their ratios of acetylated α -tubulin to actin.

Immunofluorescence

Cells grown on sterile glass coverslips were pre-extracted in microtubule stabilizing buffer (MTB) (20 mM Tris-HCl, pH 6.8, 1 mM $MgCl_2$, 2 mM EGTA, 0.5% Triton X-100) containing 4 μ g/ml paclitaxel (Cytoskeleton Inc., Denver, CO) for 90 s at 4 °C and then fixed in cold methanol at -20 °C for at least 20 min. The fixed cells were rehydrated in PBS for 15 min and incubated in primary antibodies for 1 h at 37 °C in a humid box. The coverslips were washed in PBS and then incubated for 45 min with secondary antibodies and 1 μ g/ml DAPI. After washing in PBS, the coverslips were inverted onto 5 μ l of Gel/Mount (BioMeda Corp., Foster City, CA) and examined using an Optiphot microscope equipped with a Plan Apochromat 60 $\times$, 1.4 numerical aperture oil objective (Nikon Inc., Melville, NY). Images were acquired using a MagnaFire digital camera (Optronics, Goleta, CA).

Time-Lapse Microscopy and Analysis of Microtubule Dynamics

Cells were transfected with a plasmid containing EGFP-MAP4 cDNA to visualize the microtubule network or EB1-GFP to examine microtubule nucleation. At 24 h post-transfection, cells were imaged using a DeltaVision Core equipped with a 100 $\times$ 1.4 NA oil objective (Applied Precision LLC., Issaquah, WA). The stage was enclosed in an environmental chamber and maintained at 36.5 $\pm$ 0.5 °C. Up to 50 images were acquired at 5 s intervals using a Roper RTE camera (Applied Precision LLC) driven by SoftWoRx Suite software, using 0.5 to 1 s exposure times and 1 $\times$ 1 bin. During observation, cells were incubated in modified McCoy's 5A medium (Mediatech, Inc., Herndon, VA) containing 25 mM HEPES and 10% fetal bovine serum.

Microtubule lengths were measured using Image J software and plotted as a function of time. Approximately 30 microtubules from six different cells were analyzed. Microtubule growth and shortening rates were determined by linear regression. Changes in length that were $\geq 0.5 \mu\text{m}$ between two time points were considered as true growth or shortening. Changes $< 0.5 \mu\text{m}$ were considered to be thermal movements. Dynamicity was calculated as the sum of changes in length (growth and shortening) divided by the total observation time. The catastrophe frequency for individual microtubules was determined by dividing the number of catastrophe events (transitions from pause or growth to shortening) per microtubule by the total time spent in growth or pause. The rescue frequency was determined by dividing the number of rescues (transitions from shortening to growth or pause) by the total time spent shortening. Only microtubules that were visible for $\geq 2 \text{ min}$ were included in this analysis.

Statistics

Experiments were carried out in triplicate and repeated at least 3 times. Results are reported as the Mean $\pm$ SEM. Data were analyzed by Student *t-test*. $P < 0.05$ was considered statistically significant.

Results

Isolation of Stable Cell Lines Expressing $\beta 6$ -Tubulin

G418 resistant clones of CHO cells transfected with human $\beta 6$ -tubulin cDNA were screened by immunofluorescence with a $\beta 6$ -specific antibody to identify those that had good uniformity of staining and whose expression of $\beta 6$ could be regulated by the presence of tetracycline. The level of $\beta 6$ production was determined by western blot analysis using an antibody directed against the amino terminus that recognizes all the different β -tubulin isotypes [Theodorakis and Cleveland, 1992]. Tubulin production in the clones chosen for further analysis are shown in Fig. 1. Despite having more amino acids, $\beta 6$ migrated more rapidly on SDS gels allowing its protein level to be compared to endogenous isotypes using a single antibody. The $\beta 6$ -tubulin was undetectable in all four clones grown in the presence of tetracycline but accounted for about 20-90% of total β -tubulin when tetracycline was withdrawn for 24 h.

Microtubule organization is disrupted by $\beta 6$ production

Expression of $\beta 6$ -tubulin produced dose-dependent changes in microtubule organization that differed from changes seen with any other tubulin isotype we have examined. Compared to wild-type nontransfected cells (Fig. 2A), microtubules in clones $\beta 6$ -47 (Fig. 2B) and $\beta 6$ -5 (not shown) appeared to be more curved than normal within 24 h of inducing $\beta 6$ expression. At the higher level of $\beta 6$ -tubulin expression in Clone $\beta 6$ -7 (68%), many cells exhibited microtubule fragments that were often seen to bundle at the cell periphery (arrows, Fig. 2C). These bundles became especially prominent in Clone $\beta 6$ -4 (91% expression) where they formed a marginal band-like structure around the plasma membrane similar in appearance to the marginal band characteristic of platelets that also express $\beta 6$ -tubulin as their predominant isotype (Fig. 2D). The results suggest that the expression of $\beta 6$ in megakaryocytes may be one of the major driving forces for the formation of platelet marginal bands. After 4 d of expression, microtubules in the majority of cells had reorganized into short bundles at the plasma membrane even in the two cell lines ($\beta 6$ -47 and $\beta 6$ -7) with only low to moderate levels of $\beta 6$ expression (Fig. 2E, F). We occasionally also saw cells with $\beta 6$ -tubulin aggregates but no cytoplasmic microtubules (arrow, Fig. 2F) that potentially could represent cells with even higher levels of $\beta 6$ expression. Similar peripheral microtubule bundles were also seen in HeLa and HEK cells transiently transfected with the $\beta 6$ cDNA (Supporting Information Fig. S1).

Expression of $\beta 6$ -Tubulin Interferes With Mitotic Spindle Assembly and Blocks Cells in Prometaphase

In addition to the changes produced in the microtubule cytoskeleton, even the relatively low production of $\beta 6$ in clone $\beta 6$ -47 caused the cells to become multinucleated (Fig. 2 B'). A number of cell lines such as CHO do not trigger the onset of apoptosis that normally follows a prolonged mitotic block. Instead, disruption of spindle function by drugs or mutations causes a delay in mitosis but the cells ultimately proceed to G1 phase without dividing and form irregular nuclear membranes around the scattered chromosomes [Abraham et al., 1983; Cabral and Barlow, 1991; Kung et al., 1990]. Thus, the multinucleation seen in $\beta 6$ expressing cells indicated that they had experienced problems in mitosis. A comparison of wild-type and $\beta 6$ -containing spindles (Clone $\beta 6$ -7) is shown in Fig. 3 A. Whereas wild-type CHO cells displayed all the normal stages of mitosis, expression of $\beta 6$ caused the cells to accumulate in a prometaphase-like state with asters of microtubules at the spindle poles and disorganized chromosomes. The $\beta 6$ expressing cells also had unusual γ -tubulin staining at prometaphase that we cannot currently explain. Concomitant with these changes, there was an increase in the mitotic index (Fig. 3 B) and a decrease in metaphase, anaphase, and telophase spindles (Fig. 3 C). Thus, $\beta 6$ expression, like drug treatment or mutant tubulin expression, caused cells to arrest in prometaphase.

Although it is not obvious in Fig. 3A, $\beta 6$ expressing cells had many microtubule fragments once they entered mitosis. To determine when these fragments were being formed, cells were partially synchronized using an overnight thymidine block at the same time that $\beta 6$ expression was induced. During this time, the cells were found to have normal appearing microtubules with little evidence for fragments. After release from the thymidine block, the microtubules continued to appear normal until the two spindle poles (arrows, Fig. 3D) began to move apart during prophase. At this stage, wild-type cells continued to have long intact microtubules, but $\beta 6$ expressing cells exhibited a large number of microtubule fragments (Fig. 3D). As will be discussed below, $\beta 6$ -containing fragments are very stable; they could be seen throughout mitosis (Supporting Information Fig. S2) and into the next G1 phase. Using live cell time lapse microscopy, we did not find evidence for additional fragment formation during interphase. Thus, the microtubule fragments seen in interphase cells appeared to arise from a cell cycle regulated process in mitosis that began during prophase.

Expression of $\beta 6$ -Tubulin Increases Microtubule Acetylation

Platelet marginal bands are resistant to depolymerization by nocodazole whereas microtubules in hematopoietic cells at an early developmental stage remain sensitive to the drug [Kim et al., 1987], suggesting that marginal band microtubules have increased stability. To test whether $\beta 6$ -tubulin incorporation might contribute to this increased stability, we examined the level of microtubule acetylation in cells with and without $\beta 6$ -tubulin overexpression. Acetylation of α -tubulin is specific for polymerized tubulin and increases with time; it is therefore generally regarded as an indicator of microtubule longevity and stability [Wloga and Gaertig, 2010]. Although there was some cell-to-cell variability in staining due differences in $\beta 6$ expression and other factors, fluorescence microscopy with an antibody specific for acetylated α -tubulin clearly showed that $\beta 6$ expressing cells (Fig. 4B) had more heavily acetylated microtubules than cells transfected with HA $\beta 1$ -tubulin, an abundant and ubiquitously expressed isotype (Fig. 4A). Both the HA $\beta 1$ cell line [Yang and Cabral, 2007] and Clone $\beta 6$ -7 had high levels of ectopic β -tubulin overexpression (Fig. 4C) making it unlikely that the increased tubulin acetylation observed in the latter cell line might be due to a reduction in endogenous isoforms. Microtubule fragments were also highly acetylated, suggesting that these fragments are stable as well. To obtain a more quantitative and objective measure of microtubule acetylation, the same cell lines were examined by western blot analysis (Fig. 4C). The results indicated that $\beta 6$ expression caused a 2.7-fold

increase in the amount of acetylated α -tubulin but no increase in the total α -tubulin content of the cells (Fig. 4D). Interestingly, the increase in acetylated α -tubulin reached a plateau as early as 12 h after induction, a time at which the production of $\beta 6$ was still relatively low (see Fig. 4C). It thus appears that even low amounts of $\beta 6$ are capable of causing a large increase in microtubule stability.

Cells That Express $\beta 6$ -Tubulin Are Resistant to Microtubule Inhibitors

Cell lines expressing mutant tubulin subunits that increase microtubule stability have been shown to be resistant to drugs that inhibit microtubule assembly and more sensitive to drugs that promote assembly [Cabral, 2001]. Having observed that cells expressing $\beta 6$ had increased tubulin acetylation that indicated increased microtubule stability, we reasoned that those cells should be resistant to microtubule inhibitors. Because $\beta 6$ expressing cells could not divide, we were unable to test for drug sensitivity using a standard colony formation assay. Instead, we used tubulin immunofluorescence to test for drug effects. For the experiment shown in Fig. 5, we mixed wild-type cells with $\beta 6$ -7 cells to have an internal control for drug effects on microtubule polymerization, and treated the mixed population for 3-24 h with varying doses of microtubule drugs. Because drug-induced changes in microtubule assembly occurred more slowly in $\beta 6$ -7 perhaps owing to the relative stability of microtubules in those cells, we primarily show the drug effects after 24 h in Fig. 5. For paclitaxel, a drug that binds to the β subunit and promotes microtubule assembly [Downing, 2000], we used microtubule bundling as an indicator of drug action and found no difference in the response of $\beta 6$ expressing cells compared to wild-type. For drugs that inhibit microtubule formation, however, striking differences were seen. For example, 130 nM colcemid was sufficient to eliminate microtubules from wild-type cells (arrowheads, Fig. 5), but $\beta 6$ -7 (arrows, Fig. 5) withstood up to 650 nM of the drug. Similar results were obtained with maytansine, a drug whose binding overlaps a vinca alkaloid site that is distinct from both the colcemid and paclitaxel binding sites [Gigant et al., 2005]. Interestingly, however, the $\beta 6$ expressing cells appeared to be more sensitive to vinblastine, a drug that binds directly to the vinca site. After only a 3 h treatment, $\beta 6$ -7 cells had lost many of their microtubules at 5 nM drug and microtubules were virtually absent at 10 nM even though wild-type cells in the same population still retained most of their microtubules (Fig. 5). At 20 nM vinblastine many, but not all, wild-type cells were depleted of microtubules whereas $\beta 6$ -7 cells exhibited microtubule paracrystals that are normally only seen at much higher drug concentrations in wild-type cells. The increased sensitivity to vinblastine is unexplained but could indicate that $\beta 6$, like vinblastine, favors the formation of curved protofilaments [Gigant et al., 2005; Himes, 1991]. Alternatively, increased drug sensitivity could indicate an increased affinity of $\beta 6$ -tubulin for vinblastine, but we did not find residues unique to $\beta 6$ that were close to the vinca binding site. Thus, if increased drug binding is responsible for the increased sensitivity to vinblastine, it is likely to be mediated by conformational effects. Similarly, only a leucine for isoleucine replacement at residue 368 of $\beta 6$ fell within the colchicine binding site and is unlikely to confer resistance to the drug because reduced drug binding is a recessive phenotype in diploid cells and because the $\beta 6$ producing cells were also highly resistant to nocodazole (data not shown) which has a very different structure. We thus favor the idea that the effects of $\beta 6$ on drug sensitivity and resistance are mediated by changes in microtubule assembly and stability as we have previously described for tubulin mutations [Cabral, 2001].

Incorporation of $\beta 6$ -Tubulin Suppresses Microtubule Dynamics

The increased α -tubulin acetylation in $\beta 6$ -expressing cells suggested that the microtubules had a slower turnover rate. To explore this observation in more detail, we transfected cells with EGFP-MAP4 and measured the growth and shortening rates of microtubules using live cell microscopy. MAP4 is a microtubule associated protein that is found in many cell types

and decorates microtubules without altering their assembly [Barlow et al., 1994; Wang et al., 1996]. Moreover, we previously reported that microtubule dynamics measured using EGFP-MAP4 agree well with measurements from other approaches indicating that overexpression of MAP4 does not itself alter this aspect of microtubule behavior [Yang et al., 2010]. The microtubules that we imaged were mostly near the outer periphery where the cell is relatively flat, but internal microtubules that stayed within the plane of focus had similar dynamics.

As shown in the life-history plots that measure time-dependent changes in microtubule length (Fig. 6) and in Supporting Information Movie 1, most wild-type microtubules displayed typical dynamic behavior that included episodes of growth and shortening interrupted by short pauses during which there was no change in length. There were also frequent catastrophes (transitions from growth or pause to shortening) and rescues (transitions from shortening to growth or pause). In contrast, nearly all the microtubules containing $\beta 6$ were profoundly non-dynamic with long pauses that were only occasionally interrupted by very short episodes of growth or shortening (Supporting Information Movie 2). To quantify these observations, growth rates, shortening rates, and other parameters of dynamic instability were measured and the values are compared in Table I. Compared to wild-type, $\beta 6$ -containing microtubules had lower growth and shortening rates, and had less frequent catastrophes that resulted in a large increase in the time spent in a paused state as well as a much lower dynamicity. This last parameter measures the total of changes in length (both growth and shortening) experienced by a microtubule per unit time and serves as a commonly used measure of microtubule activity [Jordan and Wilson, 1998]. It should be noted that microtubule fragments that contain $\beta 6$ exhibited similar suppressed dynamic behavior at both ends thus explaining their stable appearance in the cytoplasm. These fragments were often seen to translocate toward the cell periphery and to slide next to other microtubules. Although we don't know the mechanisms involved in these movements, they are likely to contribute to the accumulation of short bundles of microtubules at the plasma membrane seen by immunofluorescence (Fig. 2).

$\beta 6$ -Tubulin Inhibits Microtubule Nucleation

Immunofluorescence experiments indicated that $\beta 6$ expressing cells accumulated microtubules at the cell periphery as they became depleted near the cell center over time (Fig. 2). This suggested that $\beta 6$ expressing cells might have a defect in the nucleation of new microtubules by the centrosome. To test this possibility, we treated cells with colcemid to depolymerize existing microtubules, washed out the drug, and then followed the time course for the reappearance of microtubules in wild-type and $\beta 6$ expressing cells. As shown in Fig. 7 A, striking differences were seen. Significant centrosomal asters were seen in wild-type cells within 20-30 min of drug removal and a normal cytoskeleton was seen by 50 min. In contrast, $\beta 6$ expressing cells displayed a large amount of random cytoplasmic microtubule formation as early as 20 min after drug removal and, even at 50 min, microtubules could not be traced back to the centrosome. Throughout this period, only very small asters were seen at the centrosomes.

The microtubule regrowth experiment measures microtubule nucleation under abnormal conditions in which there are few existing microtubules alongside a much higher than normal concentration of unassembled tubulin subunits. Moreover, we suspected that the appearance of random microtubule fragments in the cytoplasm of $\beta 6$ expressing cells might have resulted from an incomplete disassembly of the microtubules that could then elongate and thus compete with the centrosomes by incorporating free tubulin subunits. To resolve these issues, we attempted to measure nucleation under steady state conditions by transfecting cells with EB1-GFP, a protein that is known to specifically bind the plus-ends of elongating microtubules [Akhmanova and Steinmetz, 2008]. Fig. 7B shows that the

protein bound successfully to newly nucleated microtubules in wild-type cells and produced a pattern of EB1 comets radiating out from the centrosome (Supporting Information Movie 3), but it did not bind to microtubules in clone $\beta 6$ -7 and few if any comets were seen to emerge from the centrosome (Supporting Information Movie 4). This result could potentially be explained by postulating that $\beta 6$ directly interferes with EB1 binding; but a simpler explanation, and one that is consistent with the life history plots shown in Fig. 5, is that steady state microtubules in $\beta 6$ expressing cells are seldom in a growth phase. The lack of a focus of new microtubule growth suggests that microtubules are not efficiently nucleated at the centrosome in $\beta 6$ expressing cells under steady state conditions. Although we cannot yet explain the defect in microtubule nucleation, we have noticed that spindle poles become much less focused during prometaphase in $\beta 6$ expressing cells (Supporting Information Fig. S3), suggesting the possibility that $\beta 6$ could be affecting centrosome stability.

Discussion

These studies were designed to examine the effects of $\beta 6$ -tubulin on microtubule assembly, microtubule organization, and cell division. In mammals this divergent tubulin isotype is only found in late stage megakaryocytes and platelets, cells that do not undergo cell division. Platelet formation begins with the differentiation of megakaryoblasts into large multinucleated megakaryocytes [Ravid et al., 2002]. The expression of $\beta 6$ appears to coincide with the formation of proplatelet membrane extensions from which platelets ultimately bud off and are released into the circulation [Lecine et al., 2000]. Preceding the formation of proplatelets, microtubules bundle at the plasma membrane and extend into the growing proplatelet protrusions. The platelets that bud off from these protrusions are anucleate discoid shaped cells whose microtubules are arranged into a marginal band that is composed predominantly of the $\beta 6$ isotype [Hartwig and Italiano, 2003]. Although the marginal band was originally believed to be a single microtubule that curved around the membrane several times to form a coil, recent work suggests that it actually consists of multiple overlapping microtubules [Patel-Hett et al., 2008].

Expression of $\beta 6$ in CHO cells that do not normally express this isotype recapitulated several of the events that occur during megakaryocyte differentiation. For example, even low expression of $\beta 6$ in CHO cells arrested mitosis and led to the formation of large multinucleated cells. Although mouse $\beta 6$ knockout studies indicated that $\beta 6$ is not essential for the production of multinucleated megakaryocytes [Schwer et al., 2001], our experiments suggest that the isotype could be expressed at low levels in megakaryoblasts and participate in blocking cell division, perhaps acting as a redundant mechanism to ensure morphological differentiation of the blast cells into large multinucleated cells. Our studies further indicated that incorporation of $\beta 6$ potently suppressed microtubule dynamics, inhibited centrosomal microtubule nucleation, and led to the production of relatively short microtubule bundles that accumulated at the cell cortex. Thus, expression of $\beta 6$ appears to be sufficient to reorganize microtubules into the bundles at the plasma membrane of megakaryocytes that are necessary for the elaboration of proplatelet extensions. This finding is consistent with the observation that proplatelets are deficient in megakaryocytes lacking $\beta 6$ [Schwer et al., 2001]. Because transfected CHO cells do not form proplatelets or shed platelets into the culture medium, other factors are clearly involved in the biological process; but our studies on $\beta 6$ support the hypothesis that this isotype has properties that facilitate megakaryocyte differentiation and lead to the formation of the stable marginal band that is required for discoid platelet morphology.

Earlier studies that examined the effects of $\beta 6$ transfection into cultured cell lines reported that this isotype was able to assemble into cytoplasmic and spindle microtubules without

disrupting their organization [Joshi et al., 1987; Lewis et al., 1987]. However, those experiments did not use an inducible system, transfection efficiency was low, and the levels of expression were not measured. Our demonstration that $\beta 6$ expression interferes with mitosis makes it likely that cells with high expression may have been lost in the prior studies and the authors may have examined cells with only trace amounts of the $\beta 6$ isotype. Our studies are more in concert with in vitro studies reporting that purified chicken erythrocyte tubulin formed more stable polymers with reduced elongation and nucleation rates [Murphy and Wallis, 1985].

Class VI β -tubulin represents the clearest evidence for functional variation among tubulin isotypes in mammalian cells. Of the seven β -tubulin isotypes in mammals, it is the most divergent and the most restricted in its expression, presumably because of the potent effects it has on microtubule organization and cell division. We previously reported that overexpression of $\beta 1$, $\beta 2$, or $\beta 4b$ in CHO cells had no effect on cell division, microtubule assembly, or cell sensitivity to paclitaxel [Blade et al., 1999]. We later reported that overexpression of $\beta 3$, an isotype that is largely restricted to neuronal cells, caused some inhibition of microtubule assembly and made cells marginally resistant to paclitaxel, but considerable expression was needed to see these effects [Hari et al., 2003]. Overexpression of $\beta 4a$, another brain-restricted isotype, had minimal effects on microtubule assembly but conferred increased sensitivity to paclitaxel at low levels of expression that likely was caused by increased drug binding affinity [Yang and Cabral, 2007]. Prior to the current studies, the isotype with the largest effect on microtubule assembly was $\beta 5$. Overexpression of this isotype at moderate to high levels can virtually eliminate the microtubule cytoskeleton [Bhattacharya and Cabral, 2004], yet its production at low levels is necessary for cell division [Bhattacharya et al., 2008]. At more moderate levels of overexpression, $\beta 5$ reduces microtubule assembly and confers resistance to paclitaxel in a manner that is similar to mutant forms of $\beta 1$ -tubulin that we and others have described [Cabral, 2001; Orr et al., 2003].

In all of these previous studies, even the clones with high expression that lowered the endogenous tubulin isotypes failed to produce a phenotype similar to what we found with high or low levels of $\beta 6$ overexpression. For example, $\beta 5$ and $\beta 6$ -tubulin are the only isotypes able to inhibit cell division at low to moderate levels of expression, and both of them potently suppress microtubule dynamics. Both isotypes also cause microtubule fragments to appear, but the mechanisms by which they are generated appear to differ. Our studies indicate that fragments in drug treated [Yang et al., 2010], mutant [Ganguly et al., 2010], and $\beta 5$ overexpressing cells (R. Bhattacharya, H. Yang, and F. Cabral, manuscript submitted) originate from microtubules that detach from centrosomes and spindle poles throughout the cell cycle. In contrast, an increased frequency of microtubule detachment and the generation of fragments is not seen during interphase in $\beta 6$ overexpressing cells. Instead microtubule fragmentation occurs specifically in early mitosis and appears to involve a fundamentally different process that does not involve microtubule detachment. In support of this conclusion, paclitaxel prevents the formation of microtubule fragments in $\beta 5$ overexpressing cells and in mutant cell lines that also have an increased frequency of microtubule detachment [Ganguly et al., 2010] but not in $\beta 6$ expressing cells. Moreover, $\beta 6$ expressing cells and wild-type cells appear to share similar sensitivity to paclitaxel, but mutant and $\beta 5$ overexpressing cells that produce microtubule fragments by detachment are drug resistant [Bhattacharya and Cabral, 2004; Ganguly et al., 2010].

Another unique property of $\beta 6$ is its ability to inhibit microtubule nucleation. To date, we have found no change in the rate of nucleation in mutant cell lines with altered tubulin [Ganguly et al., 2010], cells with overexpression of $\beta 5$ (R. Bhattacharya, H. Yang, and F. Cabral, manuscript submitted), or drug-treated cells (A. Ganguly, H. Yang, and F. Cabral,

unpublished studies). Although we do not know the basis for $\beta 6$ effects on nucleation, it is possible that expression of this isotype produces structural changes in the centrosome or in its ability to properly organize. Support for this speculation comes from the observation (Fig. 3A and Supporting Information Fig. S3) that the spindle poles appear to be much less focused during prometaphase in clone $\beta 6$ -7 but not in cells that express mutant $\beta 1$ tubulin or are treated with high concentrations of microtubule inhibitors. The ability of $\beta 6$ to inhibit mitosis, cause microtubule fragmentation, suppress the dynamics and thereby stabilize those fragments, and inhibit microtubule nucleation are properties that make this isotype ideally suited for megakaryocyte differentiation and platelet production.

Supplementary Material

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Acknowledgments

We thank Dr. Xiangwei He for the generous use of his DeltaVision microscope, Dr. Anthony Frankfurter for the $\beta 6$ -specific antibody D8, Dr. Joanna Olmsted for providing the EGFP-MAP4 plasmid, Dr. Jeanette Bulinski for a rabbit antibody to α -tubulin, and Dr. Don Cleveland for the β -tubulin N-terminal specific antibody 18D6. These studies were supported by grant CA85935 from the National Institutes of Health to FC.

Abbreviations

CHO	Chinese hamster ovary
HA	hemagglutinin antigen
tet	tetracycline

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Fig. 1. Expression of $\beta 6$ -tubulin

Clonal lines, derived from CHO cells transfected with human $\beta 6$ -tubulin cDNA in a tetracycline (tet)-regulated vector, were incubated in the presence (+) or absence (–) of tet for 24 h and then examined by western blots stained with an N-terminal antibody that recognizes both ectopic $\beta 6$ -tubulin ($\beta 6$) and other endogenous isoforms of β -tubulin (β). The blot was also stained for actin as a loading control. Note that tet inhibited the expression of ectopic $\beta 6$ -tubulin. The numbers at the bottom of four lanes indicate the percentage of total β -tubulin contributed by $\beta 6$ expression.

Fig. 2. Tubulin immunofluorescence in cells expressing $\beta 6$

Nontransfected wild-type (WT) CHO cells and stably transfected clones expressing $\beta 6$ tubulin, as labeled in the panels, were pre-extracted with microtubule stabilizing buffer, fixed, and then stained with an antibody to α -tubulin. For the cells in panels A and B, their corresponding nuclear staining is also shown (A' and B'). Before fixation, the transfected cells were induced to express $\beta 6$ by incubating without tetracycline (Tet) for 1 or 4 d as indicated on the figure. Arrows in panel C point to microtubule bundles near the cell periphery. The arrow in panel F points to a cell with tubulin aggregates but no microtubule cytoskeleton. Bar = 50 μ m.

Fig. 3. Effects of $\beta 6$ on mitosis

A: Tubulin immunofluorescence of CHO and $\beta 6$ -7 cells 24 h after removing tetracycline. Note that α -tubulin is green, γ -tubulin is white, and DNA is red in the merged pictures. Expression of $\beta 6$ caused the cells to block in mitosis, leading to an increase in the mitotic index (B), and to accumulate predominantly in prometaphase (C). Unlike wild-type (WT) CHO cells, $\beta 6$ -7 cells exhibited extensive microtubule fragmentation in prophase (D). Arrows in panel D indicate the location of the spindle poles. Bars = 10 μ m.

Fig. 4. Effects of $\beta 6$ on microtubule acetylation

A stable CHO cell line expressing HA $\beta 1$ -tubulin (A) and clone $\beta 6$ -7 (B) were induced for 12, 24, 36, and 48 h by removing tetracycline. Cells were stained with antibodies against α -tubulin and acetylated α -tubulin for immunofluorescence (A and B) and lysed for western blot analysis using antibodies to α -tubulin, β -tubulin, and acetylated α -tubulin (C). Time-dependent changes in the levels of α -tubulin and acetylated α -tubulin relative to the zero time point are shown in panel D, * $p < 0.05$ compared to zero time point, $n=5$. Only the 48 h images are shown in panels A and B. Bar = 10 μm .

Fig. 5. Effects of $\beta 6$ on sensitivity to microtubule targeted drugs

Wild type CHO cells were mixed with clone $\beta 6$ -7 that was induced to express $\beta 6$ -tubulin by removing tetracycline for 24 h. The indicated nM drug concentrations were added for an additional 24 h. Because the $\beta 6$ expressing cells were more sensitive to vinblastine, this drug was added for just the last 3 h of the 48 h incubation. The cells were fixed and stained with antibodies against $\beta 6$ and α -tubulin to identify $\beta 6$ expressing and non-expressing cells, but only the α -tubulin staining is shown. Arrows indicate the $\beta 6$ expressing cells and arrowheads indicate cells that do not express $\beta 6$. Bar = 10 μ m.

Fig. 6. Microtubule life history plots

Wild-type CHO (WT), clone β6-47, and clone β6-7 were transfected with EGFP-MAP4 to visualize microtubules and incubated without tetracycline for 48 h. Microtubules were imaged 5 s apart by live cell fluorescence microscopy, their lengths were measured from an arbitrary reference point, and the lengths were plotted against time. Each line represents a separate microtubule followed for up to 250 s. Note that the position of a line on the graph is arbitrary and does not represent the actual total length of the microtubule. Graphs similar to these were used to calculate growth rates, shortening rates, catastrophes, rescues, and other parameters characteristic of dynamic instability. The results from those calculations are summarized in Table I.

Figure 7. Microtubule nucleation

A: Wild-type (WT) CHO and clone $\beta 6-7$ cells were treated with 2.5 μM colcemid for 2 h to depolymerize microtubules. After washing out the drug, the cells were fixed and stained at the indicated times (min) with an antibody to α -tubulin. Bar = 10 μm . **B:** WT and $\beta 6-7$ cells were transfected with EB1-GFP and imaged by live cell fluorescence microscopy. Representative images are shown. Arrows point to the centrosome. Bar = 10 μm .

Table 1

Parameters of microtubule dynamics (Mean $\pm$ SEM)

	CHO	β 6-47 24 hrs	β 6-47 48 hrs	β 6-7 24 hrs	β 6-7 48 hrs
Growth					
Rate (μ m/min)	16.14 $\pm$ 0.77	10.07 $\pm$ 0.44 [*]	10.04 $\pm$ 0.44 [*]	10.36 $\pm$ 0.68 [*]	10.08 $\pm$ 1.06 [*]
Duration (sec)	12.88 $\pm$ 1.12	7.41 $\pm$ 0.63 [*]	6.92 $\pm$ 0.51 [*]	6.05 $\pm$ 0.35 [*]	7.85 $\pm$ 1.23 [*]
Distance (μ m)	3.16 $\pm$ 0.40	1.24 $\pm$ 0.11 [*]	1.23 $\pm$ 0.15 [*]	1.11 $\pm$ 0.10 [*]	1.37 $\pm$ 0.21 [*]
Shortening					
Rate (μ m/min)	27.80 $\pm$ 2.32	17.37 $\pm$ 1.51 [*]	15.42 $\pm$ 1.31 [*]	17.11 $\pm$ 1.85 [*]	12.00 $\pm$ 0.93 [*]
Duration (sec)	12.13 $\pm$ 1.04	12.69 $\pm$ 1.94	8.90 $\pm$ 0.99 [*]	8.00 $\pm$ 0.93 [*]	6.50 $\pm$ 0.49 [*]
Distance (μ m)	5.07 $\pm$ 0.53	3.92 $\pm$ 0.78	2.68 $\pm$ 0.44 [*]	2.74 $\pm$ 0.67 [*]	1.29 $\pm$ 0.12 [*]
Frequency (min⁻¹)					
Catastrophe ¹	1.37 $\pm$ 0.11	0.80 $\pm$ 0.15 [*]	1.09 $\pm$ 0.24	1.10 $\pm$ 0.17	0.73 $\pm$ 0.14 [*]
Rescue ²	5.95 $\pm$ 0.50	7.18 $\pm$ 0.98	8.58 $\pm$ 0.95 [*]	9.68 $\pm$ 0.60 [*]	10.02 $\pm$ 0.61 [*]
% of time in					
Growth	30.44 $\pm$ 2.36	11.23 $\pm$ 1.78 [*]	7.80 $\pm$ 1.60 [*]	8.27 $\pm$ 1.76 [*]	4.59 $\pm$ 1.09 [*]
Shortening	20.19 $\pm$ 2.09	7.88 $\pm$ 1.78 [*]	7.31 $\pm$ 1.92 [*]	10.09 $\pm$ 1.75 [*]	4.31 $\pm$ 1.10 [*]
Pause	49.17 $\pm$ 3.19	80.89 $\pm$ 3.07 [*]	84.89 $\pm$ 2.74 [*]	81.64 $\pm$ 3.07 [*]	91.09 $\pm$ 1.66 [*]
Dynamics ³ (μ m/min)	8.90 $\pm$ 0.84	2.50 $\pm$ 0.51 [*]	2.50 $\pm$ 0.49 [*]	2.75 $\pm$ 0.59 [*]	1.53 $\pm$ 0.27 [*]
Number of MTs	32	28	30	30	30

^{*} $p < 0.05$ compared to CHO control by *student t*-test

¹ Catastrophe: transition from pause or growth to shortening.

² Rescue: transition from shortening to either pause or growth.

³ Dynamics: the total of changes in length (growth plus shortening) per unit time.