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Molecular Basis for Class V β-Tubulin Effects on Microtubule Assembly and Paclitaxel Resistance*[S]

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Vertebrates produce at least seven distinct β-tubulin isotypes that coassemble into all cellular microtubules. The functional differences among these tubulin isoforms are largely unknown, but recent studies indicate that tubulin composition can affect microtubule properties and cellular microtubule-dependent behavior. One of the isotypes whose incorporation causes the largest change in microtubule assembly is β5-tubulin. Overexpression of this isotype can almost completely destroy the microtubule network, yet it appears to be required in smaller amounts for normal mitotic progression. Moderate levels of overexpression can also confer paclitaxel resistance. Experiments using chimeric constructs and site-directed mutagenesis now indicate that the hypervariable C-terminal region of β5 plays no role in these phenotypes. Instead, we demonstrate that two residues found in β5 (Ser-239 and Ser-365) are each sufficient to inhibit microtubule assembly and confer paclitaxel resistance when introduced into β1-tubulin; yet the single mutation of residue Ser-239 in β5 eliminates its ability to confer these phenotypes. Despite the high degree of conservation among β-tubulin isotypes, mutations affecting residue 365 demonstrate that amino acid substitutions can be context sensitive; *i.e.* an amino acid change in one isotype will not necessarily produce the same phenotype when introduced into a different isotype. Modeling studies indicate that residue Cys-239 of β1-tubulin is close to a highly conserved Cys-354 residue suggesting the possibility that disulfide formation could play a significant role in the stability of microtubules formed with β1- but not with β5-tubulin.

Microtubules are needed to organize the Golgi apparatus and endoplasmic reticulum, maintain cell shape, construct ciliary and flagellar axonemes, and ensure the accurate segregation of genetic material prior to cell division. These cytoskeletal structures assemble from α- and β-tubulin heterodimers to form long cylindrical filaments that exist in a state of dynamic equilibrium characterized by stochastic episodes of slow growth and rapid shrinkage (1). Impairment of normal dynamic behavior has serious consequences for cell proliferation and thus makes microtubules an attractive target for drug development (2).

Vertebrates express multiple β-tubulin genes that produce highly homologous proteins differing most notably in their C-terminal 15–20 amino acids (3, 4). These variable C-terminal sequences are conserved across vertebrate species and have been used to classify β-tubulin genes into distinct isotypes (5). In mammals, for example, there are seven known isotypes designated by the numbers I, II, III, IVa, IVb, V, and VI. The functional significance of the C-terminal sequences is uncertain, but some studies suggest that they may be involved in binding or modulating the action of microtubule-interacting proteins (6–14). Additional amino acid differences are scattered throughout the primary sequence, but the functional role of these differences, if any, has not been elucidated. Although some β-tubulin isotypes are expressed in a tissue-specific manner (3), evidence indicates that microtubules incorporate all available isotypes, including transfected isotypes that are not normally produced in those cells (5, 15–17). Genetic experiments designed to test potential functional differences among the various β-tubulin isotypes have only demonstrated isotype-specific effects on the assembly of specialized microtubule-containing structures such as flagellar axonemes in *Drosophila* or 15-protofilament microtubules in *Caenorhabditis elegans* (18, 19). Thus, the consequences, if any, of producing multiple β-tubulin isoforms in vertebrate organisms remain elusive.

Our recent work showed that conditional overexpression of isotypes β1, β2, and β4b has no effect on microtubule assembly or drug sensitivity in transfected Chinese hamster ovary (CHO)[2] cells (20). Similarly, expression of neuronal-specific β4a produced very minor effects on microtubule assembly but was able to increase sensitivity to paclitaxel, most likely through increased binding of the drug (21). On the other hand, high expression of neuronal-specific β3 reduced microtubule assembly, conferred low level resistance to paclitaxel, and inhibited cell growth (22). The most dramatic effects, however, were seen in cells transfected with β5, a minor but widely expressed isotype (23). Even modest overexpression of this isotype reduced microtubule assembly and conferred paclitaxel resistance, whereas high levels of expression (~50% of total tubulin) caused fragmentation and a near complete loss of the microtubule cytoskeleton (24). Despite the toxicity associated

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[2] The abbreviations used are: CHO, Chinese hamster ovary; αMEM, α-minimum essential medium; β1, Class I β-tubulin; β5, Class V β-tubulin; DAPI, 4′,6-diamidino-2-phenylindole; GST, glutathione *S*-transferase; HA, hemagglutinin antigen; Ptx, paclitaxel; MTB, microtubule buffer; tet, tetracycline; tTA, tetracycline-regulated transactivator; PBS, phosphate-buffered saline.

with β5 overexpression, this isotype was recently shown to be required for normal mitotic progression and cell proliferation (25).

Because of its importance for cell division, and the extreme phenotype associated with its overexpression, we sought to identify the structural differences between β5-tubulin and its more "normal" homolog, β1. Although there are 40 amino acid differences between the 2 isotypes, we report that most of the unique properties of β5 can be attributed to the presence of serine in place of cysteine at residue 239. This residue faces the colchicine binding pocket and is very close to a highly conserved Cys-354 residue. We propose that Ser-239 found in β5-tubulin may prevent formation of a disulfide bond that normally stabilizes microtubules.

EXPERIMENTAL PROCEDURES

Construction of HA-tagged β1/β5 Chimeric Tubulin Genes—The starting materials for the generation of chimeric tubulins were a CHO β1-tubulin cDNA (GenBank™ accession number U08342) and a mouse β5-tubulin cDNA (GenBank™ accession number BC008225). Both cDNAs were modified to encode a C-terminal hemagglutinin (HA) tag to facilitate separation and detection of the transfected tubulin and were cloned into a *pTOPneo* vector to permit tetracycline-regulated expression (26). Prior to the generation of chimeras, the pTOPHAβ1 was modified by site-directed mutagenesis to remove a BamHI site at the 5′-end of CHO HAβ1 cDNA, and the resulting plasmid was named pTOPHAβ1-BamHI. Subsequent steps utilized two conserved internal restriction sites, BspHI (at codon 163) and BamHI (at codon 344), to generate six distinct chimeras named according to the isotype sequences present in the N-terminal (amino acids 1–163), central (amino acids 164–344), and C-terminal (amino acids 345–444/447) regions of the protein. For example, HAβ151 contains β1 sequences at the N-terminal and C-terminal regions with a β5 sequence in the middle. Restriction enzymes were from Promega (Madison, WI). Site-directed mutagenesis of various constructs was carried out using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA).

Transfection and Isolation of Stable Transfected Cell Lines—CHO tTA cells harboring the tetracycline-regulated transactivator (26) were transfected with different chimeric β-tubulin-containing plasmids using Lipofectamine (Invitrogen) as described by the manufacturer. Following transfection, the cells were maintained in αMEM (Mediatech Inc., Manassas, VA) supplemented with 5% fetal bovine serum (Gemini Bio-Products, W. Sacramento, CA). To determine the efficiency of transfection, a sterile coverslip was placed in the dish prior to seeding the cells, and the percentage of cells expressing the transfected tubulin was determined by immunofluorescence using a rabbit antibody against the HA tag (Bethyl Laboratories, Montgomery, TX) followed by an Alexa-488-conjugated goat anti-rabbit IgG (Invitrogen). To obtain stably transfected cells, the transfected population was replated in 60-mm dishes containing αMEM, tetracycline (1 μg/ml), and G418 (2 mg/ml). After 7–8 days resistant colonies were isolated and screened for expression of the HAβ-tubulin or pooled and maintained as a total G418-resistant population.

Immunofluorescence—Cells were grown on sterile glass coverslips in αMEM for 48–72 h, washed in PBS, and extracted in MTB buffer (20 mM Tris-HCl, pH 6.8, 1 mM $MgCl_2$, 2 mM EGTA, 0.5% Nonidet P-40) containing 4 μg/ml paclitaxel for 2 min at 4 °C. Fixation was carried out in methanol at −20 °C for 15 min followed by rehydration in PBS for 10–15 min. The fixed cells were incubated in PBS containing 1:100 dilutions of mouse α-tubulin antibody DM1A (Sigma-Aldrich) and rabbit HA antibody (Bethyl) for 1 h at 37 °C in a humid chamber. The coverslips were then washed in PBS and incubated for an additional hour in 1:100 dilutions of Alexa 488-conjugated goat anti-rabbit IgG and Alexa 594-conjugated goat anti-mouse IgG (Invitrogen) as well as DAPI (1 μg/ml). After washing in PBS the coverslips were inverted onto 5 μl of mounting medium (Biomeda Corp., Foster City, CA), and were viewed by epifluorescence using an Optiphot microscope (Nikon, Inc., Melville, NY) equipped with a Plan Apochromat 60× 1.4 numerical aperture oil objective and filters to minimize cross-talk between channels.

Colony Formation Assay—Approximately 100–200 cells were seeded into the first well of a 12-well dish containing αMEM plus 1 μg/ml tetracycline to inhibit the expression of the transgene. This well was used as a control to estimate the number of viable cells plated. Two additional wells received 10 times as many cells in αMEM containing 200 nM paclitaxel with or without 1 μg/ml tetracycline. The cells were incubated until colonies were visible (7–9 days), the medium was removed, and the cells were stained with a solution of 0.05% methylene blue in water as described previously (27). The plates were rinsed gently with water to remove excess stain, dried, and photographed.

Electrophoresis and Western Blots—CHO tTA cells or the same cells transfected with HAβ-tubulin cDNAs were grown in 24-well dishes and lysed in 1% SDS. Proteins were precipitated by adding 5 volumes of acetone, resuspended in 1× sample buffer (0.0625 M Tris-HCl, pH 6.8, 2.5% SDS, 5% 2-mercaptoethanol, 10% glycerol), fractionated on a 7.5% polyacrylamide SDS minigel, and transferred to PROTRAN nitrocellulose membranes (Schleicher and Schuell, Keene, NH). The membranes were blocked by incubating in PBST (PBS with 0.05% Tween 20) containing 3% dry milk. After washing in PBST three times the membranes were incubated 1 h in a 1:5,000 dilution of mouse monoclonal antibody 18D6 specific for the N-terminal amino acids of all β-tubulin isoforms (28). A 1:25,000 dilution of actin-specific mouse monoclonal antibody C4 (Chemicon International Inc., Temecula, CA) was also added to act as an internal control. After washing 3 times in PBST, the blots were incubated 1 h in a 1:2,000 dilution of Alexa 647-conjugated goat anti-mouse IgG (Invitrogen). Immunoreactive bands were detected by capturing fluorescence emission on a STORM 860 imager (Molecular Dynamics Inc., Sunnyvale, CA). In some experiments, α- or β-tubulin was detected using mouse antibodies DM1A and Tub 2.1 (Sigma).

Measurement of Polymerized and Unpolymerized Tubulin—Cells were grown in 24-well dishes for 2 days and lysed in 100 μl of MTB buffer containing 0.14 M NaCl and 4 μg/ml paclitaxel to keep the polymerized microtubules intact (29). The solubilized cells were scraped from the wells and transferred to 1.5-ml

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           10        20        30        40        50        60        70        80
β1 MREIVHIQAGQCGNQIGAKFWEVISDEHGIDPTGTYHGDSDLQLDRISVYYNEATGGKYVPRAILVDLEPGTMDSVRSGP
β5 MREIVHIQAGQCGNQIGTKFWEVISDEHGIDQAGGYVGDSALQLERISVYYNESSSKKYVPRAALVDLEPGTMDSVRSGP

           90       100       110       120       130       140       150       160
β1 FGQIFRPDNFVFGQSGAGNNWAKGHYTEGAELVDSVLDVVRKEAESCDCLQGFQLTHSLGGGTGSGMGTLLISKIREEYP
β5 FGQLFRPDNFIFGQTGAGNNWAKGHYTEGAELVDSVLDVVRKECEHCDCLQGFQLTHSLGGGTGSGMGTLLISKIREEYP

     BspH1
       ↓
           170       180       190       200       210       220       230       240
β1 DRIMNTFSVVPSPKVSDTVVEPYNATLSVHQLVENTDETYCIDNEALYDICFRTLKLTTPTYGDLNHLVSATMSGVTTCL
β5 DRIMNTFSVMPSPKVSDTVVEPYNATLSVHQLVENTDETYCIDNEALYDICFRTLKLTTPTYGDLNHLVSATMSGVTTSL

           250       260       270       280       290       300       310       320
β1 RFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLTSRGSQQYRALTVPELTQQVFDAKNMMAACDPRHGRYLTVAAVFRGR
β5 RFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLTARGSQQYRALTVPELTQQMFDAKNMMAACDPRHGRYLTVATVFRGP

                          BamH1
                            ↓
           330       340       350       360       370       380       390       400
β1 MSMKEVDEQMLNVQNKNSSYFVEWIPNNVKTAVCDIPPRGLKMAVTFIGNSTAIQELFKRISEQFTAMFRRKAFLHWYTG
β5 MSMKEVDEQMLAIQNKNSSYFVEWIPNNVKVAVCDIPPRGLKMASTFIGNSTAIQELFKRISEQFSAMFRRKAFLHWFTG

           410       420       430       440
β1 EGMDEMEFTEAESNMNDLVSEYQQYQDATAEE EEDFGEEAEEEA
β5 EGMDEMEFTEAESNMNDLVSEYQQYQDATVNDGEEAFEDEDEEEINE
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FIGURE 1. **Alignment of β1 and β5 tubulin.** The CHO Class I (U08342) and mouse Class V β-tubulin (NP_080749) protein sequences were aligned using MacVector (Accelrys Inc., San Diego, CA). Shared BspH1 and BamH1 sites that were used in subsequent experiments are shown. Amino acids in β5 that differ from β1 are *underlined*.

microcentrifuge tubes. Residual material was washed from the wells with 100 μl of the lysis buffer and mixed with the initial lysate. To solubilize any remaining residue, 100 μl of 1% SDS was added to the wells. The lysates were briefly mixed and centrifuged at 12,000 × *g* for 15 min at 4 °C. The supernatants carrying the unpolymerized tubulin were transferred to fresh tubes. The pellets containing the polymerized tubulin were resuspended in 50 μl water and combined with the residues solubilized in SDS from the corresponding wells. To each sample (supernatant and pellet) 4 μl of a bacterial lysate containing GST-α-tubulin was added as a control for any losses incurred in subsequent steps. Proteins were precipitated using 5 volumes of acetone and resuspended in 30 μl of 1× sample buffer. Equal volumes of samples were fractionated on a 7.5% polyacrylamide SDS minigel and transferred to a nitrocellulose membrane. The membrane-bound proteins were incubated with mouse antibodies DM1A (α-tubulin) and C4 (actin), followed by Alexa 647-conjugated goat anti-mouse IgG as described above. Bands corresponding to α-tubulin, GST-α-tubulin, and actin were quantified by using ImageJ software (National Institutes of Health) using the "gelplot1" macro. The percentage of tubulin polymerized into microtubules was calculated by normalizing tubulin in the supernatant and pellet fractions to the amount of GST-α-tubulin, dividing the normalized value from the pellet by the sum of the values from supernatant and pellet, and multiplying the fraction by 100.

RESULTS

Construction of Chimeric HAβ-tubulins—We previously reported that elevated production of β5-tubulin causes microtubule disruption and paclitaxel resistance in transfected cells (24), whereas transfection of β1, β2, and β4b isotypes does not (20). In an effort to identify the structural elements that might account for the unique effects of β5, we aligned its amino acid sequence with β1, the most abundant and widely distributed isotype. Excluding the hypervariable region at the C-terminal end, 29 internal amino acid differences (conserved plus non-conserved) were found (Fig. 1).

Because we didn't know whether the phenotypes conferred by overexpression of β5 were the result of single or multiple amino acid differences, we began our search for the relevant changes by carrying out domain swapping experiments between β5- and β1-tubulin. Two conserved endonuclease sites, BspH1 (codon 163) and BamH1 (codon 344), were found that allowed us to create 6 in-frame chimeric β-tubulin cDNAs. Each chimera was named according to the isotype sequence present in each of three segments beginning with the N terminus; *e.g.* β515 has β5 at the N and C termini with a β1 sequence in the center (Fig. 2). All the different chimeric cDNAs were sequenced to make sure they were in-frame and would produce the desired fusion proteins. To aid in identifying and measuring their levels of expression, the chimeric β-tubulin cDNAs were constructed to encode an HA tag at the C-terminal end of the proteins. Production of tubulin containing the C-terminal HA tag also served as confirmation that the cDNAs were in-frame.

The Central and the C-terminal Regions of β5-Tubulin Contain Sequences That Cause Microtubule Disassembly—CHO cells stably transfected with each chimeric tubulin cDNA were selected using G418; tetracycline was also included to inhibit transcription of the transgene and thereby limit any potential toxicity. The G418-resistant cell populations were then tested for effects of chimeric β-tubulin gene expression on microtubule organization by incubating them in tetracycline-free media for 3 days to allow transgene expression and viewing the cells by immunofluorescence microscopy. As discussed in a previous publication (24), we were able to estimate the expression of ectopic β-tubulin based on dual staining with anti-HA tag (green fluorescence) and anti-α-tubulin (red fluorescence) antibodies. Using a triple bandpass filter, microtubules incorporating high levels of chimeric HAβ-tubulin appeared green, cells with intermediate levels of expression appeared yellow to orange, and cells with low expression appeared red. To maintain consistency in the analysis, we show only the cells that appeared green, *i.e.* had a high level of transgene expression, to illustrate results throughout the figures. Also note that in Fig. 2 the nuclei were pseudocolored *red* in place of the *blue* DAPI fluorescence to make them stand out more clearly.

We found that all the chimeric tubulins except HAβ511 were able to induce microtubule disruption when expressed at high levels. The cells expressing HAβ511 (Fig. 2*C*) had microtubule assembly that was indistinguishable from wild-type or HAβ1 (Fig. 2*A*)-transfected cells indicating that amino acid differences in the N-terminal region of β5 are not sufficient to cause microtubule disruption. In contrast, cells producing chimeric

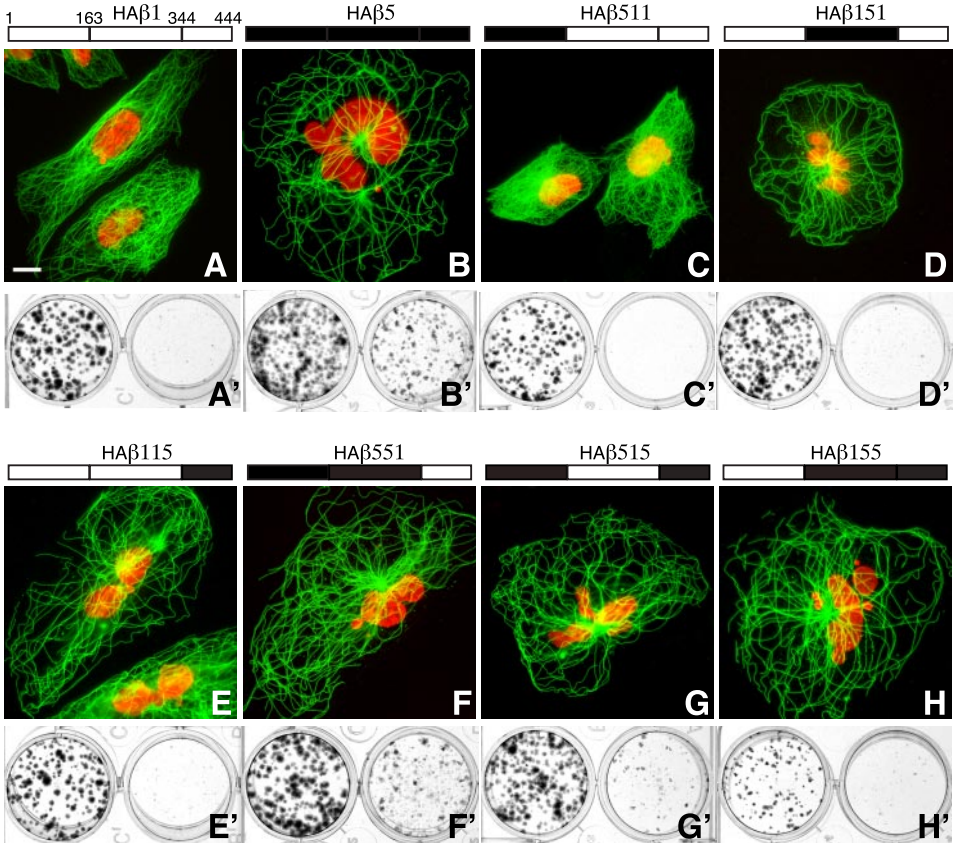

FIGURE 2. **Effects of HAβ1/β5 chimeras on microtubule organization and drug sensitivity.** CHO tTA cells were transfected with chimeric HAβ-tubulins as indicated in the *schematics* and grown for 3 days in the absence of tetracycline to induce transgene expression. Cells were then labeled for immunofluorescence with DAPI (*red*) and an antibody to the HA-tag (*green*) (*A–H*). The ability of chimeras to confer paclitaxel resistance was tested by a colony formation assay (*A′–H′*). Cells transfected with chimeric tubulins were grown with tet (*left well*) or with paclitaxel but no tet (*right well*) for 7 to 8 days, and surviving colonies were visualized by staining with methylene blue. *Bar* = 10 μm.

tubulin that contained either the central or C-terminal region of β5 had disrupted microtubules and were multinucleated (Fig. 2, *D–H*), making their appearance similar to cells transfected with intact HAβ5 (Fig. 2*B*). Low expression of these hybrids produced little or no microtubule disassembly or multinucleation, whereas moderate expression produced intermediate phenotypes, thus indicating that the effects of expression were dose-dependent (data not shown). The presence of the N-terminal region of β5 with either the central or the C-terminal part, as in constructs HAβ551 and HAβ515, did not reverse the ability of those domains to disrupt microtubules. We concluded that the central and the C-terminal regions of β5, but not the N-terminal domain, must each contain at least one or more amino acids responsible for microtubule disassembly.

The N-terminal Region of β5-Tubulin Is Required for Paclitaxel Resistance—Studies of numerous tubulin mutations have led to the observation that alterations in microtubule assembly are almost always accompanied by a change in cell sensitivity to agents that affect microtubule assembly (30, 31). Similarly, overexpression of β5-tubulin inhibited microtubule assembly and conferred paclitaxel resistance (24). To determine whether paclitaxel resistance is encoded within the same regions of β5 that encode microtubule disrupting activity, colony formation assays were performed by exposing transfected G418-resistant cell populations to a lethal dose of paclitaxel. The results summarized in Fig. 2 indicated that only cells transfected with HAβ551 (Fig. 2*F′*) and HAβ515 (Fig. 2*G′*) formed colonies in paclitaxel. Under identical conditions, cells expressing HAβ5 (Fig. 2*B′*) but not HAβ1 (Fig. 2*A′*) formed viable colonies in the drug. In all cases where tetracycline was added to inhibit expression of the ectopic tubulin, transfected cells failed to form colonies in paclitaxel indicating that transgene expression was necessary for drug resistance (data not shown). Given our previous experience, we found it surprising that three of the hybrid tubulins that disrupted microtubule assembly (HAβ151, HAβ115, and HAβ155) failed to confer resistance to paclitaxel. Although the N-terminal segment of β5 was not sufficient to cause microtubule disruption or confer paclitaxel resistance on its own (Fig. 2, *C* and *C′*), its presence in combination with either the central or the C-terminal domain was needed for drug resistance. The results suggest that interactions between the N-terminal domain and elements in the central and C-terminal domains cause differences in the response of microtubules to paclitaxel in each of the two isotypes.

The Highly Divergent C-terminal Tail of β5 Is Not Involved in Microtubule Disruption or Drug Resistance—Apart from the very divergent last 15 amino acids, β1 and β5 tubulins differ at only 4 additional amino acids in the C-terminal fragment. Because there are so many differences clustered in the last 15 amino acids (see Fig. 1), we first sought to determine whether this region by itself could cause microtubule destabilization. Hence, two further chimeric tubulins were constructed: one in which amino acids 345–417 were from β5 and the rest of the C-terminal fragment was from β1 tubulin (HAβ1151), the other in which only amino acids 417–447 were from β5-tubulin (HAβ1115) (Fig. 3). Like the parental HAβ115 hybrid tubulin, transfection of HAβ1151 caused microtubule disruption and multinucleation (Fig. 3*B*). In contrast, transfection of HAβ1115 produced no phenotype (Fig. 3*A*). We therefore conclude that the divergent amino acids at the extreme C terminus play little if any role in the ability of β5-tubulin to cause microtubule disruption. We also did not find any effect of the extreme C-terminal tail on sensitivity to paclitaxel or colcemid (data not shown).

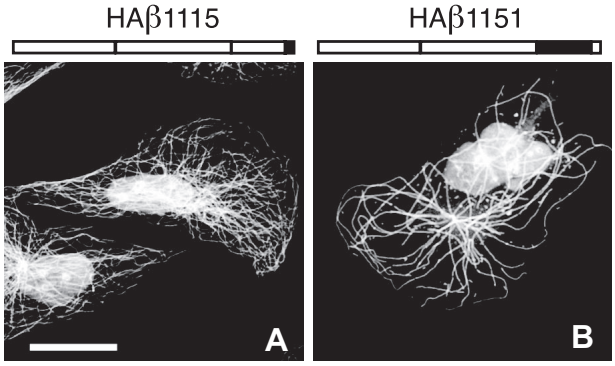

FIGURE 3. **Effects of the extreme C-terminal tail on microtubule assembly.** Hybrid HA*β*-tubulins (shown as a *schematic*) were transfected into CHO tTA cells. After 2 days without tetracycline the cells were stained for immunofluorescence with DAPI and an antibody to the HA tag. Note that overexpression of HA*β*1-tubulin containing the extreme C terminus from *β*5 (*β*1115) had no effect on microtubules or nuclear morphology (*A*), but that HA*β*1151-tubulin disrupted microtubules and produced multinucleation (*B*). *Bar* = 20 *μ*m.

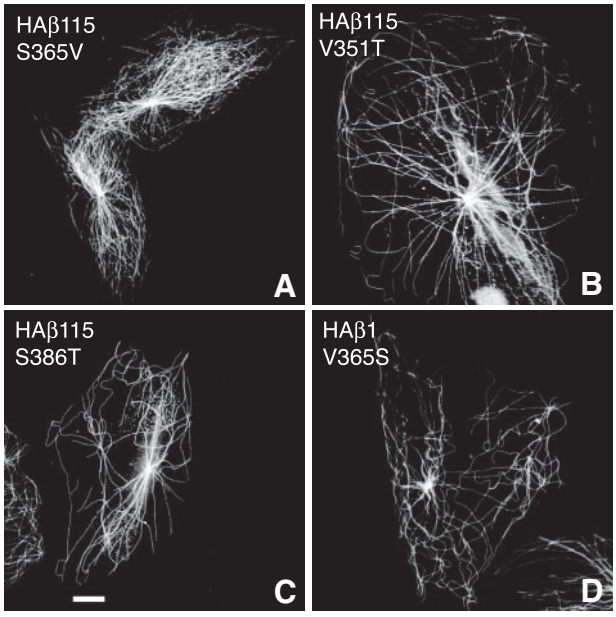

FIGURE 4. **Ser-365 of *β*5-tubulin is implicated in microtubule disruption.** Immunofluorescence with an antibody to the HA tag was used to test the ability of mutated HA*β*115 to disrupt microtubules in transfected cells. Only an S365V (*A*) mutation completely reversed the ability of HA*β*115 to disrupt microtubule assembly; V351T (*B*) and S386T (*C*) mutations did not. Transfection of HA*β*1 containing a V365S mutation strongly disrupted microtubules (*D*). *Bar* = 10 *μ*m.

Amino Acid 365 in the C-terminal Domain of β5 Is Implicated in Microtubule Disruption—The HA*β*1151 construct that retained the ability to disrupt microtubule assembly encodes only 4 amino acids (Val-351, Ser-365, Ser-386, and Phe-398) that differ from *β*1-tubulin. To determine which of them are involved in destabilizing microtubules, we altered each of those amino acids in HA*β*115 to the cognate amino acids in *β*1 tubulin, and transfected each construct into CHO cells to assess whether it was still capable of causing microtubule disruption. Of the four amino acid substitutions that were tested, only S365V caused a complete loss of the microtubule disrupting activity and left the cells with a normal diploid morphology (Fig. 4*A*). In contrast, V351T (Fig. 4*B*), S386T (Fig. 4*C*), and F398Y (not shown) substitutions did not prevent HA*β*115 from disrupting microtubule assembly and cell morphology. We thus conclude that of all the isotype-specific amino acid differences in the C-terminal segment of HA*β*115 tubulin, only Ser-365 is necessary for microtubule disruption. To determine whether alteration of residue 365 is sufficient to disrupt microtubule assembly, we produced a V365S substituted *β*1-tubulin in CHO cells and found that it potently disrupted the microtubule cytoskeleton (Fig. 4*D*).

Amino Acid 239 in the Central Domain Strongly Affects Microtubule Assembly—Similar experiments were carried out with HA*β*151 in an attempt to identify the critical amino acid residues in the central domain of *β*5 responsible for microtubule disruption. Sequence alignment between *β*1 and *β*5 identified 7 residues (170, 239, 275, 315, 320, 332, and 333) that differed between the 2 isotypes. Monosubstitution of each amino acid in HA*β*151 with the corresponding amino acid in *β*1 failed to completely abolish the ability of the chimeric tubulin to disrupt microtubules, but an S239C substitution had the largest effect on reversing the phenotype (supplemental Fig. S1).

Given the complexity of trying to eliminate the microtubule-disrupting activity of HA*β*151, we instead created mutations in HA*β*1-tubulin to look for gain of function. A C239S substitution caused the most microtubule disruption (Fig. 5*A*). It also caused problems in mitosis as evidenced by multiple large and oddly shaped nuclei in interphase cells. These multinucleated cells comprised 70% of the transfected cell population compared with only 7% for cells transfected with wild-type HA*β*1. S275A, A315T, R320P, and V333I substitutions, on the other hand, had little or no discernible effect on the microtubule network (Fig. 5, *B–E*) and did not cause any increase in the percentage of cells that were multinucleated. Finally, V170M and N332A substitutions produced minimal disruption of the microtubules; but at especially high expression, multinucleated cells become more common and comprised 18–20% of the transfected cell population (Fig. 5, *F*, and *G*). We concluded from these observations that alteration of residues 275, 315, 320, and 333 had no obvious effect on microtubule assembly or mitosis, alteration of residues 170 and 332 had weak effects, and alteration of residue 239 had the greatest effect on these phenotypes.

To more quantitatively assess the severity of the mutations that caused weak effects, stable clones of HA*β*1V170M were isolated. As an example of the results, expression of HA*β*1V170M in Clone 9 accounted for ~70% of the total *β*-tubulin (supplemental Fig. S2*A*), yet the cells retained essentially normal microtubule assembly (supplemental Fig. S2*B*). In contrast, HA*β*1 clones with amino acid substitutions at residues 239 and 365 had very low levels of microtubule polymer at similar levels of expression (described in the next section and Fig. 6). We thus conclude that amino acids residues 170 and 332 play only minor roles in the ability of *β*5-tubulin to disrupt microtubule assembly, but we can't rule out the possibility that different combinations of these and other amino acids could produce larger effects. A summary of the mutational data is provided in Table 1.

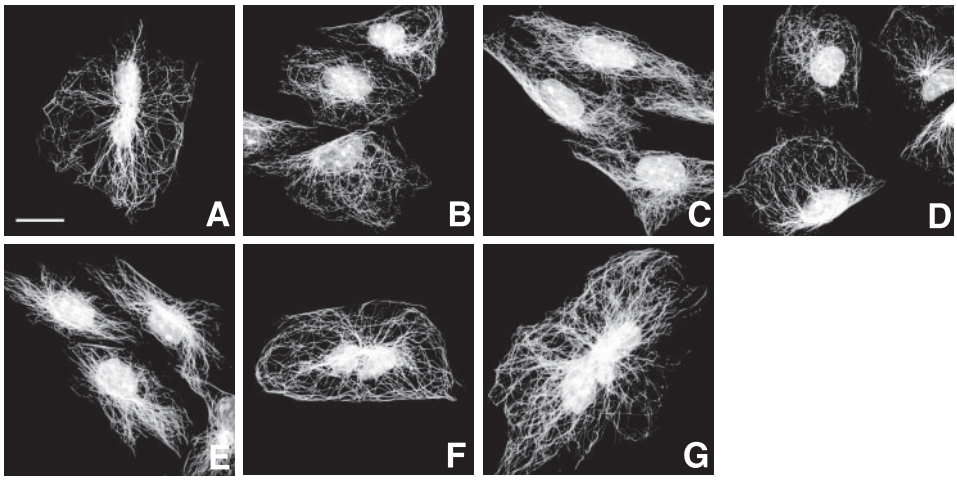

FIGURE 5. **Ser-239 of β5-tubulin is involved in disrupting microtubule organization.** The variable amino acids in the central part of β5-tubulin were introduced into HAβ1 and evaluated for their ability to cause microtubule disruption. Transfected cells were grown for 3 days without tetracycline to induce mutant protein expression and then stained with DAPI and an antibody against the HA tag. A C239S mutation (*A*) produced strong microtubule disruption and multinucleation while S275A (*B*), A315T (*C*), R320P (*D*), and V333I (*E*) had no effects. V170M (*F*) and N332A (*G*) had weak effects that were only seen at very high levels of expression. *Bar* = 20 μm.

Our previous studies showed that tubulin mutations that reduce microtubule assembly almost always confer resistance to paclitaxel. To test whether the C239S and V365S mutations act in a similar manner, transfected paclitaxel-resistant cells were grown overnight with or without paclitaxel. They were then lysed in a microtubule-stabilizing buffer and centrifuged to separate microtubule polymer from soluble tubulin. We found that resistant cells grown in paclitaxel had ~35–38% of their total tubulin in polymerized form, an amount that is similar to that found in untreated wild-type cells. In the absence of paclitaxel, however, the resistant cells had only ~15–20% of their tubulin in the polymerized form (Fig. 6*C*). Thus, in agreement with other drug-resistant cell lines (30, 31), C239S and V365S mutations confer resistance to paclitaxel by destabilizing the microtubule network and thereby opposing the action of the drug.

HAβ1 Substitutions at Residues 239 and 365 Confer Paclitaxel Resistance—To determine whether the amino acid differences in β5 that are responsible for disruption of microtubule assembly are also responsible for conferring resistance to paclitaxel, we transfected CHO cells with HAβ1-tubulin cDNA containing V365S and C239S mutations and tested the ability of the cells to form colonies in the presence of normally lethal concentrations of the drug. Cells expressing wild-type HAβ1 failed to survive in 200 nM paclitaxel, but cells producing each of the two mutant tubulins formed numerous resistant colonies. In contrast, cells transfected with HAβ1-containing mutations that did not affect microtubule assembly (V170M, A275T, T315A, R320P, and A332N) behaved like wild-type HAβ1 and did not form colonies in paclitaxel indicating that these individual mutations were not sufficient to confer drug resistance (data summarized in Table 1).

As a further test for the ability of HAβ1C239S and HAβ1V365S to confer drug resistance, we determined the ability of paclitaxel to select for cells that produce mutant HAβ-tubulin. As observed in previous studies (20, 26, 32), G418-resistant populations of mutant HAβ1-tubulin transfected cells were heterogeneous, with roughly half of the cells producing varying levels of the HA-tagged protein (*left panels*, Fig. 6*A*). We reasoned that, if the mutant tubulin is capable of conferring resistance to paclitaxel, then reselecting the G418-resistant cells in a normally lethal dose of paclitaxel should produce a population with a much larger fraction of positive cells. As predicted, immunofluorescence analysis of survivors from a 200 nM paclitaxel selection showed that 99% of the cells were positive for the ectopic protein (*right panels*, Fig. 6*A*). Consistent with these findings, Western blot analysis (Fig. 6*B*) demonstrated that the paclitaxel selected cells (*lanes 2* and *4*) had significantly higher levels of mutant HAβ-tubulin than the G418-resistant cells (*lanes 1* and *3*). Thus, by several independent criteria, C239S and V365S mutations in HAβ1-tubulin confer resistance to paclitaxel.

An S239C Mutation of HAβ5 Destroys Its Ability to Disrupt Microtubules and Confer Resistance to Paclitaxel—In contrast to the transfection of HAβ1-tubulin, which produced cells with normal microtubules and sensitivity to paclitaxel (Fig. 7, *A* and *A′*), transfection of HAβ5-tubulin produced large, multinucleated cells with a much less dense, disrupted microtubule network and the ability to grow in a normally lethal dose of paclitaxel (Fig. 7, *B* and *B′*). Although the two tubulin isotypes differ at 40 residues, only changes in amino acids 239 and 365 were found to cause a β5-like phenotype when introduced into HAβ1-tubulin. To determine whether the same two residues are required for the ability of β5 to cause the changes shown in Fig. 7, we "reverted" the amino acids at each of those two positions in β5 to the corresponding amino acids in β1. The HAβ5S365V mutant retained the ability to disrupt microtubules and confer paclitaxel resistance in transfected cells (Fig. 7 (*D* and *D′*) and Table 1), indicating that this amino acid is not essential for β5 to exert its effects. However, transfection of an HAβ5S239C cDNA produced only normal looking CHO cells with normal paclitaxel sensitivity (Fig. 7 (*C* and *C′*) and Table 1). An S239C/S365V double mutant also reverted β5 into behaving essentially like β1 (Table 1 and data not shown). These results indicate that Ser-239 is uniquely responsible for the ability of β5-tubulin to disrupt microtubule assembly and confer resistance to paclitaxel.

DISCUSSION

Class V β-tubulin is an intriguing isotype. It is one of the least studied forms of tubulin despite the fact that is found in almost all tissues except brain (23). Measurements have indicated that it is a minor isotype, comprising <10% of the β-tubulin in most tissues (23) and 10–20% of the β-tubulin in several cul-

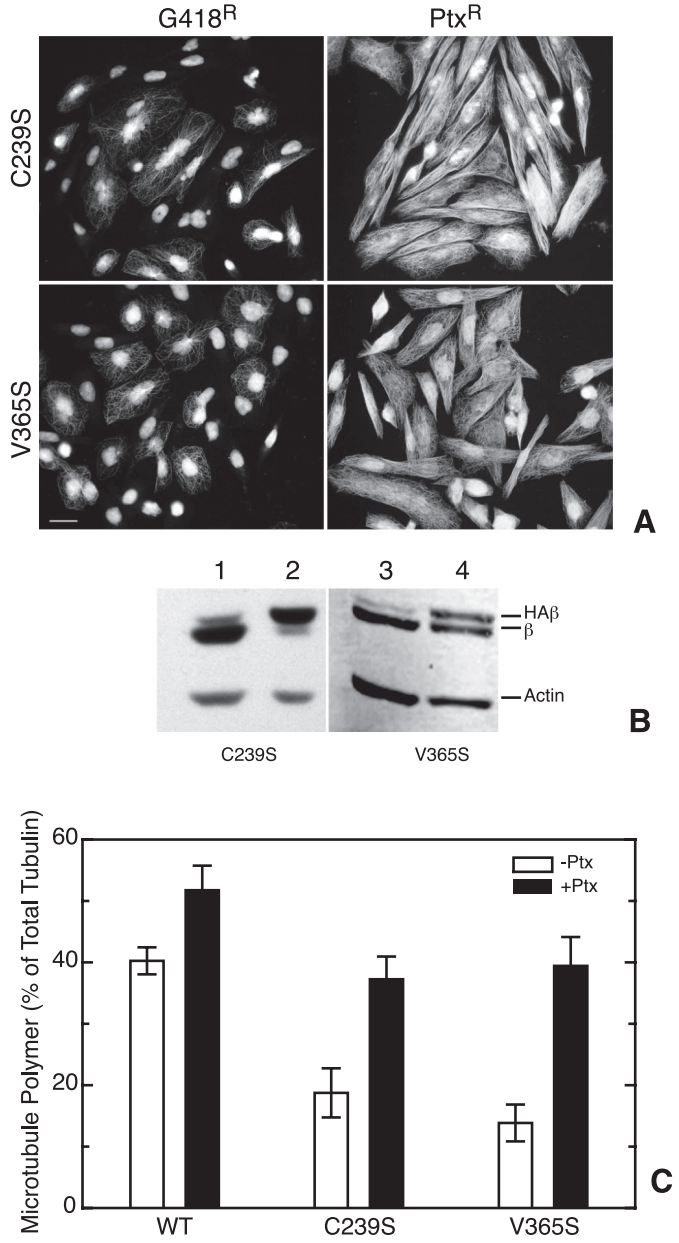

FIGURE 6. **HAβ1 C239S and V365S mutations confer paclitaxel resistance.** CHO tTA cells expressing HAβ1 with either C239S or V365S mutations were selected in the presence of G418 and tet. tet was then removed to allow transgene expression and the cells were reselected in paclitaxel. *A*, cells transfected with the indicated mutant HAβ1-tubulins and selected for G418 or paclitaxel (*Ptx*) resistance were stained with DAPI and an antibody to the HA tag. Note that the G418 survivors were a mixture of cells with or without HAβ1-tubulin expression, whereas the paclitaxel survivors were uniformly positive for mutant HAβ1-tubulin expression. *Bar* = 20 μm. *B*, cells selected for resistance to G418 (*lanes 1* and *3*) or paclitaxel (*lanes 2* and *4*) were assayed for production of the indicated mutant HAβ-tubulins on Western blots probed with an antibody to actin (loading control) and an antibody that recognizes both endogenous (*β*) and transfected (*HAβ*) β-tubulin. Note that paclitaxel-selected cells produce higher levels of mutant HAβ-tubulin than G418-selected cells. *C*, microtubule assembly in paclitaxel-resistant cells expressing HAβ1 with C239S or V365S mutations was compared with wild-type (*WT*) CHO cells. The cells were grown in the presence (*solid bars*) or absence (*open bars*) of 200 nM paclitaxel for 2 days, and the extent of tubulin polymerization (as a percentage of the total cellular tubulin) was measured.

TABLE 1
Summary of mutant effects on microtubule assembly and drug resistance in transfected cells

β1 Mutations	β5 Mutations	Microtubule disruption	Paclitaxel resistance[a]
WT		–[b]	–
V170M		+/–	–
C239S		+++	+++
S275A		–	–
A315T		–	–
R320P		–	–
N332A		+/–	–
V333I		–	ND[c]
T351V		–	ND
V365S		+++	+++
T386S		–	ND
Y398F		–	ND
	WT	+++	+++
	S365V	+++	+++
	S239C	–	–
	S365V/S239C	–	–

[a] Cells were tested for their ability to form colonies in 200 nM paclitaxel.
[b] +++, strong effect; +/–, marginal effect; –, no effect.
[c] ND, not determined.

tured cell lines (5). In CHO cells, we and others have reported that its abundance is only 5% (16, 33). Increased expression of this isotype can be very toxic (24). We have considerable experience in overexpressing various tubulin isotypes, mutant tubulins, and microtubule-interacting proteins (20–22, 26, 32, 34–37); yet of all the cDNAs we have transfected, β5-tubulin produces the most dramatic effects. The types of changes we see are similar to those we have reported for mutant forms of β1-tubulin, but the magnitude of the changes is generally much greater for β5 transfections. These changes include an altered cell morphology that results from defects in mitotic progression. As we and others have previously described, treatments that alter spindle structure or function cause a mitotic delay but do not trigger apoptosis in CHO cells. Instead, the cells escape the mitotic checkpoint and continue into G_1 without cytokinesis to form large, flat cells with fragmented nuclei that result from the inability of the mitotic cells to properly segregate their chromosomes (38–41). These cells frequently go through several aberrant cell cycles to become very large and polyploid before they die. Immunofluorescence experiments indicate that β5 overexpression strongly inhibits microtubule assembly, leaving cells with a very sparse and fragmented microtubule network (*e.g.* see Fig. 2*B*). The conclusion that β5 toxicity arises from its inhibition of microtubule assembly is consistent with the observation that the microtubule-stabilizing drug paclitaxel is able to counteract the effects of β5 overexpression; *i.e.* β5-overexpressing cells have been shown to be resistant to the effects of paclitaxel treatment and are frequently dependent on the drug for proliferation (24). Despite the toxicity associated with the overexpression of this isotype, we noted that it is present in all proliferating cells that have been examined (5, 16, 23, 33), including CHO cells, which have been in culture since 1954, and have dispensed with many nonessential genes (42–44). Attempts to deplete this isotype in mammalian cells using short hairpin RNA produced hyperstable microtubules and inhibited cell proliferation, indicating that a small amount of β5 is necessary for microtubules to function properly (25).

In an effort to understand the molecular basis for the effects of β5 overexpression, we attempted to identify sequence elements responsible for its ability to disrupt microtubules. Of the 40 amino acids in β5 that differ from β1-tubulin, an isotype whose overexpression has no effect on cells, only 2 of them,

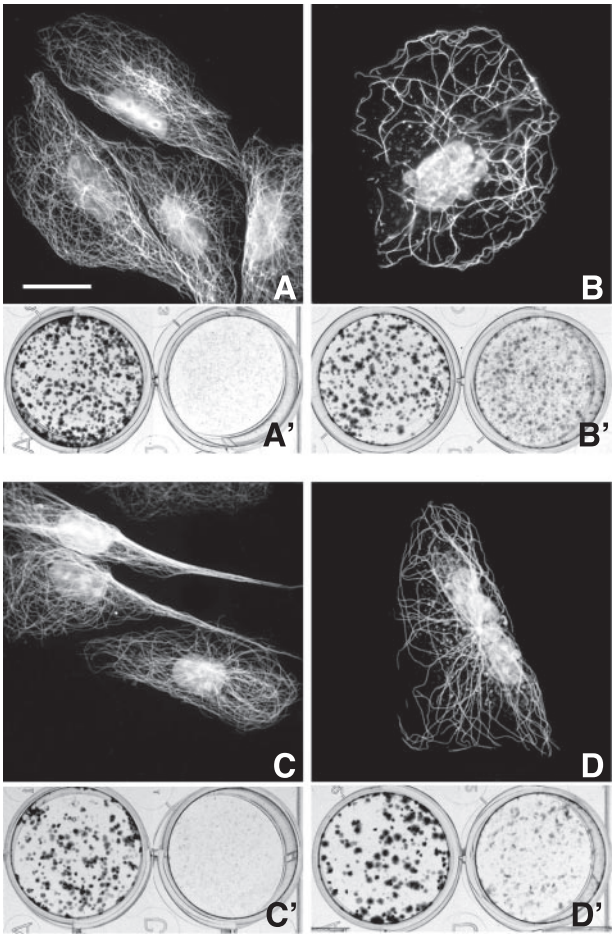

FIGURE 7. **Amino acid Ser-239 in β5-tubulin is essential for microtubule disruption and paclitaxel resistance.** HAβ5-tubulin was mutated at positions 239 and 365 and tested for its ability to disrupt microtubules and confer resistance to paclitaxel. G418-resistant populations of cells that had been transfected with HAβ1 (*A*), HAβ5 (*B*), HAβ5S239C (*C*), or HAβ5S365V (*D*) were grown for 3 days without tet, then stained with DAPI and an antibody to the HA tag. Note that the S239C (*C*), but not the S365V (*D*) substitution reversed the effects of HAβ5 on microtubules and nuclear morphology. The same G418-resistant populations were tested for their ability to survive exposure to 200 nM paclitaxel (*A'–D'*). Cells were grown with tet (*left well*) or with paclitaxel but no tet (*right well*). Note that the S239C substitution also reversed the ability of HAβ5 to confer drug resistance. *Bar* = 20 μm.

Ser-239 and Ser-365, produced significant microtubule disruption when introduced into β1. It should be noted that neither of these residues is within the extreme C-terminal region of β-tubulin. Although this region is the most variable among the various β-tubulin isotypes in a given organism, the sequence differences are highly conserved across vertebrate species. This observation has led to the idea that C-terminal sequences might define and be responsible for functional differences between tubulin isotypes (45). Our results reported here and elsewhere (20, 21) do not support this hypothesis for inherent microtubule properties such as assembly or drug sensitivity. They do not, however, rule out a possible role of C-terminal sequences in other aspects of microtubule behavior. For example, others have reported a role for this region of tubulin in ciliary beating (10, 12), sensitivity to katanin- and spastin-mediated microtubule severing (6, 14), kinesin and dynein mediated motility (13, 46), kinesin I-mediated microtubule depolymerization (8), and binding of MAP2 and Tau (7, 11).

Although Ser-239 and Ser-365 caused microtubule disruption when introduced into β1, only Ser-239 appears to be necessary for the disruptive effects of β5; *i.e.* an S239C substitution in β5 eliminated all of the effects of this isotype on microtubule assembly and response to paclitaxel, whereas an S365V substitution did not. The results therefore indicate that a serine at residue 239 is disruptive in both β1 and β5, whereas a cysteine at that position is conducive to microtubule assembly. We observed, however, that β115 and β515 constructs disrupted microtubule assembly despite having a cysteine instead of serine at residue 239. In these constructs, it is likely that Ser-365 was able to disrupt microtubules just as it did in a β1 background. This implies that even though some of the changes in β5 relative to β1 appear to be unimportant when viewed in isolation, they must somehow augment or mitigate the effects of other amino acid changes, perhaps by altering the overall conformation of a particular region. We conclude that amino acid substitutions may be context-sensitive; *i.e.* a mutation in one isotype may not necessarily produce the same phenotype when introduced into a different isotype. This will have important consequences for interpreting how tubulin mutations produce resistance to cancer drugs that target microtubules.

Several laboratories have reported that overexpression of β-tubulin isotypes can affect drug resistance (47). Our approach using conditional expression of specific cDNAs has clearly demonstrated that only a subset of β-tubulin isotypes affect the response of cells to drug treatment. Increased expression of the isotypes that have no effect on microtubule assembly (β1, β2, and β4b) also has no effect on the sensitivity of cells to antimitotic drugs (20). In contrast, increased expression of isotypes that destabilize microtubule assembly (β3 and β5) confers resistance to microtubule-stabilizing drugs such as paclitaxel (22, 24). This situation is very similar to extensive experience in several laboratories, including our own, that study mutations in β1-tubulin (30, 31, 48). In the case of these mutants, alterations that reduced the extent of microtubule assembly increased resistance to drugs that stabilize microtubules and increased sensitivity to drugs that destabilize microtubules. The converse was also true; *i.e.* alterations in β1-tubulin that increased the extent of microtubule assembly increased resistance to drugs that destabilize microtubules but increased sensitivity to drugs that stabilize microtubules (29, 49). It would appear that the effects of β-tubulin isotype expression mimic effects we and others have noted in cells that express mutant forms of β1-tubulin. Thus, we also looked at the ability of mutant β5-tubulin to confer resistance to paclitaxel. As expected, drug resistance again tracked closely with effects of missense mutations on microtubule disruption in both β1 and β5, but some of the chimeras behaved abnormally. Although β151, β115, β551, β515, and β155 all caused microtubule disruption, only β551 and β515 conferred resistance to paclitaxel. The reason why β151, β115, and β155 failed to confer resistance is unclear, but it suggests that the N-terminal region of β5 is necessary for paclitaxel resistance even though it produces no phenotype when it is fused onto β1-tubulin to produce β511 (Fig. 2). We conclude that there must be interactions of the N-terminal domain with the central and C-terminal domains of β5 that influence the ability of paclitaxel to compensate for

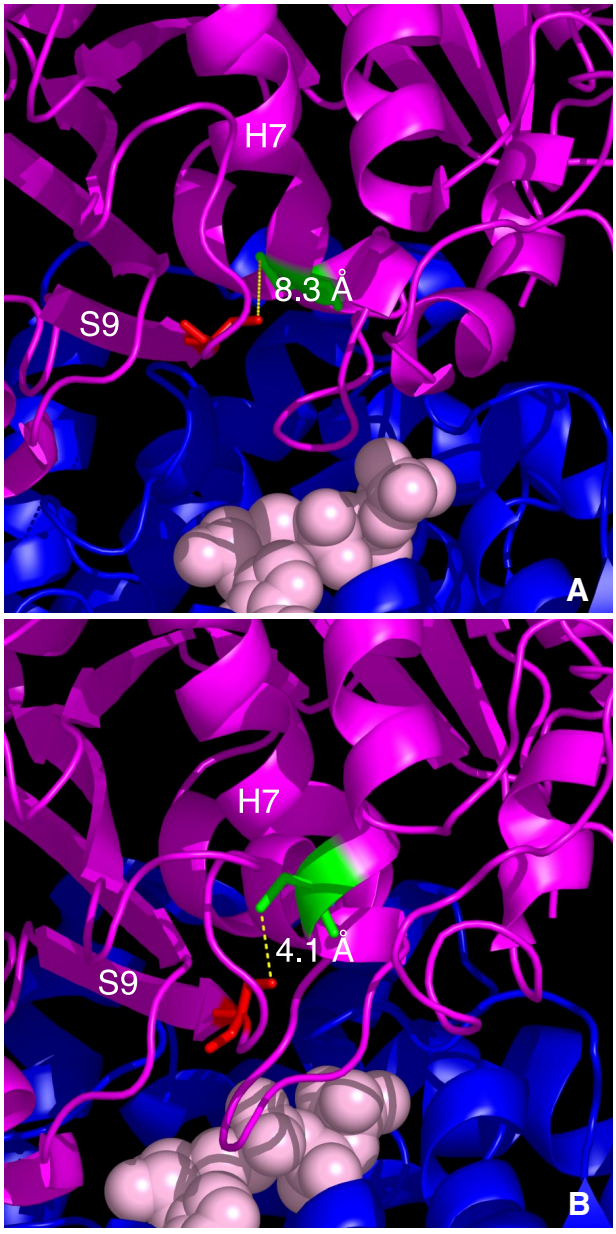

FIGURE 8. **Molecular structure of an αβ-tubulin heterodimer.** Amino acid Cys-239 (*green*) is located in the central core helix, *H7*; the conserved Cys-354 residue (*red*) is at the end of sheet *S9*. The distance between these residues decreases when tubulin moves from its curved non-assembled conformation (*A*) to the straight assembled conformation (*B*). The β-tubulin is *magenta*, α-tubulin is *blue*, and guanine nucleotides are *pink*. *A* is derived from the colcemid-bound structure 1SA0 (50), whereas *B* is derived from the paclitaxel-bound structure 1JFF (51). Calculated distances between residues 239 and 354 are shown. The structure was drawn using MacPyMOL (W. L. DeLano (2005) MacPyMOL: A PyMOL-based Molecular Graphics Application for MacOS X, DeLano Scientific LLC, South San Francisco, CA).

mutational effects on microtubule structure and assembly. These interactions may also explain why alteration of residue 365 caused severe microtubule disruption in the case of β1 and some β1/β5 chimeras, but had little effect in a purely β5 background.

The discovery that alteration of Ser-239 in β5-tubulin was sufficient to reverse the effects of β5 overexpression on microtubule disruption, mitotic progression, and paclitaxel resistance was unexpected and points to a central role of that residue in microtubule assembly. An alignment of vertebrate β-tubulins indicates that residue 239 is a cysteine in β1, β2, β4a, and β4b; *i.e.* isotypes whose overexpression has little or no effect on normal microtubule assembly. On the other hand, the same residue is a serine in β3 and β5; *i.e.* isotypes whose overexpression disrupts microtubule assembly (22, 24). Molecular modeling studies (Fig. 8) indicate that residue 239 is close to a highly conserved Cys-354 residue. Moreover, the two cysteines move closer to one another when tubulin goes from its unassembled to assembled conformation. Using the "curved" (colchicine-containing, unassembled) conformation of tubulin (structure 1SA0) (50), the distance between Cys-239 and Cys-354 is ~8.3 Å (Fig. 8*A*). In the "straight" (paclitaxel-containing, assembled) conformation (structure 1JFF) (51), however, this distance decreases to 4.1 Å (Fig. 8*B*). We propose that microtubules composed of isotypes containing a Cys-239 residue may be stabilized by the ability of β-tubulin to form a disulfide with Cys-354 that would favor the straight assembly-competent conformation. Increasing the incorporation of tubulin with an Ser-239 residue would then increasingly destabilize the microtubule lattice by diluting out the number of subunits that are capable of forming the disulfide bond. Although the 4.1-Å distance between Cys-239 and Cys-354 would appear to be too large for the formation of a disulfide, it should be noted that the crystal structure on which these measurements were based was obtained from electron diffraction patterns of zinc sheets prepared from brain tubulin that is rich in the β3 isotype (51). Thus, it is possible that the distance between Cys-239 and Cys-354 in true microtubules formed from other isotypes might actually be much closer.

Although this mechanism for the role of Cys-239 in microtubule assembly is speculative, it fits well with previous studies that have indicated a role for disulfide formation in regulating microtubule assembly. For example, studies in yeast indicate that mutations C354S and C354A result in fewer microtubules (52). Chemical approaches using mammalian tubulin have demonstrated that Cys-239 and Cys-354 are the most reactive cysteines in β-tubulin and that their modification reduces microtubule assembly (53). Similarly, inhibiting disulfide formation with dithiothreitol has been reported to reduce the ability of tubulin to assemble *in vitro* (54). Finally, it has been reported that thioredoxin and thioredoxin reductase are associated with microtubules *in vivo* (55), and this has led to speculation that thiol-disulfide exchange could play an important role in regulating microtubule assembly (53, 54).

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