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Overexpression of class I, II or IVb β-tubulin isotypes in CHO cells is insufficient to confer resistance to paclitaxel

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SUMMARY

Recent studies have suggested a correlation between increased expression of specific β-tubulin isotypes and paclitaxel resistance in drug-selected cell lines. In an attempt to establish a causal link, we have transfected Chinese hamster ovary cells with cDNAs encoding epitope-tagged class I, II, and IVb β-tubulins, as well as a class I β-tubulin with a mutation previously characterized in a paclitaxel resistant mutant. To eliminate possible toxicity that might be associated with overexpression of non-native tubulin, each of the cDNAs was placed under the control of a tetracycline regulated promoter. All transfected cDNAs produced assembly competent tubulin whose synthesis could be turned off or on by the presence or absence of tetracycline. Production of β_I, β_{II}, or β_{IVb} tubulin had no effect on the sensitivity of the cells to paclitaxel, but production of the mutant β_I-tubulin conferred clear resistance to the drug. We conclude from these experiments that simple overexpression of class I, II, or IVb isoforms of β-tubulin is insufficient to confer resistance to paclitaxel.

Key words: Paclitaxel resistance, β-Tubulin, Isotype, Tetracycline regulated expression, Mutation

INTRODUCTION

Paclitaxel is an antimitotic drug derived from the bark of *Taxus brevifolia*, a slow growing tree found in the virgin forests of the Pacific Northwest region of the United States. First discovered in 1971 (Wani et al., 1971), the drug failed to generate much interest until it was shown to have the unusual property of promoting microtubule assembly and inducing microtubule bundle formation in treated cells (Schiff et al., 1979; Schiff and Horwitz, 1980). A further resurgence of interest came from phase II clinical trials that demonstrated remarkable activity of the drug in the treatment of ovarian (McGuire et al., 1989) and breast cancer (Holmes et al., 1991). In further clinical studies, paclitaxel has also shown significant activity against non-small cell lung carcinoma, head and neck carcinomas, and melanomas (reviewed by Rowinsky and Donehower, 1995). However, as is frequently found with other chemotherapeutic drugs, many of the patients who were initially responsive to paclitaxel subsequently relapsed, presumably due to the acquisition of drug resistance by the tumor cells.

Early studies to understand the mechanisms by which cells acquire resistance to paclitaxel demonstrated that the drug is a substrate for the gP170 multidrug resistance (mdr) pump that is able to confer resistance to a wide variety of naturally derived hydrophobic and toxic substances (Gottesman et al., 1995). Thus, cells selected through multiple steps to high level resistance to paclitaxel were found to exhibit cross-resistance to a variety of other hydrophobic drugs and have elevated levels of P-glycoprotein (reviewed by Casazza and Fairchild, 1996). Conversely, cells that were selected for resistance to unrelated drugs such as doxorubicin, vinblastine, or etoposide demonstrated cross-resistance to paclitaxel. However, the observation that paclitaxel is effective in many patients with anthracycline-resistant disease suggests that mdr might not be as clinically prevalent as was initially thought (Rowinsky and Donehower, 1995).

A different mechanism of paclitaxel resistance reported by our laboratory involves alterations in tubulin that counteract the presence of the drug by affecting assembly of microtubules (Cabral and Barlow, 1989; Cabral et al., 1986). These tubulin alterations were much more common than mdr alterations in single-step selections, and gave rise to cell lines with low levels of resistance (2- to 3-fold) to paclitaxel (Schibler and Cabral, 1986). The mutations affected α- and β-tubulin with equal frequency (Schibler and Cabral, 1986) and resulted in a significant shift of tubulin from the polymer to the soluble pool at steady state (Minotti et al., 1991). We have recently sequenced the mutant β-tubulin alleles from several of these resistant cell lines and demonstrated that transfection of mutant cDNA into wild-type CHO cells is able to confer paclitaxel resistance (M. L. Gonzalez-Garay, L. Chang, K. Blade, D. R. Menick, and F. Cabral, unpublished obsevations). One of the mutations, a phenylalanine for leucine substitution at amino acid 228, served as a positive control for the experiments described here.

One recent study examining mechanisms of resistance to paclitaxel has identified mutations in β-tubulin that decrease responsiveness to the drug and may affect drug binding (Giannakakou et al., 1997); other studies have reported increased expression of specific isoforms of β-tubulin in resistant cell lines. Among the latter studies, Horwitz and colleagues first reported increased expression of β_{II}-tubulin in a paclitaxel resistant murine cell line (Haber et al., 1995) and later reported increased expression of β_I, β_{II}, β_{III}, and β_{IVa}-tubulin in drug resistant A549 human lung cancer cells as well as increases in β_I, β_{III}, and β_{IVa}-tubulin in paclitaxel resistant human ovarian tumors (Kavallaris et al., 1997). Similar studies in K562 human leukemia cells have implicated β_{IVa}-tubulin in paclitaxel resistance (Jaffrezou et al., 1995), and studies in DU-145 human prostate carcinoma cells have revealed an increase in β_{III} and β_{IVa}-tubulin expression in association with resistance to estramustine, another drug that affects microtubule assembly (Ranganathan et al., 1996).

Although these recent studies have implicated changes in tubulin isotype composition as a possible mechanism for resistance to paclitaxel, the results are correlative in nature and do not rule out other potential changes that might be responsible for the drug resistance phenotype. This point is especially troublesome because most selections were carried out in multiple steps to high levels of resistance opening up the possibility that multiple events contribute to the drug resistance of the cells. As a direct test of the ability of various β-tubulin isotypes to confer resistance to paclitaxel, we have experimentally manipulated the expression of specific β-tubulin cDNAs in CHO cells and tested the effects on cell viability and drug resistance.

MATERIALS AND METHODS

Plasmids used for transfection

CHO *Cβ1* (GenBank accession no. U08342) is a previously described class I β-tubulin cDNA (Boggs and Cabral, 1987; Boggs et al., 1996). Mouse class II and IVb β-tubulin cDNAs (Wang et al., 1986) were originally named *Mβ2* and *Mβ3* but will be called β_{II}- and β_{IVb}-tubulin according to the nomenclature described by Lopata and Cleveland (1987). All cDNAs were modified to encode a 9 amino acid hemagglutinin antigen (HA) epitope tag (YPYDVPDYA) at the C terminus of β-tubulin and were then inserted into a pTOPneo vector (M. L. Gonzalez-Garay, L. Chang, K. Blade, D. R. Menick, and F. Cabral, unpublished observations) that contains a minimal cytomegalovirus (CMV) promoter downstream from 7 copies of the *tetO* operator sequence for tetracycline regulated expression (Gossen and Bujard, 1992). A mutant form of the HA-tagged CHO β_I-tubulin cDNA was created by site directed mutagenesis using the Altered Sites II in vitro Mutagenesis System (Promega Corp., Madison, WI) according to the manufacturer's instructions. This mutant gene, $HA\beta_{I(L228F)}$, encodes a phenylalanine in place of leucine at amino acid 228, and was fully sequenced to ensure that no other alterations were introduced.

Transfection, selection, and maintenance of cell lines

The growth medium for all cells was alpha modification of minimum essential medium (αMEM) containing 5% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin (all from Gibco BRL, Gaithersburg, MD). Plasmid DNA was prepared using the QIAfilter Plasmid Maxi Kit (Qiagen Inc., Santa Clarita, CA) according to the manufacturer's instructions, and used to transfect CHO strain tTApuro 6.6a (M. L. Gonzalez-Garay, L. Chang, K. Blade, D. R. Menick, and F. Cabral, unpublished observations) that expresses the tetracycline regulated transactivator under tetracycline control (Shockett et al., 1995). Transfections were carried out using 1 μg of plasmid DNA (0.2 μg in the case of $HA\beta_{I(L228F)}$) and Lipofectamine (Gibco BRL) according to the manufacturer's instructions except that the growth medium contained 1 μg/ml tetracycline (Sigma) to prevent expression of the transfected DNA until time of analysis. Stable transfected cell populations were selected and maintained at 37°C, 5% CO_2 in growth medium that contained 1 μg/ml tetracycline and 2 mg/ml G418 (Gibco BRL).

Selection of paclitaxel resistant cells

To determine whether expression of specific β-tubulin isotypes could confer paclitaxel resistance, CHO strain tTApuro 6.6a was transfected with each of the HA tagged cDNAs in parallel. Cells from each transfection were then trypsinized and reseeded in duplicate into 6-well dishes containing normal growth medium with tetracycline (5-10×10^2 cells), medium with G418 plus tetracycline (5-10×10^4 cells), or medium with 0.2 μg/ml paclitaxel and no tetracycline (5-10×10^4 cells). The dishes were incubated at 37°C for 7 days until colonies appeared. Wells that failed to produce any visible colonies were incubated an additional week and scanned microscopically to be certain we would not miss any slow growing colonies. Cells from one set of duplicate wells were trypsinized and used for propagating the culture for futher testing, and cells in the second set of wells were stained with methylene blue as previously described (Cabral et al., 1980) to count the number of resistant colonies.

In addition to their direct selection, selections for paclitaxel resistant cells were also carried out on cell populations that had previously been selected in G418 and were therefore enriched for cells expressing the transfected β-tubulin. In this case the cells selected in G418 plus tetracycline were trypsinized and reseeded in duplicate into 6-well dishes at high (4-5×10^3) and low (150-200) cell density in normal medium without tetracycline for 6 hours to allow induction of transgene expression. Prior studies indicate that 3 hours is the earliest time that significant production of the transfected tubulin can be detected (F. Cabral, unpublished data). Following the 6 hour induction, one set of duplicate wells was changed to medium containing 0.2 μg/ml paclitaxel while the other set of wells remained in normal medium without tetracycline.

Measurement of drug resistance

To determine the dose response to paclitaxel, an equal number of cells (~100-200) were plated into 12 wells of a 24-well dish. One set of 6 wells contained medium with tetracycline, and the other set contained medium without tetracycline. In addition, each of the 6 wells in each set contained varying concentrations (0-0.3 μg/ml) of paclitaxel. After 7 days the medium was removed and the cells were stained with methylene blue. The stained plates were scanned at 300 dots per inch on an Expression 636 flatbed scanner (EPSON America, Inc., Torrance, CA) using transmitted light, and the image was saved as a Photoshop 4.0 (Adobe Systems Inc., Mountain View, CA) TIFF file on a MacIntosh 7200 Power PC computer (Apple Inc., Cupertino, CA). The digital image was imported into Illustrator 7.0 (Adobe) to add labels and was then printed on a Phaser 440 dye sublimation printer (Tektronix Inc., Wilsonville, OR).

Electrophoretic techniques

To measure the relative accumulation of transfected versus endogenous β-tubulin, transfected cell populations that had undergone selection were seeded into 24-well dishes in the continued presence or absence of tetracycline and incubated overnight (~20 hours) at 37°C. The medium was aspirated, the cells were washed 2 times with phosphate buffered saline (PBS, Gibco BRL), and the cells were then lysed by the addition of 100 μl of hot (100°C) sodium dodecyl sulfate (SDS) sample buffer (Laemmli, 1970). Protein and DNA in the lysate were precipitated by the addition of 5 volumes of cold (4°C) acetone/NH_4OH (20/1), the DNA was fished out with a thin gauge needle, and the protein was centrifuged at 12,000 ***g*** for 5 minutes.

The pellet was redissolved in 30 μl of SDS sample buffer and 5-15 μl were run on a 7.5% polyacrylamide SDS minigel (Bio-Rad Laboratories, Hercules, CA). Proteins in the gel were transferred to nitrocellulose and then incubated in a mixture of mouse monoclonal antibodies to β-tubulin (Tub 2.1, 1:2000 dilution, Sigma Chemical Co., St Louis, MO) and actin (C4, 1:5000 dilution, ICN Pharmaceuticals, Inc., Costa Mesa, CA) as previously described (Gonzalez-Garay and Cabral, 1995). Chemiluminescence detection was carried out using peroxidase conjugated goat anti-mouse IgG (1:2000 dilution, Cappel Laboratories, Cochranville, PA) followed by incubation in chemiluminescence substrate (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) and exposure to XAR-5 film (Eastman Kodak, Rochester, NY).

Immunofluorescence

Transfected cells were grown on glass coverslips to approximately 70% of confluence, rinsed in PBS, fixed in –20°C methanol for at least 10 minutes, and rehydrated in PBS. Cells were then incubated with HA-tag specific antibody 12CA5 (Boehringer Mannheim Corp., Indianapolis, IN) at 1:100 dilution in PBS for 30-60 minutes in a humid atmosphere at 37°C, rinsed 3 times in PBS, and incubated with fluorescein-conjugated goat anti-mouse IgG (Sigma) at 1:40 dilution as just described. After thorough rinsing in PBS, the coverslips were inverted over a small drop of mounting solution (90% glycerol, 10% PBS, 0.1% p-phenylenediamine, pH 8.7) and viewed by epifluorescence with a ×40 fluor objective on an Optiphot microscope (Nikon Inc., Melville, NY). Photographs were taken on TMAX 400 film (Kodak) exposed at ASA 800. The negatives were digitized using a Sprint 35 slide scanner (Polaroid Corp., Cambridge, MA) at 1350 dots per inch and saved as a Photoshop file as described above.

RESULTS

Transfected β-tubulins assemble into microtubules

Vertebrate species have been found to express at least 6 different β-tubulin genes in a tissue restricted manner, such that any given cell type expresses only a subset of the available β-tubulin genes (Sullivan, 1988). Prior work has demonstrated that microtubules in CHO cells are assembled from 3 different β-tubulin isotypes (β_I, β_{IVb}, and β_V) with relative stoichiometries of 70:25:5, respectively (Sawada and Cabral, 1989). Probing CHO cell lysates with isotype specific antisera (Sawada and Cabral, 1989), or exhaustive cloning of tubulin cDNAs (Ahmad et al., 1991) has failed to reveal the expression of any other isotypes of β-tubulin. The isolation of paclitaxel resistant CHO cells allows the isolation of mutations in α- and β-tubulin that confer drug resistance (Schibler and Cabral, 1986), and the mutations in β-tubulin invariably occur in the highly expressed β_I-tubulin gene (F. Cabral, unpublished observations). We have previously demonstrated that transfection and overexpression of an epitope tagged β_I-tubulin cDNA (HAβ_I-tubulin) has no effect on the sensitivity of CHO cells to paclitaxel (Gonzalez-Garay and Cabral, 1995), but we have subsequently found that transfection of the same cDNA containing a phenylalanine for leucine replacement at position 228 (L228F), one of the mutations we identified in paclitaxel resistant mutants, is able confer drug resistance in wild-type CHO cells (M. L. Gonzalez-Garay, L. Chang, K. Blade, D. R. Menick, and F. Cabral, unpublished observations).

With the aid of these positive and negative controls, we were able to directly test whether altering the tubulin isotype composition of CHO cells by transfection and overexpression of specific β-tubulin cDNAs would confer resistance to paclitaxel. To eliminate the possibility of toxicity associated with overexpression, 3 different β-tubulin cDNAs were each cloned into a vector that allows tetracycline regulated expression (Gossen and Bujard, 1992). In addition, each β-tubulin was tagged at the carboxyl terminus with a 9 amino acid hemagglutinin antigen (HA) sequence to facilitate analysis of the transfected cells. Previous studies have shown that this tag has no observable effect on the biological properties of the protein (Gonzalez-Garay and Cabral, 1995, 1996; Kozminski et al., 1993). The cDNAs selected for this study included CHO *Cβ1* (Boggs and Cabral, 1987; Boggs et al., 1996), which encodes class I β-tubulin, the most abundant isotype in CHO cells; mouse *Mβ3* (Wang et al., 1986), which encodes class IVb β-tubulin, an isotype that normally makes up 25% of CHO β-tubulin; and mouse *Mβ2* (Wang et al., 1986), which encodes class II β-tubulin, an isotype that is not normally expressed in CHO cells. The mutant *HA*$\beta_{I(L228F)}$ cDNA was also included in these experiments to act as a positive control.

The transfection of a β_{II}-tubulin cDNA that is not normally expressed in CHO cells raised the possibility that any expressed protein might be rapidly degraded because of an inability to find a suitable α-tubulin partner for heterodimer formation and microtubule assembly. Such a possibility would negate the rationale for our experiments and prevent us from assaying the ability of this isotype to confer drug resistance. To determine whether the transfected β-tubulins were capable of assembling into microtubules, transient transfections were carried out and the cells were stained with an antibody to the HA tag. As shown in Fig. 1, each of the 4 transfected β-tubulin

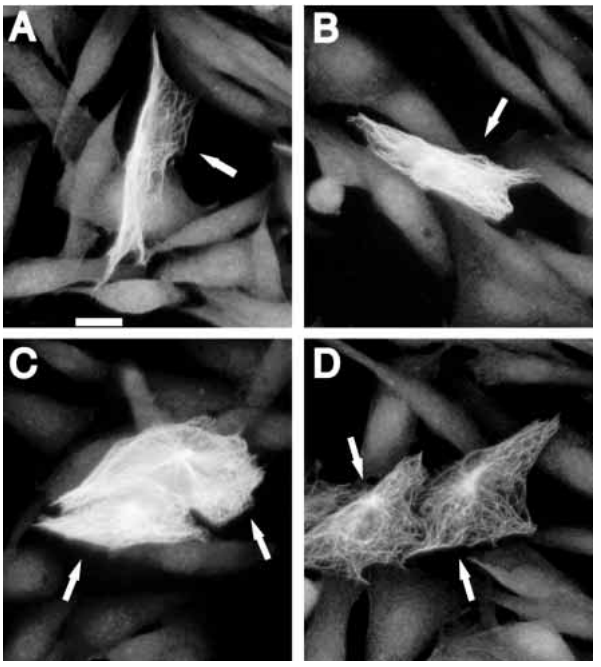

Fig. 1. Immunofluorescence of cells transfected with HAβ-tubulin. Cells from CHO strain tTApuro 6.6a were seeded onto glass coverslips and transfected with *HA*β_I (A), *HA*β_{II} (B), *HA*β_{IVb} (C), or *HA*$\beta_{I(L228F)}$ (D) cDNA. At 24 hours posttransfection, the cells were extracted with a microtubule stabilizing buffer, fixed, and stained with an antibody specific to the HA tag. Arrows indicate the positions of cells expressing the transfected HAβ-tubulin. Bar, 10 μm.

cDNAs (*HAβI*, *HAβII*, *HAβIVb*, and *HAβI(L228F)*) was expressed and the protein was able to assemble into microtubules.

Transfection with *HAβI*, *HAβII*, or *HAβIVb* does not allow the selection of paclitaxel resistant cells

To assess the ability of overexpressed HAβ-tubulin isoforms to confer paclitaxel resistance, parallel transfections were carried out using equivalent amounts (1 μg) of *HAβI*, *HAβII*, and *HAβIVb* cDNA. Only the *HAβI(L228F)* cDNA was transfected using a lower amount (0.2 μg) to ensure that its level of expression would be less than that of the non-mutated genes. Cells from each transfection were seeded onto dishes under different selective conditions. One group of cells was exposed to medium with tetracycline. These are nonselective conditions that should allow all cells to grow regardless of whether they do or do not express the transfected cDNA. Tetracycline was included to suppress transfected HAβ-tubulin expression and circumvent the possibility that the overexpressed protein might prove toxic to cell survival. This control group was used as the baseline to calculate transfection efficiencies. A second group of cells received medium containing G418 plus tetracycline. The G418 was expected to select cells that were stably transfected and expressed the neomycin resistance gene in the plasmid. Tetracycline was again included to eliminate any possible toxicity from overexpression of HAβ-tubulin. These conditions should select the total stably transfected cell population. A final group of cells received medium containing paclitaxel but no tetracycline. The expectation was that removal of tetracycline would allow expression of the transfected HAβ-tubulin, while inclusion of paclitaxel would determine whether the HAβ-tubulin could confer paclitaxel resistance. The results of this experiment are summarized in Table 1. All the transfections produced significant numbers of cells able to survive in G418, indicating that all the transfections were successful; only the population transfected with *HAβI(L228F)* contained cells that were able to survive in paclitaxel.

The tentative conclusion from this experiment was that only the mutant *HAβI(L228F)* cDNA had the ability to confer paclitaxel resistance, but it was possible that the transfections with *HAβI*, *HAβII*, and *HAβIVb* produced few cells expressing those cDNAs and this might explain our inability to select paclitaxel resistant cells. To rule out this possibility, the G418 selected cells from each transfection were examined by immunofluorescence with an antibody to the HA tag. Because G418 selects for cells that have stably integrated the transfected plasmid, we anticipated that a large fraction of these cells would express HAβ-tubulin. Indeed, it was found that approximately half of the cells in each population were positive for HAβ-tubulin expression (Fig. 2A-D). This result was similar to a variety of other transfections we have previously

Table 1. Selection of transfected cells for resistance to G418 and to paclitaxel

	HAβI	*HAβII*	*HAβIVb*	*HAβI(L228F)*
αMEM + tet	62400 (1)	70200 (1)	63700 (1)	81400 (1)
G418 + tet	242 (3.9×10^{-3})	298 (4.2×10^{-3})	240 (3.8×10^{-3})	80 (1×10^{-3})
PTX	0 ($<1.6\times10^{-5}$)	0 ($<1.4\times10^{-5}$)	0 ($<1.6\times10^{-5}$)	9 (1×10^{-4})

Cells transfected with various HAβ-tubulin cDNAs were seeded onto tissue culture dishes containing no selective drug (αMEM + 1 μg/ml tetracycline), 2 mg/ml G418 + 1 μg/ml tetracycline, or 0.2 μg/ml paclitaxel (PTX). For each selection, the number of surviving colonies is shown along with the cloning efficiency in parentheses. The cloning efficiency in αMEM + tetracycline was arbitrarily set at 1 for each transfected cell line. Note that the number of colonies in αMEM + tetracycline was multiplied by 100 because 100-fold fewer cells were seeded compared to the other selections.

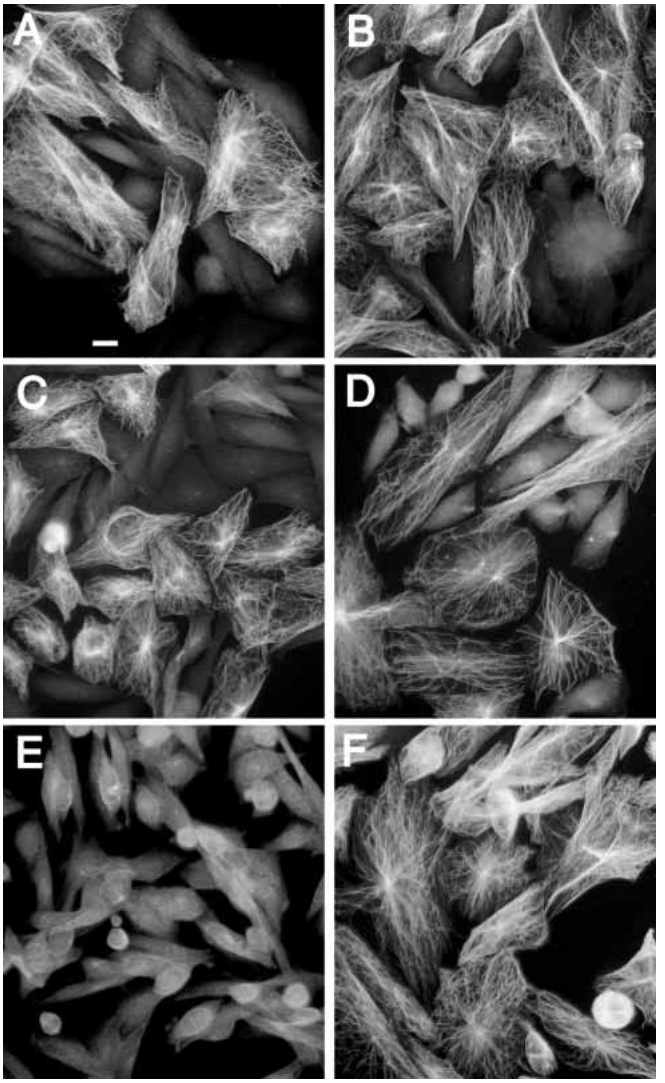

Fig. 2. Immunofluorescence of transfected cells selected in G418 or paclitaxel (PTX). Cells transfected with *HAβI* (A), *HAβII* (B), *HAβIVb* (C), or *HAβI(L228F)* (D-F) were selected for resistance to 2 mg/ml G418 + tetracycline (A-D) or to 0.2 μg/ml PTX (no tetracycline) (E,F). The resistant cells were seeded onto glass coverslips, fixed, and stained with an antibody specific for the HA tag. Cells in A-D were induced for 20 hours in medium without tetracycline before fixation. Cells in E and F were selected in PTX without tetracycline and either maintained in tetracycline-free medium (F), or incubated in medium with 1 μg/ml tetracycline for 3 days (E) prior to fixation. Note that the *HAβI(L228F)* transfected cells selected in PTX were all positive for $HA\beta_{I(L228F)}$-tubulin production (F) but the same transfected cells selected in G418 were only 50% positive (D). Also note that tetracycline effectively repressed the production of the $HA\beta_{I(L228F)}$-tubulin (compare E with F). Bar, 10 μm.

carried out (Barlow et al., 1994; Gonzalez-Garay and Cabral, 1995, 1996). The percentage of positive cells is probably not higher because in some cases, the HAβ-tubulin sequence may become disrupted when the plasmid integrates into the genome, or the plasmid may integrate into a transcriptionally silent region of the cellular DNA. Because all of the transfections had similar frequencies of G418 resistant cells (Table 1) and similar proportions of HA positive cells (Fig. 2), failure to express the transfected HAβ-tubulin cDNAs cannot explain the inability of *HAβI*, *HAβII*, and *HAβIVb* to confer resistance to paclitaxel. In fact, of the 4 transfections, the fraction of positive cells and the intensity of expression appeared to be lowest for *HAβI(L228F)*, yet it was the only one that gave paclitaxel resistant colonies.

It might also be argued that the isolation of paclitaxel resistant colonies from the *HAβI(L228F)* transfected cell population was a chance occurrence that had nothing to do with *HAβI(L228F)* expression. If true, it would be expected that the incidence of HA positive cells in the paclitaxel resistant population should be no higher than the incidence of G418 resistant cells in an unselected population (~1-4×10^{-3}, see Table 1). Fig. 2 clearly demonstrates that this is not the case. In fact HA positive cells made up essentially 100% of the paclitaxel selected population (Fig. 2F). This percentage is significantly higher than the 50% HA positive cells that were found in the G418 selected population (Fig. 2D), demonstrating that resistance must be conferred by expression of the mutant transfected cDNA. As expected, production of the HA-tagged tubulin could be repressed by tetracycline (Fig. 2E).

Frequency of paclitaxel resistance in a G418 selected cell population

Another possible explanation for the inability of overproduced $HA\beta_I$, $HA\beta_{II}$, and $HA\beta_{IVb}$-tubulin to confer paclitaxel resistance is that only certain levels of expression can confer resistance and cells with these levels of expression were not present among the relatively small number of cells used in the selection. To get around this problem, the selections in paclitaxel were repeated using G418 selected cells. In contrast to the total transfected populations in which the frequency of HAβ-tubulin expressing cells was only 1-4×10^{-3}, G418 resistant cell populations were approximately 50% positive for cells expressing the transfected genes; and, as is commonly seen in transfection experiments, we could observe a spectrum of cells expressing low to high levels of the HAβ-tubulin. Results of paclitaxel resistant selections from these G418 resistant cells are summarized in Table 2. Again, only the *HAβI(L228F)* transfected cells produced paclitaxel resistant colonies.

Table 2. Paclitaxel resistant cells in a G418 selected population

	HAβI	*HAβII*	*HAβIVb*	*HAβI(L228F)*
αMEM	190	195	197	147
PTX	0	0	0	44

Cells transfected with the various HAβ-tubulin cDNAs were first selected in G418 + tetracycline and were then replated under non-selective conditions (αMEM) or in 0.2 μg/ml paclitaxel (PTX). The number of colonies that survived under each set of conditions is shown.

This same experiment was repeated again with a 25-fold greater number of cells to ensure that any resistant cells would be detected. The results shown in Fig. 3 demonstrate that the *HAβI(L228F)* transfected cells quickly formed a confluent monolayer in paclitaxel but the other transfected populations produced no resistant colonies even though they were allowed to grow for an additional week. We conclude that paclitaxel resistance does not occur at an appreciable frequency among cells with increased production of $HA\beta_I$, $HA\beta_{II}$, or $HA\beta_{IVb}$-tubulin.

HAβ-tubulin production in G418 selected cells

Although it was possible to use immunofluorescence to get a visual impression for the relative levels of HAβ-tubulin production among different cells in a population, it was conceivable that the *HAβI*, *HAβII* and *HAβIVb* transfected cells actually produced much less transfected tubulin than the *HAβI(L228F)* transfected cells and that this might explain their failure to survive in paclitaxel. To test this possibility, the G418 selected cell populations used for the paclitaxel resistance selections were resolved on SDS gels and the relative amounts of endogenous and transfected β-tubulins were compared on western blots. The presence of the HA tag slightly retards the mobility of the HAβ-tubulin and allows it to resolve from the endogenous β-tubulin in the cell. Thus, an antibody to β-tubulin can be used to simultaneously detect both endogenous and transfected tubulin in a cell extract. Fig. 4 shows that among the G418 selected cells, those transfected with *HAβIVb* had the highest, and those transfected with *HAβI(L228F)* had the lowest production of transfected tubulin; yet $HA\beta_{I(L228F)}$-tubulin was the only one capable of producing paclitaxel resistance. Furthermore, the level of $HA\beta_{I(L228F)}$-tubulin was considerably higher in the paclitaxel selected population (right 2 lanes, Fig. 4B). This increase over the G418 selected cells (left 2 lanes, Fig. 4B) strongly argues that production of the mutant tubulin confers paclitaxel resistance. The increase is due in part to the fact that a higher percentage of the paclitaxel selected cells are positive for $HA\beta_{I(L228F)}$-tubulin expression (Fig. 2F) and in part to the fact that cells with very low levels

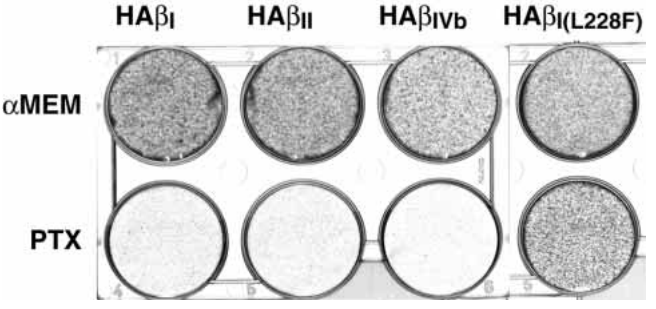

Fig. 3. Selection of paclitaxel resistant cells from populations previously selected in G418. Cells transfected with the indicated HAβ-tubulin cDNAs were selected in 2 mg/ml G418, and 4-5×10^3 of the antibiotic resistant cells were then seeded onto 6-well dishes containing αMEM or 0.2 μg/ml paclitaxel (PTX). Cells were allowed to grow 4 days for the positive wells, or 11 days for the negative wells, and were then stained with methylene blue. Note that cells transfected with *HAβI(L228F)* quickly grew to confluence in PTX, but that cells transfected with *HAβI*, *HAβII*, or *HAβIVb* formed no resistant colonies in PTX even though they were incubated an additional week.

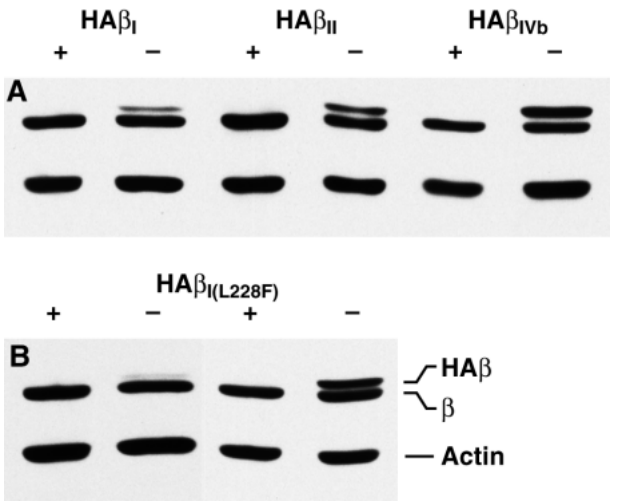

Fig. 4. Production of HAβ-tubulin in transfected cells. Cells transfected with the indicated HAβ-tubulin cDNAs and selected in G418 were run on SDS polyacrylamide gels, electroblotted onto nitrocellulose, and probed with antibodies to β-tubulin and actin. Lanes marked with a '+' represent cells grown continuously in 1 μg/ml tetracycline and lanes marked with a '−' represent cells induced overnight (20 hours) without tetracycline. The relative positions of transfected HAβ-tubulin (HAβ), endogenous β-tubulin (β), and actin are indicated in B. Also in B, the left 2 lanes represent cells transfected with *HAβ$_{I(L228F)}$* and selected in G418 while the right 2 lanes represent the same transfected cells selected in 0.2 μg/ml paclitaxel. The left 2 lanes were exposed longer to reveal the minor production of the transfected HAβ$_{I(L228F)}$-tubulin in the G418 selected cell population. Note that the transfected tubulin is dramatically increased in the cells selected in paclitaxel.

of expression are not resistant to paclitaxel and are therefore lost from the selection.

Modulation of paclitaxel resistance with tetracycline

The western blot in Fig. 4 demonstrates that HAβ-tubulin production in the transfected cells is regulated by the presence or absence of tetracycline. To address the possibility that expression of *HAβ$_I$*, *HAβ$_{II}$*, and *HAβ$_{IVb}$* might confer resistance to concentrations of paclitaxel that are lower than those that were used in the preceding selections, a dose response to paclitaxel was carried out in the presence or absence of paclitaxel for each of the G418 selected cell populations and the results are shown in Fig. 5. The cells transfected with *HAβ$_I$*, *HAβ$_{II}$*, and *HAβ$_{IVb}$* had a sensitivity to paclitaxel that was identical to non-transfected cells (data not shown), and exhibited no difference in their dose response to paclitaxel regardless of whether or not tetracycline was present to inhibit expression of the transfected cDNA. However, the cells transfected with *HAβ$_{I(L228F)}$* were clearly more resistant to paclitaxel when expression of the transfected tubulin was induced (tetracycline absent) compared to when it was repressed (tetracycline present). Although the increased survival of the *HAβ$_{I(L228F)}$* transfected cells at higher paclitaxel concentrations appears modest, it should be remembered that only half of the G418 selected population is positive for mutant tubulin expression (Fig. 2D) and that many other cells in the population express very low levels of the mutant protein (Fig. 4B, left 2 lanes). Consistent with this interpretation, it is common to isolate subclones from the population that have higher HAβ$_{I(L228F)}$-tubulin production and much more robust paclitaxel resistance (data not shown). These data provide further support for the conclusion that overproduction of normally expressed (β$_I$, β$_{IVb}$) or normally nonexpressed (β$_{II}$) isotypes of β-tubulin is insufficient to alter the sensitivity of CHO cells to paclitaxel.

DISCUSSION

Recent publications from several laboratories have implicated increased expression of specific β-tubulin isotypes as a potential mechanism for resistance to anitmitotic drugs. These include increased expression of the following β-tubulin isotypes in paclitaxel resistant cells: β$_{II}$ (Haber et al., 1995); β$_{IVa}$ (Jaffrezou et al., 1995); β$_I$, β$_{II}$, β$_{III}$, and β$_{IVa}$ (Kavallaris et al., 1997). Another publication has reported increased synthesis of β$_{III}$- and β$_{IVa}$-tubulin in estramustine resistant mutants (Ranganathan et al., 1996). The idea that the isotype composition of a cell might influence its response to chemotherapeutic drugs is an attractive one because it would open the possibility of exploiting natural differences in isotype composition among various cell types to target specific drugs to specific tumors. Additionally, it might also provide a means for circumventing the development of drug resistance in human tumors. There is some in vitro support for such a mechanism. Studies using isotype-selected subfractions of brain β-tubulin have demonstrated differences in microtubule assembly and drug binding (reviewed by Luduena, 1998). More recent studies have indicated that paclitaxel is 7 times less potent in its ability to suppress the dynamics of β$_{III}$ and β$_{IVa}$ compared to unfractionated β-tubulin (Derry et al., 1997) and that estramustine has a reduced affinity for β$_{III}$-tubulin (Laing et al., 1997).

Although there is much circumstantial evidence for this mechanism of resistance, there is no direct proof that altering the isotype composition of a cell affects its sensitivity to any antimitotic drug. Furthermore, virtually all major isotypes including β$_I$, β$_{II}$, β$_{III}$, and β$_{IVa}$ have been reported to be increased in various drug resistant mutants, and this runs counter to the idea that specific isoforms of β-tubulin confer altered sensitivity to these drugs. Finally, one reported study found alterations in expression of specific β-tubulin isotypes but the changes did not correlate with drug resistance (Dumontet et al., 1996).

In an attempt to resolve these conflicting reports and ideas, we have directly manipulated the synthesis of β-tubulin isotypes in a well characterized cell line and evaluated whether by doing so, we could affect the sensitivity of the cells to paclitaxel. We were fortunate in this endeavor to have available a mutant tubulin cDNA (*HAβ$_{I(L228F)}$*) that could act as a positive control for paclitaxel resistance. The non-mutated tubulin genes selected for this study included a CHO β$_I$-tubulin cDNA that we had previously isolated (Boggs and Cabral, 1987; Boggs et al., 1996). Since CHO cells already express β$_I$-tubulin as 70% of the total (Sawada and Cabral, 1989), it was anticipated that its overexpression would cause minimal perturbation and act as a negative control. In fact, previous transfections with this cDNA did not affect the sensitivity of the cells to antimitotic drugs (Gonzalez-Garay and Cabral,

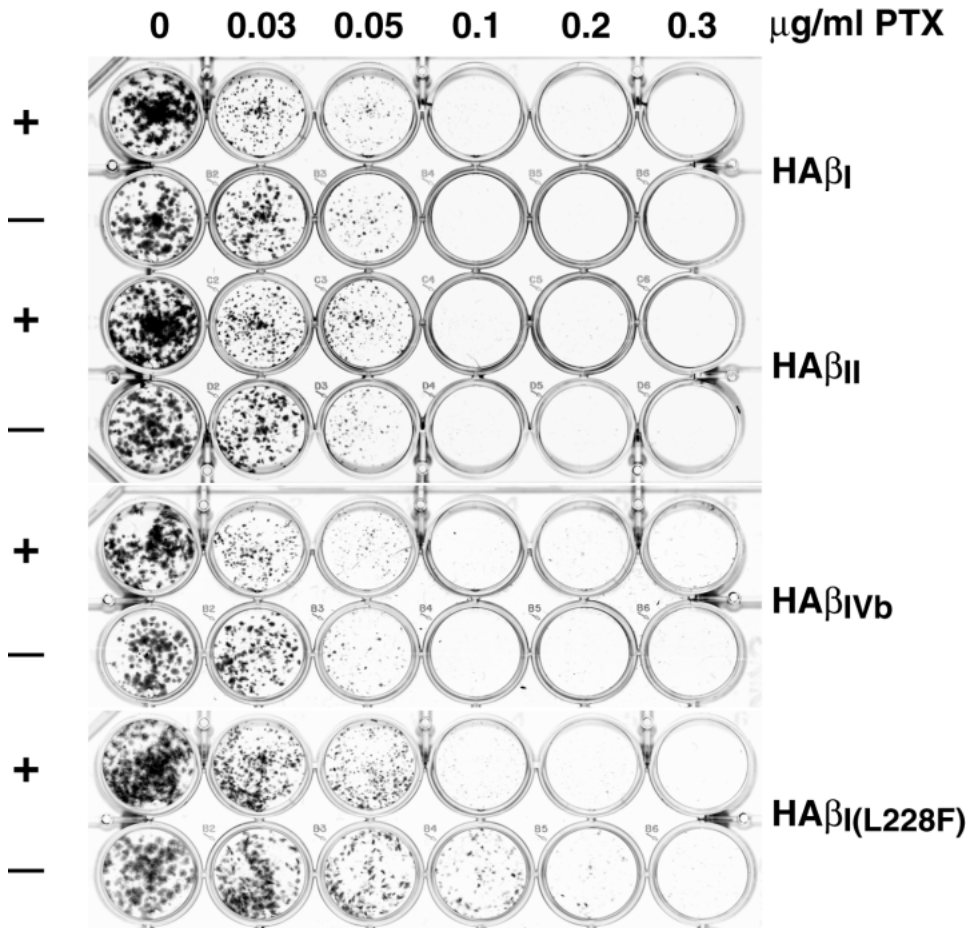

Fig. 5. Dose response of transfected cells to paclitaxel. Cells transfected with each of the indicated HAβ-tubulin cDNAs were selected for resistance to G418. Approximately 100-200 cells of each G418-resistant cell population were then seeded into replicate wells of a 24-well dish containing increasing concentrations of paclitaxel (PTX) in growth medium with (+) or without (–) 1 μg/ml tetracycline. After 1 week the cells were stained with methylene blue. Note that cells producing transfected $HA\beta_{I(L228F)}$-tubulin had increased resistance to PTX ('–' wells) compared to cells in which $HA\beta_{I(L228F)}$-tubulin was repressed ('+' wells) but that production of $HA\beta_{I}$, $HA\beta_{II}$, or $HA\beta_{IVb}$ had no effect on drug resistance.

1995). We also successfully overexpressed β_{IVb}-tubulin which normally comprises 25% of the total β-tubulin in CHO cells (Sawada and Cabral, 1989). Although the cDNA we used came from mouse, the encoded protein sequence is identical to the hamster sequence (Ahmad et al., 1991; Wang et al., 1986). In this case, overexpression allowed us to ask whether increasing production of a minor β-tubulin isotype might affect sensitivity to paclitaxel. Finally a mouse cDNA for β_{II}-tubulin was chosen for transfection in order to ask whether expression of a β-tubulin isoform that is not normally present might influence drug resistance. Increased expression of the β_{II}-tubulin isoform has previously been reported in J.774.2 mouse macrophage cells selected for paclitaxel resistance (Haber et al., 1995). Here we wanted to ask whether the simple overexpression of this isotype is sufficient to cause resistance to the drug.

One of the interesting observations that came from these studies was that tubulin from all of the transfected cDNAs was able to assemble into microtubules. This was not surprising for the β_I and β_{IVb} isotypes because they are normal constituents of CHO cells. However, it wasn't at all clear when we began these studies whether β_{II}-tubulin would be able to assemble because it is not normally expressed in these cells. Because CHO cells express β_I, β_{IVb}, and β_V isotypes at ratios of 70:25:5 (Ahmad et al., 1991; Sawada and Cabral, 1989) and express 3 α-tubulin isotypes at essentially identical ratios (Elliott et al., 1985, 1986), a case could be made for the necessity of specific αβ tubulin interactions in heterodimer formation. If true, this might have prevented β_{II}-tubulin from finding an appropriate α-tubulin partner and would have prevented its assembly into microtubules. Instead, we found that it was able to assemble, and this suggests that αβ interactions may be promiscuous.

Production of tubulin from transfected cDNA might also be expected to influence the synthesis and accumulation of endogenous tubulin protein. In previous studies, we have demonstrated that overexpression of $HA\beta_I$-tubulin produces only minor changes in endogenous β-tubulin synthesis (Gonzalez-Garay and Cabral, 1995). However, the transfected tubulin can successfully compete with endogenous tubulin for αβ heterodimer formation and any β-tubulin (transfected or endogenous) in excess of the available α-tubulin is degraded (Gonzalez-Garay and Cabral, 1995). As a result, the amount of endogenous β-tubulin at steady state decreases as the amount of transfected β-tubulin increases. More recently, we have found that the decrease in endogenous β-tubulin is not isotype specific (F. Cabral, unpublished studies) and this is consistent with the observation presented here that transfected $HA\beta_{II}$-tubulin is able to assemble into microtubules even though it is not normally expressed and would not be expected to have a specific α-tubulin partner available for assembly. Based on these observations, it is expected that the isotype composition of a cell changes as a new or pre-existing isotype is overexpressed. Despite these changes in the levels of endogenous and transfected β-tubulin isotypes, the experiments described here and previously (Gonzalez-Garay

and Cabral, 1995) indicate that the paclitaxel sensitivity of the cells remains constant.

Thus, the main question addressed in our studies, namely whether altering tubulin isotype expression in CHO cells affects sensitivity to paclitaxel, was answered. In contrast to *HAβ$_{I(L228F)}$*, transfecting cells with *HAβ$_I$*, *HAβ$_{II}$*, or *HAβ$_{IVb}$* did not allow the direct selection of paclitaxel resistant cells. Moreover, first selecting a G418 resistant population to enrich for cells that express the transfected β-tubulin, and then repeating the selection for paclitaxel resistant cells, failed to produce any viable colonies. In addition, G418 selected cells have the same sensitivity to paclitaxel in the presence of tetracycline (no transfected β-tubulin production) as they do in its absence (significant production of transfected β-tubulin). The failure of transfected β-tubulin to produce paclitaxel resistance could not be due to experimental design since transfection of HAβ$_I$-tubulin containing a known paclitaxel resistance mutation was able to confer resistance. Control experiments also ruled out the possibility that failure of HAβ$_I$, HAβ$_{II}$, and HAβ$_{IVb}$-tubulin to confer resistance could have been due to failure to express, assemble, or accumulate. Finally, the presence of the HA tag is unlikely to interfere with the ability of the transfected isotypes to confer resistance because: (1) HAβ$_I$-tubulin containing a mutation found in a paclitaxel resistant cell line was able to confer resistance when transfected into wild-type cells, and (2) a G418 selected cell population stably expressing β$_{II}$-tubulin that lacked the epitope tag still did not produce paclitaxel resistant cells even though the transfected tubulin was abundantly expressed and efficiently assembled into microtubules (data not shown). We therefore conclude that the simple overexpression of these β-tubulin isotypes is insufficient to confer resistance to paclitaxel.

How then might one explain the studies demonstrating increases in β-tubulin isotypes in paclitaxel selected cell lines? A number of possibilities exist. One is that the cell lines used for selection may naturally have considerable variation of tubulin expression on a cell-to-cell basis, and that selection of particular clones with altered expression is fortuitous and has nothing to do with the resistance phenotype. This problem is especially acute because the mutants were selected in multiple steps and could be harboring alterations in addition to the altered tubulin expression. It could be these other alterations that are actually responsible for the paclitaxel resistance. A second possibility is that the altered expression of specific β-tubulin isotypes is associated with resistance, but only because they contain mutations that have yet to be discovered. According to this scenario, a cell with a mutation in a minor isotype might not be paclitaxel resistant because that isotype is not present in sufficient quantity to impact the large mass of microtubules that exist in the cell. However, any cell able to increase its production of that mutant minor isotype might well be able to survive selection. A third possibility is suggested by a recent report that treatment of DU-145 cells with paclitaxel causes an acute and specific increase in expression of β$_{III}$-tubulin (Ranganathan et al., 1998). Although currently unconfirmed in other systems, this observation suggests the possibility that altered expression of β-tubulin isotypes is a response to drug treatment rather than drug resistance selection.

Whatever the ultimate explanation for the existence of altered tubulin expression in paclitaxel selected cells, our studies clearly demonstrate that overexpression of β$_I$, β$_{II}$, or β$_{IVb}$-tubulin isotypes is insufficient to confer resistance to the drug. However, they do not rule out the possibility that overexpression is coupled with some other change (e.g. a mutation) that does cause paclitaxel resistance. Also, our studies do not rule out the possibility that overexpression of β$_{III}$ or β$_{IVa}$-tubulin might confer resistance.

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