A highly epothilone B–resistant A549 cell line with mutations in tubulin that confer drug dependence

Chia-Ping Huang Yang,[1] Pascal Verdier-Pinard,[1] Fang Wang,[1] Eva Lippaine-Horvath,[1] Lifeng He,[1] Dansu Li,[2] Gerhard Höfle,[3] Iwao Ojima,[2] George A. Orr,[1] and Susan Band Horwitz[1]

[1]Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York; [2]Department of Chemistry, State University of New York at Stony Brook, Stony Brook, New York; and [3]Department of Natural Product Chemistry, Geselleschaft fur Biotechnologische Forschung, Braunschweig, Germany

Abstract

A 95-fold epothilone B (EpoB)–resistant, but not dependent, A549 human lung carcinoma cell line, A549.EpoB40 (EpoB40), has a Gln to Glu mutation at residue 292 that is situated near the M-loop of βI-tubulin. Further selection of this cell line with higher concentrations of EpoB produced A549.EpoB480 (EpoB480), which is ~900-fold resistant to EpoB. This cell line, like EpoB40, exhibits cross-resistance to Taxol and extreme sensitivity to vinblastine, but in contrast to EpoB40 it is unusually dependent on EpoB, requiring a minimum of 125 nmol/L EpoB to maintain normal growth. Sequence analysis of the β-tubulin and Kα1-tubulin genes in EpoB480 showed that, in addition to the β292 mutation, β60 was mutated from Val to Phe and α195 was mutated from Leu to Met. Mass spectrometry indicated that both the Val^{60}Phe and Leu^{195}Met mutations in βI- and Kα1-tubulin, respectively, were expressed at the protein level. Molecular modeling indicated that β60 is located at the end of the H1-S2 loop that has been implicated as a principal partner of the M-loop for contacts between protofilaments. A mutation at β60 could inhibit the lateral contacts between protofilaments, thereby destabilizing microtubules. α195 is located at the external surface of the microtubule that has been proposed as the domain that interacts with a variety of endogenous proteins, such as stathmin and microtubule-associated protein 4. A mutation at α195 could modulate the interactions between tubulin and regulatory proteins. We propose that the βVal^{60}Phe mutation plays a critical role in the drug-dependent phenotype of EpoB480 cells. [Mol Cancer Ther 2005; 4(6):987–95]

Introduction

The tubulin/microtubule system is a validated target for antitumor drugs. One such drug, Taxol, has been approved by the Food and Drug Administration for the treatment of ovarian, breast, and non–small cell lung carcinomas. The drug has a specific binding site on the microtubule (1, 2), and incubation of cells with Taxol results in the formation of stable bundles of microtubules (3). Low concentrations of Taxol disrupt the normal polymerization/depolymerization cycle of microtubules and suppress microtubule dynamics (4). Treatment of cells with Taxol induces aberrant mitosis at low concentrations of drug and mitotic arrest at higher concentrations of drug (5).

A new class of microtubule-stabilizing agents, the epothilones, has been identified (6). The epothilones were originally isolated from a Myxobacterium fermentation broth (7) and are currently undergoing phase II clinical trials as anticancer agents (8, 9). Like Taxol, the drug induces tubulin polymerization in the absence of GTP and causes microtubule stabilization and bundling (6, 10). Although a common pharmacophore shared by taxanes and epothilones was proposed (11, 12), recent electron crystallography studies have shown that tubulin has an indiscriminate binding pocket into which Taxol and epothilone A make unique contacts (13). Epothilone is not a substrate for the multidrug transporter P-glycoprotein, the *MDR1* gene product, and is active in Taxol-resistant cell lines and tumors that express P-glycoprotein (6, 8).

In addition to induction of P-glycoprotein, other potential mechanisms of Taxol resistance, at the level of the microtubule, have been proposed. These include altered expression of β-tubulin isotypes, point mutations in tubulin leading to alterations in microtubule dynamics, and altered expression/posttranslational modifications of tubulin regulatory proteins, such as stathmin and microtubule-associated protein 4 (MAP4; ref. 14). The Taxol dependence phenotype has also been described as cancer cells develop resistance (15–18), although the mechanism underlying Taxol dependence is not understood.

Sequence analysis of class βI-tubulin from an epothilone-resistant cell line, EpoB40, revealed a single point mutation at β292 (Gln to Glu) that is near the M-loop (19). This resistant cell line, derived from a human lung carcinoma A549 cell line by stepwise selection with EpoB,

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Requests for reprints: Susan B. Horwitz, Department of Molecular Pharmacology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461. Phone: 718-430-2163; Fax: 718-430-8959. E-mail: shorwitz@aecom.yu.edu

is maintained in 40 nmol/L EpoB and is 95-fold resistant to EpoB and 22-fold cross-resistant to Taxol. Isoelectric focusing studies showed that the mutant βI-tubulin, but not the wild type, was predominantly expressed in this cell line (20). In the present study, further selection of EpoB40 cells with higher concentrations of EpoB resulted in the cell line A549.EpoB480 (EpoB480), which is maintained in 480 nmol/L EpoB and is highly dependent on the drug for normal cell proliferation. It is proposed that a critical mutation in βI-tubulin plays a role in Taxol/EpoB dependence.

Materials and Methods

Materials and Cells

Epothilone B and Taxol were obtained as described (19). 21-[^{3}H]21-OH-EpoB was prepared by the reduction of 20-formyl-EpoB with NaB[^{3}H]$_4$. 20-Formyl-EpoB was derived from EpoB in three steps via N-oxidation with *m*-chloroperbenzoic acid (forming N-oxide), rearrangement with acetic anhydride ammonia in methanol (giving 21-OH-EpoB), and Swern oxidation. [^{3}H]Taxol was obtained from the National Cancer Institute (Bethesda, MD). Monoclonal anti–α-tubulin antibody was purchased from Sigma (St. Louis, MO) and polyclonal antistathmin antibody was from Calbiochem (La Jolla, CA). Anti-MAP4 antibody was kindly provided by Dr. Jeannette C. Bulinski (Columbia University, New York, NY). A549, a human non–small cell lung cancer line, was grown in RPMI 1640 containing 10% fetal bovine serum. EpoB480 was developed by stepwise selection of a 95-fold EpoB-resistant cell line EpoB40 (19) and was maintained in 480 nmol/L EpoB.

Cytotoxicity Assay

A methylene blue–based cytotoxicity assay was done as previously described (19) to determine drug resistance profiles of EpoB480.

[^{3}H]Taxol and [^{3}H]21-OH-EpoB Accumulation Studies

Cells (5×10^5) were plated in 35-mm culture dishes and maintained in drug-free medium for 3 to 4 hours. The medium was removed and fresh medium containing 5 μmol/L [^{3}H]Taxol (specific activity: 40 mCi/mmol) or 5 μmol/L [^{3}H]21-OH-EpoB (specific activity: 10 mCi/mmol) was added. Following incubation at 37°C for 1 hour, the cells were washed with PBS and lysed in 1 N NaOH at room temperature for 16 hours. Total protein was measured and total radioactivity was determined by liquid scintillation counting.

Sequencing of βI- and Kα1-Tubulin

Total RNA was prepared from cells as described (18) and reverse transcribed to cDNA. Human βI- and Kα1-tubulin were amplified by reverse transcription-PCR and each sequenced using four overlapping sets of primers (18). Results obtained with EpoB480 cells were compared with the sequence from drug-sensitive A549 and from EpoB40 cells.

Separation of Tubulin Isotypes by Isoelectric Focusing

Microtubule pellets prepared from A549, EpoB40, and EpoB480 cells were solubilized and loaded onto immobilized pH gradient strips at pH 4.5 to 5.5 and run on an IPGphor isoelectric focusing system. Immobilized pH gradient strips were then fixed, stained with Coomassie blue, and destained as described (20).

Analysis of Protein βI- and Kα1-Tubulin Mutations by Mass Spectrometry

Tubulin was isolated from A549 and EpoB480 cells and the masses of the tubulin isoforms were determined by liquid chromatography-mass spectrometry analysis as described (21).

Measurement of Polymerized Tubulin

Soluble and polymerized tubulin were separated as described (22). Briefly, 5×10^5 cells were suspended in 150 μL hypotonic buffer (0.5% NP40, 0.1 mol/L MES, 1 mmol/L EGTA, and 0.5 mmol/L $MgCl_2$) and incubated at 37°C for 5 minutes in the presence or absence of 10 μmol/L EpoB. Polymerized tubulin was collected by centrifugation at 16,000 × *g* for 10 minutes at room temperature. The pellet was solubilized in 200 μL SDS-PAGE sample buffer. Fifty microliters of 4× sample buffer were added to the supernatant that contained soluble tubulin. Proteins from both fractions were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted using monoclonal anti–α-tubulin antibody. The levels of tubulin in both fractions were quantitated by image analysis using Molecular Dynamics ImageQuant Software Version 3.3.

Stathmin and MAP4 Protein Analysis

For determination of stathmin levels, cell lysates were prepared as described (18). The samples were subjected to 15% SDS-PAGE, followed by Western blot analysis using a polyclonal antistathmin antibody. For MAP4 analysis, cells were lysed in a buffer containing 10 mmol/L Tris-HCl (pH 7.5) and 1% SDS. The samples were boiled and analyzed by 5% urea-PAGE. Western blot analysis was done using a polyclonal antibody to MAP4.

Modeling of the Tubulin Mutation

Molecular modeling studies were done using the DeepView/Swiss-pdbViewer v3.7 software. The structure of α- and β-tubulin that interact with one molecule of epothilone A was taken from Nettles et al. (13).

Results

EpoB480 Cells Are Unusually Dependent on EpoB for Normal Growth

As reported previously, EpoB40 cells are 95-fold resistant to EpoB (19). EpoB480 cells, which were derived from EpoB40, are ~900-fold resistant to EpoB and show substantial cross-resistance to Taxol. These cells require ~125 to 250 nmol/L EpoB to maintain normal growth, in contrast to EpoB40 cells that are not EpoB dependent (Fig. 1A). EpoB480 cells can also be maintained in a minimum of 125 nmol/L Taxol (data not shown), indicating that Taxol and EpoB are equally effective in maintaining normal cell growth for this EpoB-dependent cell line. Although both EpoB40 and EpoB480 cells are more resistant to EpoB than to Taxol, the ratio of their IC_{50}s for both drugs is approximately the same (Table 1).

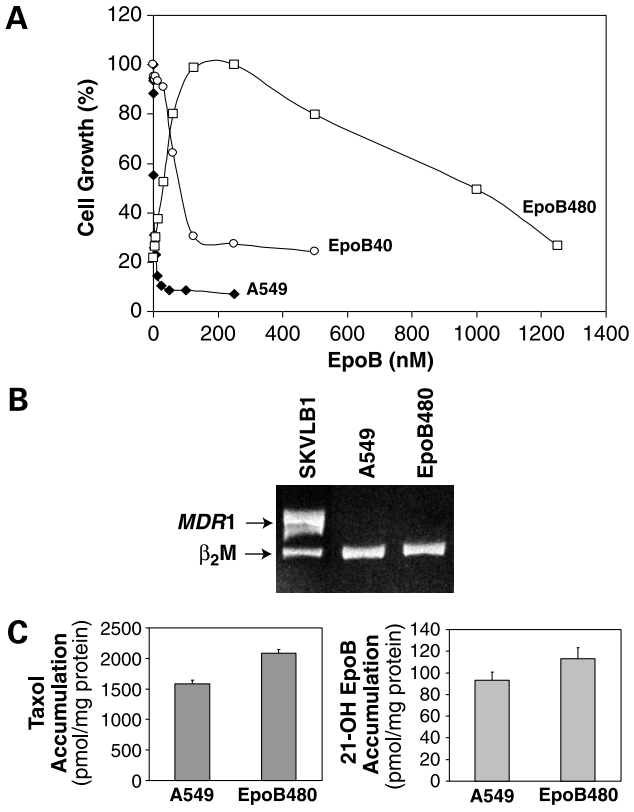

Figure 1. EpoB480 cells are dependent on EpoB for normal growth and do not express *MDR1* or other efflux pumps. **A,** cytotoxicity assays for A549, EpoB40, and EpoB480 cells were done using a methylene blue–based method as previously described (19). EpoB480 cells require ~125 to 250 nmol/L EpoB for normal growth. Error bars were omitted for clarity. SD did not exceed 5% of the mean value. **B,** reverse transcription-PCR determinations of *MDR1* gene expression were done on SKVLB1 (a highly resistant MDR cell line), drug-sensitive A549, and highly resistant EpoB480 cells. Competitive reverse transcription-PCR, involving coamplification of *MDR1* (167 bp) and control β_2-microglobulin (120 bp) gene sequences, was done for 35 cycles, and the products were separated on an 11.5% polyacrylamide gel followed by ethidium bromide staining as previously described (43). **C,** steady-state accumulation of Taxol or 21-OH-EpoB was measured as described in Materials and Methods. *Columns,* picomoles drug per milligram protein; *bars,* SD (n = 3).

Both EpoB40 and EpoB480 cell lines were unusually sensitive to vinblastine (Table 1). Although the IC_{50} value for vinblastine is slightly higher in EpoB480 than in EpoB40 cells, it should be noted that the value for EpoB480 was determined in the presence of 125 nmol/L EpoB, because EpoB480 cells are EpoB dependent.

An Efflux Pump for Epo/Taxol Is Not Present in EpoB480 Cells

It is known that epothilone does not induce P-glycoprotein nor does it serve as a substrate for P-glycoprotein (6, 8, 16). To be certain that P-glycoprotein or an unknown efflux pump was not present in the highly EpoB resistant cell line, EpoB480, competitive reverse transcription-PCR was done and it was found that *MDR1* was not expressed in this cell line (Fig. 1B). Also, steady-state drug accumulation of Taxol or 21-OH-EpoB, an analogue of EpoB that is capable of polymerizing microtubules in the absence of GTP, was not reduced in EpoB480 cells, compared with the sensitive cells (Fig. 1C). This suggests that an efflux pump for Taxol or EpoB is not present in this highly resistant cell line.

EpoB480 Cells Harbor an α-Tubulin Mutation and Two β-Tubulin Mutations

It is known that βI-tubulin in EpoB40 cells contains a Gln to Glu mutation at residue 292 (19). No α-tubulin mutations were detected in this cell line. The βI- and Kα1-tubulin genes of EpoB480 were sequenced; in addition to the mutation at β292, two new tubulin mutations were found: a heterozygous point mutation in the βI-tubulin sequence at nucleotide 178 from G to T that corresponds to a mutation from Val to Phe at residue β60 and a heterozygous point mutation in the Kα1-tubulin sequence at nucleotide 583 from C to A that corresponds to a mutation from Leu to Met at residue α195. Val^{60}, in the primary amino acid sequence of βI-tubulin, is located at the end of the H1-S2 loop (23). Leu^{195}, in the primary amino acid sequence of α-tubulin, is located just beyond the COOH-terminal end of helix 5. Therefore, this cell line contains three tubulin mutations, one at β292, one at β60, and another at α195.

$Gln^{292}Glu$ and $Val^{60}Phe$ Mutations in βI-Tubulin and $Leu^{195}Met$ Mutation in Kα1-Tubulin Are Expressed in EpoB480 Cells at the Protein Level

Mass spectrometry and isoelectric focusing are being used extensively in our laboratory to determine the expression of mutant tubulin isoforms at the protein level (20, 21). Because there is only a 1 Da mass change from Gln to Glu, the mass difference between the wild-type and mutant βI-tubulin that harbors the $Gln^{292}Glu$ mutation could not be shown by mass spectrometry. Previous isoelectric focusing studies showed that βI-tubulin is expressed predominantly as the mutant form ($\beta Gln^{292}Glu$) in EpoB40 cells (20). To determine if the $Gln^{292}Glu$ mutation in βI-tubulin was expressed in EpoB480 cells, proteins from microtubule pellets prepared from A549, EpoB40, and EpoB480 cells were analyzed by isoelectric focusing (Fig. 2A). Coomassie-stained immobilized pH gradient strips revealed identical isotype patterns for tubulins from EpoB40 and EpoB480 cells, suggesting that as in EpoB40, βI-tubulin is expressed predominantly as the mutant form in EpoB480 cells. The Coomassie-stained band comigrating with the wild-type βI-tubulin (Fig. 2A) may represent a yet unidentified protein because previous studies utilizing Western blotting with an anti-βI antibody showed that no labeling occurred at the level of wild-type βI-tubulin, suggesting that only the mutant βI-tubulin was expressed in EpoB40 cells (20). Therefore, the mutant βI-tubulin band could contain the double mutant ($Gln^{292}Glu$ plus $Val^{60}Phe$) or a mixture of the double mutant and the single mutant ($Gln^{292}Glu$).

To determine if the $Val^{60}Phe$ mutation was expressed in EpoB480 cells at the protein level, tubulin was isolated and subjected to mass spectrometry (Fig. 2B). The calculated molecular mass for wild-type βI-tubulin is 49,670 Da and we obtained an experimental mass of 49,663 Da in A549

Table 1. Characterization of the EpoB480 cell line

	A549	EpoB40	EpoB480	IC_{50} ratio
	IC_{50}, nmol/L	IC_{50}, nmol/L (fold)	IC_{50}, nmol/L (fold)	EpoB480/EpoB40
EpoB	1.06 ± 0.11	101.03 ± 4.30 (95)	942.00 ± 47.78 (889)	9
Taxol	3.30 ± 0.52	71.88 ± 16.29 (22)	803.55 ± 13.88 (244)	11
VBL	3.09 ± 0.31	1.69 ± 0.63 (0.5)	1.91* ± 0.20 (0.6)	—

NOTE: IC_{50} values were determined after 72 hours of incubation with the indicated drugs and were expressed as mean ± SE (n = 3).
Abbreviation: VBL, vinblastine.
*IC_{50} determined in the presence of 125 nmol/L EpoB.

cells. A 5 to 12 Da difference in mass between calculated and experimental tubulin mass is typically observed using our protocol (21). In EpoB480 cells, two βI-tubulin forms are present: a minor one with a molecular mass of 49,665 and a major one of 49,711. The minor peak most likely represents the βI-tubulin with a $Gln^{292}Glu$ mutation based on the isoelectric focusing results (Fig. 2A). The major peak represents a form with an additional mass of 46 Da that is close to the expected mass difference of 47 Da between βI-tubulin with a $Gln^{292}Glu$ mutation and βI-tubulin with an additional $\beta Val^{60}Phe$ mutation. This result indicates that βI-tubulin containing a $Val^{60}Phe$ mutation is expressed in EpoB480 cells. More specifically, βI-tubulin with the $Gln^{292}Glu$ plus $Val^{60}Phe$ mutation and βI-tubulin with the $Gln^{292}Glu$ single mutation are both present in EpoB480 cells.

To determine if the $\alpha Leu^{195}Met$ mutation was expressed in EpoB480 cells at the protein level, Kα1-tubulin deconvoluted mass profiles from A549 and EpoB480 cells were compared (Fig. 2C). In this study, α6-tubulin was used as the internal control and its molecular mass is essentially identical in sensitive and resistant cells. The difference in the molecular mass of Kα1-tubulin between the wild type and the mutant cell line was 19 Da. This change in mass is consistent with the predicted mass difference between wild-type Kα1-tubulin and Kα1-tubulin harboring a $Leu^{195}Met$ mutation. These results indicate that Kα1 is expressed predominantly as the mutant form in EpoB480 cells. However, it is also possible that resolution of this ion trap mass spectrometry is not sufficient to resolve wild-type and mutant Kα1-tubulin.

Polymerized Tubulin Levels Are Altered in EpoB480 Cells

In both EpoB40 and EpoB480 cells that were maintained in 40 and 480 nmol/L EpoB, respectively, there was a significant increase (~3-fold) in the polymerized tubulin levels compared with the sensitive A549 cells (Fig. 3). Ten micromoles per liter of EpoB promoted tubulin polymerization in both sensitive and resistant cell lines, but the effect of 10 μmol/L EpoB on tubulin polymerization in the resistant cells was less prominent than that seen in the sensitive cells. When EpoB was removed from the medium for 18 hours before the assay, the polymerized tubulin levels were moderately reduced by ~25% in EpoB40 cells. However, they were reduced significantly, by ~10-fold, in EpoB480 cells. The polymerized tubulin levels in EpoB480 cells growing under drug-depleted conditions were even lower than those in the parental cells (3-fold decrease). Addition of 10 μmol/L EpoB promoted tubulin polymerization by ~8-fold, but tubulin polymerization levels were still ~30% lower than those in the parental cells (Fig. 3, *bottom*).

Stathmin Levels Are Reduced in EpoB480 Cells

Levels of polymerized tubulin can be modulated by endogenous proteins, such as stathmin and MAP4. The level of stathmin, a tubulin-interacting protein that sequesters tubulin dimers, thereby destabilizing microtubules (24–26), was determined in A549 cell lines (Fig. 4, *top*). It was found that the stathmin levels were slightly reduced in EpoB40 and markedly reduced (~80%) in EpoB480 cells compared with the sensitive cells. Removal of EpoB from the medium for 18 hours did not alter stathmin expression levels significantly.

The protein expression of MAP4 that is known to stabilize microtubules (27) was determined in A549 cell lines (Fig. 4, *bottom*) using 5% urea-PAGE. Nonphosphorylated MAP4 levels were slightly increased and phosphorylated MAP4 was increased by ~4-fold in EpoB40 cells compared with the parental cells. There was a moderate increase (~4-fold) in nonphosphorylated MAP4 levels in EpoB480 compared with A549 cells. In addition, it seemed that the phosphorylated MAP4 form was increased ~5- to 6-fold in EpoB480 cells when EpoB was removed from the growth medium compared with cells grown in the presence of 480 nmol/L EpoB.

Molecular Modeling of Mutations in an α-β–Tubulin Heterodimer

In EpoB480 cells, Gln^{292} is mutated to Glu and Val^{60} is mutated to Phe in βI-tubulin and Leu^{195} is mutated to Met in Kα1-tubulin. Molecular modeling studies revealed the locations of β292, β60, and α195 in an α-β–tubulin dimer (Fig. 5). β292 is near both Thr^{274}, a key amino acid residue in the drug-binding pocket (19), and the M-loop, which is essential for interactions between adjacent protofilaments (28). β60 is at the end of the H1-S2 loop that has been reported to be involved in lateral contacts between protofilaments (29). This model also reveals that α195 is at the external surface of the microtubule.

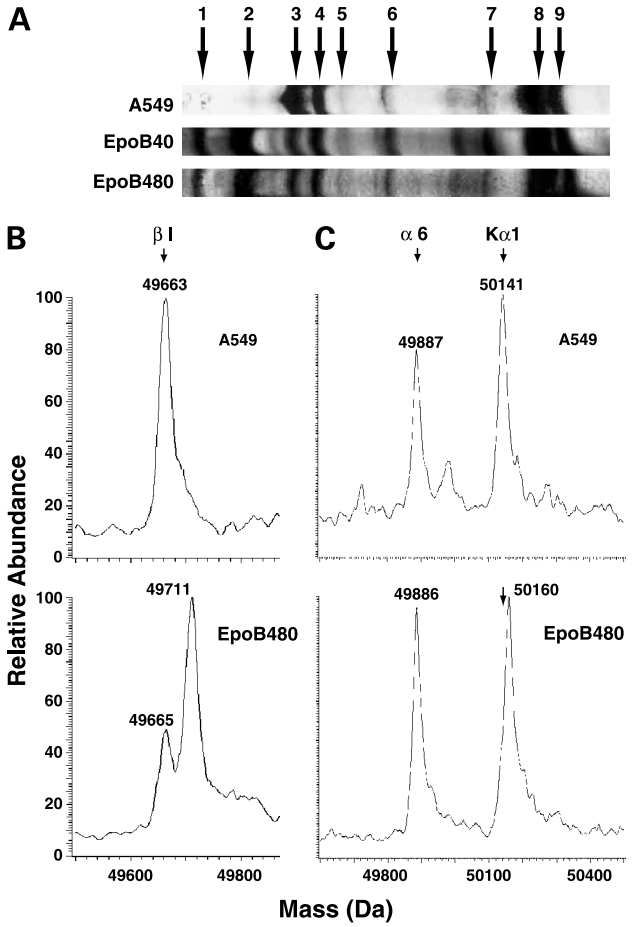

Figure 2. βI-tubulin with a $Gln^{292}Glu$, a $Val^{60}Phe$ mutation, and Kα1-tubulin with a $Leu^{195}Met$ mutation are expressed in EpoB480 cells. **A,** Taxol-stabilized microtubules from A549, EpoB40, and EpoB480 cells were analyzed by isoelectric focusing to determine if βI-tubulin with a $Gln^{292}Glu$ mutation was expressed in EpoB480 cells. *Lane 1,* monoglutamylated mutant βI; *lane 2,* mutant βI; *lane 3,* wild-type βI plus an unidentified protein; *lane 4,* βIVb; *lane 5,* monoglutamylated βIII; *lane 6,* βIII; *lane 7,* monoglutamylated Kα1; *lane 8,* Kα1; *lane 9,* α6. **B** and **C,** Taxol-stabilized microtubules from A549 and EpoB480 cells were analyzed by liquid chromatography-mass spectrometry to determine if βI-tubulin with a $Val^{60}Phe$ mutation and Kα1-tubulin with a $Leu^{195}Met$ mutation were expressed in EpoB480 cells. Deconvoluted mass spectra of βI-tubulin from A549 and EpoB480 (**B**) and deconvoluted mass spectra of Kα1-tubulin from A549 and EpoB480 (**C**) are presented. The mass of α6-tubulin is present as a marker. The arrow (**C**, *bottom*) marks the wild-type Kα1-tubulin with a molecular mass of 50,141 (calculated mass: 50,151 Da).

Discussion

The development of resistance to microtubule-interacting drugs is a multifactorial process. Induction of P-glycoprotein that maintains intracellular drug concentrations below cytotoxic levels is one mechanism involved in Taxol resistance. Interest in epothilone partly relates to the observation that the drug is active in Taxol-resistant cell lines and tumors expressing P-glycoprotein (6, 8), indicating that it is not a substrate for P-glycoprotein. The present study further shows that EpoB neither induces *MDR1* expression nor causes the expression of other drug efflux pumps even in a highly EpoB-resistant cell line, EpoB480 (Fig. 1).

The introduction of mutations in drug-targeted proteins is another mechanism of drug resistance. The unusual phenotype of Taxol/epothilone dependence has been described as cancer cells develop resistance to Taxol or the epothilones (15–19). Because abnormal microtubule dynamics impair mitotic spindle function and inhibit normal cell proliferation, it has been suggested that an alteration in microtubule dynamics may be involved in the Taxol or EpoB resistance/dependence phenotype (4, 17). Factors that influence microtubule dynamics include tubulin mutations that may affect the interaction between protofilaments or binding of regulatory proteins of different phosphorylation states that could result in microtubules with increased dynamics or decreased stability. Addition of Taxol or EpoB may restore normal dynamics to inherently unstable microtubules. However, tubulin mutations could also result in increased microtubule stability as found in a variety of cell lines that confer resistance to colcemid, vinblastine, or hemiasterlin (30, 31). In this study, it was found that tubulin polymerization increased significantly when EpoB480 cells were maintained in EpoB, whereas the levels of polymerized tubulin were reduced markedly when EpoB was removed from the medium.

Mutations in β-tubulin, many of which are not located in the drug-binding pocket, have been found in a variety of

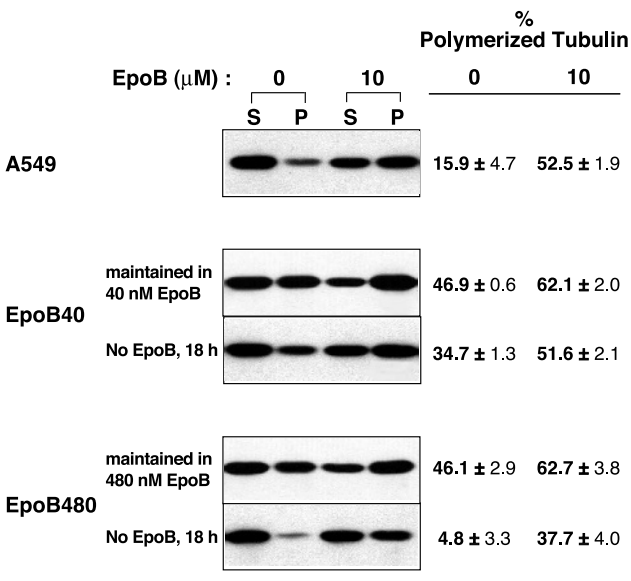

Figure 3. The level of polymerized tubulin is increased in EpoB480 cells, but decreases after EpoB is removed from the medium. Soluble (*S*) and polymerized (*P*) tubulin were separated in the absence or presence of 10 μmol/L EpoB in the lysis buffer, and relative amounts of tubulin were determined by Western blot analysis using a monoclonal antibody to α-tubulin. The percent polymerized tubulin was calculated by dividing the polymerized fraction (*P*) by the total polymerized and soluble fractions (*P* + *S*), and values are ±SE for three to four individual experiments.

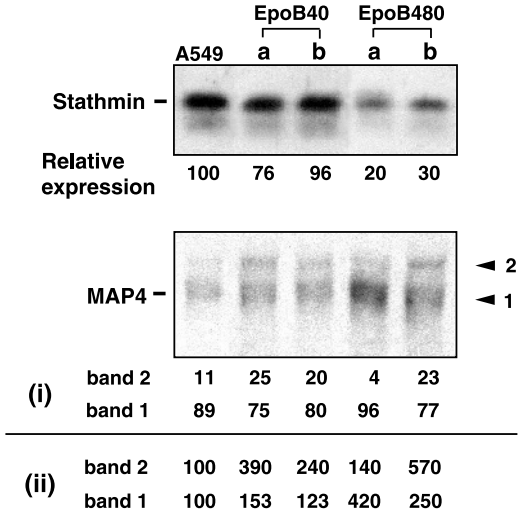

Figure 4. Stathmin levels are reduced in EpoB480 cells. Cytosolic fractions of cell lysates were prepared from A549, EpoB40, and EpoB480 cells as described (18). *Top,* samples (10 μg) were subjected to 15% SDS-PAGE, followed by Western blotting using an antistathmin antibody. Expression of stathmin was quantitated in each cell line. *Bottom,* samples (40 μg) were resolved in 5% urea-PAGE, followed by Western blot analysis using anti-MAP4 antibody. *a,* resistant cells maintained in EpoB (40 nmol/L EpoB for EpoB40 and 480 nmol/L EpoB for EpoB480). *b,* resistant cells grown in the absence of EpoB for 18 h. *i* and *ii,* relative expression of nonphosphorylated (*band 1*) and phosphorylated (*band 2*) MAP4 in each cell line. Values were determined by either assigning total MAP4 levels in each cell line as 100% (*i*) or assigning MAP4 levels in A549 cells as 100% (*ii*).

Taxol- and epothilone-resistant cells (14). In general, these resistant cells contain less stable microtubules, as evidenced by decreased Taxol/epothilone–driven microtubule assembly (11, 22, 32). It has been hypothesized that many of the mutations in β-tubulin decrease the stability of microtubules that can be compensated for by microtubule-stabilizing agents. As would be expected, these resistant cells became more sensitive to microtubule-destabilizing drugs, such as vinblastine and colchicine (19, 32).

In this study, two β-tubulin mutations were found in a highly EpoB-resistant A549 cell line, EpoB480. One is at residue 292 (Gln to Glu) that was previously reported in a less resistant cell line, EpoB40, and the other is a heterozygous β-tubulin mutation at residue 60 (Val to Phe). β292 is located near both the M-loop and Thr^{274}, a residue forming part of the binding pocket for Taxol (19). This Gln^{292}Glu mutation was also reported in cells selected for resistance with desoxyepothilone B in a human leukemia cell line, CCRF-CEM (22). In addition, a Gln^{292}His mutation has been reported to confer resistance to Taxol in a revertant of colcemid resistant Chinese hamster ovary cells (33).

Val^{60} is conserved in all β-tubulin isotypes. It is located at the end of the H1-S2 loop (N-loop) that has a substantial role in interacting with the M-loop of an adjacent tubulin molecule (29). There is a cluster of residues in the N-loop region that vary among β-tubulin isotypes and these residues are located at the position of the interprotofilament interaction with the M-loop. It has been proposed

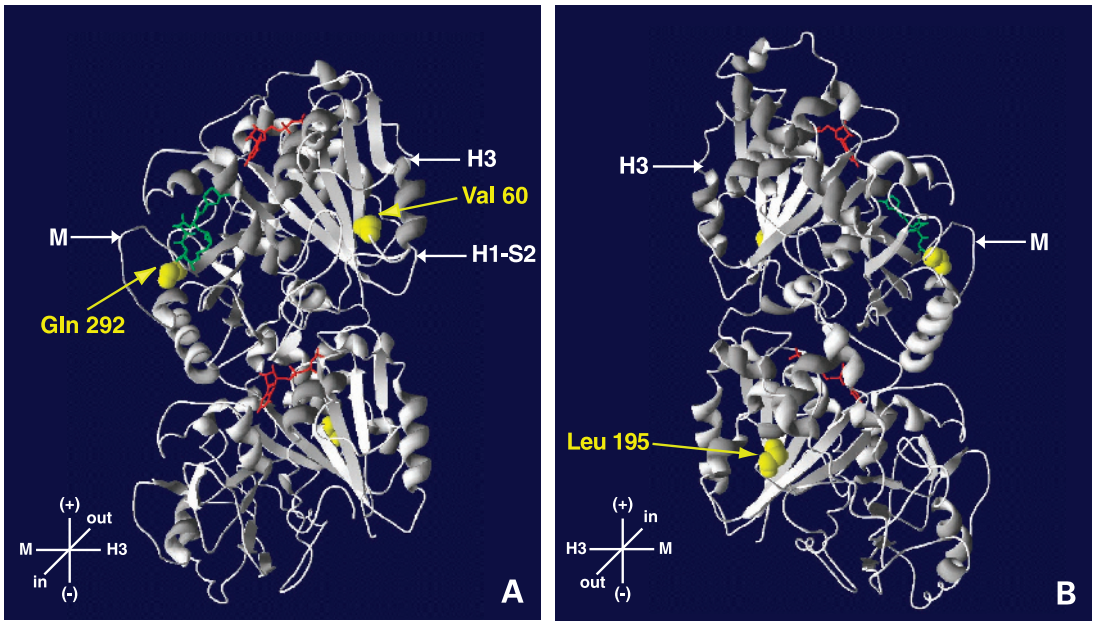

Figure 5. Structural model of a tubulin heterodimer. Helix and β-sheet diagram of a tubulin heterodimer based on the coordinates of Nettles et al. (13). Tubulin is viewed from the inside (**A**) and from the outside (**B**) of the microtubule with β-tubulin at the top toward the (+) end of microtubule (see three-dimensional axis orientation in bottom left corner). The location of Gln^{292} and Val^{60} in β-tubulin and that of Leu^{195} in α-tubulin are indicated with backbone atoms represented as yellow spheres. A molecule of GTP that binds to α-tubulin and a molecule of GDP that binds to β-tubulin are in red. Epothilone A, which interacts with β-tubulin, is in green. M-loop, helix 3 (*H3*), and helix 1 – β-sheet 2 (*H1-S2*) loop are indicated with arrows on the β-tubulin subunit.

that the amino acid differences at this contact point play an important role in determining the distinct dynamic properties of the purified tubulin isotypes and their sensitivities to Taxol (29). Molecular modeling of the tubulin α-β heterodimer indicates that Val^{60} in the H1-S2 loop of β-tubulin is close to Gln^{292}, which is near the M-loop, of the neighboring tubulin dimer. Mutations at both Gln^{292} and Val^{60} in β-tubulin would be expected to severely impair the lateral contacts between protofilaments. Such cells would require high concentrations of Taxol or EpoB to compensate for increased instability of their microtubules, thereby becoming Taxol- or EpoB-dependent. Although electron crystallography studies indicated that Taxol and epothilone exploit unique contacts in the tubulin-binding pocket (13), our results show that Taxol and EpoB are equally potent in maintaining normal cell growth for the highly EpoB-dependent EpoB480 cells. In addition, it seems that Taxol and EpoB are equally effective in compensating for the decreased microtubule stability introduced by tubulin mutations, particularly at βVal^{60} (see Table 1).

Recently, Cabral et al. (33) have selected revertants of Taxol hypersensitive, colcemid-resistant Chinese hamster ovary cells that contain a D45Y mutation in β-tubulin and have identified several *cis*-acting suppressors of D45Y. Expression of one suppressor, V60A, produced Taxol resistance and decreased microtubule assembly. Interestingly, it also produced a Taxol-dependent phenotype as the cells that harbor only the V60A mutation required Taxol for normal growth (33). This observation provides further support to our hypothesis that Val^{60} is an important residue for conferring Taxol/EpoB dependence.

We have previously reported a mutation in Kα1-tubulin at residue 379 in a Taxol-resistant A549-T12 cell line (18). These cells depend on a minimum of 2 nmol/L Taxol for normal growth and their microtubules display increased dynamics in the absence of Taxol (17). In the present study, the highly EpoB-dependent EpoB480 cells harbor an α-tubulin mutation at residue 195 (Leu to Met). Leu^{195} is conserved in all α-tubulin isotypes and is adjacent to Glu^{196}, His^{197}, and Asp^{199}. In yeast, mutations at these three residues are recessive lethal (34), suggesting that this is an important region responsible for normal growth of cells. Molecular modeling studies have indicated that αLeu^{195} is adjacent to many charged residues (data not shown) and it is also situated at the external surface of the microtubule. The charged external surface of α-tubulin is thought to be the domain that interacts with a variety of endogenous regulatory proteins, such as kinesin, stathmin, and MAP4 (25, 35, 36). EpoB480 has an α195 mutation from Leu to Met and the bulkier side chain of Met may influence the interaction between tubulin and regulatory proteins.

Interaction of endogenous regulatory proteins, such as stathmin and MAP4, has been shown to modulate microtubule stability. Stathmin (S) sequesters tubulin (T) in a T_2S complex in which it interacts with two α-β–tubulin heterodimers, and destabilizes microtubules (24–26). The NH_2-terminal region of stathmin is at the α-end of the αβ-αβ dimer (37). Stathmin plays an important role in cell division and is negatively regulated by phosphorylation (38, 39). MAP4 is the predominant nonneuronal MAP, and the microtubule-stabilizing function of MAP4 is also regulated by phosphorylation (27, 40). MAP4 can be phosphorylated by cdc2 at Ser^{696} during interphase and at Ser^{787} during mitosis (41). When MAP4 is phosphorylated, it does not bind to microtubules nor promote stabilization of the microtubule network.

In Taxol resistant/dependent A549-T12 cells, elevated levels of microtubule-destabilizing factors, including the active nonphosphorylated form of stathmin and the inactive phosphorylated forms of MAP4, were present (18). Because the region of α-tubulin that surrounds Ser^{379} is close to the COOH terminus, the proposed site of interaction with MAP4 and stathmin (25, 36), it was hypothesized that alterations in stathmin and MAP4 that resulted in increased microtubule instability could be related to the α379 mutation. These changes could be compensated for by stabilization with Taxol.

The highly EpoB resistant/dependent cell line EpoB480, grown in the presence of 480 nmol/L EpoB, did not show elevated levels of microtubule-destabilizing factors as did the A549-T12 cells that exhibit low levels of Taxol dependence (18). In EpoB480, cellular levels of stathmin were greatly reduced, suggesting that the tubulin sequestration activity of stathmin was decreased and the inactive phosphorylated form of MAP4 was not increased significantly. The unphosphorylated form of MAP4 was increased moderately. These alterations would result in an increase in microtubule stability, thereby compensating for the loss of stability by mutations. In the absence of EpoB, the steady-state levels of polymerized tubulin are reduced in these cells, indicating that microtubules are destabilized. The stabilizing properties of EpoB correct this defect. With no drug present, the inactive phosphorylated form of MAP4 was increased, compared with cells grown in the presence of EpoB (Fig. 4). This is most likely due to the accumulation of cells in aberrant mitosis similarly to what we observed with A549-T12 cells grown in the absence of Taxol (17). In the G_2-M phase, phosphorylation of MAP4 increased presumably because of the high activity of cdc2 kinase for which MAP4 is a substrate (41). The differences in stathmin and phosphorylated MAP4 levels between A549-T12 and EpoB480 cells are likely due to distinct levels of drug dependence in each cell line. In addition, these two cell lines harbor different tubulin mutations that may affect regulatory protein expression in unique ways.

Recently, the structure of tubulin at a resolution of 3.5 Å has been presented in a complex with colchicine and the stathmin-like domain of the neural protein RB3 (42). This structure visualizes the interaction of the RB3–stathmin-like domain with two tubulin heterodimers in a curved complex. One of the contact points between α-tubulin and RB3–stathmin-like domain is residue 193 (Thr)

that is very close to the α195 tubulin mutation found in EpoB480 cells. The mutation at α195 from Leu to a bulkier Met may impair the interaction between α-tubulin and stathmin and, therefore, provide a compensatory mechanism for the Val^{60}Phe mutation–induced destabilization of microtubules.

In summary, we have isolated a highly EpoB-resistant/dependent cell line, EpoB480, that harbors two β-tubulin mutations and one α-tubulin mutation. βGln^{292}Glu that is located near the M-loop is also present in the EpoB-resistant, but not dependent, cell line, EpoB40. βVal^{60} is located at the end of the long H1-S2 loop that has been implicated as the domain that interacts with the M-loop of the adjacent tubulin dimer. By two different approaches, one using a Taxol-resistant revertant derived from a colcemid-resistant cell line (33) and the other presented in this study, the conclusion emerges that the βVal^{60} mutation is important in conferring Taxol/EpoB dependence. αLeu^{195}Met may influence the interaction between microtubules and endogenous regulatory proteins, thereby affecting microtubule stability. The present studies suggest that both βVal^{60}Phe and αLeu^{195}Met have a major role in the Taxol/EpoB dependence of EpoB480 cells.

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